University of Southampton

Faculty of Environmental and Life Sciences

School of Health Sciences

Identifying early changes in skin integrity using biomarkers

by

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Thesis for the degree of Doctor of Philosophy

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Abstract

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Skin damage, in particular, those involving chronic wounds such as pressure ulcer (PU) and incontinence associated dermatitis (IAD) represent a major challenge for both vulnerable patients and health care providers. Current skin evaluation involves subjective tools, such as risk assessment scales and visual skin assessment. However, these are known to have limited reliability and diagnostic accuracy. Therefore, there is a pressing need for early detection of skin damage using novel techniques to provide timely interventions to prevent chronic wounds. Accordingly, this project is designed to identify, develop and evaluate the performance of a range of biomarkers to detect local changes in skin health.

Protocols were developed to investigate changes in skin health following different insult models, ranging from lab-based insult models to clinical cohort studies. Changes in skin parameters were investigated using a combination of prospective and secondary analysis studies. Secondary analysis involved technical evaluation of biophysical parameters measured at different anatomical regions and following different insult models. Prospective studies involved development of an efficient extraction methodology to analyse low-abundant inflammatory markers from sebum and the corresponding use of the methodology in evaluating skin's inflammatory status.

Results from the studies suggest a high level of variability in the basal values of biophysical parameters, skin surface sebum volumes and corresponding inflammatory biomarker concentrations. The secondary analysis revealed that thresholds derived from single biophysical parameters were limited in detecting skin damage following insults. Moreover, a high level of variability was observed in the basal values of sub-epidermal moisture (SEM), both at different body locations and between subjects. The prospective studies involving healthy volunteers revealed an optimum time point for sebum collection.

An optimised extraction protocol was developed resulting in an 1.5-2.0-fold increase in recovery of high and low abundant cytokines. Skin cytokine response following the use of respirators, incontinence pads and shavers revealed a selective upregulation of inflammatory biomarkers. Indeed, the findings revealed that the use of shavers and masks led to a pronounced increase in inflammatory response compared to exposure to incontinence pads. There were distinct clusters of responses within a cohort of healthy volunteers which could possibly suggest an intrinsic risk of skin damage. Skin inflammation assessed on sites presenting with Stage-I PU revealed a localized upregulation of pro-inflammatory cytokines and a downregulation of anti-inflammatory cytokine IL-1RA. Selected cytokines, namely IL-1α, IL-1RA, IL-8, G-CSF and the ratio of IL-1α/ IL-1RA offered clear delineation in the classification of healthy and Stage-I PU, with good receiver operating characteristic curves.

The findings from the studies clearly indicate that changes in inflammatory status of skin serve as promising biomarkers for detecting early and established signs of skin damage. The new extraction methodology and the identified markers have also been critical in evaluating the skin status following the use of various products, such as shavers, incontinence pads and respirators. Although further studies are required to investigate the biochemical changes occurring prior to skin damage, the current studies provide an indication for their use as a point of care diagnostic tool.

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Research Thesis: Declaration of Authorship

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I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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   Jayabal H., Bates-Jensen B.M., Abiakam N.S., et al., Anatomical variability of sub-epidermal moisture and its clinical implications, J Tissue Viability, Volume 30, Issue 3, August 2021, doi: 10.1016/j.jtv.2021.04.003

   Jayabal H., Bates-Jensen B.M., Abiakam N.S., et al., "The identification of biophysical parameters which reflect skin status following mechanical and chemical insults", Clinical Physiology and Functional Imaging, Volume 41, Issue 4, July 2021, doi: 10.1111/cpf.12707

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   Jayabal, H, Abiakam, NS, Filingeri, D, Bader, DL, Worsley, PR. Inflammatory biomarkers in sebum for identifying skin damage in patients with a Stage I pressure ulcer in the pelvic region: A single centre observational, longitudinal cohort study with elderly patients. Int Wound J.; 1- 14, 2023. doi:10.1111/iwj.14131

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Dissemination

Publications

1) **Jayabal H,** Bates-Jensen BM., Abiakam NS., et al., Anatomical variability of sub-epidermal moisture and its clinical implications, J Tissue Viability, Volume 30, Issue 3, August 2021, doi: 10.1016/j.jtv.2021.04.003

2) **Jayabal H,** Bates-Jensen BM., Abiakam NS., et al., "The identification of biophysical parameters which reflect skin status following mechanical and chemical insults", Clin Physiol Funct Imag, Volume 41, Issue 4, July 2021, doi: 10.1111/cpf.12707

3) **Jayabal, H,** Bader DL, and Worsley PR. "Development of an Efficient Extraction Methodology to Analyse Potential Inflammatory Biomarkers from Sebum." Skin Pharmacol Physiol, Volume 3, 38-50, 2023 doi: 10.1159/000528653

4) **Jayabal, H**, Abiakam, NS, Filingeri, D, Bader, DL, Worsley, PR. Inflammatory biomarkers in sebum for identifying skin damage in patients with a Stage I pressure ulcer in the pelvic region: A single centre observational, longitudinal cohort study with elderly patients, Int Wound J.; 1- 14, 2023. doi:10.1111/iwj.14131

5) Abiakam, NS, **Jayabal, H**, Mitchell, K, et al., "Biophysical and biochemical changes in skin health of healthcare professionals using respirators during COVID-19 pandemic." Skin Res Technol., 2023; 29 : e13239. doi: 10.1111/srt.13239

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Conference presentations

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Dedications

This thesis is dedicated to

Emeritus Professor Dan L Bader BSc, MSc, PhD, DSc

Definitions and Abbreviations

AD	Atopic dermatitis
ATP	Adenosine triphosphate
AU	Arbitrary units
AUC	Area under receiver operating characteristic (ROC) curve
BBJ	Barbara Bates-Jensen
BCA	Bicinchoninic Acid
BL	Baseline
BMI	Body mass index
CCD	Charge coupled device camera
CD	Corneodesmosome
CE	Cornified Envelope –
CI	Chemical irritation
CMC	Critical micelle concentration
COL	Collagen
COVID	Coronavirus disease
CSSS	Cyanoacrylate skin surface stripping
CV	Coefficient of variability
DDM	Dodecyl maltoside
DFU	Diabetic foot ulcer
DNA	Deoxyribo nucleic acid
DTI	Deep tissue injury
ECL	Electrochemiluminescence
ECM	Extra cellular matrix
ELISA	Enzyme linked immunosorbent assay
EPUAP	European Pressure Ulcer Advisory Panel
ERGO	Ethics and Research Governance Online
ESR	Early-Stage Researcher
FFP –	Filtering facepiece
FoHS-	Faculty of Health Sciences
G-CSF	Granulocyte- Cytokine stimulating factor
HCW	Health care workers
HIF	Hypoxia Inducible Factor
HRA	Health research authority
HRP	Horse-radish peroxidase
HSP	Heat shock protein
IAD	Incontinence associated dermatitis
ICC	Intra class correlation coefficient
ICG	Indocyanine green
ICU	Intensive care units
IHC	Immunohistochemistry

IL	Interleukin
INF	Interferon
IP-10	Induced protein
IRAS	Integrated Research Application System
LDI	Laser Doppler imaging
LFT	Lateral flow tests
LLC	Limited liability corporation
LLOD	Lower limit of detection
LU	Leg ulcer
MDA	Malondialdehyde
MDRPU	Medical device related Pressure Ulcers
ML	Mechanical loading
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Ribonucleic acid
MSD	Mesoscale diagnostics
NA	Nkemjika Abiakam
NHS	National Health Service
NICE	National institute for health and care excellence
NIR	Near infra-red
NMF	Natural moisturizing factor
NPUAP	National Pressure Ulcer Advisory Panel
NSS	Non-skin sensitive
OCT	Optical coherence tomography
PAI	Plasminogen activator …
PBS	Phosphate buffered saline
PC	Pakhi Chaturvedi
PCA	Principal component analysis
PP	Polypropylene
PPE –	Personal protective equipment
PPV –	Positive predictive value
PU	Pressure Ulcer
PURPOSE-T	Pressure ulcer programme of research
RA	Receptor antagonist
RAS	Risk assessment scale
REC	Research ethics committee
RHE	Reconstructed human epidermis
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
RPE	Respiratory protective equipment
RT-PCR	Reverse transcriptase – Polymerase Chain Reaction
SAP	Superabsorbent polymer
SC	Stratum Corneum

SCH	Stratum corneum hydration
SCI	Spinal cord injury
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
SEM	Sub-epidermal moisture
SFC-MS	Supercritical fluid chromatography – Mass Spectrometry
SLS	Sodium lauryl sulfate
SPV	Sulfo-phospho-vanillin assay
SS	Skin sensitive
STINTS	Skin Tissue Integrity under Shear
STROBE	Strengthening the Reporting of Observational studies in Epidemiology
SU	Synthetic Urine
SW	Saturated water pad
TA	Tibalis anterior
TAP	Transdermal analysis patch
TAS	Total antioxidant status
TCM	Transcutaneous gas monitoring
TcPCO2	Transcutaneous Carbon dioxide tension
TcPO2	Transcutaneous Oxygen tension
TEWL	Trans epidermal water loss
TGF	Transforming growth factor
THF	Tetrahydrofuran
TNF	Tumor necrosis factor
TP	Total protein
TS	Tape-stripping
UHPLC-MS	Ultra-high-performance liquid chromatography – Mass Spectrometry
UK	United Kingdom
ULOD	Upper limit of detection
US	Ultrasound
USA	United States of America
USI	Ultrasound imaging
UV	Ultraviolet
VEGF-C	Vascular endothelial growth factor

Chapter 1 : Introduction - Skin and Pressure Ulcers

The major aim of the thesis is the identification of biomarkers to provide an accurate prediction of early stages of skin damage. This chapter will summarise relevant topics, including the structure and function of skin, the nature of skin breakdown leading to the development of pressure ulcers, their prevalence and the current preventative strategies.

1.1. Skin

Skin is the largest organ of the human body accounting for about 15% of total body weight, with an average surface area of between 1.5 and 2.0 m^2 in adults (Montagna 2012). It represents the first line of defence against external insults including chemical, biological, mechanical, and thermal challenges. Skin is a complex organ made up of three main layers, namely, the epidermis, dermis, and hypodermis, each of which varies significantly in its structure and function. Furthermore, the skin also serves as an immune organ and contains different secretory glands.

1.1.1. Epidermis

Epidermis, the outermost layer is an avascularised stratified structure, that serves as a barrier to the external environment. It is about 100 μm thick, although the thickness does vary at specific anatomical locations. For example, in load-bearing regions of the body e.g., the sole of the foot, the epidermis is 1.5 mm. The sub-layers of epidermis, namely, *Stratum Corneum*, *Stratum Granulosum*, *Stratum Spinosum,* and *Stratum Basale* are made up of keratinocytes at different stages of differentiation. An additional sub-layer is sandwiched between *Stratum Corneum* and *Stratum Granulosum*, the *Stratum lucidum,* which is present in anatomical sites, such as the palm and heel (Limbert 2019). A schematic of skin structure and epidermal differentiation is represented in Figure 1-1.

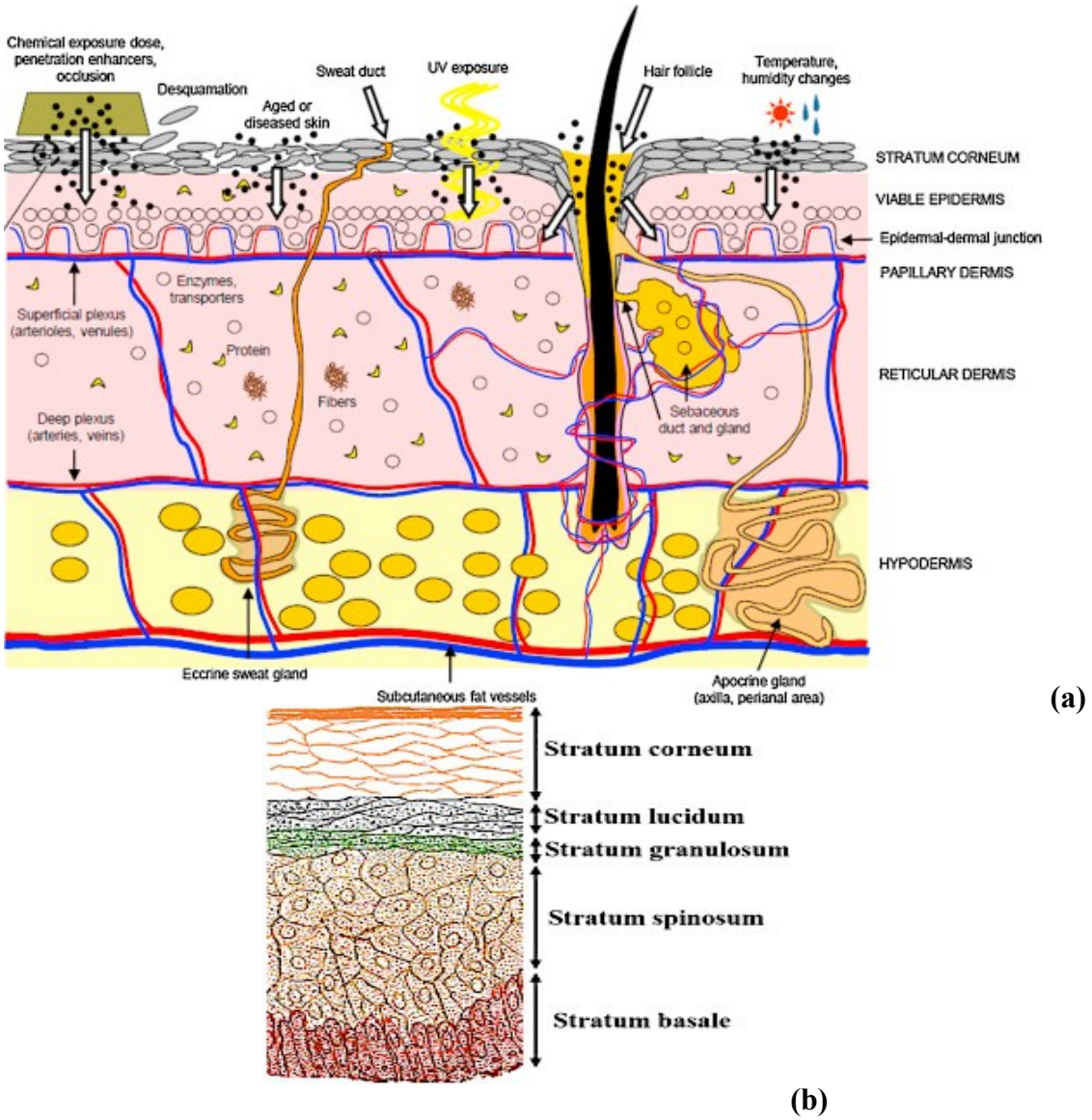


Figure 1-1 : Structure of skin depicted with everyday insults (Dancik, Bigliardi et al. 2015) (b) epidermal sub-layers (Nafisi and Maibach 2018).

The *Stratum Corneum (SC)*, the most superficial layer of epidermis, is arranged in a 'brick and mortar' like structure, wherein the terminally differentiated keratinocytes, termed as corneocytes, and lipids represent the brick and mortar, respectively. The corneocytes removed in desquamation are constantly replenished by the keratinocytes migrating from the underlying layers. Natural moisturizing factor (NMF) that attracts water molecules are present in the SC, whose primary function is to maintain the hydration levels and prevent the formation of cracks and fissures. The structural arrangement of the *Stratum Corneum* provides a chemical barrier against toxins. The key transformations of keratinocytes to corneocytes and the lipid release occurs at the subjacent layer, the *Stratum Granulosum*. The third sub-layer, *Stratum Spinosum* comprises the bulk of the epidermis wherein the cells are

connected by the adhesive structure, corneodesmosomes (CD). In addition to keratinocytes produced from the basal stem cells, the *Stratum Basale* layer consists of other cells. These include the Langerhans cells, which are vital for the immune response, the Merkel cells which serve as a sensory-receptors and Melanocytes which produce melanin, which protects the skin against ultraviolet radiation. Intrinsic and extrinsic factors influence the skin structure and morphology. As an example, intrinsic skin aging leads to remarkable reduction in the proliferation of cells in the basal layer leading to the thinning of epidermis (Zhang, Duan et al. 2018).

1.1.2. Dermis

The dermis is connected to the epidermis by an undulating dermal-epidermal junction. This connective tissue layer, which forms the major component of skin, is made of two sub-layers namely, papillary and reticular dermis. The papillary layer is composed of thin loose connective tissue that is highly vascular and provides nutrients to the connected upper layer, the epidermis. The deeper reticular dermis is thicker and is composed of a dense mesh-like network of collagen and elastin fibres (Rutter 2000), which provide the skin with its tensile stiffness and strength. With aging, the thickness of dermis decreases and furthermore, the collagen and elastin turnover are inevitably reduced (Farage, Miller et al. 2013). It is also to be noted that overexposure to UV radiation could damage collagen and elastin due to the formation of reactive oxygen species (ROS) (Gromkowska-Kepka, Puscion-Jakubik et al. 2021). Within the dermis, there are other components, such as hair follicles, sweat glands, sebaceous glands, neurons, blood and lymph vessels (Skobe and Detmar 2000). The cell population of dermis layer is primarily comprised of fibroblasts, with additional mast cells and macrophages. The primary role of fibroblasts is the synthesis of fibrous tissue proteins, collagen, and elastin. Macrophages are responsible for defense against foreign particles and mast cells are of importance in the inflammatory response and during wound repair (Prost-squarcioni, Fraitag et al. 2008). The extracellular matrix (ECM) i.e., ground substance encompasses all the structural components of the dermis and plays a critical role in maintaining the hydration of the skin. Aging leads to decrease in the cell population, primarily, mast cells and fibroblasts, which in turn decreases the amount of ground substance and hyaluronic acid (Farage, Miller et al. 2013).

1.1.3. Hypodermis

The hypodermis, otherwise known as subcutaneous tissue, is an adipose-rich layer that serves as a shock absorber, energy reserve and temperature regulator (Nafisi and Maibach 2018). The hypodermis is composed of adipocytes, macrophages and fibroblasts (Limbert 2019). Recent studies have also suggested that the thickness of the hypodermis, which varies across body locations and between genders, is also highly correlated to the body mass index (BMI) of individuals (Frank, Casabona et al. 2019). Similar to the dermis, the hypodermis also contains hair follicles, neurons and blood- and lymphatic-vessels.

1.1.4. Skin immune system

Skin contains several types of immune cells, including skin cells and immune cells, that together participate in the immune response (Figure 1-2). Some of the key immune cells in the epidermis include keratinocytes, Langerhans cells and T-cells. Strategically positioned at the surface, keratinocytes produce cytokines, such as IL-6, TNF-α, as a response to external stimuli, including chemical and mechanical stimuli, which further activates the dendritic cells present in the dermis (Lee, Stieger et al, 2013). In addition, the melanocytes have also recently been reported to be involved in the immune response (Gasque and Jaffar-Bandjee 2015). Langerhans cells, comprising 2 to 4% of the epidermal cell population, play a major role in maintaining the immune homeostasis and serve as sentinels at the skin barrier. These cells have a dendritic morphology that captures the antigen from the skin surface without disturbing its barrier function (Nestle, Meglio et al. 2009). T-cells are a central component of adaptive immunity, with memory T cells providing long-lasting immunity by producing an antigen specific response (Ho and Kupper 2019). Moreover, melanocytes have also been reported to modulate immunoactivity, in particular innate immunity through toll-like receptors. Indeed, the role of melanin synthesis and transport have influence over the immune system (Koike and Yamasaki 2020). The interplay of the complex immune system is orchestrated by the release and binding of cytokines that result in either increasing or decreasing the inflammatory response.

Cytokines/Chemokines

Cytokines, the key modulators of acute and chronic inflammation, are small glyco-proteins with molecular weights ranging between 5 to 20 kDa. Cytokines are broadly classified based on their inflammatory potential as either pro-inflammatory or anti-inflammatory. Some

examples of the former include Interleukin (IL)-1α, IL-1β, IL-6, IL-8, while anti-inflammatory cytokines include IL-1RA, IL-13, IL-4, TGF-β (Zhang and An 2007). Some of the cytokines, such as TNF- α and IL-6, have been reported to have both pro-inflammatory and anti-inflammatory activities (Fuster and Walsh 2014, Jang, Lee et al., 2021). The concentration of the cytokines varies depending on the insult to the skin, in terms of type of wounds and the course of a disease. For example, in the case of an acute wound, the cytokine release is well-established in the inflammatory phase, which can be prolonged in the case of chronic wounds (MacLeod and Mansbridge 2016).

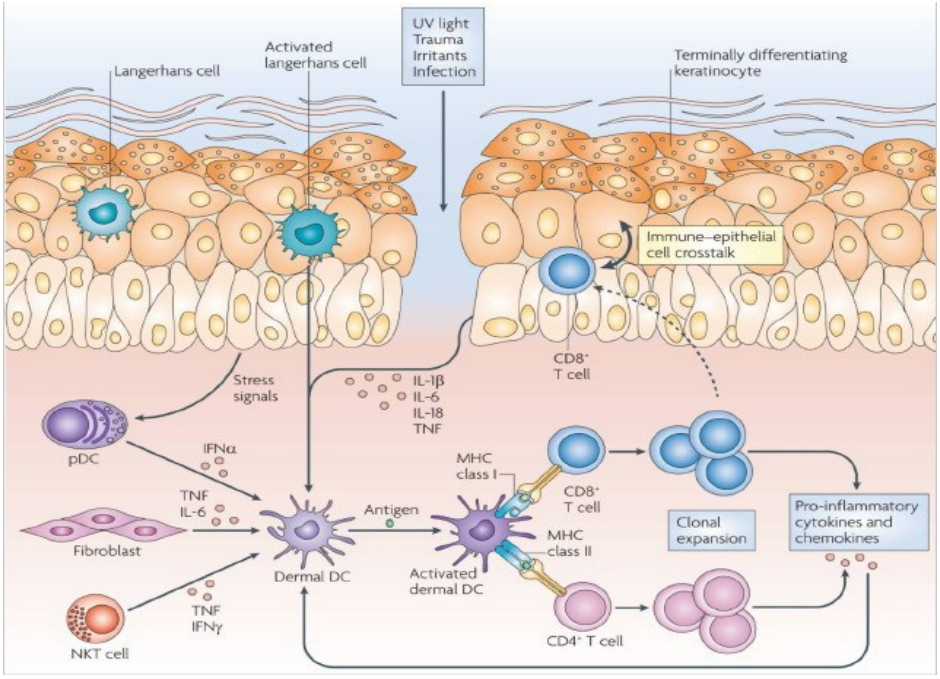


Figure 1-2: Schematic illustrating the cells and signalling cytokines involved in the skin immune system (Nestle, Meglio et al. 2009)

1.1.5. Skin glands

The function of *sebaceous and sweat glands* is to maintain skin homeostasis by the secretion of sebum and sweat, respectively. The former is a holocrine gland that produces a waxy lipid-rich substance to lubricate the skin and make it water-resistant. Typically, a sebaceous gland is present in conjunction with a follicular canal that serves as a repository to collect the sebum output. Although the number of the gland units remains fairly constant throughout an individual's lifespan, the glands tend to increase in size with age, particularly during adolescence.

Sweat glands are tubular structures present in the dermal or hypodermal layers that produce a watery fluid maintaining thermal homeostasis. There are two types, namely eccrine and apocrine sweat glands. The former is present throughout the body, whereas the latter is present in limited body locations, such as the armpit and genital regions. Previous research has demonstrated that the sweat production rate is decreased with aging and it is also highly dependent on factors such as the body and muscle mass (Larose, Boulay et al. 2013, Osayande, Ogbonmwan et al. 2016).

1.2. Skin damage – Pressure Ulcers

Chronic wounds are difficult to heal and are often associated with individuals who have multiple co-morbidities. Prolonged exposure of skin to mechanical and chemical insults can result in the formation of such chronic wounds. The most prominent forms of these wounds include Pressure Ulcers (PU), Diabetic Foot Ulcers (DFU), Leg Ulcers (LU) and Incontinence Associated dermatitis (IAD).

A Pressure Ulcer is defined as 'a localized injury to the skin and/or underlying tissue usually over a bony prominence, as a result of pressure, or pressure in combination with shear' (National Pressure Ulcer Advisory Panel 2019). PUs are associated with individuals who have limited mobility in both the community and acute health care settings. Moreover, the use of medical devices such as oxygen tubing, prosthetics, catheters and non-invasive ventilation masks, can lead to PUs that are termed as 'medical device-related pressure ulcers' (MDRPUs). These wounds lead to pain and reduced quality of life, and as such, are considered a patient safety and quality of care indicator.

COVID-19

The outbreak of COVID-2019 pandemic has caused numerous health challenges to the population worldwide. This led to an increase in patients admitted to hospitals thereby causing an increased burden on the health care workers (HCWs). Patients admitted to hospitals required attachment of medical devices for ventilation purposes. In addition, these patients were also placed in prone positioning for prolonged periods to aid ventilation (Sottile, Albert et al. 2022). The attachment of medical devices and prone positioning has resulted in the formation of PUs including MDRPUs (Martel and Orgill 2020, Challoner, Vesel et al. 2022). In addition, the mandatory use of personal protective equipments, such as

respiratory masks, by HCWs resulted in skin damage, especially at bony prominence locations, such as the nasal bridge (Hu, Fan et al. 2020). These damages result in pain and increased stress for the HCWs

1.2.1. Categorization of Pressure Ulcers

Depending on the degree of wound progression, pressure ulcers (PUs) are categorized into four stages, in addition to deep tissue injury (Table 1-1), defined by published international guidelines (National Pressure Ulcer Advisory Panel 2019). With stage I-IV PUs, the damage progresses from the skin surface to the deeper tissues (top-bottom). By contrast, Deep Tissue Injuries (DTI) are initiated in muscle layers, typically adjacent to bony prominences, and progress towards the skin surface (bottom-up). Owing to the upwards progression of damage, these ulcers are not visible until the time they appear on the surface and at this stage there is a variable prognosis in terms of healing of this full depth wound (Bouten, Oomens et al. 2003). However, it must be recognised that DTIs represent a relatively small proportion of PUs (~7%) when compared to the total number of PUs (VanGilder, MacFarlane et al. 2010, Stephenson J.; Fletcher J. 2020).

Table 1-1: Classification of PUs (Adapted from NPUAP/EPUAP guidelines (National Pressure Ulcer Advisory Panel 2019))

Stage / Category/Grade	Definition
I	Intact skin with **non-blanchable redness** of localized area, usually over a bony prominence.
II	**Partial thickness skin loss** of dermis presenting as a shallow open ulcer. Presents as a shiny or dry shallow ulcer without slough or bruising
III	**Full thickness skin loss –** Exposure of subcutaneous fat, but bone, tendon or muscle that are not exposed and depth varies depending on the anatomical location
IV	**Full thickness tissue loss** – Exposure of underlying bone, tendon or muscle.
Deep tissue injury	**Intact/ Non-intact skin with localized area of non-blanchable purple or maroon discoloration –** Preceded by pain and presents distinct differences from adjacent tissues

1.2.2. Prevalence and incidence

Prevalence measures the proportion of individuals in a population that have a particular health condition at a given point of time or over a specified period, corresponding to point prevalence and period prevalence respectively. Incidence measures the rate of new cases of a particular health condition occurring in a population at a given point of time (Noordzij, Dekker et al. 2010). As per National Institute for Health and Clinical Excellence (NICE) reports, there is no nationally collated data on the incidence of PUs due to variabilities in hospital-based studies, however, the values have been estimated to be 4-10% of patients admitted to hospitals in the UK (NICE 2014). Moreover, recent meta-analysis studies estimated the incidence rate of PUs to be 12% amongst which 45% were Stage-I PU (Borojeny, Albatineh et al. 2020). A recent review suggests the median prevalence of PUs in Europe and UK to be 10.8% and 8.4%, respectively (Moore, Avsar et al. 2019). Of the total, approximately 33% were reported as Stage-I PUs and the sacrum was reported as the most common site of PU. In a recent study at New York city in the United States, involving clinically obese individuals during the period of COVID-19, the prevalence of PUs was reported to be exponentially higher than at any recorded time in history (Trevenelli 2020). Previous reports estimate the cost of an unhealed wound to range from £831 to £7886 per patient (Guest, Fuller et al. 2020). With the high prevalence and the cost associated with PUs, they represent one of the major financial burdens to the health care system, in addition to the reduced quality of life for afflicted individuals. The mean cost of managing wounds, including PUs, has been predicted to cost the UK NHS an estimated £8.3 billion (Guest, Fuller et al. 2020).

1.2.3. Risk assessment scales

Although all individuals, if immobilised for prolonged periods are at risk of developing PUs, there are specific risk factors that deem an individual at high risk. In a clinical setting, risk assessment scales (RAS) are used as an adjunct to clinical examination to identify individuals at risk and thereby target interventions to prevent PUs. These scales are typically completed by nursing staff, with a high degree of subjectivity and variability of completion. It is also to be noted that even with significant experience, clinicians face challenges in using visual observations, such as skin redness, which can be misclassified as incontinence-associated dermatitis or moisture lesions (Kottner and Dassen 2010, Payne 2016). Moreover, it has also been reported that the redness is difficult to identify in dark skin, resulting in a

relatively high incidence of skin damage in these cohort of individuals (Clark 2010, Oozageer Gunowa, Hutchinson et al. 2018). Indeed, there are approximately 40 RAS available (Kottner and Balzer 2010). However, the most commonly employed RAS in clinical practice are the Waterlow, Norton and Braden Scales. Of these, however, the Waterlow Scale was reported to make little or no difference to the incidence of PUs (Moore and Patton 2019). Indeed, a mixed methods study in a long-term care setting suggest that health-care practitioners modified the RAS scores based on their perception of PU risk as opposed to the guidelines associated with the RAS (Baxter 2008). Moreover, the choice of cut-off score in these scales is critical in order to classify the individuals at risk with high accuracy (Schoonhoven, Haalboom et al. 2002). The features of these RAS, including the sensitivity and specificity based on previous studies are summarised in Table 1-2. The difference in findings between studies is evident, which could be attributed to the differences in both patient populations and the duration of the study periods. Recently, a new RAS tool, namely, 'PURPOSE-T' has gained attention in clinical applications. This tool uses a color-coded three-step process including an initial assessment to quickly eliminate those individuals not at risk who do not require preventative resources (Coleman, Smith et al. 2018). This new scale is based on the latest evidence of PU risk factors and expert opinion for its completion. However, there is limited widespread adoption of the scale to date.

Table 1-2: Performance characteristics and risk factors of commonly used RAS

Scale (Year)	Sensitivity	Specificity	N	Target population	Cut-off score	Score range	Risk factors
Modified Braden (1987)	57.1	67.5	6443	Generic	≤16	6-23	Sensory perception Moisture Mobility, Activity Nutrition Friction/Shear
Norton (1962)	46.8	61.8	2008	Elderly/ Generic	≤14	5-20	Physical condition Mental condition Activity Mobility Continence
Waterlow (1985)	82.4	27.4	2246	Orthopaedic/ Generic	≥10	1-64	Body mass index Skin condition Gender, Age Continence, Mobility Medication, Nutrition Surgery/Trauma

Scale (Year)	Sensitivity	Specificity	N	Target population	Cut-off score	Score range	Risk factors
PURPOSE-T (2015)	-	-	-	Generic	Orange/Pink - At risk	Colour-based	Step 1: Mobility Skin status Step 2: Sensory perception Perfusion status Detailed skin assessment Nutrition Continence Previous history Diabetes

Risk factors

International consensus has identified that PU risk can be broadly classified into intrinsic or extrinsic factors, as listed in Table 1-3. Intrinsic risk factors are related to the physical and psychological characteristics of an individual. As an example, most studies have reported that individuals with darker skin color have more severe pressure ulcers (Saunders, Krause et al. 2010). Moreover, research has also suggested that adequate fluid intake is recommended to prevent skin breakdown (Saghelini, Dehghan et al. 2018). By contrast, extrinsic risk factors are modifiable environmental loads which are placed on the skin and as such have become key targets in minimizing the risk of PUs, illustrated by the schematic in Figure 1-3. The boundary or interface conditions between a body site and the supporting surface e.g. mattress, cushion or medical device, can involve a combination of pressure, shear, friction and microclimate (temperature and humidity). Accordingly, to minimise the risk of developing PUs, surfaces have been designed to minimise pressure exposure through pressure redistribution, reduce shear through low friction materials and manage microclimate with active air flow constructs and thermal conductive gels (McInnes, Jammali-Blasi et al. 2015, Nixon, Smith et al. 2019).

Table 1-3: Risk factors associated with incidence of PUs

Intrinsic factors	Extrinsic factors
Nutritional status including hydration	Pressure
Demographics (Age, race)	Shear
BMI	Friction
Incontinence	Moisture
Immobility	

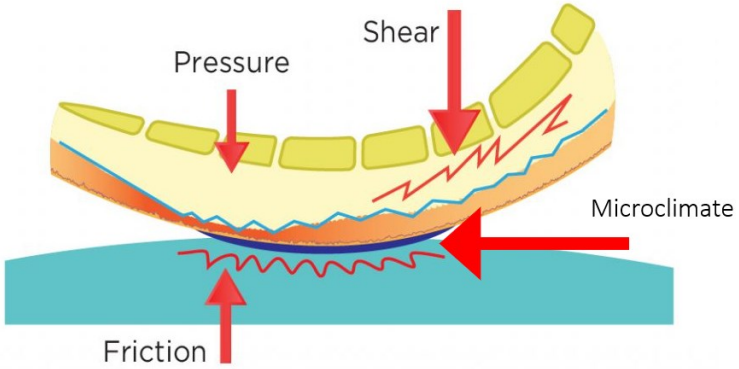


Figure 1-3: Schematic of extrinsic risk factors associated with PU incidence (Fletcher 2020)

1.2.4. Skin assessments

The use of RAS and clinical judgement based on visual observations, such as the blanchability and erythema, nutritional status and the medical history of an individual is limited in providing an accurate diagnosis of skin status. Therefore, there is a compelling need to develop objective measures of skin health and thereby design targeted prevention strategies. Various biophysical techniques have been employed to examine the tissue response when the skin is subjected to a range of external insults, such as pressure, moisture and shear. In addition, the biochemical immune response has been proposed as a means to identify early signs of skin damage although, to date, no definitive biomarkers have been established.

Of the relevant studies, cytokines such as IL-1α, IL-1RA, IL-8 and TNF-α were reported to be upregulated when skin was subjected to mechanical loading and moisture (Bronneberg, Spiekstra et al. 2007, Bostan, Worsley et al. 2019, Koudounas 2019, Soetens, Worsley et al. 2019). In addition, both an upregulation of INF-γ in plasma and decrease of INF-α in urine were reported in patients with Spinal Cord Injury (SCI), prior to the development of PUs (Krishnan, Karg et al. 2016). In another study involving cohorts of able-bodied individuals and SCI patients, differences were reported in urine myoglobin levels between the two cohorts (Traa 2019). Furthermore, previous studies have also evaluated the metabolic changes associated with ischaemic damage. The study findings suggest that, in addition to inflammatory cytokines, metabolites such as lactate, urea and pyruvate have also been found to be upregulated according to different loading regimens (Soetens, Worsley et al. 2019).

Seminal research studies have identified that skin damage leads to a change in its physical properties such as permeability, skin surface acidity (pH), skin colour i.e., erythema/redness, skin conductance and elasticity (Frankel 1973, van der Valk, Zarafonitis et al. 1995, Darlenski, Sassning et al. 2009). This has motivated the use of various biophysical techniques, such as Trans-epidermal water loss (TEWL), which is expressed in g/h m^2, to assess the integrity of the skin barrier (Antonov, Schliemann et al. 2016). Increased TEWL response has been commonly associated with impaired barrier function. Indeed, despite the inter- and intra-individual variations associated with TEWL, the technique continues to serve as the "gold-standard" to assess skin barrier integrity when measured in accordance with the recommended guidelines (Pinnagoda, Tupker et al. 1990, Grove, Houser et al. 2019). Skin hydration is often measured based on changes in skin electrical properties due to underlying moisture (Qassem and Kyriacou 2019). This indirect method results in semi-quantitative skin hydration parameters, measured in arbitrary units (AUs). The sub-epidermal moisture (SEM) meter is a commercial tool that has gained recent popularity. It is based on the measurement of non-invasive bio-impedance, which is defined as the ability of biological tissues to impede electrical current, of underlying moisture at suspected sites of compromised skin, and expressed in AUs (Moore, Patton et al. 2017). The commercial device creates a delta of sub-epidermal moisture, which the manufacturer claims is indicative of early signs of skin damage. Erythema has been investigated as an independent prognostic factor of PU incidence and recently both imaging and optical techniques have been used in its objective measurement (Vanderwee, Grypdonck et al. 2007, Shi, Bonnett et al. 2020). The surface pH of healthy skin ranges between 4.0 and 6.5 depending on the body location (Farage, Hood et al. 2018). The so-called acid mantle of skin creates a hostile environment for pathogenic microorganisms minimising the risk of infection. Transcutaneous gas monitoring (TCM) is used to monitor the partial pressures of O_2 and CO_2 at the skin. The measuring system consists of a heated electrode (43.5°C) fixed to the skin surface to achieve maximum vasodilation and monitor the transcutaneous gas levels via diffusion. On loading, the occlusion of blood vessels leads to ischaemia, reflected by a decrease in T_cPO_2 values and corresponding increase in T_cPCO_2 (Chai and Bader 2013). Although TCM systems are expensive and introduce a change in local physiology via heating, they are still commonly used in clinical scenarios to identify the ischaemic state of tissues. Previous research has suggested that the presence of prolonged pressure and/or moisture leads to a general increase in the above-mentioned biophysical parameters (Knight, Taylor et al. 2001, Bates-Jensen,

McCreath et al. 2009, He, Tang et al. 2016, Bostan, Worsley et al. 2019, Shi, Bonnett et al. 2020).

Anatomical variations

Due to differences in tissue distribution, thickness and the morphology of skin across different anatomical regions, there is a variation associated with the skin parameters from the aforementioned biophysical measurement tools (Marrakchi and Maibach 2007, Kleesz, Darlenski et al. 2012, Chirikhina, Chirikhin et al. 2020, Pan, Ma et al. 2020). As an example, the TEWL parameter measured at different body locations in a healthy cohort, has been reported to decrease in the following order, palm < nasolabial fold < forehead < neck < forearm < dorsal < leg < cheek (Firooz, Sadr et al. 2012). This variability should be considered while threshold values are chosen to differentiate healthy from compromised skin. However, limited information is available with other skin parameters, such as, sub-epidermal moisture and inflammatory markers.

1.3. Aetiology of pressure ulcers

The aetiology of PUs has been studied for many years to identify the physiological mechanisms underlying tissue damage. Hierarchical or multi-scale approaches, involving cell and tissue engineered models, animal models and human models have assisted in the identification of these mechanisms. Skin and underlying tissue damage as a result of prolonged mechanical loading is now considered to occur through the four main aetiological processes, namely,

1) Direct cell deformation damage
2) Ischaemia
3) Ischaemia-Reperfusion injury
4) Impaired lymphatic drainage

Early studies suggested that the formation of PUs is influenced by the magnitude and the duration of the loading. The well-known exponential curve, commonly known as Reswick and Rogers curve, signifying the inverse relation between these parameters, is shown in Figure 1-4 (Rogers 1976). Moreover, seminal research studies have revealed that each of the four mechanisms could be evoked at different time periods and at different magnitudes of loading (Bouten, Oomens et al. 2003, Oomens 2011).

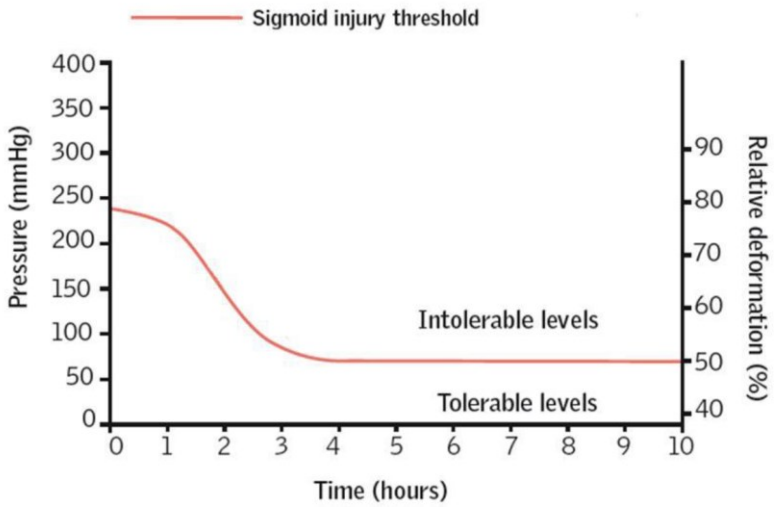


Figure 1-4: Schematic illustrating the sigmoid curve explaining the inverse relationship between pressure and time (Gefen 2009)

1.3.1. Cell deformation

Sustained cell deformation is one of the causes of cell damage, as suggested by a number of research studies (Gawlitta, Li et al. 2007, Stekelenburg, Strijkers et al. 2007, Oomens 2011). Forces applied on the surface of skin and underlying tissues lead to tissue deformation. Depending on the cell and tissue type, the tolerance for mechanical loading varies. As an example, previous experimental studies suggested that muscle cells are more susceptible to deformation damage in comparison to skin cells (Salcido, Donofrio et al. 1994).

Cell deformation leads to the disruption of cellular membrane and local cytoskeleton network triggering a mechanotransduction response, including cell death i.e., apoptosis, and altered protein expression responses. As an example, single cell studies on C2C12 mouse myoblasts subjected to unconfined compression revealed structural changes, such as the formation of bulges at the cell membrane preceding its rupture (Peeters, Oomens et al. 2005). Seminal studies involving *in vitro* 3-D engineered skeletal muscle constructs revealed cell deformation as a primary cause of cell damage under high deformation conditions at shorter time scales (Bouten, Knight et al. 2001, Breuls, Bouten et al. 2003, Peeters, Oomens et al. 2005, Gawlitta, Li et al. 2007, Stekelenburg, Strijkers et al. 2007, Oomens, Loerakker et al. 2010). For example, one such study involving strained and unstrained muscle constructs revealed that even in the presence of normal oxygen levels i.e., ~21%, strained muscle constructs displayed higher cell death than unstrained constructs (Bouten, Knight et al. 2001). In a separate study involving live/dead stain fluorescent markers, cell death was

demonstrated within 1-2h at clinically relevant yet high strains of 30 and 50% and at shorter time duration for higher strains (Breuls, Bouten et al. 2003). Further research also identified that the amount of cell damage was linearly correlated to the magnitude of applied strain (Loerakker, Stekelenburg et al. 2010). In another study, the relative contributions of compression and hypoxia in tissue damage were evaluated by employing an *in vitro* cell model wherein the engineered constructs were subjected to compression under normoxic and hypoxic conditions. Upon hypoxic conditions, cells switched to anaerobic metabolism demonstrated by an upregulated lactate production, however the study suggested that the presence of ischaemia alone did not cause damage as opposed to deformation (Gawlitta, Li et al. 2007).

To further examine the effects of deformation, animal models were employed in a series of studies at Eindhoven University of Technology (Stekelenburg, Strijkers et al. 2007, Traa, van Turnhout et al. 2019). The *in vivo* rat-model studies involved applying pressure, uniaxially directly over the tibialis anterior (TA) muscle, whose status was monitored simultaneously using magnetic resonance imaging (MRI) (Nelissen, de Graaf et al. 2017, Nelissen, Sinkus et al. 2019). The findings revealed that compressive loading (2h period) leads to irreversible damage, whereas ischaemic loading was more likely to result in reversible tissue changes (Stekelenburg, Strijkers et al. 2007). Numerical studies of these experiments using finite element models revealed that the regions associated with the greatest predicted deformations coincided with the damage regions (Ceelen, Stekelenburg et al. 2008). *In vivo* imaging studies on human volunteers in a supine/tilted position revealed that the muscle and fat regions corresponded to areas of high strain and skin region corresponded to lower strains (Oomens, Broek et al. 2016). Indeed, this study confirms the influence of intrinsic factors, such as BMI on the internal strains.

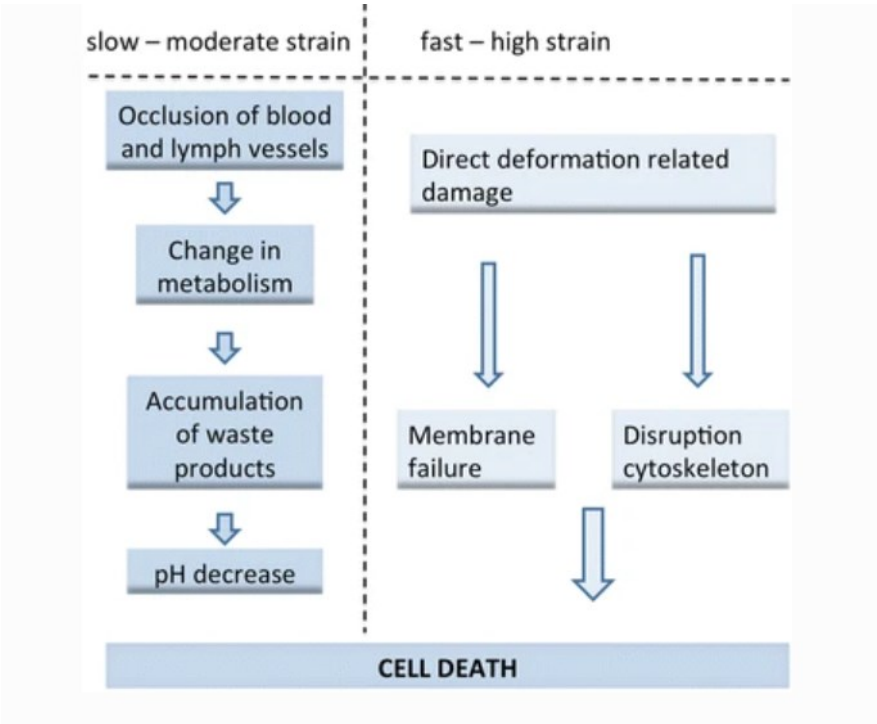


Figure 1-5: Schematic of aetiology of deep tissue injury (Oomens, Bader et al. 2015)

It is well known that the majority of pressure ulcers occur during periods of prolonged and low strain levels. However, it is to be noted that most of the experimental work focusses on indenters that elicit high strain values. It is noted that the focus on cell deformation involving muscle cells/constructs was largely driven by the recent interest in DTIs (Figure 1-5). By contrast, there has been less focus on studies incorporating skin cells/tissues, despite the fact that these contribute to the vast majority of PUs (~90%). Nonetheless, some studies have been conducted involving the mechanical loading of skin-like tissues, often in the form of commercial reconstructed human epidermis (RHE) e.g., EpiDerm models (Bronneberg 2007). These authors reported the evidence of cytoplasmic swelling associated with an upregulation of inflammatory markers following long-term loading (20h). This study examined an array of cytokines, such as IL-1α, TNF-α, IL-1RA and IL-8, which were up-regulated prior to the development of skin damage. The upregulation of cytokines with increasing magnitude of loading followed distinct profile responses. For instance, IL-1α, IL-1RA and IL-8 followed a distinct step pattern with significant upregulation for magnitudes exceeding 75 mmHg whereas TNF- α followed a linear response.

1.3.2. Ischaemia

When soft tissues are subjected to mechanical loading, the blood flow in the vessels are disrupted leading to a decreased oxygen and nutrient supply to the tissues. Accordingly, ischaemia was traditionally recognised as one of the main mechanisms leading to the development of PUs. An early study reported that an average capillary closing pressure of 32 mmHg (0.13 kPa) in nail fold capillaries (Landis 1927). Although this clearly does not represent an anatomical body site of relevance in terms of developing PUs, this pressure value has long been used erroneously as a threshold by which clinicians and companies design and select support surfaces for patients (Kirkland-Walsh, Teleten et al. 2015).

It is well established that on prolonged loading, there is a decrease in tissue oxygenation as observed with transcutaneous gas monitoring (Bader and Gant 1988, Bogie, Nuseibeh et al. 1995, Knight, Taylor et al. 2001). Early studies assessed the tissue viability by measuring the percentage of time at which the transcutaneous gas levels i.e., T_cPO_2 and T_cPCO_2 are within the acceptable thresholds (Bogie, Nuseibeh et al. 1995). Due to the lack of oxygen and depletion of adenosine triphosphate (ATP), cells switch from aerobic to anaerobic metabolism resulting in the production of by-products, such as lactic acid. The accumulation of lactic acid, as indicated in Figure 1-6, leads to a reduction of pH, which in turn, results in cell death. Indeed, studies employing muscle cells (C2C12) subjected to hypoxic conditions have reported an upregulation of lactic acid and increased cell death (Gawlitta, Li et al. 2007). In addition, several *in vivo* studies have investigated the effects of loading on lactate levels in sweat of healthy volunteers and debilitated subjects (Ferguson-Pell and Hagisawa 1988, Polliack, Taylor et al. 1993, Taylor, Polliack et al. 1994, Polliack, Taylor et al. 1997, Knight, Taylor et al. 2001, Soetens, Worsley et al. 2019).

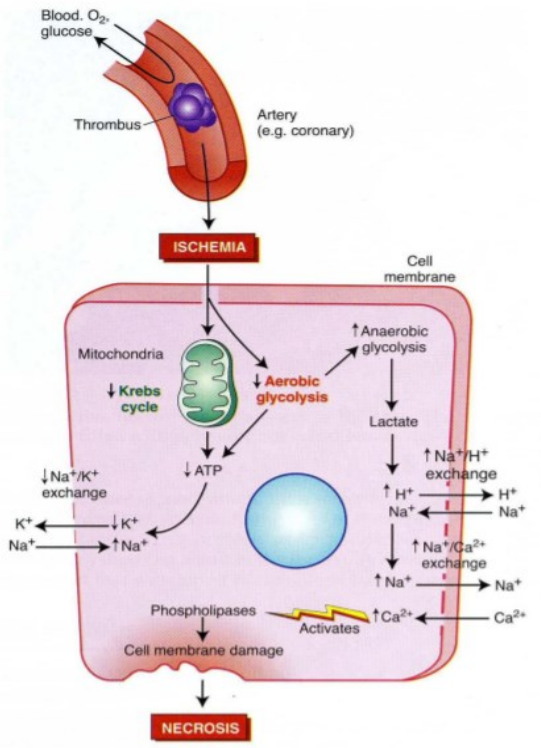


Figure 1-6: Schematic of cellular changes arising due to ischaemia (Rubin 2008)

Due to the decreased pH, the cells trigger an automated buffer response and the $Na^{+/}H^+$ exchanger excreting the excess H^+ ions and causing an increased influx of Na^+ ions, as depicted in Figure 1-6 (Kalogeris, Baines et al. 2012). The deprivation of ATP further inactivates ATPase that is important for pumping out calcium, thereby increasing the calcium loading on the cells. This inactivation of the ion pump results in an increased concentration of ions, which offsets the osmotic state of the cells leading to water inflow into the cells thereby causing intracellular oedema. The critical ischaemic time up to which ischaemic damage is reversible, is dependent on the type of tissue, as depicted in Table 1-4 based on results from previous studies (Eckert and Schnackerz 1991).

Table 1-4:Critical ischaemic time for different tissue types (Eckert and Schnackerz 1991)

Tissue	Critical ischaemic time
Muscle	4 hours
Nerve	8 hours
Fat	13 hours
Skin	24 hours
Bone	4 days

Prolonged ischaemia leads to subsequent reduction of perfusion at the microvascular level, particularly adjacent to bony prominences, which are considered to represent sites vulnerable in developing PUs. Previous studies have employed techniques such as laser Doppler imaging to evaluate perfusion in the soft tissues (Mayrovitz, Sims et al. 2002, Bergstrand, Länne et al. 2010, Karg, Ranganathan et al. 2019). As an example, one examination of the effects of sacral loading on perfusion at different skin depths involving an elderly cohort revealed that blood flow is significantly affected with each layer responding differently to externally applied pressures (Bergstrand, Länne et al. 2010).

1.3.3. Ischaemia- Reperfusion injury

Ischaemia-Reperfusion injury is defined as "the paradoxical exacerbation of cellular dysfunction and death, following restoration of blood flow to previously ischaemic tissues" (Cowled 2011). The reperfusion phase creates a reactive hyperaemic response in tissues as a direct result of the dilation of arterioles that decreases the resistance to blood flow (Rochetaing and Kreher 2003). This sudden release of blood flow leads to an excess supply of oxygen triggering the oxidative stress and the production of Reactive Oxygen Species (ROS).

ROS include free radicals, atoms or molecules containing one or more unpaired electrons (Taylor 2005). Some examples of ROS include hydroxyl radicals (OH:), hypochlorous acid (HOCl), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and Nitric Oxide (NO). During the ischaemic state, the enzyme xanthine dehydrogenase converts to xanthine oxidase and when the reperfusion phase begins, the sudden oxygen flow is utilized by xanthine oxidase to produce superoxide radicals and hydrogen peroxide (ROS) (Taylor 2005). These ROS are highly unstable due to their high reactivity causing damage to biomolecules such as proteins, lipids and DNA. As an example, the ROS attacks the membrane lipid of cells which further initiates a chain reaction destructing the fatty acids of the phospholipid membrane causing damage to membrane integrity (Yoshida, Umeno et al. 2013). Moreover, ROS also leads to activated leukocytes that adhere to the endothelium and cause disruption (Gillani, Cao et al. 2012). It is evident that the underlying mechanisms of reperfusion injury are multifactorial in nature, including the generation of reactive oxygen species (ROS), endothelial dysfunction and altered inflammatory responses, as illustrated in Figure 1-7 (Yellon and Hausenloy 2007).

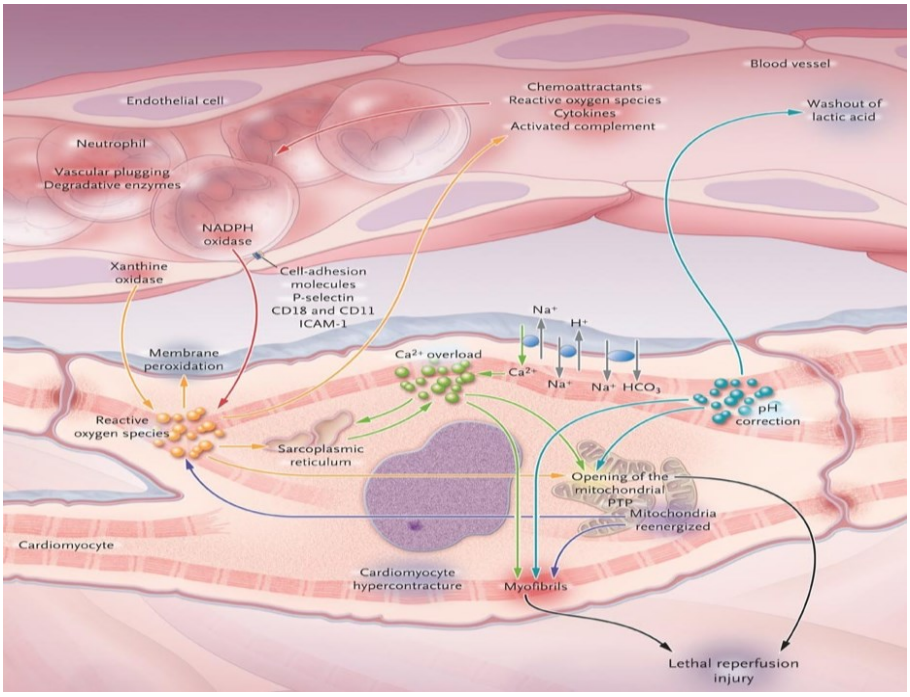


Figure 1-7: Schematic illustrating the biochemical processes associated with reperfusion injury (in myocardial cells) - (Yellon and Hausenloy 2007)

Previous studies employed rat models where the Tibialis Anterior (TA) muscles were subjected to different durations of ischaemia (1.5h, 3h, 6h) to investigate the varying time period of ischaemia on the post-ischaemic blood flow. The findings of the study suggest that excess blood flow disrupts the contractile function of the skeletal muscle (Ikebe, Kato et al. 2001). *In silico* studies on reperfusion injury suggest that beyond a critical tissue damage threshold, rapid removal of load exacerbates the damage associated with ischaemia, hence negating the potential benefits of load removal. (Bullkich, Kimmel et al. 2019). This finding was also confirmed in an animal study (Ünal, Özmen et al. 2001), comparing the effects of gradual and sudden reperfusion on the biological response of soft tissues. Moreover, research involving increased cycles of reperfusion resulted in an increase in the oxidative stress and damage to the microcirculation (Tsuji, Ichioka et al. 2005, Wang, Pu et al. 2016). Indeed, these studies indicate that both the time period of ischaemia and reperfusion and the number of cycles influence the extent of cell damage. *In vivo* studies involving patients with PUs also suggest that in addition to the systemic inflammatory response, the individuals are also presented with increased lipid peroxidation, as suggested by increased malondialdehyde (MDA) levels, as a result of oxidative stress. It is to be noted there is a paucity of research investigating oxidative stress biomarkers in comparison to that of the inflammatory markers.

Therefore, further research is required to investigate the role of this mechanism in the development of pressure ulcers. (Cordeiro, Antonelli et al. 2005).

1.3.4. Impaired lymphatic drainage

If the skin and underlying tissues are deformed, the patency of the associated lymphatic vessels will also be affected. The lymphatic system primarily constitutes a network of tissues to eliminate the toxins and unwanted biomolecules and provide a metabolic balance for the cell niche. Accordingly, the collapse of lymphatic vessels will result in an accumulation of harmful components and impairs the contractile function. It is to be noted that the anatomy of vascular and lymphatic vessels are different, with lymphatic vessels being thinner in comparison to that of the blood vessels (Reddy 1990, Bautch and Caron 2015).

Seminal studies on a canine model suggest that when external pressure was applied, the lymph flow was drastically reduced (Miller and Seale 1981). Indeed, the findings of this study suggest a non-linear relationship between applied pressure and lymph flow up to 60 mmHg, beyond which lymphatic occlusion will lead to oedema. Pressure in excess of 75 mmHg, the lymph flow completely stops due to complete lymph vessel closure (Miller and Seale 1981). In comparison with other underlying mechanisms of PU formation, the lymphatic drainage has received relatively little attention in the past. However, recent studies have began to focus on assessing lymphatic function with the advent of novel biophysical, biochemical and imaging techniques (Gray, Voegeli et al. 2016, Worsley, Crielaard et al. 2020). As an example, fluorescence lymphangiography coupled with near infrared imaging has been employed to examine lymphatic function in human volunteers subjected to mechanical loading. In one such study, a microdose of a fluorescent marker i.e. indocyanine green (ICG) was injected onto the forearms and a pressure of 60 mmHg was applied. This resulted in considerable impairment of lymph transport in some individuals but not all of the able-bodied volunteers (Gray, Voegeli et al. 2016, Gray, Worsley et al. 2016). In a further study, the lymphatic flow and the transcutaneous gas tensions were monitored in an able-bodied cohort, which was subjected to two distinct loading regimens involving incremental loading from 30 mmHg to 90 mmHg in a series of 3 cycles, and 3 cycles of constant loading of magnitude 30 mmHg. The findings revealed that a pressure of 30 mmHg lead to occlusion of lymphatic vessels, whereas the transcutaneous gas tensions were considerably influenced at a loading magnitude of 90 mmHg (Worsley, Crielaard et al. 2020). These authors suggest

a clear difference in the responses of blood and lymph vessels to mechanical load-induced occlusion.

1.4. Chapter summary

This chapter provides an overview of the structure and function of the skin, which serves as a protective barrier against challenges encountered in the external environment. There are many scenarios where external insults can compromise the skin's structure and function. Where sustained, the resulting damage may lead to chronic wounds, in the form of Pressure Ulcers, which represent a major burden on the affected individual and healthcare systems with restricted budgets. Prevalence and incidence studies suggest that these chronic wounds remain a common challenge across both hospital and community settings around the world. Prevention strategies are currently based on clinical evaluation and risk assessment scales, the latter of which is designed to identify the individual level of risk based on a set of clinical factors and previous medical history. Nevertheless, their effectiveness in reducing the incidence is generally questioned. It is accepted that there is a compelling need for objective indicators to identify skin status and create tailored preventative measures. It is undeniable that there is an increasing momentum of research in identifying objective indicators, although no single or series of objective markers has been currently proven to be reliable in a range of clinical and community settings.

Chapter 2 : Literature review – Markers of pressure ulcer (PU)

This chapter aims to provide an overview of the objective parameters, involving biophysical, biochemical, and imaging markers, that have been investigated in the literature to assess changes in the integrity of skin. This involves a narrative review approach and the results of the review have not been published separately. Biochemical markers could be measured in a range of biofluids, such as sweat, sebum and plasma. These biofluids and corresponding biomarkers of skin health are discussed separately.

2.1 Methods

A review of literature concerning PU was conducted using PubMed, Web of Science and Google Scholar with no restrictions placed on country or publication date. The search terms for this review included pressure ulcers, biomarkers, and chronic wounds. The inclusion criteria included that the article was written in English and the study focussed on biomarkers of skin health. Studies were excluded if they did not focus specifically on pressure ulcers or mechanical damage. Relevant articles were also obtained using forward and backward search, i.e., by scanning the references of found articles as well as locating newer articles that included the original cited paper. The search was invariant to the type of models used in the study and therefore included *in vitro* and *in vivo* studies. A narrative review approach was undertaken, and the review has been presented thematically in this chapter relating to biofluids and biomarkers, including biophysical, biochemical, and imaging markers.

2.2 Biofluids

Biofluids are defined as “samples that could be excreted, secreted, obtained with a needle or develop as a result of pathological processes”. The literature reveals a wide range of biomarkers relating to skin damage measured in different body fluids, namely, blood, urine, sweat, sebum and dialysate (Table 2-1). The wide range of biochemical markers involving intact skin, wounds and PUs implicated in literature are described in detail in Section 2.3.2. The choice of biomarker and their relative concentration is highly dependent on the biofluid selected for analysis. As an example, PU is a localized phenomenon and individuals at risk of these chronic wounds are typically associated with other comorbidities (Jaul, Barron et al. 2018). Therefore, an increase in a systemic inflammatory response could arise from

various underlying conditions, such as tumours, inflammatory and haematological disorders (de Mooij, Netea et al. 2017, Mantovani, Barajon et al. 2018). By contrast, a localized inflammatory response, as measured in sweat and sebum, could be attributed to skin tissue damage in the region of interest, albeit the effect of comorbidities as a confounding factor has not been fully explored previously. It is of note that the typical concentrations of biomarker candidates in sweat and sebum might be orders of magnitude less than that measured from a systemic fluid, such as blood serum, thereby demanding sensitive techniques for quantification. Moreover, the variation in secretion rate of biofluids *per se* might result in a variation of biomarker volumes and relative concentrations (Harshman, Strayer et al. 2021). Therefore, different normalization procedures must be considered to accommodate for the variations in the volume of biofluid collected and the potential for high levels of inter-subject variation (Adedeji, Pourmohamad et al. 2019, Harshman, Strayer et al. 2021). Indeed, previous studies from the host laboratory have employed different normalization methods to account for inter-individual variability (de Wert, Bader et al. 2015, Soetens, Worsley et al. 2019). Table 2-1 critiques the biofluids collected using minimally invasive techniques, in terms of their clinical utility.

Table 2-1: A critical analysis of different methods of biofluid collection

Biofluid	Advantages	Disadvantages
Sebum	1) Non-invasive 2) No qualified personnel needed 3) Samples can be collected from localized body areas	1) Sample preparation required 2) Limited sample volumes 3) Requires sensitive analytical techniques to quantify biomarkers
Sweat	1) Non-invasive 2) No qualified personnel needed 3) Samples can be collected from localized body areas	1) Variability in sample volume depending on the local sudomotor function 2) Requires sensitive analytical techniques to quantify biomarkers 3) Limited sample volumes
Dialysate (Micro dialysis)	1) Samples could be collected in localized body areas 2) Ability to sample interstitial space	1) Invasive and causes local tissue trauma 2) Need for trained personnel to collect biofluid 3) Laborious sample collection process
Blood	1) Minimal sample preparation 2) Typically collected for routine assessment in clinic	1) Minimally invasive 2) Need for clinically qualified personnel for collection 3) Biochemical response could be attributed to multiple clinical disorders
Urine	1) Non-invasive 2) No qualified personnel required i.e., the patient 3) Available in large volumes 4) Minimal sample preparation	1) Variability due to food/water intake and renal clearance 2) Inflammatory response could be attributed to multiple clinical disorders

2.3 Biomarkers

The term ‘biomarker’ derived from ‘biological marker’, is defined as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention’ (Group 2001). Examples of biomarkers range from simple measurements, such as pulse rate, to

more complex laboratory tests involving DNA and protein analysis. Although there has been a considerable emergence of biomarker research in general, there are still relatively few studies focusing on biomarkers associated with the skin (Bader and Oomens 2018). In this chapter, biomarkers have been classified based on the nature of the marker and its associated detection method, as illustrated in

Figure 2-1.

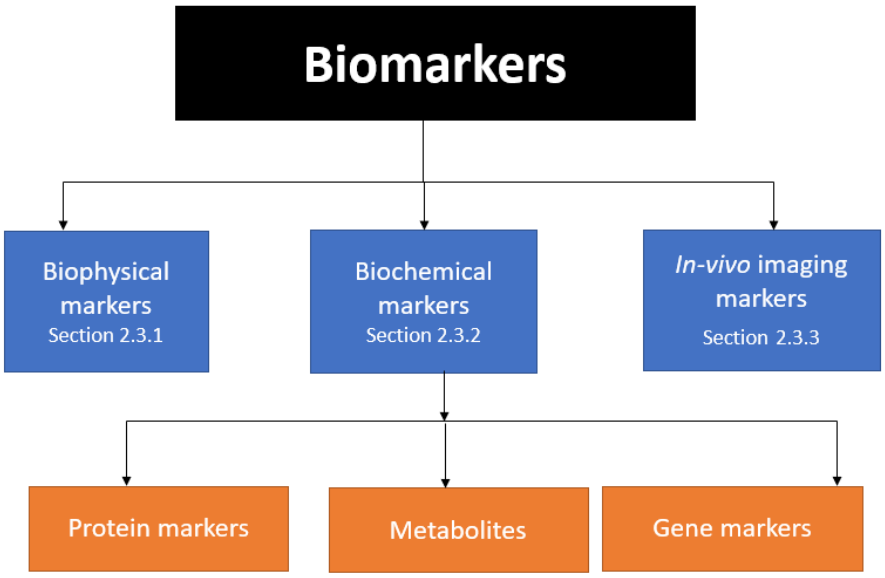


Figure 2-1: Classification of biomarkers with a specific focus on skin integrity

2.3.1 Biophysical markers

Biophysical markers are focused on quantifying the changes in the physiological parameters associated with the skin structure and function using contact probes. It is most convenient to discuss each of the parameters and their detection methods which have been primarily reported to characterize skin changes in separate sub-sections.

2.3.1.1 Trans-epidermal water loss (TEWL)

Trans-epidermal water loss (TEWL) is defined as the amount of water that passively evaporates through the skin to the external environment due to the pressure gradient of water vapour across the skin barrier (Thakur, Batheja et al. 2009). This approach has been used to characterize the barrier function provided by the stratum corneum (Honari and Maibach

2014). The water evaporating from the skin is estimated using a probe, composed of up to thirty sensors separated by a fixed distance, that is placed in close contact with the skin surface. Both open and closed chamber TEWL probes are available using the same measurement principle. A schematic representation of typical measurements in healthy and compromised skin is shown in Figure 2-2 indicating low and high TEWL values, respectively.

TEWL measurements are associated with inter- and intra-individual variations, due to intrinsic (e.g., age) and extrinsic factors (e.g., environment). In addition, studies have reported the variation in TEWL values across different anatomical locations with changes even evident across proximal and distal sites of the same forearm (Akdeniz, Gabriel et al. 2018). The range of basal TEWL values for healthy skin in sites such as sacrum and volar forearm have been reported to be 9.2-16.2 g/h m^2 and 5.3-8.8 g/h m^2, respectively (Akdeniz, Gabriel et al. 2018). Individual variability of this parameter could be attributed to intrinsic factors such as gender, age, body mass index (BMI) and ethnicity. As an example, a systematic review and meta-analysis reported that TEWL values in individuals aged 65 and above were consistently lower than the corresponding values for individuals aged between 18-64 years (Kottner, Lichterfeld et al. 2013). Moreover, the measurement is sensitive to changes in the environment such as airflow, which is often mitigated in lab-based settings. The Intra-class correlation coefficient (ICC) values for TEWL have been reported to be 0.46 (0.00-0.78) and therefore the reliability studies suggest that a mean of at least two readings is required to obtain a reliable measurement (Kottner and Blume-Peytavi 2021). Specific guidelines have recommended an acclimatization period of at least 30 minutes prior to measurements (Pinnagoda, Tupker et al. 1990, Alexander, Brown et al. 2018).

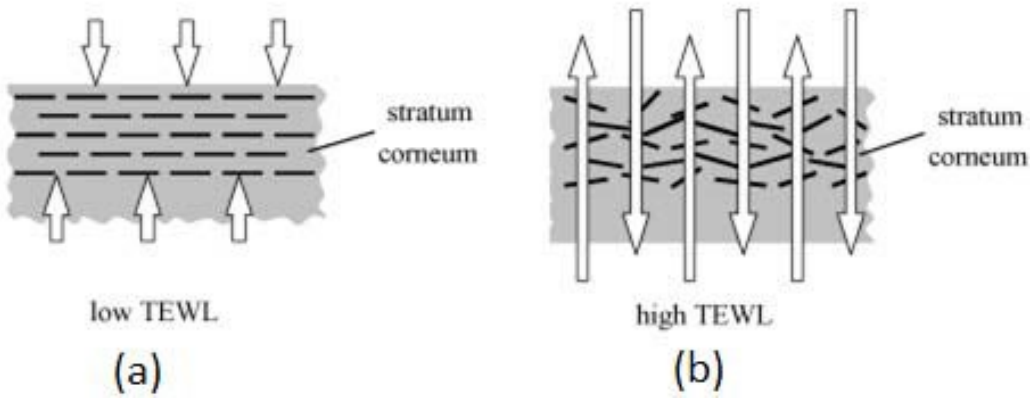


Figure 2-2: Representation of TEWL measurement in (a) healthy skin (b) damaged skin (Mündlein, Valentin et al. 2008)

TEWL has been used in research to assess skin health through its estimate of the integrity of the skin barrier (Laudanska, Reduta et al. 2003, Nikam, Monteiro et al. 2019). It has been regularly employed to assess the skin status in various dermatological conditions, such as atopic dermatitis and psoriasis (Nikam, Monteiro et al. 2019). TEWL has also been employed in studies to assess the effects of mechanical loading and incontinence. For each stimulus, a range of TEWL values ranging from 10 – 30 g/h/m^2 have been reported, which are typically restored to basal values following the removal of an insult. (Kottner, Dobos et al. 2015, Bostan, Worsley et al. 2019). Commercial TEWL measuring systems include Tewameter (Courage & Khazaka, Germany), Dermalab (Cortex Technology, Denmark), Aquaflux (Biox Systems Ltd., UK) and Vapometer (Delfin Technology, Finland). The former two systems are open-chamber based whereas the latter two systems use a closed-chamber method.

2.3.1.2 Skin hydration

Skin hydration is considered to represent an important factor in maintaining skin health. It can be measured using a range of techniques, involving Near-Infrared (NIR) spectroscopy, Raman spectroscopy and capacitance probes (Girard, Beraud et al. 2000). The latter technique, based on the change in electrical properties of skin with water content, has proved popular in dermatological research due to its low cost and ease of use. It has been developed into a number of commercial systems, such as the Corneometer (Courage & Khazaka electronic GmbH, Germany), Dermalab (Cortex Technology, Denmark) and SEMScanner (Bruin Biometrics, USA).

Corneometer

The measurement of skin hydration using a Corneometer is based on measuring capacitance of the dielectric medium within the superficial layers of the skin i.e., the stratum corneum (SC). Owing to the high dielectric constant of water (ε – 81), the device can detect even small changes in the hydration levels. The manufacturer reports that the measurement depth of the device is very shallow, between 10-20 μm, thereby excluding the influence of deeper skin layers (Khazaka). However, previous research studies have contradicted this, observing changes in hydration up to a depth of 45 μm (Clarys, Clijsen et al. 2012). Values of skin hydration has been reported to be dependent on individual demographics (Man, Xin et al. 2009, Firooz, Sadr et al. 2012). As an example, an inverse correlation has been reported with skin hydration and age (Constantin, Poenaru et al. 2014). It is to be further noted that the

device provides readings in arbitrary unit and is calibrated to range between 0-120 AU's. Despite limitations, Corneometer has been commonly used in studies to evaluate skin health for a range of pathologies and skin insult models (Phetcharat, Wongsuphasawat et al. 2015, Barone, Bashey et al. 2019, Muizzuddin and Benjamin 2020).

Sub-epidermal moisture

The sub-epidermal moisture (SEM) meter is a commercial tool, which has gained recent attention as a biophysical measure of localized oedema in the epidermal or sub-epidermal tissues (Moore, Patton et al. 2017). It is to be noted that the device is named as "Sub-epidermal moisture meter", there is less evidence on the localisation of this biophysical measure to the sub-epidermal region (Ross and Gefen, 2019). However, phantom models and *ex vivo* porcine models suggest that the SEM tool provides significant differences in the laterally localised oedematous sites and healthy skin sites (Cohan and Gefen 2019, Brunetti, Patton et al., 2023). Specific factors associated with the aetiology of PUs, namely, inflammation and impaired lymphatic drainage, could result in an accumulation of interstitial fluid and local oedema. It has been hypothesised that this results in an increase of "sub-epidermal moisture" in individuals with early features of skin damage, as shown schematically in Figure 2-3. This non-invasive method involves a bio-impedance measurement of the sub-epidermal moisture, with recorded values in arbitrary units (Wagner, Jeter et al. 1996, Swisher, Lin et al. 2015). The device uses a metric involving a differential reading in SEM values, termed the SEM delta, between a site suspected to be damaged and local sites around its circumference.

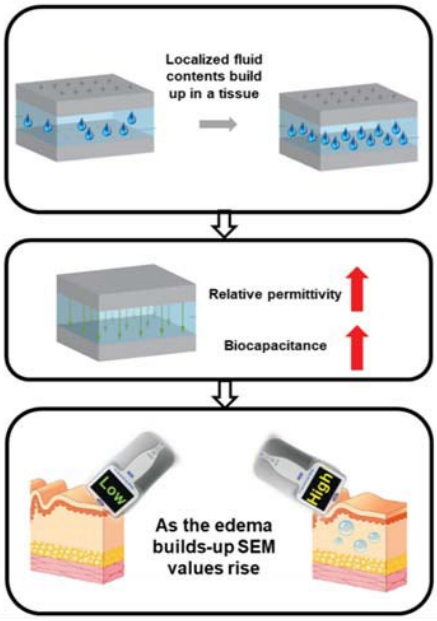


Figure 2-3:Principles of SEM measurement depicting the increase in tissue oedema

In a longitudinal clinical study, the sensitivity and specificity of the SEM values have been recently estimated as approximately 87.5% and 32.9%, respectively (Okonkwo, Bryant et al. 2020). A recent systematic review based on 17 research articles suggest that the sensitivity of SEM measurements ranged from 48.3 – 100% and the specificity ranged from 24.4 – 83.0% (Moore, McEvoy et al. 2022). The low specificity represents an indication of a high number of false positives, which inevitably limits its utility in clinical practice and results in a Positive Predictive Value (PPV) of 14% (Okonkwo, Bryant et al. 2020). Indeed, sub-epidermal moisture has also been recommended as an adjunct tool to visual assessment in clinical settings (NICE 2020).

2.3.1.3 Erythema

Conventionally considered as an early sign of skin damage (EPUAP 2019), non-blanchable erythema has been investigated as an independent prognostic factor of PU incidence (Vanderwee, Grypdonck et al. 2007, Shi, Bonnett et al. 2020). Commercial systems measuring erythema include optical probes, such as Mexameter (Courage & Khazaka GmbH, Germany) and digital skin imaging applications, such as ScarletRed (ScarletRed Holding GmbH, Austria). In recent studies, erythema was estimated objectively, using devices which measure the absorption/reflection of light on the skin surface (Borzdynski, McGuiness et al. 2016). As an example, the principle of operation of the Mexameter is shown schematically in Figure 2-4. In a cohort study, the sensitivity and specificity of using

erythema as a subjective indicator for PU has been reported to be 75% and 77%, respectively (Konishi, Sugama et al. 2008). This suggests that erythema measures are approximately equivalent in accuracy to those associated with the conventional risk assessment scales (Balzer, Pohl et al. 2007). Mexameter have been reported to be influenced by skin pigmentation (Baquié and Kasraee 2014) and previous studies have reported a wider range of ICC values ranging from -0.23 to 0.88 (Maya-Enero, Candel-Pau et al. 2020, John, Galdo et al. 2023)

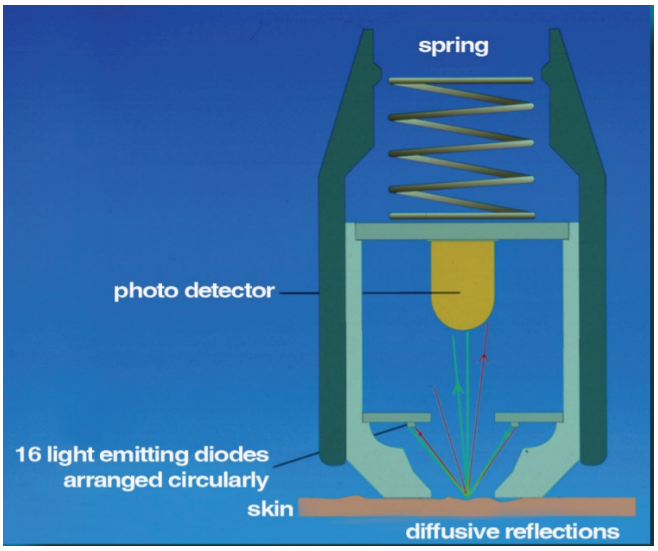


Figure 2-4: Schematic of the principle of operation of the Mexameter (Courage & Khazaka)

2.3.1.4 Transcutaneous gas monitoring

Transcutaneous gas monitoring (TCM) systems monitor the partial pressures of oxygen (T_cPO_2) and carbon dioxide (T_cPCO_2) tensions non-invasively. Sensors with heated surface electrodes are attached to the skin surface with a fixation ring and the partial pressures of the blood gases diffusing through the skin are monitored through the principles of electrolysis (Figure 2-5). Thermal contact is ensured by applying contact gel on the fixation ring. Originally used to monitor the oxygenation status of premature infants, these devices have gained popular attention in clinical settings owing to their ease of application and portability (Sandberg, Brynjarsson et al. 2011). However, it should be recognised that the TCM system requires the surface of the skin to be heated up to 43.5^{o}C to achieve maximum vasodilation, thereby increasing skin blood flow and altering normal tissue physiology. T_cPO_2 and T_cPCO_2 values of unloaded skin are established to range between 51-90 mmHg and 36-44 mmHg,

respectively (Knight, Taylor et al. 2001). Relevant research has reported that the application of prolonged pressure results in a decrease in tissue oxygenation status, which may be accompanied by an increase in $T_cPCO_{2\ levels}$ (Mirtaheri, Gjøvaag et al. 2015, Woodhouse, Worsley et al. 2015, Worsley, Rebolledo et al. 2018). The changes in TcPO2 and TcPCO2 on loading have been conveniently categorised by Chai and Bader into three categories (Chai and Bader 2013), which are:

1) Category I – Minimal changes in T_cPO_2 and T_cPCO_2 in comparison to the basal unloaded values
2) Category – II - > 25% decrease in T_cPO_2 with minimal change in T_cPCO_2
3) Category – III - > 25% decrease in T_CPO_2 associated with > 25% increase in T_cPCO_2 values

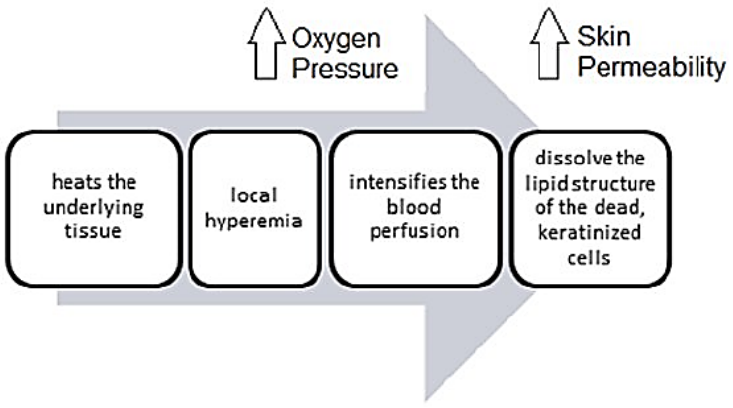


Figure 2-5– Physical principles underpinning the operation of transcutaneous gas monitoring (Worsley and Voegeli 2013)

2.3.1.5 Skin pH

The acidic pH of skin surface, in addition to playing an important role in skin defense against microorganism, also plays a vital role in the SC integrity and cohesion, as illustrated schematically in Figure 2.6. The so-called acid mantle of skin (Rippke, Berardesca et al. 2018) is also important in the regeneration of the skin barrier, which is delayed in neutral pH conditions (Schmid-Wendtner and Korting 2006, Wohlrab, Gebert et al. 2018). The device for measurement of skin surface pH is typically based on a glass H^+ ion sensitive electrode with an additional reference electrode placed in a single housing (Stefaniak, Plessis et al. 2013)

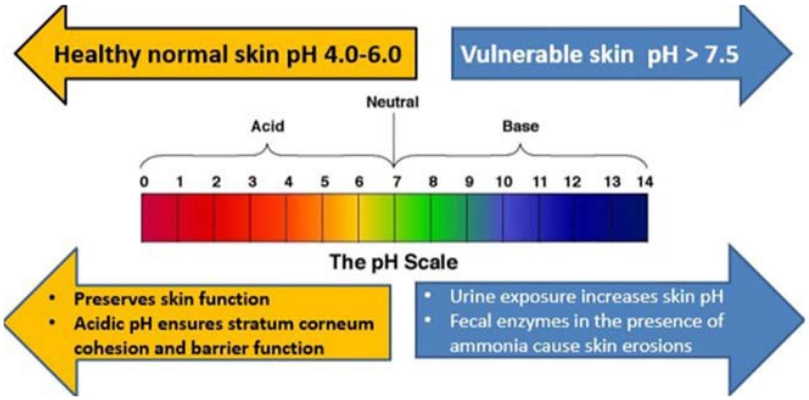


Figure 2-6- The functional consequence of skin behaviour at different pH values (McNichol, Ayello et al. 2018)

Individuals with incontinence are exposed to an altered skin pH due to the presence of urine and/or faeces, which affect the acid mantle (Figure 2-6). Indeed, recent studies have shown that individuals with IAD have higher skin surface pH compared to that of healthy individuals (He, Tang et al. 2016). In addition, placing an occlusive material onto the skin will also increase the skin surface pH (Hartmann 1983). The acid mantle of the skin provides a natural buffering capacity for pH (Larner, Matar et al. 2015, Koudounas 2019), although prolonged exposure to alkaline interfaces may not compromise skin health. A critique on these biophysical parameters is provided in Table 2-2.

2.3.1.6 Biomechanical measures

Biomechanical parameters of skin such as Young's modulus and viscoelastic parameters could be measured non-invasively involving suction, torsion and tensile forces. Conventionally, indentation tests have been employed to characterize the mechanical properties of skin with indenters of various geometries, such as conical and spherical intenders (Pailler-Mattei, Bec et al. 2008, Junker, Thumm et al. 2023). In the recent decades, *in vivo* tools have been developed, such as MyotonPRO and Cutometer, that provides the dynamic stiffness and viscoelastic properties such as the stress relaxation time (John, Galdo et al. 2023, Abbas, Lavin et al, 2022). The scope of this thesis is limited to biophysical and biochemical measures.

Table 2-2 : A critical analysis of common biophysical methods

Biophysical parameter	Advantage	Disadvantage
TEWL	a) Strongly correlated to skin barrier function b) Simple calibration checks to ensure accuracy c) Fast, non-invasive and requires minimal contact	a) Influenced by air movements and microclimate b) Not specific for SC damage c) Variability across different anatomic sites
Skin hydration (measured using electrical devices)	a) Fast, non-invasive and requires minimal contact b) Simple calibration check ensuring accuracy of the measurements	a) Typically measured indirectly (in AUs) b) Influenced by microclimate c) Limited information on depth profile of hydration
Sub-epidermal moisture	a) Clinically recommended in the UK b) Fast, non-invasive and requires minimal contact	a) Indirect measure (use of arbitrary units) b) Considerable variability c) Use of arbitrary thresholds to classify skin damage
Erythema	a) Inexpensive b) Fast, non-invasive and requires minimal contact	a) Not evident in a mixed population (esp. individuals with high melanin content) b) Limited by clinician's expertise c) Low sensitivity
Skin acidity (pH)	a) Fast, non-invasive and requires minimal contact b) Simple calibration checks to ensure accuracy of the measurements	a) Not specific for SC damage
Transcutaneous gas monitoring	a) Real time monitoring of tissue status and wound healing b) Can be quickly calibrated to ensure accuracy of the measurements	a) Requires localized heating up of tissues to 43.5ºC b) Relatively bulky instrumentation c) Expensive d) Requires approximately 20 minutes for start-up and frequent calibration

2.3.2 Biochemical markers

Biochemical markers include a range of compounds, namely, proteins, molecules, genes, enzymes and metabolites. It is well established that several biomarker candidates are regulated when skin tissues are subjected to prolonged loading and subsequent unloading process (Figure 2-7), associated with the mechanism of damage for chronic wounds e.g., pressure ulcers.

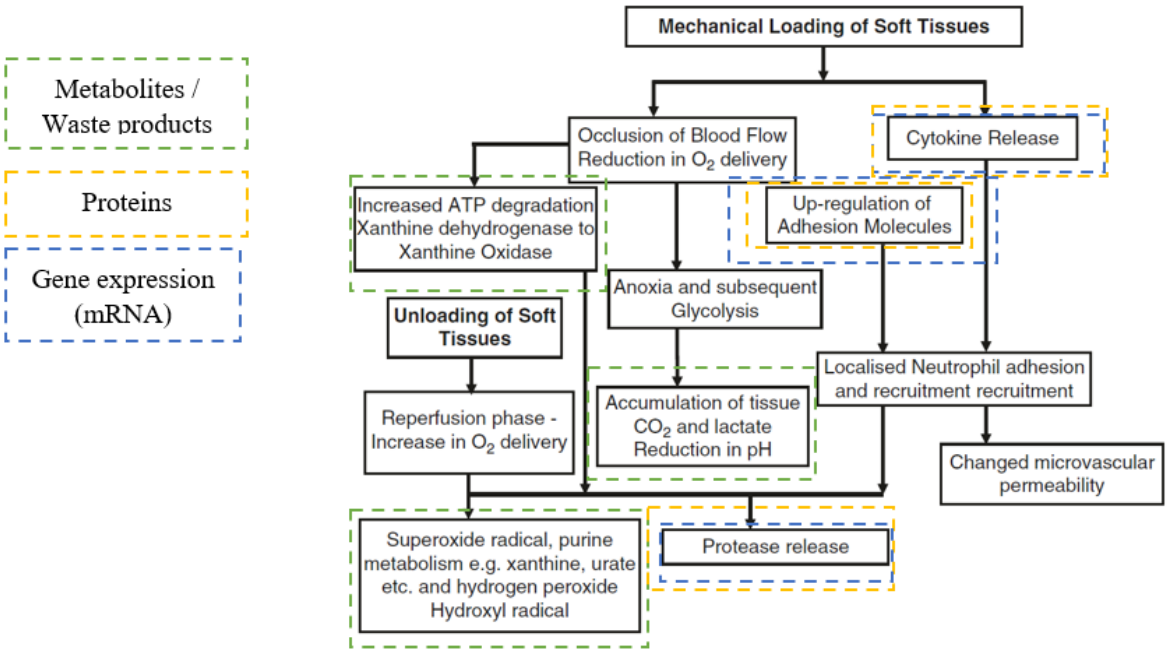


Figure 2-7:Schematic illustrating potential biomarkers regulated when skin tissues are subjected to prolonged loading and subsequent unloading process (Bader and Oomens 2018)

The biochemical markers will be discussed depending on the nature of the analyte involving both *in vitro* and *in vivo* studies on PUs and underlying mechanisms.

2.3.2.1 Protein markers

Inflammation has been recognized as one of the early responses preceding overt skin damage. Keratinocytes of the skin layer play a major role in the inflammatory processes by the production of signalling molecules, namely, cytokines and chemokines. Cytokines are typically small glycoproteins (8 – 30 kDa), that play a major role in cell-to-cell communication leading to the production of a cascade of downstream signals. Chemokines are a subgroup of cytokines, that play a vital role in cell migration through the process of

chemotaxis (Gutowska-Owsiak and Ogg 2013). The protein levels are up regulated as a result of a range of factors, including environmental stimuli, disease conditions, UV exposure, mechanical and/or chemical irritation. Moreover, multiple cascades could be triggered depending on the nature and the levels of cytokines. However, there is a limited understanding of the cell signalling relationships and temporal profiles which occur *in vivo*. Indeed, it is important to assess the biomarkers for prolonged time to understand the progression of the tissue state (healthy/damaged/healing). A schematic representation of the cells and cytokines involved in the inflammatory process is indicated in Figure 1-2 and Figure 2-8. A summary of *in vitro* and *in vivo* studies investigating protein biomarker candidates are listed in Table 2-3 and Table 2-4, respectively.

Cytokines play an important role in the inflammatory processes preceding skin damage (Zhang and An 2007). The most commonly investigated cytokines include high-abundance proteins IL-1α, IL-1β and IL-1RA (Receptor Antagonist) from the IL-1 family. Keratinocytes contain major stores of IL-1 cytokines in the cytoplasm, which normally are lost due to desquamation, but in case of injury or mechanical deformation, these cytokines are released triggering an inflammatory cascade (Uchi, Terao et al. 2000). Indeed, cytokines are also important in their role as signalling agents in the formation of skin barrier (Hänel, Cornelissen et al. 2013). As an example, IL-6 and INF-γ are responsible for maintaining the ceramide content of skin and detachment of the keratinocytes from the *Stratum Basale* layer (Sawada, Yoshida et al. 2012). Similarly, IL-1α has been reported to enhance lipid synthesis and regulates the expression of genes associated with proliferation and differentiation of epidermis (Yano, Banno et al. 2008).

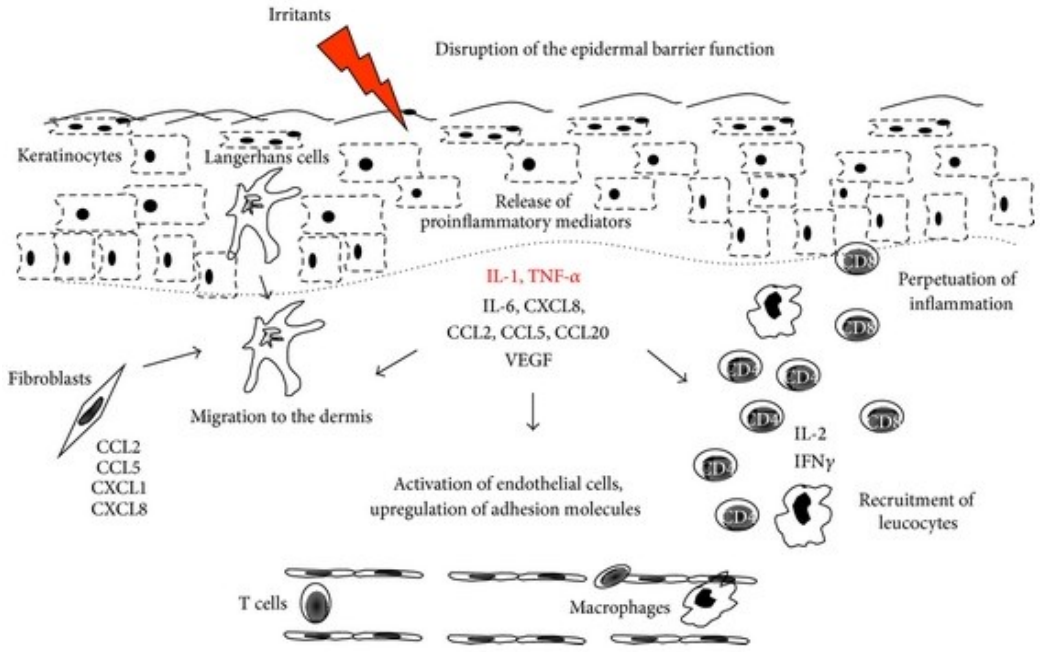


Figure 2-8: Immune mechanisms of skin disorders, such as irritant contact dermatitis, following exposure to irritants (Lee, Stieger et al. 2013)

In vitro studies have reported a significant upregulation of IL-1α and IL-1RA when the cells were subjected to cyclic mechanical strains (~33%) (Lee, Briggs et al. 1997). Subsequent research examining the effects of mechanical loading on *in vitro* tissue engineered epidermal models revealed a significant up-regulation in the levels of IL-1α, IL-1RA, TNF-α and IL-8 (Bronneberg 2007, Bronneberg, Spiekstra et al. 2007, Cornellisen 2008). One study revealed that the nature of the response, for example, the release of IL-1α, IL-1RA and IL-8 followed a distinct step pattern wherein a significant upregulation was observed when pressure exposure exceeded 75 mmHg (Bronneberg 2007). A transport model was used to predict the short-term cytokine e.g., IL-1α release from the epidermis following applied loading (Cornellisen 2008). A corresponding step response has not been observed in human volunteer studies (Worsley, Prudden et al. 2016).

Table 2-3: *In vitro* and animal studies examining the release of specific protein biomarkers

Study	Biomarkers analysed	Sample	Models used	Comments / Outcomes
Lee et al, 1997 (Lee, Briggs et al. 1997)	IL-1α and IL-1RA	*In vitro*	Cell culture	Mechanical deformation promoted secretion of IL-1α and IL-1RA
Bronneberg et al, 2007 (Bronneberg, Spiekstra et al. 2007)	IL-1α, IL-1RA, TNF-α, CXCL8/IL-8	*In vitro* – Culture supernatant	Tissue constructs	Distinct response patterns of inflammatory biomarkers to loading were identified.
Cornellisen et al, 2009 (Cornellisen 2008)	IL-1α, IL-1RA, mTNF-α, IL-8	*In vitro* model	Tissue constructs	Numerical models were employed to evaluate the transport of biochemical markers across skin layers.
Kurose et al, 2015 (Kurose, Hashimoto et al. 2015)	IL-1β and IL-6	Skin and subcutaneous tissue	Rat model	Upregulation of biomarkers in skin tissues wherein PUs was initiated using magnets.
Kimura et al, 2020 (Kimura, Nakagami et al. 2020)	IL-1α, VEGF-C, HSP-90α	Skin blotting (Skin surface proteins)	Murine model	Use of non-invasive sensitive technique to collect proteins from the skin surface

Proteins released at the skin surface can be collected locally using commercial tapes (Sebutape, Sebufix F16, Bomtech ® Sebum tape), to adsorb sebum from the skin surface. In a clinical study employing this technique, localized upregulation of IL1α/Total Protein (TP) ratio was reported at sites of a Stage-I PU compared to adjacent intact skin (van der Lans 2007). However, this was limited to a small cohort of patients, and further research is needed to explore spatial and temporal trends of these biomarkers over the site of skin damage. Moreover, the study did not explore the influences of comorbidities and extrinsic factors on the inflammatory response.

Studies have employed the Sebutape^{TM}method and skin blotting method of collection to identify potential biomarkers, such as IL-1α, IL-1β, IL-1RA, when skin is exposed to pressure, shear, moisture and other insults including tape-stripping and chemical irritation (Minematsu, Horii et al. 2014, de Wert, Bader et al. 2015, Hemmes, de Wert et al. 2017,

Bostan, Worsley et al. 2019, Nakai, Minematsu et al. 2019, Soetens, Worsley et al. 2019). In addition, these biomarkers have been analysed in examining the effects of medical device interactions with the skin (Worsley, Prudden et al. 2016, Hemmes, de Wert et al. 2017). Many of these human studies have highlighted considerable inter and intra-variability in biomarker levels. This has prompted the use of various normalization techniques to mitigate the issue of variability (de Wert, Bader et al. 2015, Soetens, Worsley et al. 2019). Recent reviews and meta- analysis have highlighted the importance of investigating inflammatory markers preceding skin damage in clinical settings (Wang, Lv et al. 2022, Chan, Avsar et al. 2023). Moreover, it is to be noted that the time scales of investigation in the experiments involving prescribed loading through indenters of medical devices are limited to a few hours (Polliack, Taylor et al. 1997, Loerakker, Huisman et al. 2012, Worsley, Prudden et al. 2016, Hemmes, de Wert et al. 2017, Soetens, Worsley et al. 2019, Soetens, Worsley et al. 2019). However, in a clinical setting it is important to assess the tissue status of immobilized patients over longer time scales.

Animal models have been commonly used to study PU formation with the use of implanted magnets or mechanical indenters to compress the skin tissues. As an example, in a rat model authors reported an up-regulation of IL-1β and IL-6 in the skin tissues (Kurose, Hashimoto et al. 2015). Recent research involving animal models indicated that in addition to commonly explored inflammatory cytokines, a number of proteins including alarmins (HSP-90α), produced by keratinocytes and epidermal cells, and protein associated with the lymphatic system (Vascular endothelial growth factor-C) are upregulated (Kimura, Nakagami et al. 2019). These authors employed a non-invasive technique, termed ‘skin-blotting’, to collect the proteins on the skin surface using a moistened nitrocellulose membrane. This semi-quantitative technique has been extended from animal studies to clinical studies involving elderly patients aged >65 years where a significant increase in IL-1α, VEGF-C and HSP-90α was reported over sites of Stage I PU. (Koyano, Nakagami et al. 2018, Nakai, Minematsu et al. 2019).

Table 2-4:*In vivo* studies investigating protein biomarker candidates

Study	Biomarker analysed	Biofluid	Population / Study	Comments/Outcomes
De Wert et al, 2015	IL-1α/Total protein	Sebum	Healthy volunteers subjected to loading, including pressure and shear	Pronounced upregulation of biomarkers in the presence of shear
Krishnan et al, 2016	IP-10, Interferon-α	Plasma, Urine	Retrospective study – Patients with traumatic SCI	Upregulation of IP-10 in plasma and decrease of INF-α in urine
Worsley et al, 2016	IL-1α, IL-1β, IL-8, IL-2, INF-γ, IL-10	Sebum	Healthy volunteers subjected to loading due to application of non-invasive ventilation masks	Up-regulation of IL-1α and significant correlations between markers of same cytokine families
Gray et al, 2017	IL-6, IL-8	Dialysate	Healthy volunteers subjected to mechanical loading	Use of micro-dialysis technique to sample the interstitial space
Hemmes et al, 2017	IL-1α	Sebum	Healthy volunteers subjected to loading on a spine board	IL-1α was used as an objective marker to evaluate the effect of spine board. Correlation between skin redness and IL-1α was revealed
Soetens et al, 2019	IL-1α	Sebum	Healthy volunteers subjected to intermittent and continuous mechanical loading	IL-1α was used to evaluate the effects of different loading patterns. Cluster analysis revealed distinct sub-groups responding differently
Bostan et al, 2019	IL-1α / Total protein	Sebum	Healthy volunteers subjected to moisture exposure and loading regimen	Upregulation of IL-1α/TP was revealed following mechanical loading independent of fluid exposure
Koyano et al, 2018	COL4, MMP-2, TNF- α	Skin blotting	Elderly patients requiring chronic medical care – Associated with skin tears	Correlations between markers and intrinsic factors e.g., age, gender, BMI

2.3.2.2 Metabolites

Research has been conducted to investigate the levels of metabolic waste products and reactive oxygen species as an indicator of skin status following mechanical loading. Most of these studies have used sweat collection non-invasively and locally, to represent a promising choice for analysis of biomarkers. Early research reported that arterial occlusion results in an increase in sweat lactate and urate concentrations (van Heyningen and Weiner 1952). Subsequent research employed iontophoresis and pilocarpine nitration to induce sweat production, which yielded a significant upregulation of lactate levels in loaded skin tissues loading (Ferguson-Pell and Hagisawa 1988). Other studies employed thermal induction methods to examine the effect of loading on the biochemical response, with a focus on the metabolites lactate, urea and urate (Polliack, Taylor et al. 1993, Taylor, Polliack et al. 1994, Polliack, Taylor et al. 1997). These authors demonstrated a significant increase in lactate and urea levels when tissues were subjected to axial mechanical loading. In a separate study, a combined approach involving sweat biomarkers and transcutaneous gas monitoring system revealed a significant correlation between the fold increases in metabolites and a reduction in T_cPO_2 tensions, beyond a threshold reduction of 60% in T_cPO_2 tensions (Knight, Taylor et al. 2001). The integration of biochemical and biophysical measurements to assess the status of loaded skin tissues provide means to examine individual tissue tolerances. Similar to IL-1α, lactate has also been employed as an objective measure to assess the effect of medical device interactions (spine board) and specific loading regimens (Hemmes, de Wert et al. 2017, Soetens, Worsley et al. 2019). It is to be noted that in both these recent studies, sebum lactate levels were measured using sensitive techniques, such as UHPLC-MS and the concentrations in sebum were observed to be 10^3-fold less than that of the sweat concentrations. Indeed, current advancements in both point-of-care diagnostics and detection techniques offer promising clinical diagnostic solutions for detection of low concentrations of biomarkers (Currano, Sage et al. 2018). Nevertheless, in order to achieve clinical translation, definitive threshold levels of biomarkers for risk stratification need to be established.

Previous research suggested that the reperfusion response as reflected in changes in purines, such as xanthine, hypoxanthine and uric acid, provides additional information to that related to ischaemic response alone (Bader, Wang et al. 2005). Although the reported concentrations were low and found to be highly variable, this research has provoked the need to examine the influence of factors, such as the magnitude and time period of loading and the number

of loading/unlading cycles on the purine response as an indicator of tissue status. These factors have been investigated using animal models as, for example, in a study, where the effects of reperfusion in rat hind limbs on the malondialdehyde (MDA) concentrations in blood were measured (Ünal, Özmen et al. 2001). The study revealed that gradual reperfusion leads to significantly lower concentrations of MDA levels compared to those following sudden reperfusion. With respect to cycles of reperfusion, authors reported a significant increase in tissue homogenate MDA after 5 cycles of ischaemic/reperfusion injury when compared to 3 cycles, using a rat model (Wang, Pu et al. 2016). In a clinical study involving patients with PUs, an increase in serum MDA (nM) was measured accompanied by a decrease in α-tocopherol and ascorbic acid, the latter two of which act as antioxidants (Cordeiro, Antonelli et al. 2005). By contrast, a study involving a large cohort of patients with PUs(n-100) and healthy controls (n-213), revealed a significant decrease in serum MDA levels normalized to total protein in the health control cohort (Khlifi, Graiet et al. 2019). Nevertheless, the authors also reported a significant decrease in total antioxidant status (TAS) demonstrating the oxidative stress in patients with PUs. Clearly, further investigations are warranted to understand the relevance of MDA levels in conditions of skin compromise.

2.3.2.3 Genetic markers

Genetic transcriptomics have gained recent popularity in diagnostics owing to its ability to detect low amounts of biomarkers. There is limited information available about gene markers as possible indicators of skin damage (Table 2-5). Early research in the late ‘90s on cell models revealed that mechanical strain induces IL-1 (α and β) mRNA expression in keratinocytes (Takei, Kito et al. 1998). There has been a paucity of research since a number of gene markers present in little amounts could not be detected using techniques such as northern blot analysis. The development and advancement of Reverse Transcriptase – Polymerase Chain reaction (RT-PCR) techniques offering high sensitivity have rekindled the interest of gene markers in the case of PUs. As an example, Kurose et al investigated the changes in a wide array of inflammatory cytokines using a rodent model (Kurose, Hashimoto et al. 2015). The authors reported that most of the pro-inflammatory cytokines (IL-1α, IL-1β, IL-6) from skin and subcutaneous tissue were elevated for a shorter time period (12 hours) whereas MMP-3 and INF-γ remained elevated for a longer time period (3 days). It is worthy to note that the protein analysis from the same study revealed significant changes only in IL-1β and IL-6 levels. This study reinforces the need to investigate the temporal profile of genes/proteins to identify markers that are released at early stage of skin damage.

Further, few studies investigated the role of hypoxia in deep PUs by monitoring levels of HIF-1α mRNA using animal models (Kaneko, Minematsu et al. 2015, Wang, Pu et al. 2016). Recent studies with murine models have also focussed on identifying microRNAs that are regulated following mechanical damage (Hsu, Minematsu et al. 2022). These studies rather focussed on providing an understanding about the underlying mechanisms governing PUs.

Table 2-5: Studies investigating gene expression markers

Study	Biomarker analysed	Biofluid
Takei et al, 1998 (Takei, Kito et al. 1998)	IL-1α mRNA and IL-1β mRNA	*In vitro* studies
Kurose et al, 2015 (Kurose, Hashimoto et al. 2015)	mRNA - INF-γ, IL-1β, IL-6 Matrix metalloproteinase – 3 (MMP-3)	Animal study – Tissue homogenates
Wang et al, 2015 (Wang, Pu et al. 2016)	HIF-1α	Murine model - Muscle tissue
Kaneko et al, 2015 (Kaneko, Minematsu et al. 2015)	Plasminogen activator inhibitor (PAI -1) / HIF-1α	Murine model – Tissue homogenates
Hsu et al, 2022 (Hsu, Minematsu et al. 2022)	miR92b and miR-877	Murine model – Tissue homogenates

2.3.2.4 Biochemical markers selection and evaluation

As detailed in this section (Section 2.3.2), there are several biochemical markers that could be investigated for their potential in identifying skin damage. Indeed, it is important to choose a panel of biomarkers amongst the cytokines identified from literature and evaluate their performance as objective markers of skin health. Moreover, these biomarkers should be present in sufficient quantities for them to be detected from skin surface using non-invasive means. Cytokines, such as IL-1α, IL-1RA and IL-8 have been identified to be highly relevant in the context of PUs owing to their upregulation following mechanical stimuli in tissue models. In addition, other cytokines, namely INF-γ, IL-6, TNF-α, G-CSF have been highlighted for their role in the inflammatory cascade following insults and in the formation of skin barrier. Cytokines such as IL-33 have been identified for their role in other inflammatory conditions such as atopic dermatitis and psoriasis and therefore might offer promising potential in identifying skin damage due to mechanical and chemical insults (Fania, Moretta et al. 2022). Therefore, the panel of cytokines investigated in this thesis would majorly include high-abundant cytokines such as IL-1α and IL-1RA and also low abundant cytokines, such as IL-6, IL-8, TNF-α, IL-33 and INF-γ. Moreover, the signalling

cascade of inflammatory cytokines is an interactive process, and their connecting network needs to be considered in order to choose a biomarker that could detect skin damage at an early stage as well as to prevent choosing cytokines that provide redundant information. Concurrent analysis of biomarkers is challenging owing to different requirements for sensitive analysis of target molecules as well as to the low volume of sample available for analysis. As an example, surfactants are required for extracting protein molecules from the samples whereas the introduction of detergents renders the identification of metabolites a challenging process as they are incompatible with the instrumentation of chromatographic and spectrometric techniques.

2.3.3 *In vivo* imaging markers

Imaging technologies has played a major role in the advancement of modern health care, particularly in the diagnostics field. Several imaging techniques including Optical coherence tomography, laser Doppler perfusion imaging, ultrasound, microscopy and magnetic resonance imaging have been proposed to evaluate the integrity of skin and sub-dermal structures. Imaging provides the means to assess different morphological and functional skin characteristics in both healthy skin and skin presenting with disease and/or damage (Aspres, Egerton et al. 2003). As these techniques are non-invasive and *in vivo*, they can be readily translated onto clinical settings and can be employed to monitor skin status, monitoring the treatment and the status of healing wounds. In addition to the abovementioned imaging techniques, there are different *ex vivo* techniques involving immunohistochemistry (IHC) staining of skin biopsies that could be employed to study the inflammatory and structural changes in skin. However, the scope of this thesis is restricted to *in vivo* non-invasive imaging markers.

Optical coherence tomography (OCT) represents a contact-free imaging modality providing a detailed view of skin structures. Although conventional OCT offers higher resolution into the superficial layers, it is limited in assessing features in the dermis and deeper tissues (Schuetzenberger, Pfister et al. 2019). OCT has been examined for its potential application in diagnosing skin tumours and inflammatory diseases which are accompanied by swelling/oedema (Kato, Kawaguchi et al. 2019, Li, Li et al. 2019). Early research studies assessing the potential of dynamic OCT, a recent variant of OCT that permits depth-resolved imaging, in identifying chronic wounds have shown promising results with certain features,

such as vessel morphology and density in the superficial dermis, being different in healthy and damaged skin (Holmes, Schuh et al. 2019).

Laser Doppler imaging *(LDI)* has been used for real-time *in vivo* assessment of blood flow in dermal tissues (Leutenegger, Martin-Williams et al. 2011). For example, it has been extensively used in burns clinics to assess depth of damage and wound healing (Hoeksema, Baker et al. 2014). The resolution of ultrasound (US) imaging is highly dependent on the frequency of the applied probe (Mlosek and Malinowska 2013). Accordingly, a high frequency scanner (>25MHz) is required to estimate the structural properties of skin including the thickness of the epidermis, dermis and hypodermis. Ultrasound imaging has been employed in the dermatology area in order to assess melanoma skin cancers and scleroderma (Levy, Barrett et al. 2021). Ultrasound elastography can be used to estimate tissue stiffness, typically by means of tracking a shear wave in the underlying tissues. Its application in diagnosing breast cancer has high sensitivity and specificity (Youk, Gweon et al. 2017), although its utility in skin imaging has been limited to date. Magnetic Resonance Imaging (MRI) determines the change in hydrogen density and magnetic relaxation times within different tissues. This provides information on properties, such as mobility of water in the skin layers, and has proved effective in identifying diseased tissues, such as tumours from normal tissues (Stefanowska, Zakowiecki et al. 2010, Kato, Kawaguchi et al. 2019). MR elastography that combines MRI imaging with low-frequency vibrations provides a visual map of the stiffness of body tissues. These optical elastography techniques provide information about local skin mechanical properties (Kirkpatrick, Wang et al. 2006). Recently, MRI has been employed in conjunction with biochemical markers to study the tissue status following compression (Traa, van Turnhout et al. 2019). Typical images of the skin using various modalities are illustrated in Figure 2-9. The features of each of these distinct imaging techniques have been summarised in Table 2-6.

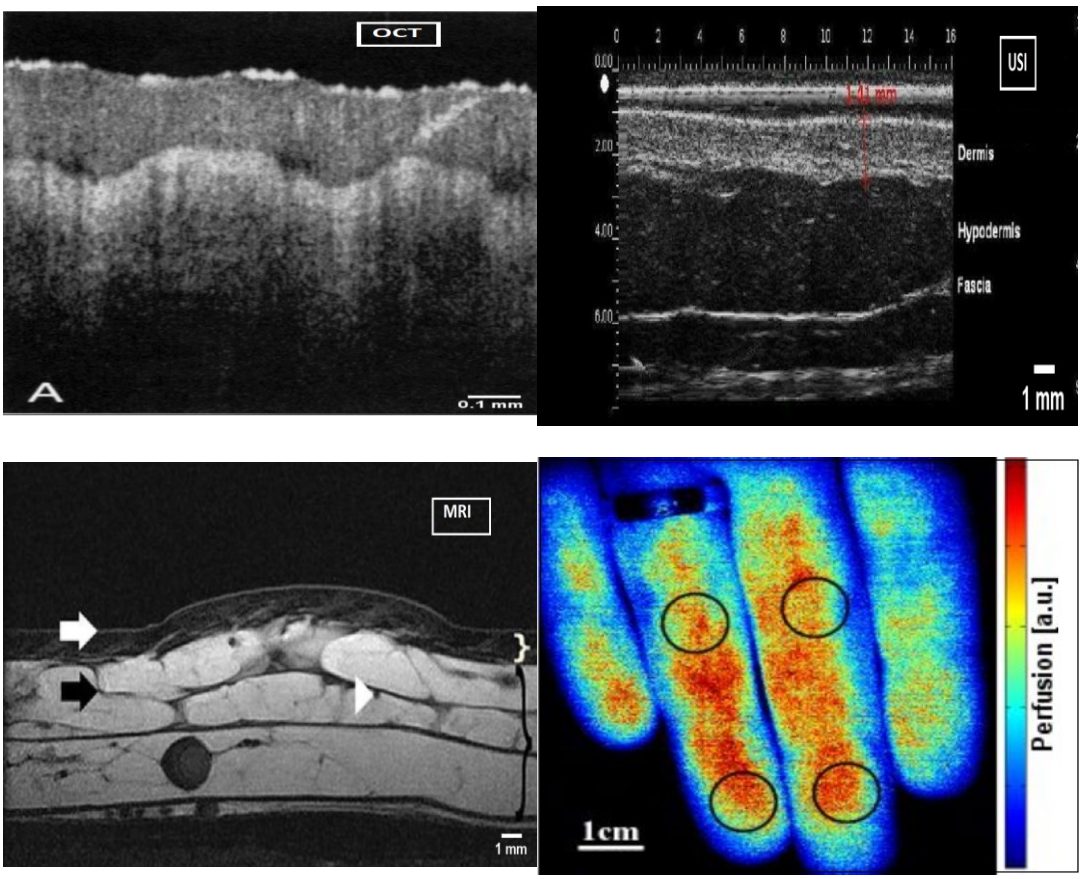


Figure 2-9 : Prominent skin imaging modalities (Clockwise from top-left) OCT: Optical coherence tomography (Cross sectional view); USI – Ultrasound imaging (Cross sectional view); LDI – Laser Doppler perfusion imaging (Top-view) ; MRI: Magnetic Resonance Imaging (Cross sectional view); Adapted from (Welzel, Lankenau et al. 1997, Stefanowska, Zakowiecki et al. 2010, Leutenegger, Martin-Williams et al. 2011, Wortsman 2012, Navarini, Meier et al. 2013)

Table 2-6 : Critical features of imaging modalities for skin

Imaging modality	Resolution (µm)	Imaging depth (mm)	Parameters derived	References
Optical coherence tomography (OCT)	5.8 (Axial), 4.1 (Lateral)	0.4–2.0	Morphological parameters, Thickness of layers, Elasticity measurements	(Welzel 2001, Alex, Weingast et al. 2011, Koehler, Lange-Asschenfeldt et al. 2011, Li, Li et al. 2019, Schuetzenberger, Pfister et al. 2019)
Ultrasound (>20 MHz) (USI)	66 (Axial), 63 (Lateral)	5	Morphological parameters, Thickness of layers, Stiffness (Elastography techniques)	(Oh, Kim et al. 2019, Schuetzenberger, Pfister et al. 2019)
Laser Doppler perfusion imaging (LDI)	No axial resolution, 40 (Lateral)	≈1.0–2.0	Cutaneous blood flow (measured in arbitrary units of flux)	(Rajan, Varghese et al. 2009, Leutenegger, Martin-Williams et al. 2011)
Magnetic resonance imaging (MRI)	≈100 (Spatial resolution)	Entire body	Hydration of skin layers, Tissue type	(Rallan and Harland 2004, Oh, Kim et al. 2019)

2.4 Motivation of the present study

The existing risk assessment methods, as described in Chapter 1, is limited by its subjectivity and poor predictive capability. There exists a number of objective tools, as outlined in this chapter, to monitor the status of skin health. However, the performance of these objective markers in classifying skin health and differentiating skin insults have not been investigated. Moreover, a single biomarker might not be efficient in an accurate diagnosis of compromised skin (Bader and Worsley 2018). Therefore, a complementary approach involving the biophysical, biochemical and imaging methods could provide an objective measure of skin integrity. There has been a paucity in literature in investigating cytokines owing to the low-

abundance of selected cytokines in skin surface and therefore efforts will be focussed on the optimization of the extraction of inflammatory biomarkers from samples. In addition, studies investigating the spatial and temporal profile of the biomarkers would provide a deeper understanding about the progressive development of damage leading to chronic wounds. Indeed, there is a lack of such studies in the literature to understand the temporal release of biomarker. Accordingly, the present work will focus on identifying biomarkers and studying their temporal profiles in different cohorts, including healthy volunteers, participants vulnerable to skin damage, and those patients with clinically diagnosed sites of early damage.

2.5 Aims and objectives

The overall goal of this PhD project is to identify early changes in skin integrity using a combination of biomarkers, with the use of prospective and retrospective studies. A part of the studies (Aim 4) is planned in collaboration with STINTS (Skin Tissue INTegrity under Shear) early-stage researchers, Nkemjika Abiakam (NA, ESR-3) and Pakhi Chaturvedi (PC, ESR-7). With respect to clinical studies, the participant recruitment and sample collection will be carried out by the clinical researcher (NA). In this thesis involving exploratory studies, the focus was on testing the hypotheses as detailed below.

Aim 1: Assess appropriate biophysical and biochemical techniques and protocols to characterise the integrity of skin tissues (Chapter 2 and Chapter 4)

Objectives

(1) Critique existing biomarkers, derived from both biophysical and biochemical techniques, previously reported in the literature

(2) Examine the performance parameters of biophysical and biochemical tools and associated parameters used in previous research conducted within the host laboratory

(3) Develop test protocols, which can be used to assess a range of insults and characterise the integrity of skin tissues

Aim 2: Assess the intra- and inter-variability associated with anatomical locations and intrinsic factors, such as gender, age and BMI, on normative biochemical and biophysical parameters. (Chapter 4)

Objectives

a) Analyse retrospective data, including TEWL, SEM, Erythema and LDI of healthy volunteers
b) Identify associations between the intrinsic factors e.g., gender, age, BMI, and skin status, including biochemical and biophysical parameters.
c) Investigate the variations associated with biofluid i.e., sebum
    i. Develop a test protocol to measure biofluid, in particular, sebum from the skin surface
    ii. Recruit a cohort of able-bodied volunteers and collect biofluids from different anatomical locations
    iii. Analyse the sebum content and optimize the time period of sebum collection

Aim 3: Develop an efficient extraction methodology to analyse inflammatory biomarkers from sebum (Chapter 5)

Objectives

a) Prepare standards of biomarker in biofluid surrogates, typically synthetic sebum
b) Estimate the influence of following processes on protein extraction efficiency
    i. Detergent used and its concentration for extraction
    ii. Extraction volume
    iii. Sonification and centrifugation time
c) Identify optimum process parameters based on collected datasets
d) Identify the differences in the concentration of biomarkers collected using different sampling approaches

Aim 4: Identify biomarker features across different insult models, ranging from controlled lab-based stimuli to functional stimuli (Chapter 6)

The participant recruitment and sample collection will be carried out by ESR-3 and ESR-7

a) Analyse a set of biochemical markers in the different set of studies conducted at the host laboratory

b) Collect the different parameters of the study, namely the anatomical location, nature of the cohort (healthy volunteers/patients with vulnerable skin) and the type of irritation model employed
c) Identify associations between the biochemical markers and the parameters of each of the studies

Aim 5: Identify distinct thresholds of biochemical and biophysical parameters for classifying changes in skin integrity in individuals with early signs of skin damage (healthy/damaged/healing) (Chapter 7)

The participant recruitment and sample collection will be carried out by ESR-3.

Objectives

a) Analyse the collected biofluids for biomarkers from control and target sites from cohorts of vulnerable volunteers at distinct time points
b) Compare relative change in biomarker status of healthy and at damage sites.
c) Identify biochemical parameters for early detection of skin damage.
d) Evaluate the sensitivity and specificity of identified biochemical and biophysical parameters

A schematic of the studies described in this thesis is briefly described in Figure 2-10.

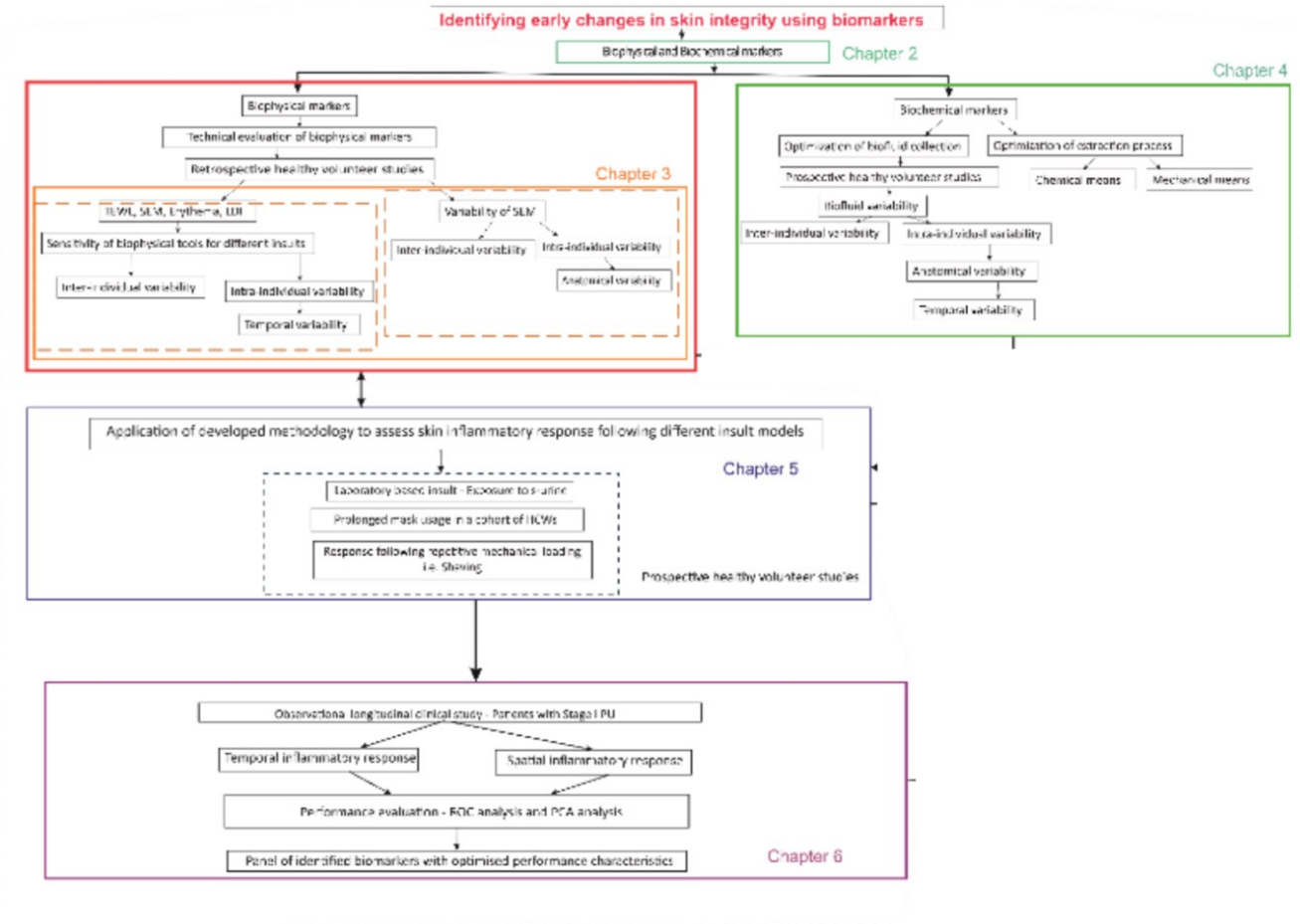


Figure 2-10: Schematic of the studies conducted to test the hypotheses described in the thesis

Chapter 3 Framework for biomarker identification and study design

Skin integrity is challenged due to various types of insults such as prolonged pressure, shear, friction, and moisture, which can lead to skin and sub-dermal damage. New technologies could reduce the socioeconomic burden of skin damage by establishing a comprehensive assessment of human skin integrity using non-invasive biomarkers and physical sensors. An overview of candidate skin parameters, including biophysical and biochemical markers, in the context of identifying changes in skin integrity have been detailed in Chapter 2. For the biomarkers to qualify as viable candidate to diagnose early skin damage, certain universal characteristics have been defined in literature. In this chapter, an overview of these characteristics in addition to the framework typically employed for identification of a biomarker have been summarised. Indeed, changes in skin health can involve various factors, which may influence non-invasive measurements (Section 2.3). Therefore, studies were designed to understand the factors influencing the response thereby aiding in identifying appropriate markers and methodology. These are achieved using prospective and retrospective studies that have been detailed. Furthermore, different skin insult models employed in the studies to simulate real-life conditions are briefly described.

3.1 Biomarker Evaluation

For a biomarker to be considered for diagnostic potential, several characteristics have been defined in the context of various diseases (Bennett and Devarajan 2011, Davis, Aghaeepour et al. 2020). Moreover, the context of use for the biomarker should also be clearly defined. The biomarker should be

a) measured easily and where possible non-invasively
b) in sufficient quantities for reliable detection
c) highly sensitive thereby aiding in early detection
d) highly specific in the response clearly distinguishing healthy state from the disease condition as well as specific to a given pathology
e) biologically relevant providing insight about the pathophysiology of the condition
f) reflective of the severity of the condition, including recovery and further progression of the condition

With respect to the current studies, the biofluid of interest i.e., sebum could be collected easily and non-invasively. The context of use for the biomarkers that will be identified in this thesis is to aid clinicians in making informed decisions in classifying the skin status i.e., damaged, healthy and in recovery. Further studies in this thesis aim to investigate the above defined parameters for an ideal sebum biomarker.

3.2 Framework for biomarker identification

Biomarkers are typically identified and validated in a three-step process, involving discovery, verification, and validation (Figure 3-1). In the discovery phase, many candidates are identified either based on critical understanding of the aetiology of the disease and a critical appraisal of the literature, or based on an untargeted approach, involving measuring all the proteins or genes, using proteomic or transcriptomic techniques. With the recent advance of unparalleled technologies, the omics strategies have gained recent popularity (Günther, Chen et al. 2012, Davis, Aghaeepour et al. 2020) as they could be used for screening significant numbers of protein and gene candidates. Furthermore, the advancement in mass spectrometric techniques such as trapped ion mobility spectrometry and time-of-flight spectroscopy provide sensitivity and the ability to even measure post-translational modifications of proteins. However, these technologies lack the understanding of how these markers are relevant to the pathophysiology of a given condition. In addition, these processes involve digesting the proteins which would therefore lead to loss of information. It is to be noted that proteomics techniques involve complex sample preparation as well as complex data analysis.

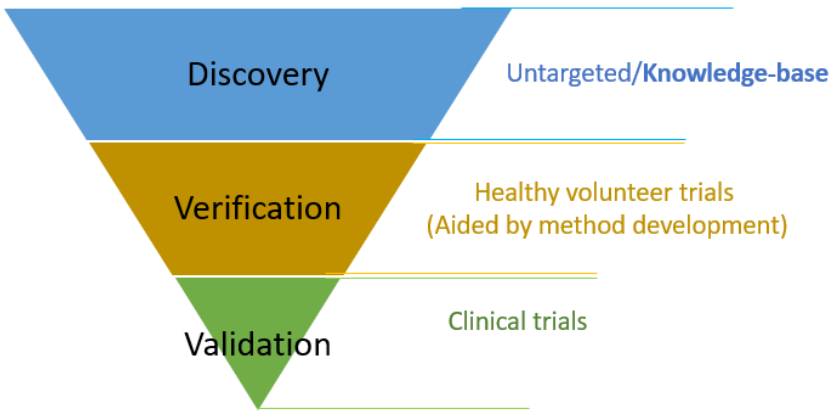


Figure 3-1: Schematic of phases of biomarker identification pipeline in this thesis with each phase requiring different trials

In the present studies, several biomarkers, including biophysical markers, protein markers and metabolites, were evaluated for their performance based on the existing knowledge base and their underlying aetiology (Chapter 2) as detailed in Figure 3-2. A panel of markers were chosen for the next phase of the pipeline, i.e., verification, in this study. This selection offers an insight into the aetiology of the disease and the biological relevance, as an example, cytokine levels are regulated as an immune response to external insults (Section 2.3.2.4).

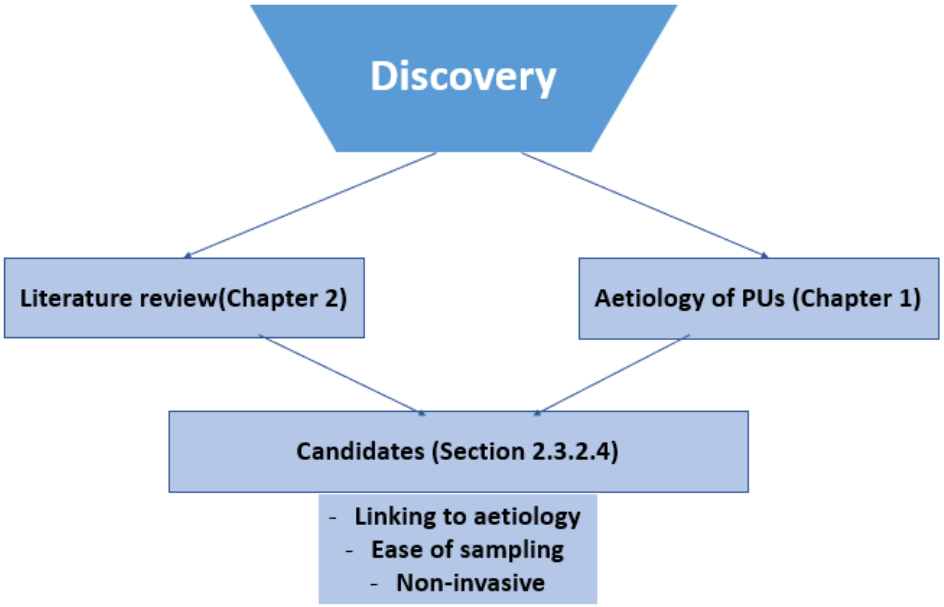


Figure 3-2: Schematic illustrating the discovery process employed in the thesis

In the second phase, involving verification, the candidate markers selected from literature were evaluated using a series of studies (Figure 3-3). As it is important to be able to measure low amounts of candidate biomarkers, initial evaluations were focussed on developing efficient methods for extracting biomarker candidates, including low-abundance biomarkers to provide a comprehensive evaluation of skin health (Chapter 5). Therefore, a series of healthy volunteer studies were designed to verify the potential of the markers to identify early signs of skin damage, through temporary insult models (Chapter 4 and Chapter 6). Moreover, these studies would also provide means to identify the variabilities associated with skin response following insults and the ability of the parameters to detect temporal changes in skin structure and function i.e., response and recovery characteristics. These are summarised in the schematic below:

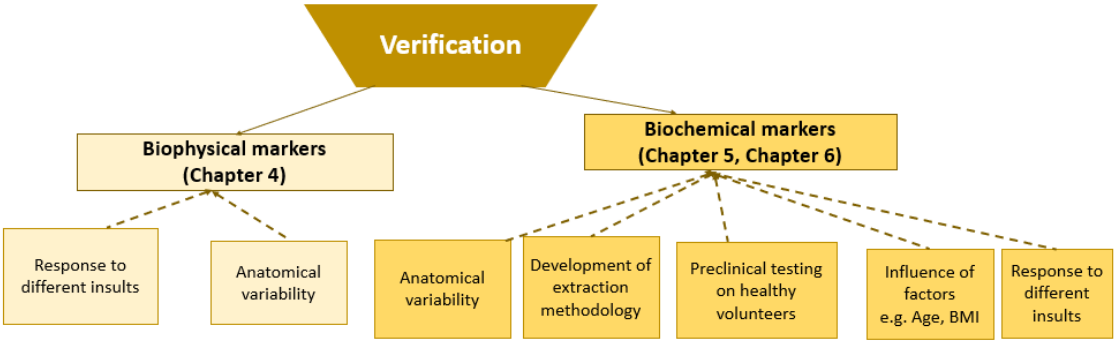


Figure 3-3: Schematic illustrating the studies designed as part of the verification phase of the biomarker discovery framework

As the anatomy of the skin and soft tissues change with different body location (Section 1.1), there is considerable variation in skin response at different anatomical sites (Man, Xin et al. 2009, Robertson and Rees 2010, Pan, Ma et al. 2020). It is important to account for these variabilities for biomarker candidates to develop an understanding of confounding factors for their application, for which, the changes could be attributed to the processes underlying skin damage. Therefore, in this thesis, the following studies focussed on investigating the anatomical variability of different parameters, as follows:

1) Anatomical variability of sub-epidermal moisture to establish the basal values and investigate the recommended thresholds for distinguishing skin status – Chapter 4, Section 4.1
2) Anatomical variability of biofluid, i.e., sebum secreted at different relevant locations of skin damage – Chapter 5, Section 5.2.1

To study the ability of the parameters to distinguish between different skin insult models as well as to study the temporal profiles of skin parameters following insults, studies were designed as described below:

1) Evaluation of biophysical parameters following mechanical and chemical insults - Chapter 4, Section 4.2
2) Evaluation of biochemical parameters following mechanical insults, including loading (use of respirators), friction (shaving), and moisture insults (s-urine) - Chapter 6

In the third phase of biomarker discovery, the candidate markers are evaluated in a clinical study wherein the skin response is measured at the healthy and damaged sites of a Stage 1 PU (Chapter 7) (Figure 3-4). Spatial and temporal profiles of the biomarkers as well as their

consistency are investigated. The performance of these markers, i.e., sensitivity and specificity can then be assessed for their ability to distinguish the sites efficiently. In addition, the influence of comorbidities and intrinsic factors on the biochemical response are also investigated.

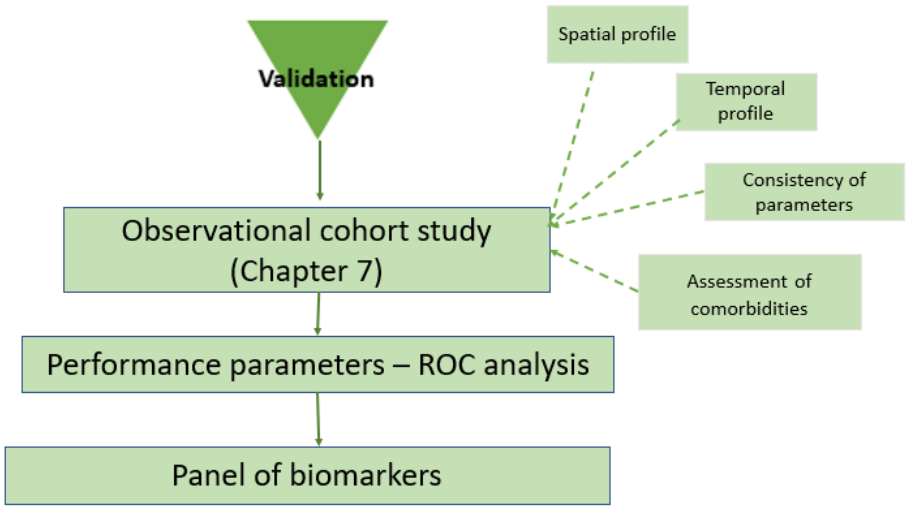


Figure 3-4: Schematic illustrating the study and analysis conducted as part of the validation phase

3.3 Skin Insult Models

A range of skin insults, which are mechanical, and chemical in nature, have been typically employed in research studies to simulate common clinical conditions relevant to skin health. Some of the common insult models selected to correspond with both mechanically induced skin damage and moisture/chemical induced damage include:

i) Tape-stripping
ii) Mechanical loading (axial and tangential)
iii) Chemical irritation (Sodium lauryl sulfate-induced)
iv) Incontinence insults – Exposure to S-urine/Moisture

Tape-stripping is an insult used to simulate the peeling and tearing of skin, typically used as a positive control for skin barrier disruption and local inflammation. Mechanical loading (pressure and shear forces) simulates the clinical condition of axial and tangential loads applied to the skin during static lying and sitting postures, representing the primary cause of pressure ulcers. SLS-induced chemical irritation and the incontinence insults have

commonly been used in literature to simulate contact dermatitis and incontinence-associated dermatitis, respectively.

3.3.1 Tape-stripping

Tape-stripping is a simple method that is widely employed as an irritant model in skin research (Zhai, Poblete et al. 1998). It has also been extensively used to evaluate the transport of different formulations applied on the skin surface (Sobiepanek, Galus et al. 2019). In this method, an adhesive tape is used to repetitively remove successive cell layers of the Stratum Corneum from an anatomical site (Figure 3-5).

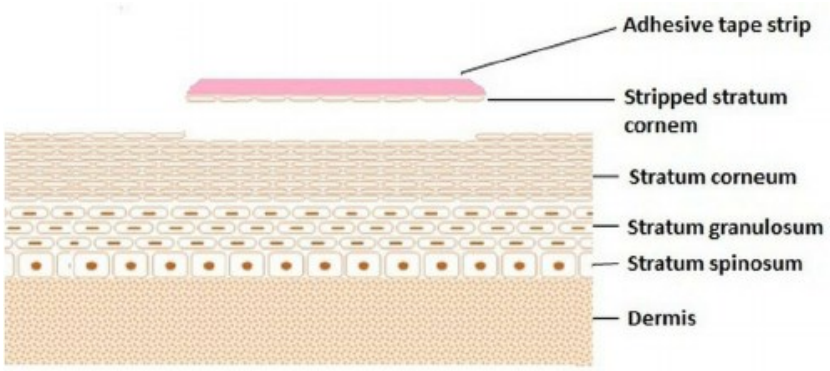


Figure 3-5 : Schematic of the tape-stripping process wherein an adhesive tape is used to remove the SC (Nair, Jacob et al. 2013)

Studies have identified that the effect of tape-stripping on the skin response is associated with inter-individual variability and depends on various factors, such as the skin thickness and the cohesion between the corneocytes of the SC (Breternitz, Flach et al. 2007, Berthaud and Boncheva 2011). In a study involving 70 volunteers, the thickness of skin layer removed at successive tape-stripping events was measured (Berthaud and Boncheva 2011). It was reported that the number of events required to remove 5µm of skin thickness varied between individuals. In addition, the corresponding change in TEWL values following the removal of 5 µm skin thickness was highly variable, as illustrated in the results in Figure 3-6.

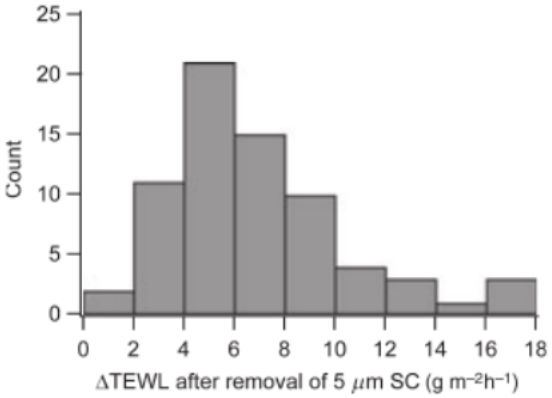


Figure 3-6 : Frequency histogram demonstrating change in TEWL values following the removal of 5um of the epidermal layer in a cohort of volunteers (Berthaud and Boncheva 2011)

It is well established that the tape-stripping process is also affected by several extrinsic factors as listed below (Breternitz, Flach et al. 2007, Lademann, Jacobi et al. 2009)

1) Type of tape
2) Angle at which tape is removed
3) Velocity of tape removal
4) Application of tape
5) Site of application
6) Duration of tape application
7) Contact pressure of application
8) Number of tape stripping

In the current project, studies included tape-stripping as a positive control to simulate mechanical-induced skin damage. Owing to the cost and ease of availability, Sellotapes^{TM} were employed for mimicking insult models. 40 fresh tape strips were employed in the studies to ensure sustained skin damage. It is of note that these tapes differ to those which are designed to analyse the constituents of the tape, such as D-Squames.

3.3.2 Mechanical loading

Mechanical loading has been recognized as one of the leading insults which can result in the development of pressure ulcers. Therefore, it has been employed in previous research to simulate local loading and corresponding deformation of the underlying tissues (Bronneberg

2007, Bostan, Worsley et al. 2019, Soetens, Worsley et al. 2019). In this thesis, the influence of loading due to postures, attachment of medical devices as well as controlled loading regimens on skin response were investigated. The protocols involving loading due to postures and attachment of medical devices have been detailed in the respective chapters (Chapter 6). These loads range up to 100 mmHg based on previous studies (Worsley, Prudden et al. 2016, Worsley, Rebolledo et al. 2018).

In the current work, involving evaluation of skin response at forearm locations and sacrum, controlled static uniaxial loading was employed. The selection of these sites was deliberate, with the sacrum chosen because it is the most common location for pressure ulcer incidence, and the forearm selected for its ease of accessibility. At forearm locations, this was achieved using an indenter perpendicular to the skin surface with an in-built load cell, as shown in Figure 3-7(a). For sacral loading, free standing weights as shown in Figure 3-7b were employed. To prevent stress concentrations, a 3D printed plastic surface with rounded edges was placed at the interface between the loading element and the skin surface (Figure 3-7b). The magnitude of loading was set equivalent to a pressure of 11.0 kPa (80 mm Hg) by adding weights to the indenter of diameter 42.4 mm (Soetens, Worsley et al. 2019). The protocols involving loading due to postures and attachment of medical devices have been discussed in detail in Chapter 6. In all of the studies described in Chapter 4 and Chapter 6, the loading was controlled rather than the displacement due to loading.

(a) (b)

Figure 3-7: (a) Uniaxial loading device with in-built load cells (b) (Left) Representative image of uniaxial loading on the sacrum with the participant lying in a prone position (Soetens, Worsley et al. 2019). (Right) Closer view of the indenter with 3D printed polymer cap surface with curved edges at its base to minimise stress concentrations during uniaxial loading.

3.3.3 Chemical irritation

Sodium-lauryl sulfate (SLS) represents one of the commonly employed surfactants in dermatological research to simulate irritant contact dermatitis (Törmä, Lindberg et al. 2008, Leoty-Okombi, Gillaizeau et al. 2021). It is deemed safe for use in research and consequently been employed in a number of studies (Bergfeld, Belsito et al. 2005). Previous studies used concentrations ranging from 0.1-2% SLS with varying exposure times ranging from hours to days (de Jongh, Verberk et al. 2006). Studies revealed that the skin response, in the form of both visual observations, such as erythema, and objective measurements involving TEWL and the inflammatory response, have indicated clear irritation and barrier disruption to the skin (de Jongh, Jakasa et al. 2006, de Jongh, Verberk et al. 2006, Marrakchi and Maibach 2017). However, variability in skin response was evident across several studies, which could have been attributed to exposure time, concentration, and application method. Intrinsic factors such as anatomical location, age and gender also influenced findings (Marrakchi and Maibach 2017). In order to model skin irritation, 0.5% SLS was employed in the studies for a period of 24 hours based on previous guidelines (Tupker, Willis et al. 1997).

3.3.4 Incontinence insults

Skin can be exposed to different moisture sources, involving sweat, urine, and faeces. Research studies have employed several models to simulate these exposures to biofluids. In this thesis, two separate moisture-based insults were considered involving water and synthetic urine (S-urine). Cotton pads of 20 mm diameter were saturated with water and applied onto the skin surface. A non-permeable tape (Tegaderm Film, 3M Healthcare, US) was used to seal the saturated pad in place and keep it occluded during the test period of the study. Similarly, cotton pads saturated with S-urine were used to simulate urinal incontinence. S-urine, composed of a mixture of commonly found salts in urine, was prepared as previously detailed (Mayrovitz and Sims 2001). The composition of the formulation, maintained at a pH of 7.9, is summarized in Table 3-1.

Table 3-1: Composition of S-urine used to simulate conditions of urinary incontinence (Mayrovitz and Sims 2001)

Compound	Concentration (% w/v)
Urea	2.5
Sodium chloride	0.9
Ammonium chloride	0.3
Sodium sulfite	0.3
Anhydrous disodium hydrogen orthophosphate	0.25
Creatinine	0.2

3.4 Summary

This chapter provides an overview of the characteristics of a discovery, verification and validation of candidate biomarkers using a standardised framework. These have been briefly detailed in the context of skin damage and the aims of the thesis. In the light of the biomarker discovery framework, the schematic illustrates the basis for each of the chapters in the thesis (Figure 3-2, 3-3 and 3-4). An overview of the insult models employed in the thesis are also detailed.

Chapter 4 Technical evaluation of biophysical skin parameters– A retrospective data evaluation

As access to laboratories were limited due to COVID-19, the exploitation of existing data sets provided an opportunity to explore skin's response to insults and factors affecting skin parameters. Thus, retrospective data collected at the host laboratory by a visiting USA researcher, Professor Barbara Bates-Jensen (BBJ), were analysed by the doctoral researcher (Hemalatha Jayabal) to evaluate i) the variability associated with biophysical parameters to different skin insults and ii) the effects of anatomical location on skin parameters. This retrospective evaluation informed the scientific community about the performance of the biophysical parameters in distinguishing varying nature of insults and the importance of choice of thresholds for classifying skin status. The findings from the retrospective evaluation have now been published as two separate manuscripts (Jayabal, Bates-Jensen et al. 2021a, Jayabal, Bates-Jensen et al. 2021b) and are discussed in separate sub-sections (Section 4.1 and 4.2)

With respect to biochemical parameters, there are emerging approaches of non-invasive sampling of biofluids from the skin surface to monitor inflammatory and ischemic responses following skin insults. The prospective evaluation focussed on investigating the biofluid variability at relevant anatomical locations (Chapter 5) for which a methodology (Sulfo-phospho-vanillin assay) has been discussed and optimised in this chapter.

4.1 Anatomical variability of sub-epidermal moisture

Article: Jayabal H., Bates-Jensen B.M., Abiakam N.S., et al., Anatomical variability of sub-epidermal moisture and its clinical implications, J Tissue Viability, Volume 30, Issue 3, August 2021

The experiment design and data collection for this study were carried out by the visiting researcher (BBJ) and the data-analysis and interpretation were carried out by the doctoral researcher (HJ). Sub-epidermal moisture was measured using a handheld, portable device (SEM Scanner, Bruin Biometrics LLC, USA) (Section 2.2.1). The device measures the bio-capacitance of soft tissues which is converted into arbitrary units (AUs). All measurements were taken according to the manufacturer's guidelines. To review briefly, light skin pressure was applied at an optimal level indicated by the device prior to each recording. The basal

SEM measurements were collected from 27 anatomical sites involving bony prominences, which included the right (R) and left (L) sides of the shoulder, scapula, hip, trochanter, buttocks, ischial tuberosities, medial knee, lateral knee, medial ankle, lateral ankle, lateral heel, posterior heel, as well as the middle of the back, sacrum and coccyx (Figure 4-1). SEM readings were taken in triplicate by an experienced nurse (BBJ) and a mean of the three values was calculated for each body location. Participants either adopted a supine, prone or side-lying posture, ensuring that the sites were unloaded prior to taking the measurements. This was carried out by giving sufficient time for recovery after loading as informed by previous studies (Bates-Jensen, McCreath et al. 2018). All the measurements were performed in a controlled lab environment set at a temperature of 23 ± 2 °C and relative humidity of 42 ± 6%.

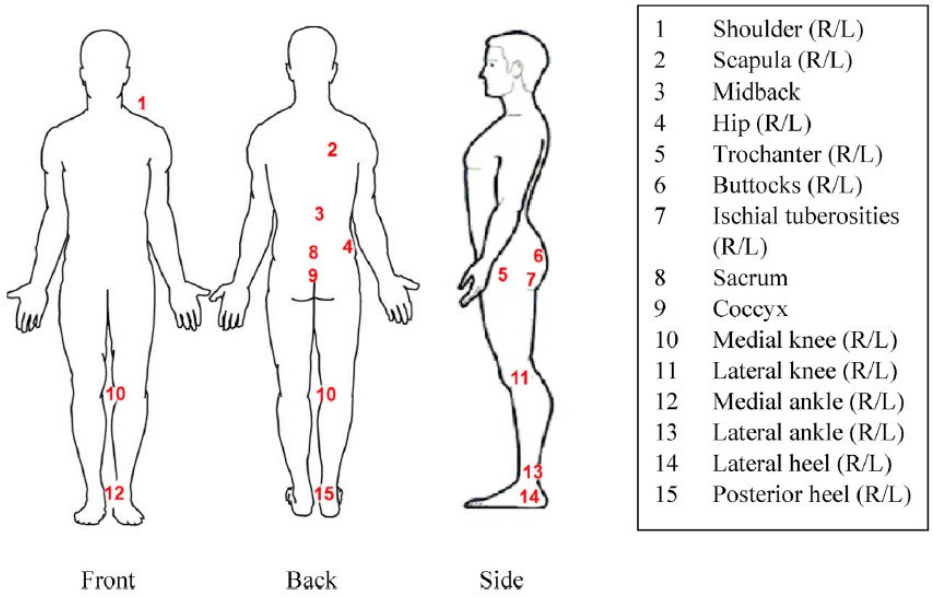


Figure 4-1: Location of 27 anatomical sites at which SEM measurements were performed.

The demographics of the participant cohort are detailed in Table 4-1. A total of 24 healthy participants (10 males and 14 females) were recruited from the local community. They were aged between 23 and 82 years (mean age 48±17 years) with a mean height and weight of 1.7±0.1m and 72.7±13.8kg, and a corresponding mean BMI of 25±4 kg/m^2.

Table 4-1: Summary of participant demographics and anthropometric data

Participant ID	Sex	Age	Weight (kg)	Height (m)	BMI (kg/m2)
1	F	57	48	1.63	18.18
2	F	56	52	1.65	19.10
3	M	41	55	1.69	19.26
4	F	51	50	1.60	19.53
5	F	28	63	1.74	20.81
6	F	68	59.5	1.66	21.59
7	F	28	61	1.68	21.61
8	M	37	76	1.83	22.69
9	M	73	78	1.82	23.55
10	M	28	67	1.67	24.02
11	F	59	62	1.60	24.22
12	M	64	92	1.93	24.70
13	M	24	83	1.83	24.78
14	F	73	70	1.68	24.80
15	M	23	85	1.85	24.84
16	M	82	74	1.71	25.31
17	F	49	80	1.76	25.83
18	M	55	80	1.73	26.82
19	F	37	85	1.75	27.76
20	M	31	85	1.73	28.40
21	F	50	72	1.52	31.00
22	F	26	95	1.75	31.02
23	F	50	82	1.63	31.05
24	F	52	89	1.62	33.91

The absolute SEM values were presented with respect to the BMI of the individual participants in the form of heat map in Figure 4-2.

Participant ID	BMI (kg/m^2)
1	18.2
2	19.1
3	19.3
4	19.5
5	20.8
6	21.6
7	21.6
8	22.7
9	23.6
10	24.0
11	24.2
12	24.7
13	24.8
14	24.8
15	24.8
16	25.3
17	25.8
18	26.8
19	27.8
20	28.4
21	31.0
22	31.0
23	31.1
24	33.9

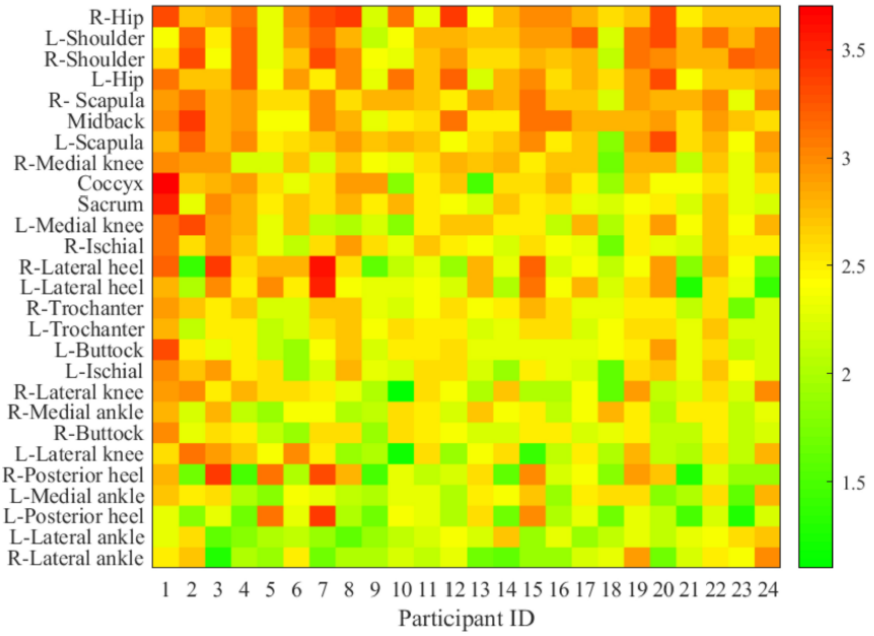


Figure 4-2 : Heat map of baseline SEM values for individual participants (1-24) across 27 anatomical sites in increasing BMI order from left to right

The mean and co-efficient of variation (CV) of the SEM values for different anatomical locations are presented in Table 4-2. Comparisons between right and left body sites revealed similar trends in each anatomical region, showing a level of consistency between sides. Indeed, in all cases where right and left SEM values were recorded, the corresponding mean values were within 0.1 AU. However, it is clear that certain anatomical locations, such as shoulder, scapula, and mid-back, corresponding to the cephalad (towards the head) locations presented with inherently higher basal values compared to those in caudad (towards the feet) locations. In addition, site-specific variability across the cohort, as determined by the CV, ranged from 8% to 27%, with the highest degree of variability associated with the sites adjacent to the heels.

Table 4-2: Mean and coefficient of variation (CV) of baseline SEM values measured which was rank ordered to different anatomical sites

Location	Mean	CV (%)
R - Hip	2.8	12.6
L - Shoulder	2.8	12.2
R - Shoulder	2.8	12.0
L - Hip	2.8	10.6
R - Scapula	2.8	8.1
Midback	2.8	9.8
L - Scapula	2.7	10.9
R - Medial knee	2.6	12.4
Coccyx	2.6	16.7
Sacrum	2.6	11.9
L - Medial knee	2.5	14.7
R - Ischial	2.5	11.9
R - Lateral heel	2.5	23.2
L - Lateral heel	2.5	20.2
R - Trochanter	2.4	11.0
L - Trochanter	2.4	8.5
L - Buttock	2.4	11.8
L - Ischial	2.4	13.9
R - Lateral knee	2.4	19.2
R - Medial ankle	2.4	11.3
R - Buttock	2.4	11.1
L - Lateral knee	2.3	20.5
R - Posterior heel	2.3	26.2
L - Medial ankle	2.3	14.7
L - Posterior heel	2.2	22.9
L - Lateral ankle	2.2	14.4
R - Lateral ankle	2.2	19.3

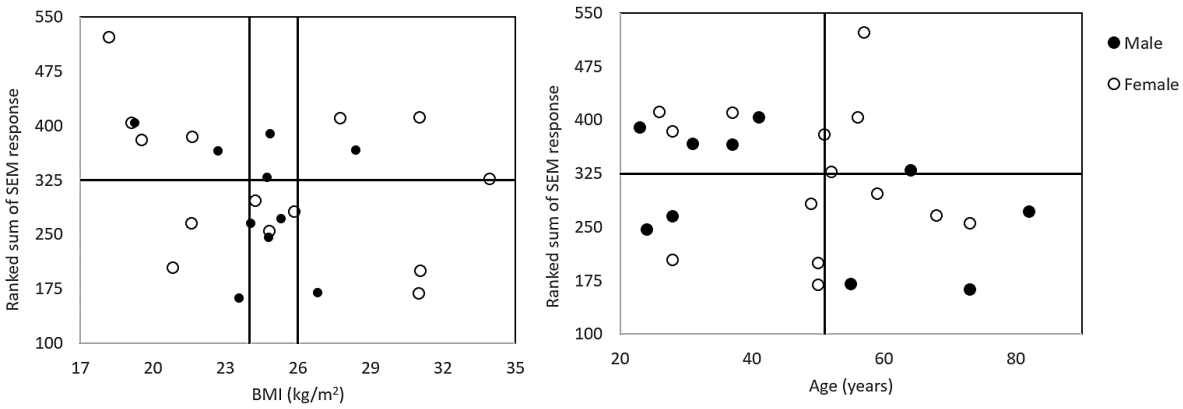
An alternative analysis was conducted to examine the variability associated with the two intrinsic factors, namely age and BMI, which involved ranking the SEM values for each location. The ranks were summed across all the locations for each of the participants as indicated in Figure 4-3, with a maximum rank sum of 648. Close examination of the rank-sum analysis with respect to BMI revealed considerable variability (Figure 4-3a). Adopting a cluster analysis approach with an arbitrary rank-sum threshold value of 325 i.e., 50% of the maximum rank-sum value, and three categories of BMI groups, a few tentative observations revealed:

i) Of the nine participants with BMI < 24 kg/m^2, six of them (67%) demonstrated elevated SEM values as evidenced by high rank-sums,

ii) Of the eight participants with BMI ranging from 24-26 kg/m^2, two (25%) demonstrated high SEM values,

iii) Of the seven participants with BMI > 26 kg/m^2, four of them (57%) revealed elevated SEM values.

Similar analysis with respect to age revealed no consistent trends. As an example, with participants aged under 50 years of age, 7 of the 13 (54%), demonstrated increased SEM values over baseline, compared to a corresponding 45% for those over 50 years (Figure 4-3b). There were no significant differences between genders at any of the locations, with the exception of the lateral knee, for which males exhibited higher SEM values, with a mean value of 2.6 and 1.9 for males and females respectively.



(a) (b)

Figure 4-3: Rank sum analysis of individual SEM values as a function of (a) BMI and (b) Age

The findings of the study revealed distinct differences in SEM values recorded at 27 different anatomical sites of the body, with a clear trend in decreasing values from the head to the feet. This anatomical variability warrants further examination of the thresholds used to define tissue damage, currently set by the manufacturers at 0.6. The relative change in absolute values to achieve the SEM delta threshold indicative of damage would be significantly different dependent on the specific site of interest. In addition to this, there was inter-individual variability, with associations between the SEM basal values at selected locations and intrinsic factors. As an example, there appears to be some association between BMI and SEM values, with those individuals presenting with a low BMI (<20 kg/m^2)

generally exhibiting higher SEM readings. Although these inter-individual variations are accommodated to some extent by the use of a 'delta' parameter normalized to values at the local site, the use of a single "universal threshold" for all individuals and anatomical locations could limit the predictive potential of SEM to identify early signs of skin damage. Monitoring the temporal changes in the SEM parameter is recommended to reliably capture the early signs of skin damage.

4.2 Sensitivity of different biophysical parameters to skin insults

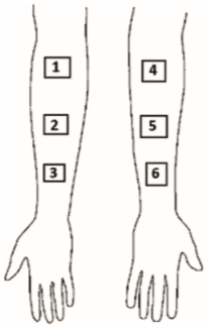
Article: Jayabal H., Bates-Jensen B.M., Abiakam N.S., et al., "The identification of biophysical parameters which reflect skin status following mechanical and chemical insults", Published in Clinical Physiology and Functional Imaging, Volume 41, Issue 4, July 2021

Retrospective data collected at the host laboratory by Prof. Barbara Bates Jensen (BBJ) was used to evaluate the sensitivity of biophysical parameters to detect local skin changes following a range of insults, including chemical, mechanical and tape stripping. The experiment design and data collection for this study were carried out by the visiting researcher (BBJ) and the data-analysis and interpretation were carried out by the doctoral researcher (HJ).

An array of measurement techniques, including biophysical and imaging techniques, were employed to assess the skin response to a selection of insults (Chapter 2, Section 2.3.1 and 2.3.3). Skin barrier function was measured using an open-chamber TEWL probe (TEWL, MPA9, Courage & Khazaka, Germany). Skin blood flow was measured using Scanning Laser Doppler Imaging (Scanning LDI, Moor Instruments Ltd, Axminster, UK), with a helium neon red gas laser (wavelength, 635 nm). Sub-epidermal moisture was measured using a hand-held, portable, diagnostic device (SEMScanner, Bruin Biometrics LLC, USA) and erythema was evaluated using a mobile imaging application (ScarletRed Holding, GmbH, Austria).

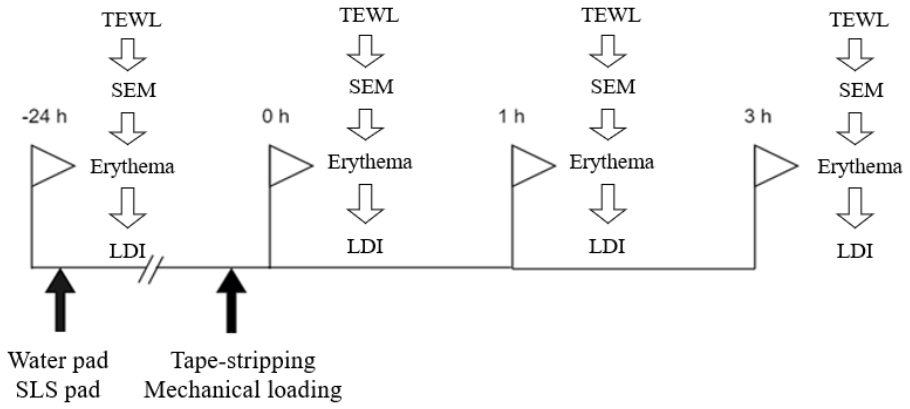
In this study, eleven healthy participants (6 males and 5 females) were recruited from the local community. The participants were aged between 23 and 64 years (mean age 37 years) with a mean height and weight of 1.7±0.1 m and 76±14 kg, respectively, and a corresponding mean BMI of 25±5 kg/m^2. Three assessment zones were marked on each of the forearm,

with four separate insults imposed on four sites and the other two sites serving as negative controls. The insults include tape stripping, exposure to 0.5% SLS, exposure to water and mechanical loading of magnitude 11kPa (Chapter 3, Section 3.3). Changes in biophysical parameters were monitored at different time points, namely, baseline and immediately after removal of insults (0 h). Subsequent measurements were repeated at 1 hour (1 h) and 3 hours (3 h) to monitor recovery characteristics, as illustrated in Figure 4-4.



Site	Insults
1	Saturated water pad (24 hours)
2	Control site 1
3	Mechanical loading (11 kPa – 30 minutes)
4	Saturated pad with 0.5% Sodium lauryl sulfate (SLS) (24 hours)
5	Control site 2
6	Tape-stripping (40x using medical tape)

(a)



(b)

Figure 4-4: a) Schematic representation of the experimental protocol performed on the volar aspects of both forearms b) Timeline of the experimental protocol. The flags indicate the time-point of skin measurements, and the black arrows indicate the point when insults were applied. At each time point, the measurements are taken in the order as shown in the schematic.

The findings of the study with respect to each of the biophysical parameters are summarised as follows:

TEWL (Figure 4-5a)

An increase in TEWL was observed throughout the time period of the study following tape-stripping and exposure to SLS. With respect to mechanical loading, minimal changes were observed at the sites (except one participant), similar to that of the control sites. Although increase was detected immediately after exposure to saturated water pad, the values returned to baseline after a period of 1 hour. Close examination of responses following tape-stripping and chemical irritation (SLS) revealed distinct differences in individual response, with one sub-group exhibiting a very high increase in TEWL values while another exhibited a moderate response

SEM (Figure 4-5b)

Control sites revealed minimal variability in SEM delta parameter, with values ranging from -0.25 to 0.25. A small subgroup of participants exceeded the SEM recommended threshold (0.6) following mechanical loading, however this was not sustained throughout the test period. Following exposure to moisture, a high degree of variability was reported with values ranging from -0.5 to 0.5. The responses to chemical irritation and tape-striping were similar to mechanical loading, with selected individuals (Tape stripping - #1, #4, #5, #6; Chemical irritation - #4, #6, #9) exceeding the recommended threshold.

LDI (Figure 4-5c)

Similar to the other biophysical parameters, there were clusters of individuals demonstrating distinct responses following exposure to moisture pads and chemical irritation. There were no remarkable changes in the parameter following mechanical loading and at the control sites.

Erythema (Figure 4-5d)

The response following insults revealed a high degree of variability and few clear temporal trends. A dichotomy of responses were observed between those with elevated erythema post insult and those with minimal change from basal values.

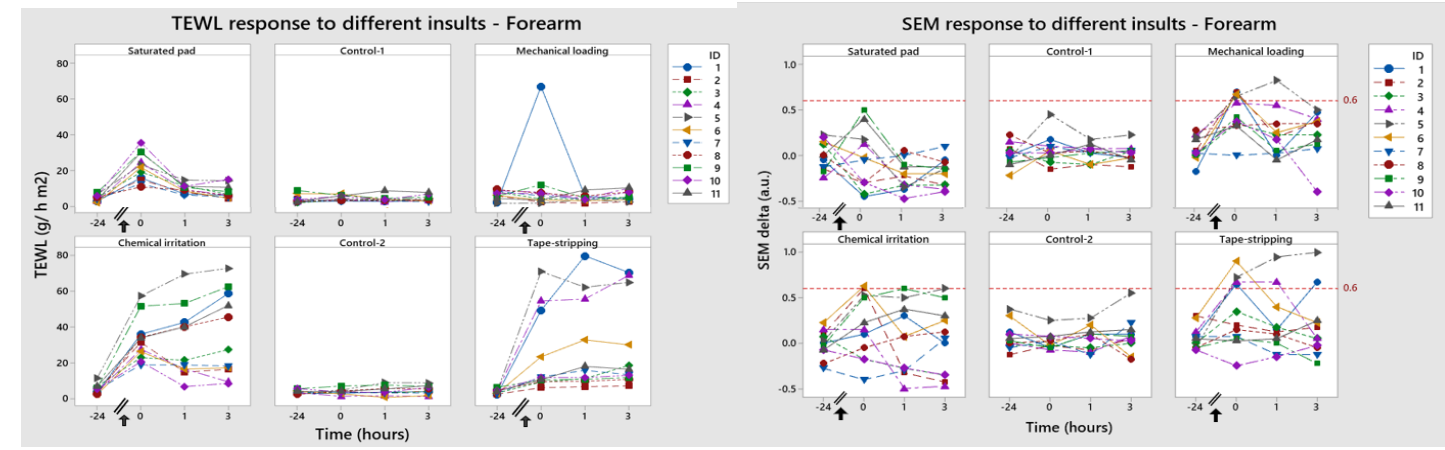


Figure 4-5: a) TEWL (b) SEM (c) LDI and (d) Erythema response to different skin insults. The black arrows indicate when the insults were applied. The red dotted lines in SEM plots indicate threshold value recommended by the manufacturer.

The choice of thresholds is critical to ensure performance of a biomarker candidate in clinical settings. In this study, thresholds for each of the biophysical parameters were estimated based on a value of 2 standard deviations above the mean at the control sites. Table 4-3 summarizes the thresholds and the number of participants with response exceeding the threshold values. From the table, it is clear that no single parameter was sensitive across all the insults. Temporal nature of responses indicating skin recovery was evident with the TEWL response. As an example, sites exposed to moisture and mechanical loading recovered back to baseline within the time period of the investigation as compared to chemical irritation and tape-stripping.

Secondary analyses were performed to examine the sensitivity of SEM delta thresholds, with values ranging between 0.1 – 0.7 (Figure 4-6). Relative sensitivity of each threshold was examined for three insults namely mechanical loading, exposure to chemical irritation and tape-stripping. For each threshold at the three insults, there was a decrease in the percentage of participants exceeding each threshold from immediately post insult (0h) to three hours post insult (3h). Interestingly, even at the lowest threshold of 0.1, the number of participants did not exceed 65% in detecting skin changes following tape-stripping and chemical irritation. By contrast, the thresholds were more sensitive to mechanical loading, particularly at delta value ≤0.3, a value which represented 50% of that recommended by the manufacturers.

Table 4-3: Descriptive summary of skin output parameters for a range of insults

(TS- Tape stripping, ML-Mechanical loading, CI- Chemical irritation, SW – Saturated water pad)

Parameter (Units)	**Range**		**Threshold**	**Immediate response after insult (0h)**				**1-hour post-insult (1h)**				**3-hours post-insult (3h)**			
				No. of participants exceeding the threshold				No. of participants exceeding the threshold				No. of participants exceeding the threshold			
	Control	Insult		TS	ML	CI	SW	TS	ML	CI	SW	TS	ML	CI	SW
TEWL ($g/h\ m^2$)	0.8 — 8.9	1.6 —79.7	≥ 8	10	2	11	11	10	1	10	8	10	2	9	3
SEM - delta (AU)	-0.28 — 0.55	-0.5 — 1.0	≥ 0.6	4	4	2	0	2	1	1	0	2	0	1	0
LDI flux (AU)	52— 216	48 — 453	≥ 177	4	1	7	5	3	1	5	3	2	1	5	3
Erythema (AU)	0.004 — 0.054	0.001 - 0.093	≥0.06	1	6	4	1	3	1	5	3	2	4	5	2

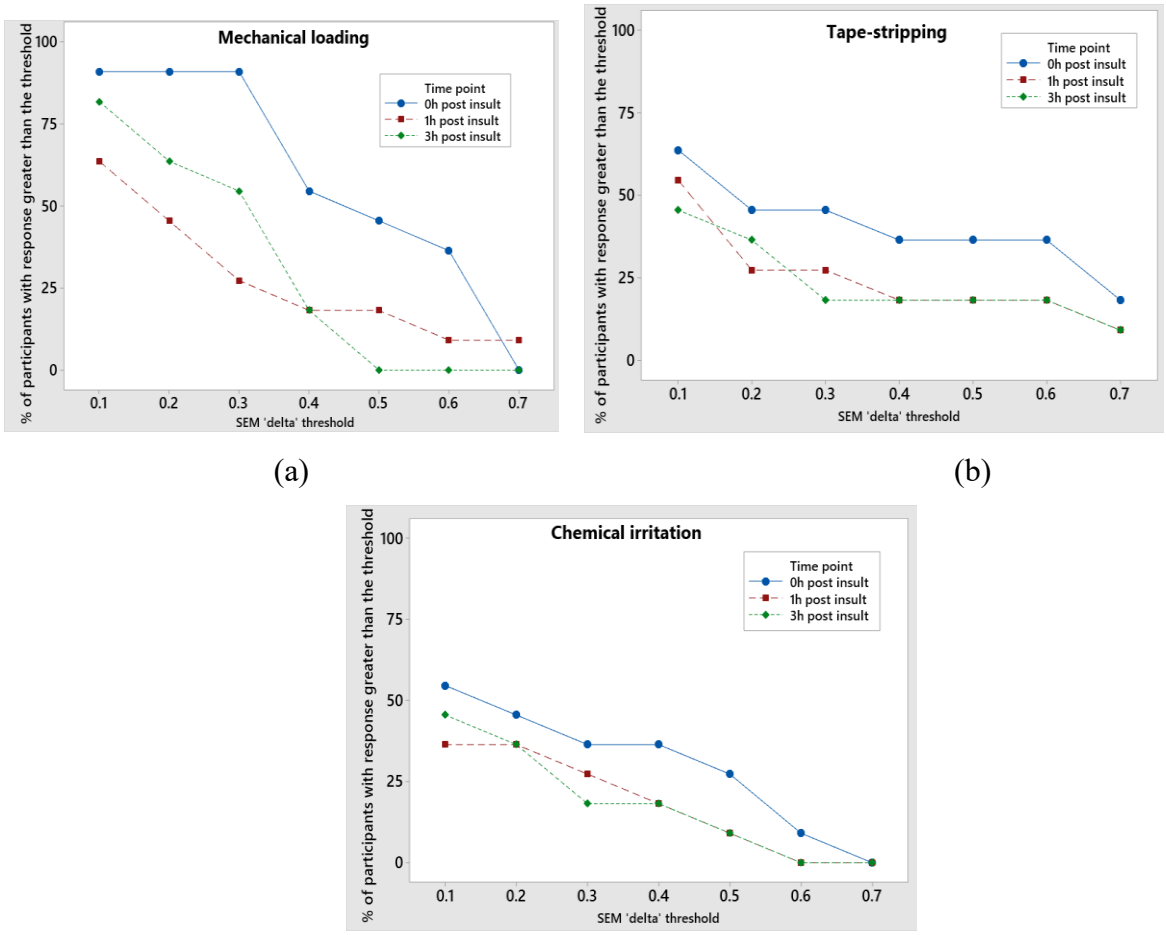


Figure 4-6: Sensitivity analysis of SEM 'delta' parameter following mechanical loading, tape-stripping and chemical irritation.

Associations between the different biophysical parameters revealed statistically significant trends (Table 4-4). Following tape-stripping and chemical irritation, TEWL and SEM 'delta' parameter were found to be significantly correlated at each of the three time points. In addition, TEWL was also significantly correlated with LDI parameter immediately following tape-stripping, whereas these parameters were statistically correlated at 1h and 3h following chemical irritation.

Table 4-4: Summary of significant correlation coefficients between skin output parameters (ns – not significant, * -p<0.05, ** - p<0.01, *** - p< 0.001)

Insult	Parameter	Parameter	Correlation coefficient		
			Immediately after insult (0h)	1 h post-insult (1h)	3 h post-insult (3h)
Tape-stripping	TEWL	SEM	0.72*	0.65*	0.70*
	TEWL	LDI	0.64*	ns	ns
Chemical irritation	TEWL	SEM	0.62*	0.87***	0.78**
	TEWL	LDI	ns	0.62*	0.78**
Saturated water pad	TEWL	LDI	ns	ns	0.61*

Bubble plots were created by integrating parameters presenting with significant correlation, namely, TEWL, SEM and LDI, in order to compare characteristics of individuals to a composite biophysical response (Figure 4-7). Although, a sub-group of individuals exhibited an elevated response for all three parameters throughout the test period, there were no clear influences of BMI and age.

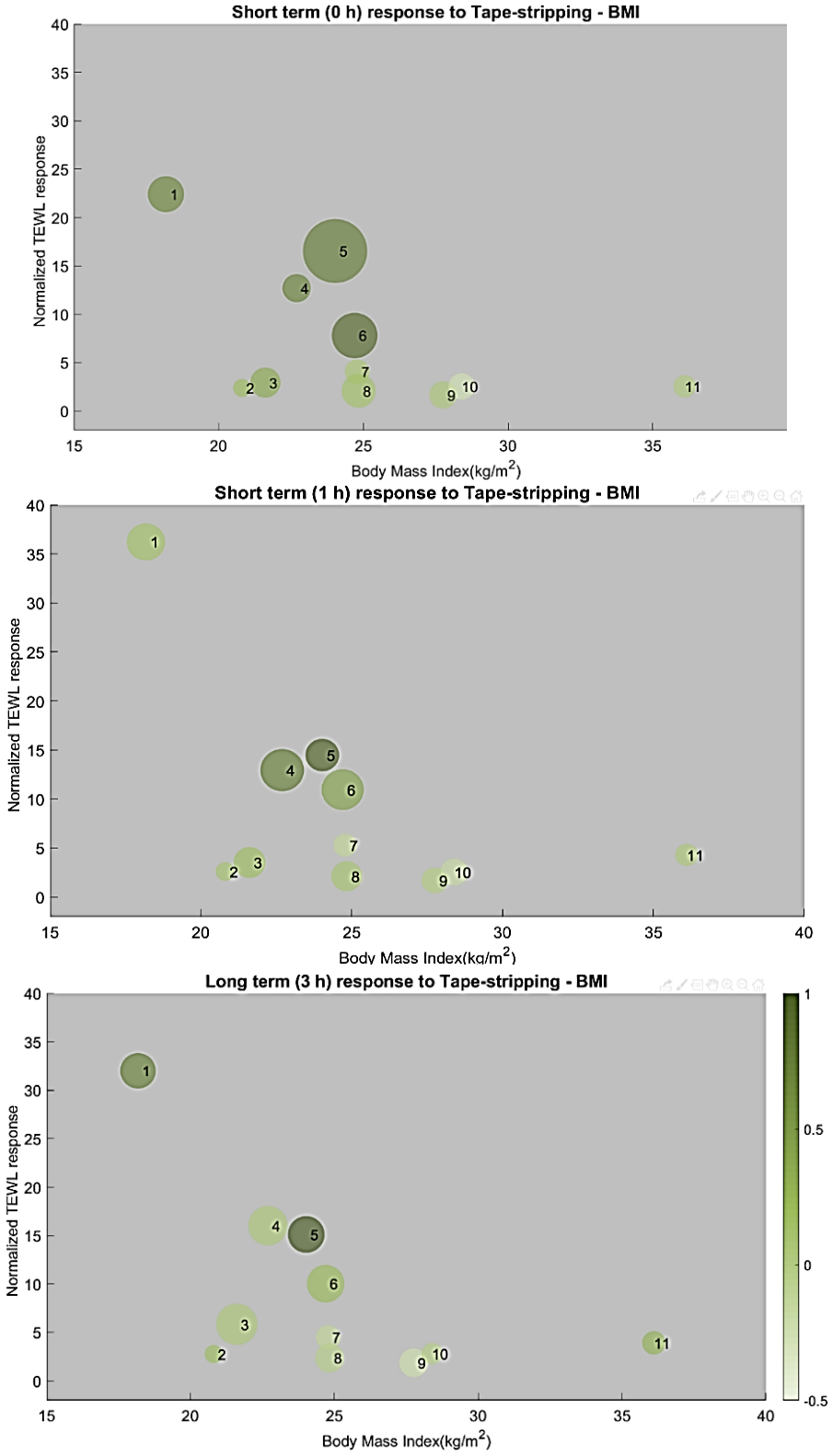


Figure 4-7: Representative bubble plot depicting BMI on the x-axis and normalised TEWL response on y-axis. The size of each bubble is proportional to normalised LDI response, and the bubble colour is given by SEM delta response. The data labels on the bubble indicate the participant IDs. (a) Immediately after the insult (0 h), (b) 1h post insult and (c) 3 h post insult (3h)

The findings of this study revealed differences in temporal response of the biophysical parameters depending on the type and degree of the insult. With the exception of TEWL, the other parameters, namely SEM, LDI and erythema revealed considerable variability limiting clear delineation of responses. It is also clear that thresholds indicative of skin changes lacked sensitivity in a number of the parameters, for example, the SEM 'delta' parameter affected by both time and threshold magnitude.

4.3 Biochemical markers

Skin surface sampling could be achieved through various methods, namely, tape-stripping, cyanoacrylate skin surface stripping, skin blotting, reverse iontophoresis, transdermal analysis patch (TAP) and commercial adhesive tapes for sebum collection (Perkins, Osterhues et al. 2001, Lee, Carson et al. 2009, Minematsu, Horii et al. 2014, Piérard, Piérard-Franchimont et al. 2014, Clausen, Slotved et al. 2016, Falcone, Spee et al. 2017, Nakai, Minematsu et al. 2022). These different techniques are critically appraised in Table 4-5. Based on the advantage and disadvantages, it was decided to employ commercial tapes for sampling biofluid from the skin surface. In this study, the difference in the protein uptake from skin surface using Sebutapes and D-Squame tapes were investigated.

Table 4-5: Critique on different skin sampling techniques

Method	Advantage	Disadvantage
Tape-stripping	1) Easy sampling 2) Useful for sampling corneocytes, protein markers and metabolites 3) Proteins are found in abundance for quantification	1) Minimally invasive 2) High protein could be a result of stripping corneocytes 3) Trigger inflammatory response
Cyanoacrylate skin surface stripping (CSSS)	1) Useful for the analysis of follicular contents 2) Proteins are found in abundance for quantification	1) Invasive especially in hairy areas 2) Inadequate quality in case of erratic contact of glue
Skin blotting	1) Non invasive 2) Inexpensive	1) Semi-quantitative 2) Depends on various factors such as the curvature of the region 3) Can only be used to analyse proteins
Reverse iontophoresis	1) Useful for non-invasive monitoring	1) Prolonged exposure of skin to electrical current 2) Requires specialised technology development for different markers
Transdermal analysis patch (TAP)	1) Non-invasive 2) Quantitative	1) Expensive 2) Can be employed only for a selective list of markers
Lipophilic tapes **e.g., Sebutape**	1) Non-invasive 2) Inexpensive 3) Quantitative 4) Can collect proteins and metabolites	1) Low amount of biomarkers collected as no cells are collected.

The process for sampling sebum as a biofluid to assess skin inflammatory markers was first established in 2001 (Perkins et al 2001). The methodology was originally designed to assess inflammatory changes in the skin following in uncompromised skin, as well as to compromised (diaper or heat rash), chemically treated (sodium lauryl sulfate), or sun-exposed skin. However, there remains questions regarding the efficiency of the approach to extract sebum as a biofluid and the selection of the corresponding biomarkers most relevant to specific skin insults (Section 2.3.2.4). To this effect, a combination of spiked samples from synthetic sebum and human sebum samples were employed in this study to evaluate and optimise the efficiency of the extraction method. Synthetic sebum was prepared as

described by a previous protocol (Lu, Valiveti et al. 2009). The composition of the synthetic sebum is detailed in Table 4-6. Subsequently a systematic study was designed to improve the extraction efficiency of Sebutapes and analyse the factors associated with changes in the corresponding biomarkers (Chapter 5).

Table 4-6:Composition of sebum (Weight of components required for a total of 5 g of sebum) (Lu, Valiveti et al. 2009)

Compound	Manufacturer	Mass (g)	Weight (%)
Squalene	MP Biomedicals	0.75	15
Paraffin wax	Aldrich	0.5	10
Jojoba oil (Spermaceti)	Sargent-Welch	0.75	15
Olive oil	EMD Chemicals	0.5	10
Coconut oil	Aldon Corporation	0.5	10
Cottonseed oil	MP Biomedicals	1.25	25
Oleic acid	Aldrich	0.07	1.4
Palmitoleic acid	MP Biomedicals	0.25	5
Palmitic acid	EMD Chemicals	0.25	5
Cholesterol	Sigma Aldrich	0.06	1.2
Cholesterol oleate	Tokyo Kasei Kogyo Co.,	0.12	2.4

A series of steps were involved in the analysis of biochemical markers from the skin, including the collection, extraction and the analytical technique, as shown schematically in Figure 4-8. In addition to the individual steps, this section includes details of the normalization technique employed and the methods to analyse the biofluid.

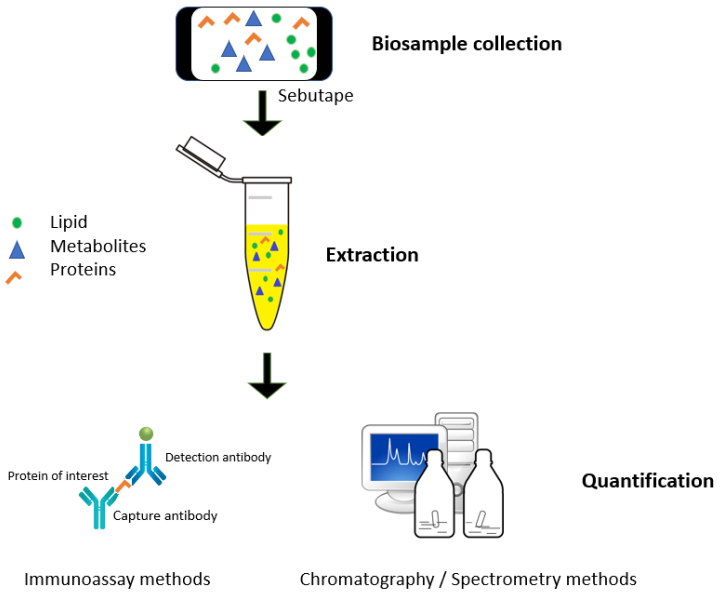
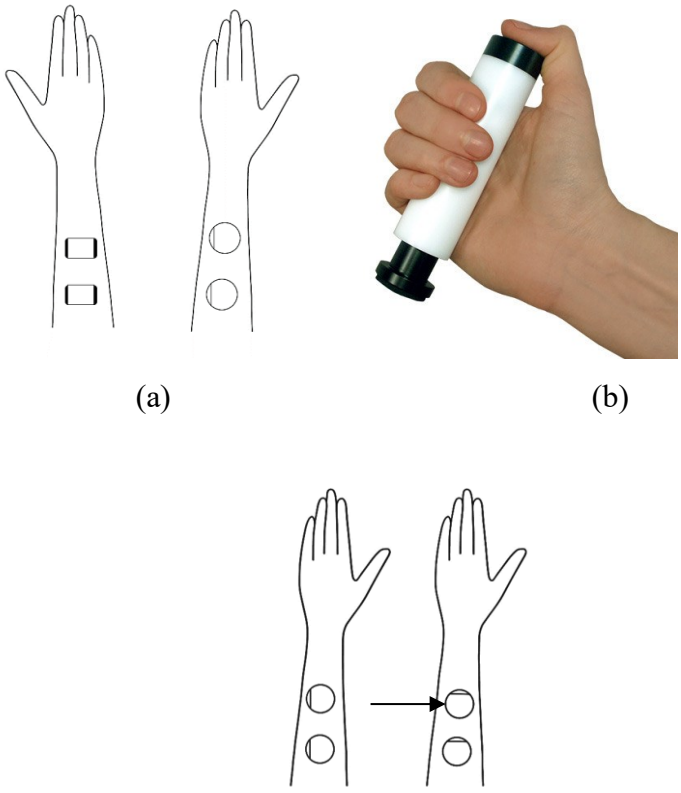


Figure 4-8: Schematic of processes involved in the analysis of biochemical markers.

4.3.1 Collection of protein using different adhesive tapes

To evaluate the difference in protein uptake with adhesive tapes, namely Sebutapes and D-Squame, tape samples were analysed for IL-1α and soluble protein content. Two sets of Sebutapes and D-Squame tapes were collected randomly from either the right or left forearm (Figure 4-9). One set of Sebutapes and D-Squame tapes were extracted for analysis of a representative inflammatory cytokine, IL-1α, and soluble protein content. The other set of tapes were analysed for RNA as potential biomarkers. The preliminary panel of mRNAs analysed include Filaggrin, Involucrin, IL-1α and IL-8.



(a) (b)

(c)

Figure 4-9(a) Schematic of sample collection from the volar aspects of forearm. Each of the forearm was randomized for the application of Sebutapes and D-Squame (b) Spring-loaded pressure device for D-Squame, as provided by manufacturer (c) Re-orientation of D-Squame to ensure complete saturation of tapes

In this pilot study, a cohort of 6 healthy volunteers were recruited at Essity, Sweden (as a part of a secondment) and the measurements were carried out in a laboratory environment controlled at a temperature of 23 ±2°C and a relative humidity of 42±6%. Ethics approval was received from the company and informed consent was received from the participants. Prior to collection of tapes, the skin surface was gently blotted to remove any moisture or contaminants present on the surface, including sweat. Different application techniques for the tapes were employed according to manufacturer recommendations. Sebutapes were placed on the skin for 2 minutes using blunt forceps and a roller was gently rolled five times over the Sebutapes to ensure complete contact with the skin surface. D-Squame was applied using blunt forceps and uniform pressure was applied for five seconds using a spring-loaded device as provided by the manufacturer to ensure complete adhesion (Figure 4-9). The tapes were re-oriented to 90^{o} and re-applied in the same location to ensure maximum absorption

of protein by the tapes (Figure 4-9c)]. Sebutapes and D-Squames collected from the participants were stored at -80°C until analysis. The tapes were extracted using a standardized protocol adapted for use of D-Squame (Perkins, Cardin et al. 2002), as described in Figure 4-10.

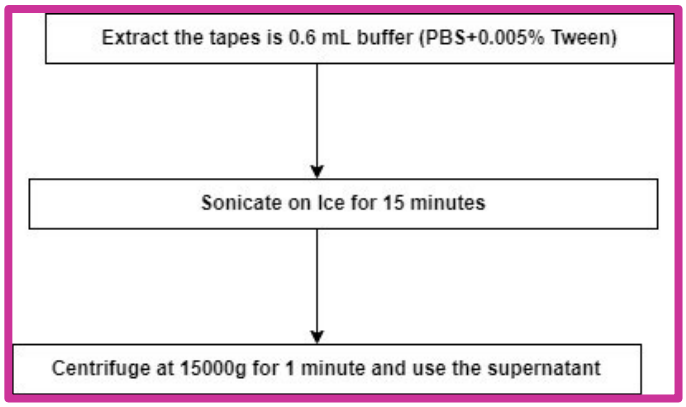


Figure 4-10: Schematic of the extraction process followed for both Sebutapes and D-Squames

Concentrations of IL-1α and protein content extracted from Sebutapes and D-Squame tapes are illustrated in Figure 4-11. It is clear that D-Squame was able to collect increased amounts of inflammatory marker, IL-1α, as well as protein content when compared to Sebutapes. With respect to the gene markers, the concentration of RNA was below the limit of detection for further analysis, for both D-Squame as well as Sebutape.

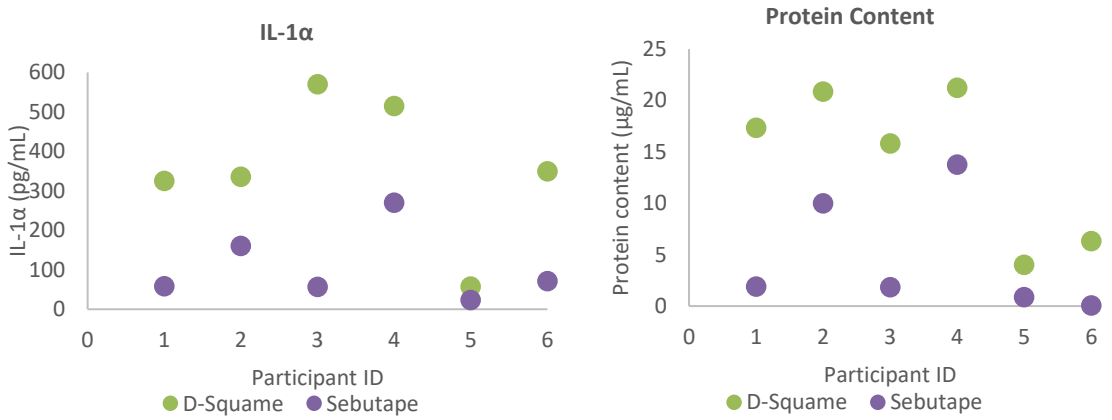


Figure 4-11: Concentration of (a) IL-1α and (b) Protein content extracted from different commercial sampling tapes, namely, D-Squame and Sebutape

It is to be noted that in this study, the concentration of proteins in supernatants were investigated. As centrifugation is a process of relative separation based on molecular

weights, the analysis of supernatants with D-Squame might have warranted efficient analysis of proteins owing to the collection of high molecular weight of cell debris. However, the usefulness of sampling supernatants with Sebutapes is yet to be investigated. Despite the increased concentrations of protein collected from D-Squame, it has been reported that the use of D-Squame disrupts the skin barrier function, as measured using TEWL, unlike Sebutapes that has no influence on the skin barrier function (Clarys, Lambrecht et al. 1997, Breternitz, Flach et al. 2007). Therefore, in studies involving vulnerable participants and repeated sampling, Sebutapes offer non-invasive means to investigate the inflammatory processes. Indeed, Sebutape™ (Cuderm Corporation, USA) which are lipophilic in nature have been extensively used to collect biomarkers as well as lipids from the skin surface (Perkins, Osterhues et al. 2001, Fabbrocini, Cameli et al. 2014, Soetens, Worsley et al. 2019). However, it is to be noted that Sebutapes are not suitable for the analysis of gene markers or very low-abundance markers. The patches are 28.58 mm x 19.05 mm in size, with a black tabbing material at the edges, as shown in Figure 4-12.

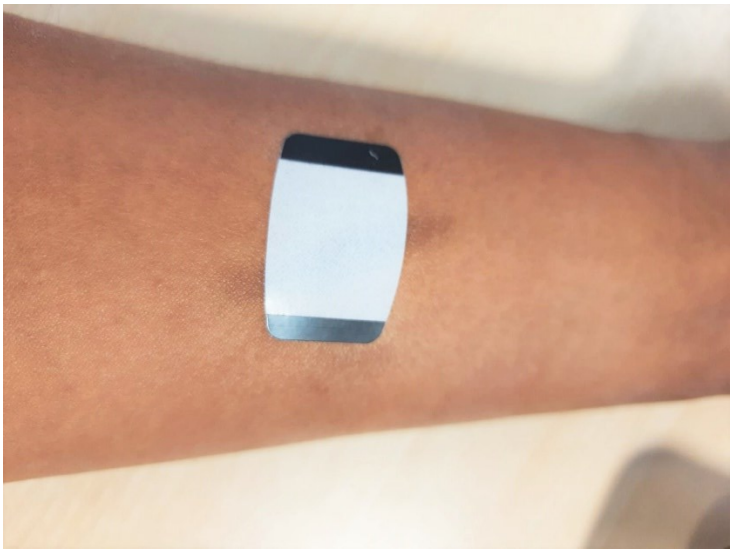


Figure 4-12: Representative image of Sebutape™ applied on the forearm

4.3.2 Extraction methodology

In the present studies, as Sebutapes were used for the inflammatory analysis, the extraction methodology employed in the studies are described. Sample preparation was based on a previously detailed protocol (Perkins, Osterhues et al. 2001). To review briefly, the tapes were extracted in 1 mL of phosphate-buffered saline (PBS) with 0.05% Tween as a surfactant. Typically, PBS is employed as a buffer for proteins due to their buffering capacity

and their stabilizing capacity. On the other hand, increased salt concentrations could lead to potential aggregation of proteins (Pavani, Kumar et al. 2021). Each tape was kept in solution for a period of 1 hr and then sonicated for 10 minutes at 20^{o}C followed by a brief vortexing for 2 minutes. After removing the tapes, the extracted solution was either used immediately or aliquoted at -80^{o}C for further analysis. Indeed, it should be noted that there is a combination of factors, including chemical and mechanical factors, that could aid in enhancing the extraction of biomarkers from the tape. As an example, the type and concentration of detergent is instrumental in maintaining the protein structure in solution whereas mechanical stimuli such as shaking, and centrifugation are essential in the extraction of proteins from the tape. Further studies were conducted with spiked samples in synthetic sebum to investigate the influence of these parameters on the extraction efficiency will be detailed in Chapter 5.

4.3.3 Analysis methodology

Following the extraction of the sebum, promising analytical techniques are required to measure the concentration of biomarkers. Such sensitive analytical methods could include techniques such as ELISA (Enzyme-Linked Immunosorbent Assays) and HPLC (High-Performance Liquid Chromatography)

4.3.3.1 ELISA

In the present work inflammatory markers, including the cytokines such as IL-1α, IL-1RA and IL-6, were quantified through conventional ELISA and electro-chemiluminescent (ECL) multiplex assays. The ELISA and ECL multiplex assays follow the same principle, although they differ in their detection methodology. A schematic of the ELISA technique is illustrated in Figure 4-13 and is briefly detailed as follows:

1) The plates were coated with a capture antibody that has high specificity for the target protein i.e., the analyte. Commercial manufacturers also provide coated plates wherein the capture antibody has already been coated.
2) A defined volume of standard, as prescribed by the ELISA kit manufacturer, was pipetted onto the wells of the plates.
3) The plates were washed three times in between each step with a wash buffer to prevent any non-specific binding

4) The detection antibody was then added to the plates. In the case of a conventional ELISA, the detection antibody is a complex that is conjugated with an enzyme, for example, horse-radish peroxidase (HRP) or fluorescent tags such as Sulfo-Tag.
5) After incubation with the detection antibody and washing of the plates, the substrate/read buffer was then added. The substrate reacts with the enzyme leading to a colour formation, that is proportional to the quantity of analyte, that is then read by a spectrophotometer. In the case of the ECL kits, the plate reader applies an electric current across the electrodes situated at each well. This leads to the emission of light by the Sulfo-Tag labels. The intensity of the emitted lighted was then measured by a sensitive charge-coupled device (CCD) camera.
6) Calibration curves from the software were obtained and the data from the samples were then overlaid on the calibration curve to ensure that they corresponded to the linear region of the calibration curve and above the detection limits. This report was then exported and saved for further analysis.

Owing to the expensive cost of the multiplex plates, some of the preliminary investigations in the present work employed the use of conventional ELISA technique, while in the latter investigations the multiplex ECL technique was used.

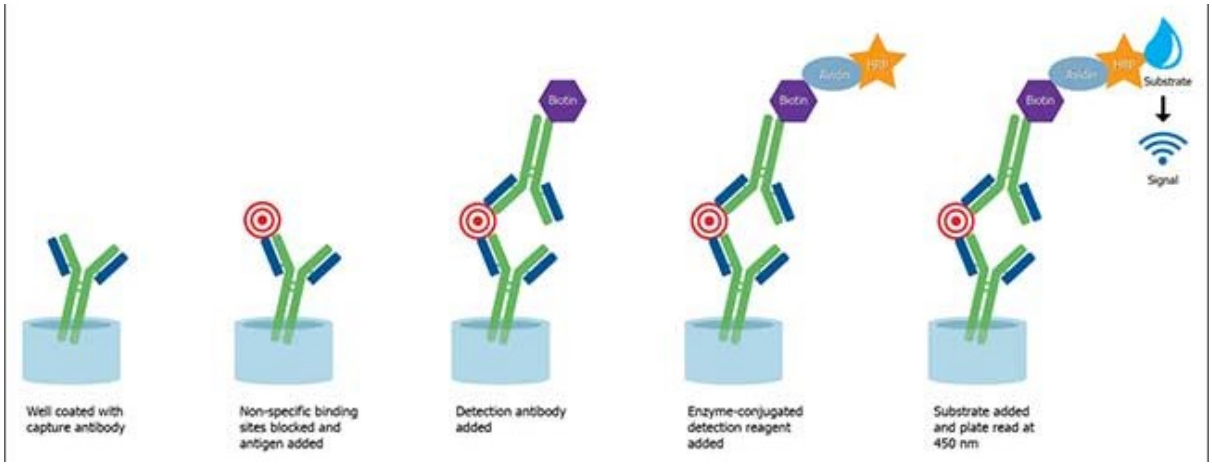


Figure 4-13: Schematic of a conventional ELISA technique (Source : Optofluidic bioassay)

4.3.3.2 HPLC

High-Performance liquid chromatography (HPLC) is a versatile analytical technique that has commonly been employed to separate, identify and measure the amount of analyte present in a sample. The technique involves two phases, namely the mobile phase and the stationary phase. The mobile phase is a solvent mixture, which plays an important role of transporting

the analyte solution to the stationary phase and the detector. Typically, the stationary phase is a column packed with porous solids, such as glass, silica or alumina. Due to the difference in the polarity of the individual components present in the analyte solution with the mobile and stationary phases, they spend different times in the separation column. This difference in the retention times lead to the separation of the constituents of the sample solution. This technique is highly sensitive but involves complex sample preparation and analysis steps (Vogeser and Seger 2008). Previous research studies conducted in the host laboratory have employed chromatographic techniques to measure the quantity of metabolites, namely lactate and pyruvate, from both sebum and sweat collected at the skin surface (Soetens, Worsley et al. 2019, Herniman, Worsley et al. 2022). This method offers a potential to detect metabolites, however, the analysis of markers is still not fully established.

4.3.4 Normalization methods

Normalization steps are important in order to account for variations in levels of molecules (metabolites/proteins) recovered from each Sebutape^{TM} as well as the differences in different anatomical regions (Soetens, Worsley at al. 2019). For example, concentrations of biomarkers recovered from Sebutape^{TM} have previously been reported by normalizing the amount of biomarker to the total amount of protein on the tape (Perkins, Osterhues et al. 2001). Indeed, the constituents of the tape could be analysed for soluble protein as well as total protein using microBCA and Bradford assay, respectively (Bradford 1976, Smith, Krohn et al. 1985). Owing to the ease of use, the stability of the protein-dye complex and its high reproducibility, the use of Bradford assay is typically employed for measuring the total protein content (Bonjoch and Tamayo 2001). Total protein content measured using the Bradford assay kit (Pierce, Coomassie, USA), is based on the colour change from brown to blue when the acidic reagent reacts with protein present in the sample. The intensity of the blue end product is proportional to the concentration of protein (Bradford 1976). In longitudinal studies, normalization of time points to baseline has been reported to allow for reliable analysis providing a relative change i.e., up/down regulation of specific biomarkers over a given site (Worsley, Prudden et al. 2016, Soetens, Worsley et al. 2019). Although a number of normalization approaches exist, there has been no universal normalization methods that have been reported to be efficient (Qin, Kim et al. 2012, O'Rourke, Town et al. 2019). Therefore, it is important to investigate different normalization approaches based on the study protocol as well as the aims of the study.

4.3.5 Sebum quantification – SPV assay

Given the varying nature of the density and productivity of sebaceous glands there is a need to quantify how much sebum is recovered on tapes from different skin sites. To quantify the sebum levels, Sulfo-Phospho-Vanillin (SPV) assay was employed. The assay is a colorimetric technique that has typically been used to quantify the amount of lipids in samples ranging from microalgae to human serum (Knight, Anderson et al. 1972, Pinger, Copeman et al. 2022). The assay is particularly appropriate for use with sebum with its high lipid content (Ashraf, Pasha et al. 2011). The SPV assay is performed in three steps, with the first step involving extraction of the lipids using an appropriate solvent, followed by the reaction of lipids with concentrated sulfuric acid at high temperature of 95ºC. The final step involves the reaction of the derived products with vanillin in the presence of phosphoric acid. This assay is based on the formation of a stable carbonium ion in the first step, which then leads to the generation of a pink chromophore that is obtained on adding vanillin. Previous studies employing the SPV assay in Sebutapes are limited by the time required for quantifying large number of samples (Ashraf, Pasha et al. 2011). Therefore, the assay has been adapted to enable quantification of large number of Sebutape samples at a time by conducting the analysis using a 96-well microplate (McMahon, Lu et al. 2013). In the present work, the volumes were doubled to ensure sufficient quantity for running duplicates. The protocol involved:-

1) Each Sebutape™ was placed in a scintillating glass vial and covered in 2 mL of tetrahydrofuran and methanol (3:1 v/v) mixture.
2) The contents were subjected to vigorous shaking and sonicated briefly in a nitrogen fume hood.
3) Sebutape™ was removed from the vials using sterile forceps and the contents of the vial were dried using a rotary evaporator for a period of 30 minutes at 40ºC (CentriVap evaporator, LabConco, USA). In the case of any remaining solvents in the vial, the evaporation was carried out for further 10 minutes until the vials are dry. The evaporator system is illustrated in Figure 4-14
4) Subsequently, 240 µL of 95% sulfuric acid (Sigma Aldrich, UK) was added to the vial and the contents were heated using a block heater at 95ºC for a period of 20 minutes.
5) The reaction mixture was rapidly cooled by placing the tubes on an ice pack, following which the vial was vortexed briefly.

6) 100 µL of sample was aliquoted into 96-well plates in duplicate and the initial pre-vanillin absorbance was measured at 535 nm using a microplate reader (SpectraMax, Molecular Diagnostics, USA)
7) 50 µL of 0.2 mg/mL vanillin (Sigma Aldrich, UK) in 17% aqueous phosphoric acid (Sigma Aldrich, UK) was added to each of the microwells
8) The microplate was incubated for 10 minutes at room temperature in dark conditions.
9) The post-vanillin absorbance was measured at 535 nm using the microplate reader and the difference between post- and pre-vanillin absorbances was calculated. The mean absorbance from the blanks was subtracted from the readings to correct for the background signal

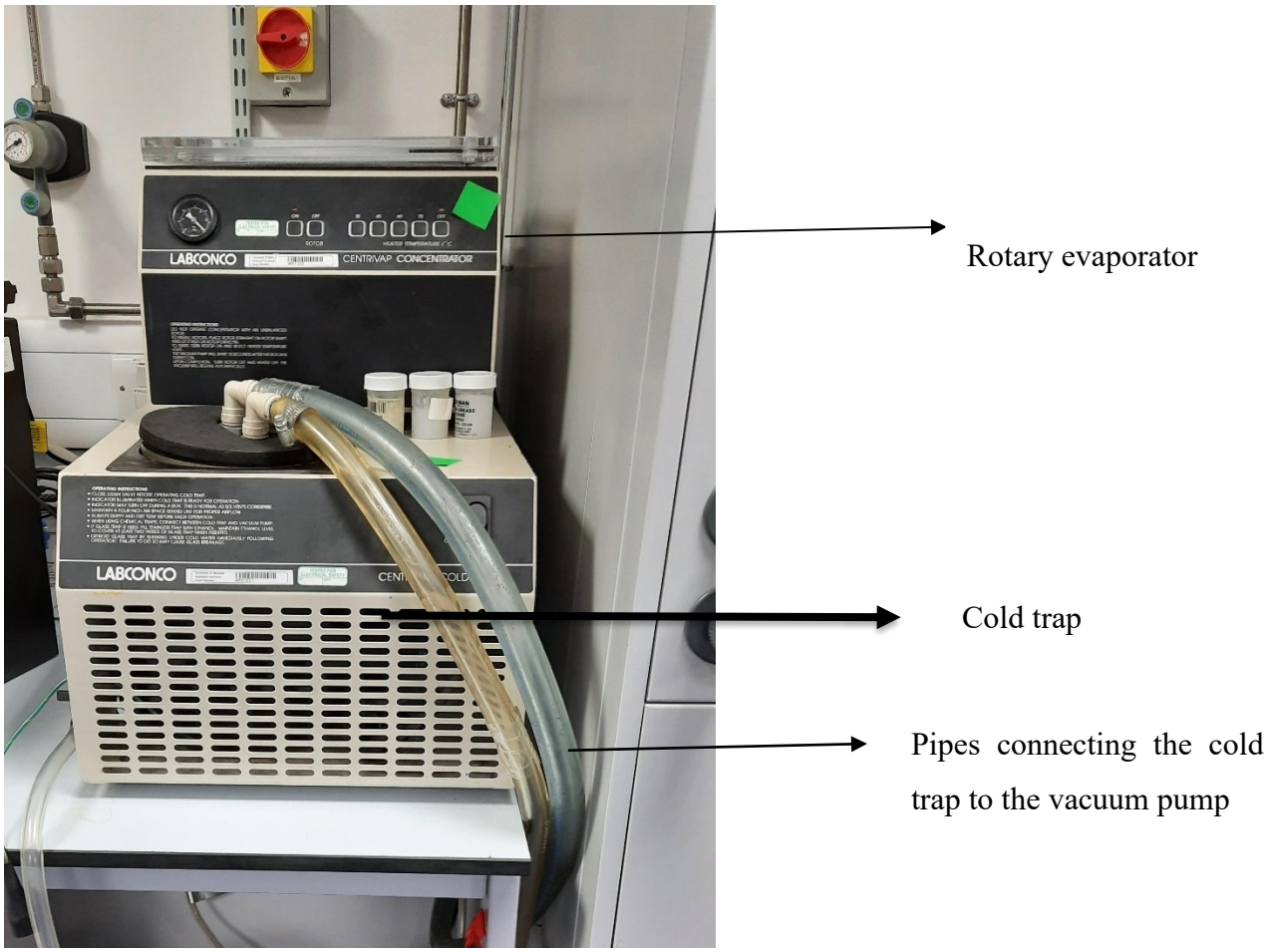


Figure 4-14: Evaporator arrangement for drying the solvent from the vials

Further work was undertaken to adapt the SPV assay for measuring the amount of sebum collected using Sebutape™. This involved the use of synthetic sebum as a standard and was prepared as described before (Table 4-6). It was important to assess the use of Sebutape™ with organic solvents. Accordingly, Sebutape™ was immersed in tetrahydrofuran and

methanol solvent mixture (3:1 v/v) for a period of 20 mins and after vigorous shaking was removed. This resulted in degradation of the black tabbing part of the Sebutape™, as illustrated in the Figure 4-15. Therefore, for analyses involving the use of organic solvents, the black tabbing material of the Sebutape™ was removed by cutting before each experiment.

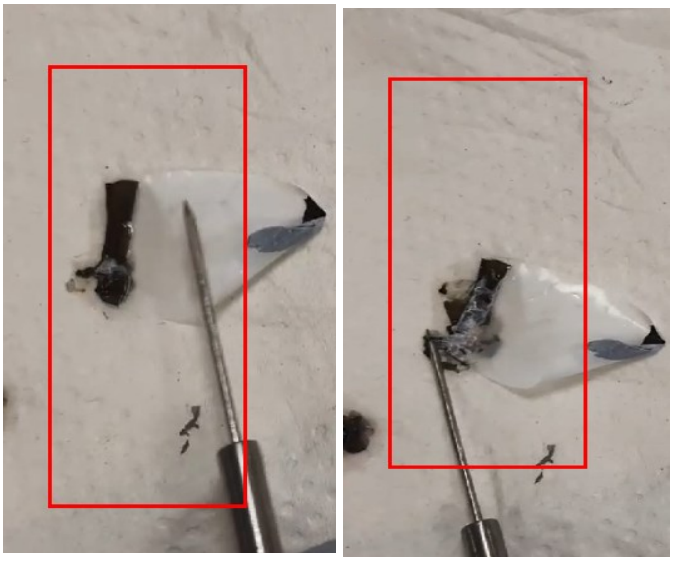


Figure 4-15: Degradation of the black tabbing material when Sebutape™ was extracted using organic solvents

Preliminary experiments were conducted to investigate two parameters, namely the effect of sulfuric acid concentration on the assay and the extraction of lipids from the Sebutape™. A range of concentrations of sulfuric acid, from 75% to 95%, were used in the previously described protocol. These high concentrations range had been reported to be optimal for the assay performance (Knight, Anderson et al. 1972). To examine lipid extraction using the solvents, two sets of controls were used. In the first set, known amounts of synthetic sebum (1 µL) were coated on the Sebutape™ while the other set of controls involved the use of identical amounts of synthetic sebum in the absence of the Sebutape™.

The results, as illustrated in Figure 4-16, indicate that for standards coated on to the Sebutape™, the post-vanillin absorbance values were ranging from 0 to 4, for samples involving higher concentrations of sulfuric acid, such as 85% and 95%. It is to be noted that an absorbance value of 4 is the maximum value that could be read by the spectrometer, therefore revealing saturation. Accordingly, a sulfuric acid concentration of 75% was selected as the optimum for further studies. However, it was also noted that the standards

without Sebutape™ revealed high absorbance values irrespective of the concentration of sulfuric acid (Figure 4-16). This also suggests that the lipids coated in the Sebutape™ were not completely extracted with the solvents.

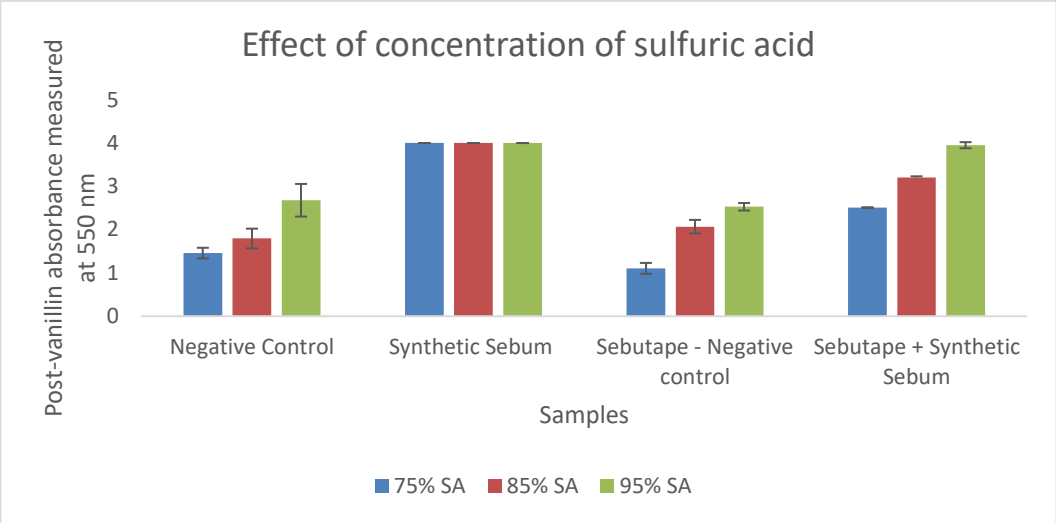


Figure 4-16: Bar chart illustrating the effect of concentration of sulfuric acid on the post vanillin absorbance

4.4 Chapter Summary

In this chapter, a retrospective evaluation of biophysical parameters and the preliminary investigations for the measurement of biochemical markers were detailed. With respect to biophysical parameters, it is clear that there are anatomical differences with Sub-epidermal moisture (SEM) (Section 4.1). Moreover, single biophysical parameters were deemed insufficient to differentiate skin insults (Section 4.2). Similar investigations with biochemical parameters are critical for estimating the performance of biomarkers in diagnosing early changes in skin health. Indeed, combination of biophysical and biochemical parameters could provide a comprehensive understanding of skin health and therefore aid in reducing the incidence of PUs or IAD by enabling early diagnosis. Accordingly, prospective studies investigating variability of biochemical parameters with respect to different anatomical locations and insult models will be detailed in Chapter 5 and 6, respectively. A summary of the methods and parameters employed for the analysis of biochemical parameters are detailed in Table 4-7.

Table 4-7: Summary of the methods and parameters employed in the current study

Methods	Parameters employed in this study
Biofluid	Sebum
Biofluid analysis	SPV assay
Biomarker sampling	Sebutape™
Biochemical marker analytical technique	ELISA Multiplex assays
Normalization	Total Protein normalization Baseline normalization

Chapter 5 Optimization and method development of biochemical analysis

Biochemical markers, such as proteins and metabolites, are present in varying concentrations in a range of biofluids (Xu and Veenstra 2008). In order to investigate the potential of different biomarker candidates in skin damage, it is imperative to study the spatial and temporal profiles in a range of skin damage models. However, some biomarkers are inherently found in relatively small concentration depending on the biofluid of interest (Gramolini, Lau et al. 2016). Therefore, this chapter describes the development of methodology and protocols to collect and extract protein biomarkers from the skin surface efficiently.

Although several skin biofluid sampling approaches exist, as described in Chapter 3, only few methods offer non-invasive sampling from the skin surface without damaging the skin barrier. Indeed, Sebutapes have been employed over several decades to non-invasively collect sebum from the skin surface to examine the concentration of potential biomarkers in various conditions such as acne, dermatitis and pressure ulcers (Perkins, Cardin et al. 2002, de Wert, Bader et al. 2015, Hemmes, de Wert et al. 2017, Bostan, Worsley et al. 2019). However, the amount of sebum collected at various anatomical locations has not been studied, despite the well-defined variations in sebaceous gland density and productivity. Moreover, the time taken by the skin to regenerate sebum has not been investigated. The extraction of inflammatory markers from Sebutape has generally been performed using a standard protocol developed two decades ago (Perkins, Cardin et al. 2002). The efficiency of the extraction method has not been fully reported. Previous studies have traditionally reported only high-abundance proteins, namely, IL-1α and IL-1RA, in participant cohorts subjected to a range of external insults. By contrast, other low-abundance cytokines, namely TNF-α, IL-6 and IL-8 are often cited as being below the limit of detection from commercial ELISA kits (Koudounas, Bader et al. 2021). Therefore, the potential of these low-abundance cytokines as biomarkers have rarely been investigated. However, the inflammatory signally process is represented by both high and low-abundance markers, requiring an array of biomarker analysis to explore the phenomenon of local skin inflammation.

5.1 Aims and Objectives

This study aimed to optimize the protein extraction process to measure low-abundance cytokines with the use of chemical and mechanical stimuli. In addition, a study was conducted to investigate the temporal profile of sebum collected at various locations in a cohort of participants. This will be achieved through the following objectives:

1) To analyse factors affecting sebum sampling involving the temporal release of sebum at relevant body sites (Study 5-1). This will involve:
    a. Quantifying the sebum collected at relevant body locations in a cohort of healthy volunteers
    b. Monitoring the temporal release of sebum and identify the optimal time points for biofluid collection
2) To optimise the extraction protocol by evaluating the influence of extraction parameters, including chemical and mechanical stimuli on the extraction efficiency (Study 5-2). This will involve:
    a. Assessing the influence of chemical reagents, including the type and concentration of detergent, on the extraction efficiency of a range of cytokines
    b. Assessing different mechanical stimuli, such as shaking and centrifugation as well as the extraction volume on the extraction efficiency of a range of cytokines

5.2 Materials and methods

A series of studies involving human sebum sampling and synthetic sebum was conducted to achieve the aims.

5.2.1 Study 5-1: Analysis of factors affecting Sebum sampling

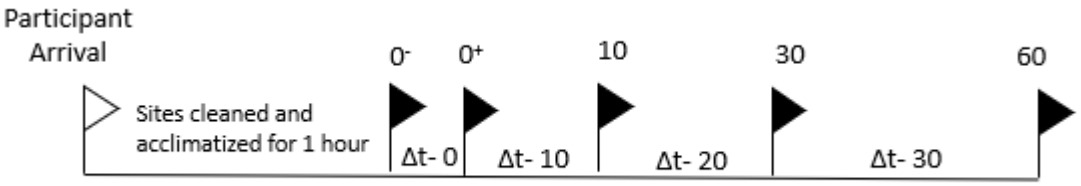
In this thesis, we aim to investigate potential biomarkers by sampling skin surface sebum using Sebutapes. It is well known that the amount and composition of sebum secreted is a function of the anatomical site, due to difference in sebaceous gland density, as well as the characteristics of the individual, such as age, gender and their harmonal changes (Kim, Choi et al. 2006, Ludovici, Kozul et al. 2018). Moreover, for experiments involving subsequent sample collection from the same investigation site, optimal time points are to be identified

ensuring minimal variability in the collected biofluid amount. In order to study the variabilities associated with different anatomical locations and varying time points on sebum release, a pilot study was conducted. The quantification of sebum collected with Sebutapes was carried out using a chemical colorimetric assay i.e., Sulfo-phospho-vanillin (SPV) assay.

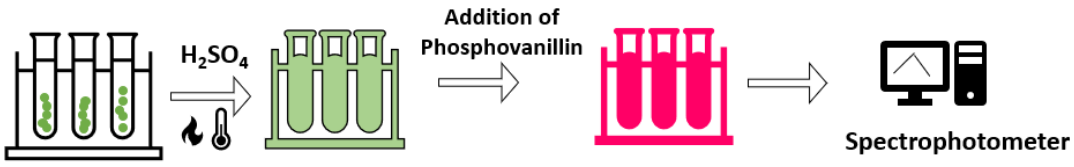
5.2.1.1 Sebum collection

All the measurements were carried out in the Biomechanics Testing Laboratory in the Clinical Academic Facility in Southampton General Hospital, with the environment controlled at a temperature of 23 ±2°C and a relative humidity of 42±6%. Institutional ethics were granted for the study (ERGO-FoHS-54362). Exclusion criteria included a history of skin-related conditions that could affect sebum sampling. Participants were requested to wear comfortable loose-fitting clothing when attending the data collection sessions and were asked not to apply moistening products prior to sample collection. Each of the measurements was performed with the participant in a sitting position. In this pilot study, samples of sebum were collected from 5 healthy volunteers at 6 different locations, namely the forehead, nasal bridge, forearm, sacrum, coccyx and the plantar/posterior of the heel (Figure 5-1a). The sites, forehead and nasal bridge falls under the widely known T-zone where high sebum levels are typically reported, whereas forearm has been commonly employed as test site owing to ease of access (Bazin and Fanchon 2006, Okoro, Bulus et al. 2016). The other sites of investigation, such as sacrum, coccyx and the heel are most commonly reported locations of chronic wounds (Bhattacharya and Mishra 2015).

(a)



(b)



(c)

Figure 5-1: (a) Representative image of locations from which Sebutapes were collected for lipid analysis (b) Timeline of sample collection from the skin sites and (c) Schematic of SPV assay employed for lipid analysis

The schematic of the protocol for sample collection is illustrated in Figure 5-1b. To review briefly, each of the site was marked with a non-permanent marker for accurate placement.

Prior to sampling, the sites were cleaned with 70% isopropyl alcohol injection wipes to remove any build-up of sebum and dust on the body sites. The black ends of the Sebutape known as the tabbing ends were cut before collecting sebum. The pre-cut Sebutapes were carefully placed on the skin for 2 minutes using blunt forceps. Sebum was collected at 5 time points from each of the six locations. The baseline measurements were collected from each site after 1 hour from when the sites were cleaned (0^- min). Subsequent collections were performed immediately at (0^+ min), 10 minutes (10 min), 30 minutes (30 min) and 60 minutes (60 min) to evaluate the temporal release of sebum from each site. The samples at 0^- and 0^+ are collected consecutively with no time gaps. The sample at 0^- and 0^+ would therefore serve as a reference to measure the build-up of sebum over 1 hour and 0 hour respectively. Upon removal, each Sebutape sample was immediately frozen at -20ºC prior to lipid analysis to ensure stability of the lipids. Alternatively, they can also be stored at -80ºC or snap-frozen with liquid nitrogen.

5.2.1.2 Quantification of sebum lipids

Sebum was quantified using a Sulfo-Phospho-Vanillin (SPV) assay, adapted from literature, to quantify lipids that constitute 99% of sebum (Ashraf, Pasha et al. 2011, McMahon, Lu et al. 2013). Solvents and reagents, namely tetrahydrofuran (THF), chloroform, methanol, sulfuric acid, phosphoric acid and vanillin were purchased from Merck Millipore, UK. Solvent evaporation was carried out using a CentriVap concentrator coupled with a CentriVap cold trap (LabConco Corporation, USA). All the lipids were handled using glass or stainless steel and stored in scintillating glass vials with Teflon-coated caps. An optimized protocol, as described in Section 4.3.5, measuring lipids from Sebutape samples using a microplate reader was employed (Figure 5-1c). To review briefly, 2 mL of tetrahydrofuran and methanol (3:1) mixture was added to each of the Sebutapes in a glass vial. After shaking and sonication, the Sebutapes were removed followed by the evaporation of the solvents. 240 µL of 75% sulfuric acid was then added to each of the vials and heated to 95ºC for 20 minutes. On cooling, 100 µL of the sample solution was aliquoted into the microplates in duplicate and the pre-vanillin absorbance was measured at 535 nm. After the addition of 50 µL of vanillin reagent (0.2 mg/mL in 17% aqueous phosphoric acid) and incubation of the sample for 10 minutes, the post-vanillin absorbance was measured. The difference between the post- and pre-vanillin absorbances were calculated. The SPV reactive lipids, in mg/mL, calculated from the analysis were plotted across the time periods for each of the participants. The constituents of synthetic sebum are detailed in Table 4-6 and synthetic sebum solutions

of known concentrations were prepared in a solvent mixture of Tetrahydrofuran and methanol (3:1) to generate a calibration curve, which was used to estimate the concentrations of the unknown samples.

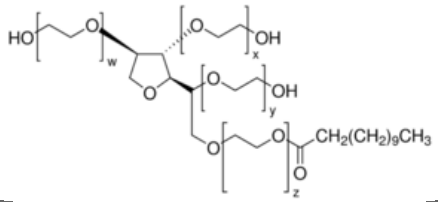
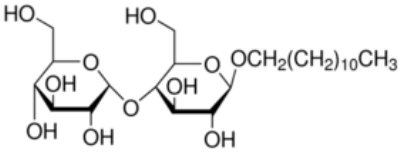
5.2.2 Study 5-2 - Extraction optimization

In this thesis, we aim to investigate the potential biomarker candidates for identifying skin damage. In order to inquire a range of biomarkers, including high-abundance and low-abundance markers, sensitive analysis and efficient extraction procedures are required. Sensitive analysis could be achieved by the use of chemi-luminescence immunoassay kits, which can measure concentrations, up to 1 pg/mL. However, the efficiency of the extraction process has not been studied and optimised. Indeed, external stimuli, including chemical and mechanical stimuli, could be introduced to increase the extraction efficiency of the process. Therefore, in this study, we aim to investigate the influence of different stimuli in a panel of high-abundance and low-abundance cytokines in a systematic study.

To measure the protein concentrations, the proteins must be liberated from the tapes and maintained in a soluble, native, and functional form. The parameters investigated in the study include chemical and mechanical stimuli as well as buffer volume and subsequent extraction process.

Chemical agents e.g., detergents are added to the extraction buffer, to improve the extraction of the proteins. The detergents partition into the biological membranes owing to their amphiphilic nature thereby extracting and maintaining the proteins in solution (Arachea, Sun et al. 2012). Depending on the ionic charge and the degree of hydrophobicity of the detergent, the interaction with membrane proteins is influenced thereby affecting the extraction process. Non-ionic detergents have been reported to extract proteins and prevent their aggregation compared to the ionic detergents (Andersen, Oliveira et al. 2009, Kaspersen, Søndergaard et al. 2017). For example, Dodecyl maltoside (DDM) has been reported to be stabilizing and provide good extraction potential (Arachea, Sun et al. 2012, Mahjoubi, Fazeli et al. 2017). In addition, Tween is a detergent commonly employed in protein extraction studies owing to its cost and stabilizing properties (Kerwin 2008). This has motivated the use of two non-ionic detergents, namely Tween and DDM, in the present set of experiments.

Table 5-1: Properties of detergents employed in this study

Detergent	Structure	Molecular weight	CMC	Concentrations employed
Tween-20		1228	0.06 mM	≈ 5- 15 * CMC
n-dodecyl maltoside (DDM)		510.62	0.15 mM	≈ 5- 15 * CMC

The term *Critical micelle concentration* (CMC) of a detergent represents the minimum concentration required for its molecules to form micelles and encapsulate the protein/lipid molecules (Table 5-1). Higher concentrations have been reported to form aggregates and denature the protein (Palazzo, Lopez et al. 2010, Yang, Wang et al. 2014). Therefore, in the present study, the influence of non-ionic detergents was investigated with working concentrations of each detergent ranging from 5-fold to 15-fold CMC.

5.2.2.1 Reagents and test equipment

An array of selected cytokines, including IL-1α, TNF- α, IL-6, IL-8 and INF-γ, were quantified using multiplex kits (MesoScale Diagnostics LLC, Rockville, US). The panel of cytokines include high-abundance and low-abundance cytokines that have been proposed to be involved in the inflammatory processes underlying skin damage (Section 2.3.2.4). A combination of single cytokine immunoassay kits (Peprotech EC Ltd, UK and MesoScale Diagnostics LLC, Rockville, US) were employed for the preliminary experiments. The electro chemiluminescent spectra were recorded with Meso QuickPlex SQ 120 (MesoScale Diagnostics LLC, Rockville, US). Dulbecco's Phosphate Buffered Saline (PBS) was purchased from Thermofisher Scientific, UK. Individual lipid standards for the preparation of synthetic sebum, as described in Table 4-6, were purchased from Sigma Aldrich, MP Biomedicals and EMD Chemicals Ltd. The detergents n-dodecyl β-D maltoside (DDM) and Tween-20 were purchased from Merck Millipore and milli-Q-water was employed for the

preparation of reagent solutions. Shaking and centrifugation were performed in a Grant Ultrasonic bath and Eppendorf refrigerated centrifuge, respectively.

5.2.2.2 Preparation of samples

Sebutapes were coated with 0.5 µL of freshly prepared synthetic sebum (Section 4.3.5) and allowed to uniformly distribute over the surface, using a sterile glass rod on the surface of the adhesive side of the Sebutape, ensuring complete transparency of the tape after 10 minutes. The synthetic sebum coated Sebutapes were spiked with known concentrations of selected cytokines to evaluate the extraction efficiency for a range of mechanical and chemical stimuli.

Previous studies in the host lab have reported that the cytokine, IL-1α, is evident in considerable quantities before and after skin loading (Worsley, Prudden et al. 2016, Bostan, Worsley et al. 2019, Soetens, Worsley et al. 2019). Therefore, preliminary experiments employed the use of single cytokine kits, to evaluate the extraction efficiency of IL-1α. It is well known that mechanical stimuli such as centrifugation affects protein separation based on the molecular weights (Lebowitz, Lewis et al. 2002). In this study, the effect of centrifugation was assessed in a panel of proteins with molecular weights ranging between 10 kDa to 65 kDa. Subsequently, a multiplex kit including cytokines of a range of molecular weight, as described in Table 5-2 was used to evaluate an array of protein biomarkers. 100 µL of known concentrations of cytokine standards were dispensed as two 50 µL droplets, as shown in Figure 5-2.

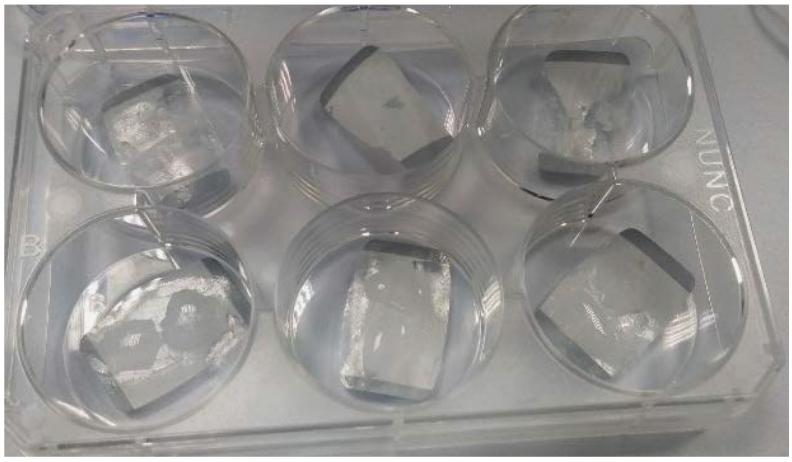


Figure 5-2:Sebutapes with synthetic sebum and cytokine solution dispensed as two 50 µL droplets

The cytokines involved those abundantly reported in sebum, namely IL-1α and IL-1RA as well as low-abundance cytokines, such as IL-6, IL-8, INF-γ and TNF- α on the coated Sebutapes. These cytokines have been reported in previous studies to be involved in the inflammatory processes preceding skin damage (Worsley, Prudden et al. 2016, Gray 2017). In the case of multiplex kits, the calibrator standard solution constituted a mixture of each cytokine at appropriate concentrations. Accordingly, the coating concentration of each cytokine was specified by the calibrator standard provided by the immunoassay manufacturer. The tapes were further incubated at 4^{o}C for 14 hours to ensure that the Sebutapes were completely dry.

Table 5-2: Inflammatory cytokines assessed and their molecular weights

Cytokine	Molecular weight (Da)*
IL-1RA	65402
IL-1α	30607
TNF-α	25644
IL-6	23718
INF-γ	19348
IL-8	11098

* Molecular weights of the cytokines as retrieved from phosphosite.org

The type of kit employed for analysing each of the cytokine and the limits of detection as provided by the manufacturer are summarised in Table 5-3.

Table 5-3: Cytokines, the type of kits employed, and their median limits of detection as provided by manufacturer specifications

Cytokine	Kit type	Lower Limit of Detection (LLOD) (pg/mL)	Upper Limit of Detection (ULOD) (pg/mL)
IL-1α	U-Plex, Multiarray V-Plex	0.98	5100
TNF-α-	U-Plex	0.54	3700
IL-6	U-Plex	0.33	2000
IL-8	U-Plex	0.15	2200
INF-γ	U-Plex	1.7	17000
IL-1RA	Multiarray V-Plex	1.12	650

5.2.2.3 Extraction parameters

5.2.2.3.1 Surfactants

Preliminary experiments (**Study S1**) investigated the influence of a range of working concentrations using the single cytokine IL-1α kit. Further studies (**Study S2**) were conducted using multiplex kits to assess the influence of optimized concentrations of each detergent on the recovery of a range of cytokines. The details of other extraction parameters are comprehensively detailed in Table 5-4. Moreover, the denaturing effect of the detergent was investigated in a separate study (**Study S3**). This involved mixing cytokine solutions of known concentrations with extraction buffers containing different concentrations of detergent. The corresponding control samples were made by preparing the cytokine solutions with the diluents provided by the manufacturer. The samples were then quantified using electrochemiluminescence (ECL) multiplex kits.

5.2.2.3.2 Mechanical stimuli

Mechanical stimuli play a major role in breaking up the protein-tape interactions. Although immunoassay protocols constantly employ the use of sonication, vortexing, shaking and centrifugation for protein extraction and separation, there has been no systematic evaluation to examine the influence of each of these parameters on the extraction process. This will be investigated in a separate set of experiments **(Studies S4, S6, S7).** As each parameter was optimised systematically, it was then made constant in subsequent analysis to reduce risk of bias.

Table 5-4: Summary of the studies investigating the influence of a set of parameters in optimizing the recovery of proteins from Sebutape

Parameter assessed	Study ID	Parameter values	Cytokine	Concentration of coating solution (pg/mL)	Other extraction parameters				
					t_{son} (minutes)	t_{vort} (minutes)	Sur, C_{sur}	V_{buffer} (mL)	t_{cent} (minutes)
Surfactant (Sur, C_{sur})	**S1**	Tween - 0.025 – 0.1% DDM – 0.075-0.175%	IL-1α	500,750	10	2	-	1.7	0
	S2	0.05% Tween, 0.1% DDM	IL-1α IL-6 IL-8 TNF-α INF-γ IL-1RA	177.5 - 1420 63.4 - 507.5 32.2 - 515 114.4 - 915 743.8 - 5950 22.9 - 366	10	2	-	1.7	0
Protein denaturation	**S3**	Tween - 0.025 – 0.05% DDM – 0.03%, 0.07%, 0.1%	IL-1α** IL-6** IL-8** TNF-α** INF-γ**	1420 507.5 515 915 5950	-	-	-	-	-
Sonication time	**S4**	0,5,10,15 minutes	IL-1α	750	-	0	DDM, 0.1%	1.7	0

Parameter assessed	Study ID	Parameter values	Cytokine	Concentration of coating solution (pg/mL)	Other extraction parameters				
					t_{son} (minutes)	t_{vort} (minutes)	Sur, C_{sur}	V_{buffer} (mL)	t_{cent} (minutes)
(t_{son})									
Vortex time (t_{vort})	**S4**	0,0.5,1,2,3 minutes	IL-1α	750	0	-	DDM, 0.1%	1.7	0
Buffer volume (V_{buffer})	**S5**	0.7mL, 1.0mL, 1.7mL 1.mL, 1.7mL	IL-1α IL-6	560,280 250,80	5	0	DDM, 0.1%	-	0
Centrifugation (S_{cent})	**S6**	1000g, 10000g, 15000g	IL-1α IL-6 IL-8 TNF-α INF-γ IL-1RA	177.5 - 1420 63.4 - 507.5 32.2 - 515 114.4 - 915 743.8 - 5950 22.9 - 366	5	0	DDM, 0.1%	0.7	10
Shaking	**S7**	0, 1 hour	IL-1α IL-6 IL-8 TNF-α INF-γ	1420 507.5 515 915 5950	5	0	DDM, 0.1%	0.7	10

Parameter assessed	Study ID	Parameter values	Cytokine	Concentration of coating solution (pg/mL)	Other extraction parameters				
					t_{son} (minutes)	t_{vort} (minutes)	Sur, C_{sur}	V_{buffer} (mL)	t_{cent} (minutes)
Subsequent extraction	**S8**	One-off, Two-step	IL-1α IL-6 IL-8 TNF-α INF-γ	1420 507.5 515 915 5950	5	0	DDM, 0.1%	0.7	10

Sur – Surfactant, Csur- Concentration of surfactant, tson – Time of sonication, tvort – Time of vortexing, Vbuffer - Volume of extraction buffer, tcent - Time of centrifugation, Scent – Speed of centrifugation

*This study does not involve coating of the tapes and the extraction of cytokines

**The concentrations are not coating concentrations, but the concentrations of cytokine solution used for studying protein denaturation

*a. Sonication and Vortexing (**Study S4**)*

The influence of sonication and vortexing on the extraction efficiency were examined. Following extraction using the buffer, a set of Sebutapes were vortexed for different time periods, ranging from 0 to 3 minutes with no sonication and a further set were sonicated for different time periods, ranging from 0 to 15 minutes, with no vortexing. Furthermore, two sets of control samples were employed, one set of which was not subjected to either of the mechanical stimuli, serving as a negative control. The other set was subjected to sonication for 10 minutes and vortexing for 1 minute, thereby serving as a positive control (Table 5-4).

*b. Centrifugation (**Study S6**)*

To investigate the influence of centrifugation, the Sebutapes were placed in small square polypropylene boxes (MB26P009) and extracted in a volume of 700µL. The Sebutapes were then sonicated, and the tapes were removed from the boxes using clean forceps. The resultant solution was centrifuged in refrigeration conditions at 4ºC, to prevent protein denaturation due to the heat generated. After centrifugation, 200 µL of the cytokine solution was left in the vials as pellets while the rest of the volume was removed as a supernatant without disturbing the pellet in the vial. Preliminary experiments to investigate the influence of speed of centrifugation was performed using single cytokine kits, IL-1RA. The coated Sebutapes were then extracted using the buffer, sonicated and centrifuged at different speeds, ranging from 1000g to 15000g. The uncentrifuged samples serving as a control, the supernatant and the pellet were then quantified using immunoassays. Subsequent experiments were performed at the optimised speed with multiplex kits to study the influence of centrifugation on a range of cytokines with different molecular weights.

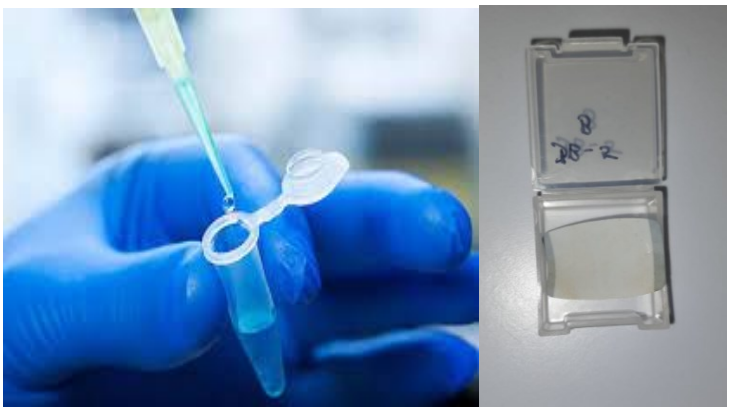
*c. Shaking (**Study S7**)*

In this study, a set of Sebutapes placed in the box with 700 µL of extraction buffer (PBS+0.1% DDM) were shaked for 1 hour. The other set of Sebutapes with the same volume of extraction buffer were placed unperturbed for 1 hour. The tapes were then sonicated and vortexed with tapes *in-situ,* as described in Table 5-4. Following centrifugation, the concentration of the pellet was analysed and reported.

5.2.2.3.3 Other factors

*a. Buffer volume (**Study S5**)*

The protein concentration also influences their stability in a given volume of solution. Higher protein concentrations have been reported to be relatively stable as the formation of aggregates minimising denaturation (Hauptmann, Podgoršek et al. 2018). Therefore, the influence of extraction volume was investigated. The Sebutapes were placed with the adhesive side upwards in square polypropylene boxes (MB34P009, Mocap Limited, UK) of dimensions 34 x 34 x 13 mm. The use of these boxes ensures that the adhesive part of Sebutape is in contact with the extraction buffer ensuring maximal recovery of cytokines. An image of the employed Eppendorf tubes (1.7mL) and the square boxes (1 mL) have been illustrated in Figure 5-3



(a) (b)

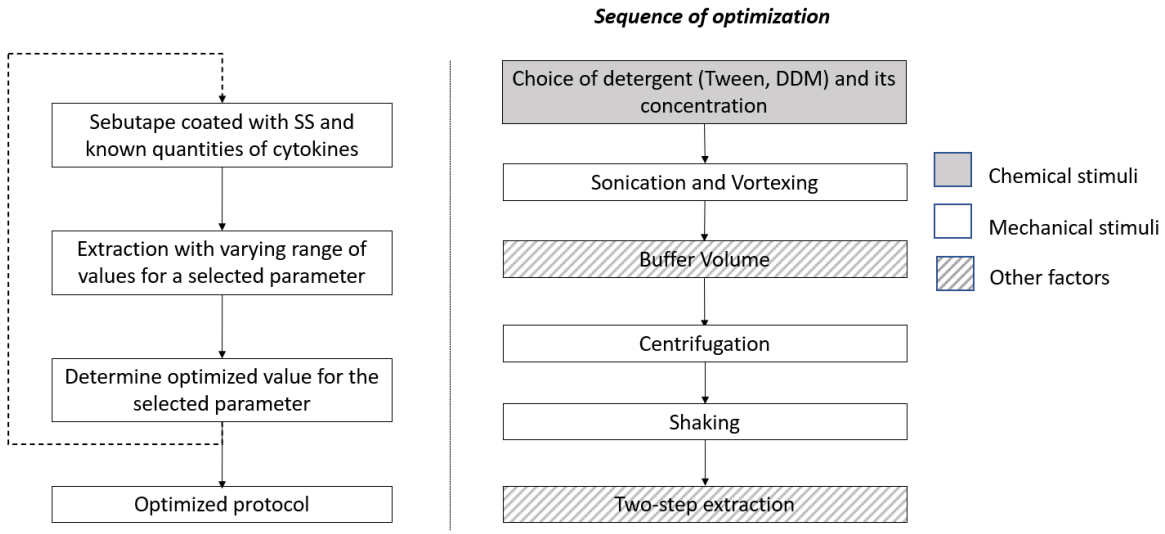
Figure 5-3: Storage containers used for extraction (a) Eppendorf vials (b) Square Boxes

The effect of buffer volume was assessed with a representative high abundant cytokine, IL-1α and a low-abundant cytokine, IL-6. With respect to IL-6, two sets of Sebutapes were extracted with 1.7 and 1.0 mL of the extraction buffer. In addition to the investigated buffer volumes, IL-1α was further investigated for a reduced buffer volume of 0.7 mL. These samples were sonicated for 5 minutes, and the extraction efficiencies were examined. The other extraction parameters were kept constant, as described in Table 5-4.

*b. Secondary extraction (**Study S8**)*

The Sebutapes that were extracted using the optimized parameters informed from studies 1-7 were subjected to a subsequent extraction process to investigate if there is unextracted protein remaining on the surface of the tape to be quantified. Figure 5-4 provides an overview

of the various stages employed to optimise the extraction efficiency. In each of these studies, the parameter of interest was varied across a range of values while the values of the other extraction parameters were kept constant.



(a) (b)

Figure 5-4 (a) Schematic of the experimental protocol illustrating the stepwise process implemented to develop and optimize the protocol and (b) the sequence in which the parameters were investigated

5.2.2.4 Data analysis

Raw data were imported into Excel (Microsoft Office,2019, USA) for analysis. The normality of the data was assessed using Shapiro-Wilk test. Each condition was performed using 4 replicates (n=4) and the mean + SD was calculated. The recovery percentage was calculated using the following formulae:

$$\text{Recovery (\%)} = \frac{\text{Amount of cytokine recovered x 100}}{\text{Amount of cytokine coated}}$$

$$= \frac{\text{Concentration obtained from the calibration curve x Volume of extraction buffer x 100}}{\text{Coating concentration x Volume coated}}$$

The data followed a normal distribution. Accordingly, pairwise t-test was used to compare the test conditions. Differences in recovery were considered to be statistically significant at the 5% level ($p < 0.05$).

5.3 Results

5.3.1 Lipid quantification

The participant cohort in this pilot study consisted of 2 females and 3 males, aged between 31-40 years, with a BMI range from 17.1-32.1 kg/m^2, as summarised in Table 5-5.

Table 5-5: Summary of participant demographics in the lipid quantification study

Participant ID	Gender	Age	BMI (kg/m^2)
P1	36	M	32.1
P2	39	F	17.1
P3	32	F	21.3
P4	31	M	22.3
P5	38	M	27.2

A representative calibration curve is illustrated in Figure 5-5. The concentration of the SPV reactive lipids collected from each of the six locations for the five participants are illustrated in Figure 5-6. There are clearly high inter-individual differences in values. As an example, at the time point 0-, the SPV reactive lipids from the site of the nose for participants P1 and P5, are 3.8 mg/mL and 0.34 mg/mL respectively. It is clear that 2/5 participants (#1, #2) had relatively higher sebum levels compared to the other participants (#3, #4, #5). Moreover, intra-individual variability of SPV reactive lipids was also observed across the different sites. For example, in the case of participant 2, it can be observed that the SPV reactive lipid concentration at the nose is 10-fold higher than that of the heel location. Indeed, the forehead and the nose locations yielded high concentrations of SPV reactive lipids when compared to the heel and the forearm locations across all participants. It is evident that the sebum regeneration process takes time, as shown by the decrease in sebum levels at the time point 0+ collected immediately after the previous time point. SPV reactive lipid concentrations were restored to baseline values at the final 60-minute collection, suggesting a period of 30 minutes is required to restore sebum levels on the skin surface.

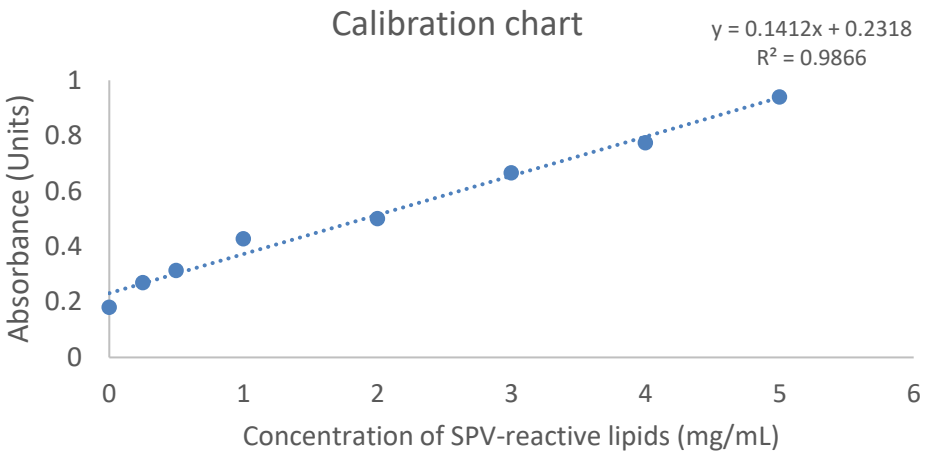


Figure 5-5: Calibration curve for SPV-reactive lipids using synthetic sebum solutions as standards

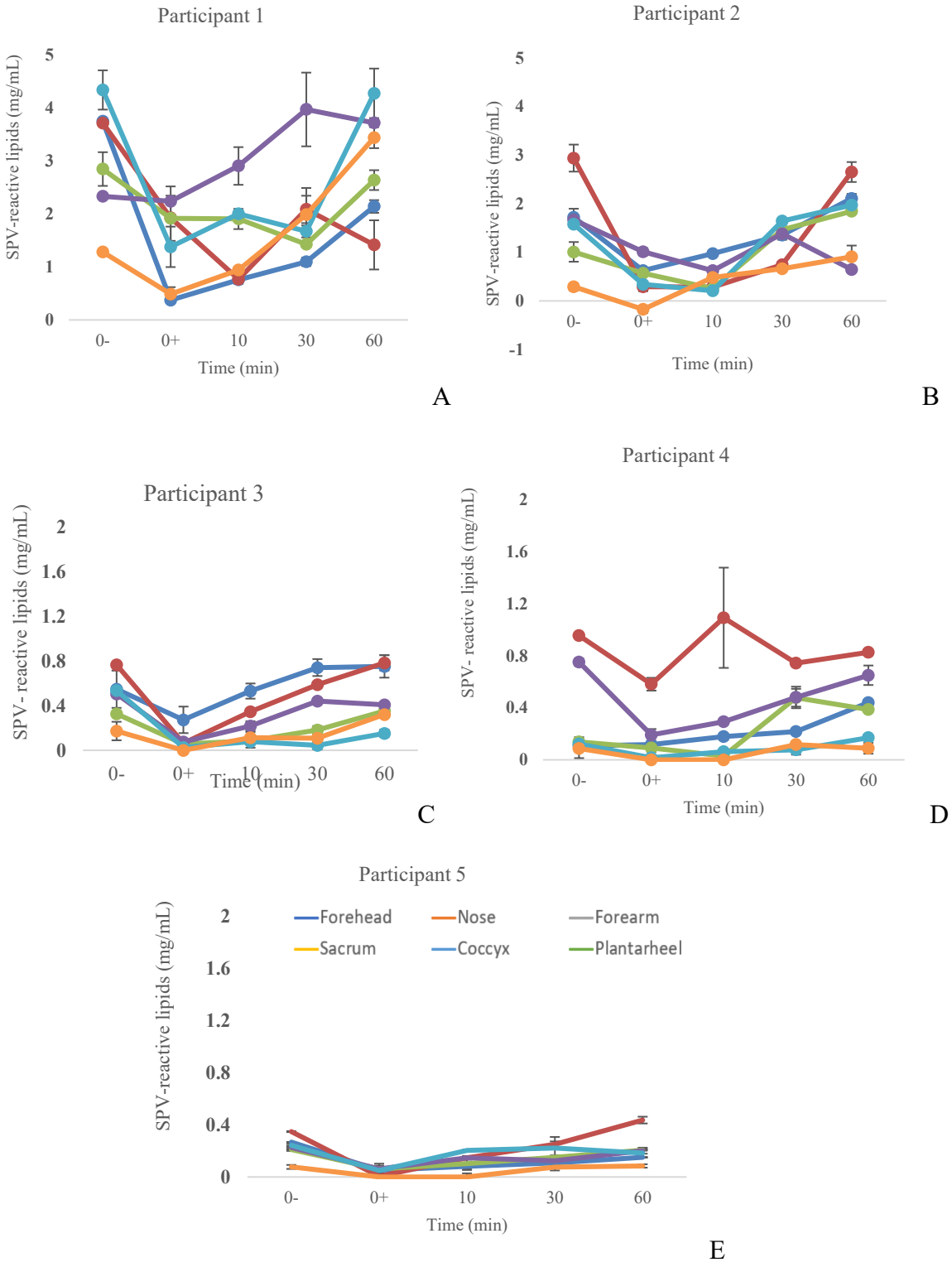


Figure 5-6 : Temporal profile of SPV reactive lipids for a cohort of 5 healthy volunteers (A-E). The legends indicate the anatomical locations of sebum collection

5.3.2 Extraction optimization

5.3.2.1 Chemical agents

In this study (S1), IL-1α single kits at two different cytokine concentrations, i.e.,500 pg/mL and 750 pg/mL were employed and a range of concentrations of two detergents, namely Tween and DDM in PBS on extraction efficiency were investigated. The control samples were extracted with PBS buffer. The recovery of the cytokine for each of the test conditions is illustrated in Figure 5-7. The use of both chemical agents, Tween and DDM, increased the recovery of cytokines by 20-25%. It is also clear that 0.1% DDM yielded the highest values of IL-1α recovery, ranging between 70-80%. In addition, DDM recovery varied little with the different concentration of proteins used in this experiment. By contrast, the recovery efficiency for Tween was more dependent on IL-1α concentration.

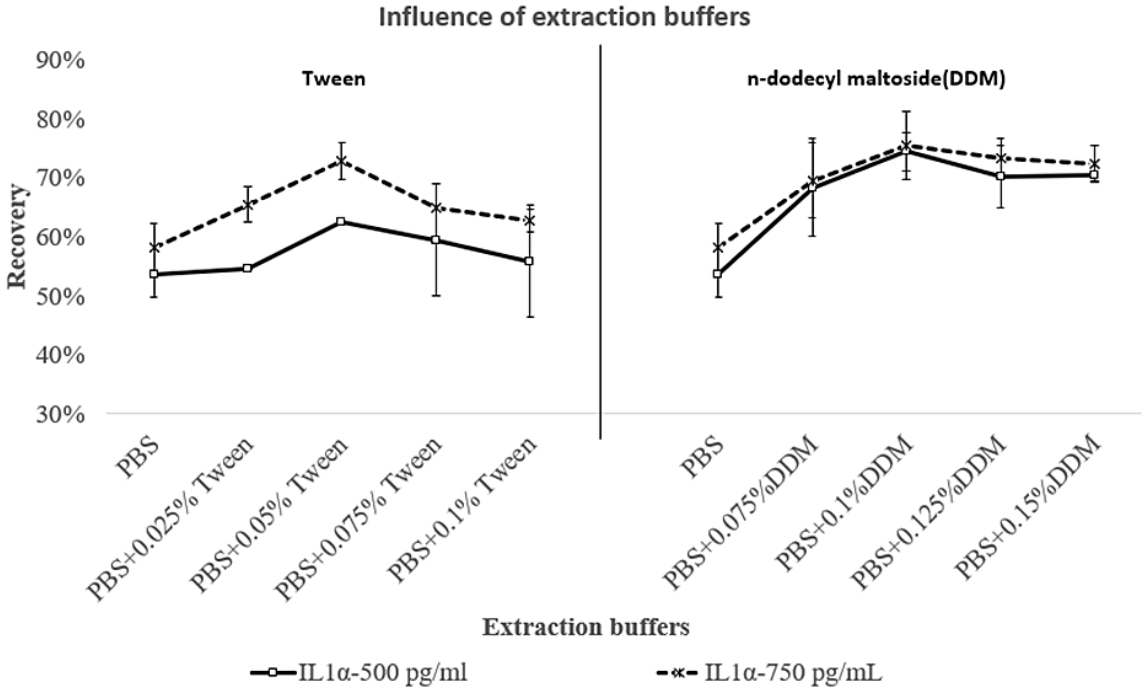


Figure 5-7: Recovery (%) of IL-1α vs range of extraction buffers employed for two different concentrations of cytokine

From the data in S1, the two optimum concentrations of detergent i.e., 0.05% Tween and 0.1% DDM were used to examine their performance with multiplex cytokine kits (S2). The results are illustrated in Figure 5-8, indicates that the maximum recovery of each cytokine for each of the test conditions is highly variable. As an example, the maximum recovery achieved in the case of IL-1α, was 65% whereas the maximum recovery achieved with TNF-

α was 27%. The recovery of each cytokine is highly dependent on the concentration of the coating protein and, except for IL1-RA, the recovery was greatest at the highest cytokine concentrations. Amongst the three extraction buffers investigated i.e., PBS, PBS and Tween, PBS and DDM, it has been observed that 0.1% DDM PBS buffer has higher recovery with each of the cytokines in comparison with the other buffers.

Protein denaturation study

From Figure 5-9, it is evident that there is no single extraction buffer that stabilizes all the concentrations of the six cytokines. Nonetheless, there is compelling evidence that there is only minimal influence, about 15 - 25%, of the effect of detergent on the stability of three of the selected proteins i.e., IL-1α, IL-6 and IL-8 (Figure 5-9 a-c). Moreover, it can be observed that TNF-α is stabilized by 0.07% DDM and 0.1% DDM (Figure 5-9 d), whereas different concentrations of Tween treatment were associated with a decrease in protein stability. In addition, there was a degree of denaturation with INF-γ ranging from 15% to 65%, for the different test conditions irrespective of the extraction buffer employed (Figure 5-9 e). The two sets of experiments confirm that treatment with 0.1% DDM provide a significant increase in extraction efficiency and do not produce marked denaturation in most target proteins. Therefore, this concentration of DDM detergent was selected for subsequent experiments.

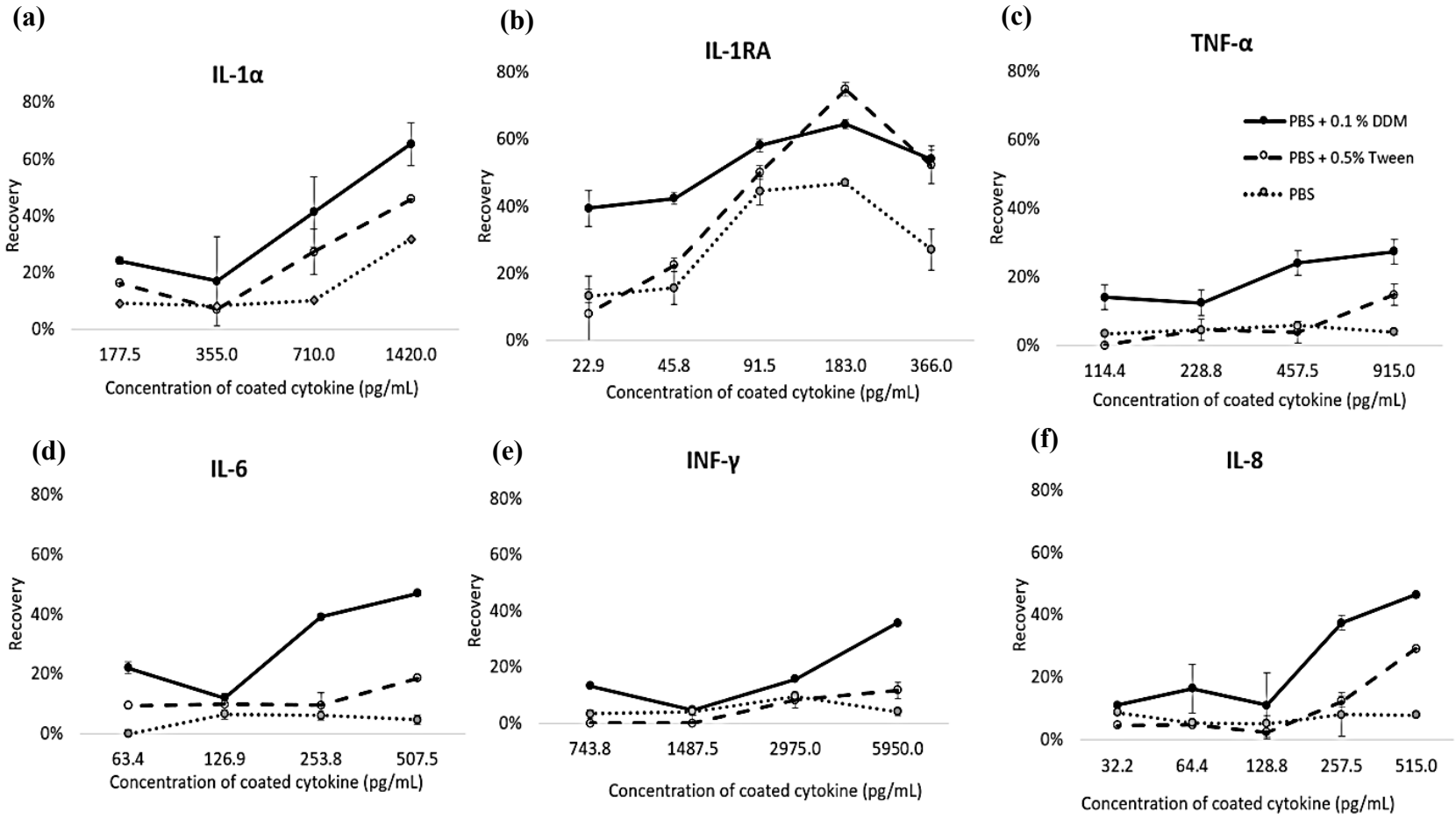


Figure 5-8 : Recovery (%) of cytokines, namely (a) IL-1α (b) IL-1RA (c) TNF-α (d) IL-6 (e) INF-γ (f) IL-8 from coated Sebutapes vs concentration of cytokine coating solutions extracted using different buffers, namely, PBS, PBS +0.05% Tween, PBS + 0.1% DDM

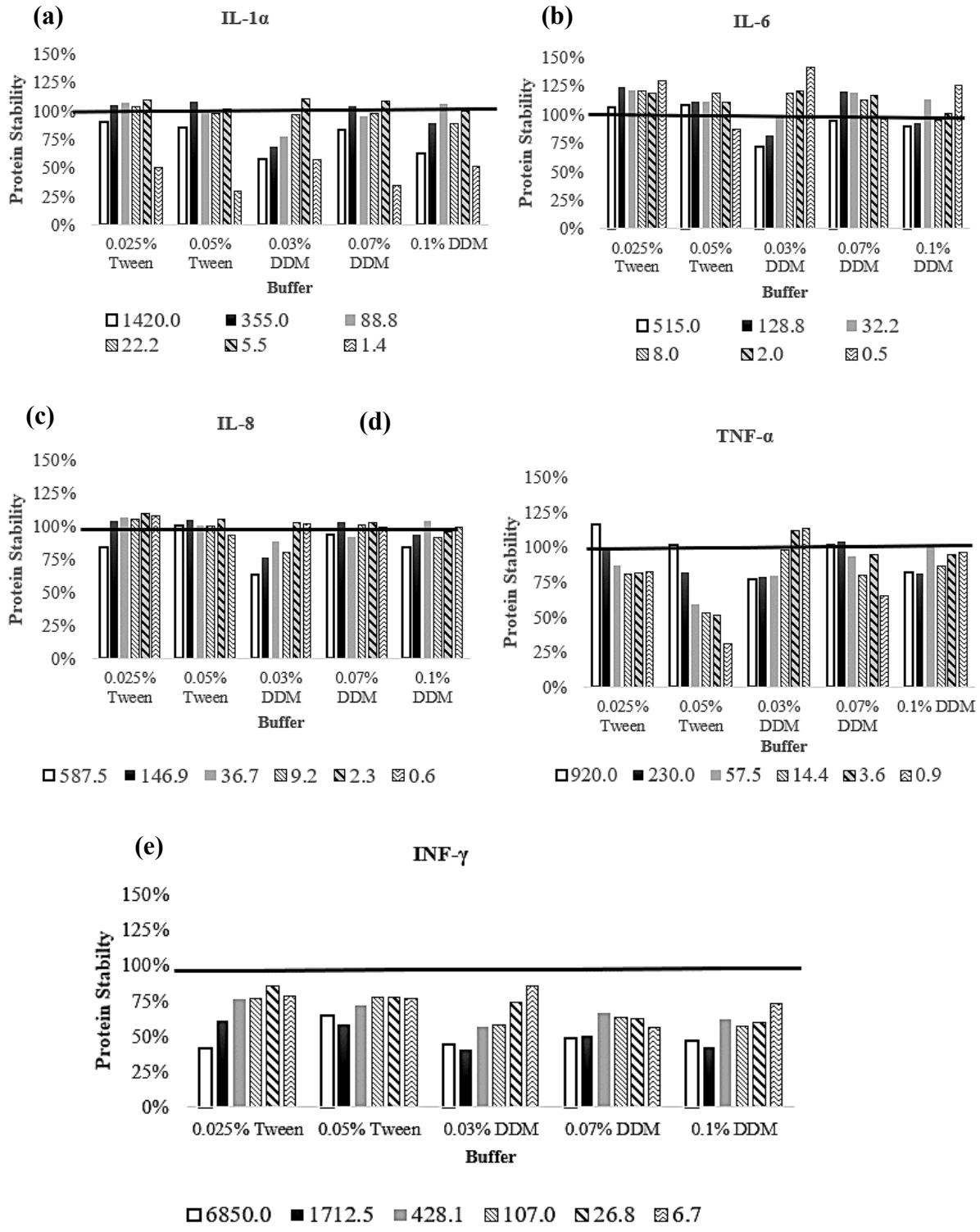


Figure 5-9: Effect of detergents on the stability of varying concentrations of cytokines, namely(a) IL-1α (b) IL-6 (c) IL-8 (d) TNF-α (e) INF-γ Legends represent the protein concentration of each of the cytokines. The blue bar at 100% indicates that values below and above the line represents protein denaturation and stabilisation respectively.

5.3.2.2 Mechanical stimuli

*Sonication and Vortexing (**Study S4**)*

Results of the mechanical stimuli, in the form of sonification and vortexing, revealed an increased recovery of IL-1α of approximately 10% from the coated Sebutapes, as shown in Table 5-6. However, when each of the methods was investigated separately, vortexing in isolation had a minimal effect on the extraction process. Indeed, with respect to sonication over the period of 5 to 15 minutes, an increased recovery of cytokines was observed. This suggests that the vortex process could be eliminated, and the time of sonication could be reduced to 5 minutes in further experiments whilst retaining recovery efficiency.

Table 5-6 : Recovery (%) of IL-1α from coated Sebutapes for different time periods of mechanical stimuli

Sonication time –t_{son} (minutes)	**Vortex time – t_{vort} (minutes)**	**Recovery - mean±SD (%)**
0	0	66.5 ± 2.0
5	0	77.2 ± 0.8
10	0	75.1 ± 2.1
15	0	77.6 ± 0.2
0	0.5	67.2 ± 0.5
0	1	67.0 ± 1.8
0	2	67.2 ± 3.0
0	3	66.5 ± 2.0
10	1	75.0 ± 4.0

*Buffer volume (**Study S5**)*

Figure 5-10 indicates the percentage recovery of both a high-abundant cytokine i.e., IL-1α and a low-abundant cytokine i.e., IL-6 in different volumes of optimised extraction buffer (DDM, 0.1%). It is evident that there was an increased recovery in lower extraction volumes for varying concentrations of both cytokines with the exception of IL-6 at 80 pg/mL. Moreover, there was limited variability associated within the replicates, as denoted by the error bars (representing the standard deviation). For example, the replicates for IL-1α with a

coating concentration of 560 pg/mL extracted with a buffer volume of 0.7 mL displayed recovery values ranging between 83-92%.

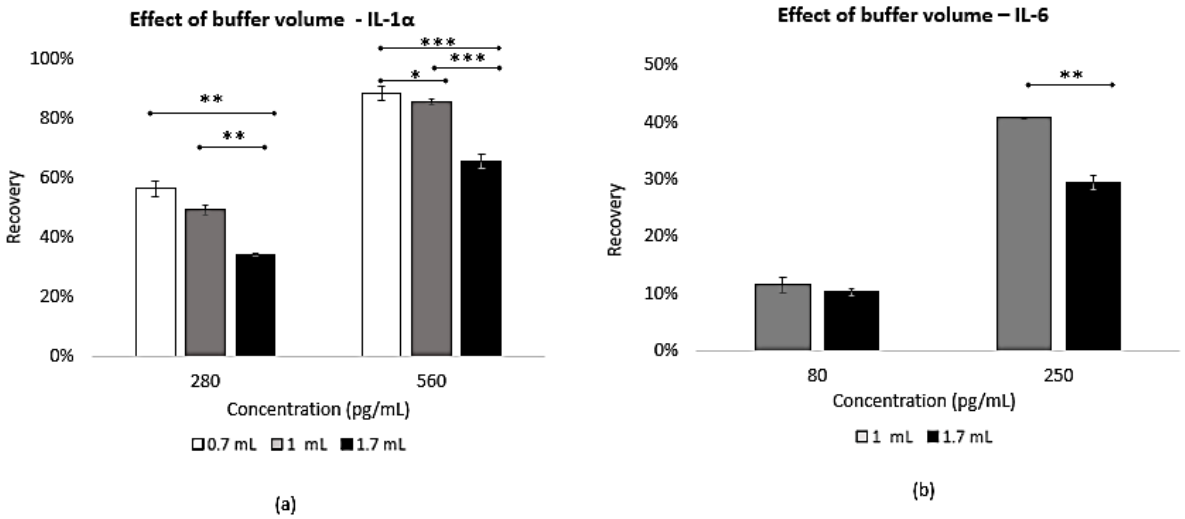
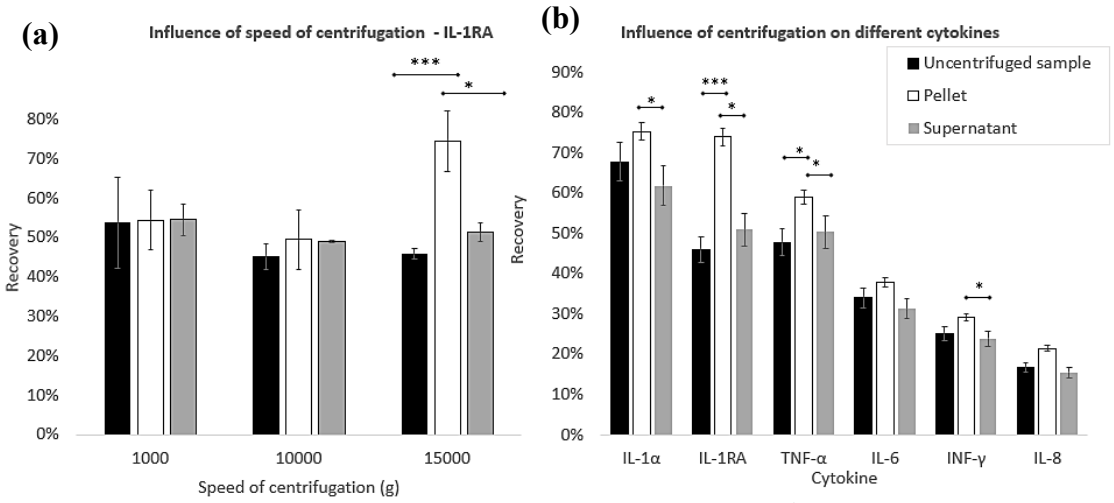


Figure 5-10: Recovery of a high abundant cytokine (IL-1α) and low abundant cytokine (IL-6) extracted with different volumes of extraction buffer (PBS+0.1% DDM)

*Centrifugation (**Study S6**)*

The recovery and the absolute concentrations were plotted in the y-axis for coated samples (Figure 5-11). The results detailing the supernatant, pellet and the uncentrifuged samples at three different speeds of centrifugation are plotted in Figure 5-11. It was evident that at higher speeds of centrifugation, i.e., at 15000 g, the concentrations of the cytokine (IL-1RA) in the pellet are increased by 30% compared to that of the uncentrifuged samples, as shown in Figure 5-11.



Cytokine	IL-1α	IL-1RA	TNF-α	IL-6	INF-γ	IL-8
Molecular weight (Da)	30607	65402	25644	23718	19348	11098

*-p<0.05, **-p<0.01, ***-p<0.005

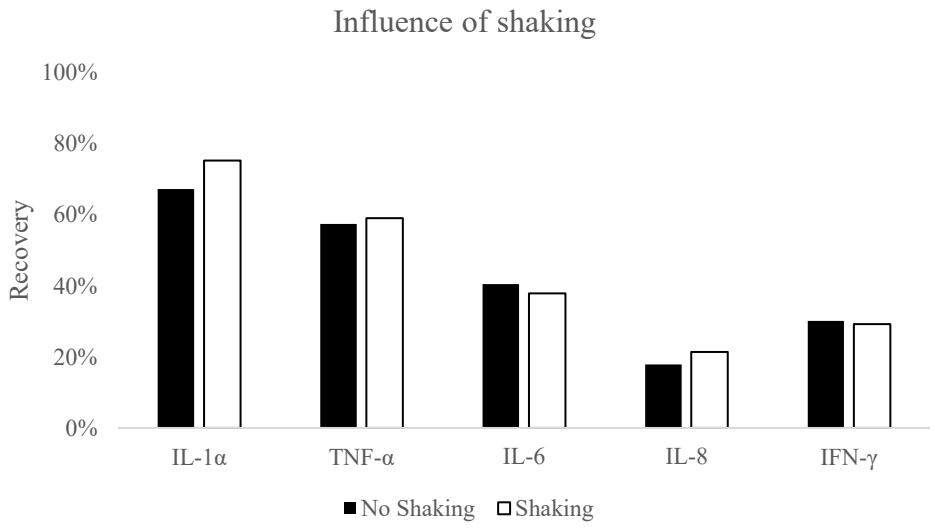
Figure 5-11: Plots illustrating (a) the influence of centrifugation speeds on recovery (%) of IL-1RA and (b) the influence of centrifugation on recovery (%) of different cytokines with their molecular weights listed (Molecular weights retrieved from phosphosite.org).

As the centrifugation process is highly dependent on the molecular size of the cytokine, a selection of cytokines was analysed using a multiplex kit. The centrifugation speed was set at the optimized speed, as informed by the preliminary experiments, i.e., 15000 g (Figure 5-11a). The data revealed that for all cytokines, that the percentage recovery of cytokines in the pellets are higher than that of the uncentrifuged samples (Figure 5-11b). On closer examination, the recovery of cytokines in the pellet, supernatant and the uncentrifuged samples increased with respect to the individual molecular weights. As an example, low-molecular weight cytokines namely, IL-8, INF-γ and IL-6 demonstrated recovery values in the pellet of less than 40%, whereas the recovery of high molecular weight cytokines, namely IL-1α and IL-1RA demonstrated corresponding values in excess of 70% (Figure 5-11). However, the relative increase in pellet recovery when compared to uncentrifuged samples was comparable for each of the cytokines. These values ranged from ~5% to 15% with the exception of IL-1RA, wherein the recovery increased from 45% in the uncentrifuged samples to 75% in the pellet.

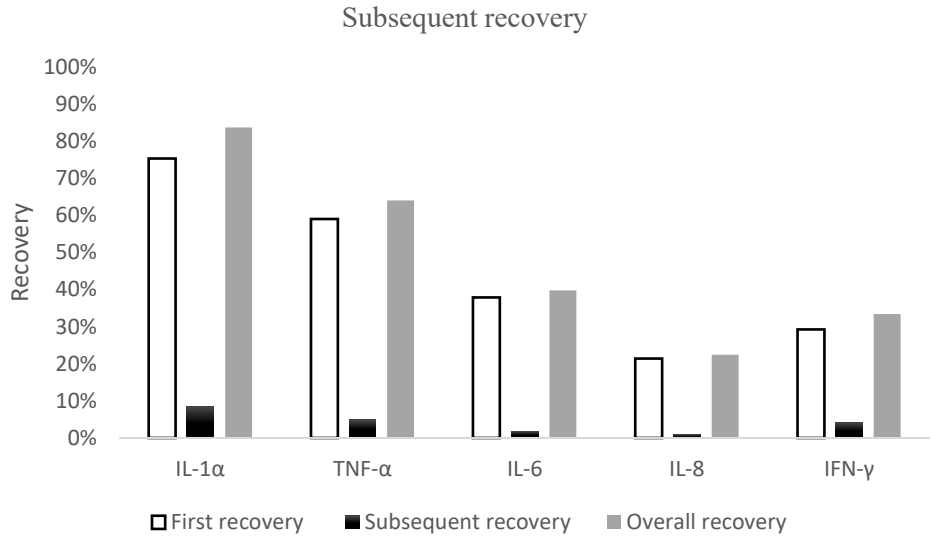
*Shaking and Subsequent recovery (**Study S7 and Study S8**)*

In the studies to investigate shaking (**Study S7**), it was evident that the relative increase in recovery for a range of cytokines due to the shaking process was minimal (Figure 5-12a). The extraction process requires the Sebutapes to be in the extraction buffer for 1 hour to

ensure sufficient time for extraction. As there was no destabilizing effect of shaking as well as no additional time taken, the shaking process was continued to be carried out.



(a)



(b)

Figure 5-12: Influence of (a) shaking and (b) subsequent recovery on the extraction of cytokines from Sebutape

With respect to a secondary extraction, as illustrated in Figure 5-12b, it is evident that the recovery of cytokines is equivalent to less than 10% of the overall for all the cytokines. It is noted that for those cytokines where the recovery is small e.g., IL-6 and IL-8, the subsequent extraction process yielded only approximately 2%.Thus, there appears to be no additional benefit of secondary extraction and the assumption is that the vast majority of proteins are extracted in the initial process.

5.4 Discussion

The aim of this chapter was to study and optimise the biofluid collection process and extraction of the associated cytokines to enable quantification of high and low-abundance biomarkers. In the first phase, the variability associated with sebum volumes sampled from different anatomical locations over a defined time-series was investigated in healthy volunteers. The study informed the variability in biofluid i.e., sebum for studies involving sample collection from different sites and repetitive time points. The findings from the study suggest that a minimum of 30 minutes is required for regeneration of sebum levels. In addition, the study emphasizes the importance of choosing appropriate control sites owing to the variability of biofluid collected in different anatomical locations. The panel of cytokines investigated in the thesis, as detailed in Section 2.3.2.4, include both high-abundance and low-abundance cytokines. In order to analyse these panel of biomarkers, an efficient extraction protocol was developed. To this end, in the second phase of this chapter, a step-by-step optimization process was performed to enable maximum recovery of cytokines from the Sebutape by use of chemical and mechanical stimuli. The findings from these studies will inform an optimized protocol for the analysis of sebum samples using Sebutape from a series of studies involved lab-based skin insults and patient cohorts with early signs of skin damage.

Previous studies have highlighted the variability in sebum content and sebum gland density using tools, such as the Sebumeter and Chromatography (Man, Xin et al. 2009, Kleesz, Darlenski et al. 2012, Ludovici, Kozul et al. 2018). In this study, a chemical assay, i.e., SPV assay, has been employed to quantify the lipids that constitute 98-99% of sebum composition (Picardo, Ottaviani et al. 2009). From the present study, the SPV-reactive lipids measured at the forehead was observed to be between 5 and 15-fold higher than that collected at the forearm, which supports previous findings which report 8-to-10-fold differences between equivalent locations (Man, Xin et al. 2009).The findings of the present study confirm that the facial locations exhibit the highest concentrations of SPV-reactive lipids, followed by the mid back locations and the limb locations. This is associated with high sebaceous gland density at the facial locations (Thody and Shuster 1989, Ludovici, Kozul et al. 2018). Indeed, the forehead and the nose region belong to the widely known ‘T-zones’ that are reported to produce high amounts of sebum (Seo, Li et al. 2014). This difference suggests that the locations associated with mechanical damage, such as the heel and the midback locations, are inherently associated with low sebum secretions. Furthermore, the interpretation of biomarker quantities at these locations requires appropriate controls adjacent to the sites of

investigation, which provide equivalent sebum yields. Indeed, it is widely known that sebum is generated on the skin surface to maintain skin homeostasis (Niemann and Horsley 2012). However, there is limited information on the temporal profile of sebum release over the time. Based on the novel findings from the present study, it is evident that a minimum of 30 minutes is required between subsequent sebum collection to restore levels to the normal sebum release.

The present findings revealed that high recovery could not be consistently achieved for all the selected protein markers. As an example, the recovery of high-abundance markers, such as IL-1α and IL-1RA, ranged between 70-80%. By contrast, the recovery of low-abundance, low molecular weight protein markers ranged between 20-30%. This reduced recovery in samples containing low protein concentrations could be attributed to their inherent thermal instability, in addition to their inability to maintain their functional form at low concentrations (de Jager, Bourcier et al. 2009, Simpson, Kaislasuo et al. 2020). It should also be noted that in five of the six cytokines, the maximum recovery was observed for highest concentrations (Figure 5-9). By contrast, this was not observed with IL-1RA, which presents with a high molecular weight (60 kDa), where the protein aggregates would tend to form at lower concentrations in comparison to the other low molecular weight cytokines (Krishnan and Raibekas 2009). It was not in the scope of the study to further investigate the dependence of molecular weight on cytokine stability and recovery, as a larger panel of cytokines with a wide range of concentrations would have to be investigated. Increased extraction could be achieved with ionic detergents, although this might be associated with the denaturation of proteins (Yang, Wang et al. 2014)

Previous studies investigating the influence of detergents on proteins suggests that DDM offers a combination of high extraction efficiency with enhanced protein stability when compared with Tween (Arachea, Sun et al. 2012, Mahjoubi, Fazeli et al. 2017). For example, a study investigating protein extraction from cells using SDS-PAGE (poly acrylamide gel electrophoresis) indicated that DDM yields improved extraction when compared with Tween (Arachea, Sun et al. 2012). This was supported in the present studies which showed that the use of PBS with 0.1% DDM as an extraction buffer yielded improved extraction to the selected cytokines (Figure 5-8). However, the studies indicate that there is no significant difference in the protein stabilization properties between the two detergents under investigation. It should be noted that SDS-PAGE is a technique to identify protein based on molecular mass that does not account for protein denaturation, whereas ELISA technique employed in the present study incorporates the effects of protein denaturation (Kresge,

Simoni et al. 2006). The extent of denaturation for each protein was different (Figure 5-9), which could possibly be attributed to the protein structure and the tendency of the protein to maintain its functional form (Goldenzweig and Fleishman 2018).

Furthermore, it was observed that mechanical methods, such as centrifugation and sonication, offer a considerable increase in the recovery of cytokines when compared to other mechanical stimuli such as vortexing (Figure 5-11). It is to be noted that the process of centrifugation is dependent on the molecular weight of proteins and therefore there would be differences in recovery for proteins of different molecular weights. Moreover, ultracentrifugation could further provide increased separation for low molecular weight proteins. The process of shaking ensured a more uniform contact of the buffer volume with the tapes. To assess whether the extraction process removed all proteins from the Sebutape surface, the present study also conducted a two-stage extraction process and evaluation of the subsequent recovery. The results yielded only minimal additional protein recovery from the second extraction, indicating almost complete protein recovery from the first extraction (Figure 5-12b). The original evaluation of sebum extraction from Sebutapes used sonication alone (Perkins, Osterhues et al. 2001) and reported a 100% recovery of IL-1α. However, in the present study, a maximum recovery of up to 80% was observed (Figure 5-10). This difference could be a direct result of the use of synthetic sebum in the present study to simulate real-life conditions in addition to the extended drying period of Sebutapes (>14 hours). Recent studies employing the established extraction protocol have revealed that the detection of low-abundance proteins, namely, IL-6, IL-8, INF-γ and TNF-α, was challenging owing to their low concentrations, which were often below the detection limit of commercial immunoassay kits (Gray 2017, Koudounas, Abbas et al. 2020, Koudounas, Bader et al. 2021). Low-abundance of certain markers on the skin surface could be attributed to the transport properties of the protein molecules as well as the barrier function of skin (Wei, Haridass et al. 2018, Jankovskaja, Engblom et al. 2021, Schaap, Bruins et al. 2021), thus making them an important set of cytokines to analyse.

It is recognised that the sebum quantification study was limited by the small size of the cohort, and the influence of intrinsic factors such as gender, age and ethnicity was not possible to investigate. Indeed, these factors have been reported to influence the sebum content as well as other biophysical parameters (Man, Xin et al. 2009, Sugawara, Nakagawa et al. 2019). As an example, a study investigating sebaceous gland using ultrasound microscopy revealed that young females have increased sebum production compared to elderly females, suggesting shrinkage of sebaceous glands with age (Sugawara, Nakagawa

et al. 2019). However, with appropriate controls the issues associated with inter- and intra-individual variability could be mitigated. In addition, the temporal profiles were only examined up to 1 hour, a period considered practical for participant and clinical studies. In the extraction optimization study, the use of a selected panel of cytokines and the detergents precludes generalising the findings to all potential biomarker candidates. Nonetheless, the studies did include those cytokines recently implicated in the inflammatory processes preceding skin damage (Worsley, Prudden et al. 2016, Gray 2017, Bostan, Worsley et al. 2019, Soetens, Worsley et al. 2019). It is to be noted the recovery of cytokines from each of the studies was also influenced by the incubation time required to dry the coating solution. However, each phase of the present study was conducted with separate controls to establish the relative change in recovery. It should be noted that the preliminary studies were conducted with high-abundance biomarker IL-1α as the immunoassay kits (Peprotech Limited.,) employed for the study are not sensitive enough at very low concentrations. In addition, although sebum could be collected locally and non-invasively, it is only available in relatively small volumes, particularly at skin sites with limited density of sebaceous glands. For example, sites such as the plantar foot has low gland density (1-34 $\mu g/cm^2$) in comparison to the nasal bridge of the face (146-231 $\mu g/cm^2$) (Sheu, Chao et al. 1999, Ludovici, Kozul et al. 2018). It should also be noted that the use of detergents in the extraction process restricts the concurrent analysis of metabolites, using sensitive techniques such as chromatography and spectrometry. Further studies are recommended to include other protein biomarkers using a similar experimental approach and protocol.

In conclusion, the improved extraction methodology from this study has enabled the detection of low-abundance protein markers, with 1.5 – 2.5-fold increases in extraction from the previously established protocol. The identification of such predictive biomarkers of skin integrity requires further evaluation of the spatial and temporal profiles to establish thresholds by which changes in skin status can be established. This could provide the basis for more objective monitoring of skin health and provide a complementary assessment to support clinical decision making.

5.5 Summary

The sebum quantification and the extraction optimization study are critical in the analysis of the profiles of low-abundance proteins (Figure 5-13). Based on the results from the first part of the study, future studies involving biomarker sampling will ensure the use of suitable

controls and a minimum time period of 30 minutes between samples. The optimised extraction protocol is summarised as follows:

1) Sebutapes are extracted in 700 µL of the extraction buffer (i.e., PBS+0.1% DDM) and the samples are shaken in a shaker for a period of 1 hour at room temperature.
2) The samples are then sonicated by placing the boxes on pre-cut holders for a period of 5 minutes
3) The contents are then completely transferred into Eppendorf tubes for centrifugation. The Sebutapes are removed carefully from the boxes using clean forceps
4) The vials were centrifuged at 15000 g in a refrigerated centrifuge for a period of 10 minutes
5) 500 µL of supernatant was removed carefully with a side motion without disturbing the transparent pellet and the pellet was then stored for analysis using ELISA kits.

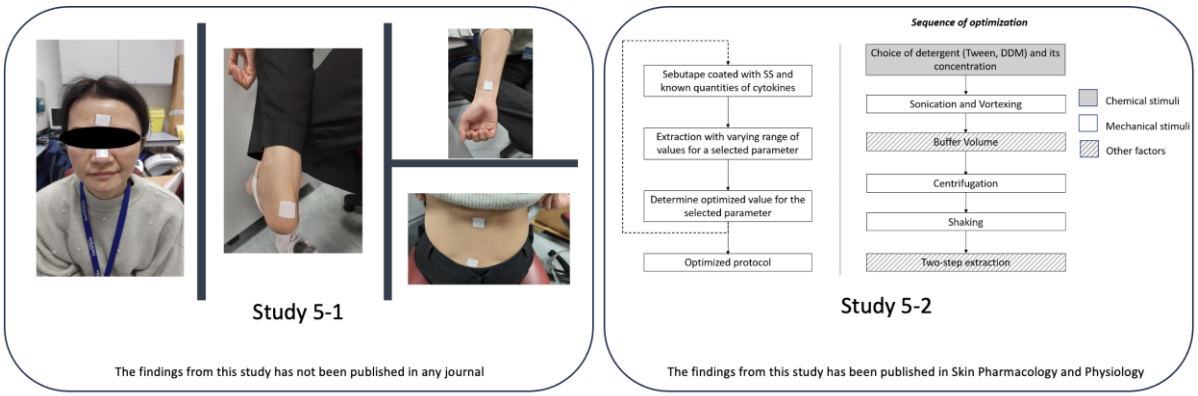


Figure 5-13:Schematic of studies undertaken and published from Chapter 5

This optimized protocol was employed to analyse Sebutape samples from future studies (Chapter 6 and Chapter 7). The results from this chapter involving the optimized methodology to extract cytokines from the Sebutape has been published at Skin Pharmacology and Physiology.

Chapter 6 Skin biochemical response following different skin insults

The exposure of skin to a range of insults, namely, mechanical, chemical and thermal, leads to different skin and sub-dermal responses. Indeed, the biophysical response of skin, including TEWL, LDI, skin hydration and erythema, to a range of varied insults, such as mechanical loading, chemical insults and tape-stripping have been investigated and detailed in Chapter 4. However, these biophysical parameters have a number of limitations and none of which represent a direct measurement of inflammation which is one of the primary signalling processes following skin damage (Figure 2-7). In this chapter, the inflammatory biochemical response of skin to different insult models were studied in three different lab-based controlled studies.

Insult models were selected to reflect real-world scenarios in which skin is exposed to mechanical and chemical loads. For example, incontinence is a common scenario faced in clinical and community settings, especially with aging individuals. Research studies have reported the prevalence of urinary incontinence to be approximately 40% with female patients (Cooper, Annappa et al. 2015). In addition, elderly individuals are subjected to prolonged periods of sitting or lying down due to their limited mobility. Urinary incontinence combined with mechanical loading can potentially affect skin homeostasis and lead to IAD (Koudounas 2019). In this chapter, skin's inflammatory response was studied, when exposed to synthetic urine (S-urine) and mechanical loading, in a cohort of healthy young and elderly individuals (Study 6-1).

Another relevant scenario where the skin can be exposed to high mechanical loads comes from consumer products in the form of shavers. Pressure ulcers are a manifestation of different mechanical forces, mainly, pressure and shear whereas mechanical damage due to shaver is predominantly due to shear and friction. Moreover, there is also a pulling effect with shaving wherein the hair follicles are sheared. Removal of unwanted facial hair *via* shaving represents a technical challenge wherein hair removal efficacy should be increased without causing damage to the skin surrounding the hair. Indeed, poor device design and poor shaving techniques, such as repetitive shaving and applying high force, would lead to a change in the inflammatory response of skin (Cowley and Vanoosthuyze 2016). In this chapter, the effect of shaving on the inflammatory response of skin was investigated (Study 6-2).

The final skin insult model is associated with the recent pandemic of COVID-19, where healthcare workers (HCWs) were required to use personal protective equipment (PPE), such as filtering facepiece (FFP) respirators to prevent the spread of the virus (Howard, Huang et al. 2021). Here healthcare workers are mandated to use respirators for prolonged clinical shifts (up to 12 hours) in order to carry out their clinical duties. Due to the increased pressure and frictional forces applied on the skin interface coupled with the change in microclimate, adverse reactions of skin have been reported in survey studies in addition to the biophysical changes observed following prolonged mask usage (Hu, Fan et al. 2020, Abiakam, Worsley et al. 2021, Han, Shin et al. 2021). Thus, to compliment the self-reported skin reactions, a study was conducted to assess the local inflammatory response following prolonged respiratory protective equipment (RPE) usage in healthcare workers (Study 6-3).

This chapter comprises of three different studies, corresponding to the three aforementioned skin insults. To summarise:

1) The first study investigated the inflammatory response following exposure to S-urine and loading
2) The second study investigated the inflammatory response of skin following a shaving insult
3) The third study would investigate the inflammatory response following prolonged use of RPE in a cohort of health care workers

The combined studies would also provide a means to identify the differences in skin inflammatory response following different insults. It is to be noted that data regarding biophysical parameters, namely, TEWL and Skin hydration, were collected in all of the studies, but their assessment was beyond the scope of the thesis.

6.1 Aims and objectives of the study

1) To investigate the temporal inflammatory response following exposure to S-urine and loading
    a. Collect Sebutapes at the sacral region in a cohort of healthy volunteers in the presence and absence of S-urine following periods of clinically relevant postural loading as well as a control site

   b. Analyse the Sebutapes for a panel of inflammatory biomarkers, including high-abundance and low-abundance markers, and study the temporal response
   c. Investigate the influence of demographic factors on the inflammatory response
2) To investigate the inflammatory response following shaving insults on the cheek and the neck regions
   a. Collect Sebutapes at the sites of investigation, including cheek, neck and the forearm regions
   b. Investigate the inflammatory response to a prescribed shaving load on high-abundance and low-abundance biomarkers
3) To investigate the inflammatory response following the prolonged use of RPE in a cohort of healthcare workers
   a. Collect Sebutapes from the facial locations, including the site of loading due to device and a control site, in a cohort of healthcare workers
   b. Investigate the inflammatory response, including both high-abundance and low-abundance biomarkers
   c. Investigate the influence of intrinsic, such as demographic factors as well as extrinsic factors, such as the duration of mask usage

6.2 Materials and methods

A series of studies were conducted investigating the inflammatory response. The study protocol of sample collection is more conveniently described separately for each of the studies. The biochemical analysis of the samples collected from the studies followed the same procedure as detailed in the following section.

Biochemical analysis

Inflammatory skin biomarkers were evaluated non-invasively by collecting sebum from the skin surface of each participant, using commercial Sebutape^{TM} patches (32x19 mm) (CuDerm, Dallas, TX, USA). The Sebutapes were attached to the skin, using a tweezer and gloved hands, and held in place for 2 minutes prior to removal. Subsequently, they were placed in appropriate labelled sterile containers and stored at -80ºC until biochemical analysis.

The extraction of skin inflammatory biomarkers was performed following an optimised protocol as detailed in Chapter 5, (Jayabal, Bader et al. 2022) with the use of chemical and mechanical stimuli to improve the extraction efficiency. To review briefly, the Sebutapes were extracted with 0.85 mL of extraction buffer, i.e., PBS + 0.1% Dodecyl maltoside. The tapes were shaken with the buffer for 1 hour followed by 5 minutes of sonication. A 0.35 mL aliquot was then used for total protein analysis. The remaining 0.5 mL was centrifuged for 10 minutes at a speed of 15000 g at 4^{o}C. The supernatants were discarded and the remaining solution with the pellet was briefly vortexed and used for immunoassay analysis. The immunoassay was performed as prescribed by the manufacturer using MSD U-Plex kits (MesoScale Diagnostics, USA). The panel of cytokines investigated in each of the studies include high-abundance markers, namely, IL-1α and IL-1RA and low-abundance markers, namely, IL-8, IL-6, TNF-α and INF-γ. Total protein was measured using the Bradford assay (Bradford 1976).

6.2.1 Inflammatory response following exposure to S-urine and loading

The findings of the study, including biophysical and biochemical parameters, have now been accepted in Journal of Wound Ostomy and Continence Nursing in a manuscript titled, “The effects of moistened incontinence pads on loaded skin with reference to biophysical and biochemical parameters”

Participants

The studies were carried out in the Biomechanics Testing Laboratory in the Clinical Academic Facility in Southampton General Hospital, maintained at a temperature of 22.5± 0.7 ºC and relative humidity of 42± 6%. Participants were recruited from the local community and the exclusion criteria was as follows:

1) Participants with diabetes
2) Participation with active skin diseases on the test sites
3) Participants with intake of non-steroidal anti-inflammatory drugs in the last 7 days of testing
4) Participants with back pain

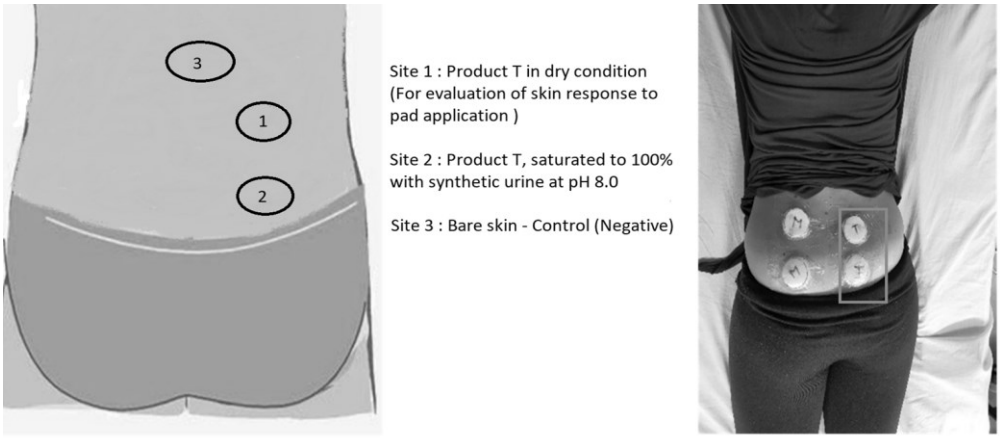
Protocol

For the purpose of investigating inflammatory markers, one incontinence product was chosen which was provided by Essity AB, Sweden. The incontinence pads were fabricated to be 40 mm circular pads (Figure 6-1). They constituted three layers, namely,

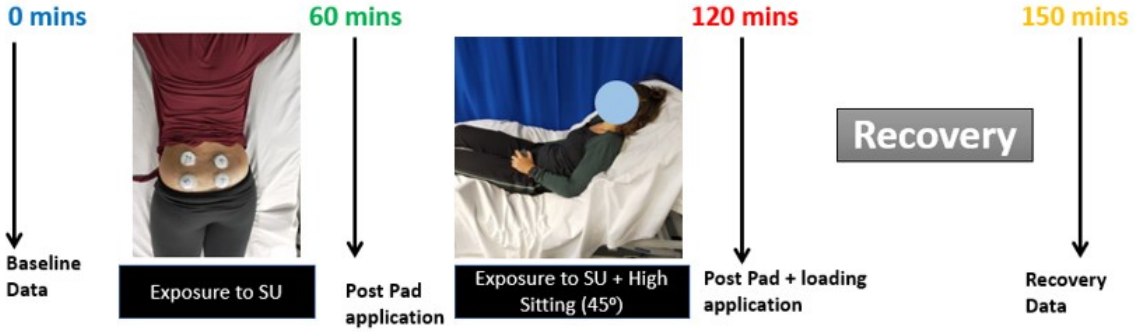
1) Acquisition layer made up of non-woven polypropylene (PP)
2) Core section made up of cellulose fibres and super absorbent polymer (SAP) particles
3) Back sheet made up of Polyethylene (PE) and non-woven laminated fabric

The pads were saturated with 15 mL S-urine (pH 8.0) corresponding to 100% saturation levels of the absorbent material. S-urine was prepared as suggested by Mayrovitz et al (Mayrovitz and Sims 2001) and stored at 4^{o}C. The constituents of S-urine are detailed in Chapter 3 (Section 3.3.3). The S-urine was allowed to acclimatize to room temperature before pipetting onto pad. A period of 15 minutes was provided for the pad to equilibrate with the S-urine.

Three sites (1,2 and 3) on the sacrum and lower lumbar spine of each participant were selected. Sites 1 and 2 were exposed to the dry and saturated incontinence products, respectively, and Site 3 was chosen as the control site. Impermeable adhesive dressing (3M Tegaderm) was employed to secure the pads in place throughout the test period. The test protocol is summarised in Figure 6-1. To review briefly, following 15 minutes of acclimatization, Sebutapes were collected for baseline value at the three test sites. Subsequently, the sites were each exposed to the respective test conditions whilst the participant adopted a prone position, ensuring the sites were unloaded for a period of 60 minutes. The incontinence products were then removed for intermediate Sebutape sample collection. This was then followed by 60 minutes of exposure to mechanical loading, which was applied by ensuring the participants were adopting a high sitting position (semi-Fowler's position) on a commercial hospital bed frame with visco-elastic foam mattress (Virtuoso, wissner-bosserhoff, Germany), in addition to the test conditions involving exposure to S-urine. In this posture, participants were positioned on their back with the head and trunk raised to a 45 degrees bed angle, with the knees raised to flex the legs. At the end of this test phase, equivalent to 120 minutes (Figure 6-1b), the incontinence pad samples were removed, and the skin of the participants blotted to remove an excess of fluid. Sebutapes were collected at this time point as well as after a recovery period of 30 minutes.



(a)



(b)

Figure 6-1: Schematic of the test protocol detailing (a) experimental test sites on the sacral region and (b) time scale of skin challenges and data collection

Data analysis

Raw data from the plate readers were exported from softwares (MSD Discovery Workbench and SoftMax Pro) to Excel and assessed for normality using Shapiro-Wilk test. The concentrations of inflammatory biomarkers were normalised to a baseline value (Initial time point) to accommodate individual variations as described previously (Bostan, Worsley et al. 2019, Henshaw, Bostan et al. 2020). Based on the distribution of the data, appropriate statistical tests were employed. A statistical significance level of 5% was prescribed for the tests.

6.2.2 Inflammatory response following shaving insult

In this study, the participant recruitment and sample collection were carried out by another researcher (ESR-6, Pakhi Chaturvedi, Philips, NL). I collaborated in the analysis of collected samples for inflammatory proteins as well as total protein content, data analysis and interpretation of the results. The study aimed to explore perceptual and observed responses to repetitive shaving insults on the face. A modified electric shaver was used to deliver the stimuli to the skin, assessing the response on the neck and cheek of a healthy cohort of volunteers.

Participants

This study was approved by the local Faculty Ethics committee of the University of Southampton (FoHS -Ethics -71825). The test protocol was performed in the Biomechanics Testing Laboratory in the Clinical Academic Facility in Southampton General Hospital, Southampton, UK. The room temperature was maintained at 24°C ± 2°C.

A convenience sample of participants were recruited via email advertisements and word of mouth. Each potential participant was given an information sheet and requested to confirm their decision if they wished to participate. The participant inclusion criteria were as follows:

1. Healthy volunteers
2. Minimum age of 18 years
3. Males who can clean shave
4. Individuals presented with the mental capacity and English proficiency to provide informed consent
5. Individuals with availability for at least 2 hours on two consecutive days.
6. No history of systemic skin disease.

On arrival, the participants were given the chance to ask questions regarding the study and requested to sign the consent form. Using a stencil (70 mm x 50 mm) with a cut-out (15 mm x 15 mm), squares were drawn on cheek, and neck for each participant. The region of interest was marked at skin areas away from bony prominences. As such, first, a scale ruler was aligned from the top of the ear to the corner of the participant's mouth. Then, a point was marked at a distance of 5cm from the top of the ear. The square was outlined on the cheek skin using the cut-out of the stencil as a boundary. For the neck, the scale ruler was used to mark a point vertically below the square draw on the cheek, such that the point was located

1cm above the end of the hairline. Using the stencil, the square was drawn on the neck by aligning a corner of the cut-out with the identified point (Figure 6-2).

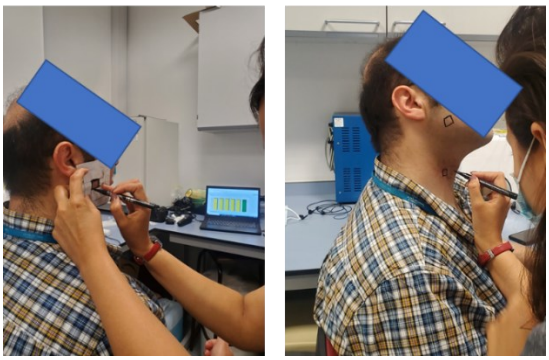


Figure 6-2: Skin areas investigated in the shaver study

Measurement protocol

Shaving was performed on the cheek and neck to investigate different facial sites. The shaver was modified to include a load cell in the handle to provide biofeedback to the user and raw load values sampled at 1Hz via a Bluetooth connection. Participants were requested to apply a shaving contact pressure such that the light ring on the shaver handle was orange, indicating force values greater than 4N at the skin/device interface for 60 seconds.

Participants were requested to attend two sessions over three days. However, for the biomarker analysis, the samples from the second session were analysed to have a preliminary understanding of the inflammatory profile following shaving at different facial locations. Therefore, the measurement protocol for the second session has been detailed. For the second session, as a prerequisite, participants were asked to clean shave 72 hours before the baseline measurement, using their preferred method of shaving at home. Participants baseline skin was assessed using a combination of biophysical and biomarker techniques (according to modified protocol from Chapter 5), and each individual was given a questionnaire to characterise their skin sensitivity. The skin subjective questionnaire employed in the study was a standardized questionnaire employed by Philips which was adapted from previous literature (Richters, Uzunbajakava et al. 2017). Based on their skin sensitivity scores, participants were classified to three categories, namely, individuals with mild, moderate and high skin sensitivity. Following baseline measurements, the mechanical stimulus was applied and subsequent samples were collected after 20 minutes and 24 hours (Figure 6-3). The scope of this analysis was limited to biochemical parameters.

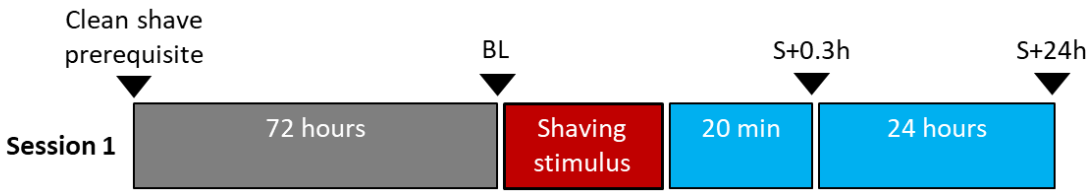


Figure 6-3 - Labels for the timepoints of the data collection on the cheek and neck: BL → Baseline; S+0.3hr → 20 minutes post first stimulus; S+24hr → 24 hours post first stimulus.

Data analysis

Raw data from the plate readers were exported from softwares (MSD Discovery Workbench and SoftMax Pro) to Excel and assessed for normality using Shapiro-Wilk test. Skin sensitivity scores and labels were estimated using methods established and validated by Philips. These have been detailed in Appendix C. The concentrations of inflammatory biomarkers were normalised to a baseline value (Initial time point) to accommodate individual variations as described previously (Bostan, Worsley et al. 2019, Henshaw, Bostan et al. 2020). In order to compare the individual temporal changes following shaving from baseline as well as recovery following the insult, Wilcoxon signed-rank sum tests were employed.

6.2.3 Inflammatory response following prolonged use of RPE in a cohort of HCWs

The findings of this study have now been published in the journal of Skin Research and Technology in a manuscript titled, "Biophysical and biochemical changes in skin health of healthcare professionals using respirators during COVID-19 pandemic"

Participants

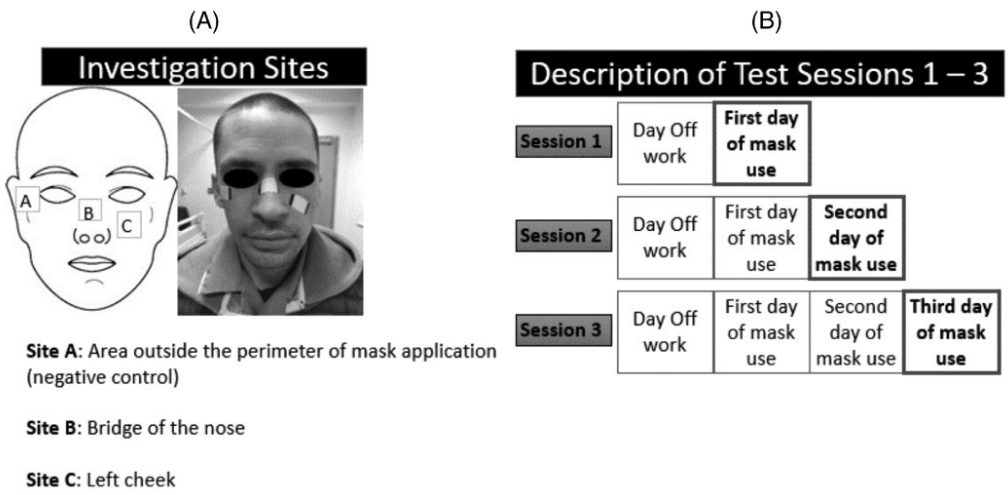
Healthcare workers were recruited from COVID-19 high-risk departments of one UK University Hospital healthcare provider via poster advertisement and gatekeeper communication. Inclusion criteria consisted of individuals over 18 years of age, who employ FFP2/3 masks on a daily basis while attending to clinical commitments, who worked a minimum of three consecutive clinical shifts per week. Exclusion criteria included individuals with open wounds of active systemic skin conditions at the facial sites of investigation, allergies or sensitivity to adhesive tape and the inability to attend a minimum of two out of the three assessment sessions. The study was approved by the UK Health

Research Authority committee (IRAS 285764) and written informed consent was obtained from participants prior to commencing the study. Participant information sheet employed for this study has been detailed in Appendix A.

Test Protocol

The study was conducted during the second wave of the COVID-19 pandemic in the UK (December 2020 to March 2021). Three anatomical locations on the face, namely an area outside the perimeter of respirator application (negative control denoted A), and two at the respirator interface, namely the bridge of the nose (B) and the left cheek (C), were investigated (Figure 6-4 a). Participants who agreed to take part in the study were tested on three different occasions based on a standardised protocol, as summarised in Figure 6-4b. Participants were requested to avoid the application of any moisturizer and/or cosmetics on the face on each of the assessment days. During the test session, each participant acclimatized to an indoor environment and their face dried with paper towels (Tork®, Bedfordshire, UK) prior to commencing skin assessments. All test sessions were conducted in a temperature and humidity-controlled laboratory (room temperature of 22.5± 0.7ºC and relative humidity of 42± 6%) before and after the participant's working shift. Sebutape samples were collected generally after 10 minutes following the removal of RPE, which corresponded to the time taken for the HCWs to transfer from their various hospital departments to the controlled lab settings. Three distinct data collection sessions were used:

- Session 1: participant first day of mask usage following return to work after a period of absence (minimum of 24 hours)
- Session 2: second consecutive day of mask usage in a given working week
- Session 3: third consecutive day of mask usage in a given working week



(a) (b)

Figure 6-4 : Schematic of the (a) investigation site and (b) test protocol detailing the time scale of sample collection

Data analysis

Raw data from the plate readers were exported from softwares (MSD Discovery Workbench and SoftMax Pro) to Excel and assessed for normality using Shapiro-Wilk test. The concentrations of inflammatory biomarkers were normalised to a baseline value (Initial time point) to accommodate individual variations as described previously (Bostan, Worsley et al. 2019, Henshaw, Bostan et al. 2020). Cluster analysis was conducted for normalised inflammatory biomarkers where combined cytokine rank for each participant was collated for each test session. The integrated cytokine response was estimated by ranking the absolute values of all four cytokines for each time point and summing them across all participants in the cohort (Bostan 2019). This would result in a maximum rank sum of 204, based on the number of time points (3), participants (17) and cytokines (4).

6.3 Results

6.3.1 Inflammatory response following exposure to S-urine and loading

6.3.1.1 Participants

The cohort included 12 healthy volunteers, four males and eight females, with a body mass index (BMI) ranging from 18.5-37.7 kg/m^2 (Table 6-1). Participants were purposely sampled from two different age groups ranging from 32 to 39 years old (#1- #6) and from 50 to 62 years old (#7 – #12). The cohort included three ethnic backgrounds, although White ethnicity represented the most predominant (10/12).

Table 6-1:Demographic and anthropometric data of the participants

Participant ID	Gender	Age (years)	Ethnicity	BMI kg/m^2
#1	Female	32	White	21.9
#2	Male	36	White	32.1
#3	Female	32	White	19.8
#4	Male	39	Black	23.6
#5	Male	39	White	29.4
#6	Female	35	Mixed Ethnicity	29.8
#7	Female	59	White	24.1
#8	Female	62	White	18.5
#9	Female	54	White	34.7
#10	Male	51	White	25.7
#11	Female	56	White	37.7
#12	Female	50	White	25.1

6.3.1.2 Biomarker response

Changes in biomarker response were evaluated in the sebum sampled at sites 1, 2 and 3 (Figure 6-6). The analysis revealed considerable variability within and between the inflammatory biomarkers with, for example, absolute concentrations for the pro-inflammatory cytokine IL-1α and the anti-inflammatory cytokine IL-1RA ranging between 13 - 2008 pg/mL and 68 to 1585 pg/mL, respectively (Figure 6-6). The corresponding values for the low-abundance proteins, namely IL-6, IL-8, INF-γ and TNF-α ranged from 0.12-7.18 pg/mL, 0.13-7.32 pg/mL, 1.69-144.53 pg/mL and 0.5-25.0 pg/mL, respectively. It is to be noted that the measurements for some of the low-abundance proteins were at the lower end of the calibration curve. A representative image of the calibration curve generated by the MSD Discovery Workbench Software is shown in Figure 6-5. The total protein content

ranged between 23 – 130 µg/mL, however, the normalisation of the inflammatory markers to total protein did not provide any useful information. (Figure 6-7).

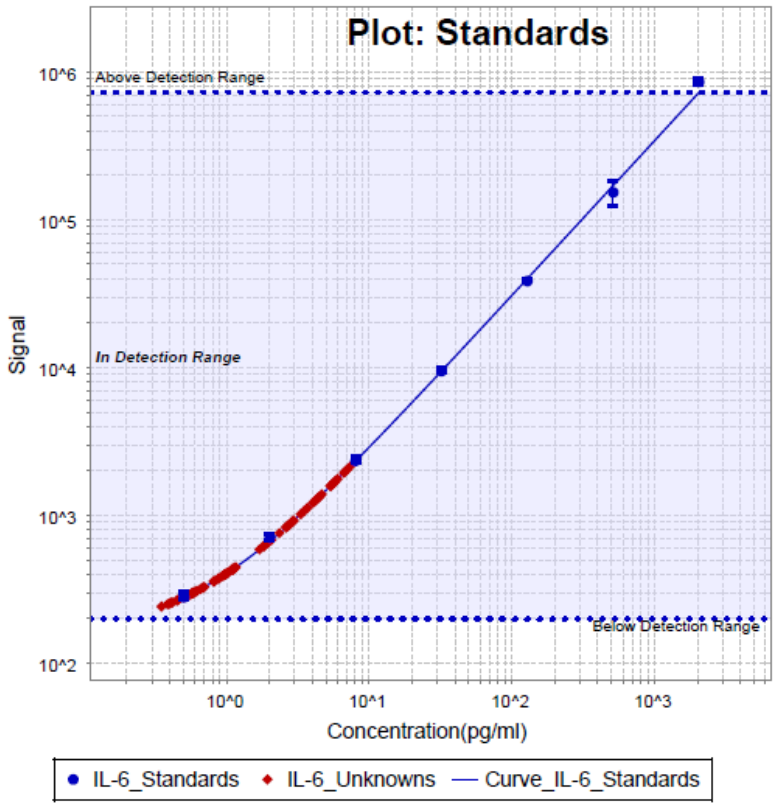


Figure 6-5: Representative calibration curve generated by the MSDWorkbench software indicating the detection limits for the cytokine and the concentrations of the unknown on the calibration curve

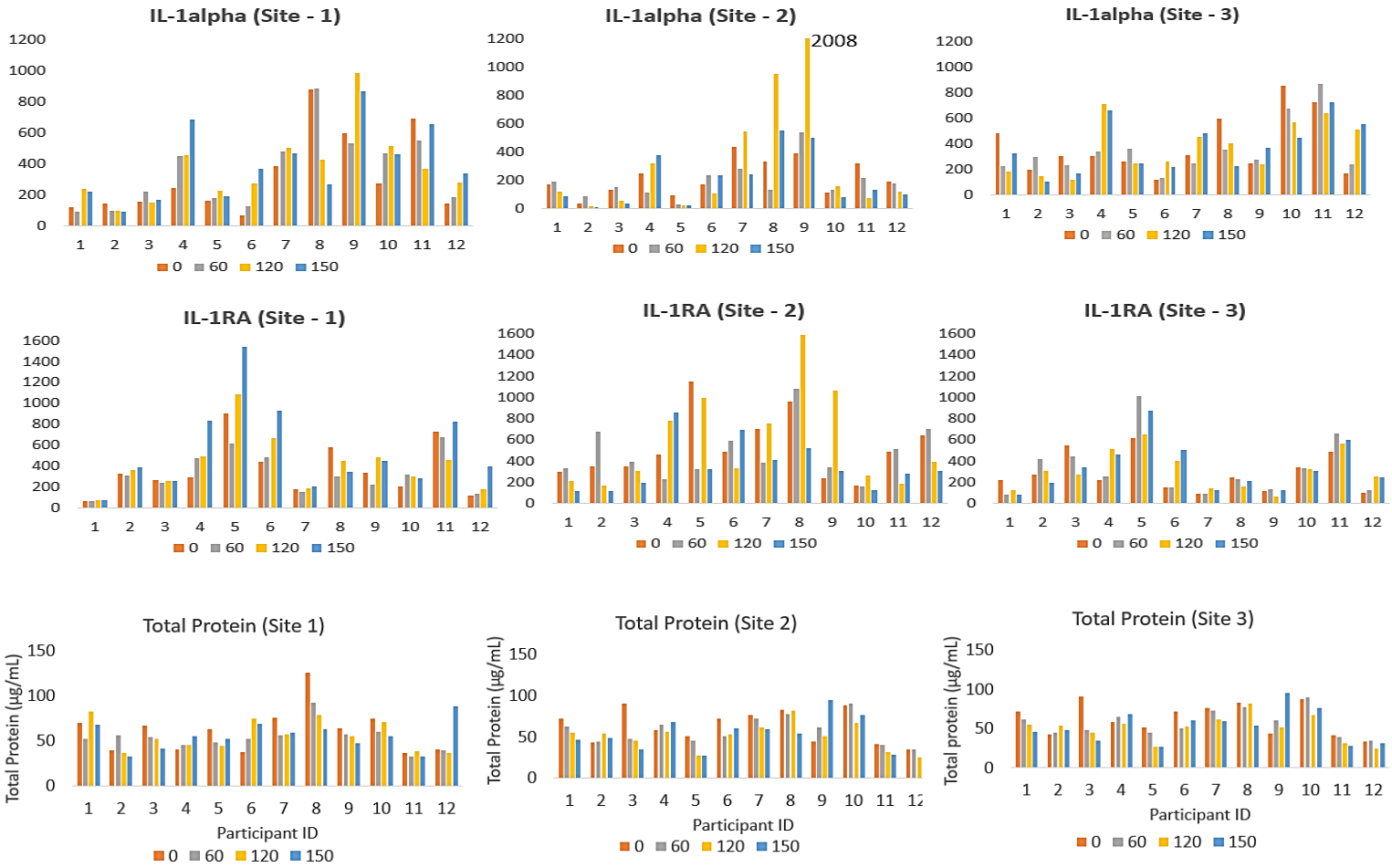


Figure 6-6: Absolute concentrations of high abundant cytokines IL-1α, IL-1RA and total protein at the three sacral sites

A summary of the post to pre-insult ratios of the six cytokines is presented in Table 6-2. With respect to sites 1 and 2, no consistent temporal trends across the cohort were observed. However, there were statistically significant temporal differences in Site-2 for the low-abundance biomarkers, such as IL-6, TNF-α and INF-γ. Close examination of the data revealed variable temporal changes in cytokines relative to basal values, as an example, with INF-γ, IL-6 and TNF-α ratios increasing at time points 60 and 120 at site 2 (median ratios of 1.27-1.94). However, IL-1α, IL-8 and IL-1RA displayed more modest changes in ratios at the same site (median ratios of 0.54-1.10). Moreover, it was observed that at the control site 3 all the cytokines displayed variable changes in the ratio values (median ratios of 0.53 -1.16), with the exception of IL-6. It was also noted that with respect to the low-abundance proteins, IL-8 presented with the lowest median ratio change at each timepoint. Normalised ratio of pro-inflammatory cytokines to its corresponding anti-inflammatory cytokine, i.e., IL-1α/IL-1RA revealed modest changes in Site 1, with a maximum ratio of 2.5, indicating an upregulation of the pro-inflammatory response for selected participants. However, majority of the participants exhibited a ratio value of 1 at Site 2 and Site 3 (Figure 6-7 and Table 6-2).

Table 6-2 Descriptive statistics of the ratio changes of each cytokine at the three sites of investigation over the test sessions

Post to pre-insult ratio	Time point (minutes)	Site 1	Site 2	Site 3
IL-1α – Median (range)	60	1.19 (0.69-1.87)	1.03 (0.34 – 2.53)	1.09 (0.47-1.51)
	120	1.52 (0.49-4.01)	0.67 (0.23 – 5.16)	0.91 (0.38 – 2.96)
	150	1.33 (0.30- 5.39)	0.54 (0.28 – 1.64)	0.97 (0.38 – 3.25)
INF-γ – Median (range)	60	1.28 (0.67 – 5.10)	1.94 (0.36 – 11.60)*	1.12 (0.25 – 6.28)
	120	1.49 (1.00 -4.08)	1.69 (0.09 – 10.35)*	0.94 (0.08 – 4.15)
	150	1.32 (1.09 – 10.94)	1.01 (0.10-4.74)*	0.91 (0.08 – 3.89)
IL-6 – Median (range)	60	1.37 (0.67-5.19)	1.89 (0.42-8.46)*	1.23 (0.26 – 2.33)
	120	1.35 (0.95-3.94)	1.78 (0.10 – 7.19)*	1.03 (0.10-3.43)
	150	1.29 (1.19 – 11.16)	1.08 (0.11 – 3.20)*	1.42 (0.09-2.83)
IL-8 – Median (range)	60	0.98 (0.34 – 2.90)	1.00 (0.16 – 7.40)	0.61 (0.28 – 2.99)
	120	1.23 (0.62 – 2.44)	1.00 (0.22-12.00)	0.53 (0.12 – 2.68)
	150	1.25 (0.45 – 5.74)	0.70 (0.11-3.90)	0.54 (0.12 – 1.92)
TNF-α – Median (range)	60	1.18 (0.85 – 3.50)	1.27 (0.56 – 4.14)*	1.15 (0.37 – 2.16)

Post to pre-insult ratio	Time point (minutes)	Site 1	Site 2	Site 3
	120	1.48 (0.81 – 2.90)	1.60 (0.32 – 2.87)*	0.91 (0.13 – 2.05)
	150	1.45 (0.95 – 4.26)	0.94 (0.30 – 2.26)*	1.06 (0.06 – 3.24)
IL-1RA – Median (range)	60	0.94 (0.52 – 1.66)***	1.10 (0.28 – 1.91)	1.05 (0.40 – 1.64)
	120	1.16 (0.62 – 1.71)***	0.86 (0.38 – 4.53)	1.09 (0.50 – 2.60)
	150	1.26 (0.60 – 3.40)***	0.56 (0.28 – 1.86)	1.16 (0.38 -3.29)
IL-1α/IL-1RA – Median (range)	60	1.24 (0.74-1.93)	1.05 (0.36-1.33)	0.97 (0.62 – 1.18)
	120	1.15(0.60-2.63)	0.97 (0.28-1.72)	0.87 (0.64 – 1.69)
	150	1.01(0.51-2.54)	0.99 (0.51 – 3.01)	0.5 (0.43 – 1.82)

Significant differences between the three test sessions evaluated using Friedman's test * - $p<0.05$, **-$p<0.01$, ***-$p<0.001$

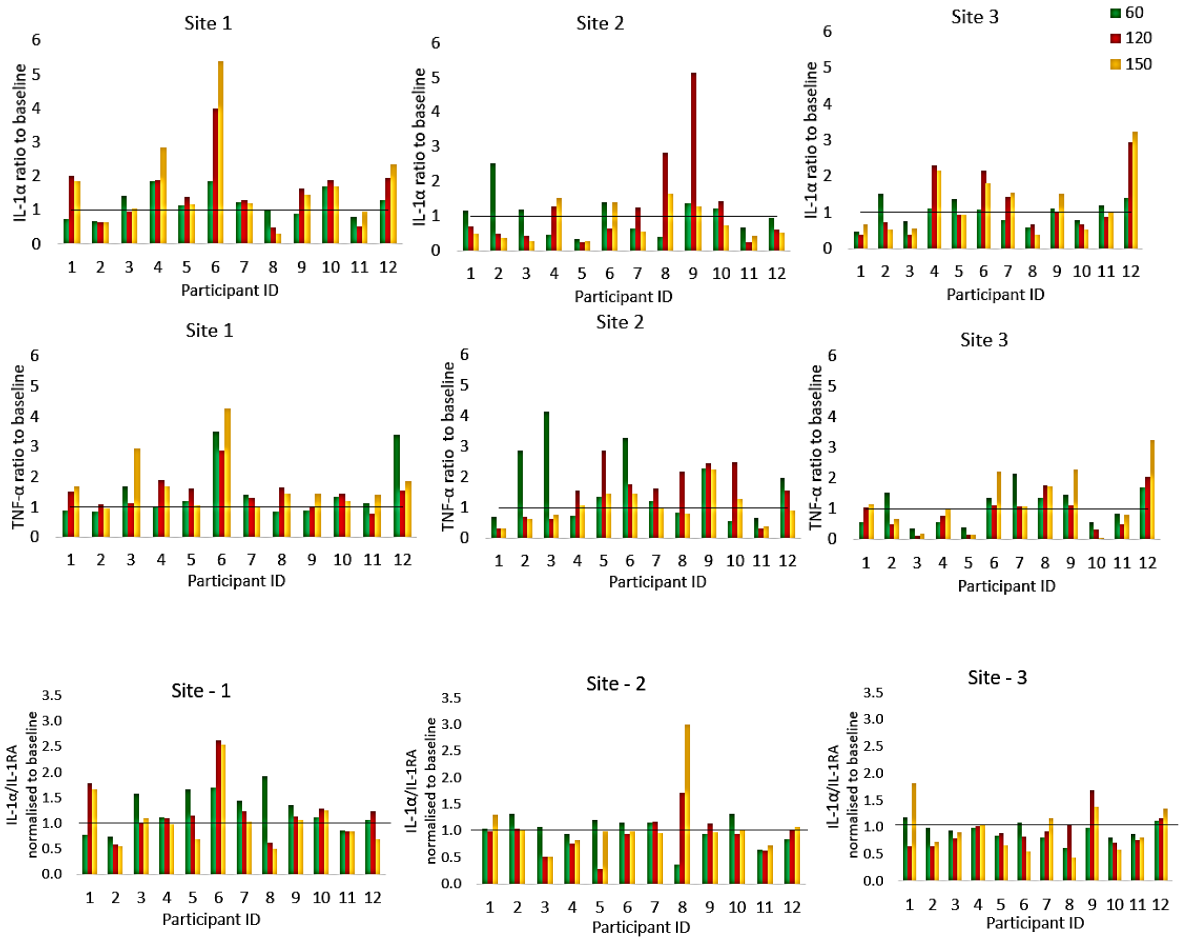


Figure 6-7: Ratio changes in high-abundance marker, IL-1α and low-abundance marker, TNF-α and the ratio of IL-1α/IL-1RA for each participant at the sites of investigation over the test sessions.

6.3.1.3 Influence of intrinsic factors

The role of the age of the participants in relation to biomarkers responses was evaluated with respect to the absolute values of IL-1α and TNF-α at site 2 (Figure 6-8). Data revealed high variability in absolute values across the cohort at the different time points. Some statistically significant correlations were observed with age and selected biomarkers (IL-1α, TNF- α and INF-γ). As an example, a positive correlation was observed with IL-1α and age at selected time points whereas a negative correlation was observed with TNF-α. Interesting trends within the age groups were observed for the two cytokines, with older individuals presenting overall higher upregulation in IL-1α compared to younger participants, with values reaching a maximum of 985 pg/mL. By contrast, an opposite trend was detected for TNF-α, with younger individuals presenting with higher biomarker expression up to 18 pg/mL, while the values for older participants were below 4 pg/mL. It is to be noted that there were no trends observed with BMI. In case of other cytokines, such as IL-1RA, there were no considerable trends with age (Figure 6-8)

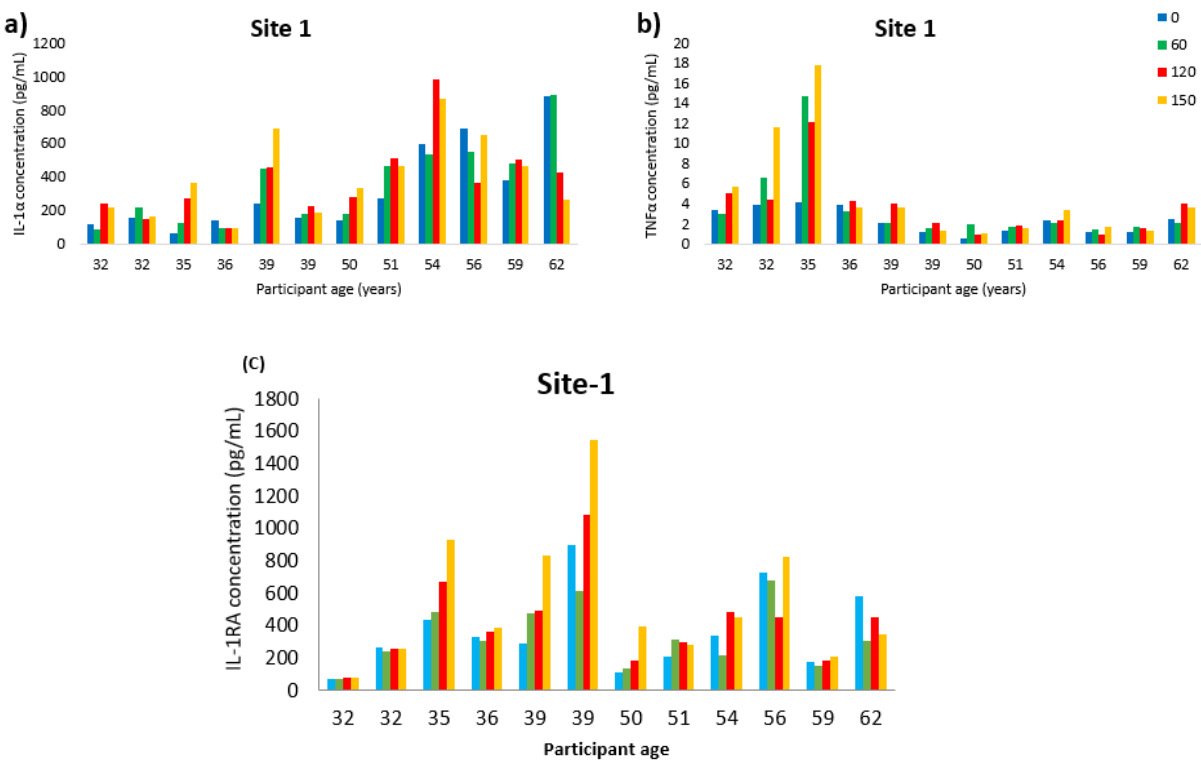


Figure 6-8: The influence of age on the absolute concentrations of (a) IL-1α (b) TNF-α and (c) IL-1RA at site 1 over the test session

6.3.1.4 Discussion

The absolute concentration of the biomarkers highlighted differences in responses between individuals, however consistency in response was observed for a given individual (Figure 6-6). In order to enable comparison at different time points, normalisation to baseline was carried out. Moreover, there were no temporal changes in total protein content and therefore the normalization of inflammatory markers to total protein for each of the sites did not provide any further useful information. Inflammatory skin biomarkers highlighted considerable variability across the cohort with large inter-individual differences (Table 6-2). Nevertheless, upregulation of both high and low-abundance proteins was observed for some participants at both the dry and SU-saturated pad sites (Figure 6-7). Increased expression of high-abundance proteins IL-1α and IL-1RA following skin exposure to moisture has been previously reported (de Jongh, Lutter et al. 2007, Bostan, Worsley et al. 2019). A recent study showed an increase in IL-1α when skin was in contact with pads moistened with 0.9% saline solution alone. However, the study did not detect differences in the expression of low-abundance proteins, namely TNF-α, IL-6 and IL-8 (Koudounas, Abbas et al. 2020). This could possibly be attributed to the low levels of the biomarkers that were possibly below the limit of detection of the immunoassay techniques. By contrast, the present study used an optimised method of extraction from the Sebutapes involving both chemical and mechanical stimuli. As a result, the analysis was able to quantify these low-abundance markers within the calibrated range of commercial ELISA kits. Furthermore, the evaluation of absolute biomarker concentrations also highlighted trends with respect to the two age groups.

It has been reported that ageing induces a dysregulation in cytokine expression, with a progressive tendency toward a pro-inflammatory phenotype, as well as a progressive decline in immune responsiveness to environmental agents (Rea, Gibson et al. 2018). Linear correlations were observed between the high-abundant cytokine, IL-1α and age for selected sites, in particular, Site 1 (Figure 6-8). However, there was no linear relationship between IL-1RA and age (Figure 6-8).

6.3.2 Inflammatory response following shaving insult

6.3.2.1 Participants

Ten male healthy volunteers were recruited onto the study and their age ranged between 24 and 41 years (mean age 34±6 years). Majority of the participants (8/10) were classified under

the mild skin sensitivity category based on their responses to the subjective questionnaire. The skin sensitivity scores, and the participant related information are tabulated in Table 6-3.

Table 6-3 Demographics and subjective sensitivity scores

Participant ID.	Age	Q-score	S-Score	SS Label
P1	35	30	29%	Mild SS
P2	28	96	52%	Mild SS
P3	25	20	26%	Mild SS
P4	37	35	40%	Mild SS
P5	24	25	27%	Mild SS
P6	38	25	52%	Mild SS
P7	36	66	54%	Mild SS
P8	37	61	62%	High SS
P9	41	100.74	59%	High SS
P10	41	62.3	43%	Mild SS

6.3.2.2 Biomarker response

Changes in skin response were evaluated in the sebum sampled at two different facial sites, namely, cheek and neck. Similar to previous study (Section 6.3.1), there were considerable variability between and within the shaving time points (Table 6-4). Moreover, the variability between the anatomical locations was evident. The absolute concentration for the high abundant cytokines, ranged between 32.8 – 6545.4 pg/mL and 201.4 – 19219.3 pg/mL for IL-1α and IL-1RA respectively. The corresponding values for low-abundance proteins, namely, IL-6, IL-8, TNF-α and INF-γ, ranged between, 0.12 – 3.53pg/mL, 0.36 – 186.8 pg/mL. 0.12- 59.65 pg/mL and 2.54 – 256.7 pg/mL respectively. It is to be noted that for some of the low abundant cytokines, such as, IL-6 and TNF-α, the cytokine concentrations were below the limit of detection. In order to compare the skin response between individuals, the inflammatory cytokines were normalised to their baseline. A summary of the post to pre insult ratios of the investigated cytokines are presented in Table 6-4 and Figure 6-9.

Table 6-4: Summary of post to pre-insult ratio following shaving at two anatomical locations

Location	**Cheek**		**Neck**	
Time point	**20 mins post stimulus to baseline**	**24 hours post stimulus to baseline**	**20 mins post stimulus to baseline**	**24 hours post stimulus to baseline**
IL-1α	2.83 (1.81-4.80)	0.93 (0.36-2.96)	2.27 (1.19-9.16)	0.73 (0.49-1.91)
IL-1RA	2.46 (1.66 – 5.17)	1.24 (0.75- 4.96)	3.19 (1.28-11.8)	0.78 (0.56-1.93)
IL-6	1.76 (1.57 – 5.5)	1.07 (0.56-1.69)	2.99 (1.68 – 5.02)	1.17 (0.67 – 2.80)
IL-8	3.74 (0.89 – 9.74)	3.95 (0.59– 12.12)	3.90 (0.76 – 9.56)	8.89 (1.63 – 169.8)
TNF-α	1.51 (0.27 – 2.51)	0.63 (0.06 – 1.85)	4.96 (0.24 – 163.4)	0.92 (0.43 – 88.12)
INF-γ	2.94 (1.23 – 3.67)	0.99 (0.07 - 2.09)	1.81 (1.22 – 6.81)	1.52 (0.56 – 2.73)
IL-1α/IL-1RA	0.87 (0.57-1.91)	0.74 (0.49 – 1.27)	0.74 (0.29 – 3.74)	0.99 (0.46 – 1.73)

Close examination of the absolute concentrations of the data revealed that there was a statistically significant increase in the inflammatory response of most of the investigated cytokines, including IL-1α (p-0.009), IL-1RA (p-0.009) and INF-γ (p-0.009), following shaving (S+20 min) at both the anatomical locations (Figure 6-11 and Figure 6-12). Moreover, these responses reduced towards baseline after 24 hours ($p<0.05$) (Figure 6-11 and Figure 6-12). It is interesting to further note that for a number of individuals (#2, #4, #8), the IL-8 response at the neck location continued to increase at 24 hours following the shaving insult (Figure 6-9). However, this trend was not observed at the cheek location.

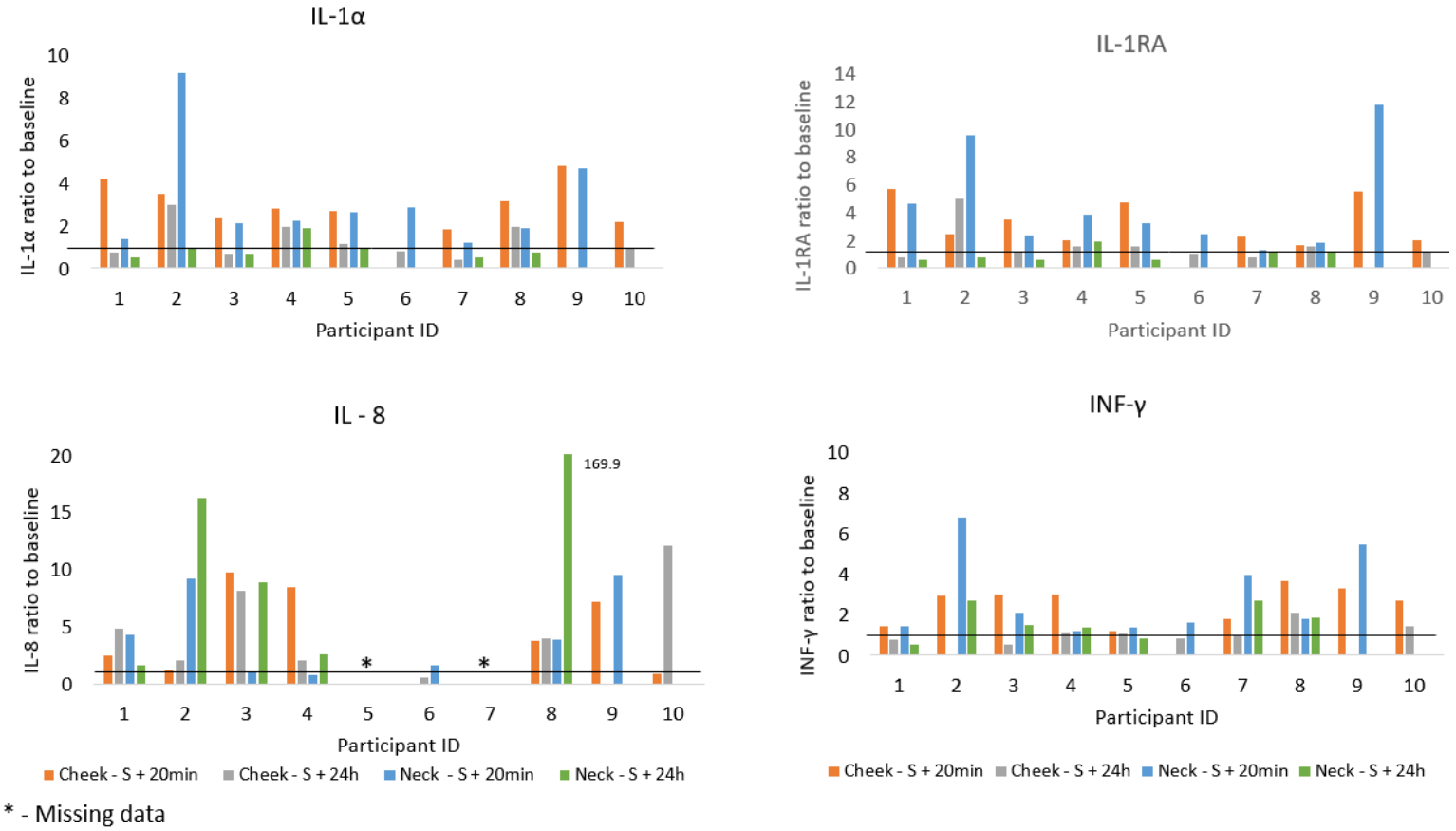


Figure 6-9: Ratio changes in high-abundance markers (IL-1α and IL-1RA) and low-abundance markers (IL-8 and INF-γ) for the cheek and neck locations following shaving

The median values suggest that the inflammatory response is highly elevated at 20 minutes following shaving, with median ratio values ranging between 1.5-4.0 and 1.8-5.0, at the cheek and neck, respectively. Of particular note was the ratio change in IL-8, which revealed an increase in median value from 3.9 to 8.9 corresponding to 20 mins and 24 hours after shaving. Moreover, there were no consistent trends between the two anatomical locations. Despite the increase in the absolute concentrations of IL-1α and IL-1RA, it is interesting to note that the median IL-1α /IL-1RA ratios were less than 1 at both the locations and timepoints.

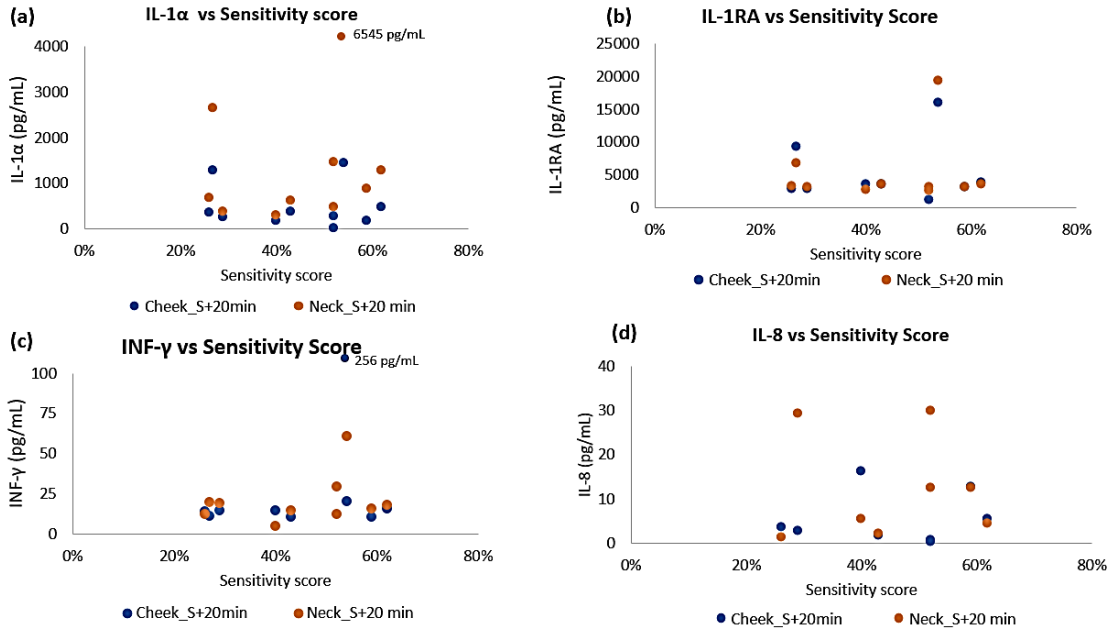


Figure 6-10: Changes in biomarker response as a function of sensitivity score derived from subjective questionnaires

Close examination of skin biomarker response with respect to sensitivity scores derived from the subjective questionnaires revealed no clear trends (Figure 6-10). As an example, two individuals with different sensitivity scores, i.e. 29% (P1- Mild SS) and 52% (P9 – High SS) had an elevated IL-8 response with values ranging up to 30 pg/mL.

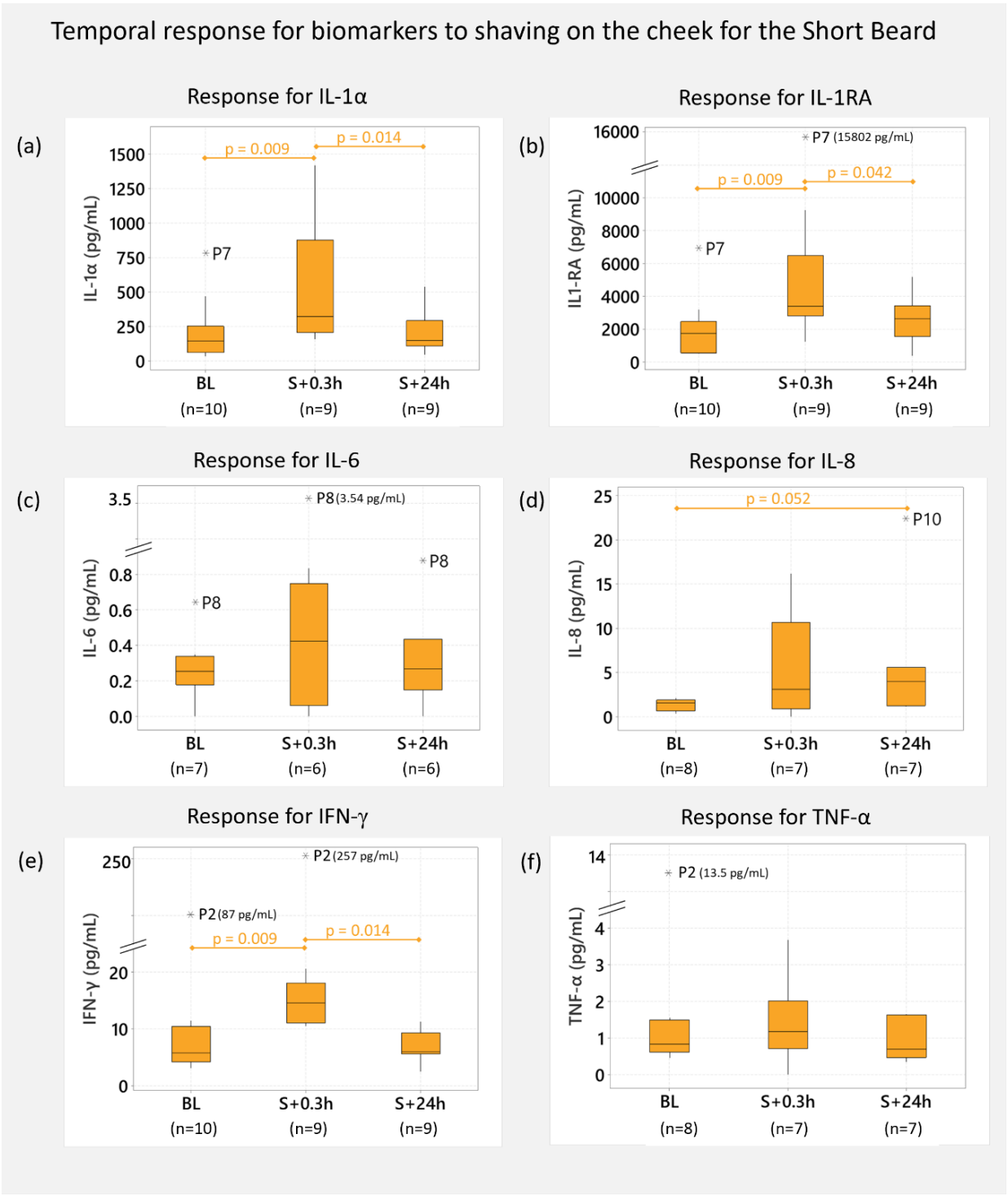


Figure 6-11: Temporal inflammatory response following shaving on the cheek. Significant changes, assessed using the Wilcoxon test, have been indicated ($p<0.05$)

6.3.2.3 Discussion

This study examined the inflammatory response following shaving in a cohort of healthy volunteers. There was variability in the inflammatory response at the cheek and the neck locations. Nevertheless, statistically significant temporal trends were observed following the insult at both the locations, with up to five-fold median changes from baseline (Figure 6-11 and Figure 6-12).

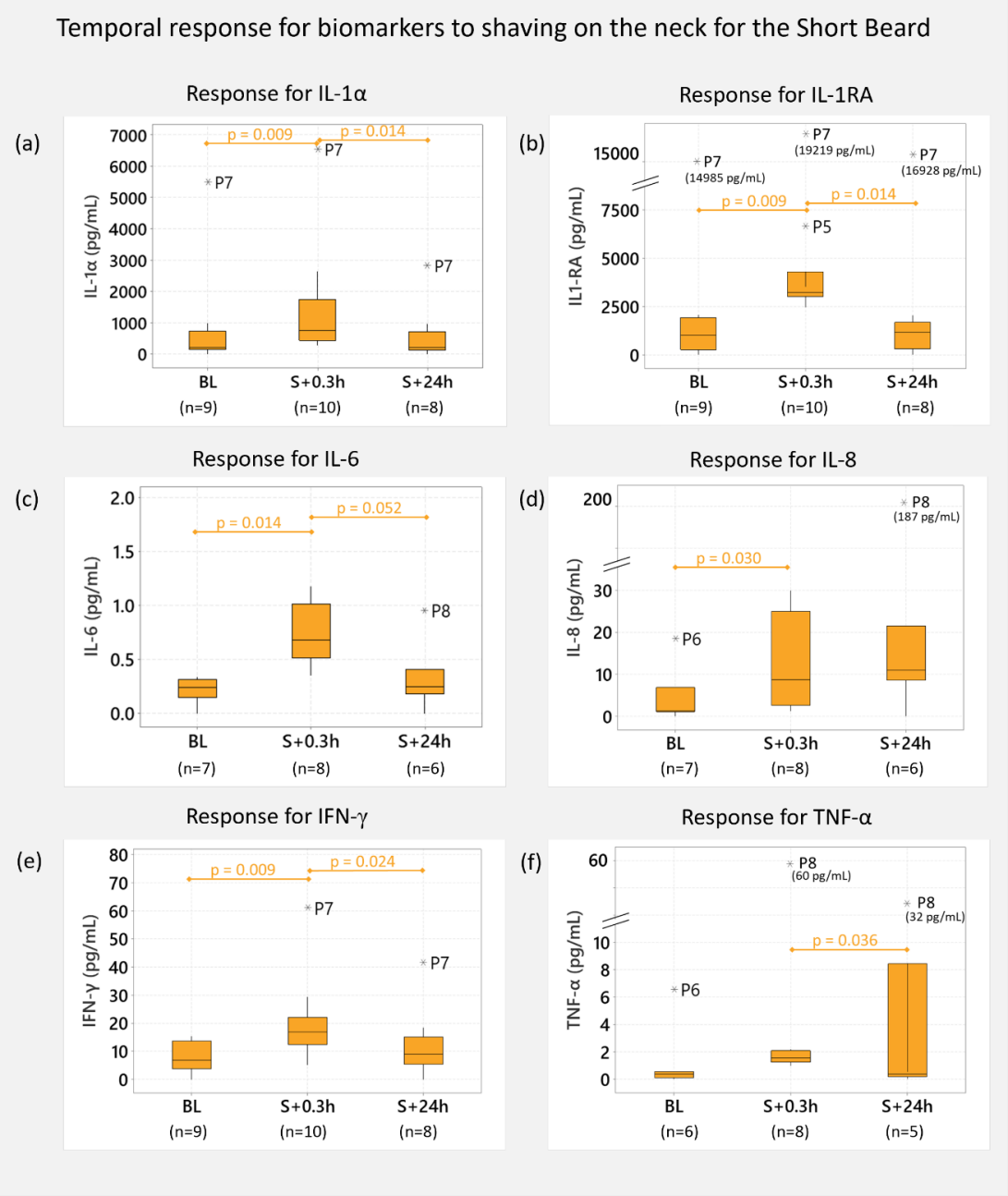


Figure 6-12: Temporal inflammatory response following shaving on the neck. Significant changes, assessed using the Wilcoxon test, have been indicated ($p<0.05$)

Shaving as an insult is composed of a perpendicular force involving the loading of device onto the skin as well as a drag force (frictional) on the skin surface. In addition, the process of the blade cutting through the hair during shaving translates to pulling forces experienced by the hair follicles (Cowley and Vanoosthuyze 2012). It is to be noted that these could also influence the inflammatory process associated with shaving. Indeed, inflammatory response has been reported to be upregulated following mechanical insults, including loading and shear insults (de Wert, Bader et al. 2015, de Wert, Geerts et al. 2019, Soetens, Worsley et al. 2019). Previous studies have reported a 6-fold increase in IL-1α/TP ratio from baseline following 30 minutes after pressure and shear application. In this study, a 5-fold increase in median IL-1 α ratio values were reported at 20 minutes following shaving. There were selected individuals who displayed an increased response in the inflammatory response (IL-1α and IL-1RA) as well as visible hyperaemic response (Figure 6-13). However, they did not correspond to the individuals with high/low sensitivity scores (Figure 6-10). Moreover, it was also interesting to note that the pro-/anti-inflammatory ratio values were less than 1. This could be attributed to the sustained increase in both the pro- and anti-inflammatory response (Table 6-4). Previous studies have explored the differences in the gene response, involving markers of different cell types (T-cells, Dendritic cells, Langerhans cells), of sensitive skin (SS) individuals and non-sensitive skin (NSS) individuals using transcriptomic studies (Richters, Uzunbajakava et al. 2016, Bataille, Le Gall-Ianotto et al. 2019). However, there is limited literature investigating the inflammatory response distinguishing skin types (SS vs NSS). Perhaps, other biophysical parameters, such as TEWL and imaging techniques, coupled with the inflammatory markers could provide further information to highlight the individuals with high skin sensitivity. The analysis of biophysical parameters and imaging parameters are beyond the scope of this thesis.

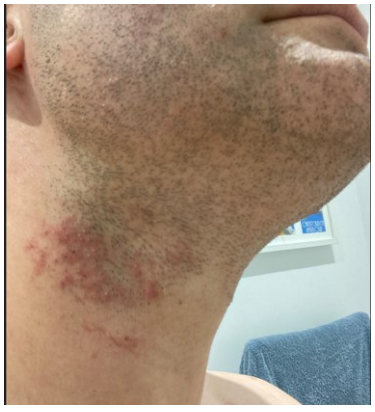


Figure 6-13: Increased hyperaemic response of a participant (P-8) following shaving

6.3.3 Inflammatory response following prolonged use of RPE in a cohort of HCWs

6.3.3.1 Participants

17 HCWs, including 15 females and 2 males, who use RPE (FFP2 or FFP3) on a regular basis during their clinical shifts were recruited onto the study (Table 6-5). Time period between consecutive test sessions varied for practical reasons, ranging between 1 to 8 weeks. The participants' age ranged between 22 and 61 years (mean age 33 ± 11 years), with a mean height and weight of 1.70 ± 0.1 m and 69.7 ± 17.1 kg, respectively. The mean corresponding body mass index (BMI) was 25.1 ± 5.4 kg/m². Participants included nurses (n=8), doctors (n=2) and other health-related professions (n=7). All participants were fit tested using a standardised procedure (HSE 2021) prior to employing respirators, except for the two who used the N95 device. Approximately one half of the participants (9/17) reported pain when employing RPE during clinical duties. The approximate frequency of breaks recorded by participants were similar for each session of data collection, as they followed an established working pattern.

6.3.3.2 Biomarker response

Changes in skin cytokine response were evaluated in the sebum sampled at the bridge of the nose (Site B). Figure 6-14 reveals a considerable intra- and inter-inflammatory marker variability, with absolute cytokine concentrations for IL-1α and IL-1RA ranging from 10 to 347 pg/mL and 375 to 15591 pg/mL, respectively. The corresponding value for the low-abundance proteins, IL-8 and TNF-α, ranged from 0.2 to 63.3 pg/mL and 0.3 to 12.3 pg/mL, respectively (Figure 6-14). Amongst the use of absolute data, normalisation to baseline and normalisation to total protein, it was found that normalisation to baseline provided means to compare the changes in inflammatory response following the use of respiratory masks. Close examination of the data highlighted that some individuals (#2, #10, #17) presented with higher ratio changes for each of the four cytokine biomarkers. By contrast, other participants (#1, #13, #15) exhibited no changes in the ratio values for any of the cytokines and the test sessions. Moreover, selected individuals (#2, #4, #5, #9, #10) revealed an upregulation of the ratio of pro-inflammatory to anti-inflammatory cytokines (Figure 6-14)

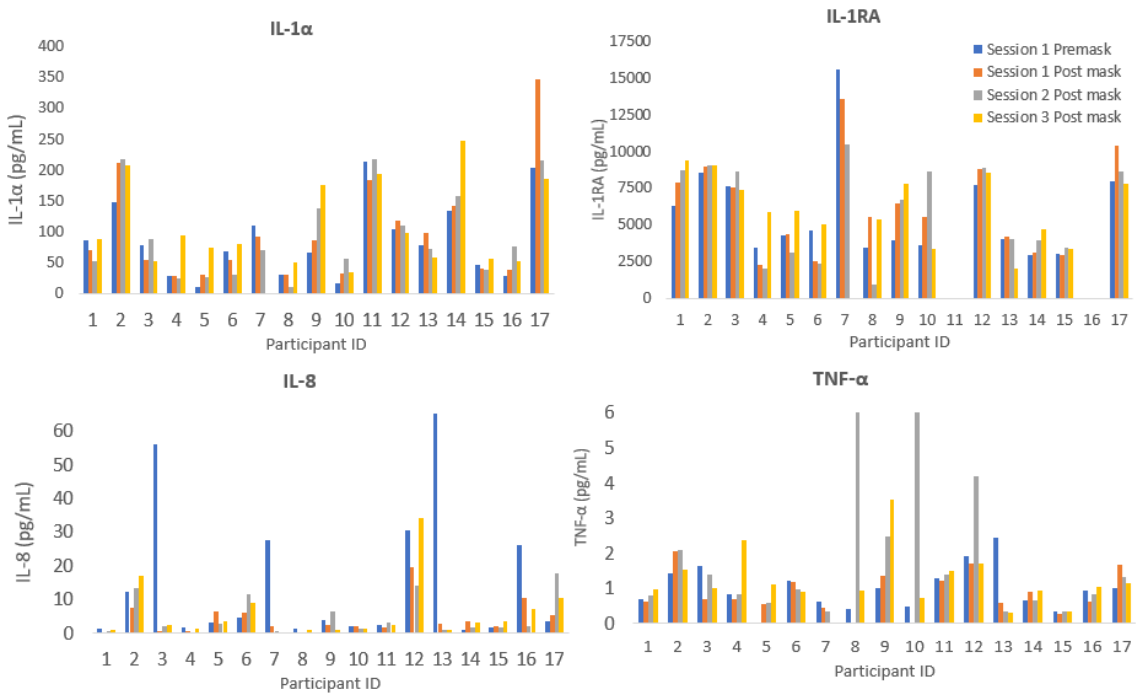


Figure 6-14: Absolute concentrations of biomarkers, namely, IL-1α, IL-1RA, IL-8 and TNF-α for each of the participants at the nasal bridge

Table 6-5 : Summary of demographics, working pattern, type of mask employed and their reactions to PPE

ID	Profession	Gender	Ethnicity	Age (Years)	BMI (kg/m²)	Mask make	Working hours	Breaks from mask	Adverse reactions to RPE
1	Nurse	Female	White	29	20.3	Aura 1863+	12	4	Spots, dry skin
2	Nurse	Female	White	28	22.5	Aura 1863+	12	2	Itchiness, excessive sweating
3	Doctor	Female	White	41	23.1	Aura 9330+	8	2	Spots, itchiness
4	Nurse	Female	White	61	24.6	Aura 1863+	8	2	None
5	Nurse	Female	White	33	39.4	Aura 1863+	12	3	Spots, lumps
6	Other	Female	White	40	34.5	Alpha Solway 3030v	10	3	None
7	Nurse	Female	White	28	22.3	Aura 9330+	12	3	Spots, itchiness
8	Other	Male	White	30	23.0	N95	10	4	Excessive sweating
9	Other	Female	White	22	25.1	Aura 1863+	7.5	1	Spots, dry skin
10	Other	Female	Asian	26	25.0	3M 8835+	8.5	1	Spots, dry skin, excessive sweating, headaches
11	Nurse	Female	White	28	20.3	Aura 9330+	12	2	Itchiness, spots, excessive sweating
12	Other	Female	White	30	19.8	N95	8	4	None
13	Other	Female	White	26	25.1	Aura 9330+	8	1	Spots
14	Nurse	Female	White	57	25.2	Aura 9330+	10	1	None
15	Other	Female	White	23	33.5	Aura 1863+	12	3	Dry skin, rashes, spots, itchiness
16	Doctor	Female	Asian	35	20.2	Aura 1863+	9	4	Dry skin, spots, itchiness, rashes, excessive sweating
17	Nurse	Male	White	31	22.5	Aura 9330+	12	3	Dry skin

Sensitivity analysis with respect to ratio change in biochemical markers are also summarised in Table 6-6. Differences were identified between specific biomarkers and the test sessions. For example, with IL-1α, IL-1RA, TNF-α and IL-1α/IL-1RA, ratios were higher in test sessions 2 and 3 compared with test session 1. By contrast, there was little difference in the IL-8 response across the three sessions. In addition, a subset of participants demonstrated elevated biomarker responses i.e., >1.5 fold-change, for some of the cytokines. For example, a ratio change of 1.5 was exceeded during session 2 in 35%, 33% and 41% for IL-1α, IL-1RA and TNF-α, respectively (Table 6-6).

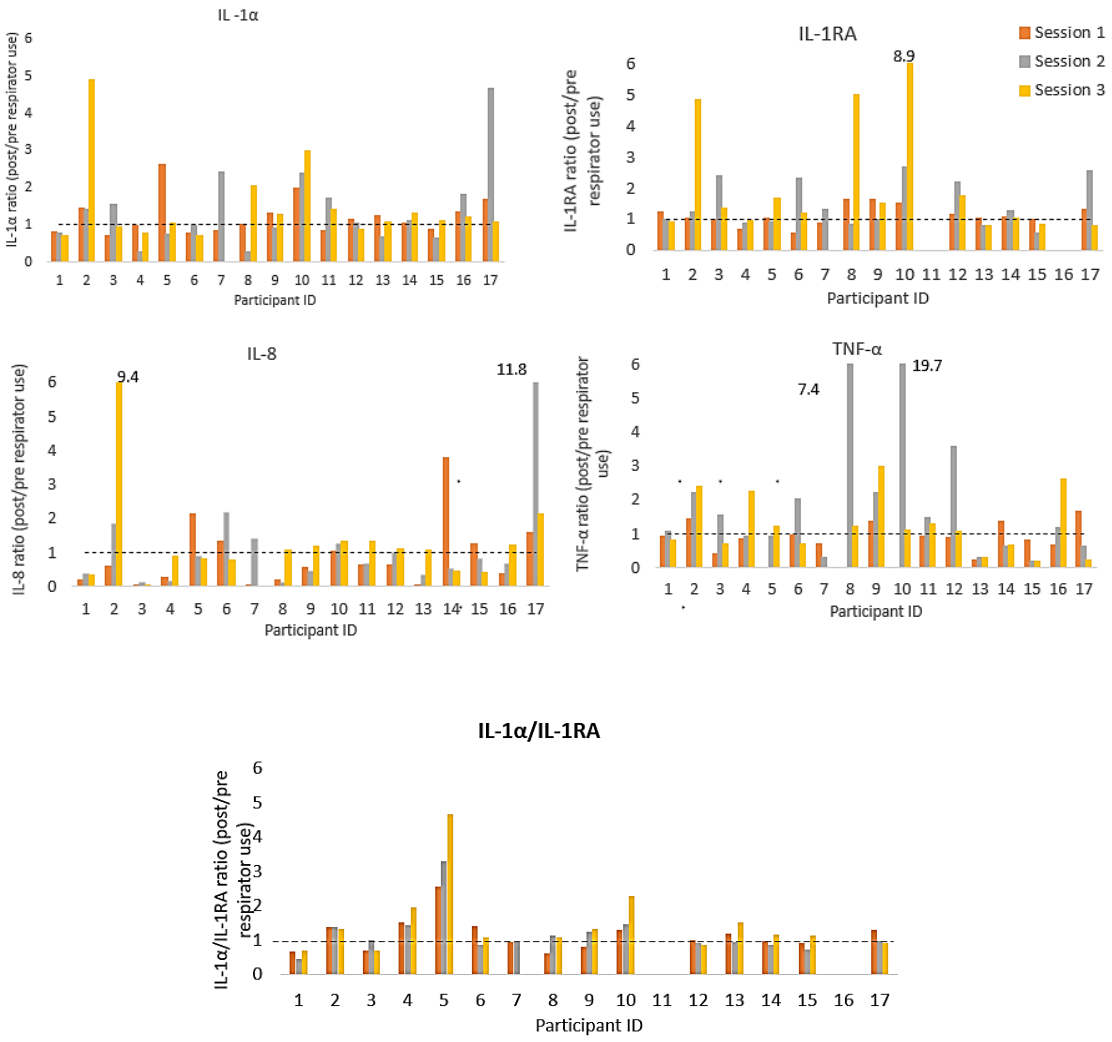


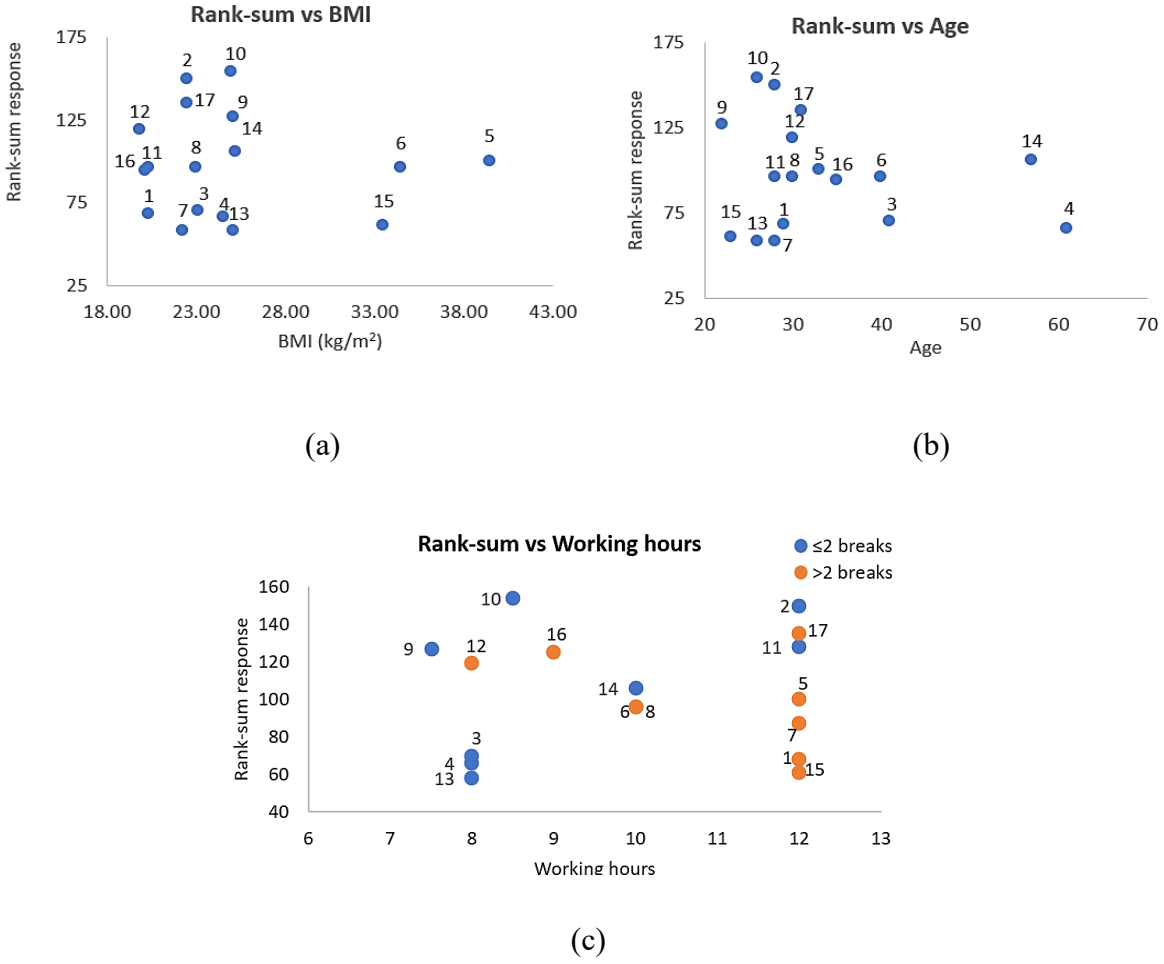
Figure 6-15 : Ratio changes in biomarkers for each participant at the nasal bridge site on the three test sessions (* indicates missing data)

Table 6-6: Sensitivity analysis of the ratio changes in biomarkers at the nasal bridge for each test session

Parameter	Threshold	Site B		
		% of participants according to the threshold		
		Session 1	Session 2	Session 3
IL-1α	< 1.0	41	47	31
	≥ 1.0	59	53	69
	≥ 1.5	18	35	19
	≥ 2.0	6	18	19
	≥ 2.5	6	6	13
	≥ 3.0	0	6	6
IL-1RA	< 1.0	33	40	36
	≥ 1.0	67	60	64
	≥ 1.5	20	33	43
	≥ 2.0	0	33	21
	≥ 2.5	0	13	21
	≥ 3.0	0	0	21
IL-8	< 1.0	65	65	44
	≥ 1.0	35	35	56
	≥ 1.5	18	18	13
	≥ 2.0	12	12	13
	≥ 2.5	6	6	6
	≥ 3.0	6	6	6
TNF-α	< 1.0	76	41	44
	≥ 1.0	24	59	56
	≥ 1.5	6	41	25
	≥ 2.0	0	35	25
	≥ 2.5	0	18	13
IL-1α/IL-1RA	< 1.0	53	53	29
	≥ 1.0	47	47	71
	≥ 1.5	13	7	29
	≥2.0	7	7	14
	≥ 2.5	7	7	7

6.3.3.3 Influence of intrinsic and extrinsic factors

The influence of intrinsic (BMI, age) and extrinsic factors, such as, the working hours, on skin biomarker response was investigated. There was no significant linear relationship ($p>0.05$) between the intrinsic factors, namely, BMI and age, and the rank-sum response of the cytokines. Indeed, it is also recognized that there were very small variations with respect to the age and BMI of the participants. The analysis also confirmed a high degree of variability in the biomarker response with respect to the duration of the working hours and the number of breaks taken within a shift (Figure 6-16).



(a) (b)

(c)

Figure 6-16: Relationship between (a) BMI (b) Age (c) Working hours on rank-sum response of the four biomarkers at the nasal bridge site on the three test session

6.3.3.4 Discussion

Biochemical marker analysis highlighted considerable variations in the ratio response between participants and across the test sessions (Figure 6-16). While some individuals expressed consistently higher responses in the candidate biomarkers in each of the test

sessions, others showed minimal up-regulation following respirator usage. This suggests that individual sites of mechanical insult evoke a variable number of macrophages, which are responsible for the production of cytokines (Zhang and An 2007). In addition, the up-regulation of IL-1 family of cytokines, namely IL-1α and IL-1RA, could be a direct result of their early synthesis and storage as precursor proteins which are released following inflammatory events. By contrast, IL-8 and TNF-α are mainly associated with dendritic cells, and thus require the recruitment and the migration of these to the site exposed to external stimuli prior to being expressed (Feldmeyer, Werner et al. 2010). Accordingly, these cytokines are expressed in smaller concentrations. However, these low-abundance cytokines were investigated for their potential in providing a differential diagnostic following various skin insults. It is to be further noted that the interplay of pro- and anti-inflammatory markers is important in the process of skin inflammation (Jensen 2010). Therefore, the analysis of pro- and anti-inflammatory markers would provide more information on the inflammatory status of the skin. The present study indicated equivalent up-regulation in the pro-inflammatory (IL-1α) and the anti-inflammatory receptor antagonist (IL-RA) for sessions 1 and 2 (Figure 6-15). In session 3, however, there is a higher proportion of participants who demonstrated an up-regulation in IL-RA. This might indicate that more time is required in order for this cytokine to migrate and be detectable at the skin surface. The biochemical parameters have highlighted the importance of cluster analysis, where sub-groups within a healthy cohort respond differently to given external stimuli, as has been demonstrated by previous studies from the host laboratory (Bostan, Worsley et al. 2019, Jayabal, Bates-Jensen et al. 2021b).

Table 6-7: Summary of the differences in the features of the studies and corresponding results

	Study 6-1 **Study investigating response following exposure to chemical insults**	**Study 6-2** **Study investigating response following exposure to shaving**	**Study 6-3** **Study investigating response following exposure to prolonged use of RPE**
Number of participants	12	--	17
Nature of insults	Exposure to dry and saturated incontinence pads (S-urine) in addition to postural loading	Exposure to rigorous shaving	Exposure to prolonged FFP3 mask usage
Stimuli	Semi-controlled stimuli	Semi-controlled stimuli	Functional stimuli
Cohorts	Healthy volunteer cohort purposely sampled from two different age groups Group 1 – 32 - 39 years Group 2 – 50 - 62 years	Healthy volunteer cohort Age range -	Healthcare workers (HCW) Age range – 22-61 years
Site of investigation	Sacrum and lower back	Cheek and Neck	Bridge of nose
Time scales assessed	0 - 150 minutes	0 - 24 hours	0 - 3 days of mask usage according to the work pattern of HCWs
IL-1α range (pg/mL)	13 - 2008	32-6545	10 - 347
IL-1RA range (pg/mL)	68 - 1585	201-19219	375 - 15591
IL-1α/IL-1RA range (no unit)	0.03 - 4.00	0.04-0.82	0.002 - 0.06
IL-1α/IL-1RA (Normalised to baseline) (no unit)	0.28 - 3.01	0.28 – 2.85	0.43 - 4.66
Other low-abundance cytokines assessed (pg/mL)	IL-6 – 0.03 - 7.18 IL-8 – 0.13 - 7.32 TNF-α - 0.16 - 25.67	IL-6 – 0.13 – 3.53 IL-8 – 0.36 – 186.79 TNF-α – 0.12 – 59.65	IL-6 – 0.08 - 0.67 IL-8 – 0.18 - 324.65 TNF-α – 0.29 - 10.66

	Study 6-1 **Study investigating response following exposure to chemical insults**	**Study 6-2** **Study investigating response following exposure to shaving**	**Study 6-3** **Study investigating response following exposure to prolonged use of RPE**
	INF-γ – 1.02 - 144.53	INF-γ – 2.54 – 256.67	INF-γ – 0.36 - 12.44
Control site - IL-1α Range (pg/mL)	104 - 873	32-5492[#]	11 – 214[#]
Control site - IL-1RA Range (pg/mL)	70 – 1016	255-14984[#]	2918 – 15591[#]
Control site - IL-1α/IL-1RA Range (no unit)	0.3 – 4.0	0.04-0.69[#]	0.003 - 0.050[#]
Other risk factors investigated	BMI Age	-	BMI Age Number of breaks Working hours

[#] - Baseline measurement at the site of investigation

6.4 General discussion

Prolonged mechanical and chemical stimuli affect the integrity of skin leading to conditions such as incontinence-associated dermatitis and pressure ulcers. In this chapter, the biochemical response of skin to various stimuli, ranging from a controlled chemical stimulus in a lab setting to a functional stimulus encountered by healthcare workers, have been investigated. Indeed, biophysical response following various stimuli have been discussed in Chapter 4. The studies in the present chapter involve different sites of investigation and wide demographics of the cohort, based on the nature of the stimuli to reflect real-time conditions (Table 6-7). As an example, the effect of shaving as a mechanical stimulus was studied at the facial locations, in particular, cheek and neck, as the shaving stimulus is typically applied in these facial sites.

Cytokines measured from Sebutapes serve as a promising tool to investigate different anatomical sites and stimuli. There is upregulation of selected cytokines following insult within the timescales of the study, ranging from hours to days, suggesting that the inflammatory signalling process is triggered within hours and remains upregulated depending on the nature of the insult (Study 6-1). Even in the absence of external stimuli, it is interesting to note that the facial location with high sebaceous gland density (Study 6-3) had considerably low amounts of IL-1α with a maximum value of 214 pg/mL, relative to that of the sacral locations with a maximum value of 873 pg/mL (Study 6-1). With respect to IL-1RA, the maximum concentrations observed at the bridge of the nose and sacrum were 15591 pg/mL and 1016 pg/mL respectively. Previous studies involving healthy volunteers have reported similar trends with the mean concentrations of IL-1α/Total protein at the facial and sacral location to be 34 pg/µg and 235 pg/µg respectively. The corresponding values for the trunk location were 1421 pg/µg and 326 pg/µg respectively (Terui, Hirao et al. 1998). The present study and previous literature confirmed that the normative levels of biomarkers are variable for different locations irrespective of the sebaceous gland density at a given location. The findings highlight the importance of choosing appropriate controls adjacent to the site of investigation while designing study protocols. With respect to the low-abundance biomarkers, it was found that the use of the optimised extraction protocol ensured that the cytokines were in the detectable range, however, the concentrations remained on the lower end of the calibration curve (Table 6-7 and Figure 6-5). The findings further revealed that IL-8 was upregulated considerably following the use of shavers and respiratory masks than following exposure to s-urine. An increase in IL-8 has been reported to promote keratinocyte

proliferation and moreover, IL-8 has been reported to be a potent chemoattract for neutrophils triggering an inflammatory response (Murata, Kaneko et al. 2021). This suggests that these low abundance biomarkers could possibly be employed to distinguish different skin insults.

Depending on the magnitude and the time of load application, the insults investigated in the study, namely, the incontinence loads (Study 6-1), exposure to RPE (Study 6-3) and shaving loads (Study 6-2) could be categorised as mild, moderate and high loads. It is interesting to note that the IL-1α/IL-1RA ratio is not exclusively dependent on the loading magnitude and duration. Indeed, it is widely acknowledged that the IL-1α/IL-1RA ratio is a function of the load magnitude and duration (Bronneberg, Spiekstra et al. 2007, Soetens, Worsley et al. 2019), however the studies suggest that the ratio is also highly dependent on the anatomical location and individual characteristics (Table 6-7). Moreover, the range of absolute cytokine concentrations, such as IL-1 α and IL-1RA

The balance between the inflammatory mediators, in particular, pro-inflammatory cytokine IL-1α and corresponding anti-inflammatory cytokine IL-1RA, affects the inflammatory homeostasis of the tissues and therefore influences the susceptibility of tissue to disease / damage. Indeed, previous research has suggested that the overproduction of IL-1 cytokines and/or downregulation of IL-1RA predisposes the individual to develop a condition (Arend 2002). From the present studies, the ratio of IL-1α/IL-1RA at the sacral locations are 2 folds greater than that of the facial location (Study 6-3) in the absence of external insults (Table 6-7). Similar trends were reported in previous studies with the ratio values higher at the trunk and forearm locations relative to that of the facial location (Terui, Hirao et al. 1998). It is to be noted that there is limited literature reporting the ratios of inflammatory markers at different anatomical locations. Furthermore, on application of external stimuli, including mechanical and chemical stimuli, the changes in the ratio values are not statistically significant (Table 6-7) suggesting that the inflammatory homeostasis is unaffected in the timescales of the studies.

The present study suggests that there is selective upregulation of inflammatory markers following mechanical and chemical insults. However, the inflammatory homeostasis, as observed with the ratio of IL-1α/IL-1RA, is unaffected at the locations investigated in the study. Indeed, there were distinct clusters of responses, even within a healthy cohort of volunteers, as previously reported from studies at the host laboratory (Bostan, Worsley et al. 2019). The biochemical parameters would provide a complementary evaluation of skin

health along with the biophysical parameters collected from the present studies (Abiakam, Jayabal et al. 2022, Abiakam, Jayabal et al. 2022). The clinical recommendations arising from the biophysical and biochemical evaluation would include regular changing of incontinence products to the reduce the time of interaction and maintaining skin health. Moreover, caregivers must implement an efficient continence management regimen, as well as regular checks to enable skin off-loading, particularly in elderly immobile individuals who might present with impaired tolerance to moisture and mechanical loads.

6.5 Conclusion

In this chapter, the biochemical parameters to monitor the inflammatory changes in skin following a range of mechanical insults, including loading and shaving, as well as chemical challenges were evaluated. The analysis of biomarkers highlighted difference in inflammatory responses at various sites of investigation even in the absence of external stimuli. Moreover, subject specific responses were observed following exposure to chemical insults, loading and shaving. Changes in inflammatory responses were modest in a cohort of healthy volunteers. It is important to develop site-specific thresholds to monitor any changes in the inflammatory status of skin. The studies also suggested that there are some influences of intrinsic factors, such as age as well as extrinsic factors, such as duration of mask usage.

Chapter 7 Biochemical markers for identifying Stage I PU – An observational clinical study

Prolonged mechanical loading could result in the formation of chronic wounds such as Pressure Ulcers. Indeed, the economic and social burden caused by Pressure Ulcers have been described in detail (Chapter 1). The analysis from Chapter 6 highlights the application of inflammatory biomarkers in monitoring the skin response following a range of lab-based and real-world insults in cohorts of volunteers. The studies provide an understanding of the factors affecting inflammatory response, such as the type of insult, time and anatomical site (Chapter 6). With an understanding of these factors, there is a need to evaluate the inflammatory biomarkers in distinguishing sites of skin damage (Stage I PU) from healthy sites. Moreover, the sensitivity and specificity of inflammatory biomarkers in characterising the skin damage has not been investigated.

7.1 Aims and objectives of the study

This study aimed to compare the spatial and temporal biochemical skin responses at both compromised PU skin site and an adjacent healthy site. This was achieved through the following objectives:

1) Collect sebum samples from the sites of skin damage and a healthy site in a cohort of patients with Stage-I PU, as defined in Chapter 1, Section 1.2.1.
   Participant recruitment and sample collection was carried out by ESR 3 from STINTS Consortium
2) Quantify a panel of relevant pro- and anti-inflammatory cytokines, including high-abundance and low-abundance cytokines, from the Sebutape samples
3) Examine the sensitivity and specificity of biomarkers and identify biomarkers using different approaches, namely, Receiver Operating Characteristic Curves (ROC)
4) Identify thresholds to differentiate the sites of skin damage from the healthy sites
5) Combined biomarkers and identify discriminatory features of the biomarkers using Principal Component Analysis (PCA)
6) Investigate the influence of intrinsic and extrinsic factors on the skin inflammatory response

The findings from the study have been published in the "International Wound Journal", with a manuscript titled, "Inflammatory biomarkers in sebum for identifying skin damage in

patients with a Stage 1 pressure ulcer at the pelvic region– A single centre observational longitudinal cohort study with elderly patients". The study has been reported according to STROBE guidelines (Cuschieri 2019).

7.2 Materials and methods

7.2.1 Study design and setting

An observational longitudinal cohort study was carried out with patients presenting with Stage-I Pressure Ulcer at the pelvic region from four geriatric departments at one large University Hospital in the UK between March'22 – July'22. The study was conducted by collaborating with clinicians, in particular, ward nurses who approached potential participants based on their voluntary consent. The study received ethical approval from the UK Research Ethics Committee (REC) and the Health Research Authority (HRA) (IRAS 301685). Participant information sheet employed for this study has been detailed in Appendix B. Signed informed consent was received from each participant on the day of screening.

7.2.2 Participants

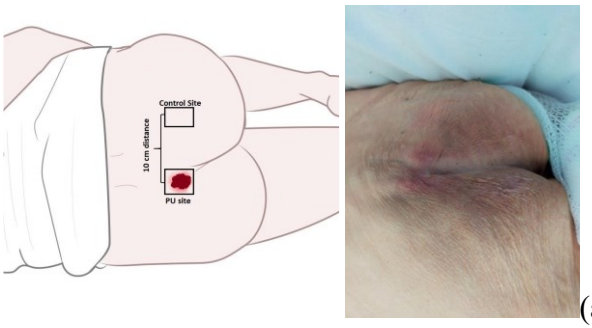
Participants were purposefully recruited who satisfied the inclusion and exclusion criteria. The inclusion criteria and exclusion criteria are listed in Table 7-1.

Table 7-1: Inclusion and exclusion criteria for participant recruitment

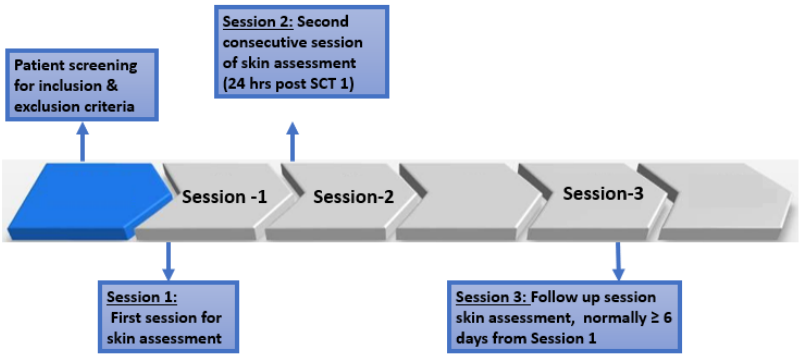
Inclusion criteria	Exclusion criteria
Patients above 18 years of age	Patients with broken skin or with an active skin condition
Patients of all genders and ethnicity	Patients approaching the end of life
Patients presenting with Stage-I PU confirmed by non-blanchable erythema	Patients who cannot be repositioned due to medical reasons
	Patients in COVID-19 departments
	Patients who are unable to understand the study protocol and provide informed consent

Patients with Stage I PU were identified by nurses from the wards and further assessed by ESR-3 for non-blanchable erythema, using a standardised skin tolerance test (Whitlock

2013). An adjacent site, 10 cm laterally away from the site of skin damage, was chosen as appropriate control site to provide comparison (Figure 7-1a). This was considered to be both sufficient to demonstrate spatial changes in skin inflammation, whilst mitigating differences between skin morphology and sebaceous gland density. The spatial and temporal differences of inflammatory biomarkers were evaluated by assessing two distinct sites, namely, the site presenting with Stage I Pressure Ulcer and a healthy adjacent site, 10 cm lateral from the site of damage (Figure 7-1). Stage I pressure ulcer (PU) was assessed as per international guidelines, confirmed by locating an area of redness and non-blanchable erythema (EPUAP/NPIAP/PPPIA 2019). The two sites were monitored over consecutive timepoints, namely, the first session following the confirmation of non-blanchable erythema and the second session 24 hours later (Figure 7-1b). Skin biomarkers were also sampled at a third session, approximately the day before their hospital discharge (between 5-9 days) in a small sub-group of participants.



(a)



(b)

Figure 7-1: Schematic representation and (a) an image of the sites of investigation (with permission and consent) and (b) timeline of study protocol involving three sessions

7.2.3 Study protocol

Prior to the attachment of Sebutapes, the skin surface was gently blotted to remove any moisture or contaminants present on the surface, including sweat, stool or urine. Biophysical measurements using TEWL (Tewameter, CK electronics, USA) and hydration (Corneometer, CK electronics, USA) probes were performed, the analysis from which was conducted by another early-stage researcher (NA). Inflammatory skin biomarkers were evaluated non-invasively by collecting sebum from the identified skin sites of each participant, using commercial Sebutape^{TM} patches (32x19 mm) (CuDerm, Dallas, TX, USA). To review briefly, the Sebutapes were attached to the skin, using a tweezer and gloved hands, and held in place for 2 minutes prior to removal. Subsequently, they were placed in appropriate labelled sterile containers and stored at -80ºC until biochemical analysis.

7.2.4 Biochemical analysis

The extraction of skin inflammatory biomarkers was performed following an optimised protocol (Jayabal, Bader et al. 2022) with the use of chemical and mechanical stimuli to improve the extraction efficiency (Chapter 5). To review briefly, the Sebutapes were extracted with 0.85 mL of buffer, which consisted of PBS + 0.1% Dodecyl maltoside. The tapes were shaken with the buffer for 1 hour followed by 5 minutes of sonication. A 0.35 mL aliquot was then used for total protein analysis. The remaining 0.5 mL was centrifuged for 10 minutes at a speed of 15000 g at 4ºC. The supernatants were discarded and the remaining solution with the pellet was briefly vortexed and used for the immunoassay analysis, as prescribed by the manufacturer using MSD U-Plex kits (MesoScale Diagnostics, USA). The light intensity for standard concentration of reagents were measured through electro chemiluminescent readers and a standard curve was plotted to determine the limits of detection. Based on the standard curve and the light intensity measured for the unknown samples, the concentration of the samples was quantified for each of the cytokine in this lab-based approach. In addition to the panel of cytokines investigated in Chapter 6, a few cytokines, namely, IL-33 and G-CSF, were added to the investigation panel as they have been identified for their role in other inflammatory conditions such as atopic dermatitis and inducing anti-inflammatory cytokines, respectively. Indeed, the role of these cytokines in other inflammatory conditions indicate promising potential in distinguishing skin status (Section 2.3.2.4). Therefore, the panel of cytokines investigated in the study included high-abundance cytokines, namely, IL-1α and IL-1RA, as well as low-abundance markers,

namely, IL-6, IL-8, IL-1β, G-CSF, TNF-α, IL-33 and INF-γ. The total protein was measured using the Bradford assay (Bradford 1976).

7.2.5 Variables

Concentrations of inflammatory biomarkers from the sebum samples were the primary output variables. Patient demographics, including age, gender and BMI, as well as information about the intrinsic factors, such as PU history, mobility, incontinence, nutrition status was also collected. The time points of data collection were also recorded to investigate the temporal changes associated with skin damage.

7.2.6 Study size

In this exploratory study, a convenience sample of hospitalised patients were recruited. With previous studies identifying a non-normal data distribution in previous sebum biomarker data, no formal power calculations were completed. Patients who satisfied the eligibility criteria and were willing to participate in the study were recruited from March 2022 to July 2022. The target sample size was 30, where patients acted as their own controls when comparing between stage 1 PU and healthy control sites.

7.2.7 Bias

Patient selection in the study was based on testing for non-blanchable erythema which was confirmed by an experienced researcher and ward nurse. Biomarker sampling and biomarker analyses were carried out independently with researchers blind to whether it was the PU or healthy control site.

7.2.8 Data analysis

Data from the ELISA plate readers (MSD Discovery Workbench and SoftMax Pro) were exported to Excel and assessed for normality using a Shapiro-Wilk test. Accordingly, non-parametric descriptors and inferential tests were employed for analysis. Comparisons between the different sites were tested using Mann Whitney tests and comparison between the two different sessions, namely Session 1 and Session 2, were tested using Wilcoxon signed-rank tests). A group-level analysis was carried out using a Friedman test to assess the independent effect of time on skin parameters across the three sessions. The influence of

intrinsic and extrinsic factors on the biochemical responses were assessed using Mann-Whitney and Kruskal-Wallis tests. A 95% confidence interval for statistical significance ($p<0.05$) was used for all evaluations. To evaluate the agreement of the measurements between the first two sessions, Intraclass Correlation Coefficient (ICC) estimates were calculated using SPSS statistical package based on absolute-agreement, single-rater, 2-way mixed-effects model. Based on the ICC, the agreement was defined as poor, moderate, good and excellent for values <0.5, 0.5-0.75, 0.75-0.9 and >0.9, respectively (Koo and Li 2016).

Principal Component Analysis (PCA) was conducted using MATLAB (Mathworks, UK) with the inflammatory panel of cytokines and the ratio of IL-1α/IL-1RA. PCA involves Eigen decomposition of the covariance matrix of the data through which the principal components that explain the largest percentage of variance could be identified (Tharwat 2016). PCA was used to combine the biomarker responses and assess discriminating features from the dataset. The resulting eigen values and eigen vectors were estimated. In addition, the variance explained by each of the principal components and the corresponding contribution of each of the markers to the principal components were also assessed. This data will inform about the usefulness of each of the biomarker in identifying the skin status.

To measure the ability of each of the potential biomarker to distinguish between healthy site and the site of Stage-I PU, data from all the sessions were pooled, compared between the sites of investigation and values for sensitivity and specificity at different thresholds were estimated. Receiver Operating Characteristic (ROC) analysis was performed for the panel of cytokines, as well as the ratio of selected cytokines. The area under the ROC curve was estimated for each biomarker combination (SPSS Software, IBM SPSS Statistics) to assess the aggregate performance. An AUC value in the range of 0.6-0.7, 0.7-0.8, 0.8-0.9 and 0.9-1.0 are considered acceptable, fair, good and excellent for classification, respectively (Pitamberwale, Mahmood et al. 2022). Optimum thresholds were identified for biomarkers with AUC value greater than 0.6 (Pitamberwale, Mahmood et al. 2022) based on sensitivity and specificity using two different methods, namely, by estimating the maximum Youden's Index, and the threshold corresponding to minimum distance from (0,1) (Pepe 2003, Perkins and Schisterman 2005).

7.3 Results

The demographics and the risk factors, defined based on a conceptual framework (Coleman, Nixon et al. 2014), of the patient cohort are detailed in Table 7-2. This group represented an elderly cohort (aged between 71 – 95 years), who presented with stage 1 pressure ulcers on the sacrum or buttock area. A high proportion of the individuals had mobility restrictions and several comorbidities (Table 7-2).

Table 7-2: Summary of demographics and intrinsic factors of the cohort, namely mobility status, incontinence, diabetic status and the medication history of the participants

Participant ID	Gender	Age (years)	Body Mass Index (kg/m²)	Location of PU	History of PU	Mobility Status	Incontinent	Diabetic	Number of medications
#1	Female	79	34.6	Buttock	No	Mobile with assistance	No	Yes	12
#2	Male	78	19.1	Sacrum	No	Mobile with assistance	Yes	No	5
#3	Male	88	24.0	Sacrum	No	Mobile with assistance	Yes	No	8
#5	Male	94	23.1	Sacrum	No	Immobile	Yes	No	11
#6	Male	80	16.3	Sacrum	No	Mobile with assistance	No	No	9
#7	Male	93	20.6	Sacrum	No	Immobile	No	No	6
#8	Male	88	32.4	Buttock	No	Mobile with assistance	No	No	N/A
#9	Female	83	14.8	Sacrum	No	Immobile	Yes	Yes	5
#10	Male	75	27.7	Sacrum	Yes	Immobile	Yes	Yes	11
#11	Male	77	22.1	Sacrum	No	Independent	No	Yes	12
#12	Male	93	17.4	Sacrum	Yes	Immobile	Yes	No	9
#13	Female	95	30.0	Buttock	No	Immobile	Yes	No	8
#14	Male	94	18.3	Sacrum	No	Immobile	Yes	No	12
#15	Male	84	27.8	Sacrum	No	Mobile with assistance	No	No	12
#16	Male	95	21.3	Buttock	Yes	Mobile with assistance	No	No	9
#17	Male	89	21.4	Sacrum	No	Mobile with assistance	Yes	No	7
#18	Female	71	26.8	Sacrum	No	Immobile	No	Yes	14

Participant ID	Gender	Age (years)	Body Mass Index (kg/m²)	Location of PU	History of PU	Mobility Status	Incontinent	Diabetic	Number of medications
#19	Female	93	19.5	Sacrum	No	Immobile	No	No	16
#20	Female	82	N/A	Buttock	No	Immobile	Yes	Yes	18
#21	Female	83	26.7	Buttock	No	Mobile with assistance	No	Yes	14
#22	Female	92	45.9	Sacrum	No	Immobile	Yes	No	15
#23	Female	91	30.3	Sacrum	No	Mobile with assistance	Yes	No	6
#24	Female	85	35.4	Buttock	No	Mobile with assistance	Yes	No	11
#26	Female	86	17.0	Buttock	No	Mobile with assistance	Yes	Yes	7
#27	Female	89	16.4	Sacrum	No	Immobile	No	No	4
#28	Female	90	19.4	Buttock	Yes	Immobile	Yes	No	12

N/A – Data not available

Changes in the high-abundance cytokines, their ratios and the low-abundance cytokines are most conveniently described separately. It was observed that the levels of total protein were in a similar range for most sites and the sessions and therefore the absolute values of cytokines are presented in the results section (Table 7-3). The intra-class correlation coefficient for each of the cytokines based on the two sessions are detailed in Table 7-4

7.3.1 An up-regulation in IL-1α

There were distinct differences in IL-1α between the control and the PU sites estimated on Session 1 with median values of 2400 pg/mL (142 – 13850 pg/mL) and 7200 pg/mL (633 – 26044 pg/mL), respectively (Figure 7-2a). The corresponding values at Session 2 were 2401 pg/mL (296 – 14315 pg/mL) and 6388 pg/mL (438 – 21855 pg/mL), respectively (Figure 7-2d). The differences between the two sites were statistically significant for both test sessions ($p < 0.05$). However, it was also evident that there was inter-individual variability in IL-1α within the cohort of patients depicted by the range values. Nevertheless, most of the individuals revealed an upregulation at the site of damage compared to the control site at Session 1 (18/26), Session 2 (19/26) and Session 3 (7/9). There were no statistically significant differences in the absolute values between Session 1 and Session 2. Moreover, in the subset of patients (n=9) followed up for Session 3 (Figure 7-3), there was an increase in the response relative to Session 1 and Session 2 for selected participants (#10, #11) whereas there was no change in response for others (#12, #21, #22, #24, #27). There were no statistically significant changes in the temporal changes of IL-1α at the control and the PU site ($p>0.05$) for the three sessions with the small-cohort of patients (n-9/30). In addition, it was clear from Figure 7-3 that there were no consistent trends between the inflammatory response and the duration of the measurement from when the redness was reported ($p>0.05$).

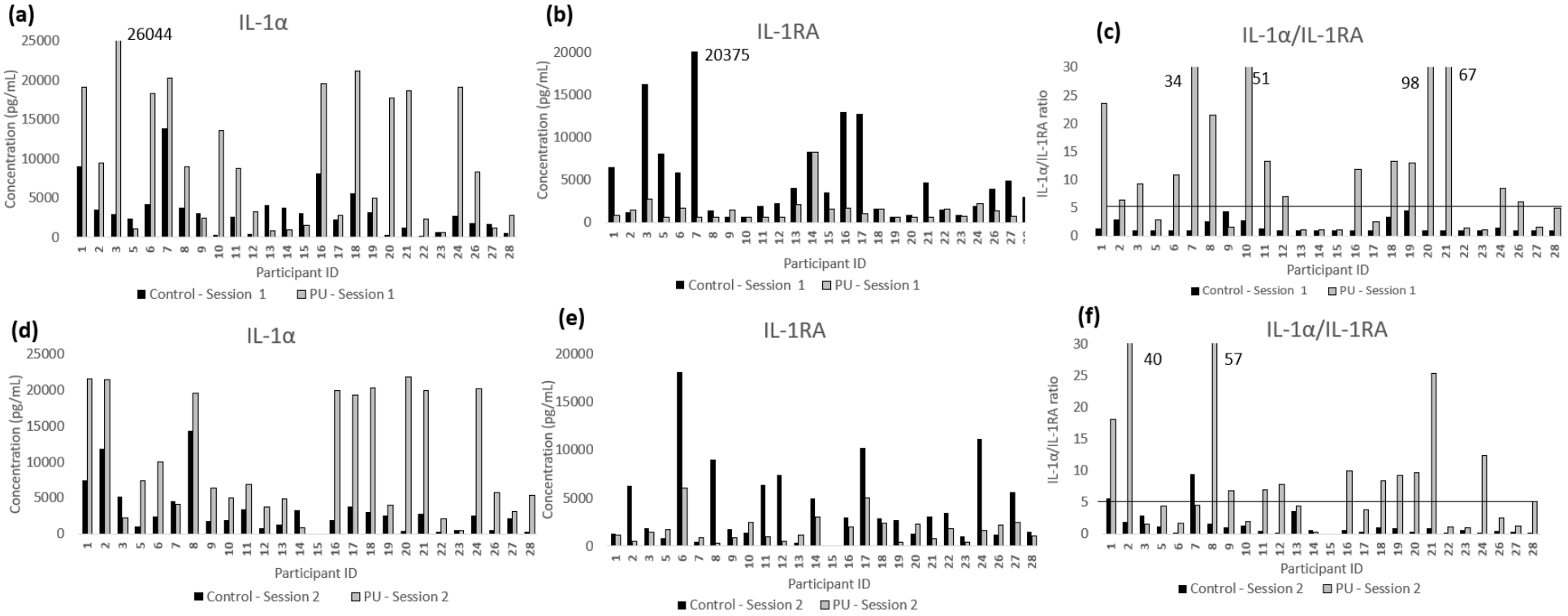


Figure 7-2: Concentrations of IL-1α (a,d), IL-1RA (b,e) and ratio of IL-1α/IL-1RA (c,f) at the sites of investigation for Session 1 and Session 2.

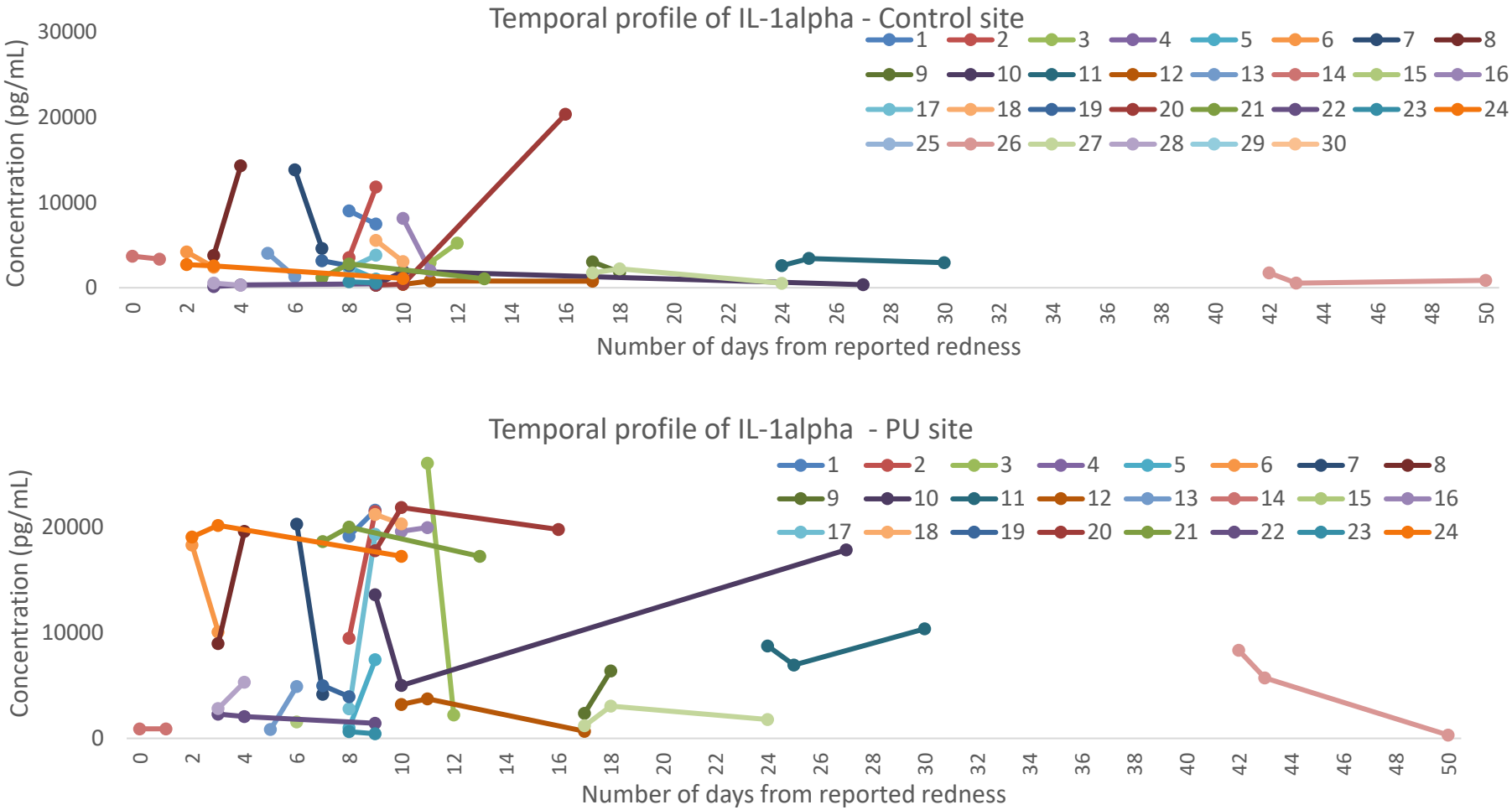


Figure 7-3: Temporal profile of IL-1α at the control (a) and PU sites (b)over the three test sessions

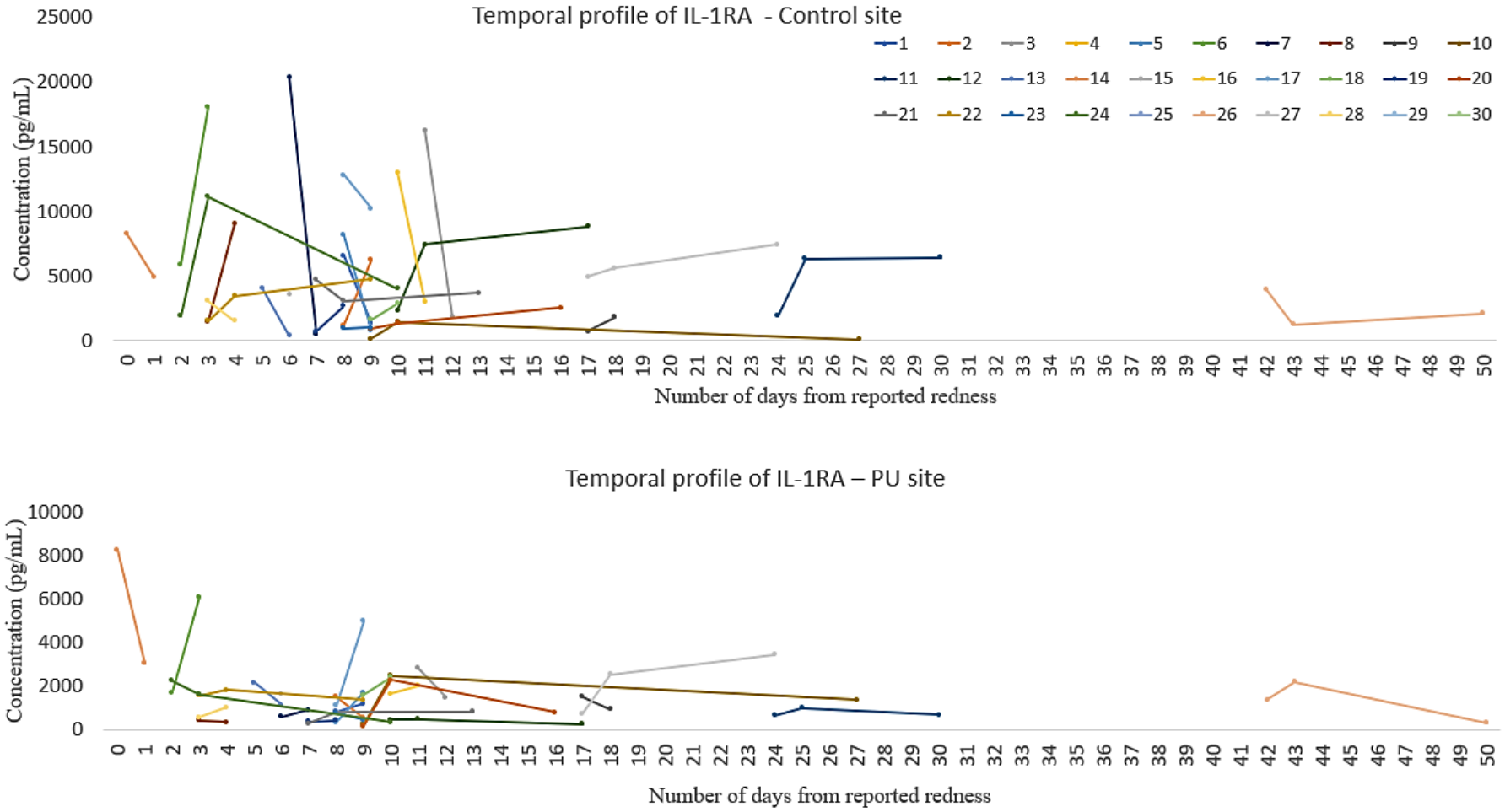


Figure 7-4 : Temporal profile of IL-1RA at the control (a) and PU sites (b)over the three test sessions

7.3.2 A down-regulation in IL-1RA

The anti-inflammatory cytokine, IL-1RA, for the control and PU sites revealed distinct differences with median value of 3312 pg/mL (107–20375 pg/mL) and 959 pg/mL (181–8245 pg/mL) at Session 1 (Figure 7-2b) and 2859 pg/mL (361–18094 pg/mL) and 1491 pg/mL (345–6094) at Session 2 (Figure 7-2e), respectively. Despite a high degree of inter-individual variability in IL-1RA within the cohort, there was a significant down-regulation ($p<0.05$) of the anti-inflammatory cytokine at the PU site in comparison to the control site for both sessions. There were no significant differences between sessions 1 and 2 at the control and PU site ($p>0.05$). However, on closer examination, there was variability between the sessions for several participants. For example, the IL-1RA concentration of #1 at the PU site was reported to be 6550 pg/mL on Session 1, whereas it was 1200 pg/mL on Session 2. Indeed, the poor agreement between the sessions are reflected by the low intra-class correlation coefficient (ICC= 0.24, -0.34 – 0.57). It should also be noted that 8/9 participants showed a marked down-regulation of IL-1RA at the site of PU on Session 3 (Figure 7-4), also reflected by statistically significant differences observed at the PU site for the three sessions within the sub-cohort of patients ($p<0.05$) (n-9/30).

7.3.3 IL-1α/IL-1RA ratio

Ratio of pro-inflammatory cytokine IL-1α to corresponding anti-inflammatory cytokine IL-1RA revealed clear significant differences in the control and PU site at all three sessions ($p<0.001$) (Figure 7-2c and f). Moreover, it was apparent that the ratio was less than 5 at the control site for majority of the patients (25/26) at all the sessions. By contrast, it was observed that the ratio values at the PU site ranged between 1 to 98, with a median of 7. Indeed, 16/25 patients had an IL-1α to IL-1RA ratio >5. On closer examination, there were some variabilities in this ratio value between the three sessions, although the differences were not statistically significant ($p>0.05$).

7.3.4 Low-abundance cytokines

Low abundant cytokines, including IL-1β, TNF-α, IL-8, INF-γ, IL-33, IL-6 and G-CSF were also investigated in the study. The median and range of concentration values from the different cytokines at the control and PU site are summarised in Table 7-3. Similar to the pro-inflammatory cytokine IL-1α, most of the low-abundant cytokines, including IL-8, G-CSF and IL-1β revealed significant upregulation at the PU site for one or more of the test sessions (Figure 7-5). Other cytokines, namely,

INF-γ, TNF-α and IL-33 revealed considerable variability and no significant difference between the PU and control sites. It is also noted that the concentrations of IL-6, although above minimum detection limits, were generally very low (0.6-52.1 pg/mL) (Table 7-3). It is to be noted that there were no significant temporal differences among the three sessions ($p<0.05$) in the small sub-cohort of patients (n-9/30)

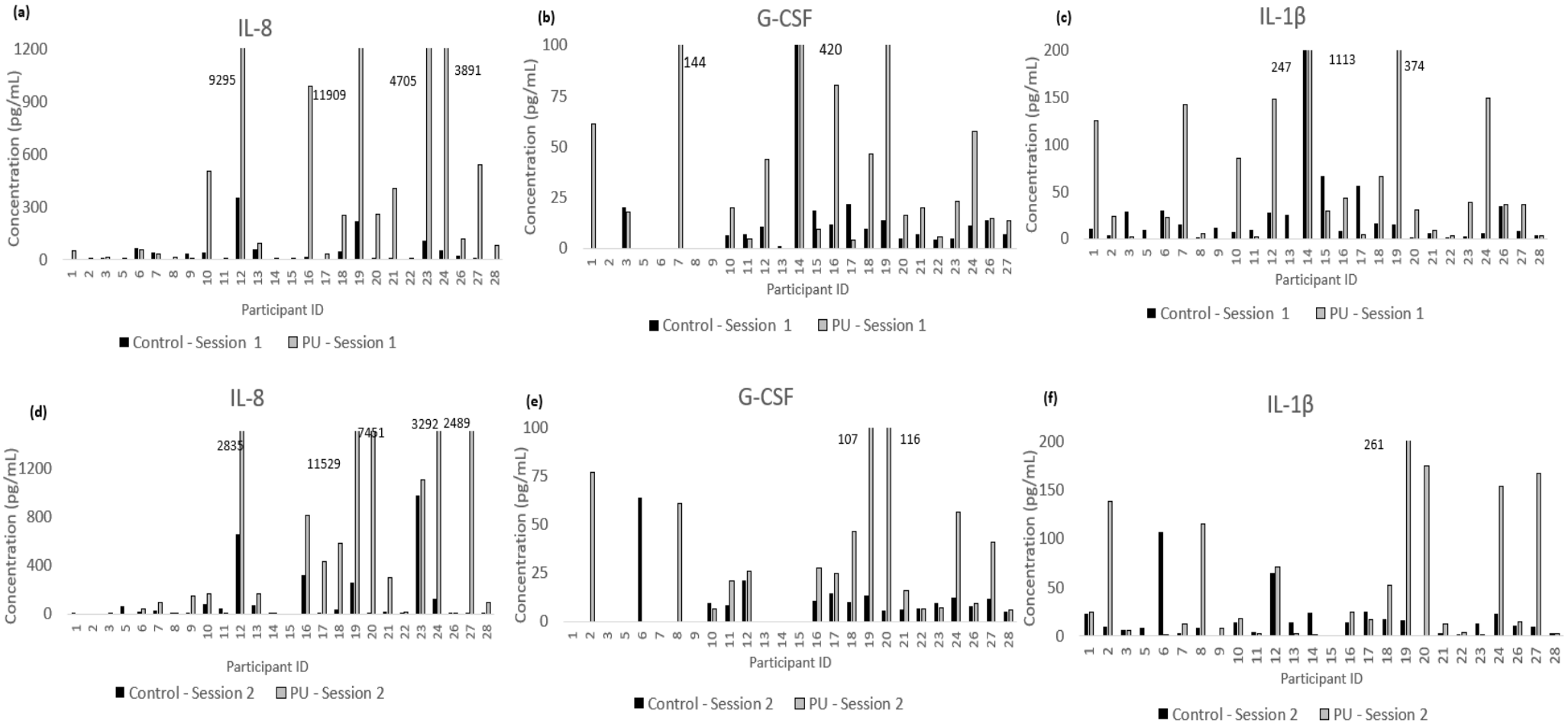


Figure 7-5 : Concentrations of IL-8 (a,d), G-CSF (b,e) and IL-1β (c,f) at the sites of investigation for Session 1 and Session 2.

Table 7-3: Median and range of the cytokine concentrations at the two sites for the test sessions.

Significant changes in cytokine concentrations between the control and PU sites have been annotated as follows: * - p<0.05, ** - p<0.01, *** - p<0.001

Cytokine	Session 1 (n - 26/30)				Session 2 (n - 25/30)				Session 3 (n - 9/30)			
	Control (pg/mL)		PU (pg/mL)		Control (pg/mL)		PU (pg/mL)		Control (pg/mL)		PU (pg/mL)	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
IL-1α	2829	142 - 13850	8545**	633 - 26044	2402	296 - 14315	6389***	438 - 21855	874	360 - 20347	10368	314 - 19784
IL-1RA	3312	107 - 20375	959***	181 - 8245	2860	361 - 18094	1491**	345 - 6094	4012	98 - 8837	791**	249 - 3443
TNF-α	7	2 – 77	9	1 – 67	8	1 – 17	12	2 – 76	7	3 - 22	5	2 - 20
IL-8	14	1 – 354	69*	1 – 11909	20	2 – 978	164*	2 – 11530	16	3 - 2184	58	3 - 4949
INF-γ	15	5 – 91	25	5 – 472	14	7 – 47	18	2 – 417	15	6 - 228	15	8 - 79
IL-33	7	2 - 34	9	2 – 28	9	2 - 27	13	2 - 43	11	2 - 24	11	2 - 21
G-CSF	9	1 – 146	20*	4 – 421	10	5 – 64	26*	6 – 117	8	6 - 82	6	3 - 107
IL-1β	10.2	1.1 – 247.2	35.8*	2.0 – 1114.0	11.2	1.2 – 106.7	16.6	0.4 – 261.1	6.0	3.4 - 124.3	7.7	2.7 - 466.9
IL-6	1.6	0.9 - 12.2	2.3	0.8 - 52.1	1.4	0.7 - 14.7	3.0*	0.7 - 34.8	1.4	0.6 - 13.8	1.3	0.8 - 5.0
IL-1α/IL-1RA#	0.7	0.1 - 4.6	7.8***	0.1 - 98.0	0.7	0.1 - 9.5	5.1***	0.3 - 5.1	0.3	0.1 - 8.0	13.1***	0.5 - 50.2
Total Protein^	45.3	20.6-78.7	44.5	26.5 - 76.4	42.2	29.0 - 67.2	63.8	16.8 - 136.5	48.2	25.2 - 146.9	50.8	36.4 - 76.9

- Dimensionless unit; ^- µg/mL

7.3.5 Agreement assessment

The agreement between the two consecutive sessions, as measured by the intra-class correlation co-efficient for each of the cytokines and the pro-inflammatory to anti-inflammatory ratio is detailed in Table 7-4. From the ICC classification, it could be seen that IL-1RA, IL-1β and the ratio of IL-1α/IL-1RA offer poor agreement between the sessions whereas the other cytokines, in particular, IL-1α, INF-γ and G-CSF offer good agreement between the sessions. It is to be noted that the intra-class correlation coefficient is not highly dependent on the abundance and offers an understanding of the temporal profile of the inflammatory cytokines.

Table 7-4: Intra-class correlation coefficient (ICC) between the two sessions for each of the cytokines

Marker	ICC	Confidence intervals	Classification
IL-1α	0.76	0.57-0.86	Good
IL-1RA	0.24	-0.34-0.57	Poor
TNF- α	0.73	0.48-0.86	Moderate
IL-8	0.86	0.73-0.92	Good
INF-γ	0.95	0.90-0.97	Excellent
IL-33	0.67	0.30-0.84	Moderate
G-CSF	0.78	0.54-0.90	Good
IL-1β	0.17	-0.51-0.53	Poor
IL-6	0.72	0.39-0.87	Moderate
IL-1α /IL-1RA	0.45	0.01-0.69	Poor

7.3.6 Principal Component Analysis (PCA)

PCA with the entire panel of inflammatory cytokines and IL-1α/IL-1RA revealed that the first two principal components explained 99% of the variability in data (Figure 7-6a). Eigenvectors confirmed that these first two PCs were primarily associated with the high-abundance proteins IL-1α and IL-1RA. The data projected to the first two principal components reveal that there is some overlapping of the control and PU data points to the left of the plot. However, the cluster to the right of the graph reveals clear separation of PU data points (Figure 7-6b).

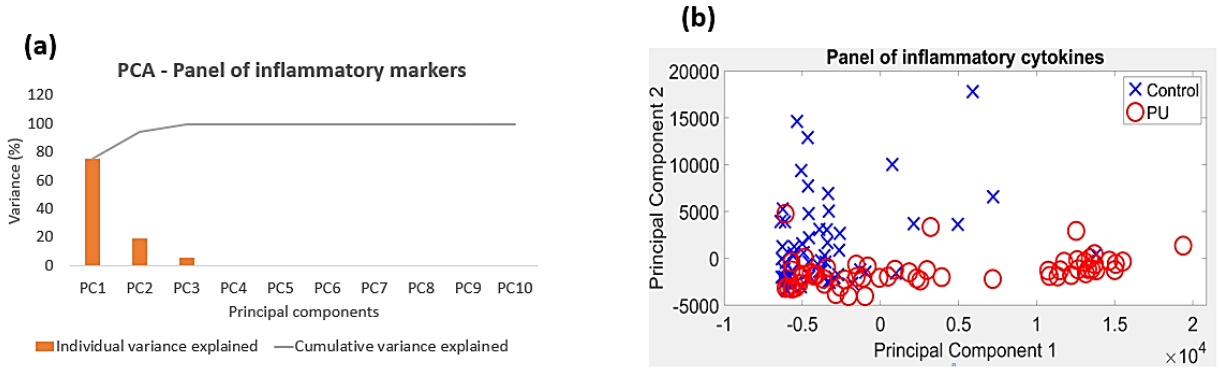


Figure 7-6:Variance explained by the principal components (a) and projection of data onto the first two principal components for the entire panel of biomarkers (b)Sensitivity and Specificity

To evaluate the ability of each biomarker to differentiate between PU and control site, the receiver operating characteristic curve was plotted, with true positive rates (sensitivity) against the false positive rate (1- specificity), for a range of threshold values. Table 7-5 provides the AUC values for the biomarkers and their significance in classifying skin damage. Selected biomarkers, namely IL-1α and IL-1RA provided fair classification whereas IL-8 and G-CSF provided an acceptable classification. The corresponding ROC curves for high and low abundant biomarkers with AUC value greater than 0.6 are presented in Figure 7-7a and b, respectively. It is interesting to note that the ratio of IL-1α/IL-1RA produced the most effective performance at both the low and high thresholds when compared to the individual cytokines, with an AUC value of 0.87 (Figure 7-7 and Table 7-5). Visual inspection of the Receiver Operating Characteristic Curve also resulted in the same threshold values corresponding to minimum distance from (0,1). The thresholds for selected biomarkers calculated using both methods are summarised in Table 7-6. Youden's index resulted in a wide range of sensitivity and specificity with values ranging between 47% to 90% and 58% to 87%, respectively. The corresponding values for minimum distance method range between 50% to 82% and 68% to 82%.

Table 7-5: Biomarkers and the corresponding area under the curve of the ROC curve

Biomarker	AUC
IL1α/IL1RA	0.87***
IL-1RA	0.77***
IL-1α	0.75***
IL-8	0.65**
G-CSF	0.61*
IL-1β	0.58
INF-γ	0.57
IL-33	0.54
IL-6	0.52
TNF-α	0.51

(*** -$p<0.005$, ** - $p<0.01$, *-$p<0.05$)

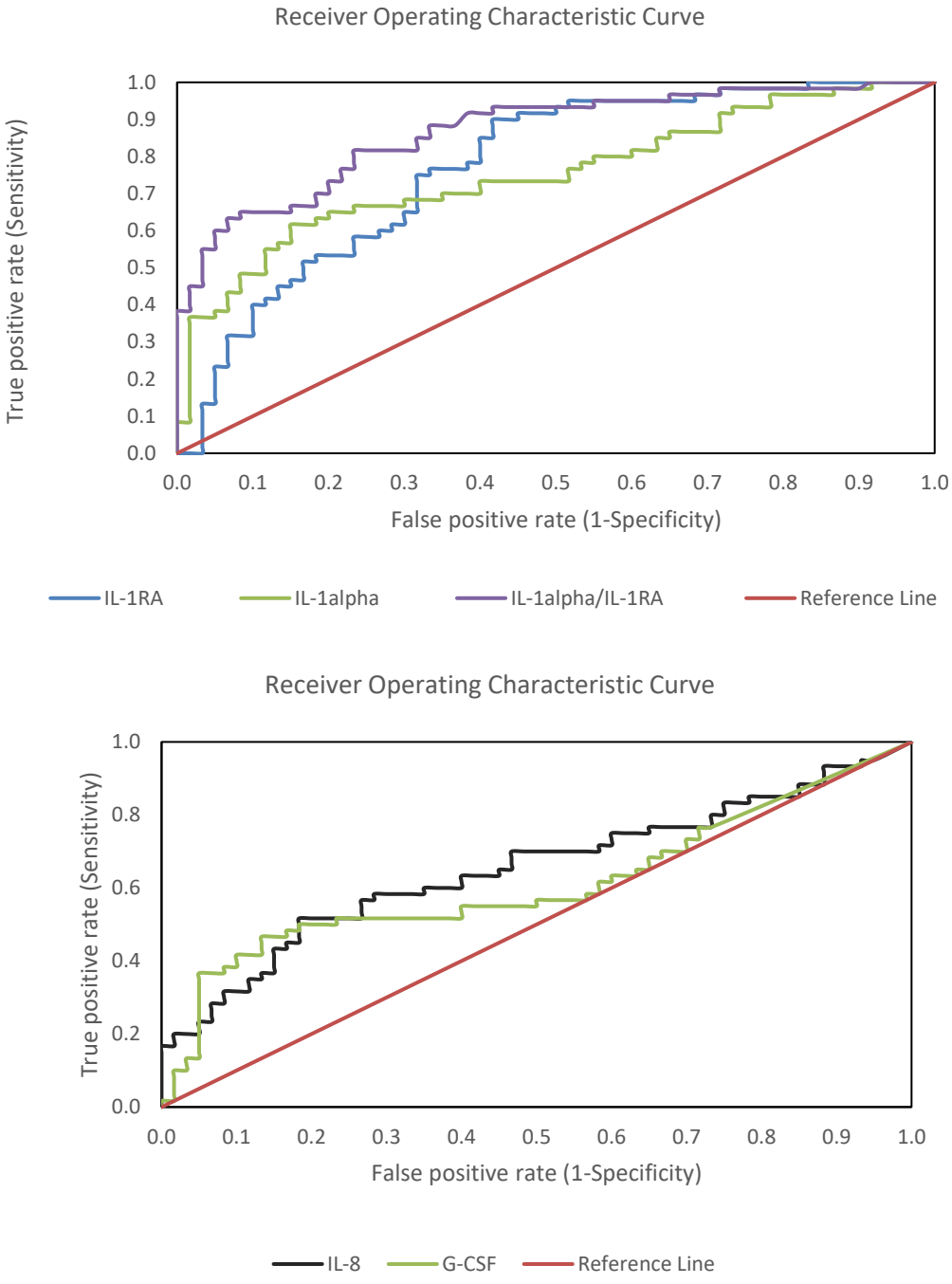


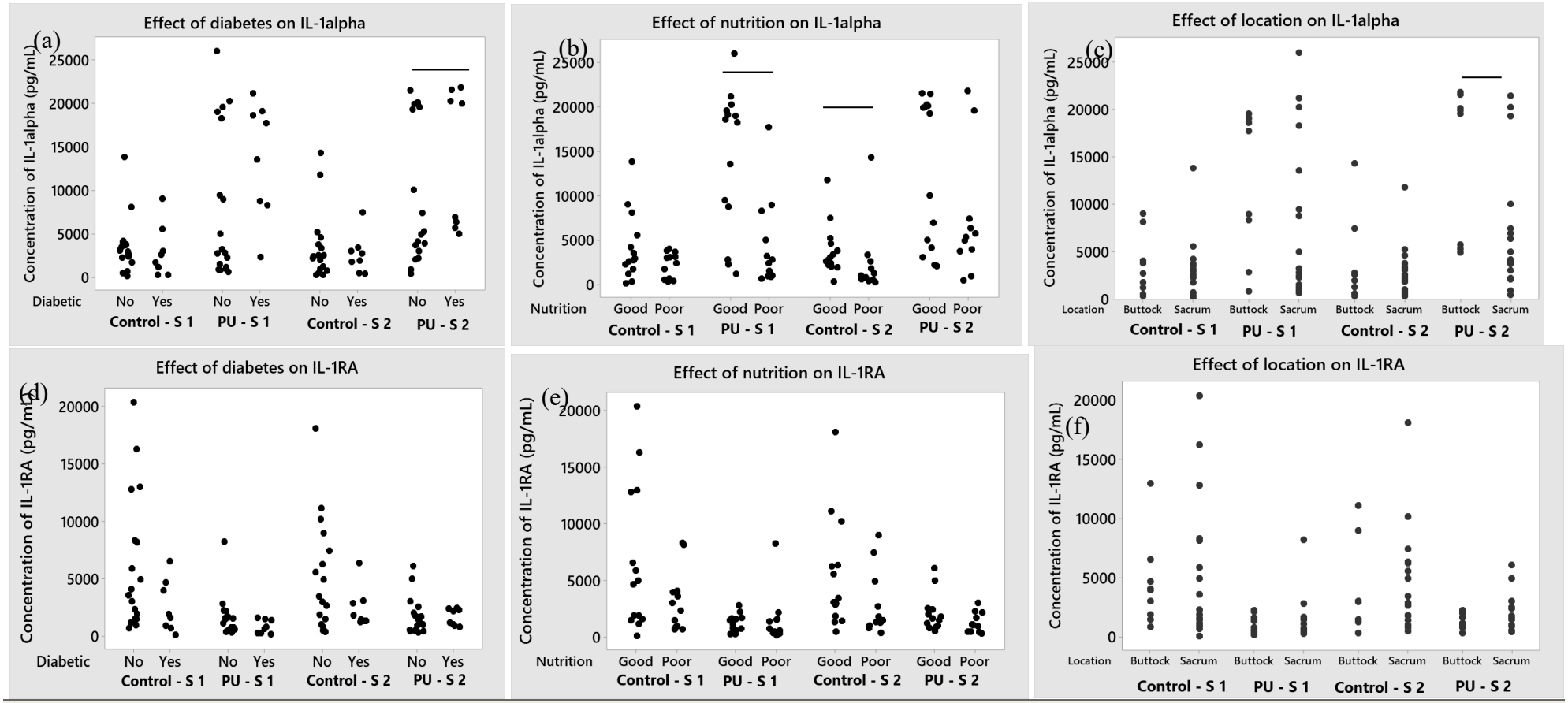
Figure 7-7: Representative Receiver Operating Characteristics (ROC) Curves for (a) high abundant and (b) low abundant proteins with AUC greater than 0.6.

Table 7-6 : Thresholds of biomarkers identified using two different methods and the corresponding sensitivity and specificity

Biomarker	Method to determine thresholds					
	Youden's Index			Minimum distance from (0,1)		
	Threshold	Sensitivity	Specificity	Threshold	Sensitivity	Specificity
IL-1α / IL-1RA	1.45	82	77	1.45	82	77
IL-1RA (pg/mL)	2550	90	58	1750	75	68
IL-1α (pg/mL)	4800	62	85	3900	65	80
IL-8 (pg/mL)	80	52	82	52	58	72
G-CSF (pg/mL)	15.7	47	87	13.7	50	82

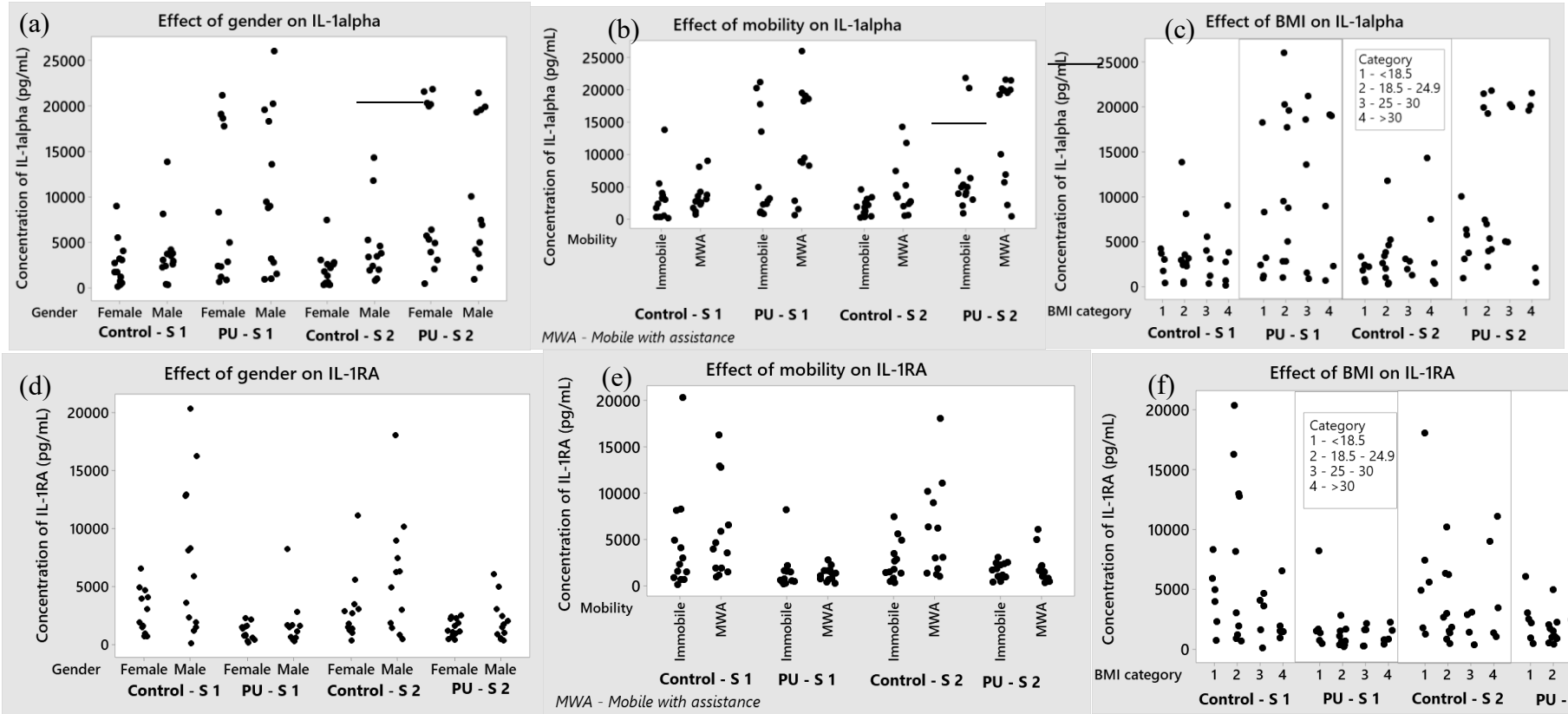
7.3.7 Influence of intrinsic and extrinsic factors

The influence of gender, diabetic status, continence, location of skin damage, PU history and BMI on the biochemical response of the high-abundance cytokines (IL-1α and IL-1RA) was examined. It was observed that there were no significant differences at the control site with respect to the intrinsic factors for both sessions for most of the parameters (Figure 7-8a-f and Figure 7-9a-f). Although the differences at the site of skin damage suggest that the non-diabetic individuals displayed an elevated response which was statistically significant for IL-1α at Session 2, the corresponding range for patients with and without diabetes were similar, ranging between 5007 – 21855 pg/mL and 438-21478 pg/mL, respectively (Figure 7-8a). It was also observed that at the PU site there were significant differences ($p<0.05$) between the diabetic and non-diabetic individuals at Session 2 (Figure 7-8a). Similar to diabetes, the corresponding range for the locations, buttocks and sacrum, were fairly similar (Figure 7-8c) There were no clear differences between the different BMI categories (Figure 7-9c and f). Interestingly, there were some differences with nutrition, gender and mobility on IL-1α at the control site for Session 2 ($p<0.05$) (Figure 7-8b and Figure 7-9a and b). It was observed that males typically had increased IL-1α and IL-1RA compared to females at the control sites. Moreover, individuals with good nutrition exhibited a significant increase in IL-1α ($p<0.01$) at the PU site for Session 1 compared to those with poor nutrition (Figure 7-8b and e). However, it is recognised that these are generally isolated occurrences and are highly variable.



	Diabetes		**Nutrition**		**Location**	
	No	Yes	Good	Poor	Buttock	Sacrum
Number of patients in each category	18	8	14	12	9	17

Figure 7-8 : The influence of diabetes (a, d), nutrition (b, e) and location (c, f) on the absolute concentrations of IL-1α and 1L-IRA on both sites in sessions 1 and 2.



	Gender		Mobility		BMI Category			
	Male	Female	Immobile	Mobile with assistance	1	2	3	4
Number of patients in each category	15	15	14	16	8	10	5	6

Figure 7-9: The influence of gender (a, d), mobility (b, e) and BMI category (c, f) on the absolute concentrations of IL-1α and 1L-IRA on both sites in sessions 1 and 2.

7.4 Discussion

This study was designed to compare the spatial and temporal differences in the expression of pro- and anti-inflammatory cytokines over the site of a Stage I PU. Nine cytokines extracted from Sebutapes were measured in this study. The results revealed distinct spatial differences for selected cytokines (IL-1α, IL-1RA, IL-8, G-CSF, IL-1β) over the localised site of the PU for majority of the patients. However, with some low-abundance cytokines, namely, IL-6, IL-33 and TNF-α, there was considerable variability between individuals as well as the sites of investigation. The performance of the cytokines as potential biomarkers was assessed by ROC analysis, which revealed that cytokines namely IL-1α, IL-1RA and IL-8 offered encouraging potential in classifying the skin status, with the ratio between IL-1α to IL-1RA providing the best discrimination. There were limited influences of intrinsic and extrinsic factors on the biochemical response (Figure 7-8 and Figure 7-9). Thus, the change in inflammatory status of the local skin tissues can be attributed primarily to the damage caused over the stage 1 pressure ulcer. Furthermore, it was also found that there was good agreement between the sessions for most of the cytokines, with the exception of IL-1RA and IL-1β.

Previous studies involving pre-clinical evaluations of tissue and animal models, in addition to healthy volunteers have highlighted the upregulation of cytokines when tissue was subjected to mechanical loading (Bronneberg, Spiekstra et al. 2007, Soetens, Worsley et al. 2019, Kimura, Nakagami et al. 2020). Pilot studies conducted with a small patient cohort (n=6) of Stage I PU revealed an upregulation of IL-1α/TP at the localised site of skin damage. However, other cytokines, namely IL-1RA and IL-8, were not detectable in this study (Bronneberg 2007). Other sampling techniques, such as skin blotting, have also reported an upregulation of IL-1α over the site of a PU, although these methods remain semi-quantitative and requires standardization owing to the inflexibility of nitrocellulose membranes (Nakai, Minematsu et al. 2019). Indeed, in the present study, we have for the first time reported a significant upregulation of IL-1α as well as the low abundant cytokines, IL-8, IL-1β, G-CSF at the site of Stage I PU relative to that of the control site. This corresponded with a down-regulation of IL-1RA, which is an anti-inflammatory cytokine. The study demonstrated that for most test sessions, the level of total protein was similar between skin sites, thus enabling direct comparison of cytokine concentration without normalising to TP value.

In a recent study conducted with patients at intensive care units (ICU), it was reported that there was an increase in IL-1α/TP at the control site, chosen to be the head of humerus, relative to that of the sacrum. The present study chose a control site close to the PU because it was considered to present with similar anatomy and the sebaceous gland density. Indeed, it is well known that the inflammatory response is highly site-specific (Terui, Hirao et al. 1998). The study conducted in ICU also hypothesised that the IL-1α measured from Sebutapes were influenced by systemic inflammation (McEvoy, Patton et al. 2022). However, from the present study, the localised upregulation could be predominantly attributed to the skin damage, with other intrinsic factors known to cause metaflammation e.g., diabetes, revealing limited influence on the findings. There were also minimal variations in the cytokines for majority of the cytokines between both sessions of skin assessment thereby demonstrating a degree of agreement in the concentration values (Figure 7-2 and Figure 7-5).It is interesting to note the temporal changes in the IL-1RA and IL-1β between the two sessions (ICC <0.5). Perhaps, this could be attributed to the IL-1β – IL-1R signalling pathway that has been highlighted to play an important role in autoinflammatory skin disease such as psoriasis (Cai, Xue et al. 2019). However, further research is required to investigate the temporal signalling pathways associated with these two cytokines of IL-1 family.

The inflammatory response is mediated by pro-inflammatory and anti-inflammatory cytokines. Indeed, a balance between these cytokines have been reported to play a major role in susceptibility to disease conditions (Arend 2002). Previous studies investigating UV - effects on skin and inflammatory skin diseases, such as atopic dermatitis (AD), has reported a decrease of IL-1α to IL-1RA ratio as well as an increased IL-1RA response at the exposed sites relative to control sites (Hirao, Aoki et al. 1996, Terui, Hirao et al. 1998). By contrast, the present study has reported an increase in the ratio of IL-1α to IL-1RA, in addition to the down-regulated IL-1RA response, with ratio values greater than 5 for majority of the participants, at the site of skin damage (Figure 7-2c and f). These differences in response could be attributed to the nature of the skin damage, with the present study investigating mechanical damage at localised areas developed at within relatively short periods whereas the literature investigated systemic diseases presented with metaflammation from AD. Indeed, it is to be noted that these ratios are site-specific, as an example, previous studies have shown high values of the ratio i.e., IL-1α/IL-1RA at the trunk, hand and feet relative to the facial locations (Terui, Hirao et al. 1998). Nevertheless, it is clear from the present as well as the previous studies that skin's inflammatory homeostasis is affected at the sites of

PU damage. This corresponds to the emerging evidence derived from mechanobiology studies, that inflammation is one of the primary signalling opportunities within pressure ulcer research (Gefen, Brienza et al. 2022).

Area under the ROC curve to assess the diagnostic value for distinguishing between PU and healthy site were investigated, with a perfect value equal to 1 (Hajian-Tilaki 2013). In the present study, the ratio of IL-1α/IL-1RA offered the highest performance with an AUC value of 0.87. The combination of IL-1α and IL-1RA offered an improved performance than the individual cytokines, reflective of the balance between pro- and anti-inflammatory biomarkers in the sebum. Low abundant cytokines, namely IL-8 and G-CSF, offered significant classification, albeit with a lower AUC value to distinguish between the skin sites providing further information on the localised inflammation (Table 7-5). In addition to the ROC analysis, PCA analysis also revealed IL-1α, IL-1RA and IL-8 as primary contributors for the principal components offering separation between the control and PU data (Figure 7-6). As highlighted in a review, integration of multiple biomarkers would offer better prediction potential (Bader and Worsley 2018). The analyses from the present study identified the markers that could be potentially combined as a biomarker for PU diagnosis. Indeed, larger data sets are required to validate these findings. Moreover, one of the challenges in choosing a threshold in biomarker studies is the compromise between sensitivity and specificity. The Youden method offered a wide range of sensitivity and specificity whereas the minimum distance method offered optimal sensitivity and specificity in the present study (Table 7-6).

Previous studies investigating the cytokines profiles following insults have reported change in high abundant cytokines, such as IL-1α and IL-1RA (de Wert, Bader et al. 2015, Bostan, Worsley et al. 2019). Those studies that have attempted to assess low-abundance markers have often failed to achieve the LOD (Koudounas, Bader et al. 2021). However, with the use of the new extraction protocol, as detailed in Chapter 5, we were able to quantify low-abundant cytokines, such as IL-8 and G-CSF that could provide classification of damaged skin from healthy skin (Jayabal, Bader et al. 2022). It is well known that a variety of skin insults (mechanical, chemical and thermal) alters the production of the high-abundant cytokines, namely, IL-1α and IL-1RA (Lee, Briggs et al. 1997). Indeed, this has also been observed in studies detailed in Chapter 6, wherein skin inflammatory response was investigated following various insult models. In addition, each of the low-abundant cytokines play a unique role in maintaining the barrier function, as an example, TNF-α, INF-γ and IL-1β are important in the lipid synthesis, whereas IL-8 and G-CSF are associated with

dendritic cell migration and neutrophil regulation (Jensen 2010, Hänel, Cornelissen et al. 2013, Wolk, Brembach et al. 2021).

It is of note that other biomarker candidates have been proposed to monitor pressure ulcer cites, with reference to the known aetiology (Bader and Oomens 2018). Indeed, when tissue is subjected to mechanical loading, ischaemia, i.e., loss of blood flow leads to the production of metabolites such as lactate, pyruvate and purines, and on unloading, the reperfusion phase leads to the production of oxidative stress markers (Khlifi, Graiet et al. 2019, Soetens, Worsley et al. 2019). These metabolic markers, purines and oxidative stress markers could provide classification of the skin status and therefore future studies are required to investigate the potential of the metabolic biomarkers. Biophysical parameters, such as trans-epidermal water loss (TEWL), erythema and skin hydration, have been previously reported to be upregulated following loading and insults to skin (Abiakam, Jayabal et al. 2022, Abiakam, Jayabal et al. 2022, Völzer and Kottner 2022). With respect to the present study, biophysical parameters were also investigated in conjunction with the biochemical markers, the results of which have been reported separately (Abiakam, Jayabal et al. 2022). Here it was demonstrated that parameters, such as TEWL, offer clear distinction between healthy and damaged skin sites. There is potential to combined biophysical and biomarker parameters to provide a detailed objective means of describing skin structure and function, with further studies required to assess their diagnostic and prognostic capability in a range of skin of skin damage models.

In the current study, the influence of intrinsic factors on cytokine response was evaluated. Indeed, a number of factors, including, immobility, moisture status, diabetes, nutrition status and PU history have been identified to predispose individuals to pressure ulcer development (Coleman, Nixon et al. 2014). In the present study, there were no clear differences in cytokine response between individuals with comorbidities, for example diabetic and non-diabetic patients. This is in contrast to a previous study wherein individuals with diabetes showed more expression of pro- and anti-inflammatory cytokines, albeit in the sole of the foot known to be at risk of tissue damage in this population (Henshaw, Bostan et al. 2020). There were also no clear differences between the different locations, i.e., sacrum and buttocks, of the skin damage as well as BMI and gender. Owing to the pragmatic approach of the study there were differences in the number of days between the first sebum sampling and the day redness was first reported by the clinicians, this ranged from 0 to 50 days (Figure 7-3). However, there were no influences of this time period on the cytokine profiles (Figure

7-3). This suggests that the inflammatory cytokines are upregulated at the PU site for a prolonged period and an active physiological response continues to take place.

For the first time, this cohort study has identified distinct difference in cytokine profile at the PU site relative to that of the nearby control site, irrespective of other intrinsic and extrinsic factors. Therefore, these cytokines offer promising potential for adaptation to clinical settings to identify skin damage. The low volume of samples extracted from the present study would be sufficient for the assessment of a single cytokine using lateral flow tests (≈100-150 μL), however, for concurrently analysing a panel of cytokines, more sample volume would be required. In addition, the samples extracted from the current study were also highly viscous and therefore might require an additional sample preparation step within the device that could be facilitated by the selection of appropriate membranes and viscosity modifiers for the lateral flow device (Posthuma-Trumpie, Korf et al. 2009, Guo, Hansson et al. 2018). With the advancement in technology, Point of Care (PoC) testing has become a reality in diagnosis, monitoring and screening for a range of disease conditions. Indeed, PoC tools for the detection of interleukins are in their early phase of development (Corstjens, de Dood et al. 2011, Eiras 2020, Rahbar, Wu et al. 2021). Monitoring the temporal changes in the biochemical status using PoC tools as an adjunct to the routine risk assessment employed in clinical settings would aid in identifying individuals at risk as well as ensuring appropriate resource allocation. The use of non-invasive biomarker analysis could provide meaningful improvements to practice which relies on visual assessment, for example when assessing black skin where redness is not apparent.

7.4.1 Limitations

The study is limited by the small sample size and the preponderance of elderly Caucasian participants that limits the generalisability of the findings. The limited data set also precluded the potential to include regression analysis to integrate multiple biomarkers. However, ROC analysis and PCA analysis has highlighted the inflammatory biomarkers offering predictive capabilities (Section 7.3.5 and Section 7.3.6). Patients from the study were administered regular medications for their underlying pathologies, which includes a list of anti-inflammatory drugs, vitamins, anticoagulants, diuretics, analgesics etc., Although it is known that some of the drugs, such as, anti-inflammatory drugs would cause a change in the systemic inflammation, the effects of such medications have not been investigated in the study. The present study was limited to the sacrum and buttock site, further studies are required to identify thresholds for various skin sites, such as heels, which vary in sebaceous

gland density and skin morphology, as detailed in Chapter 4. It is important to further investigate the predictive and prognostic capability of these cytokines in a longitudinal clinical study, in which stage 1 pressure ulcer sites may heal or progress to wounds.

7.5 Conclusion

This study investigated the differences in biochemical response at two different skin sites, namely a stage 1 pressure ulcer and an adjacent healthy site. The results revealed that there were significant spatial differences between the two sites, with upregulation of pro-inflammatory cytokines and down-regulation of anti-inflammatory cytokines at the site of skin damage. There were limited influences in the cytokine response due to other factors, namely nutrition, mobility and location. ROC analysis identified a panel of biomarkers, including IL-1α, IL-1RA, the ratio of IL-1α/IL-1RA, IL-8 and G-CSF offered moderate to high classification of the damaged sites from the healthy sites. Performance measures, such as sensitivity and specificity were calculated for different thresholds and optimum thresholds were identified for the biomarkers using different approaches. Further research is required to investigate the predictive and prognostic capability of these biomarkers and the corresponding thresholds.

Chapter 8 Discussion

This chapter reviews the important findings of this thesis and the contribution to the scientific literature. Furthermore, it summarises key research findings for each of the aims and hypotheses for the research project, the limitations of the thesis, and future research avenues are detailed.

8.1 General overview – Addressing research aims and hypothesis

Diagnosing skin status objectively represents an important challenge in the prevention of chronic wounds, particularly, pressure ulcers. Current practice of skin assessment involving risk assessment scales (RAS), visual assessment, medical history and physical examination aims to identify individuals at risk of skin damage (Defloor and Grypdonck 2004, Kottner and Balzer 2010, Moore and Patton 2019). However, the subjective nature of these assessments has several limitations, as described in Chapter 1, Section 1.2.3, thus it is imperative to employ objective measures to support clinical decision-making and personalised interventions. Accordingly, this project focussed on investigating the skin status using novel biomarker candidates to detect skin damage at an early stage thereby aiding the implementation of targeted prevention strategies to reduce the incidence of chronic wounds.

Various approaches could be employed to diagnose skin status, including biophysical, biochemical, and imaging approaches, as highlighted in Chapter 2. Indeed, the chosen biomarker candidates were measured easily and non-invasively, as described in Section 3.1. In this thesis, biomarkers, in particular, biophysical and biochemical markers, were identified and validated by using a three-step framework, involving, discovery, verification, and validation (Section 3.2). There are several commercial biophysical tools, such as trans epidermal water loss (TEWL) and sub-epidermal moisture (SEM), which have a variety of evidence supporting their use to monitor skin status following insults. With respect to biochemical markers, there has been a considerable momentum in research in the past few years. However, there are no biochemical markers that have been validated in clinical settings. The literature suggests that there is no single or multiple combination of biomarkers that could clearly identify the changes in skin integrity (Bader and Worsley 2018). Moreover, these tools have not been evaluated for their performance in clinical settings and distinguishing skin responses following different insults. Accordingly, this PhD project aimed to identify novel biomarker candidates, with a

particular focus on biochemical markers, and evaluate their performance of biomarkers in identifying early signs of skin damage. The aims and objectives of the PhD project, as detailed in Section 2.4, were achieved by prospective experiments from lab-based insults to clinical situations as well as analysis of retrospective data (Figure 8-1).

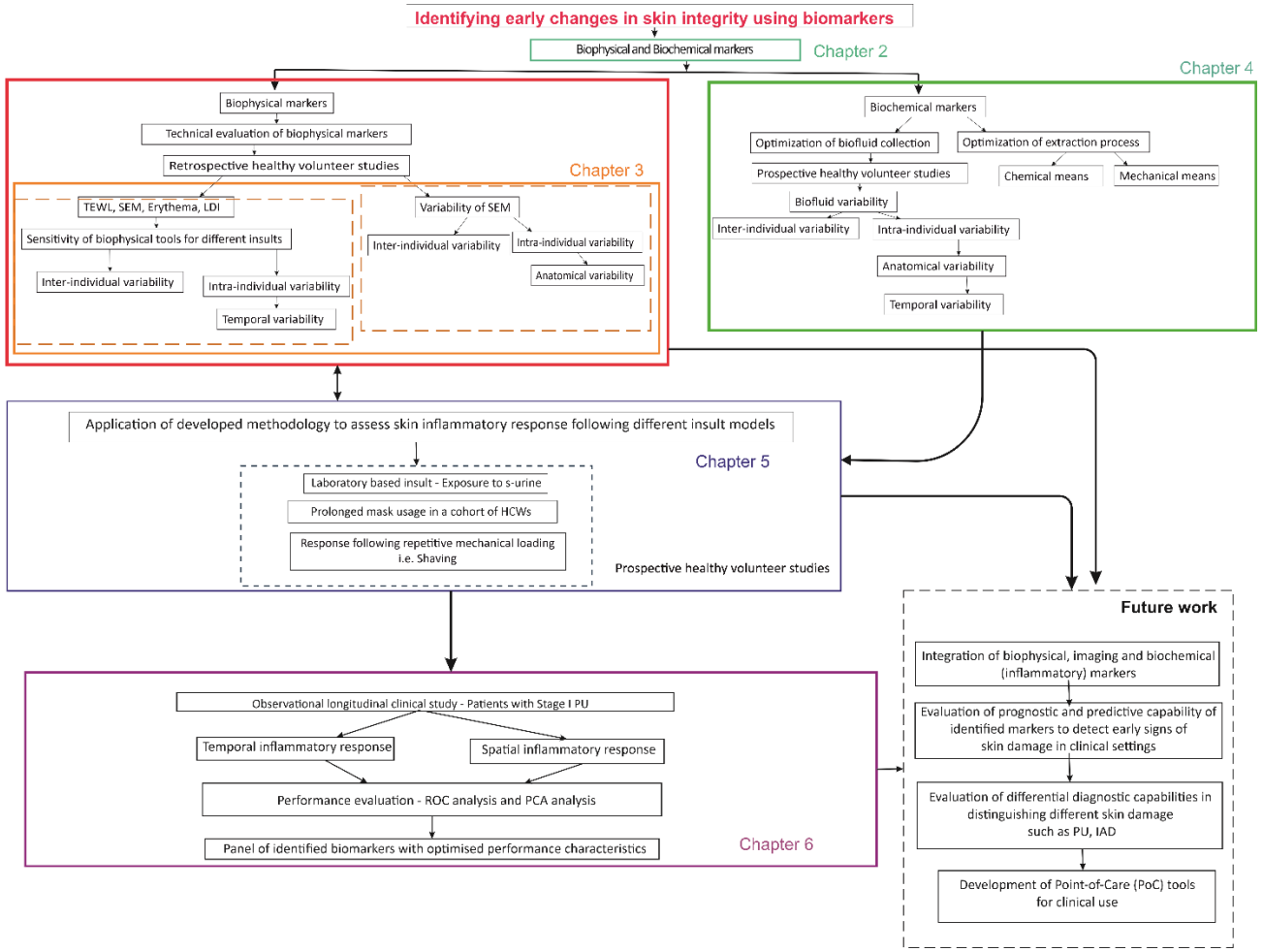


Figure 8-1:Schematic of the studies conducted to achieve the aims and objectives of the thesis.

Aim 1: Assess appropriate biophysical and biochemical techniques and protocols to characterise the integrity of skin tissues

With respect to biochemical and biophysical parameters, there exists several potential candidate biomarkers (Chapter 2). Protocols were developed and employed to measure skin parameters, which are reflective of skin status, following a range of skin insult models. Moreover, standard skin insult models, including tape-stripping, moisture exposure and chemical irritation, were developed and tested (Chapter 3). The changes in parameters, including spatial and temporal changes, were assessed following established skin insult models as well as new skin insult models, such as the use of masks.

With respect to biochemical markers, the inflammatory status of an individual could also be characterised by sampling different biofluids, such as blood, sebum and sweat. These biofluids were appraised for their use in clinical settings as well as in measuring skin integrity (Chapter 2). Sebum was chosen as a biofluid because it could be collected locally and non-invasively with minimal discomfort to the patients (Section 3.1). In the discovery phase of biomarker discovery framework (Chapter 3), the literature was appraised with an understanding of the aetiology of PUs to identify candidates of interest. Integrating the biophysical and biochemical parameters, protocols were developed in collaboration with an ESR partner (ESR-3 NA) to evaluate skin integrity in patient cohorts.

Aim 2: Assess the intra- and inter-variability associated with anatomical locations and intrinsic factors, such as gender, age and BMI, on normative biochemical and biophysical parameters

The aim was achieved in two phases using retrospective technical evaluation of the biophysical parameters during pandemic limited working and in a subsequent technical evaluation on healthy volunteers. In the verification phase (Figure 3-3), the retrospective evaluation was instrumental in assessing the variabilities associated with the biophysical parameters, which include anatomical variability, temporal variability following different insult models as well as inter-individual differences at baselines and following insults. Retrospective normative data involving sub-epidermal moisture revealed considerable variability in the parameter between different sites of investigation across the transverse plane of the body (Section 4.1). Moreover, the findings from the first phase of the study revealed the influence of intrinsic factors (age and BMI) on normative skin parameters (SEM). The effects of anatomical location and demographics needs to be considered when applying thresholds for the detection of skin

damage. Indeed, the results from this study indicate that a SEM delta parameter of 0.6, would not be efficient in distinguishing skin damages at all commonly reported sites of pressure ulcer, such as sacrum, heels, occiput and ischial tuberosities (Jayabal, Bates-Jensen et al., 2021a).

The second phase of the technical evaluation was focussed on evaluating whether biophysical parameters were sensitive and specific to mechanical and chemical challenges to the skin. The findings from the study revealed that certain insults, such as mechanical loading and exposure to moisture, led to a transient response (< 1hour) in selected biophysical parameters (TEWL and LDI), whereas insults such as tape-stripping and chemical irritation led to a long-lasting response (>3 hours). Moreover, in the presence of these defined insults, there was considerable inter-subject variability with some of the biophysical parameters, such as sub-epidermal moisture and erythema (Section 4.2). Indeed, these findings suggest that perhaps a single parameter will not be sufficient to provide a comprehensive evaluation of skin integrity. Interestingly, cluster analysis has demonstrated distinct responses to skin challenges even in a cohort of healthy volunteers suggesting that there could be a group of individuals who could intrinsically be at higher risk of skin damage. It was evident from the findings that although objective parameters are clearly beneficial in evaluating skin health, there is a need to explore other non-invasive methods of monitoring changes in skin health (Jayabal, Bates-Jensen et al, 2021a). The implications from the retrospective analysis on the advantages and disadvantages of biophysical tools are summarised in Table 8-1.

Table 8-1: Summary of advantages and limitations of biophysical parameters investigated in complementary studies

Parameter	Advantage	Limitations
TEWL	1. Able to detect time-dependent changes 2. Able to detect spatial changes 3. Able to distinguish healthy sites from Stage-I PU sites	1. Unable to differentiate between the causes of skin damage (loading, moisture, chemical irritation) 2. Influenced by intrinsic factors and anatomical variability 3. Need for a specific threshold to classify skin status
Stratum corneum hydration (SCH)	1. Highlighted variations from baseline when exposed to moisture 2. Able to detect time-dependent changes	1. Unable to distinguish healthy skin sites from Stage-I PU 2. Influenced by intrinsic factors 3. Need for a specific threshold to classify skin hydration status
Mexameter	1. Aids in visual inspection of skin status	1. Variations between individuals 2. Influenced by intrinsic factors, more particularly, skin tone 3. Unable to distinguish healthy and damaged skin sites through objective measures
pH	1. Provides information about the acidic mantle	1. Unable to detect spatial/temporal changes 2. Unable to distinguish healthy skin sites from sites presenting with Stage-I PU
Corneocytes	1. Provides information about the structural changes to Stratum Corneum 2. Provides understanding about mechanisms leading to loss of skin integrity	1. Variations between individuals 2. Influenced by anatomical variability 3. Semi-quantitative method 4. Findings did not correlate with TEWL warranting further research

Aim 3: Develop an efficient extraction methodology to analyse inflammatory biomarkers from sebum

Prior to evaluating the performance of biochemical parameters in identifying skin status, it is important to investigate the variabilities associated with anatomical locations as well as the efficiency of the existing extraction process. It was hypothesised that biofluids secreted at local skin areas subjected to mechanical and moisture loads would provide a more accurate method of assessing skin damage than those found in systemic biofluids (blood and urine). Therefore, sebum was considered as it is readily available for collection on the skin surface and could reflect the physiological status of the local dermal and epidermal tissues. The findings from the prospective study, as detailed in Chapter 5, revealed that certain anatomical locations relevant to skin damage, such as the heel and the back exhibited lower sebum levels when compared to facial locations, corresponding to higher density of sebaceous glands (Camera, Ludovici et al. 2010, Ludovici, Kozul et al. 2018). Indeed, facial locations have become highly relevant with the advent of COVID-19 pandemic due to the use of respiratory protective equipments (RPE) (Abiakam, Worsley et al. 2021). Moreover, the studies further revealed that approximately 30 minutes is required for the sebum to be restored to its basal level when sampled with Sebutape (Figure 5-6). Although sebum is a useful biofluid as it could be collected locally and non-invasively, it is also acknowledged that the protein levels are low in comparison to systemic fluids such as blood plasma (Corrie, Coffey et al. 2015). Therefore, a systematic approach was adopted to examine the influence of various stimuli, mechanical and chemical in nature, on the extraction efficiency from the Sebutapes. The findings from the study revealed that a concentration of 0.1% DDM as a chemical stimulus offered better stability and increased recovery of relevant cytokines (Section 5.3.2).Moreover, selected mechanical stimuli as well as lower extraction volume further offers means to assess a range of protein biomarkers and represents an optimal protocol for the extraction and subsequent analysis of sebum. To our knowledge, there is no scientific literature on a complete systematic evaluation of the extraction of inflammatory markers from sebum and the current study offered a 1.5 – 2.5-fold increase in extraction efficiency compared to the conventionally employed protocol (Perkins, Osterhues et al. 2001).

Aim 4: Identify biomarker features across different insult models, ranging from controlled lab-based stimuli to functional stimuli

The performance of biophysical parameters in evaluating skin damage following various insult models were detailed in Chapter 4. Similarly, the biochemical response of parameters following controlled lab-based stimuli including moisture exposure (Study 6-1), mechanical loading due to the use of shavers (Study 6-2) and functional loads encountered in clinical settings (Study 6-3) were investigated in Chapter 6. The exploratory nature of the project informed the design and statistical approach of the studies. It is to be noted that the introduction of the optimised protocol further allowed the quantification of low-abundance cytokines in these studies. Indeed, there were differences in cytokine concentrations dependent on the anatomical location and study design. As an example, even within the facial locations, such as the cheek and the neck, there were clear differences in the inflammatory response (Section 6.3.2). These findings further emphasised the importance of the choice of control sites in evaluating the skin response, with a local site preferable to provide normative ranges. The balance between the pro-inflammatory cytokine and anti-inflammatory cytokine of IL-1 family, i.e., (IL-1α /IL-1RA) provided surrogate of the inflammatory status of the tissues and was observed to be significantly changed in skin damage models. Interestingly, it was observed that with defined insults, there was selective upregulation of the ratio in participants. There was considerable variability with low-abundance cytokines with respect to different stimuli, as an example, IL-8 response following prolonged mask usage was higher relative to that of a lab-based stimuli involving exposure to postural loading and moisture. Perhaps, these low-abundance biomarkers could provide further specificity thereby providing a differential diagnostic measure. However, further research is required to investigate the performance of these markers in clinical settings to differentiate skin conditions.

Aim 5: Identify distinct thresholds of biochemical and biophysical parameters for classifying changes in skin integrity in individuals with early signs of skin damage (healthy/damaged/healing)

As part of the validation phase of the framework, the final aim of the thesis were to investigate the potential of inflammatory biomarker candidates in identifying signs of skin damage in clinical settings. For the first time, spatial differences in inflammatory response between the site of skin damage, in particular, Stage-I Pressure Ulcer and a healthy site has been reported in a substantial cohort (n=27) of elderly inpatients from a single hospital site (Chapter 7). The

analysis of the biomarker candidates using ROC identified a panel of markers, namely, IL-1α, IL-1RA, IL-8 and G-CSF as well as the ratio of IL-1α/IL-1RA, that could detect localised skin damage. The optimised extraction protocol has provided the means to measure low-abundance markers that could distinguish skin damage which in-turn could possibly provide more specificity to the biomarker panel. Optimum thresholds were identified in this study based on the specificity and sensitivity of each of the biomarker ROC values. Although localised control sites were chosen, it is to be noted that the thresholds were calculated based on the anatomical location that included sacral and ischial tuberosities and further investigations are warranted to identify site-specific thresholds. There is also a need to study other forms of early skin damage from different mechanisms e.g., incontinence associated dermatitis, to establish if the panel of biomarkers are specific to PU or upregulated with other types of skin damage.

8.2 Contributions of thesis to scientific literature

The current work has provided novel contributions to the domain of skin health research, evidencing the changes in biochemical parameters. Below is a summary of the main contributions:

1. Reported distinct spatial inflammatory responses, involving significant upregulation of pro-inflammatory markers and down-regulation of anti-inflammatory markers at the site of Stage-I PU, for the first time in a clinical study of substantive cohort (n-27)
2. Identified candidate biomarkers from the panel of investigated inflammatory through sensitivity and specificity analysis. The selected biomarkers were IL-1α/IL-1RA, IL-1α, IL-1RA, IL-8 and G-CSF.
3. Achieved a 1.5-2.5-fold increase in extraction efficiency of low-abundance cytokines compared to a previously established protocol by systematically optimising the detergent concentration (0.1% DDM) and mechanical means (centrifugation and sonication)
4. Determined that 30 minutes of time period was required between sampling for optimal sebum collection from skin surface using a modified SPV assay
5. Identified cluster of inflammatory response with high-responding individuals and low-responding individuals following skin insults in a cohort of healthy volunteers suggesting inherent vulnerability of selected individuals to skin damage.

6. Based on the retrospective evaluation, TEWL was recognized to be the sensitive parameter to various skin insults, with other biophysical parameters having limited time dependent changes
7. Tape-stripping and chemical irritation led to prolonged changes in skin barrier function (TEWL) and blood flow whereas the changes following mechanical loading and moisture-exposure were transient with the parameters returning to baseline in duration less than 3 hours.
8. Identified the variabilities associated with sub-epidermal moisture at different anatomical locations and as such, it was recommended that a fixed 'delta' threshold of 0.6 would not be optimal for identification of skin damage at all locations.

The novel findings of this research suggest promising potential for the inflammatory biomarkers to identify the early signs of skin damage both in pre-clinical and clinical damage states. Furthermore, the developed methodology for sampling inflammatory markers could be employed to study a diverse range of scenarios including devices/products on skin integrity, such as incontinence pads, masks, shavers, respiratory tubes and wound dressings. These findings could contribute to better design of devices without compromising skin health, through inflammatory monitoring. However, for this technique to be used in point-of-care (PoC) settings, future work should focus on the translation of conventional immunoassays to a lateral flow assay format.

8.3 Complementary analyses

In the prospective studies conducted to achieve the aims of the PhD, focus has been on biochemical markers. However, biophysical markers were also collected from the studies by collaborating with a Marie-Curie Research Fellow (ESR-3, NA). In addition, the properties of superficial corneocytes were investigated by collaborating with another Marie-Curie Research Fellow (ESR-1, AE) from University of Birmingham. In these studies, I collaborated in the data analysis, interpretation of the results and publication of research articles.

8.3.1 Skin biophysical markers

As detailed in the retrospective studies, changes in skin response could also be measured using skin biophysical parameters, such as TEWL, SEM and erythema. These parameters have been reported to monitor the status of loaded soft tissues non-invasively (Worsley and Voegeli 2013,

Bader and Worsley 2018). The retrospective studies have investigated the sensitivity and specificity of these parameters following skin insults. Data from selected parameters, such as TEWL, Skin hydration, pH and erythema were collected for the studies described in Chapter 6 and Chapter 7 by another researcher (NA).s The findings from the lab-studies, including biophysical and biochemical data, have also been published (Abiakam, Jayabal et al. 2022, Abiakam, Jayabal et al. 2022). With respect to the clinical studies involving patients with Stage-I Pressure Ulcer, data from biophysical findings have been submitted for publication separately (Abiakam, Jayabal et al. 2022). The combination of biophysical and biochemical markers would provide a holistic understanding of the changes preceding skin damage both in terms of barrier function, hydration, and local physiology.

8.3.2 Analysis of superficial corneocytes

In addition to evaluating the biophysical and biochemical parameters, it is essential to understand the implications of external insult on the properties of skin, particularly stratum corneum (SC) and its primary cellular component, i.e., corneocytes. These cells undergo a complex maturation process which involves the cross-lining of certain precursor proteins, such as involucrin and loricrin, and the covalent attachment of lipids to produce a rigid and hydrophobic cornified envelope (CE) (Évora, Adams et al. 2021). Moreover, gradual degradation of corneodesmosomes occur in the central cell region leading to a honeycomb formation of cell junctions. Corneodesmosomes (CDs) are observed indirectly by immunostaining of desmoglein (Dsg1) which is a cadherin-type cell-cell adhesion molecule found in stratified epithelial desmosomes, that is expressed in the suprabasal layer of the epidermis. Corneocytes collected from the studies (Chapter 6, Study 6-3) were analysed by the collaborating researcher to measure the properties of SC corneocytes, in particular CE maturation and indirect visualization of corneodesmosomes by immunostaining of Dsg1. The findings highlighted a considerable inter-subject variability for both immature CEs and Dsg1. Nonetheless, a high level of CDs and immature CEs were detected at the insulted/impaired anatomic locations compared to the control sites and low levels of immature CEs were associated with higher TEWL values after prolonged pressure application. In addition, a greater amount of Dsg1 was detected at the compromised sites. These findings suggest the need for more evidence-based research pertaining to the possibility of using surface corneocytes as biomarkers for the early detection of skin compromise. These findings have now been published at the Journal of Tissue Viability (Evora, Abiakam et al. 2023).

Chapter 8

Evora, A., N. Abiakam, **H. Jayabal**, P. R. Worsley et al., (2023). "Characterisation of superficial corneocytes in skin areas of the face exposed to prolonged usage of respirators by healthcare professionals during COVID-19 pandemic." J Tissue Viability.

8.4 Limitations

One of the limitations of the studies described in this thesis were the relatively smaller number of participants in lab-based studies (n=10-12) as well as clinical studies (n=30) due to the exploratory nature of the research. This would limit the potential to analyse the influence of intrinsic factors such as gender, age, body mass index (BMI) and nutrition status. It is reported that it is difficult to detect skin damage in individuals with darker skin (Section 1.2.3). However, the vast majority of the participants from the present studies were of White ethnicity. This further precludes the generalisability of the findings and identification of whether the biophysical and biomarker parameters could offer improved evaluation of darker skin types. Another limitation of the study is the use of less defined insults such as respirator protective masks as well as postural loading in incontinence pads. These insults would possibly lead to variability in the magnitude of insults depending on the individual characteristics, such as mask tension, facial shape as well as the BMI of the individual. However, these situations reflect the real-time scenarios commonly observed in clinical settings.

With respect to the extraction process, despite the efficient optimization process developed as part of the thesis (Chapter 5), some of the inflammatory markers are still observed in low quantities that are below the limit of detection and therefore restricts the potential to investigate them in the context of skin damage. Moreover, other candidate biomarkers including metabolites lactate and pyruvate were below the limit of detection even with the use of sensitive detection techniques such as SFC-MS (Supercritical fluid chromatography – Mass Spectrometry). This may have been due to the delay between storage and analysis or the use of new detergents, wherein previous studies were able to quantify the metabolites following mechanical insults (Soetens, Worsley et al. 2019). Indeed, the use of other sample extraction techniques, such as tape-stripping, skin biopsies could overcome this limitation, but these methods could also trigger the inflammatory process owing to their invasive nature. When assessing vulnerable skin, methods which are minimally invasive are preferrable and most likely to be used in practice.

The study is also limited by the selected panel of cytokines and chemokines that were investigated based on literature review as well as aetiology of pressure ulcer. A number of other signalling molecules could be analysed with respect to skin damage models. With respect to the biofluid, sweat could also be employed to locally assess the skin status.

However, it is to be noted that the amount of sweat collected is a function of the environmental conditions as well as individual's sudomotor function (Baker 2017, Buchmann, Penzlin et al. 2019). Indeed, there is also a possibility of saturation effects with both sweat and sebum, wherein the biomarker secretion tends to reduce after repeated number of cycles within minutes/hours. Moreover, the anatomical variability associated with the inflammatory markers makes it challenging to develop thresholds which could be applied universally over the body. Indeed, further research is required to develop site-specific thresholds for commonly reported pressure ulcer locations.

8.5 Clinical implications

The findings from this project provide initial direction on skin's inflammatory response which will inform further work on pressure ulcer diagnostics. The present research has highlighted the importance of choice of thresholds and the influence of these on the sensitivity and specificity of biomarkers in lab-based and clinical models of skin damage. The present research has developed efficient means of extracting biomarkers from skin surface non-invasively using systematic optimisation process, which is a major advancement from the conventional protocol developed a decade ago. The proposed extraction methodology could be employed in the identification of biomarkers for other skin conditions, such as atopic dermatitis, psoriasis etc., There remains a challenge to the translation of this approach with regards to time and expense of extracting and analysing biomarkers through ELISA. Therefore, it remains a research tool at present, with the need to develop systems of real time acquisition of sebum and subsequent analysis of candidate markers. This has recently been highlighted through the routine use of lateral flow devices (LFTs) during the COVID-19 pandemic through the use of saliva biomarkers (Le Page, 2020).

Moreover, to compare the inflammatory profiles between individuals, various normalization procedures are often employed. Amongst the different normalization approaches, which include normalization to total protein, sebum content and baseline, it was found that baseline normalization provided a more accurate overview of the changes in temporal profile in clinical settings. The comparison to a relative near (within 10cm) control site was also seen as key in determining the local changes in skin health. One of the most important implications of the present work is the successful identification of panel of inflammatory markers that reflect skin damage, i.e., Stage-I Pressure Ulcers. Furthermore, the ranking of the biomarkers provided an overview of the usefulness of the biomarkers for which lateral flow devices could be developed for use in point of care settings. In clinical settings, sebum

testing could be added to routine blood/urine testing commonly employed to monitor the skin status.

8.6 Future work

8.6.1 Clinical application

The encouraging results from the findings of the studies suggest that skin damage could be identified using the inflammatory markers. Despite these findings, the major question of whether these biomarkers could predict skin damage even before they could occur still remains unanswered. Similarly, limited data was available on evaluating the ability of the biomarkers in predicting the progression/healing status of Stage-I Pressure Ulcers. Therefore, further longitudinal studies are required in clinical settings to assess the prognostic potential of the biomarkers.

In addition, for more widespread use in practice, further studies are required to assess the feasibility of sampling sebum in other care settings. This would include the barriers and facilitators in practice, healthcare workers perception on its ease of use and how logistics of storage and analysis could be performed in practice. There is also the key question of economic evaluation. If point of care systems were to be developed, it would need to be cost effective in relation to its ability to prevent wounds which are known to cost the NHS substantive sums of money to treat (Section 1.2).

8.6.2 Biomarker selection

The present studies include a panel of pro-inflammatory and anti-inflammatory markers which were selected based on an exhaustive literature review in the domain of pressure ulcer research as well as other skin conditions. However, there are a large number of inflammatory markers that have not been investigated for their diagnostic potential. In addition to the inflammatory markers, metabolites, such as lactate and pyruvate, as well as oxidative stress biomarkers have not been investigated in the present studies owing to their low-abundance despite the use of sensitive analytical techniques (Soetens, Worsley et al. 2019). In this thesis, efforts were focussed on developing a methodology for the quantification of an oxidative stress marker, i.e., malondialdehyde. However, owing to the irreproducibility of derivatizing malondialdehyde, sensitive quantification was not achieved. Therefore, further work on developing appropriate sensitive analytical techniques and using appropriate

surfactants could lead to the measurement of these markers thereby providing increased specificity of the diagnostic measure (Dalrymple, McEwan et al. 2022).

8.6.3 Translation to point of care settings

In the current studies, Sebutape samples were collected during the participant data collection. However, the samples were batch processed owing to the complexity of the process, the length of the process (2 days) as well as the requirement of minimum number of samples to process each immunoassay plate (96-wells). However, in clinical settings, typically user-friendly prompt technologies are required to implement preventive strategies. Therefore, there is a need for translation of the lab-based technologies to point-of-care settings for the use of clinicians and patients to regularly monitor the skin health. There exists a huge momentum of research in the development of wearable technologies as well as point of care solutions. As an example, recent studies have focussed on lateral flow devices that have been developed for the detection of inflammatory proteins, such as interleukins (Rahbar, Wu et al. 2021, Klebes, Kittel et al. 2022). However, considerable efforts are required for the point of care devices that could measure high-abundance markers as well as low-abundance markers (Mondal, Zehra et al. 2021, Zhang, Zeng et al. 2021).

Appendix A Participant Information Sheet for study involving health care workers

Chapter 6 – Study 3 - Skin response following the use of RPE

Study Title: Monitoring skin health of clinical staff during COVID-19 pandemic

Researcher: Nkemjika Abiakam, Hemalatha Jayabal, Dr Peter Worsley, Professor Dan Bader

ERGO number: 57311/ IRAS 285764

You are being invited to take part in the above research study. To help you decide whether you would like to take part or not, it is important that you understand why the research is being done and what it will involve. Please read the information below carefully and ask questions if anything is not clear or you would like more information before you decide to take part in this research. You may like to discuss it with others, but it is up to you to decide whether or not to take part. If you are happy to participate you will be asked to sign a consent form.

What is the research about?

This study is part of a PhD project funded by the EU-Marie Sklodowska-Curie "Skin Tissue Integrity under Shear" (STINTS) programme.

The project focuses on evaluating the health of facial skin and assessing measures which could indicate early signs of skin damage. Since the outbreak of COVID-19 pandemic clinical staff has been forced to use Personal Protective Equipment (PPE) for prolonged periods whilst treating patients with COVID19 or attending other duties within the hospital setting. As a consequence, many have reported damage to the skin from wearing these devices. In particular, the prolonged use of FFP2 and FFP3 respirator masks has been associated with facial indentation marks and erythema (redness), events that if not attended to could possibly lead to skin breakdown. Prior to this occurring, the skin and underlying soft tissues will undergo a number of changes, for example it will become inflamed. This study aims to better understand how the skin changes before and after using respirator masks in order to create methods of detecting skin damage before wounds form. We will use non-invasive measures of skin health including physical sensors and sampling fluids from the skin surface.

Why have I been asked to participate?

Appendix A

You have been asked to take part in this study as you are a member of the hospital staff that is required to wear respirator (FFP2 or FFP3) mask while carrying out your duties, due to the outbreak of COVID-19. In addition, you meet the following inclusion criteria:

– You are over 18 years of age

– You are a clinical staff at the University hospital Southampton (UHS)

– You work at least 3 days per week

– You work at least 2 consecutive days in a week

What will happen to me if I take part?

After you have had the time to read the participant information sheet and ask questions, you will be asked to sign a consent form if you wish to participate. A copy of your signed consent form and of the information sheet will also be provided to you. If you decide to take part in the study, the researcher will collect some of your demographic information (such as age, height, weight, gender etc) and with your consent, images of your nasal bridge area. The images will capture solely this area and will not include your eyes. Where these are captured by mistake, they will be 'blacked out' in order to ensure you are not identifiable in any manner.

You will be asked to attend the Clinical Academic Facility at the UHS on three different days. These days will be scheduled to match with the days that you on duty in the hospital. You will be asked to attend two different assessment sessions on the same day. These sessions are planned to last approximately 10 minutes and we will ensure that they do not interfere with your clinical commitments.

At the beginning of each session, the researcher will obtain verbal consent of your willingness to continue with the study and answer to any questions that you might have. We will then take two biophysical measurements and collect sebum from the surface of the nasal bridge and the cheek of your face. We will require you not to apply cosmetics on these sites of your face. The non-invasive biophysical measurements will include:

– the amount of water which is lost through the skin

– the level of erythema (redness)

The amount of water loss and skin erythema (redness) will be measured at each session using wireless handheld devices, Tewameter and Mexameter, which will gently come in contact with your skin for 1 minute and 30 seconds, respectively, for each investigation site Each

probe will be cleaned in-between assessments in accordance with the NHS infection control policies. Samples of skin sebum will also be taken at each assessment from the areas of interest using Sebutapes, which will be gently applied to your skin for 2 minutes before removal.

Are there any benefits in my taking part?

There will be no direct benefit to you in taking part of this study. However, you will be helping to further the understanding regarding how research can support the early detection of skin damage, enabling clinicians to optimise preventive approaches.

Are there any risks involved?

There are no anticipated physical or psychological risks involved in this testing, your safety will be ensured at all times and the researcher will remain with you throughout.

During data collection, in order to minimise the contact between you and researcher, Standard Infection Control policy will be followed. The researcher will wear a long-sleeved disposable fluid repellent gown (covering the arms and body), a filtering face respirator mask, eye protection and disposable gloves. He will ensure to wash his hands prior and after carrying out the assessment. To ensure your safety, you will be provided with personal protective equipment (PPE) consistent of disposable plastic apron and a pair of disposable nitrile gloves. You will be gently asked to wash or sanitise your hands and you will be assisted in donning, doffing and disposal of the PPE equipment. Besides you and the researcher, no one else will be allowed into the session room during the assessments. All the devices used for data collection will be thoroughly disinfected prior and after each session, following the NHS infection and prevention policies.

You will be removed from the study if you feel any of the common symptoms associated with COVID-19 (loss of smell and taste, temperature or a continuous dry cough). The researcher will also monitor their health and suspend the study immediately if any of the aforementioned symptoms are present.

What data will be collected?

Skin measures and biomarkers will be collected by the researcher. Participants samples will be anonymised, via a unique identifier in order to remove any traceable information. Clinical information and demographic data will also be collected. These will include age, gender,

ethnicity, height, weight, body mass index (BMI) and working hours, and with your permission, images of your nasal bridge area.

Sebum collected from your skin surface will be stored in -80°C freezer, located at the chemical laboratory at the South Academic Block, Southampton General Hospital. The researcher will be the only one with access to your samples and will be responsible for custodial. Your samples will be disposed of immediately after the measurements have been recorded.

Will my participation be confidential?

Your participation and the information we collect about you during the course of the research will be kept strictly confidential.

Only members of the research team and responsible members of the University of Southampton may be given access to data about you for monitoring purposes and/or to carry out an audit of the study to ensure that the research is complying with applicable regulations. Individuals from regulatory authorities (people who check that we are carrying out the study correctly) may require access to your data. All of these people have a duty to keep your information, as a research participant, strictly confidential.

Your data will be anonymised immediately after collection. If any hard copies are made of the data, these will be stored in a locked cabinet in a research office, which is staffed during the day and locked out of hours. The anonymised data will only be seen by the research team while the study is ongoing and will be kept confidential throughout. When the study is completed, in accordance with data protection policy of the University of Southampton and in compliance with the General Data Protection Regulation (GDPR) 2018, all anonymised data will be stored in the University of Southampton's repository for 10 years. At the end of the study, your personally identifiable data (consent form) will be kept securely for further 6 months prior to being destroyed.

Do I have to take part?

No, it is entirely up to you to decide whether or not to take part. If you decide you want to take part, you will need to sign a consent form to show you have agreed to take part. A signed copy of your consent will be also provided to you

If you would like to participate, please contact the researchers using the details at the end of this information sheet.

What happens if I change my mind?

You have the right to change your mind and withdraw at any time without giving a reason and without your participant rights being affected.

If you wish to withdraw, please contact the researchers using the provided details. If you withdraw from the study, we will keep the anonymised information about you that we have already obtained for the purposes of achieving the objectives of the study.

What will happen to the results of the research?

Your personal details will remain strictly confidential. Research findings made available in any reports or publications will not include information that can directly identify you without your specific consent.

Your anonymised analysed data will be used for scientific publications, presentations as well as part of PhD qualification. If you would prefer your data to not be included in research publications at any time during or after the study, please inform the researcher.

Where can I get more information?

If you would like to receive more information about the study, please contact the researchers using the following details:

Nkemjika Abiakam: n.s.abiakam@soton.ac.uk (Researcher)

Dr Peter Worsley: P.R.Worsley@soton.ac.uk (Supervisor).

What happens if there is a problem?

If you have a concern about any aspect of this study, you should speak to the researchers who will do their best to answer your questions.

If you remain unhappy or have a complaint about any aspect of this study, please contact the University of Southampton Research Integrity and Governance Manager (023 8059 5058, rgoinfo@soton.ac.uk).

You can also contact our research team at the following address:

School of Health Sciences |Faculty of Environmental and Life Sciences | Clinical Academic Facility |University of Southampton | Level A, Room AA97 | South Academic Block (MP11) | Southampton General Hospital Tremona Road | Southampton | SO16 6YD

Appendix A

Tel: +44(0)2381 208287 (718287)

Data Protection Privacy Notice

The University of Southampton conducts research to the highest standards of research integrity. As a publicly funded organisation, the University has to ensure that it is in the public interest when we use personally identifiable information about people who have agreed to take part in research. This means that when you agree to take part in a research study, we will use information about you in the ways needed, and for the purposes specified, to conduct and complete the research project. Under the General Data Protection Regulation (GDPR) 2018, 'Personal data' means any information that relates to and is capable of identifying a living individual. The University's data protection policy governing the use of personal data by the University can be found on its website (https://www.southampton.ac.uk/legalservices/what-we-do/data-protection-and-foi.page).

This Participant Information Sheet tells you what data will be collected for this project and whether this includes any personal data. Please ask the research team if you have any questions or are unclear what data is being collected about you.

Our privacy notice for research participants provides more information on how the University of Southampton collects and uses your personal data when you take part in one of our research projects and can be found at http://www.southampton.ac.uk/assets/sharepoint/intranet/ls/Public/Research%20and%20Integrity%20Privacy%20Notice/Privacy%20Notice%20for%20Research%20Participants.pdf

Any personal data we collect in this study will be used only for the purposes of carrying out our research and will be handled according to the University's policies in line with the General Data Protection Regulation (GDPR) 2018. If any personal data is used from which you can be identified directly, it will not be disclosed to anyone else without your consent unless the University of Southampton is required by law to disclose it.

General Data Protection Regulation (GDPR) 2018 requires us to have a valid legal reason ('lawful basis') to process and use your Personal data. The lawful basis for processing personal information in this research study is for the performance of a task carried out in the public interest. Personal data collected for research will not be used for any other purpose.

For the purposes of General Data Protection Regulation (GDPR) 2018, the University of Southampton is the 'Data Controller' for this study, which means that we are responsible for looking after your information and using it properly. The University of Southampton will keep identifiable information about you for 6 months after the study has finished after which time any link between you and your information will be removed. Anonymised data will be kept for 10 years in the University's repository at the end of the study.

To safeguard your rights, we will use the minimum personal data necessary to achieve our research study objectives. Your data protection rights – such as to access, change, or transfer such information - may be limited, however, in order for the research output to be reliable and accurate. The University will not do anything with your personal data that you would not reasonably expect.

If you have any questions about how your personal data is used, or wish to exercise any of your rights, please consult the University's data protection webpage (https://www.southampton.ac.uk/legalservices/what-we-do/data-protection-and-foi.page) where you can make a request using our online form. If you need further assistance, please contact the University's Data Protection Officer (data.protection@soton.ac.uk).

Thank you very much for taking the time to read the information sheet and considering taking part in the research. We really appreciate your interest in our study. If you would like more information, please do not hesitate to contact us at the following details:

Nkemjika Abiakam: n.s.abiakam@soton.ac.uk (Researcher)

Dr Peter Worsley: P.R.Worsley@soton.ac.uk (Supervisor)

Kay Mitchell: kay.mitchell@uhs.nhs.uk (Primary Investigator

Appendix B Participant Information Sheet for patient study

The following is the participant information sheet provided for the patient study (Chapter 7)

Study Title: Detecting changes in Skin status over the site of a stage 1 pressure ulcer using biophysical sensors and biomarkers.

Researcher: Nkemjika Abiakam, Hemalatha Jayabal, Dr Peter Worsley and Prof Dan Bader

ERGO number: 54362/IRAS 301685

You are being invited to take part in the above research study. To help you decide whether you would like to take part or not, it is important that you understand why the research is being done and what it will involve. Please read the information below carefully and ask questions if anything is not clear or you would like more information before you decide to take part. You may like to discuss it with others, but it is up to you to decide whether or not to take part. If you are happy to participate you will be asked to sign a consent form. Thank you for reading this.

What is the research about?

This study is part of a PhD project funded by the EU-Marie Sklodowska-Curie "Skin Tissue Integrity under Shear" (STINTS) programme.

The project wishes to evaluate changes in skin properties after the development of stage 1 pressure ulcers, commonly known as bedsores. Indeed, during your hospital stay, your skin can be exposed to various insults including forces from sitting or lying and attachment to different medical devices. Your skin remains healthy through regular movements to remove pressure from the skin surface. However, sometimes if the skin is exposed to these insulting factors for a prolonged period, this can take the form of wounds termed pressure ulcers (PUs) or bedsores. Before the ulcers get to severe stages and become wounds, the skin and underlying soft tissues will undergo some changes, for example, they will become inflamed. This study aims to better understand how the skin changes at the body sites compromised by bedsores compared to healthy sites, to create strategies of detecting skin damage before wounds form. We will use non-invasive measures of skin health including physical sensors and sampling fluids and cells from the skin surface. The knowledge acquired from this project will help healthcare professionals to adopt the appropriate preventive strategies to avoid skin damage and subsequent wounds.

Why have I been asked to participate?

You have been asked to take part in this study as you have a stage 1 pressure ulcer (PU) due to prolonged sitting and/or lying or because of prolonged attachment to a medical device. In addition, you meet the following inclusion criteria:

- You are over 18 years of age
- You are a patient at the University Hospital Southampton (UHS)

Appendix B

What will happen to me if I take part?

After you have had the time to read the participant information sheet and ask questions, you will be asked to sign a consent form if you wish to participate. A copy of your signed consent form will be provided to you for your reference. The decision to take part will not affect any of your routine healthcare. If you decide to take part in the study, the researcher will collect some of your demographic information (such as age, height, weight, gender, etc), your current medications, current skincare regime, relevant medical history, and with your consent, images of your skin. The images will be taken by the researcher and stored in a password-protected laptop. The pictures will capture exclusively the compromised areas of your skin and will not capture identifiable features. If by mistake any of these are captured within the photograph, these will be blacked out to ensure you are unidentifiable. The images will not be shared with anyone and will be deleted at the end of the study.

You will be asked to provide an appropriate time for the research assessment while you are at the hospital. We can coordinate this with your healthcare team to ensure it does not affect your routine care. You will be visited on three different occasions for your skin assessment. The first two visits will be on 2 consecutive days, while the last visit will be the day before your discharge. We will ensure that the sessions, which will last approximately 15-20 minutes, will not interfere with any of your commitments.

At the beginning of each session, the researcher will obtain verbal consent of your willingness to continue with the study and answer any questions that you might have. We will then take different biophysical measurements, sebum (oily substance) from the surface of your skin as well as corneocytes (outermost skin cell layer) from the compromised and healthy areas of your skin. The measurements will include:

1. the pH (acidity) at the skin surface
2. the amount of water which is lost through the skin
3. skin hydration

The pH and water loss measurements will be taken at each visit using two non-invasive, harmless wireless probes (Figure B1 A and B) which will be placed gently in contact with your skin for a 1-minute duration. Skin hydration will be assessed at each visit using a non-invasive device (Figure B1- C), which will come in contact with your skin for 30 seconds. Each probe will be cleaned in-between assessments following the NHS infection control policies.

Skin sebum will also be taken at each assessment from the areas of interest using Sebutapes, which will be gently applied to your skin for 2 minutes before removal.

Corneocytes will be taken only at the first visit from both sites of investigation using two strips of standard Sellotape *(*Figure B 1*)*. These will be applied to the skin surface and slightly pressed for 5 seconds before removal.

Privacy and dignity will be ensured at all times during the assessments. During the assessment, bedsheets or blankets will be used to cover exposed areas of your body other than the sites under investigation in order to ensure your comfort. The curtains of your bed area will be used to provide a private enclosure. You will be given the option to have a

hospital staff supervise the session and you will be talked through the process while the researcher performs the skin assessment.

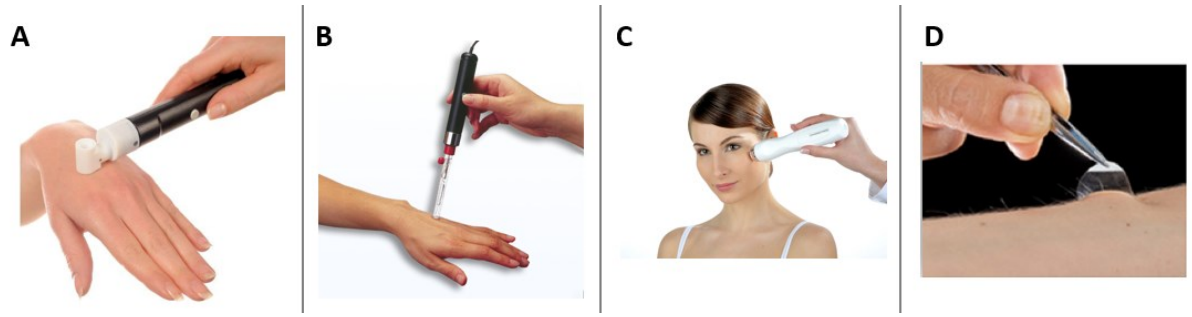


Figure B 1. Illustrative images of biophysical and biochemical measurement techniques. A) Tewameter for the measure of skin water loss B) pH meter C) Corneometer for skin hydration evaluation, and D) corneocytes collection using standard Sellotape.

Are there any benefits in my taking part?

There will be no direct benefit to you in taking part in this study. However, you will be helping to further the understanding regarding how research can support the early detection of skin damage, enabling clinicians to optimise preventive approaches.

Are there any risks involved?

There are no anticipated physical or psychological risks involved in this testing, your safety will be ensured at all times and the researcher will remain with you throughout.

COVID Specific Measures: During data collection, in order to minimise the contact between you and the researcher, Standard Infection Control policy will be followed. The researcher will wear a disposable fluid repellent gown, surgical mask and disposable gloves. He will ensure to wash his hands before and after carrying out the assessment. To ensure your safety, you will be provided with a surgical mask, if you wish. You will be gently asked to wash or sanitise your hands. All the devices used for data collection will be thoroughly disinfected prior and after each session, following the NHS infection and prevention policies.

You will be removed from the study if you feel any of the common symptoms associated with COVID-19 (loss of smell and taste, temperature, or a continuous dry cough). The researcher will also monitor their health and be tested on a regular basis using the Southampton Saliva test facility. The study will be suspended immediately if any of the aforementioned symptoms are present.

What data will be collected?

Skin measures and biomarkers will be collected by the researcher. Your samples will be anonymised, via a unique identifier in order to remove any traceable information. Clinical information and demographic data will also be collected. These will include age, gender, ethnicity, height, weight, body mass index (BMI), medical history, current medication and

with your permission, images of any areas of the skin which might be compromised by bedsores.

Sebum collected from your skin surface will be stored in a -80°C freezer, located at the chemical laboratory at the South Academic Block, Southampton General Hospital. The researcher will be the only one with access to your samples and will be responsible for their storage. Your samples will be disposed of immediately after the measurements have been recorded. Your corneocytes, labelled anonymously, will be stored in a -20ºC freezer before being transferred to a facility at the School of chemical engineering at the University of Birmingham for analysis. These samples will be disposed of immediately after analysis. If you wish for your corneocytes anonymous samples not to be shared with our Birmingham partners, please inform the researcher.

Will my participation be confidential?

Your participation and the information we collect about you during the course of the research will be kept strictly confidential. Your data will be anonymised immediately after collection. If any hard copies are made of the data, these will be stored in a locked cabinet in a research office, which is staffed during the day and locked out of hours. Only members of the research team and responsible members of the University of Southampton may be given access to data about you for monitoring purposes and/or to carry out an audit of the study to ensure that the research is complying with applicable regulations. All of these people have a duty to keep your information, as a research participant, strictly confidential.

When the study is completed, in accordance with data protection policy of the University of Southampton and in compliance with the General Data Protection Regulation (GDPR) 2018, all anonymised data will be stored in the University of Southampton's repository for 10 years. At the end of the study, your personally identifiable data e.g., consent form, will be kept securely for further 6 months prior to being destroyed.

The school of chemical engineering at the University of Birmingham will dispose of your unidentifiable samples immediately after the study has finished.

Do I have to take part?

No, it is entirely up to you to decide whether or not to take part. If you would like to participate, please contact the researchers using the details at the end of this information sheet.

What happens if I change my mind?

You have the right to change your mind and withdraw at any time without giving a reason and without your participant rights and your medical care being affected. If you wish to withdraw, please contact the researchers using the provided details. If you withdraw from the study, we will keep the anonymised information about you that we have already obtained for the purposes of achieving the objectives of the study.

What will happen to the results of the research?

Your personal details will remain strictly confidential. Research findings made available in any reports or publications will not include information that can directly identify you without your specific consent.

Your anonymised analysed data will be used for scientific publications, presentations as well as part of PhD qualification. If you would prefer your data to not be included in research publications at any time during or after the study, please inform the researcher.

Where can I get more information?

If you would like to receive more information about the study, please contact the researchers using the following details:

Nkemjika Abiakam: n.s.abiakam@soton.ac.uk (Researcher)

Dr Peter Worsley: P.R.Worsley@soton.ac.uk (Supervisor)

What happens if there is a problem?

If you have a concern about any aspect of this study, you should speak to the researchers who will do their best to answer your questions.

If you remain unhappy or have a complaint about any aspect of this study, please contact the University of Southampton Research Integrity and Governance Manager (023 8059 5058, rgoinfo@soton.ac.uk).

You can also contact our research team at the following address:

School of Health Sciences |Faculty of Environmental and Life Sciences | Clinical Academic Facility |University of Southampton | Level A, Room AA97 | South Academic Block (MP11) | Southampton General Hospital Tremona Road | Southampton | SO16 6YD

Tel: +44(0)2381 208287 (718287)

Data Protection Privacy Notice

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Appendix B

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The school of Chemical Engineering at the University of Birmingham will not have any identifiable information about you, and they will destroy your samples at the end of the study.

To safeguard your rights, we will use the minimum personal data necessary to achieve our research study objectives. Your data protection rights – such as to access, change, or transfer such information - may be limited, however, in order for the research output to be reliable and accurate. The University will not do anything with your personal data that you would not reasonably expect.

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Dr Peter Worsley: P.R.Worsley@soton.ac.uk (Supervisor)

Appendix C Skin sensitivity questionnaire and scores

To identify associations between the perceived and observed parameters of skin sensitivity, participants were purposefully selected based on a questionnaire. The short version of the questionnaire was developed at Phillips Consumer Lifestyle (PCL) from a more detailed version previously published (Richters et al., 2017).

C.1 Q-Score

To review briefly, the original questionnaire contained 32 questions including details of the presence and extent of typical responses associated with SS following interaction with various stimuli, such as toiletries and weather conditions. This questionnaire was developed based on inputs obtained from 481 responders and was validated in the clinical studies based on both non-invasive and histological data obtained from human volunteers. Benchmarking against the score derived from the original questionnaire, the PCL team aimed to reduce the number of questions related to the various stimuli. Subsequently, models were developed to evaluate the effects of reducing the number of input parameters to few questions as shown in Table C 1. The predictive performance of these models was then assessed in terms of the r-squared values (goodness of fit), percentage of classification errors, and their ability to derive the pre-determined class sizes. The classification error calculation estimated how many individuals with true "very SS", as responded in the original questionnaire, were re-classified as "normal". Thus, stating the desired class sizes, threshold values were chosen, which should be reconsidered for new studies with different observations. Based on the results of the performance indicators, the following model was selected to estimate the skin sensitivity score (i.e., Q-score):

$$\text{Q-score} = 81.02 + 4.93 * \text{Q1} + 36.89 * \text{Q2} + 27.77 * \text{Q3} - 35.2 * \text{Q4} \quad \text{(Equation C1)}$$

where the variables were coded as shown in Table C 1.

Considering the specific focus of the research, an explicit question regarding the skin response to mechanical stimulation (Q5) was added to the questionnaire and subsequently to the selection criteria.

Table C 1 Coding values for the responses from the SS Questionnaire

Appendix C

Q. No.	Questions from the screener	Answers	Coding values
Q1	Which skin type best describes you? (Fitzpatrick Skin Type)	1, 2, 3, 4, 5, 6	1, 2, 3, 4, 5, 6
Q2	How frequently does your skin breakout?	Acne Sometimes No	1 0 0
Q3	What is your facial skin like in the morning (before washing)?	Tight Comfortable Oily	1 0 0
Q4	Do you think you have sensitive skin?	Yes A bit No	0 1 2
Q5	Does your skin react during or after shaving or contact with fabrics like clothes or towels?	1, 2, 3, 4, 5 (Never to Always)	-

C.2 Skin sensitivity label assignment

To understand the group skin characteristics of individuals with Low, Mild, and High Skin Sensitivity, the participants needed to be assigned SS labels based on thresholds from their questionnaire responses. It has already been established that the Q-score did not account for the participants answer to the question explicitly enquiring the skin response to mechanical stimulation (i.e., Mech SS Score). Thus, a new criterion was developed to assign the SS labels based on the following S-score:

$$S\text{-}Score\% = {}^{1}/_{4}\left(\frac{Q\text{-}score\ July}{Highest\ possible\ Q\text{-}score} + \frac{Q\text{-}score\ Dec}{Highest\ possible\ Q\text{-}score} + \frac{Mech\ SS\ score\ July}{Highest\ possible\ score} + \frac{Mech\ SS\ score\ Dec}{Highest\ possible\ score}\right) x\ 100$$

(Equation C2)

By normalising the Q-scores and Mech SS scores to the highest possible values that can be achieved respectively, both variables have been reduced to a scale from 0 to 1. Thus, this approach assigns equal weights to the Q-scores and Mech SS scores while ranking the respondents in an ascending order from Low to High skin sensitivity. Identifying the first and third quartile values for the S-score distribution obtained, the participants were categorised as follows:

- Low SS – individuals with S-score lesser than the first quartile value

- Mild SS – individuals with S-score greater than the first quartile value, but lesser than the third quartile value
- High SS – individuals with S-score greater than the third quartile value

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