Delivering proteins to human skin to alter its identity, via a pH responsive Microneedle.

PhD Candidate: Attiya Anna Abbas

Student ID: 01102890

Department of Bioengineering,

Faculty of Engineering,

Imperial College London.

Supervisor: Dr Claire Higgins, Reader in Tissue Engineering

This thesis is submitted for the degree of

Doctor of Philosophy

2024

Declaration of Originality

The presented work in this thesis is original and a result of my work. Every contribution made to the presented work has been acknowledged. Pieces of this body of work will be submitted for publication which has been indictated at each chapter.

Attiya A. Abbas,

2024

Copyright Declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution Non-Commercial 4.0 International Licence (CC BY-NC). Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose. When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes. Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Acknowledgments

Firstly, I would like to express my deepest gratitude to my PhD primary supervisor Dr Claire Higgins. The continued guidance and valuable mentorship of my supervisor has encouraged me to persevere and overcome every experimental drawback and find resolutions to each obstacle. My supervisor's experience and knowledge has been of immense help in developing me professionally as an independent scientist. I thank Dr Higgins for allowing me to design and steer the project to my interest. I would also like to thank my secondary supervisor Dr Ben Almquist, for his helpful guidance during my PhD journey. Many thanks to Mr Abdullah Bukhamsin for providing training on how to make a microneedle and perform axial compression testing, and to Dr Muhsin Ali for providing training and assistance on the scanning electron microscope (SEM) (Chapter 2). I would also like to thank the clinical collaborator, Dr Mohammed Akavani, Dr Summik Limbu and Mr Jia Jun Lee for kindly providing several skin and keratinocyte samples respectively that were essential to this project and my investigation (Chapter 3). Lastly and most importantly, I want to thank my wonderful parents who are my source of inspiration and the reason behind my every accomplishment.

Abstract

Human skin is heterogeneous. As the interface between the outside world and our internal organs it is a vital barrier to external damage. On a single person, skin varies in thickness and stiffness across body sites, thus possessing various susceptibility to injury. From individual to individual, skin varies in colour and biological age further influencing disease risk and regeneration capacity. In this body of work, I discuss the nuances of skin properties impacted by body location, age, and colour. I present the development of a therapeutic device to prevent skin injury.

Firstly, it is known that skin thickness can vary depending on body site notably differing the most between plantar and nonplantar tissue. Nonplantar human skin is more susceptible to skin injury than plantar skin which is present on palm of hands and soles of feet. This is a critical concern for limb amputee patients that exhibit nonplantar stump site tissue. I investigated whether skin properties could be transformed with protein therapy; Wnt5a, by modulating the specific gene expression of nonplantar tissue to become plantar. I designed and engineered a microneedle (MN) device for optimal delivery of the Wnt5a protein and other drug candidates to the epidermal layer of the human skin as a potential therapeutic.

Next, in recent years the definition of ageing has become more convoluted with biological ageing being characterised as a scale of ageing that considerably varies depending on the individual. Several internal and external factors can drive biological ageing of the skin which impacts the properties of this vital organ. I explored the effect of a physiologically relevant environmental stimuli, UVA, on *ex vivo* human skin to assess changes in biological age and how that in turn influences skin injury. I investigated the preventative capacity of an anti-ageing protein, klotho, on UV impaired healing.

Table of Contents

DECLARATION OF ORIGINALITY **2**

COPYRIGHT DECLARATION **2**

ACKNOWLEDGMENTS **3**

ABSTRACT **4**

LIST OF ABBREVIATIONS **15**

LIST OF FIGURES **16**

LIST OF TABLES **18**

MANUSCRIPTS IN PREPARATION: **19**

CHAPTER 1 ..... **20**

1.1 INTRODUCTION; CHAPTER AIMS **20**

1.1.1 THE STRUCTURE OF THE HUMAN SKIN **20**

THE EPIDERMIS IS CONSTANTLY REGENERATED VIA UPWARD KERATINOCYTE MIGRATION 21

THE COMPOSITION OF EXTRACELLULAR MATRIX. 24

THE DERMIS SUPPORTS THE SKIN EPIDERMIS AND AIDS IN SENSATION. 25

THE HYPODERMIS ENCOMPASSESS CONNECTIVE TISSUE AND BLOOD VESSELS 28

EPITHELIAL MESENCHYMAL SIGNALING BETWEEN KERATINOCYTES AND FIBROBLASTS 29

1.1.2 WOUND HEALING AND SKIN CELLS. **29**

THE ROLE OF KERATINOCYTES IN WOUND REEPITHELISATION 31

THE ROLE OF FIBROBLASTS IN SKIN WOUND HEALING 32

1.1.3 HETEROGENEITY IN HUMAN SKIN 33

SKIN VARIES IN THICKNESS DEPENDING ON BODY SITE 33

TRANSFORMING NONPLANTAR TO PLANTAR SKIN 35

SKIN PIGMENTATION INFLUENCES SKIN HEALTH AND AGEING. 35

CHRONOLOGICAL AGEING ALTERS SKIN INTEGRITY. 36

UVA DRIVES AGEING OF HUMAN SKIN 36

ULTRAVIOLET RADIATION 37

1.1.4 THE WORLD IS AGEING 39

IMPLICATIONS OF AN AGEING POPULATION 41

1.1.5 THE PROCESS OF AGEING 43

CHRONOLOGICAL VS BIOLOGICAL AGEING 44

SKIN AS A MODEL OF AGEING 45

1.1.6 CHANGES TO THE SKIN WITH INCREASING AGE 46

CELLULAR SENESCENCE 46

STEM CELL DECLINE 48

DELAYED HEALING IN AGED SKIN 49

1.1.7 PREVENTATIVE MEDICINE AS AN EMERGING SOLUTION TO SKIN RELATED DISEASE. 50

1.1.8 DERMATOLOGICAL TREATMENT OPTIONS 50

DRUG DELIVERY. 50

1.2 AIMS OF THESIS 53

CHAPTER 2 54

2.1 INTRODUCTION; CHAPTER AIMS 54

2.1.1 TRANSDERMAL DEVICES 55

MICRONEEDLES OVERCOME CONVENTIONAL FORMS OF DRUG DELIVERY. 55

2.1.2 MICRONEEDLES AS DRUG DELIVERY DEVICES 56

TYPES OF MICRONEEDLES 56

LIMITATIONS OF CURRENT COMMERCIALLY AVAILABLE MICRONEEDLE DEVICES 57

2.1.4 ENGINEERING A MICRONEEDLE DEVICE FOR TARGETED DELIVERY TO THE SKIN EPIDERMIS 58

GEOMETRY OF A DESIRABLE MN DEVICE....58

FABRICATION METHOD OF A DESIRABLE MN DEVICE 59

2.1.4 DESIGN CRITIERA OF MY MN DEVICE....59

2.1.5 CASES OF LIMB AMPUTATION 60

2.1.6 DERMATOLOGIC ISSUES ASSOCIATED WITH AMPUTEE PATIENTS 61

2.1.7 RECONSTRUCTING THE SKIN FLAP FOR LIMB AMPUTEE PATIENTS 62

2.1.8 CURRENT OPTIONS TO REDUCE SKIN INJURY. 62

2.1.9 AIMS OF CHAPTER: DESIGNING A DEVICE TO OVERCOME CURRENT LIMITATIONS AND OFFER A THERAPEUTIC OPTION FOR AMPUTEE PATIENTS 63

2.2 MATERIALS AND METHODS....65

2.2.1 MICRONEEDLE FABRICATION 65

MASTER MOLD 3D MICROFABRICATION....65

POLYDIMETHYLSILOXANE (PDMS) EMBOSSING 66

PEGDA MICROMOLDING 66

2.2.2 MECHANICAL AND TENSILE STRENGTH TESTING OF MICRONEEDLE. 67

AXIAL COMPRESSION TEST 67

PARAFILM INSERTION POINT. 67

SWELLING STUDY. 67

2.2.3 SCANNING ELECTRON MICROSCOPY (SEM) 68

2.2.5 COATING OF MICRONEEDLE: DIP COATING....68

FINE TUNING COATING OF MICRONEEDLES TIPS 69

2.2.6 TESTING VISCOSITY OF PROTEINS IN PAA 69

2.2.7 PROTEIN RELEASE 69

FLUORESCENCE INTENSITY 69

CONFOCAL MICROSCOPE 70

FLUORESCENCE CALIBRATION CURVE 70

NANODROP ABSORBANCE MEASUREMENTS 70

ASSESSING PH SENSITIVITY OF COATED MICRONEEDLE DEVICE 70

2.2.8 GELATIN METHACRYLATE (GELMA) MODEL ESTABLISHMENT 71

2.2.9 HUMAN SAMPLES 71

ISOLATION OF PRIMARY KERATINOCYTES 71

SKIN PENETRATION TEST 72

2.2.10 MEASURING FLUORESCENCE INTENSITY (%) BEFORE AND AFTER MICRONEEDLE APPLIED SKIN. 72

2.2.11 WNT5A TO HUMAN KERATINOCYTE CELLS. 72

2.2.12 WNT5A TO *EX VIVO* SKIN. 73

BIOACTIVITY OF WNT5A IN PAA 73

POLYMERASE CHAIN REACTION.....74

IMMUNOSTAINING 75

2.2.13 STATISTICS AND REPRODUCIBILITY 77

2.3 RESULTS 78

2.3.1 PRELIMINARY: CHARACTERIZING DIMENSIONS OF BIOMAD MANUFACTURED MICRONEEDLE.....78

2.3.2 OPTIMIZATION: FINE-TUNING THE MECHANICAL AND SWELLING PROPERTIES OF POLYETHYLENE GLYCOL DIACRYLATE (PEGDA)- BASED MN BY MOLECULAR WEIGHT. 79

PEGDA OF DIFFERING MOLECULAR WEIGHT CONFERS DIFFERENT MECHANICAL PROPERTIES. 79

2.3.3 DI(TRIMETHYLOLPROPANE) TETRA ACRYLATE ADDITIVE ENHANCES THE MECHANICAL PROPERTIES OF POLYETHYLENE GLYCOL DIACRYLATE (PEGDA).....81

A TETRA CROSSLINKER ADDITIVE ENHANCES COMPRESSION FAILURE POINT AND REDUCES SWELLING THRESHOLD OF PEGDA MN 700.....81

2.3.4 OPTIMIZATION: A VARIABLE HEIGHT MICRONEEDLE DEVICE DESIGNED TO ELUDE THE 'BED OF NAILS' EFFECT.....84

A VARIABLE HEIGHT MN DEVICE OVERCOMES THE 'BED OF NAILS' PHENOMENON.....84

2.3.5 DESIGN OF COATING, AND VALIDATION OF RELEASE USING ARTIFICIAL SKIN MODEL, AND PH-SENSITIVITY.....86

A PAA HYDROGEL COATING ON THE MN BASE ALTERS THE MORPHOLOGY OF THE MICRONEEDLE DEVICE. 86

2.3.6. INVESTIGATING THE PH RESPONSIVENESS OF THE MN COATING 91

PAA HYDROGEL IS PH RESPONSIVE ENSURING ENCAPSULATED PROTEIN IS ONLY RELEASED IN NEUTRAL AND HIGH PH ENVIRONMENTS. 91

2.3.7. DESIGNING A GELMA ARTIFICIAL SKIN MODE FOR DRUG DIFFUSION VALIDATION STUDIES. 93

GELATIN METHACRYOLATE EXHIBITS COMPATIBLE PROPERTIES TO HUMAN SKIN AND THUS IS AN EFFECTIVE MODEL FOR DRUG DIFFUSION STUDIES.....93

GELMA MODEL CONFIRMS PH RESPONSIVE RELEASE OF DRUG OF INTEREST FROM PAA COATED MICRONEEDLE ARRAY.....95

INSERTION POINT RESULTS OF MICRONEEDLE ON PARAFILM TO EMULATE STRATUM CORNEUM. 96

2.3.8 ASSESSING THE BIOLOGICAL EFFICACY OF MY MICRONEEDLE ON *EX VIVO* HUMAN SKIN.....98

VALIDATING BIOLOGICAL ACTIVITY OF WNT5A ON KERATINOCYTES IN UPREGULATING KRT9 98

2.3.9 THE CONSTRUCTED MICRONEEDLE IS SIGNIFICANTLY MORE EFFECTIVE IN DELIVERING A DRUG OF INTEREST TO EPIDERMIS THAN TOPICAL TREATMENT. 99

2.4 DISCUSSION.....104

CHAPTER 3.....108

3.1 INTRODUCTION; CHAPTER AIMS 108

3.1.1 AN AGEING POPULATION 109

3.1.2 UVA ACCCELERATES SKIN AGEING 111

3.1.3 CPG METHYLATION 112

METHYLATION CLOCKS TO ASSESS AGE IN RESPONSE TO UVA 113

3.1.4 UVA INDUCES SENESCENCE 114

3.1.5 AGEING AND WOUND HEALING, ... 116

3.1.6 UVA IMPAIRS WOUND HEALING 117

3.1.7 ANTI-AGEING COMPOUNDS 118

SENOLYTICS 118

THE KLOTHO PROTEIN 119

3.1.8 STUDYING THE ROLE OF UVA IN IMPAIRED REGENERATION WITH AGEING 120

3.2 MATERIALS AND METHODS 121

3.2.1 SKIN AND CELL SAMPLES 121

PREPARATION AND PROCESSING OF HUMAN SKIN SAMPLES 121

ISOLATION AND CULTURE OF KERATINOCYTES AND FIBROBLASTS FROM WHOLE SKIN 122

3.2.2 UVA EXPOSURE REGIMEN 123

OPTIMIZATION OF UV DOSES 123

3.2.3 WOUND HEALING ASSAYS *EX VIVO* AND *IN VITRO* 124

ESTABLISHMENT OF WHOLE *EX VIVO* SKIN WOUND HEALING ASSAY 124

IN VITRO WOUND HEALING ASSAY 125

CULTURING KERATINOCYTES WITH UV CONDITIONED FIBROBLAST MEDIUM 125

EFFECT OF IL-12 ON KERATINOCYTE WOUND REPOPULATION 126

CELL AND TISSUE ANALYSIS 126

QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR) 126

IMMUNOSTAINING....129
BIOLOGICAL AGE....131
CYTOKINE ASSAY....132
ANTI-AGEING THERAPEUTIC STRATEGY....133
ADMINISTRATION OF KLOTHO IN MEDIA....133
KLOTHO IN DOWNREGULATING SENESCENCE OF KERATINOCYTES AND FIBROBLASTS....133
KLOTHO IN SCRATCH ASSAY....134
KLOTHO IN EPITHELIAL MESENCHYMAL SIGNALING....134
KLOTHO EFFECT ON WOUND HEALING....135
BIOACTIVITY OF KLOTHO IN PAA....136
KLOTHO COATED MN PENETRATION OF GELMA® SYNTHETIC MODEL....136
KLOTHO COATED MICRONEEDLE APPLICATION ON *EX VIVO* SKIN....137
STATISTICS AND REPRODUCIBILITY....137

3.3 RESULTS....137

3.3.1 OPTIMISATION OF UV DOSES AND WOUND HEALING ON *EX VIVO* SKIN....138
3.3.2 YOUNG AND OLD SKIN HEAL DIFFERENTLY....139
3.3.3 UVA AGE ACCELERATES HUMAN SKIN AND IMPAIRS WOUND HEALING *VIA* DNA DAMAGE INDUCTION....142
3.3.4 UVA EXPOSURE MODIFIES THE PHENOTYPE OF KERATINOCYTES AND FIBROBLASTS IN YOUNG SKIN....145
3.3.5 UVA EXPOSED FIBROBLASTS SIGNAL TO KERATINOCYTES VIA IL12, TO REDUCE REPOPULATION TO INJURY SITE....152
3.3.6 THE KLOTHO PROTEIN PREVENTS UVA-AGE ACCELERATION OF PRIMARY EPIDERMAL AND DERMAL CELLS....156
3.3.7 THE KLOTHO PROTEIN REDUCES P21 EXPRESSION AND ENHANCES LAMINB1 EXPRESSION IN KERATINOCYTES TREATED WITH UVA CONDITIONED FIBROBLAST MEDIUM....161

3.3.8 KLOTHO PREVENTS UVA INDUCED DAMAGE ON YOUNG SKIN. 165

3.3.9 A PH RESPONSIVE MICRONEEDLE EFFICIENTLY DELIVERS KLOTHO TO HUMAN SKIN AS A PREVENTATIVE THERAPEUTIC. 169

3.4 DISCUSSION....176

CHAPTER 4....180

4.1 INTRODUCTION; CHAPTER AIMS....180

4.1.1 THE DISREPENCY IN DIAGNOSIS OF SKIN DISEASES IN DIFFERENT ETHNIC COHORTS 181

4.1.2 MELANOCYTES IN SKIN HOMEOSTASIS....181

MELANOCYTES IN HUMAN SKIN 182

4.1.3 MELANIN PRODUCTION AND PHENOTYPIC DIVERSITY IN SKIN COLOUR....182

SPECTRUM SCALES TO CLASSIFY SKIN COLOUR FROM LIGHT TO DARK. 182

GENETIC POLYMORPHISMS CAN DRIVE PIGMENTATION. 184

THE INFLUENCE OF THE ENVIRONMENT ON SKIN PIGMENTATION 185

THE DOUBLE-EDGED SWORD OF UV EXPOSURE....187

MELANOGENIC ACTIVITY INFLUENCES SKIN COLOUR. 188

MELANOCYTES PRESENT REGIONAL DIVERSITY ACROSS THE HUMAN BODY....190

MELANOCYTES IN AGEING. 191

4.1.4 MELANOCYTES AND SKIN DISEASE....192

DISEASES CAUSED BY VARIATIONS IN MELANOGENIC ACTIVITY. 193

SKIN MELANIN CONTENT AND DISEASE SUSCEPTIBILITY 196

4.1.5 WOUND REPAIR AND SCARRING DIFFER ACROSS RACIAL GROUPS. 201

LACK OF IDENTIFICATION OF PRESSURE ULCERS IN ERS....201

KELOIDS ARE TYPICALLY PRESENTED IN TYPE VI SKIN. 202

SKIN CANCERS ARE MOST PREVALENT IN TYPE 1 SKIN BUT SEVERE IN TYPE VI SKIN. 203

MELANOMA: LATE-STAGE MELANOMA IS PREVALENT IN TYPE III-TYPE VI SKIN 203

SQUAMOUS CELL CARCINOMA: TYPE III-IV SKIN IS NOT IMMUNE TO SKIN CANCER SUSCEPTIBILITY. 206

BASAL CELL CARCINOMA: IS PREVALENT IN SUN EXPOSED COUNTRIES. 207

RARE SKIN CANCERS: LACK OF DIAGNOSTIC UNDERSTANDING IN TYPE III-TYPE VI SKIN TYPES. 208

4.2 DISCUSSION 209

CHAPTER 5 211

5.1 DISCUSSION 211

5.1.1 CHAPTER 2 211

5.1.2 CHAPTER 3 212

5.1.3 CHAPTER 4 214

5.2 FUTURE WORK 215

5.2.1 CHAPTER 2 215

TOXICITY EFFECTS OF MICRONEEDLES, I.E. SKIN IRRITATION OR ALLERGY 217

SPATIOTEMPORAL MICRONEEDLES. 218

5.2.2 CHAPTER 3 219

OTHER INDICATORS OF AGEING I.E. TELOMERE SHORTENING, DNA DAMAGE, METABOLISM 219

ASSESSING THE SYSTEMIC EFFECTS OF UV BEYOND THE HUMAN SKIN. 220

COMPARING THE EFFECTS OF UVA AND UVB ON *EX VIVO* SKIN. 224

EXAMINING THE ROLE OF ENDOTHELIAL CELLS IN WOUND HEALING. 224

KLOTHO AND UV PREVENTION 226

ANTI-AGEING COMPOUNDS FOR SKIN THERAPY 228

5.2.3 CHAPTER 4 229

5.3 CONCLUDING REMARKS 229

6.0 REFERENCES 230

7.0 APPENDIX.....286

.....291

Keywords: Skin, Ageing, Ultraviolet radiation (UV), Microneedle, polymeric, pH-responsive, wound-healing, keratinocytes, fibroblasts, melanocytes,

List of Abbreviations

PAA	Polyacrylic Acid
MN	Microneedle
DMSO	Dimethylsulfoxide
PBS	Phosphate Buffered Saline
PEG	Poly(ethylene glycol)
SEM	Scanning electron microscope
UV-A/B/C	Ultraviolet-A/B/C
ECM	Extracellular Matrix
THBS	Thrombospondin
FBS	Fetal Bovine serum
RT	Room Temperature
IL	Interleukin
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
EDX	Energy dispersive Xray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
MW	Molecular weight
FITC	Fluorescein isothiocyanate
THBS(1,2,4)	Thrombospondin
SASP	Senescence associated secretory phenotype.
HS	Hidradenitis Suppurativa
PPG	Polypropylene Glycol
TETRA	di(trimethylolpropane) tetra acrylate
Short-cross linker	4-Butanediol diacrylate crosslinker
HMGCR	3-hydroxy-3-methylgutaryl-CoA reductase
FASN	Fatty acid synthase
FADS2	Fatty acid desturase 2
ELN	Elastin
FBLN2	Fibulin 2
FLBN5	Fibulin 5
COL5A1	Collagen V
COL1A1	Collagen I
COL3A1	Collagen III
DEJ	Dermal-epidermal junction
MCSP	Melanoma Chrondroitin sulphate proteoglycan
ERC	Eumelanin Rich Skin
ELOVL2	Elongation of very long chain fatty acids-like 2)
DWAT	Dermal white adipose tissue
LBL	Layer-by-Layer
HCL	Hydrochloric acid

List of Figures

Figure 1.1: The structure of the human skin.. 21
Figure 1.2: The transformation of keratinocytes within different layers of the skin epidermis.....22
Figure 1.3: The structure of the skin dermis.....27
Figure 1.4: The phenotype of a fibroblast cell……29
Figure 1.5: The hypodermis structure... 29
Figure 1.6: Stages of wound healing……....31
Figure 1.7: Phenotypic changes in skin with old age……...37
Figure 1.8: Penetration effects of UV on skin…… 39
Figure 1.9: Global population ageing…….. 41
Figure 1.10: Age related disease heightens with age…….. 44
Figure 1.11: Biological ageing clock……... 46
Figure 1.12: Cellular senescence as a hallmark of aging……48
Figure 2.1: Microneedles as drug delivery devices... 56
Figure 2.2: Types of Microneedles.. 57
Figure 2.3: Design and construction of pH responsive Microneedle for epidermal delivery of proteins……....65
Figure 2.4:Characterisation images of Manufactured MN from BioMAD group..... .79
Figure 2.5: Stiffness and swelling properties of PEGDA differ according to molecular weight ……..81
Figure 2.6: The addition of di(trimethylolpropane) tetra acrylate to PEGDA Mw 700... 84
Figure 2.7: Variable height design of microneedles.....86
Figure 2.8: PAA forms a filmy layer over needle tips to encapsulate drug of interest ……....88
Figure 2.9: PAA is pH responsive, and drug of interest only diffuses out of hydrogel in slightly neutral or alkaline conditions.....90
Figure 2.10: PAA is pH responsive,(BSA).....92
Figure 2.11: PAA is pH responsive,(Avidin).....93
Figure 2.12: Microneedles pierce into GelMA.....95
Figure 2.13: Wnt5a coated MN diffuses up to >90% into synthetic skin materials……96
Figure 2.14: Parafilm insertion point……..…….98
Figure 2.15: KRT9 expression of Wnt5a treated keratinocytes.....……99
Figure 2.16: Biological function of wnt5a coated MN on ex vivo human skin.....100
Figure 2.17: Immunostaining of Keratin9. 102
Figure 2.18: Fine-tuning coating of MN array.....104
Figure 3.1: The aging population across the world. ……. 110
Figure 3.2: Illustration of the many ways UVA and B may impact human health.……....112
Figure 3.3: Illustration of DNA damage and epigenetic changes……...114
Figure 3.4: Illustration of Senescent cell. ……..116
Figure 3.5: Illustration of wounded skin model…..……..118
Figure 3.6: Schematic of project plan……..…….. 120
Figure 3.7: Effect of klotho on scratch assay……134
Figure 3.8: Effect of klotho on epithelial signalling…….……135
Figure 3.9: Effect of klotho on wound healing….. …….……136
Figure 3.10: UVA optimisation. ……..…..138

Figure 3.11: Baseline differences in wound healing in young vs elderly skin. ……....140
Figure 3.12: Changes in wound healing in old skin…….……..141
Figure 3.13: UV injury accelerates aging of young skin impairing wound healing ..……...143
Figure 3.14: UV injury induces DNA damage in young skin…….…...144
Figure 3.15: Assessing viability and metabolic activity of UV exposed Keratinocytes and Fibroblasts. ……146
Figure 3.16: Effect of UVA dose (0.05, 0.1 and 0.15 J/cm^2) on keratinocyte cells ……...148
Figure 3.17: Effect of UVA dose on gene expression of keratinocyte cells …….....149
Figure 3.18: The effect of UVA dose (2.0 J/cm^2) on fibroblasts.....150
Figure 3.19: The effect of UVA dose on gene expression of fibroblasts. 151
Figure 3.20: The mechanism behind UVA impaired migration of keratinocyte cells.. …….153
Figure 3.21: Effect of klotho on UV exposed keratinocytes…….……156
Figure 3.22: Effect of klotho on UV exposed Fibroblasts..……. …….158
Figure 3.23: Effect of klotho on UV exposed Fibroblasts gene expression...……....159
Figure 3.24: Effect of klotho on epithelial mesenchymal network …….....…163
Figure 3.25: Effect of klotho on ex vivo human skin.……....166
Figure 3.26: Changes in gene expression in elderly skin.…….168
Figure 3.27: Bioactivity of Klotho in polyacrylic acid material……170
Figure 3.28: Effect of klotho coated microneedle on ex vivo human skin.…….. …172
Figure 3.29: The effect of klotho coated MN on photoaging and biological ageing gene expression……...174
Figure 4.1: Schematic of the FST (left) and Eumelanin skin score (right) scales……..183
Figure 4.2: The distribution of melanocytes across the body.……190
Figure 4.3: Illustration of vitiligo presentation on skin…….……..……...193
Figure 4.4: Illustration of vitiligo presentation on skin….……194
Figure 4.5: Illustration of dermatitis on skin……..……199
Figure 4.6: Illustration of keloid presentation on skin**.** …….……202
Figure 4.7: Illustration of pathogenesis of melanoma..……..204

List of Tables

Table 2.1: Primer sequencing list of KRT9, KRT14 and GAPDH. 76

Table 2.2: List of Primary and Secondary Antibodies……..77

Table 3.1: Information on the time exposure and intensity of UVA doses. .. **Error! Bookmark not defined.**

Table 3.2: Primer sequencing list of Senescent, wound healing makers, and SASP markers. ..... **Error! Bookmark not defined.**

Table 3.3: Primer sequencing list of photoaging and biological aging genes..... **Error! Bookmark not defined.**

Table 3.4: List of Primary and Secondary Antibodies 131

Table 3.5: Primer sequencing of ELOVL2 age-related marker. ..... **Error! Bookmark not defined.**

Manuscripts in preparation:

Abbas AA., Steer I., Teenan O., Hanif W., Akhavani M., Hasan E., Salama K., Almquist B., Higgins CA., and Alsulaiman D. “Reprogramming skin identity through protein delivery via a smart microneedle device” (Research article)

Abbas AA., Tan D., Akhavani M., Limbu S., Lee JJ., Muttaghabani K., Alsulaiman D., and Higgins CA. “UVA age acceleration of human skin and impeded wound healing is protected by the klotho protein” (Research article)

Abbas AA., and Higgins CA. “The Impact of Skin Colour on Skin Health” (Literature Review)

Chapter 1

The hetereogeneity of human skin

1.1 Introduction; Chapter Aims

In this chapter, I introduce the structure and function of human skin. I describe the intrinsic formation and heterogenity of human skin that is influenced by body location and modified by ageing and how that in turn impacts skin injury susceptibility. I discuss how the functional role of epidermal keratinocytes and melanocytes as well as dermal fibroblasts are altered with ageing and skin colour. I also highlight the importance of dermatology ageing research in the coming decades as population ageing persists. I conclude by highlighting the need for personalised, rather than reactionary, dermatology therapeutics within the public health sector.

1.1.1 The Structure of the human skin

The human body is intricately composed of several cross talking, interconnected systems which all function with the overall goal for maintaining health. The body is cloaked by a pivotal protective barrier to the outside stimuli, known as skin [Figure 1.1]. Human skin is the largest organ of the body, amounting on average to 9 lbs., [Gallo. 2017; de-Souza et al., 2019] accounting for up to 15% of total body weight [Richardson. 2003; Kanitakis. 2002].

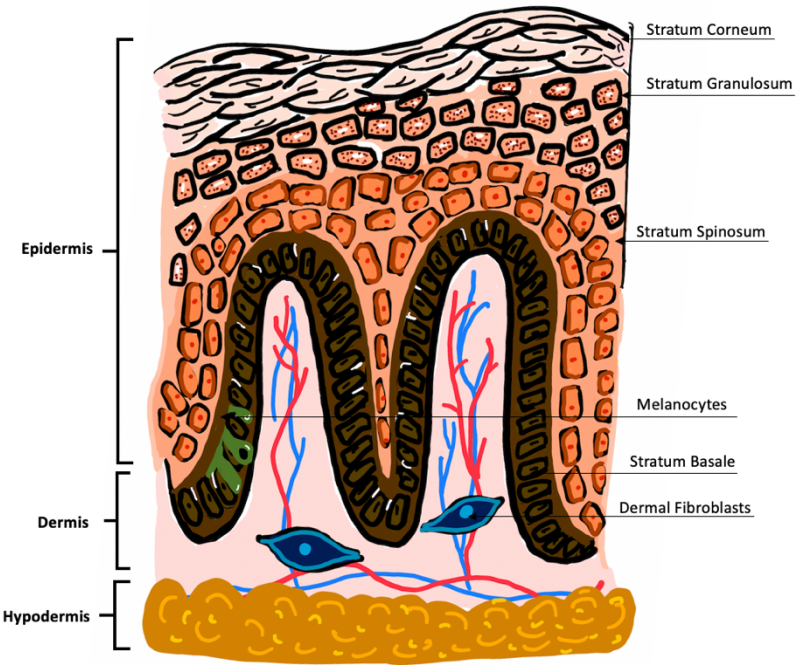


Figure 1.1: The structure of the human skin. The human skin is composed of the epidermis and the dermis. The epidermis is composed of the stratum corneum, stratum granulosum, stratum spinous, where Vitamin D is synthesised, and the stratum keratinocyte basale layer where melanin is synthesised by melanocytes.

Our skin is a vital structural aspect of the human system, responsible for defending against external chemical, mechanical, and biological injury, preventing excess water loss from the body and crucially for thermoregulation [Kanitakis 2002]. The skin forms a continuous layer across the body's surface and is lined with the mucous membrane. The organ is composed of multiple layers, home to various cells with specialized activities, and distinct in thickness and barrier functions [Figure 1.1]. Human skin is diversified by age, and colour leading to intrinsic variances, and thus modifying susceptibility to disease and vulnerability to external stimuli, which I discuss and explore in this body of work.

The epidermis is constantly regenerated via upward keratinocyte migration

Fundamentally, human skin is primarily composed of three essential layers, the epidermis (derived from ectodermal lineage, connective tissue), dermis and hypodermis (derived from mesodermal lineage, epithelial tissue) [Figure 1.2].

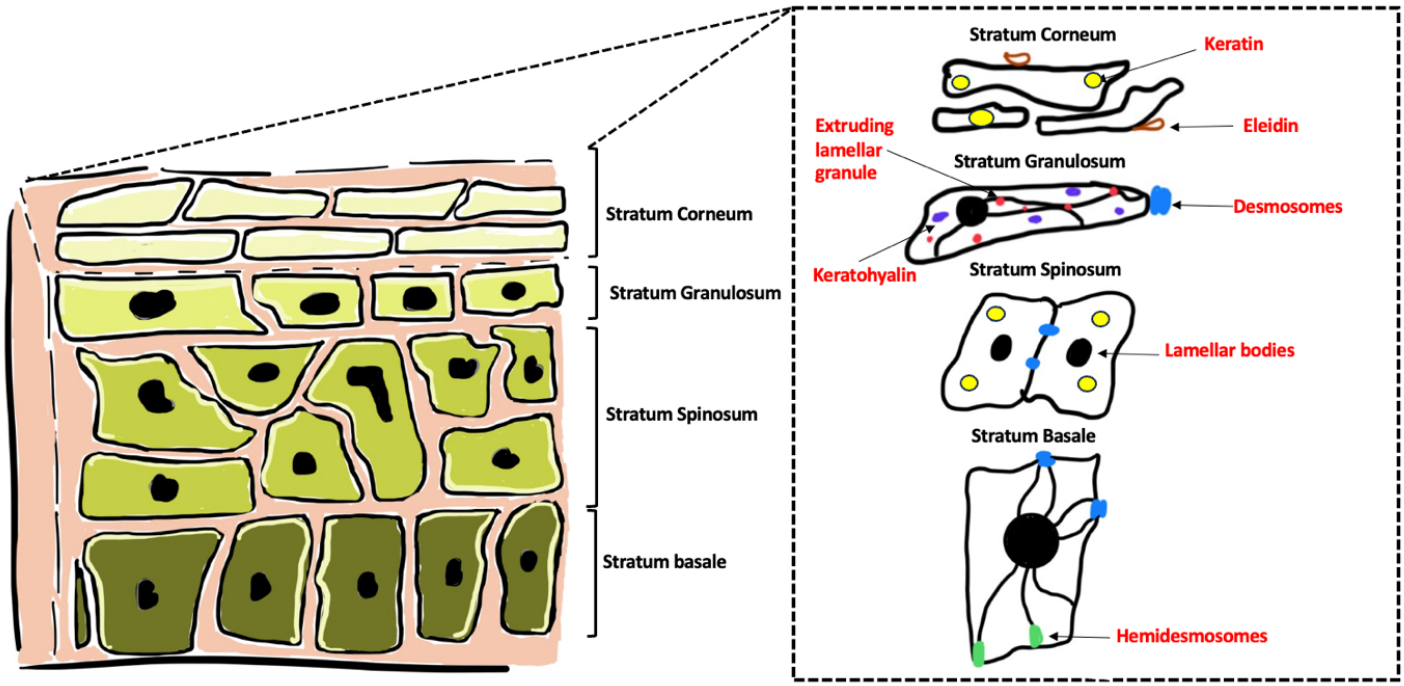


Figure 1.2*:* The transformation of keratinocytes within different layers of the skin epidermis. Keratinocytes are created from basal stem cells in the stratum basale, where they start to assemble fibres of keratin. Keratinocytes move upwards to the stratum spinosum layer where they connect to each other via desmosomes. In the stratum granulosum keratinocytes are tightly connected to each other via desmosomes with lamellar bodies, keratohyalin and keratin molecules embedded within the cytoplasm. In the stratum corneum, dead keratinocytes become corneocytes which are filled with keratin and gradually shed off the skin, where they are no longer connected to one another.

The epidermis is compiled of several sub layers including the stratum basal, stratum spinosum, stratum granulosum, stratum lucidum (in palmoplantar skin only), and stratum corneum [Figure 1.2] [Yousef et al., 2022].

The stratum basale is the innermost layer of the skin epidermis and is separated from the dermis via the basement barrier and adheres to the basement membrane via hemidesmosomes [Yousef et al., 2022] [Figure 1.2]. The basal layer is also home to melanocytes which give rise to skin pigmentation. Basal keratinocyte markers include Keratin-(KRT)-14, Krt5, and CDH3. These keratinocytes undergo mitosis, and thus can divide, allowing them to be pushed upwards into the outermost layers of the epidermis [Wang et al., 2020].

The stratum spinosum is located above the stratum basale layer and is where basal cells have been pushed upward once delaminated. They are now characterised as squamous cells [Abdo et al., 2020]. The stratum spinous layer is characterized as the prickle cell layer, typically 5-10 cell layers thick, which possess dendritic cells and irregular polyhedral cells with cytoplasmic processes, referred to as spines, that extend upwards and adhere to neighbouring cells via desmosomes [Yousef et al., 2022]. Keratinocytes from the squamous spinous layer are differentiated as they are moving up through the skin and become the known as the stratum granulosum and lucidium (only present in palmoplantar skin). Spinous keratinocyte markers include KRT1, KRT10, DSG1, and CDH1 which are also continually expressed in granular keratinocytes [Wang et al., 2020].

The stratum granulosum, of the skin epidermis, is formed of up to 3-5 cellular layers, possessing diamond shaped cells with keratohyalin granules and lamellar granules. These keratohyalin granules carry keratin precursors that aggregate, cross link and form bundles over time. The lamellar granule contains glycolipids that are secreted to the surface of the cells and create adhesion between cells. At this skin layer, the keratinocytes become bigger and flatter and once they adhere become dehydrated and die which results in tough, durable layers of cellular skin. The granular layer can confer barrier function to skin as it is composed of occluding tight junctions [Bäsler et al., 2014]. Granular keratinocytes express the differentiation gene markers DSC1, KRT2, IVL and TGM3 [Wang et al., 2020].The secondary layer, stratum lucidum, present only in thick skin sites such as palm sites or soles of feet, is of

3 cellular layers, forming a thin translucent coating established by eleidin; a transformation product of keratohyalin.

The outermost epidermal layer is the stratum corneum, a pivotal barrier to large molecules, over the size of 500 Da due to the tight packing of keratinocytes and the lipid layers which restrict passage [Bos et al., 2000]. The stratum corneum is composed of up to 20-30 cellular layers of keratin in plantar skin, (3-5 layers on average in non-plantar skin) and horny scales of dead shedding keratinocytes, characterized as anucleate squamous cells. Involucrin, is a reported differentiated marker typically expressed by irreversibly differentiated keratinocytes in the stratum corneum [Lee et al., 2014]. Each corneocyte within the stratum corneum, is embedded in the lipid matrix produced by the lamellar granules. Cells bathed in the lipid matrix form the 'Brick and Mortar' structure where nonviable corneocytes represent bricks and intercellular lipids present mortar. The lipids present in the stratum corneum are important for preventing excessive water loss through the epidermis and preventing compounds from the environment from permeating into the viable epidermal and dermal layers [Smeden et al., 2016]. This crucial layer differs vastly in thickness, critically in calloused skin [Yousef et al., 2022]. The stratum corneum is divided into two distinct layers, the stratum compactum and the stratum disjunctum [Murphrey et al., 2022]. The stratum compactum is a deep, dense, cohesive layer, while the stratum disjunctum, present in all skin types, is looser and lies superficially to the stratum compactum. As the stratum disjunctum gradually loses adhesiveness following decreased inter-corneocyte adhesion, cells begin to desquamate [Murphrey et al., 2022]. The stratum corneum is continually sloughed off as old cells are replaced by new long-wearing cells; however, this shedding process does gradually decline with ageing. The cell turnover of the stratum corneum takes 28-30 days in young adults, but 45-50 days in elderly adults [Grove et al., 1983].

Epidermal Keratinocytes

Keratinocytes are the predominant cell type of the epidermis and are derived from the basal layer, where they produce keratin [Chan. 2023]. As keratinocytes move upwards to the stratum corneum they differentiate and stratify, producing keratin which is the primary component of the stratum corneum. These epidermal cells are responsible for the production of the epidermal water barrier by forming and secreting lipids. In addition to maintaining adequate skin hydration by secreting keratin, these epidermal cells are essential for defending against

microbial invasion and shielding against UV exposure. Keratinocytes are fundamental to the epithelization process, as the primary cells of the epidermis, whereby they migrate, proliferate, and differentiate to restore the epidermal barrier [Landen et al., 2016]. Keratinocytes are responsible for producing keratin, a robust and protective protein that forms the structure of skin, hair, and nails [Abdo et al., 2020]. Keratin is also responsible for the creation of the epidermal water barrier via secretion of lipids in plantar skin [Yousef et al., 2022]. In skin epithelia, keratin protects the epithelial cells from mechanical and non-mechanical stresses that induce cell rupture and death [Gu et al., 2006].

The Composition of extracellular matrix.

The extracellular matrix (ECM) is a dynamic 3-dimensional network of macromolecules that offers structural support for cells and tissue [Karamanos et al., 2021]. The ECM is important for orchestrating cell signalling, functions, properties and morphology. Collagens, proteoglycans (PGs), glycosaminoglycans (GAGs), elastin, elastic fibres, fibronectin, laminins, and glycoproteins i.e. matricellular proteins all compose the ECM [Theocharis et al., 2016; Theocharis et al., 2014]. ECMs function as communication mediators between cells in organs and tissues by orchestrating signalling inside-out or outside in commands. ECMs guide tissue morphogenesis, development and haemostasis, by modulating cellular physiology, growth, survival, differentiation and adhesion [Manou et al., 2019; Theocharis et al., 2012].

Collagen type I is the dominant form found in the skin, produced from endothelial cells and epithelial cells. In the ECM, collagen is arranged into fibrils to ensure structural integrity for tissues, and fibril formation is restricted to collagen types I, II, III, V and XI. The fibril formation offers strength to connective tissues that are needed to withstand different mechanical stresses like tension, shear and pressure [Badylack et al., 2009]. It has been indicated that collagen fibril formation is regulated by decorin and fibromodulin. Integrin cell surface receptors are present on the surface of collagen, i.e. $\alpha_1\beta_1$ controls production of collagen through negative feedback and $\alpha_2\beta_1$ which increases collagen matrix synthesis and turnover [White et al., 2004].

Elastin is another structural protein present in the ECM, providing dermis of the skin, and allows recovery from persistent stretching in tandem with glycoproteins i.e. fibrillin and fibulin [Eckes et al., 2010; Frankz et al., 2010]. Fibronectin is located within the basement membrane

(BM) of the ECM and is important for cell adhesion and wound healing response to injury. Fibronectin is arranged into a mesh of fibrils similar to collagen and is linked to integrins. Fibronectin is produced int the form of disulphide-bonded dimer that can be broken down into subunits, of which there are three different types (types I, II, and III) that are repeated within each subunit [Mao et al., 2005]. Laminins are another type of glycoprotein with a trimeric structure, made up of three different chains, α, β and γ, which exist in several genetically distinct forms [Aumailley et al., 2005]. Laminins are important for cell adhesion, and cell processes such as differentiation and migration via integrins [Eckes et al., 2010]. Laminins intercede between cells and the underlying matrix, the BM, where they reside. Lastly, tenascins are a group of ECM proteins and exist as five different manifestations TN-C, TN-R, TN-W, TN-X and TN-Y/ Tenascins are commonly found within connective tissue where load bearing is required and have been linked to mechanical activity [Bosman et al., 2003; Chiquet et al., 2003].

The dermis supports the skin epidermis and aids in sensation.

The dermis is majorly composed of fibrous tissue, which consists of collagen and elastin fibres [Brown and Krishnamurthy. 2022]. The BM separates the dermis from the epidermis and consists of two layers: basal and reticular lamina [Pozzi et al., 2017; Wade et al., 2014]]. The basal lamina sits closest to the epithelium and is composed of fine fibrils of mostly laminin and type IV collagen, whereas the basal reticular layer lies beneath the lamina and is thicker, more diffuse and fibrous with a higher concentration of proteoglycans present a as well as type III collagen.

The dermis, characterised as a connective tissue layer of mesenchymal origin [Figure 1.3], is located underneath the epidermis [Lopez-Ojeda et al., 2022].

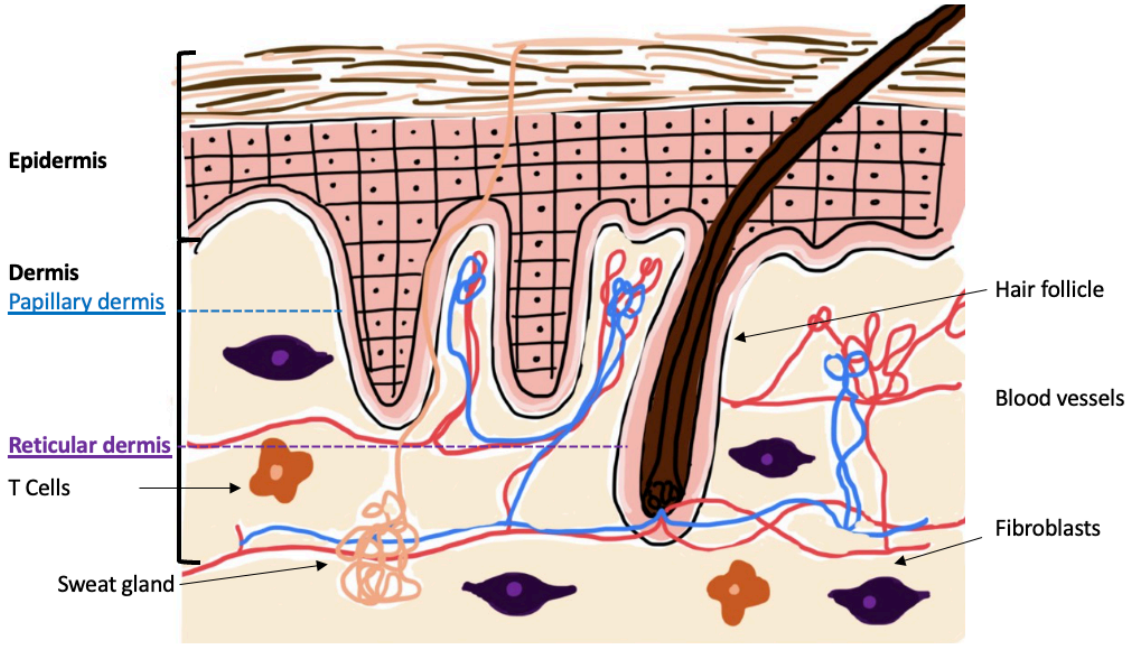


Figure 1.3: The structure of the skin dermis. The dermis is derived from mesodermal tissue and sits beneath the skin epidermis. The dermis is composed of collagen and elastin fibres, and home to blood vessels, connective tissue, hair follicles, nerve fibres and sweat glands. The dermis is divided between the papillary and reticular dermal layers.

Between the fibrous components of the dermis resides an amorphous extracellular 'ground substance' which encompasses glycosaminoglycans, i.e. hyaluronic acid, proteoglycans, and glycoproteins. The papillary dermis is the superficial layer, sitting beneath the epidermis [Figure 1.4]. This layer contains highly vascular and loose connective tissue. The reticular layer sits deep in the dermis, creating a dense layer of connective tissue that forms the bulk of the dermis. Collagen, specifically Type I (COL1A1) and Type III (COL3A1), is the primary component of the dermis. Elastic fibres are composed of fibrillin microfibrils and elastin, which plays a fundamental role in the maintaining the structural integrity of the dermis via gliding, stretching, and recoiling of its fibres [Green et al., 2014]. Elastic fibres are comprised of two subtypes: elaunin and oxytalan fibres [Cotta-Pereira et al., 1976]. The elaunin fibres are horizontally arranged and reside near the junction of the papillary and reticular dermal layers. The oxytalan fibres are perpendicular elastic fibres that reside within the papillary dermis [Uitto et al., 2013]. The papillary dermis is composed of loose connective tissue, and form papillae that interwine with rete ridges of the epidermis. The reticular dermis is acellular and exhibits a denser meshwork of thicker collagen and elastic fibres than papillary dermis [Falanga et al.,

2014]. The dermis is also home to blood vessels, hair follicles, nerve endings and glands. Within the connective tissue of the dermis there are a variety of cell types including fibroblasts, macrophages, adipocytes, mast cells, Schwann cells, and stem cells [Prost-Squarcioni et al., 2008]. The dermis is integral in protecting the body from mechanical injury, binding water, aiding in thermal regulation, and encompasses receptors for sensory stimuli [Chu et al., 2008]. The dermis interacts with the epidermis in the morphogenesis of the dermal-epidermal junction (DEJ), epidermal appendages, and in repairing and remodelling the skin in wound healing.

Dermal fibroblasts

The dermis is home to several types of cells but primarily possess fibroblasts, papillary and reticular fibroblasts. Papillary fibroblasts present a spindle-shaped morphology, whereas reticular fibroblasts are characterised by a flattened appearance and possess a higher percentage for cells expressing myofibroblast marker α-smooth muscle actin (α-SMA) [Janson et al., 2012]. Fibroblasts are terminally differentiated into myofibroblasts upon skin injury, cells important for the inflammatory response to injury, and undergo apoptosis once they have competed remodelling of the extracellular matrix [Baum et al., 2012; Hinz et al., 2020].
As the principal cell of the dermis, fibroblasts are imperative for the formation of the connective tissue, the fibrous cellular material that supports and connects tissues or organs to the body [Figure 1.4]. Fibroblasts secrete collagen proteins, that maintain the structural framework of tissues with proteoglycans, elastin, fibronectin, microfibrillar proteins and laminins, forming a ‘matrisome’ [Pilkus et al., 2022]. Fibroblasts are responsible for remodelling the extracellular matrix microstructure via covalent cross linking, protein glycosylation and controlled proteolysis through the secretion of modifying enzymes such as lysyl oxidase, matrix metalloproteinases (MMPs) and MMP inhibitors [Lu et al., 2011].

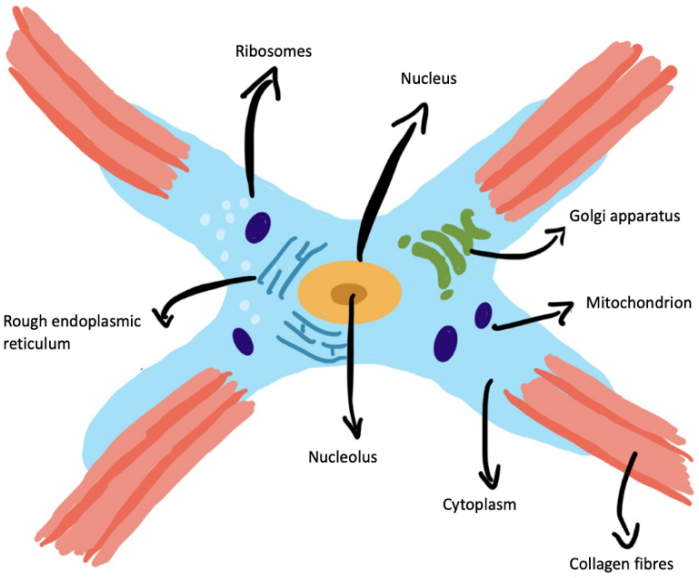


Figure 1.4: The phenotype of a fibroblast cell. Fibroblasts are the predominant stromal cell type in soft connective tissue. They present as plump spindle shaped or stellate shaped cells (active myofibroblasts) with a centrally round nucleus. Fibroblasts are characterised by the expression of COL1A1, and mesenchymal cellular markers like vimentin. Fibroblasts lack the molecular markers found in other cell lineages.

The hypodermis encompassess connective tissue and blood vessels

The hypodermis is the deepest layer of the skin [Figure 1.5] which lies beneath the dermis [Yousef et al., 2022].

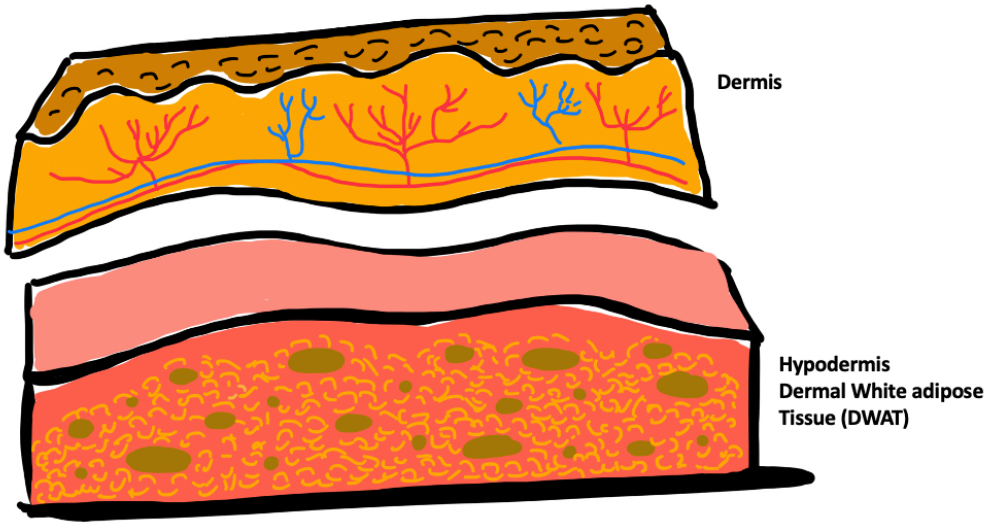


Figure 1.5: The hypodermis structure. The hypodermis is derived from the mesoderm and is the deepest portion of the skin. The hypodermis is composed of adipose lobules, but has within it sensory neurons, and blood vessels. The DWAT sits on the fascia muscle.

The hypodermis, also known as dermal white adipose tissue (DWAT) is comprised of adipose lobules and bursa (lubricant sac that protects bones from rubbing against tendons, muscles, or

skin). Within the hypodermis is the blood vessels, neurons, and lymphatic vessels. The base of hair follicles sits in the adipose tissue of the hypodermis [Figure 1.5]. The hypodermis is essential for storing energy via adipocyte production, in providing insulation to the body, and in protecting the body from harm through shock absorption [Kim et al., 2023].

Epithelial Mesenchymal signaling between keratinocytes and fibroblasts

It is well known that in healthy skin, keratinocytes maintain skin homeostasis by actively cross-talking with fibroblasts. Therefore, if the mesenchymal signalling network between both cell types is affected this will disrupt the delicate physiological balance of the skin. Majority of studies that explore the interaction between keratinocytes and fibroblasts, have identified that in physiological conditions keratinocytes in fact stimulate fibroblasts through the release of interleukin 1, in turn inducing keratinocyte growth factor (KGF) and metalloproteinases (MMPs) in fibroblasts [Russo et al., 2020]. MMPs are important in remodelling the ECM during morphogenesis, repair, and angiogenesis [Chang. 2023; Cabral-Pacheco et al., 2020]. It is important to note that although most MMPs are secreted proteins, there is also a subgroup of membrane-type (MT)-MMPs that form a subgroup of the MMP family, which are subclassified into type I transmembrane type and glycosylphosphatidylinositol-anchored type MMPs [Itoh. 2015]. Keratinocytes in fibrotic tissue, including hypertrophic scars, keloids, and scleroderma, have been shown to augment the ECM deposition [Russo et al., 2020]. Keratinocyte proliferation, differentiation, and production of soluble mediators in response to fibroblast signalling has shown that fibroblasts regulate keratinocyte viability, proliferation, and differentiation [Russo et al., 2020]. This further reinforces the epithelial mesenchymal cross talk between keratinocytes and fibroblasts in the human skin.

1.1.2 Wound healing and skin cells.

After injury to skin, wound healing is a physiological response to injury that culminates in wound closure. [Goodell & Rando. 2015] and inflammaging [Carrasco et al., 2022]. The healing capacity of human skin declines drastically with age, disproportionately affecting old vs young individuals [Ding et al., 2021]. Wounds are categorized into two types, acute and chronic, chronic wounds entail pressure ulcers, venous ulcers, and diabetic foot ulcers, none of which arise due to UV as they present on regions unexposed to the sun i.e legs, buttocks region [Bowers et al., 2020].

Normal wound healing encompasses four highly programmed phases: haemostasis, inflammation, proliferation and remodelling [Figure 1.6].

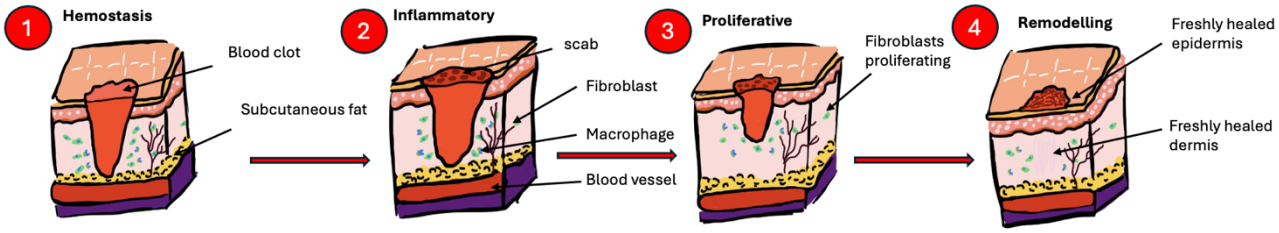


Figure 1.6: Stages of wound healing. Healing begins with haemostasis, followed by the inflammatory phase, the proliferative phase and lastly the remodelling phase.

Haemostasis is immediately initiated following wounding [Guo et al., 2010]. To prevent a haemorrhage, the injured micro vessels constrict, the coagulation cascade is initiated, platelets aggregate, and a fibrin clot, which acts as a provisional matrix, is formed, essentially forming a temporary seal of the wound area [Sgonc and Gruber. 2013]. This prevents further blood loss [Gosain and Dipetro. 2004]. An abundance of secretory factors is released to promote migration of inflammatory cells to the injury site, i.e. Transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblasts growth factor (FGF), and epidermal growth factor (EGF). Once bleeding is controlled, the inflammatory phase is initiated. The inflammatory phase is depicted by the successive infiltration of neutrophils, monocytes, and lymphocytes [Gosain and DiPietro. 2004; Broughton et al., 2006; Campos et al., 2008]. Neutrophils clear invading microbes and cellular debris in the wound area. Macrophages release cytokines that promote the inflammatory response by recruiting and stimulating additional leukocytes. Macrophages are also important for clearing apoptotic cells i.e. neutrophils to bring the end to the inflammatory phase.

Next is the proliferation phase, which follows the replacement of the provisional fibrin matrix with the granulation tissue, beginning 72 hours upon injury and lasts on average for 14 days. This phase can overlap with the inflammatory phase and entails epithelial proliferation and fibroblast migration over the provisional fibrin matrix within the wound, essentially re-epithelisation. It is important to note that epithelisation is a critical aspect of successful wound closure as a wound cannot be healed in the absence of re-epithelisation. Endothelial cells and fibroblasts support capillary growth, collagen formation and the formation of granulation tissue at the injury site. Within the wound site, fibroblasts generate collagen, glycosaminoglycans, and proteoglycans to synthesises the ECM. After 8 days (depending on size of wound and healing speed which can vary from person-to-person), the wound-healing process transitions

to the remodelling phase and can persist for 1 year or even longer. During this phase, collagens form cross linking matrixes, and is critical for restoring the architecture of the normal tissue. The wound undergoes physical contraction throughout the complete wound-healing process mediated by the contractile fibroblasts, myofibroblasts that are present in the wound [Gosain and DiPietro. 2004; Campos et al., 2008] This final stage of the wound healing process is critical for the restoration of full tissue functionality.

Disruption of the strict regulation of the phases [migration, proliferation, phagocytosis, and synthesis of the extra cellular matrix (ECM)] [Sgonc and Gruber. 2013] of wound healing, impacts numerous cell types: cytokines, chemokines, growth factors and proteolytic enzymes [Sgonc and Gruber. 2013] thus leading to delayed repair response and eventually impaired healing [Ding et al., 2021]. Impaired healing drives chronic wounds, infection, pathological scarring, and in some cases cancer formation, leading to morbidity. Morbidity associated with delayed wound healing confers an immense social and financial burden on the healthcare system [Ashcroft et al., 1995; Guest et al., 2017]. As human skin acts as the interface between the outside world and internal organs, it plays an imperative role in defending from external damage. Maintaining efficient wound repair is critical for survival and is obtained via the orchestration of several pivotal cellular and molecular events in a temporal sequence.

The role of Keratinocytes in wound Reepithelisation

Keratinocytes serve a fundamental role in the restoration of tissue through reepithelization [Raja et al., 2007; Gilchrest et al., 1983]. Reepithelization is the process by which new epithelial cells will migrate and proliferate to repair a wound area, thus restoring skin integrity [Raja et al., 2007]. The reepithelization process is impaired in all types of chronic wounds [Pastar et al., 2014]. Keratinocytes are essential for conducting reepithelization of an injury site, essentially restoring the epidermis. Several modulating factors i.e. integrins, cytokines, keratins, metalloproteinases (MMPs), chemokines, and the extracellular matrices regulate keratinocyte migration during wound closure. Moreover, microRNA and histone modifications are also important for re-epithelisation.

In addition to reepithelization, keratinocytes synthesise antimicrobial peptides that can directly kill the invading pathogens and contribute to wound repair in various ways [Piipponen et al., 2020]. In fact, keratinocytes actively cross talk with immune cells during wound repair, mediated via the secretion of several signalling proteins including cytokines, chemokines, and

extracellular vesicles. During the first line of defence, keratinocytes sense the pathogen-associated molecular patterns (PAMPs) i.e. lipopolysaccharides (LPS), endotoxins, or viral-derived nucleic acids, are detected by pattern-recognition receptors (PRRs) [Chen et al., 2018]. Keratinocytes express many types of PRRs, including toll-like receptors (TLRs), C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors, and retinoic-acid inducible gene I (RIG-I)-like receptors [Lebre et al., 2007]. The ligand recognition via PRRs stimulate the downstream activation of specific signalling pathways and the production of inflammatory cytokines, chemokines, and host antimicrobial molecules [Jiang et al., 2020].

Once released from keratinocytes, these pro-inflammatory molecules are critical in activating the skin-resident immune cells and recruiting circulating immune cells to the wound site [Chen et al., 2018]. The termination of the wound healing process requires a delicate balance between the ECM disposition and degradation. To preserve this fine balance, efficient information exchange between keratinocytes and fibroblasts must be maintained. Disrupting the epithelisation during the process of wound healing enhances the frequency of developing chronic wounds [Huang et al., 2013; Zhu et al., 2016]. There is strong evidence that suggests that keratinocytes activate fibroblasts to synthesize growth factors, which in turn stimulates keratinocyte proliferation in a double paracrine manner [Werner et al., 2007; Amiri et al., 2021].

The Role of Fibroblasts in Skin Wound Healing

Double paracrine communication regulates fibroblasts during the healing process. Following injury, fibroblasts are stimulated to release ECM factors such as collagen which mediate tissue repair. Fibroblasts tend to remain quiescent until tissue repair is induced. To undergo tissue repair, quiescent cells are transformed to myofibroblasts, which through the production of contractile expression proteins i.e. αSMA, can drive biochemical remodelling as well as skin contraction via traction force [Pakshir et al., 2019]. As the healing process proceeds, fibroblasts transform to a myofibroblast phenotype under the control of keratinocytes via a transforming growth factor (TGF)-β-dominated environment [Werner et al., 2007]. Fibroblasts switch from ECM synthesis toward an increase in MMP release thus inducing degradation and tissue remodelling, all mediated by keratinocytes. In addition, the fibroblast growth factor (FGF)-2 can accelerate wound closure by stimulating vascular endothelial cells and fibroblasts [Koike et al., 2020]. Once the wound is fully re-epithelised, mature keratinocytes can reduce ECM

synthesis and increase its degradation via the release of various factors i.e. MMP-1 expression in fibroblasts, or downregulating COL1A1 and COL3A1 in fibroblasts [Amiri et al., 2021].

1.1.3 Heterogeneity in human skin

Skin heterogeneity can be affected by various factors including body site, chronological age, or skin pigmentation. I will discuss how each factor influences skin identity in this following section.

Skin varies in thickness depending on body site

The thickness of each layer can vary based on the body region and is classified according to the thickness of the epidermal and dermal layers [Yousef et al., 2021]. The hairless palmoplantar skin of palms and soles (also known as volar skin) of the hands and feet are the thickest regions and encompasses an additional layer of cornified stratum lucidum (3 cells thick) [Yousef et al., 2022; Vela-Romera et al., 2019; Boyle et al., 2019]. The upper back is also considered one of the thickest regions due to the thick dermal layer, though in comparison to palmoplantar tissue is characterized as histologically thin not only due to the absence of the stratum lucidum but notably because of a thinner stratum corneum [Yousef et al., 2022; Barbieri et al., 2014]. Moreover, plantar skin possesses a unique composition and structure of keratin filaments with a high content of KRT1 and KRT10 intermediate filaments for example that provide structural support and strength to skin [Egelrud and Stigbrand. 1989; Swensoon and Eady. 1996; Swensson et al., 1998] Recently, a porcine model has showcased the distinct properties of plantar skin from non-plantar skin [Fuchs et al., 2024]. For example, plantar tissue can present hypertrophic keratinocytes, spongiosis, flattening of the epidermal calcium gradient, downregulation of stromal interaction molecule 1 (STIM1), differentiation of KRT14 in the suprabasal epidermis, aggregated keratin filaments and reduction in KRT10 in stress keratins [Fuchs et al., 2024].

Plantar skin is considered a highly specialised skin type with a complete lack of hair and sebaceous glands, and an accelerated epidermal turnover rate as skin is sloughed from the palms 35 times faster than nonplantar skin [Golschmidt and Kligman. 1963]. Due to the thickened stratum corneum of plantar tissue, it is a 100-fold less effective permeability barrier than nonplantar tissue [Baker and Kligman. 1967; Blank. 1953]. Essentially as the plantar stratum corneum is thicker, it holds higher water content, making it less permeable than the thinner and drier nonplantar stratum corneum. Plantar skin has adapted to stresses and injuries

by stimulating a series of injury response phenotypes. For example, the stress keratins present in plantar tissue are anti-proliferative and anti-migratory thus conferring increased mechanical resilience to withstand physical pressures within a wound environment but with delayed epithelisation [Rotty and Coulombe. 2012; Wawersik et al., 2001; Wong and Coulombe. 2003]. These features of plantar tissue confer heightened mechanical strength, as the skin type can withstand pressure 50 times that of other load-bearing locations of the body [Gyi et al., 1999; Crossland et al., 2024]. Notably, plantar skin deforms much less under load than nonplantar skin [plantar skin deforms 1.6-fold less than nonplantar skin] [Boyle et al., 2019]. Therefore, pressure ulcers, characterised as tissue necrosis, arise in non-plantar tissue exposed to considerable deformations because of a series of overlapping pathways such as ischemia, reperfusion, poor lymphatic drainage, and direct cell deformation [Bouten et al., 2003]. As plantar skin is accustomed to high mechanical loads, pressure ulcers rarely form in this skin type. Pressure ulcers have been found to severely affect the quality of life of its patients [Roussou et al., 2023] with Grade four pressure ulcers presenting a high risk of progressing into a life-threatening infection [Bhattacharya and Mishra. 2015].

Regionally, keratin composition within the human plantar skin is heterogeneous in keratin composition, with the presence of friction ridges. The epidermis underlying the main body of each friction ridge expresses the plantar marker KRT9, and the differentiation markers KRT1/10 but no KRT6/16. Within the furrow between friction ridges there is a substantial reduction in differentiation-associated markers KRT1, KRT9 and KRT10 while stress keratins KRT6/KRT16 are specifically expressed in these regions [Egelrud and Stigbrand. 1989; Swensson et al., 1998; Schweizer et al., 1989;]. Moreover, the basal marker KRT14 also presents similar friction-ridge based variations, with irregular persistence in the suprabasal epidermis, only in the secondary ridges.

Single cell RNA sequencing data has revealed the distinct keratinocytes and fibroblast populations in plantar tissue [Wiedemann et al., 2023]. The expression of KRT9 in the suprabasal layers of the palmoplantar epidermis has been identified to be required for maintaining the structural integrity and terminal differentiation of the skin type [Fu et al., 2014]. Fu et al., found that KRT9 deficient mice developed calluses marked by hyperpigmentation and are exclusively localised to stress0bearing footpads. The authors reported that the complete ablation of KRT9 is not tolerable in vivo and K9 is needed for terminal differentiation and for preserving the mechanical integrity of palmoplantar epidermis

[Fu et al., 2014]. Notably, the thickened epidermal phenotype of palmoplantar skin can be achieved via co-culture of keratinocytes with palmoplantar fibroblasts [Yamaguchi et al., 1999]. This epidermal-mesenchymal signalling enhances the expression of KRT9 of keratinocytes. Moreover, it has been found that within plantar tissue there is a significant lack in basal keratinocytes which express KRT14 with differentiated KRT9 expressing keratinocytes being markedly abundant [Wiedemann et al., 2023].

Transforming nonplantar to plantar skin

My lab has previously proposed that plantar fibroblast implantation could protect amputees from skin injury [Boyle and Higgins. 2021] by inducing expression of plantar-specific KRT9 in the epidermis. KRT9 is a type I intermediate filament protein which is specifically expressed in the suprabasal layers of the thick palmoplantar epidermis [Fu et al., 2014; Boyle et al., 2019; Knapp et al., 1986; Swensson et al., 1998], therefore, it is perceived as a differentiation marker of palms and soles [Yamaguchi et al., 1999]. KRT9 has been identified as an essential protein for terminal differentiation and for ensuring mechanical integrity of palmoplantar epidermis [Fu et al., 2014; Kim et al., 2016]. Extrinsic KRT9 regulation is signaled from dermal fibroblasts via epithelial mesenchymal signaling [Yamaguchi et al., 1999] and therefore expression can be induced via co-culture of keratinocytes with palmoplantar fibroblasts [Boyce et al., 2016]. The Wnt5a signalling protein, released from fibroblasts, can induce expression of KRT9 in keratinocytes, a foot skin-specific keratin, in keratinocytes [Rinn et al., 2008].

Skin pigmentation influences skin health and ageing.

Ethnicity confers immense effects on skin health and ageing via variances in pigmentation [Farage et al., 2008]. High levels of pigmentation elicit protective effects against photoaging, with African Americans showcasing little cutaneous difference between UV exposed and unexposed sites [Robinson. 1999; Chien et al., 2018; Young et al., 2010]. Moreover, African American skin exhibits reduced sensitivity to skin cancer incidence which can be equated to 500-fold level protection from UV radiation [Rees. 2004]. African American skin is more dense than Caucasian skin, with increased intercellular lipid content, which likely contributes to heightened resistance to ageing [Robinson. 1999; Vashi et al., 2016]. In addition, wrinkling in Asian skin is delayed and less severe in comparison to Caucasian skin, although, the reasons for this have not been widely explored [Martini. 2004; Sudel et al., 2005]. Skin heterogeneity

regarding skin pigmentation and melanocytes will be discussed more extensively in chapter 4 of this thesis. Chapter 4 is a review written during my PhD.

Chronological ageing alters skin integrity.

Internal factors that influence skin integrity and function include hormones and genetic factors i.e. mutation in the WRN gene in Werner syndrome, a progeria disease characterised as premature ageing [Sinha et al., 2014] as well as specific conditions i.e. diabetes. Moreover, chronological age alters skin integrity, as age-related epidermal changes encompass a series of cellular morphological and functional changes [Figure 1.7].

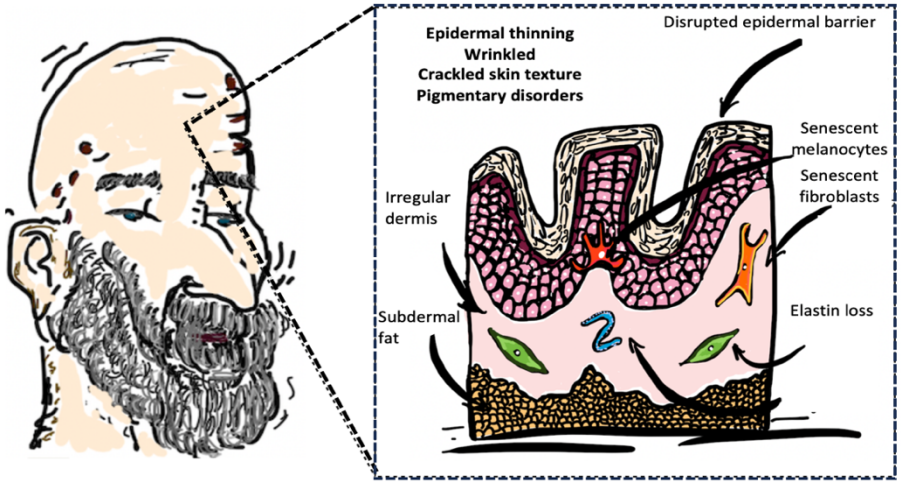


Figure 1.7: Phenotypic changes in skin with old age. Over time, skin epidermis becomes thinner, with flattened DEJ junctions, skin wrinkling, elastin, and collagen loss.

This includes a decrease in the number of Langerhans and melanocyte cells, flattening of the dermal-epidermal (DEJ) junction as well as reduced keratinocyte proliferation [Sgonc and Gruber. 2021] [Figure 1.6]. Ageing of skin influences thickness as epidermal thinning occurs over time due to reduced cell proliferation, changes to the ECM and via external damage, largely driven by prolonged chronic UVR exposure [Lopez-Ojeda et al., 2022]. Notably, the turnover time (the number of days for keratinocytes to migrate from the basal layer to the skin surface) is increased by 50% in aged skin, meaning that though the overall epidermis is thinner in old skin, the stratum corneum is in fact thicker [Sgonc and Gruber. 2013].

UVA drives ageing of human skin

Whilst chronological ageing can influence skin integrity, biological age can also have an impact. External factors that affect biological ageing includes sun exposure, smoking, and air

pollution [Vierkötter and Krutmann. 2012; Parrado et al., 2019]. Air pollution is of significant interest as in 2018, the World Health Organisation (WHO) reported that nine out of ten people breathe high levels of air pollutants [World Health Organisation. 2018]. In 2016, the deaths per year, because of ambient air pollution, were reported to reach 4.2 million. Sustained exposure to the sun triggers the formation of free radicals.

Ultraviolet radiation

Ultraviolet (UV) radiation is the most physiologically relevant stimuli that drives ageing of human skin [Figure 1.7]. It is an environmental stimulus that every individual is habitually exposed to, irrespective of location or demographic, whether the region is seasonal or tropical. In addition, climate change is causing striking increases in UV radiation which means its detrimental effects on human skin will only heighten in coming decades. Sustained exposure to the sun and thus UV triggers the formation of free radical which destroy the skin's collagen, leading to wrinkling, dark spots, fine lines, and loose saggy skin [Poijšak et al., 2012].

The most damaging and age accelerating external factor to human skin is UV which consists of three subtypes: UVA, UVB and UVC [Figure 1.8]. These rays differ in the length of their respective wavelengths. UVA possesses the longest wavelength from 315 to 400 nm, however UVB is most potent at 280-315 nm and UVC is the shortest from 200-280 nm [Rünger et al., 2007; Sanches Silveira. 2014; Bosch. 2015]. UVC is completely filtered by the stratospheric ozone layer, UVB is majorly filtered at up to 90%, and UVA is partially filtered at 50% [Gruijl and van der Leun. 2000]. UV can be regarded as a double-edged sword in that it elicits both benefits and detriments to human health.

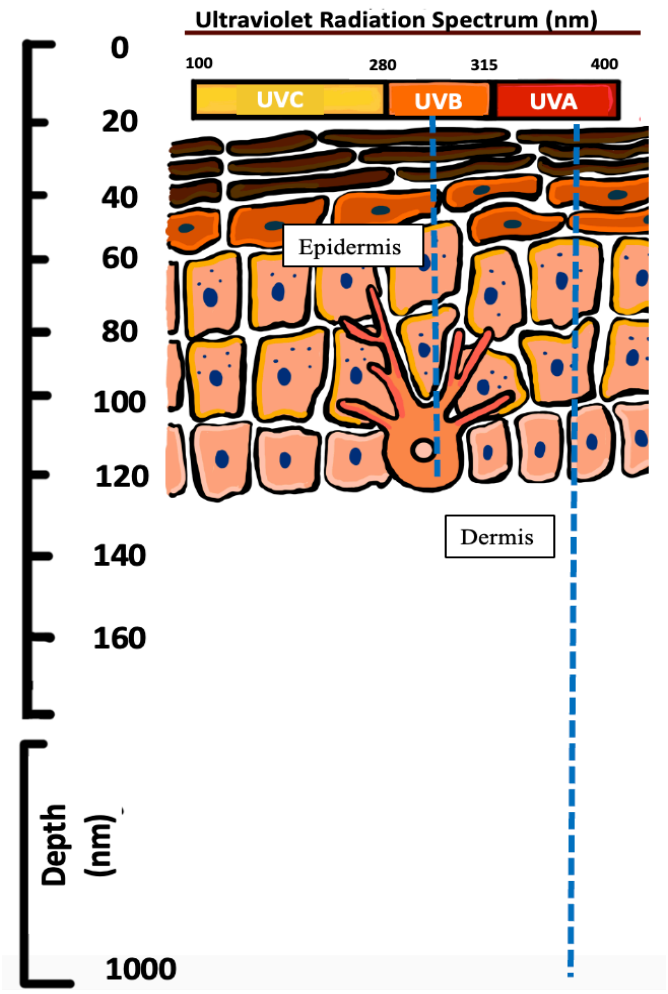


Figure 1.8: Penetration effects of UV on skin. UVA penetrates the dermal layer of the skin via its wavelength of 315-400 nm (blue line) whereas UVB penetrates the epidermal layer via a wavelength of 280-315 nm (blue

In moderation, short term, consistent exposure to UV is largely beneficial and necessary for human health for two main purposes. Firstly, it is needed to produce melanin, which in turn elicits protective effects for cells against UV exposure by filtering the rays [Brenner et al., 2008]. In addition, UV exposure is the primary mode of elevating serum vitamin D levels which possess numerous health benefits. UV stimulates the production of the pre-vitamin D from 7-dehdroxycholesterol [Erem et al., 2021]. Keratinocytes are important for modifying the calcium absorption via the stimulation of the cholesterol precursors by UVB light to form vitamin D. Vitamin D deficiency is implicated in many known diseases, specifically in type 2 diabetes and chronic kidney disease (CKD).

UVA radiation modifies the activity of epidermal keratinocytes and dermal fibroblasts and induces long term changes. The key result from photoaging is less dermal fibre expression, and the formation of reactive oxygen species (ROS) which damage the skin collagen and elastin [Gromkowsak-Kępka et al., 2021]. Notably, photoaged skin is associated with a significant loss in mechanical resilience, and skin elasticity [Langton et al., 2019]. Photoaged skin (young and old) has been shown to present flattening of rete ridges and disrupted arrangement of elastic fibres. This highlights the impact sun exposure, specifically UVR, has on the biochemical properties of human skin as alterations in skin integrity induces functional decline. A characteristic feature of photoaged skin is solar elastosis within the dermis. Elastoses are a dystrophic elastic material and upon degradation of the elastic fibers via ROS, solar elastosis can arise. Prolonged UVR exposure can cause significant DNA damage; UVA and B induce

cyclobutene pyrimidine dimers (CPD), and 8-hydroxy-2-deoxyguanosine (8-OHdG) is induced by UVA only, which is a common marker for the estimate of DNA damage from UVA and is implicated in premature ageing syndromes [Hussain et al., 2021; Wlaschek et al., 2001; Gromkowska-Kępka et al., 2021].

With depleting ozone layers, UV penetration of the Earth's surface is expected to continue to rise immensely in coming decades. In fact, scientists from the National Aeronautics and Space Administration (NASA) have identified a striking increase in UVR levels across the last 30 decades [Herman. 2010]. Therefore, as this physiological stimulus continues to increase in the future, UV driven disease and effects on skin, will also likely continue to rise, and with population ageing UV will detrimentally affect this pool of people, the elderly, due to intrinsic changes in their skin integrity.

1.1.4 The world is ageing

Population ageing refers to the increase in the age composition toward an older generation [Land et al., 2008]. By the year 2050 the world's population is predicted to succeed 9.7 billion people [United Nations. 2017] with almost 2 billion people living over the age of 65 [United Nations. 2022]. The United Nations (UN) has estimated that the global population will rise from 8 billion from the year 2022 reaching 10.4 billion by the next century [Roser and Rodés-Guiro. 2019]. However, the global population growth rate is declining from 2% per year 50 years ago to slightly under 1.0% per year at present [Roser et al., 2019].

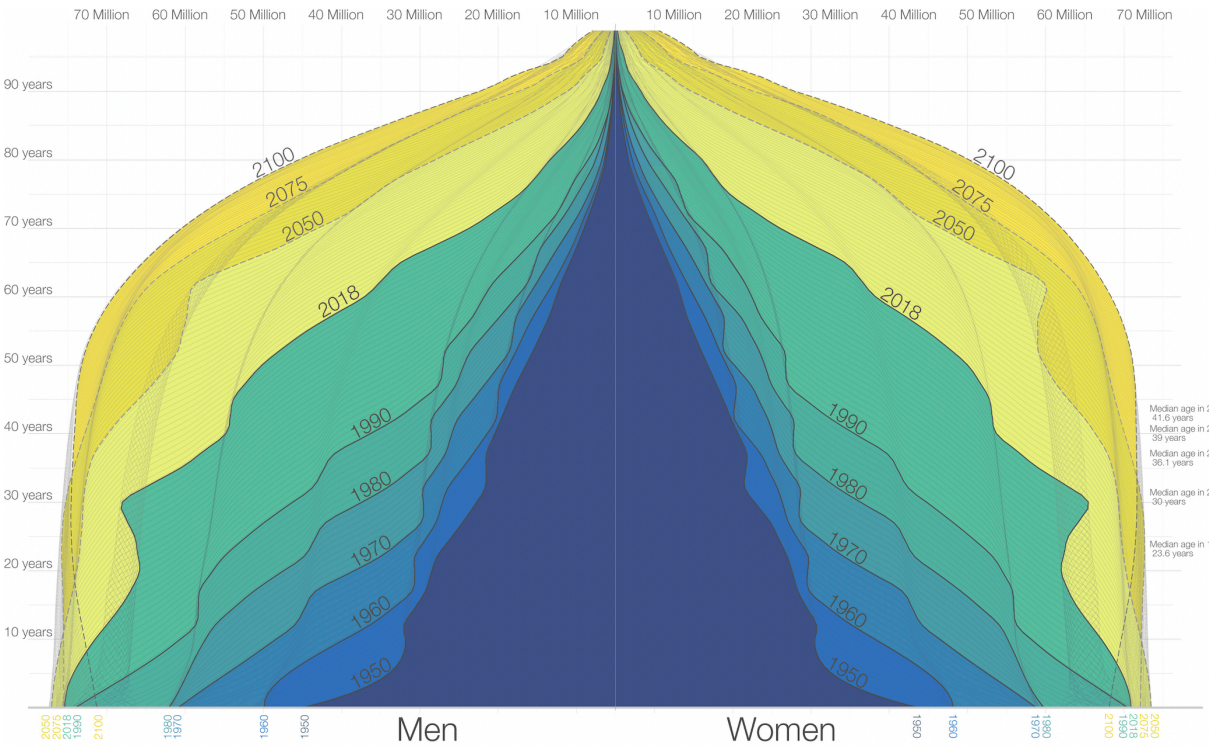


Figure 1.9: Global population ageing. The demography of the world population from 1950-2100. Dark blue presents global population in 1950. Shades of blue and green represent the world population structure over last several decades up to 2018. Yellow presents predicted global population age up until 2100. The 'fill up' of the population above the base, indicates fertility rates declining but old age substantially increasing. Image extracted from [Ritchie and Roser. 2019].

A large proportion of the western hemisphere is undergoing a demographic shift toward a majority middle-aged or elderly population [Figure 1.9]. However, there is disparity in the population distribution from country to country, particularly within developing vs developed countries. The United States (US) straggles behind many developed nations in terms of health and longevity e.g. the increase in life expectancy, observed in the last several decades, have not been on par with other developed countries. Notably, since 1980, the US has, on average, amassed five years in life expectancy from birth, whilst comparable nations have amassed eight [Rakshit et al., 2022]

In the United Kingdom (UK) it is estimated that there are 11.8 million people over the age of 65 years old, constituting up to 18% of the population [Office for National Statistics. 2018]. By 2030, it is projected that 1 in 5 people in England will be over the age of 65 years old. The UN has projected for rapid world population growth to come to an end by 2100 [Roser and Rodés-Guiro. 2019]. Notably, Bangladesh is the most densely populated region with around 1,315 people per square kilometre: three times as dense as India. This is followed by Rwanda (558), Lebanon (537), South Korea (531) and the Netherlands (522) making the top five most

densely populated countries [Roser et al., 2019]. There are immense regional differences in ageing patterns, for example in North America the median age adjusted for life expectancy has declined throughout a large part of the century [Lutz et al., 2008]. Japan is home to a largely elderly population and is accelerating toward a super aged society. Several countries with the Middle Eastern and North African (MENA) region have previously possessed majorly young populations but are now in the emerging stages of a demographic transformation, in fact Saudi Arabia, a part of the G20, has already begun investing in policies to regulate this future challenge [Salam et al., 2023].

Recently, in Iran a qualitative content analysis study on male and females was performed to implement strategies of the Iranian health system in dealing with future ageing [Samouei and Keyvanara. 2022]. One study has implemented an Ageing Society Index, to identify countries that are being productive in policy as society ages [Goldman et al., 2018]. The index has revealed that the United States (US) lags in equity and longevity, that Japan has the longest life expectancy, Germany currently has the same age distribution as the US will have in 2030, the Nordic countries have strong social support systems in place, and Spain has implemented innovative pension reforms.

Implications of an ageing population

The rate at which the global population age is increasing is of crucial importance to policymakers attempting to navigate the complex issues of pensions and healthcare of today and tomorrow's climate [Lutz et al., 2008]. Through studying trends in population age one can anticipate any disruptive changes where adjustments would need to be made. For example, a key concern with population ageing is accommodating the number of persons requiring extended care because of disability which would likely heighten in all countries. Therefore, it is also important for the national healthcare system (NHS) to promote healthy ageing and for research to be carried out into how that can be done efficiently and effectively, to thus ameliorate age-related disorders that become present and can be exacerbated in later years of life. Moreover, as the world undergoes this immense demographic change toward population ageing, it is imperative that global policymakers ensure economical plans are put in place in preparation for the impact this will have on fiscal balance, investment savings, labour supply, and the welfare system [Yoshino et al., 2019].

A population with an extended life expectancy reflects many positive points about a region's infrastructure and society. Ultimately a long-life expectancy signifies an advanced economy, and sufficient health care systems leading to reduced mortality rates which are allowing for longer living. In the context of epidemiology, once a region begins experiencing reduced infant mortality and birth rates, that is when the population begins to stagnate at an older population. This is occurring in many parts of the developed world and is largely attributed by more women progressing in their careers and thus leading to delayed marital age, or reduced birth rates.

Elderly are commonly referred to those reaching retirement age, typically >65 years old [Tinker. 2002]. It is important to acknowledge and understand how social attitudes will change or vary as populations shift toward an elderly pool of people. Notably, as countries become richer, the burden of diseases associated with wealth will become more prevalent which includes smoking and obesity in developing regions [Tinker. 2002]. Age discrimination, also known as 'ageism', is a growing concern particularly within public health forums and areas of employment [Bytheway. 1995; McEwen. 1990; Walker and Maltby. 1997]. A major social implication of an ageing society is on the labour force as the size of the population that are of working age will shrink. This will impact pensions and benefit schemes for those who have retired, thus raising concerns of intergenerational fairness. This will see a change in the transfer of resources from old to young in inheritance to the other way. In terms of family dynamics intergenerational links may be disrupted with an ageing population as social support systems become more of a necessity to enhance the ability of families to accommodate an older person.

Overall, a demographic shift toward an elderly population has positive reflections in terms of signifying an improved economic infrastructure. Notably, as mortality rates decline and fertility rates increase, a growing population toward the elderly will be the likely outcome. The global transition to an aged society confers severe financial obstacles to every institution across all economies [Ogura and Jakovlijevic. 2018; Cylus and Tayara 2021]. Developed countries are experiencing these challenges despite foreseeing population ageing two decades in advance, struggling to obtain enough money to cover the amassing retirement income and health care costs for the rapidly growing elderly population [Ogura and Jakovlijevic. 2014]. Societal ageing has and will continue to impact and elicit a strain on economic growth, work, retirement rates, familial dynamics, and the capacity for governments and communities to accommodate for the elderly and majorly alleviate the accumulation of chronic disease and disability that arises with an ageing society.

Extending longevity will allow for people to work longer, as an aged population does tend to reduce the functioning workforce of a region which can have immense economic impact. An ageing population that not only lives longer but also healthier will conserve a region's labour force with increased productivity as well as positively influence one's mental health in providing them with a sense of purpose, reducing loneliness and giving more time to build financial security [Lang et al., 2019]. With biomedical advancements, the prospect of extending healthy life span without pathology becomes more and more plausible and achievable. Interestingly, one study explored the extent to which population ageing will be detrimental to the economy through study of the impact of age-specific Years Lived with Disability (YLDs) in 180 countries from 1990 to 2017 on labour force and real per capita gross domestic product (GDP) growth. The authors found that through modelling simulations that an increase in the 55–69-year-old cohort of the total population is associated with a reduction in real per capita GDP growth. However, importantly, this decline in economic growth attributable to population ageing is avoidable through policy interventions that support healthy ageing.

1.1.5 The process of ageing

The very definition of ageing has evolved in recent years, becoming more convoluted in present day. Traditionally, ageing is simply defined as to 'grow old or older'. This infers it to be a natural state, one that all living things are vulnerable to with time. In a biological context the mechanism of ageing is perceived with much more complexity, as a deteriorative physiological process heavily influenced by time. Professor David Sinclair, [Lifespan: Why We Age – and Why We Don't have To. David Sinclair, 2019] a frontier in the biology of ageing field articulately defines ageing as being a state at which an individual is brought to the precipice of a cliff, at which point their susceptibility to several age-related diseases is heightened, ultimately bringing about their downfall from said cliff [Figure

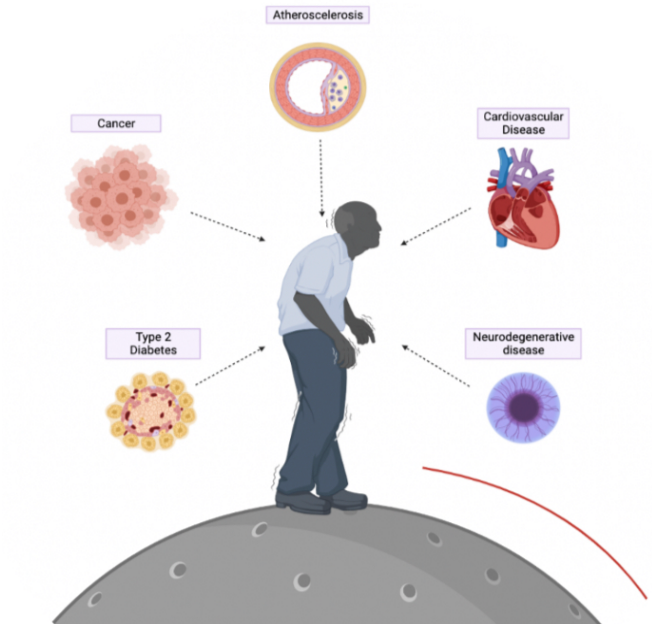


Figure 1.10: Age related disease heightens with age. Schematic presents the rise in age-related disease susceptibility that occurs in old age.

1.10]. Ageing is largely believed to be irreversible however with amassing interest and research in the field the prospect of this process being reversible, or preventative is gaining more and more potential, moving away from what was one a fictitious claim. In 2018, the World Health Organisation (WHO) implemented an extension code that was to be updated to the International Classification of Diseases for 'age-diseases (ICD-11), defined as pathological processes that drive an organism's loss in adaptation, which augments with age [World Health Organisation. 2022]. From January 2022, every country across the globe was initially encouraged to report using the ICD-11 code to diagnose those with 'old age' however, this was withdrawn due to unclear, and erroneous use [Rabbheru et al., 2022]. The terminology has changed to 'Ageing associated decline in intrinsic capacity' [World Health Organization. 2022]. Across the world the way ageing is perceived and tackled is varied though it is continuing to emerge as a global issue.

Overall, rather than aiming for longevity, my focus in this project is promoting healthy ageing, as despite humans living longer, that does not equate to living better. There has been an observed influx of diseases, which continues to amass a burden on the NHS. Therefore, if biological ageing could be regulated or monitored, this could drive healthier ageing, ameliorating symptoms of age-related diseases and lightening the economic burden that longer life expectancy inflicts on the healthcare sector [Menassa et al., 2023; Abud et al., 2022].

Chronological vs biological ageing

To be able to tackle ageing, one must first understand that humans age differently. There is a chronological and a biological age. The chronological age being a measurement of the number of years an individual has been alive, whereas the biological age represents how old an individual's cells and tissues are based on physiological evidence [Figure 1.11].

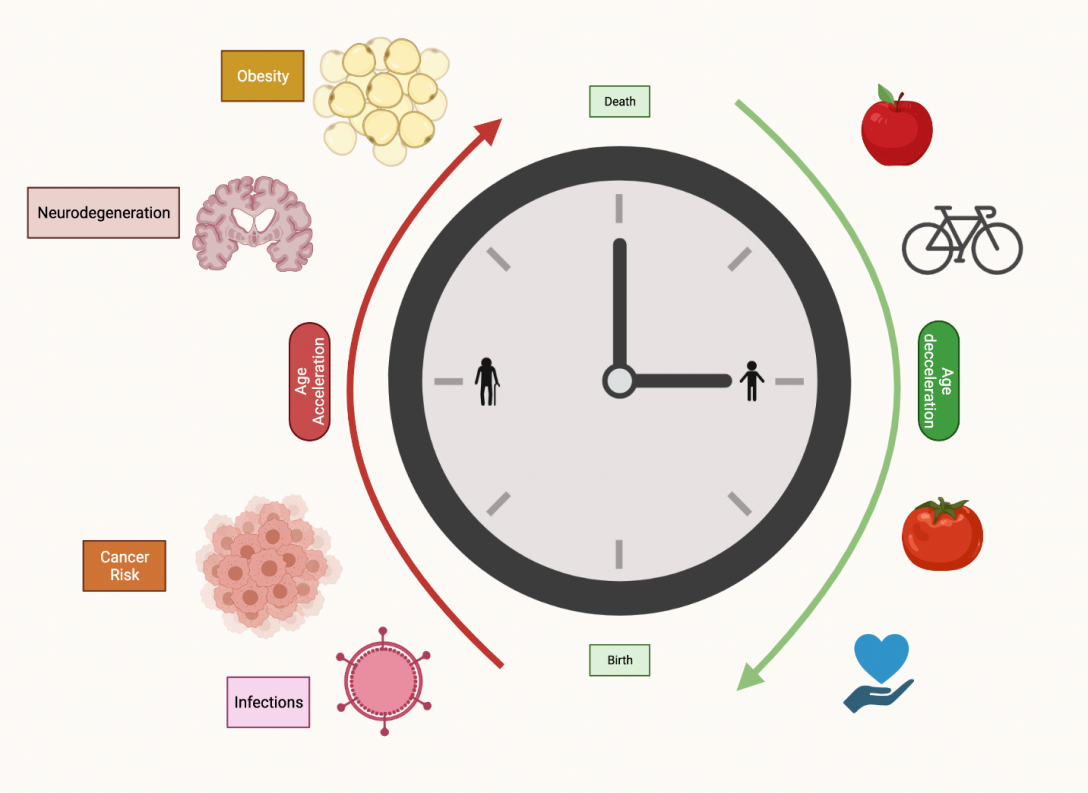


Figure 1.11: Biological ageing clock. The Schematic illustrates the contributing factors that give rise to age acceleration or age deacceleration including genetic and choices in lifestyle.

In simple terms, chronological age can be compared to the year of production of cars whereas biological age is comparable with mileage. Therefore, whereas chronological age will always increase at a linear rate, biological age is variable and differs from individual-to-individual dependent on numerous internal and external factors [Ahadi et al., 2020]. As the world is continually ageing, greater emphasis is being applied on promoting a "sense of well-being", "having good physical health", and "preserving good mental health", via the strong encouragement for an active lifestyle, and implementation of healthy eating habits [Halaweh et al., 2018; Charles and Carstensen. 2014; Hudges and Touron. 2021]. This highlights that healthy ageing is an adaptive process that can be regulated despite chronological age being a progressive linear process. If biological age were to be regulated, disease susceptibility can potentially be assessed therefore, one way to tackle the root of age-related disease is at the root itself which is biological ageing.

Skin as a model of ageing

It is important to note that although skin is heterogenous in nature, presenting variations with ageing, across skin pigmentations, and body sites it remains as a physiological relevant model that presents the first signs of ageing. The skin is the mirror of our inner health, as well as our interface to the world. Visually human age is observed through changes on the skin surface,

which includes the presence of wrinkles, ageing sunspots, skin thinning and skin translucency. With ageing, certain skin conditions become prevalent, including dermatosis, skin tags, warts, seborrheic keratoses (depicted as rough brown patches), actinic keratosis and skin cancers [Blume-Peytavi et al., 2016]. This presents with chronological ageing, the number of years linearly lived by an individual since birth which is the same measure for every person. However, these phenotypic changes can present differently among people of various skin colour which I will discuss more in chapter 4. Particularly, with an ageing world, skin is an interesting and phenotypically representative model for age related changes. Skin is dramatically influenced by chronological ageing as well as external factors i.e. one's lifestyle such as smoking, and diet and notably UVA which everyone is exposed to and accelerates the biological age of the tissue.

1.1.6 Changes to the skin with increasing age

Cellular senescence

Cells are naturally programmed to divide, and multiply as well as implement fundamental biological functions. However, the more that cells divide, the older they get. Several mechanisms are behind human ageing, including metabolism [van Beek et al., 2015], dysfunctional autophagy of misfolded or damaged proteins [Hartl et al., 2011], genomic instability with accumulating DNA damage [Roos et al., 2016; Nicolai et al., 2015], telomere degradation [Aubert et al., 2008], epigenetic changes [Horvath 2013], mitochondrial dysfunction [Vermulst et al., 2008], cellular senescence [Childs et al., 2017], and stem cell exhaustion. Cellular senescence is considered one of the nine hallmarks of ageing [Figure 1.12]. In 1961, Hayflick and Moorhead discovered that primary fibroblasts in culture present diminished proliferative capacity following successive packages [Hayflick and Moorhead. 1961]. This is a result of sequential telomere shortening following each cycle of cell division.

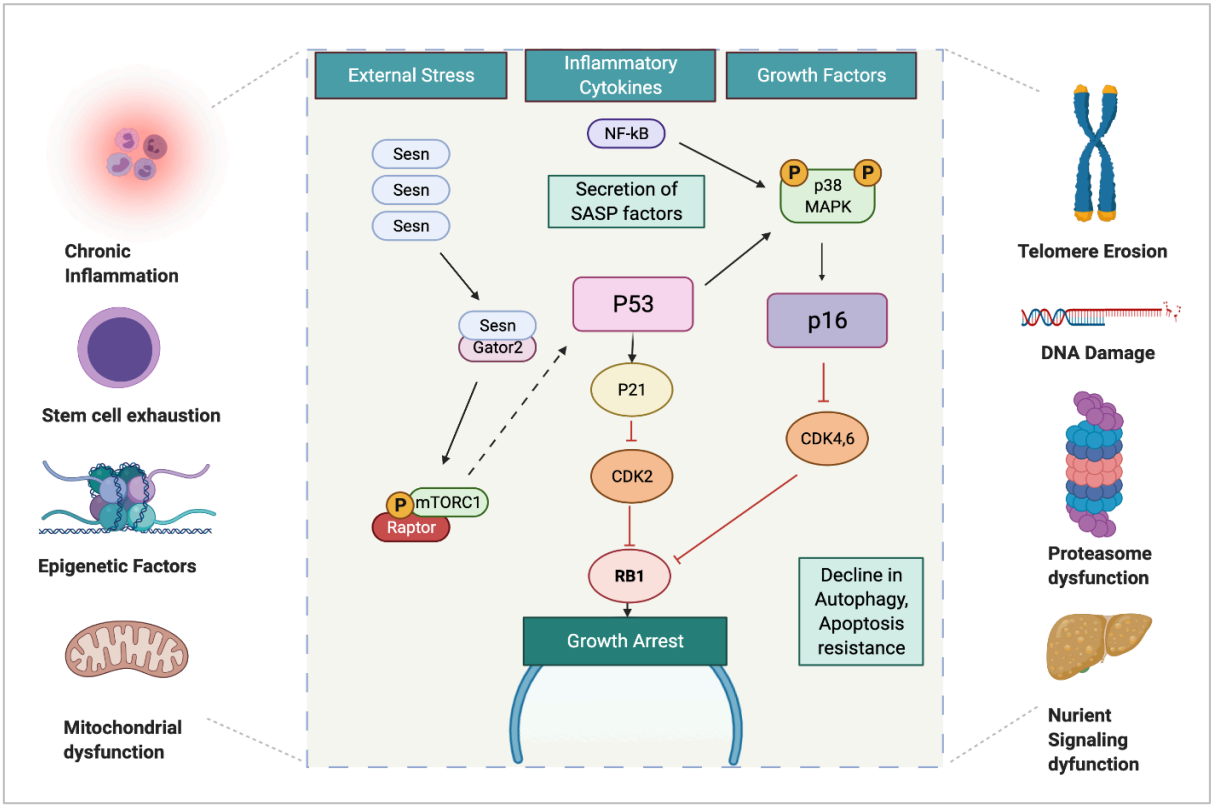


Figure 1.12: Cellular senescence as a hallmark of aging. The diagram illustrates the key pathways involved in inducing cellular senescence notably the p53 pathway. Stress inducible proteins i.e. Sestrin, responds to stress signals from multiple stimuli e.g. DNA damage or mitochondrial dysfunction. This causes the downstream activation of the p53 pathway by overstimulating p21 which inhibits CDK2 and thus causes overactivation of Retinoblastoma-1 (RB1) leading to growth arrest. This leads to senescent stationary state cells that can release senescence associated secretory phenotype (SASP) factors i.e. pro-inflammatory cytokines.

These non-proliferative cells may act as a deterrent for cancer induction however in many cases senescent cells can actively or indirectly induce or promote cancerous cell spreading through the secretion of pro-inflammatory factors that heavily contributes to chronic inflammation, also known as ‘inflammaging’. Senescence is induced by several different external factors ranging from replicative exhaustion [Figure 1.11], genotoxic drugs, ionizing and non-ionizing radiation, demethylating and acetylating agents as well as oxidative stress [Hernandez-Segura et al., 2018]. Senescent cells accumulate in the skin with time and is a physiological indicator of skin ageing and photoaging [Wang and Dreesen. 2018]. With ageing, as senescent keratinocyte cells accumulate this can contribute to the loss of regenerative capacity [Andrade et al., 2022]. Notably, p63 deficiency has been shown to induce cellular senescence in proliferating keratinocytes, giving rise to accelerated aging in vivo, as shown in p63 heterozygous mice [Keyes et al., 2005].

Stem cell decline

Stem cells reside in the basal layer of the skin epidermis. These cells are essential for conserving normal tissue homeostasis and serve a pivotal role in tissue repair and regeneration in response to damage [Alonso and Fuchs. 2003]. Stems cells constitute a small pool of cells within any tissue, and is projected to comprise 2-7% of the basal layer cells [Ahmadi-Ashtiani et al. 2020; Alonso and Fichs. 2003]. Lineage tracing has revealed contrary findings to a proliferative hierarchy of assymetrically dividing stem cells that trasnmit amplifying cells that undergo a fixed number of division before differntiation. Lineage tracing however, has revealed that mean clone size has been found to increase progressively with time [Piedrafita et al., 2020]. With ageing, stem cells lose their renewable capacity as their ability to differentiate into other cell types is majorly impacted [Ahmed et al., 2017; Murai et al., 2018]. The depletion of stem cells with age can lead to a loss in regernative capapcity [Flores et al., 2005; Rando et al., 2006; Sharpless and Depinho. 2007; Rossi et al., 2008; Nishimura et al., 2011; Giangreco et al., 2010]. The deleterious effects of ageing on stem cell function hugely impacts the genome, epigenome, and proteome. Cellular turnover of stem cells (on average in humans, of the order of 20-30 days) in the skin is very high but this declines with ageing (to 40 days) and as a result of external damage [Farage et al., 2013; Alberts et al., 2002].

UV is a major cause of stem cell DNA daamge, driving the depletion of such cells, and initiating damage to the stem cell niche [Panich et al., 2016]. With ageing, epidermal stem cell markers, CD49f and CD34 [Lavker et al., 2000; Kretzschmar et al., 2014; Negri et al., 2022; Tani et al., 2000], and melanoma chondroitin sulphate proteoglycan (MCSP) decline in expression which is associated with reduced rete ridge height, and basal cell density [Giangreco et al., 2010]. Notably, as stem cells are able to proliferate, faciliate tissue repair, and possess the capacity for multidirectional differentiation, stem cell therapy using for example induced pluriopotent stem cells (iPSCs) in human clinical trials [Schwartz et al., 2015; Sundberg et al., 2013; Stoddard-Bennett et al., 2019; Mensaché et al., 2019; Mandai et al., 2017], mesenhchymal stem cells, or embryonic stem cells though controversial due to the ethnical concerns, have been utilised to improve skin texture, maintain skin rejuventation, and regress the ageing process [Hoang et al., 2022; Chang et al., 2014; Liu and Rando. 2011].

Delayed healing in aged skin

Increased cellular senescence, and reduced stem cell numbers associated with increased ageing contribute to declining skin function. Therefore, as skin ageing is associated with a decline in tissue integrity, function, and regenerative function, chronic wounds are more likely to present in the elderly vs the young as supported by considerable clinical and experimental findings [Ashcroft et al., 2002, 1997a; Goodson and Hunt, 1979]. Changes in wound healing with age have also been presented. With the elderly exhibiting a slower healing process, they notably present a slower healing process with all phases of wound healing being impacted [Gerstein et al., 1993]. This includes a delay or reduced inflammatory and proliferative response, delayed angiogenesis, delayed tissue remodelling and slowed epithelisation. Wound healing in aged skin is associated with prolonged inflammation, impaired immune cell signalling and function, enhanced cellular senescence, as well as delayed re-epithelisation, re-vascularization, granulation tissue formation, and wound closure [Vu et al., 2022]. Recently, using the computational tool, CellChat, on single cell RNA sequencing data (scRNA) researchers were able to study the cellular variances between unwounded and wounded skin of young and aged mice [Vu et al., 2022]. The authors found that $Arg1^{Hi}$ and $C1q^{Hi}$ (Cd206-expressing) macrophages to be present within distinct regions of the wound site indicating that skin wounds resemble cancer in its apical demarcation of the M1/M2 macrophages and that macrophages become stuck in the inflammatory phase within aged skin wounds. Moreover, the authors found CD19 expression (marker for B cells) in aged skin to be reduced and theorised that its deficiency may contribute to delayed wound healing. Notably, skin fibroblasts are a primary target of $Arg1^{Hi}$ macrophage-originated IL-1 signalling in aged skin wounds. Moreover, Fibroblast-derived CCL19, implicated in age-related conditions i.e. arthritis, atherosclerosis, and neurodegeneration, has been found to accelerate wound closure in human *ex vivo* models [Topouzi et al., 2020]. Notably, the incidence of frailty (at risk or vulnerable skin that is thin or tears easily e.g. elderly skin) and non-healing wounds is reported to rise with a patient's age [Espaulella-Ferrer et al., 2021]. From a total of 51 consecutive patients wound healing rate was reported to be significantly higher in non-frail patients (8.9% wound reduction/day) in comparison with frail patients (with 3.26% wound reduction/day) [Espaulella-Ferrer et al., 2021]. Overall, this highlights that elderly skin undergoes delayed healing because of aging attributed by the intrinsic changes to its structural integrity and cell functionality.

In addition, elderly skin is more susceptible to injury than young skin. The DEJ of the human skin undergoes age related changes which contributes to increased fragility of the skin,

increasing risk of trauma and shear-type injuries [Hatje et al., 2015]. Flattening of the DEJ, because of the retraction of the epidermal papillae, diminishes the proliferative capacity of the epidermal cells and causes vulnerability to skin tears and ulcers [Kurban and Bhawan. 1990; Desai. 1997; LeBlac et al., 2018; LeBlanc et al., 2016]. Therefore, the combined threat of delayed healing observed in elderly skin coupled with increased susceptibility to injury makes this age demographic very vulnerable to wounds, cases of which will only grow more pronounced with an ageing population as will the economic burden.

In 2012-2013, the NHS managed an estimated 2.2 million patients with a wound, with an annual cost projected to be between 4.5 billion and 5.1 billion pounds [Guest et al., 2015]. In 5 years, there was a reported 71% increase (between 2013-2018) in wound cases across the UK, [Guest et al., 2020]. In addition, chronic wounds confer a major socioeconomic threat in the US, affecting over 6.5 million people, which amasses an economic burden of $25 billion annually [Roy et al., 2011; Banerjee and Sen. 2015].

1.1.7 Preventative medicine as an emerging solution to skin related disease.

As population ageing is emerging as a global concern for future generations, public health policies are shifting, and their medical health systems are adapting their approaches from conservatively reactionary treatments to preventative therapeutics. Western medicine is a prevailed model for reactive rather than proactive medicine. Reactive medicine is characterised as medical care activation in response to an adverse disease case initiation, acute condition, or symptom, whereas preventative medicine is aimed to implement medical treatment specifically designed to reduce, reverse, or elude the causes of disease [Badimon et al., 2023; Waldman et al., 2019]. Primary prevention also possesses the benefits of not only reducing the quality-of-life decrements but also reducing costs relative to early detection [Gordon et al., 2019]. In recent years, preventative medicine has evolved as a viable solution for extending longer and healthier life span by translating a biological discovery to a therapeutic intervention.

1.1.8 Dermatological treatment options

Drug delivery.

The most common routes of drug delivery are oral and parenteral routes. Small molecule drugs are typically delivered orally [Anselmo and Mitragotri. 2014; Han and Das. 2015]. Oral drug delivery possesses the benefits of using pre-determined doses, portability, and patient self-administration, therefore it is regarded as a convenient delivery route [Brambilla et al., 2014;

Ita. 2014]. However, proteins and peptides if delivered orally are highly susceptible to rapid degradation via the gastrointestinal tract. In addition, if delivered topically to the skin, proteins and peptides will be unable to surpass the epidermal barrier, the stratum corneum, due to size limited transport across the epithelium (<500 Da can pass) [Schoelhammer et al., 2014]. Therefore, hypodermic needles are typically used for the delivery of macromolecules to the skin [Anselmo and Mitragotri. 2014; Schoelhammer et al., 2014; McCrudden et al., 2013]. However, this is an invasive form of drug delivery, that requires health care assistance, and is inadequate for the delivery of compounds with a short-half life that would travel through the blood circulatory route.

Transdermal delivery has increasingly become an attractive alternative to oral, topical, and hypodermic drug delivery in recent years [Guy & Hadgraft. 2003; Williams. 2003; Prausnitz et al., 2004; Bronaugh & Maibach 2005]. The first transdermal device for systematic drug delivery was a three-day patch that delivers scopolamine to treat motion sickness, which was approved in the US in 1979. In 2008 there were 19 transdermal delivery devices available for drugs including estradiol, fentanyl, lidocaine and testosterone [Prausnitz and Langer. 2008]. Transdermal drug delivery possesses many benefits compared with traditional drug delivery options. In contrast with oral route drug delivery, transdermal delivery can overcome the significant first-pass effect of the liver which can prematurely metabolize drugs. In comparison with hypodermic needles, transdermal devices offer a non-invasive alternative, reduced medical waste, ease of use without assistance of a healthcare professional and notably eliminate risk of disease transmission by needle re-use, a common occurrence in developing countries [Miller and Pisano. 1999].

Transdermal delivery allows for the drug of interest to penetrate through the stratum corneum and thus pass through the deeper epidermis and dermis without drug accumulation in the dermal layer, and ensuring localized drug delivery [Donnelly et al., 2012; Krestos et al., 2007]. Once the target drug reaches the dermal layer, it can be systematically absorbed via the dermal microcirculation. As transdermal delivery is a non-invasive alternative to hypodermic needles, it can pose as a convenient alternative especially for those with needle phobia [Han and Das. 2015]. A large surface area of the skin and ease of access makes transdermal delivery a versatile platform for a variety of drugs to be transdermally absorbed [Schoelhammer et al., 2014]. Moreover, the pharmokinetic profiles of drugs tend to be uniform which minimises risk of toxic side effects [Han and Das. 2015]. This can in turn improve patient compliance because of

reduced dosing frequencies. Transdermal drug delivery is also especially appropriate for patients who are unconscious or prone to vomiting. Transdermal delivery eludes the issue of pre-systematic metabolism, showcasing improved bioavailability to more common drug delivery routes [Han and Das. 2015; Ita. 2014].

1.2 Aims of Thesis

The overarching aim of this thesis is to explore human skin heterogeneity with particular focus on three main areas in which skin varies: location on body, with ageing, and in colour and how that in turn influences skin disease susceptibility, notably on wound healing. I have focused on developing a preventative therapeutic microneedle (MN) device for drug delivery to the epidermal or dermal layers of the human skin for skin rejuvenation or skin transformation, respectively.

Overall hypothesis of thesis: Human skin is heterogenous in its location, colour and ageing which can influence dermatological disease susceptibility, namely wound injury.

Chapter Aims:

1. **Chapter 2:** Design, develop and engineer a pH responsive, non-invasive MN for the optimal drug delivery of compounds to human skin. *(Experimental)*

   ***Hypothesis*:** A variable height MN design formed of polymeric materials can penetrate human skin to improve protein delivery to the epidermis vs topical delivery.

2. **Chapter 3:** Develop and establish a UVA age-accelerated *ex vivo* human skin model to explore effect of UVA on wound healing and the potential amelioration of this effect by an anti-ageing compound. *(Experimental)*

   ***Hypothesis*:** UVA age accelerates human skin by inducing DNA damage and in turn impairing wound healing capacity.

3. **Chapter 4:** Explore and discuss the impact variable skin colour will have on skin disease susceptibility, UVA/B injury and on wound healing. *(literature review)*

Chapter 2

Reprogramming epidermal skin identity through protein therapy using a Smart Microneedle device.

2.1 Introduction; Chapter Aims

In this chapter I showcase the engineering process of an epidermal drug delivery device. I present the design critieria necessary for the establishment of a non-invasive MN patch to ensure efficient and targeted delivery of proteins to human skin. This device was constructed with the purpose of providing a preventative therapeutic for skin tissue regeneration or transformation. In my investigation, I deliver the Wnt5a protein to human skin using my designed MN to modify gene expression of epidermal keratinocytes for the purpose of skin transformation from a nonplantar to a plantar identity.

Aims:

1. Design a MN device with variable needle dimensions that ensure penetration to the epidermal or suprabasal regions of the human skin, and elusion of "bed of nails" effect.
2. Select appropriate materials for the MN base that ensures high mechanical properties (>0.3N per needle breakage), and low swelling threshold (<2% change in mass (mg)), and high stiffness properties (young modulus of >3MPa).
3. Efficiently coat the MN base with a pH responsive material that can encapsulate compunds of choice for delivery to the human skin.
4. Assess and validate the effect of the Wnt5a compound in transforming human skin from nonplantar to plantar via upregulation of KRT9 expression.
5. Deliver the Wnt5a compound to the epidermal keratinocytes using the coated MN device and assess gene expression changes via KRT9 expression.

The work presented in this chapter was conducted in collaboration with BioMad, and Sensors Groups, at King Abdullah University of Science and Technology (KAUST).

*Parts of this chapter are taken verbatim from the manuscript in preparation:

Abbas AA., Steer I., Teenan O., Hanif W., Akhavani M., Hasan E., Salama K., Almquist B., Higgins CA., and Alsulaiman D. "Reprogramming skin identity through protein delivery via a smart microneedle device"

2.1.1 Transdermal devices

Microneedles overcome conventional forms of drug delivery.

Transdermal devices are an attractive platform for drug delivery to the skin [Figure 2.1]. Microneedles are non-invasive devices that can address many obstacles facing conventional drug delivery tools including intravenous, oral, and topical routes of drug delivery [Spahn et al., 2008]. Drug efficacy can be affected by several factors from low aqueous solubility along the gastrointestinal (GI) tract, possibility of degradation in the acidic milieu of the stomach, limited permeated drugs in the upper GI tract, and systemic side effects [Amarnani and Shende. 2022; Rizwan et al., 2017]. Hence, one of the main advantages of a microneedle device for transdermal delivery is the ability to surpass the stratum corneum, which is a largely impermeable barrier to large molecules (>500 Da) and a key limitation to topical therapeutics abundantly used for the skin [Zhao et al., 2007; Bariya et al. 2011]. In addition, microneedles overcome the issue of first-pass effect typically encountered with oral routes of delivery. Moreover, microneedle patches can be applied by oneself without the assistance of healthcare specialists, and thus can be administered with ease and repeatedly over time [Waghule et al., 2019]. When developing a microneedle device for drug delivery, one must consider many factors: (i) ability to control and potentially maximize drug/cargo and its bioactivity, (ii) the design and mechanical properties of the device to effectively penetrate skin (without breakage and without ‘bed of nails’ effect), and (iii) physiological differences related to patients’ skin hydration, stiffness and thickness [Bariya et al., 2011; Avcil and Çelik. 2021; Chandran et al., 2019].

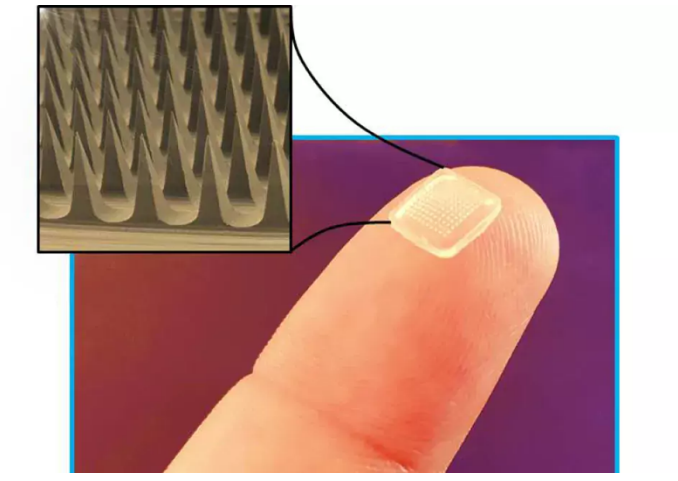


Figure 2.1: Microneedles as drug delivery devices. These transdermal devices are composed of microscale needles that are designed to penetrate the epidermal layer of skin without penetration of blood vessels or pain sensing neurons hence characterised as non or minimally-invasive. Image taken from [Amani et al., 2021].

2.1.2 Microneedles as drug delivery devices

Types of Microneedles

Microneedles are typically classified into four types: solid, coated, dissolvable and hollow MNs.

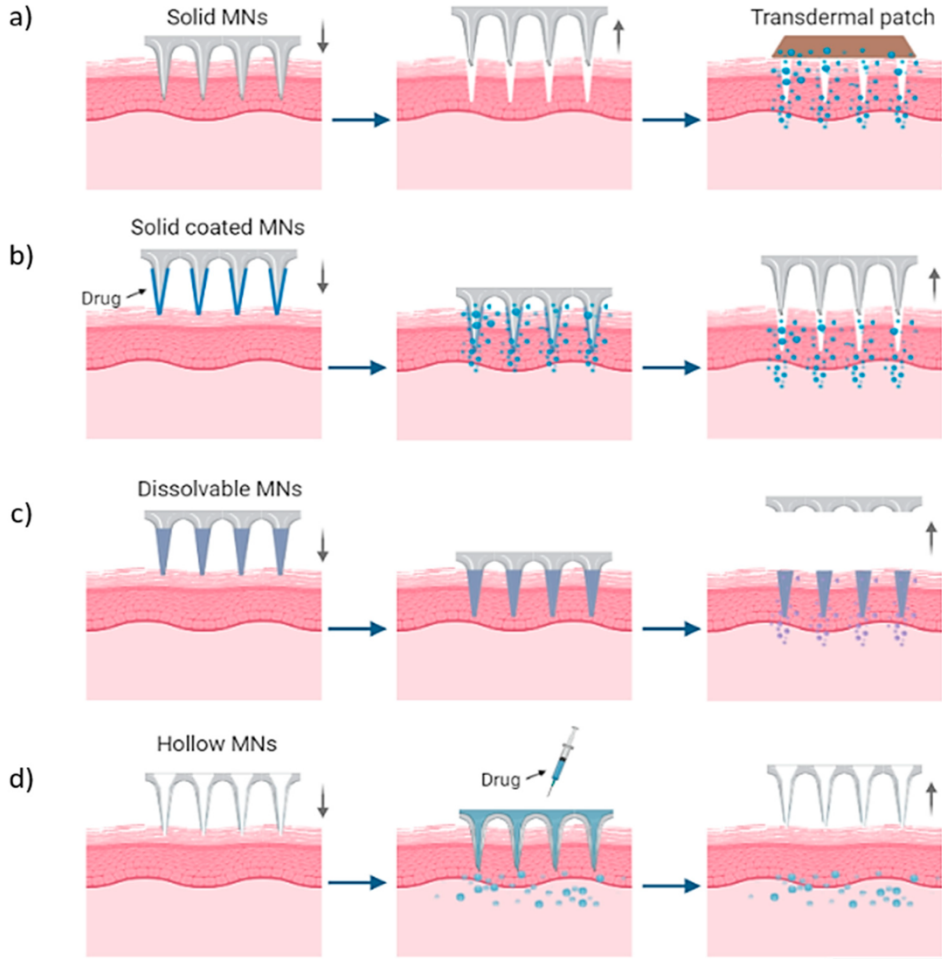


Figure 2.2: Types of Microneedle devices and drug delivery routes. (a) The 'poke-detach-diffuse' approach whereby solid MNs penetrate holes into the skin stratum corneum, and drugs are thus diffused through the holes. (b) The 'coat and poke' approach whereby the drug is coated onto the needles and thus once penetrated the skin the drug is diffused through. (c) The "Poke and release" method for dissolvable needles which once penetrated the skin are dissolved into the skin. (d) The "poke and flow" approach where the drug is loaded through hollow needles and delivered into the skin. Figure is taken from [Sargioti et al., 2022].

Each of the four types of MNs has their own benefits and detriments [Figure 2.2]. A solid MN punctures the skin surface and deposits the cargo within the skin layer, thus allowing the drug to gradually diffuse through the pierced holes of the skin. This possesses the benefit of preventing pathogenic infection however the drug delivery effect is typically low [Sargioti et al., 2022]. The dissolving MN is composed of water-soluble materials. Once the MN pierces the skin, the MN melts into the tissue, thus releasing the drug. An advantage of this route is the transfer of large doses of drug, but it is difficult to deliver a fixed amount of drug to the skin.

Hollow MNs are comparable to hypodermic needles in shape and allow for liquid medication to be directly injected into the skin. Lastly, coated MNs are array coated with an encapsulated cargo. Coated MNs ensure high drug loading volumes, notably controlled drug release, with conserved bioactivity of the drug in an encapsulated material as well as prevention of cargo biowaste as it is separate from the manufacturing of the MN itself [Gill et al., 2007; Haj-Ahmad et al., 2015; Sargioti et al., 2022; Kwon et al., 2017]. The fact that coated MNs ensure that manufacturing of the needles is distinct from drug coating, also means that the mechanical strength of the needles is retained.

Limitations of current commercially available Microneedle devices

Many advanced drug delivery MNs have been proposed in the literature, with most commercially available designs being based on metallic materials. While these MNs address mechanical criteria, they often suffer poor biomimetic properties and susceptibility to fractures and corrosion [Guillot et al., 2020]. Polymer-based MNs can address these challenges and have been used for both biosensing [Alsulaiman and Chang et al., 2019] and drug delivery applications [Mandal et al., 2018]. Coated microneedles offer versatility and reusability as the microneedle can be re-loaded with more drug after administration and utilized for a broad spectrum of materials including small molecules, proteins, DNA, viruses, and nanoparticles [Haj-Ahmed et al., 2015; Gill and Prausnitz. 2007]. Furthermore, the bioactivity of the drug can be controlled and more likely preserved in the coating when compared to if it were incorporated into the microneedle base, where it may be degraded during the fabrication procedure [Avcil and Çelik. 2021].

While these technologies are promising, proposed solutions can occasionally suffer from the following limitations: (i) fabrication strategy is often complex and multi-step requiring ovens and large equipment which is costly, (ii) high susceptibility for drug loss through contact with skin or other surfaces during handling, and (iii) ensuring the drug viability and functionality within the coating after administration [Bariya et al., 2011; Gill and Prausnitz. 2007; Jung and Jin. 2021]. Some of these issues have been addressed via layer by layer (LBL) coating of microneedle devices [Yuan et al., 2023; Yanpu et al., 2018; Brito et al., 2024]. LBL polymeric coating of MNs possesses the benefits of precision assembly, versatile inclusion of bioactive molecules and conformality to complex architectures of implantable devices. However, challenges remain with inconsistent drug delivery and needle breakage into skin [Avicil and Çelik. 2021].

2.1.4 Engineering a Microneedle device for targeted delivery to the skin epidermis

Geometry of a desirable MN device

When engineering a MN device, it is important to consider the length of the needles. It has been reported that needles with a shank length of 300 μm cannot penetrate the human skin whilst needles with a length of 550 μm can penetrate the human skin [Verbaan et al., 2007]. An increase in MN length is found to increase penetration depth [Donnelly et al., 2010]. However, longer needles may lead to molecular release into the reticular dermis and their absorption by skin microcirculation [Davidson et al., 2008; Badran et al., 2009]. Too long needles will stimulate pain receptor, or may puncture capillaries, causing bleeding, if they were to reach the reticular dermis or hypodermis. Notably, an increase in MN length from 480 μm to 1450 μm confers a more than seven-fold increase in pain (5-37% in participants) [Gill et al., 2008]. Interestingly, needle spacing (2z to 6x base diameter) has also been shown to elicit increased force of application per needle, resulting in higher skin (from rat abdomen) penetration depth [Kochlar et al., 2013].

To prevent microneedle's fracturing while piercing the skin, MN design must take into account the tip radius, angle and length/base diameter ratio [Prausnitz. 2004; Davis et al., 2004]. The MN tip's diameter affects the contact area between the MN and the skin, and subsequently the insertion force. Therefore, areas will result in smaller insertion force, which leads to a smaller skin deformation and so a higher chance of penetration [Kochar et al., 2013; Davis et al., 2004; Bal et al., 2010; Park et al., 2007]. Moreover, a small needle tip diameter (of <5 μm) results in smoother penetration in ex vivo human skin [Römgens et al., 2014]. Moreover, a study has revealed that reduction in the force required to pierce the stratum corneum from 3.04 to 0.08 N per needle, when the tip radius was reduced from 80 to 30 μm. Kochar et al., also identified the importance of aspect ratio between the base diameter and length of the MNs [Kochar et al., 2013]. The researchers found that a length of 827 μm and a base diameter of 200 μm resulted in a slower penetration rate compared to a base diameter of 300 μm and a length of 400 μm. The authors suggest that a greater aspect ratio correlates with inferior mechanical properties of both the needle tip and base. Bending occurs before reaching the insertion force during the piercing process, resulting in reduced penetration rate and so a lower piercing rate [Gittard et al., 2013].

Needle geometry influences penetration of the MNs in the skin, being a critical factor in drug loading, stress distribution, and mechanical properties [Bauleth-Ramos et al., 2023]. It has been reported that square-based MNs have a greater penetration depth than hexoagonal-based MNs at a constant MNs length and spacing as hexagonal-based MNs start penetrating the startum corneum less easily [Loizidou et al., 2016]. A desired design of the MN can be generated on a computer-aided design (CAD) software to predefine the shape, geometry and dimensions. The Microfabrication will outline the design with a CO_2 laser beam, after which the needles are cleaned in hot water, bent at 90 degrees, vertically from the plane of the base. This cleanroom-free, and low-cost fabrication method allows for rapid manufacture of microstructures compared to conventional time-consuming photolithography processes.

Fabrication method of a desirable MN device

To fabricate a master template with a desired design, 3D printing is typically used. However, this method, despite offering a high degree of control over the geometry of the MNs with high-aspect ratio, is restricted by the resolution of the printer. Low-cost 3D printers can overcome this limitation [Krieger et al., 2019], but they do fabricate MNs with a tip of 20-30 μms which rises the force needed for insertion. Two-photon polymerisation (TPP) based laser lithography, there is finer control over the scalable production of the needles and the MNs design and geometry. TPP has a resolution limit of 900 nm [Rad et al., 2017], however, there is a limitation in that the lateral bending force applied for demolding can reduce the achievable geometry of the MNs [Moussi. 2018]. The support bed of the MNs influences the conformability and weight of the array on soft tissue. As a solution, an elastomeric intermediary mold, fabricated using embossing of PDMS on a master mold can be fabricated using TPP-based laser lithography [Bukhamsin et al., 2021]. PEGDA is then cast over this intermediary mold to fill the pyranmical cavirites and is sequentially UV-cross linked. To carefully release the polymeric MNs, the elastomeric molds can be submerged in a chlorinated hydrocarbon solvent which causes swelling in ghte PDMS matrix, conferrig volume expansion of the matrix. Following expansion of the matrix, the mold releases the MNs from the pyramidal cavities, pushing them out of the intermediary mould. This faciliates release with minimal bending force.

2.1.4 Design critiera of my MN device

During my project I designed a stimuli-responsive, polymer-coated microneedle device for pH-sensitive and non-invasive delivery of protein therapeutics to the basal layer of the skin

epidermis. Importantly, I ensured fabrication relied on low-cost equipment based on a photopolymerization strategy. I also designed the MN properties to penetrate non-plantar skin as epidermal thickness ranges from 75.5 μm to 100 μm for non-plantar forearm skin [Lintzeri DA et al., 2022; Sandby-Møller et al., 2003]. The microneedle geometry and dimensions are designed to elude the 'bed of nails' effect. This 'bed of nails' phenomenon is theorized to restrict skin penetration efficiency when MN density is high; <150 μm spacing between MNs [Groves et al., 2013; Giannakopoulos et al., 2009; Li et al., 2017] and needle height is low, which I overcame by designing equal spacing >300 μm individual needle based dimensions, >600 μm distance between each needle base and alternating needle heights of 500 and 700 μm.

To optimize its performance, I developed a simple *in vitro* skin model to emulate the epidermal layers, dermal layers, and the stratum corneum. The microneedle device is made completely of photopolymerized polymeric materials, which ensures optimal biomimetic properties, ease of fabrication, cost effectiveness, and mass production [Lee et al., 2013]. Moreover, with a coated microneedle design, I ensured reusability of the device as coatings can be washed off and a new or different coating of interest can be applied. To maintain coating stability and specific stimulus-triggered degradation/delivery, the microneedle is coated with a pH-responsive polymer to immobilize the drug and to ensure the cargo is delivered only under specific appropriate conditions upon skin penetration, thus preventing issues of drug desorption or loss during handling. As proof of concept, I coated the MN array with wnt5a, a protein that can switch on gene expression (KRT9) in the stratum basal layer to confirm that my device can successfully the compound to the basal epidermis [Kim et al., 2016]. The delivery of wnt5a via a MN can thus be used to potentially transform human skin from nonplantar to plantar by selectively enhancing KRT9 expression I theorize would be difficult to achieve via topical treatment. This treatment option would be beneficial for patients with prosthetic limbs where the skin tissue is nonplantar and thus susceptible to wounding [Boyle et al., 2019].

2.1.5 Cases of limb amputation

In 2017, 57.7 million people across the globe lived with limb amputation due to traumatic causes [McDonald et al., 2020]. A retrospective cohort study from 2003 to 2009 on patients aged 50-84 years old across England found there to be 25,312 amputations [Ahad et al., 2014]. Across the black sea, in the Kingdom of Saudi Arabia (KSA), 325 amputations are likely to occur annually in Jeddah, 741 In Riyadh, and 3970 in the whole of KSA [Alzahrani. 2012].

Half a million diabetes-related amputations of the lower extremities are projected to occur in KSA and middle eastern and north African (MENA) countries in the coming decade [Alzahrani. 2012]. Peripheral arterial disease (PAD) impacts the lower limbs and is a critical healthcare issue for developed nations. Over 90% of the annual 5000 major leg amputations processed in England are attributed to arterial disease [Norgren et al., 2007] and almost 8000 major diabetes lower limb amputations were reported in England from 2017 to 2020. Moreover, the cost of healthcare for ulceration and amputation in diabetic patients has been projected to be almost one billion pounds per year [Nicole Mottolini. 2022]. The burden of diabetes mellitus (DM) is prevalent in low- and middle-income countries (LMICs). A recent study on 358 patients with DM in Jeddah, KSA, identified that 84.9% of the cohort underwent amputation, with 40% having experienced major amputations [Almohammadi et al., 2022].

2.1.6 Dermatologic issues associated with amputee patients

Patients experiencing lower extremity amputation (LEA) exhibit 65% more dermatologic issues than non-amputees, with skin issues occurring in 75% of LEA patients who use prostheses [Highsmith et al., 2016]. Repeatedly referring LEA patients to a dermatologist for every stump-related skin issue is rather impractical. Skin issues of the stump in lower limb amputees are a common occurrence in the regular rehabilitation practice, tending to prevent prosthetic use. This impairment can elicit an immensely negative impact on daily life [Meulenbelt et al., 2006]. A major factor contributing to the high risk and probability of skin injuries is that skin on the residual limb cannot adequately accommodate the mechanical loads of ambulation [Boyle and Higgins. 2021]. Dermatological issues can prevent normal use of a prosthetic limb [Lyon et al., 2000]. Stump ulcers are a frequent issue in amputees and can interfere with the day-to-day use of artificial limbs and thus impact the independence and lifestyle of amputees [Salawu et al., 2006]. Mean sizes of ulcers at first and second observations can range from 0.70 cm^2 to 3.30 cm^2 respectively.

The stump in lower limb amputees is susceptible to dermatologic issues because the area of the skin is exposed to many conditions that the soles of our feet encounter, such as shear and stress forces as well as elevated humidity when a prosthesis is used [Meulenbelt et al., 2007]. The fragile cutaneous landscape of a stump site is a result of amputation damage to the cutaneous, nervous, musculoskeletal, vascular, and lymphatic structures [Buikema et al., 2014]. The altered skin is placed in a socket of a prosthesis, and thus must adapt to a humid environment and withstand the compressive and frictional forces for which it is not adapted to. Moreover, a

history of vascular disease or diabetes can make the stump skin site more susceptible to immunologic and tissue destruction. This in turn can lead to local immune dysregulation, also known as an immunocompromised district [Ruocco et al., 2009; Ruocco et al., 2011]. Unlike plantar skin, nonplantar stump skin is not adapted to withstand the compressive forces that arise from prosthesis during movement, specifically ambulation [Meulenbelt et al., 2007; Schwartz et al., 1991; Buikema et al., 2014]. In some cases, the severity of skin disease and pain at stump site can lead to prosthesis abandonment, which can have an immense impact on an amputee's quality of life.

2.1.7 Reconstructing the skin flap for limb amputee patients

Researchers are exploring the ways in which adaptation and durability of stump skin site could be improved, which would reduce skin breakdown, infections, tumors, and malignancies typically presented on a fragile site [Buikema et al., 2014]. Reconstruction of nonplantar tissue around the stump site is a deeply challenging task [Kadam. 2010]. Microsurgical tissue transfer is a common salvation method. Local and regional flaps can, however, be associated with increased morbidity to the afflicted limb and can be restricted by tissue availability and composition [Herter et al., 2007]. Free tissue transfer has been utilized as an alternative to restore anatomy to near normal, with the least amount of trauma and scarring. However, microsurgical free tissue transfer is a technically demanding process which demands meticulous exercise to planning and execution [Koul et al., 2013]. Furthermore, free tissue transfer is an invasive and costly method that requires significant resources, specifically the need for skilled microsurgeons, microsurgical supplies, operating room equipment, as well as close post-operative monitoring [Wan et al., 2019]. There is a 5-10% flap failure rate with free tissue transfer, typically due to thrombosis, which would necessitate a second operative procedure as well as subsequent donor site morbidity if an additional flap is needed [Wan et al., 2019; Dibbs et al., 2019].

2.1.8 Current options to reduce skin injury.

There are several treatment options available to reduce skin injury for limb amputee patients. For example, adequate prosthetic fitting to ensure that the prosthetic limb correctly fits which can thus alleviate skin irritation and pressure sores [Luigi et al., 2014; Ajua et al., 2018; Highsmith et al., 2016]. Maintaining regular skin care to inspect stump sites for signs of irritation can ensure skin health is conserved. Pressure relief techniques i.e. cushioning materials or padding to limit pressure on bony prominences can inhibit pressure ulcers from

forming. Regular repositioning of the prosthetic limb can help to avoid prolonged pressure on the stump site to reduce skin damage. Moreover, prosthetic liners that wick away sweat and reduce fiction provide a moisture-wicking barrier to keep the skin dry and limit friction between the residual limb and prosthetic socket [Davies et al., 2020; Lannan et al., 2021]. By maintaining a dry environment, liners can limit skin irritation, chafing and pressure sores from developing that would occur as a result of prolonged wear of a prosthetic limb. Although, these forementioned approaches can help to reduce friction and improve comfort and mobility for limb amputee patients there are no currently available treatment options to prevent injury from occurring. There are only existing options to reduce skin injury or treat skin wounds [Kwasniewski et al., 2022; Choo et al., 2022] once they have occurred.

Developing an efficient and effective approach for transdermal delivery of these protein therapeutics to the amputation site could serve as a transformative strategy to prevent complications from arising in amputee patients. Herein, I develop a novel approach to non-invasively reprogram amputation sites to a KRT9 expressing identity by engineering a responsive microneedle device to deliver Wn5a to nonplantar skin. This strategy presents a refined, robust, minimally invasive, and economical approach to prevent skin injury in amputee patients, by transforming nonplantar tissue into a KRT9 expressing epidermis by delivering Wnt5a, a protein therapy that targets epidermal basal layers, as a therapeutic drug.

2.1.9 Aims of Chapter: Designing a device to overcome current limitations and offer a therapeutic option for amputee patients

Microneedles (MNs) have emerged as a promising medical device for the delivery of therapeutics to skin due to their non-invasive properties, ease of use, and capacity to surpass the epidermal barrier. In this chapter I present my MN device from design to the construction to the application [Figure 2.3].

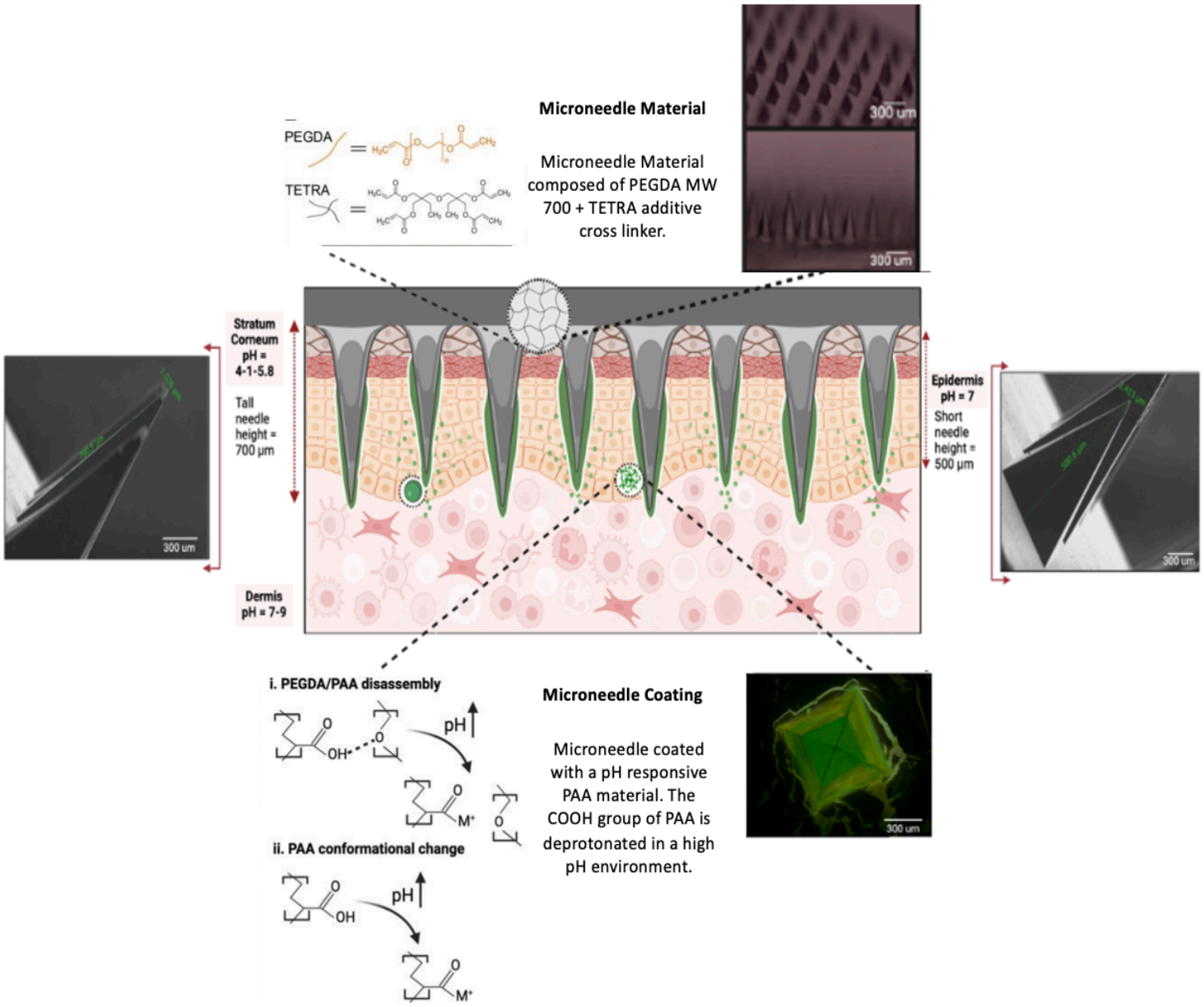


Figure 2.3: Design and construction of pH responsive Microneedle for epidermal delivery of proteins. 1) The MN base was composed of PEGDA Mw 700 with di(trimethyolpropane) TETRA acrylate additive. 2) The base was coated with a PAA (0.035% wt) coating encapsulating drug of interest.

Despite the potential of MN devices, many conventional designs face limitations with re-usability, drug biowaste, variable mechanical properties and limited penetration of human skin depending on thickness. Herein, I present my design and construction of a therapeutic MN patch which accounts for these flaws, with a variable needle height design to elude the 'bed of nails' effect. The reduced needle insertion is theorized to reduce drug delivery [Makvandi et al., 2021] therefore penetration rather than compression is imperative. The MN wall thickness at the tip ranged from 1-2 µm to ensure sharpness at the tip for effective skin penetration [Figure 2.3-SEM] in addition to the variable needle design to elude 'bed of nails effect'. I aimed for

high mechanical properties (>0.6 N failure point per needle) for consistent penetration of skin of all types, and pH responsive material (>80% drug release in >pH 7 environment) to prevent biowaste of the drug of interest [Figure 2.3].

I have composed my established MN device from completely biocompatible polymeric materials that ensures reusability, and photopolymerized to ensure efficient and cost-effective curing with acrylated precursors. As proof of concept, I highlight the success of the fabrication and design by delivering proteins of variable size and charge to human skin, notably using Wnt5a to transform human skin from nonplantar to plantar via the upregulation of KRT9. This biological application demonstrates the device's capacity and specificity for localized drug delivery to the basal layer of the epidermis.

2.2 Materials and Methods

2.2.1 Microneedle Fabrication

Master Mold 3D MicroFabricationA MN 10 x 10 array template with 100 pyramids based MNs, with alternating heights of 700 µm and 500 µm, spacing of 300 µm on a flat rectangular 2 by 2 mm^2 bed that is 100 µm thick. was designed using AutoCAD® software suite (AutoDesk). The CAD file was converted to a general writing language (GL) file using Describe software (Nanoscribe, GmbH) [Bukhamsin et al., 2021; Fakeih et al., 2023]. The software split the structure in the horizontal and vertical axes, characterized as slicing and hatching distances, to generate the exposure path of the laser. The slicing distance and hatching distance were set to 0.5 µm. A shell and scaffold configuration was selected to accelerate the TPP-direct laser lithography process. The outermost layer of the structure, characterized as the shell, lines demarcating its slices, are revealed by the laser. The thickness of the shell was selected to be 10 µm. The interior of the structure encompassed a triangular scaffold, also hatched and sliced with distances of 0.5 µm. The writing field and piezoelectric motor selected to articular the laser in the instrument restricts the voxel size to 300 by 300 by 300 μm^3. A secondary galvo motor is engaged to move the laser between each voxel size, which facilitates stitching of the voxels. To achieve adhesion of the individual voxel, a block overlap distance of 2 µm is chosen. At the interface of the voxels, a stitching line is observed due to the minute misalignments during movement of the stage and exposure. A 25 by 25 μm^2 single-sided polished silicon die was subsequently cleaned in acetone, isopropanol, and DI water. IP-S resin was decanted into the center of the dye, which was loaded into the Photonic Professional GT

system (Nanoscribe, GmbH). The exposure process of the master template was completed in 2 hours and 35 minutes. Once the polymerization process had completed, the die was submerged in mer-Dev 600 (MicroChem) to remove the uncured resin and then washed in isopropanol.

Polydimethylsiloxane (PDMS) Embossing

An acrylic rectangular 20 by 20 mm^2 frame was cut using a CO_2 laser [Buckhamsin et al., 2021]. The frame was placed around the master mold and attached to the Silicon die using drops of chloroform released from the nozzle of a hypodermic needle. PDMS (Sylgard 184) was mixed in a 10:1 ration (monomer to curing agent) vigorously and sequentially outgassed for 45 minutes. The PDMS was then cast on the master mold and the excess was removed with a surgical blade. The. Mold was subsequently outgassed for a further 20 minutes before moving to a convention over set at 60°C to cure the PDMS, for 40 minutes. To remove the replica from the master mold, a surgical blade was used to trim the edges and released the PDMS mold [AlSulaiman and Chang et al., 2019; Lin et al., 2016].

PEGDA Micromolding

Next, the MN was engineered using the PDMS mold, using commercially available materials. First, 1 mL solution of PEGDA (Polyethylene Glycol diacrylate) Mn 700, or 250 (Sigma Aldrich, St. Louis, MO, USA) with 5% photo-initiator, Darocur© 1173 (2-Hydroxy-2-methyl-1-phenyl-propan-1-one) and 47.5% of di(trimethylolpropane) tetra acrylate cross-linker was pipetted into the mold. The Darocur© liquid photo-initiator was used to initiate the photo-polymerization of the chemically unsaturated pre-polymer PEGDA (Polyethylene Glycol diacrylate) solution [Dardano et al., 2015]. This 1 mL mixture was desiccated in a vacuum chamber for 30-40 minutes to remove air bubbles [Agrawal et al., 2017]. The material was cured for 3 minutes on the top, then 3 minutes on the bottom, using a UV lamp (6-Watt, 365 nm) to create enhanced chemical durability that is insoluble and infusible [Shirai. 2014; Mellottv et al., 2001]. The final photopolymerized structure is the MN, images of which were taken using a TG-5 Olympus digital camera at 4 x magnification. After being used to make a MN, MN moulds were cleaned using methanol, prior to being ultrasonicated to clean off any remaining PEGDA following photopolymerization. This ultrasonication step was repeated with acetone, before being left to desiccate overnight. The mould was then suitable for reuse to make another MN.

2.2.2 Mechanical and tensile strength testing of Microneedle.

Axial Compression Test

An axial compression test was used to assess the maximum axial force which induces MN failure and measure young modulus and peak load [Park and Prausnitz. 2010]. A 5966 Instron® series compression platens instrument was used to perform this mechanical test. The MN was attached to the base of the loading cell with double-sided tape and a starting force of 0 N was used, up until failure point after balancing to calibrate the instrument. The force (N) linearly increased as the instrument ran at an extension rate of 10, 25 or 30 µm/seconds until needles broke (identified by a 40% peak drop in the needles from breaking under force). A range of extension rates (µm/second) were explored to identify the optimal rate for highest failure force (N). Once a 40% peak drop in the rate of loading was achieved, the experiment was ended. The failure point (N) of the MN array was divided by 100 to normalize per needle (there are 100 MNs per patch). The Sigma, also known as the compression stress, was first calculated by dividing the failure force (N) of the needle array over the surface area (µm) (0.5 x 471 µm (legth) x 10^6 x 250 (diameter of base) $5.8875x10^{8}$ m^2) of the MNs. The length (500 µM) of the MNs subtracted by the compressed length of the MNs (300 µM) was divided over the original length (500 µM) of the MN was used to measure the Epsilon, also known as the compression strain. The compression stress was then divided over the compression strain to calculate the Young Modulus (MPa) [Park and Prausnitz. 2010; Du et al., 2021].

Parafilm insertion point.

Parafilm M PM-996 all-purpose Laboratory Film, (1.5mm width, 120 µm thickness) was clamped by two adjacent 3D printed PDMS cuboids (5 cm by 5cm) (designed on Autocad®) with 10 cm distance between both blocks. The same axial compression test method and parameters as mentioned above were used in this test. The Microneedle was attached to the load cell via double sided tape and at a rate of 10 µm/seconds descended to pierce the parafilm material. The force (N) at which the needles inserted into the parafilm was recorded. This model was used to emulate stratum corneum, modified from the literature (parafilm was placed on plastic block for support) [Larrañeta et al., 2014].

Swelling study.

The change in MN mass of MNs before and after being submerged in an aqueous environment was observed to assess the material's swelling properties. A high cross-linking density confers

a lower swelling capacity. After being weighed, the MN array was submerged in 1 mL of PBS with 1 mM FITC-tag used for visualization in imaging (Thermofisher®, Waltham, Massachusetts, USA) for 60 minutes at room temperature. The MN array was then dried with a kimiwipe and cleaned using nitrogen air. Next, the mass (mg) of the MN array was measured again and the change in mass (%) normalized by initial mass was calculated (divided by the entire array to obtain % change in mass (mg) per needle. MN arrays were all imaged before and after being submerged in PBS using a TG-5 complexus digital camera at 4 x magnification.

2.2.3 Scanning Electron Microscopy (SEM)

The Quattro environmental scanning electron microscope (SEM), (Thermofisher®, Waltham, Massachusetts, USA), was used to access the dimensions of the MN array, as well as morphology changes after PAA coating [Roxhed et al., 2007]. All MN samples were sputtered using a magnetron sputter instrument to platinum coat (3 nm thickness coating) the MN array. This was done to transform the polymeric MN to a conductive material to thus minimize the charging effects of the material and make visualization easier on the SEM [Fisher et al., 2012]. MNs were imaged at the tip to determine width of needle tip, and along the shank of the needle to measure height of needle.

2.2.4 Electron Dispersive X-ray spectroscopy (EDX)

The Quattro EDX technique was used to determine the chemical or elemental composition of a sample. Uncoated and BSA-FITC PAA coated MN arrays were used to compare the elemtal composition. The x-ray fluorescence spectroscopy analyses the x-rays emitted by a sample when it's hit by charged particles. This culminated in a map of elemental distribution [Sciemeca et al., 2018].

2.2.5 Coating of Microneedle: Dip coating

Fabricated MNs were coated with polyacrylic acid (PAA) (Sigma Aldrich, St. Louis, Missouri, United states) *via* a dip coating method. To accommodate for the surface area of the MN array, 30 µL of PAA (0.035% wt. in PBS), with FITC dye (1 µM), bovine serum albumin (BSA)-FITC (1 µM), Avidin-FITC (1 µM) (Sigma Aldrich, US) or Wnt5a-FITC (1 µM) (Abcam, Cambridge. The MN array (tips facing down) was dipped into the reservoir of solution for 1 minute to ensure the whole array was coated with solution [Zhang et al., 2011]. The MN array was then removed from the reservoir and desiccated in a vacuum chamber for 30 minutes to

remove air bubbles and create a thin film of coating over each needle. The MNs were then imaged using a a TG-5 Olympus digital camera at 4x magnification.

Fine tuning coating of Microneedles tips

To reduce biowaste of protein of interest and optimise deposition of different cargo onto alternate needles, a GIX SonoPlot® Microplotter II was used (SonoPlot, Middleton, United States). The instrument allowed for precision picolitre fluid dispensing for a microarray as well as non-contact deposition [Allanurov et al., 2014]. The dispenser of the microplotter was loaded with a total volume of 30 μL (0.3 μL per needle) so 300000 pL per needle. The dispenser utilises controlled ultrasonics to deposit 1 μM of Cy3 (red) (Sigma-Aldrich, St.Louis, Missouri, United States) over 700 μm needles only. The method entailed loading the dispenser with the loading solution and via connected camera, the dispenser was navigated over the 700 μm needles a drop was loaded per needle. Images of the coated MN were captured using a Typhoon Gel Image Scanner (Cytiva Life Sciences, Little Chalfont, Amersham, England).

2.2.6 Testing viscosity of proteins in PAA

The solution viscosity of 1 mL of Avidin or BSA diluted (to 1 μM in varying % wt of (100, 000 MW) polymeric PAA, 3.5% wt, 0.35% wt 0.035% wt, with PBS and water as controls) was assessed using the ViscoSystem AVS 470 (Xylem, Washington DC, United States). The samples in pneumatic tubes were pumped by the ‘suction’ mode into the measuring bulb of the viscosimeter to generate the viscosity reading.

2.2.7 Protein Release

Fluorescence Intensity

To measure the fluorescence intensity of BSA-FITC embedded PAA coated MNs before and after MN buffer wash, or penetration to GelMA or human skin model, the ZEISS Axio Observer microscope was used with ZEN microscopy software (ZEISS, Oberkochen, Germany). For fluorescence excitation, an X-cite XYLIS broad spectrum LED illumination system (Excelitas, Waltham, Massachusetts) was used coupled with a FITC filter (Fluorescein isothiocyanate) (**λ(**ex)citation maximum – 495 nm; **λ(**em)ission maximum – 525 nm). Images were taken at 10x magnification. Images were analyzed using ImageJ [Schneider et al., 2012].

Confocal microscope

A Leica SP8 TCS (Technical Services) STED 3X Confocal microscope at 10x or 20x magnification was used to image MN arrays, individual MNs, and the gelatin methacrylate (GelMA) artificial skin model after needle compression to its surface. A Z-stack was taken (10 μm per step) to assess the diffusion penetration of FITC and BSA-FITC (Alexa 488) (ThermoFisher®, Waltham, Massachusetts, USA) both on the MN surface, and on the GelMA model, respectively [Alsulaiman et al., 2019; Mansoor et al., 2015]. Images were analyzed using ImageJ [Schneider et al., 2012].

Fluorescence calibration curve

A Varioskan LUX microplate reader (ThermoFisher®, Waltham, Massachusetts, USA) was used to generate a standard calibration curve of BSA-FITC (Excitation maximum - 495 nm, Emission maximum - 519 nm). The wash solution left after placing the MN array in a buffer solution, was loaded into a 96 well plate (100 μL per well) and placed into the microplate reader and fluorescence readings were measured. The generated calibration curve was used to quantify the percentage of protein released from the MN device following 15 minutes, or 2-hour buffer washes with MES, PBS or 1xTE buffers.

Nanodrop Absorbance Measurements

Absorbance measurements were conducted on a Nanodrop One (ThermoScientific® Waltham, Massachusetts, USA) Spectrophotometer following zeroing with an appropriate “blank” solution i.e., PBS (phosphate buffered saline), polyacrylic acid, MES (2-ethanesulfonic acid) buffer or 1xTE buffer. Next, 2 μL of the sample was pipetted onto the nanodrop spectrophotometer, and the absorbance values (A260/A280) were measured. Three readings were taken for each coated MN sample and the average was calculated to obtain the mean value reported.

Assessing pH sensitivity of coated microneedle device

To assess pH sensitivity of the PAA coating, the MN device was washed with various buffers at different pH values, including MES (compound 2-ethanesulfonic acid) at pH3, PBS (with addition of Hydrochloric acid (HCL) at pH7, and 1xTE (Tris & EDTA) at pH 8.8. The PAA coated MN was dipped needle tip face down into a reservoir holder of 30 μL of the buffer wash (1:1 ratio), for 15 minutes, or 2-hours at room temperature 37°C. Once the device was removed

from the reservoir, the protein or fluorescence release was quantified *via* fluorescence intensity measurements (a), the calibration curve (c) or through absorbance measurements (d).

2.2.8 Gelatin methacrylate (GelMA) model establishment

To construct an artificial skin model, I used a Photogel®-IRG, Methacrylate Gelatin Hydrogel kit (Sigma Aldrich, St. Louis, MO, USA) [Bedell et al., 2020; Piao et al., 2021; Bupphathong et al., 2022; Sun et al., 2018]. Up to 5 mL sterile Milli-Q water was warmed to 60°C for 40 minutes, before the solution was used to solubilize 250 mg of lyophilized gelatin methacrylate. The mixture was stirred using a spinner whilst left on a hot plate (at 60°C) until it was completely solubilized. Next, 1 mL of methanol was used to solubilize 50 mg of Irgacure-2959 photo-initiator (Sigma Aldrich, St. Louis, MO, USA). 1 wt% of Irgacure was directly added to the 5 mL of GelMA mixture which was then thoroughly mixed. 300 µL of the mixture was added to a 2 cm glass bottom well dish creating a mound before it was photopolymerized under irradiation by a UV curing lamp (6-Watt, 230 V, 365 nm) for 3 minutes (top exposure) and 3 minutes (bottom exposure) of GelMA.

2.2.9 Human samples

Abdominal skin was acquired from patients (30-40 years old) after receiving their written informed consent, using Imperial College Research Ethics (ethics approval number: 21IC6723), committee approved consent forms. To sterilise the human skin samples and prevent risk of contamination, the skin samples were trimmed of fat, and the epidermis was septically cleaned by wiping with alternating betaiodine, then cutiwipes (70% ethanol), three times [Nasir et al., 2019]. The skin was placed on gauze, and DMEM medium (10% FBS) and stored at 4ºC temperature to prevent drying out.

Isolation of primary keratinocytes

To extract primary keratinocytes from human skin, the skin was submerged in Dulbecco's Modified Eagle Medium (DMEM)+ Glutamax (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 3x Gibco™Antibiotic Antimycotic (ABAM) for 1 minute. Skin was cut into 1x1 cm pieces and placed in a petri dish containing 1x dispase® (MilliporeSigma, Burlington, Massachusetts) at 4°C overnight. The next day, the epidermis was separated from the dermis using forceps with the former placed in a falcon tube of 10-15 mL 1x trypsin, EDTA (Thermo Fisher Scientific, Waltham, Massachusetts, United States) incubated in the water bath (37°C) for 30 minutes with gentle agitation (shaking) conducted

every 5 minutes. Following incubation, to neutralize the trypsin, 10-15 mL 1x DTI, EDTA (Thermo Fisher Scientific, Waltham, Massachusetts, United States) incubated in the water bath (37°C) for 30 minutes with gentle agitation (shaking) conducted every 5 minutes. Following incubation, to neutralize the trypsin, 10-15 mL of Gibco™ defined trypsin inhibitor (DTI) was added to the epidermal pieces, which were then passed through a 70 μM cell filter to remove tissue debris. The filtered supernatant containing cells was centrifuged at 300 xg for 10 minutes to pellet cells. These were resuspended in keratinocyte serum free media (KSFM) with bovine pituitary extract (BPE) and epidermal growth factor (EGF) supplements, and 1x ABAM (St. Louis, Missouri, United States, Sigma Aldrich). These cells were seeded into flasks pre-coated for 1 hour with 1 mg/mL fibronectin in PBS and incubated at 37°C with 5% CO_2.

Skin Penetration test

To assess the efficacy of penetration of the variable height MN design in piercing the epidermal layer of the skin and eluding the 'bed of nails effect' a penetration test on *ex vivo* skin was performed. A droplet of 0.4 % trypan blue was applied to the surface of *ex vivo* abdominal skin. The MN was pressed onto the trypan blue coated surface and held for 1 minute before being removed. The trypan blue was absorbed using a chem wipe and tissue was then washed with PBS. The MN pierced or compressed area was then imaged from above using a stereo microscope at 0.7 x magnification.

2.2.10 Measuring fluorescence intensity (%) before and after Microneedle applied skin.

To assess the pH-responsiveness of our constructed MN materials on human epidermal skin (pH 7), I measured the fluorescence intensity % of MNs before and after the FITC-tagged Wnt5a coated MN was applied on *ex vivo* skin (5, 10 and 15 minutes, N=4 biological replicates).

2.2.11 Wnt5a to human keratinocyte cells.

Wnt5a was chosen as the protein of interest to deliver to the basal keratinocytes as it can induce the expression of KRT9 in epidermal keratinocytes, a specific gene, not normally expressed within abdominal skin keratinocytes [Yamaguchi et al., 1999]. In addition, Wnt5a is 42.3 KDa, with a short half-life of 140 minutes (about 2 and a half hours), so topically would be unable to surpass the stratum corneum and exert its biological function, hence epidermal delivery via a MN is more suitable [Abedini et al., 2020]. Moreover, Wnt5a is a challenging molecule to deliver to the human skin due to its large size, hydrophobic nature, and instability. As a member

of the Wnt signalling pathway, it is susceptible to degradation by proteases in the extracellular environment [Zhu et al., 2014]. This instability can restrict the bioavailability and effectiveness of Wnt5a delivery to skin tissue [Bhatt et al., 2014]. Therefore, the encapsulation of this compound in PAA can preserve its biological activity and facilitate its delivery to the epidermis.

The biological function and bio activity of Wnt5a was assessed comparing topical delivery and delivery with a MN. RNA extraction and a qPCR was performed on wnt5a administered biological samples. Primary confluent keratinocytes were seeded at 100,000 cells per 3.5 cm^2 well in a 12-well plate, (seeded via drop-by-drop method) [Garza et al., 2011]) [Rinn et al., 2008]. The drop-by-drop method involved seeding 100,000 cells in a 100 µL in in non-supplemented KSFM against untreated controls. The 100 µL was pipetted into the well drop by drop across the surface area of the well and left to incubate (37°C and 5%) for 2 hours before 1 mL of supplemented KSFM was administered to all wells. Once cells were confluent (3-5 days after seeding), recombinant human Wnt5a protein (Biotechne, Minneapolics, Minnesota, United States) (0.3 µg/mL) was added to wells against untreated controls [Rinn et al., 2008].

2.2.12 Wnt5a to *ex vivo* skin.

On fresh *ex vivo* skin (following processing and sterilization), Wnt5a protein was either topically administered to 1x1 cm skin pieces (in a 6-well plate against respective controls, in media (DMEM+glutamax, 10% FBS (Fetal Bovine Serum) and 1% ABAM)), or a Wnt5a in 0.035% wt coated MN was applied to the surface and in both cases left for 15 minutes, before being washed way or removed respectively. Subsequently, tissue samples were left to incubate (37°C) in medium for 24 hours, before epidermis separated from skin tissue, homogenized, extracted of RNA which was then converted to cDNA and qPCR was finally performed.

Bioactivity of Wnt5a in PAA

To assess the bioactivity of the wnt5a protein in 0.035% wt (100, 000 MW) PAA (Sigma, St Louis, Missouri, United States), the protein was diluted in freeze dried 0.035% wt PAA (with keratinocyte medium (KCSFM). To assess bioactivity of long term stored freeze dried Wnt5a in 0.035% wt PAA, a sample that had been stored at 4°C for 3 months was administered to confluent keratinocytes and following 24-hour incubation (37°C and 5% CO_2) of the cells, RNA was extracted and underwent cDNA conversion and qPCR was performed.

Polymerase chain reaction

RNA extraction

The biological function and bio activity of Wnt5a was assessed comparing topical Wnt5a delivery and Wnt5a coated MN delivery. RNA extraction and a qPCR was performed on Wnt5a administered biological tissue and keratinocyte samples. The RNA was extracted from cells or homogenized skin (according to the instructions of the RNeasy® Plus mini kit (Qiagen, Holden, Germany), then converted to cDNA using TaqMan^{TM} Reverse Transcription Reagents kit, (ThermoFisher Scientific,Waltham, Massachusetts, United States).

cDNA conversion

A total of 200 ng RNA (diluted in H_2O) per sample was converted to cDNA using Oligo and dNTP mix (Master Mix (MM) I), DTT, 5 x superscript IV Reverse transcriptase (RT) buffer, DTT, RNase out and Superscript IV RT (MM II). Up to 2 µL of MMI (for 1 reaction: 1 µL of OligoDT, and 1 µL of dNTP mix) was pipetted into each PCR tube followed by 11 µL of the RNA and H_2O mix and placed into a PCR block. During the cycle the mixture was heated at 65°C for 5 minutes and colled down to 4°C for 1 minute. After the has ended, 7 µL of MMII (for 1 reaction: 4 µL of 5x SuperScript IV RT buffer, 1 µL of DTT, 1 µL of RNase OUT and 1 µL of Superscript IV RT) was pipetted into each sample tube and incubated at 52.5°C for 10 minutes, then the reaction was inactivated by heating to 80°C for 10 minutes before finally cooled down to 4°C.

qPCR reaction

The qPCR (QuantStudio® Real-time PCR (Polymerase Chain Reaction)) was next performed. To perform qPCR, per sample a master mix of 10 µL SYBR green, 1 µL primer of interest, 0.5 µL DMSO and 7.5 µL RNase free water was made up, and 19 µL of the master mix solution was pipetted into each well in a 96 well plate along with 1 µL of the corresponding cDNA for a total volume of 20 µL. Negative controls with negative 1 µL cDNA or RNase water were added to the 19 µL master mix solution on the 96 well plate. The qPCR parameters were set up with SYBR green reagent with a standard cycling of : holding stage (50°C for 2 minutes, followed by 95°C for 2 minutes) and cycling stage (95C for 15 seconds and 60°C for 1 minute). Primers of interest that were used in context of both primary cell and *ex vivo* skin, were KRT9 and Keratin 14 (KRT14), to identify supra-basal palmoplantar keratinocytes ($KRT9^{high}$)

[Yamaguchi et al., 1999; Alam et al., 2011] and basal (keratin14^{high}) keratinocytes. GAPDH was used as a baseline reference gene against KRT9 and KRT14. The delta-delta ct (also known as $2^{-\Delta\Delta Ct}$) method was used to quantify the CT values generated from the qPCR instrument to calculate the relative fold gene expression of samples.

Table 2.1: Primer sequencing list of KRT9, KRT14 and GAPDH.

Gene	Sequence	Product Size (base pairs)
KRT9 [Sloan et al., 2004]	**FW**CTTCTTCCTCAAAATCTGGTGACC **REV**TCCAAAGCCAAGCAGGGTTG	251
KRT14 [designed in lab]	**FW** AGA CCA TTG AGG ACC TGA GG **REV** CCT TCA GGC TCT CAA TCT GC	231
GAPDH [Origene, Rockbille, Maryland, United States]	**FW** GTCTCCTCTGACTTCAACAGCG **REV** ACCACCCTGTTGCTGTAGCCAA	235

Immunostaining

To explore protein expression of KRT9 in Wnt5a administered primary keratinocytes and *ex vivo* skin, immunostaining was performed.

Preparing Keratinocytes for immunostaining

Keratinocytes in culture were seeded over 12 plate compatible coverslips (that had been coated prior with fibronectin). 24 hours following UV exposure of the keratinocytes, the plate was removed from incubation (37°C and 5% CO2) and aspirated of media. Each sample well was washed with 1x PBS and kept on shaker for 3 minutes, which was repeated thrice. The samples were then fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature in the fume hood. The samples were washed once more with PBS for 3 minutes, before 1 mL of 0.3% Triton in 5%. Goat serum (diluted in PBS) was added to each sample which were then left for 30 minutes at room temperature. Following incubation, the primary antibodies: 1:1000 anti-chicken KRT14 (Catalogue No: SIG-3476), and 1:50 anti-mouse KRT9 (Catalogue No: GPHK9-TY1) (ThermoFisher, Waltham, Massachusetts, United States) were diluted in 5% goat to each sample which were then left overnight at 4°C [Table 2.2]. Following overnight incubation, the wells were washed in 1x PBS and left on shaker for 3 minutes, which

was repeated thrice. The secondary antibodies (1:1000 goat anti-chicken Alexa Fluor 647 and 1:50 goat anti-mouse 568 (ThermoFisher, Waltham, Massachusetts, United States) were diluted in PBS with 5% goat serum and added to samples before incubation for 1 hour at room temperature in the dark. The samples were then washed with 1xPBS three times for three minutes each shaking cycle.

Finally, glass slides were prepared with a drop of Mounting Medium with 4',6'-diamidino-2-phenylindole (DAPI)(ab104139) (ABCAM, Cambridge, United Kingdom). Each coverlip from the plate well was carefully removed with forceps and the cell facing side was placed over the mounted DAPI on the slides. The slides were left to dry for 48 hours at 4°C before imaging on Leica SP8 Confocal Microscope instrument, at 10 x magnification for keratinocytes.

Table 2.2: List of Primary and Secondary Antibodies

Target	Host	Company	Dilution
Primary			
KRT9	Mouse	Thermofisher	1:50
KRT14	Chicken		1:1000
		Thermofisher	
Secondary			
Alexa Fluor 568	Goat		1:50
Mouse		Thermofisher	
Alexa Fluor 647	Goat		1:1000
Chicken		Thermofisher	

Preparing Skin for immunostaining

Fresh *ex vivo* skin samples (after processing and sterilization) were cut into 1x1cm pieces and placed in media (DMEM+glutamax, 10% FBS (Fetal Bovine Serum) and 1% ABAM) on a 6-well plate. Wnt5a diluted in 0.035% wt PAA coated MN or topically applied Wnt5a was applied to respective skin pieces for 15 minutes before removed or washed off respectively (against respective controls) and left in incubation (37°C) for 5 days, with media changes every 2 days. Following a 5-day incubation, skin samples were embedded in ThermoScientific™ OCT

embedding matrix and sectioned onto glass slides at 14 μM using a Cryostar^{TM} NX70 Cryostat instrument (Histo-line, Milan, Italy).

For skin samples, the slides were washed in 1xPBS for 3 minutes before fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature in the fume hood. The samples were washed once more with PBS for 3 minutes, before 1 mL of 0.3% Triton in 5%. Goat serum (diluted in PBS) was added to each sample which were then left for 30 minutes at room temperature. The primary antibodies: 1:1000 anti-chicken KRT14 (Catalogue No: SIG-3476), and 1:50 anti-mouse KRT9 (Catalogue No: GPHK9-TY1) (ThermoFisher, Waltham, Massachusetts, United States) were diluted in 5% goat to each sample which were then left overnight at 4°C [Table 2.2]. Following overnight incubation, the slides were washed in 1xPBS three times for three minutes per shaking cycle.

The secondary antibodies (1:1000 goat anti-chicken Alexa Fluor 647 and 1:50 goat anti-mouse 568 (ThermoFisher, Waltham, Massachusetts, United States) were diluted in PBS with 5% goat serum and added to samples before incubation for 1 hour at room temperature in the dark. The samples were then washed with 1xPBS three times for three minutes each shaking cycle.
Finally, coverslips were mounted onto slides with Fluoroshield Mounting Medium with 4',6'-diamidino-2-phenylindole (DAPI)(ab104139) (ABCAM, Cambridge, United Kingdom). The samples were left for 48 hours at 4°C before imaging on the Leica SP8 Confocal Microscope instrument, 20 x magnification for skin tissue sections.

2.2.13 Statistics and Reproducibility

All experiments were performed with n=3 or 4 technical repeats (MNs, tissue and cells) and where mentioned N=3 (indicate experimental repeats). Mechanical test results were analyzed and plotted using MATLAB, (`The MathWorks Inc. (2022). Optimization Toolbox version: 9.4(R2022b), Natick, Massachusetts: The MathWorks Inc. https://www.MathWorks.com). All statistical tests, including unpaired two-tailed T-Test, and one-way ANOVA, were performed using Graph Pad Prism Version 10, MAC10, (GraphPad Software, San Diego, California USA, www.graphpad.com). (*P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001). Statistical details for each experiment can be found in the corresponding figure legends. Confocal and fluorescent images (Zeica) were analyzed using ImageJ (Rasband WS., ImageJ, US. National Institutes of Health, Bethesda Maryland, USA).

2.3 Results

2.3.1 Preliminary: Characterizing dimensions of BioMAD Manufactured Microneedle

Polymeric MNs have become an increasingly attractive platform for epidermal drug delivery owing to their non-invasive, biocompatible, and biodegradable nature, in addition to being efficient drug loading tools [Gao et al., 2018]. Among the biocompatible polymeric materials, PEGDA offers a hydrophilic prepolymer solution that can be used to form cross-linked polymeric systems and biocompatible hydrogels, making them suitable materials for MN fabrication [Cavallo et al., 2016; Alafnan et al., 2022]. Moreover, this material is appropriate for the delivery of therapeutic proteins due to its tunable physiochemical properties, and resistance to non-specific protein absorption. Furthermore, the mesh size of PEGDA-based networks can be regulated by varying the monomer molecular weight and concentration as well as by the addition of crosslinker groups [Lee et al., 2016; Peppas and Hilt. 2006; Drury and Mooney. 2003; Peppas et al., 1999; Lin and Anseth. 2009]. Initially I characterised the properties of an available MN mould made by the BioMad Group with an array of 80x 500 µm needles composed of PEGDA (MW 250) [Figure 2.4].

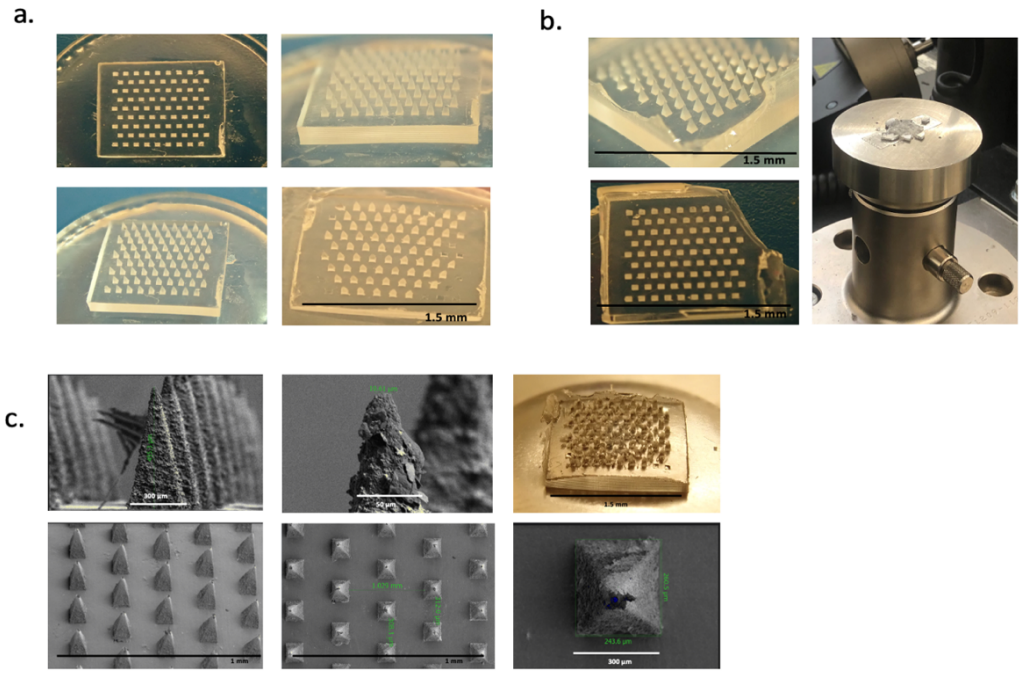


Figure 2.4: Characterisation images of Manufactured MN from BioMAD group. (a) Images at 4x magnification show 80 needles across the array with 1.5x1.5 base dimension. (b) Highlights the poor stability and stiffness of the MN array with missing needles in the final cured product and poor mechanical properties as the device easily breaks upon handling. (c) The SEM images present the dimensions of the needle shank and tip, and the rough morphology of the needles.

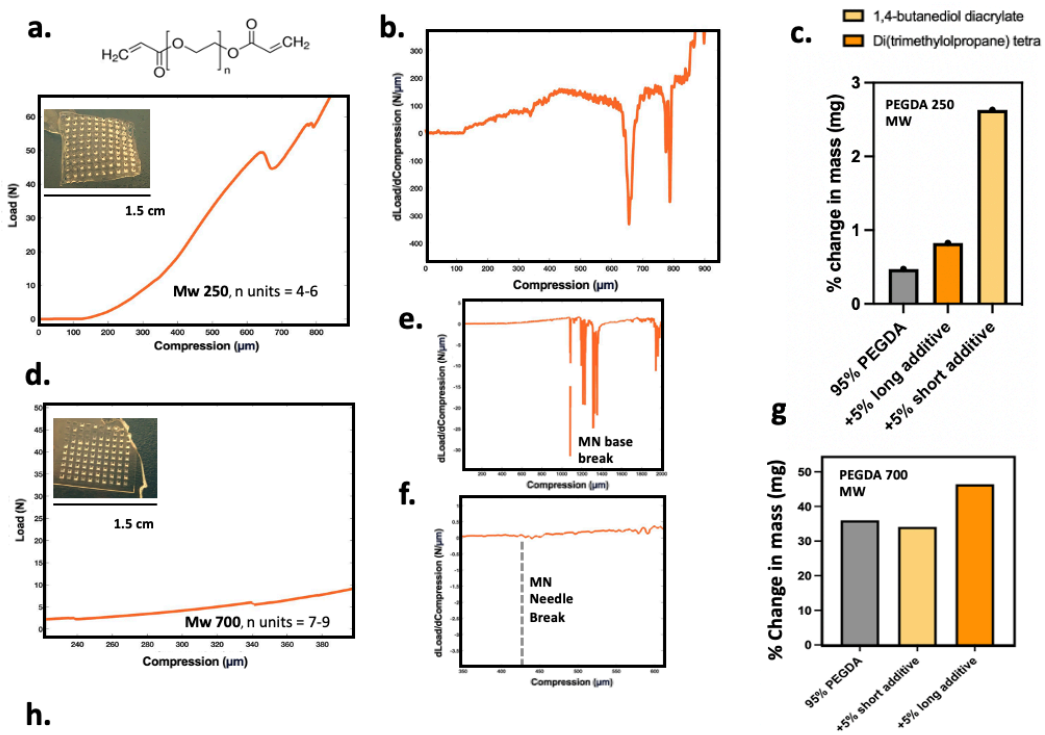
The MN arrays, composed of PEGDA, were photopolymerized in the presence of the photo-initiator Darocur® 1173 [Hien et al., 2018; Ouali et al., 2017] under ultraviolet (UV) light exposure at 365 nm [Shirai et al., 2014] for 3 minutes above and below surface resulting in the cross-linking of the MN array. This strategy has been previously exploited as a highly efficient method of LBL curing of polymeric material [Datta et al., 2021; Shirai et al., 2014]. The BioMAD design exhibited inadequate dimensions that were unsuitable for penetrating the basement membrane of the human skin with short needle shanks and wide needle tips. Therefore, I redesigned the MN following training in this area from the Sensors group at KAUST.

Herein, I designed a MN array of 100 needles alternating in short and tall needle heights (500 μm and 700 μm) [Figure 2.4]. I developed a polymeric MN base made of crosslinked polyethylene glycol-diacrylate (PEG-DA), coated with a layer of pH-sensitive polyacrylic acid (PAA) in which the drug is loaded [Figure 2.3]. I will discuss the design criteria and characterization of my MN device in subsequent sections of this chapter as I sought to achieve optimal mechanical properties, penetration of the human skin, and successful biological application of Wnt5a to skin via my device.

2.3.2 Optimization: Fine-Tuning the mechanical and swelling properties of Polyethylene Glycol diacrylate (PEGDA)- based MN by molecular weight.

PEGDA of differing molecular weight confers different mechanical properties.

When designing and optimising the MN polymeric material, two main design criteria points were critical for optimal performance: sufficient mechanical properties to surpass the stratum corneum, penetrate the basement membrane and epidermal barrier of skin [Donnelly et al., 2010] and minimal swelling ratio to maintain the array geometrical and mechanical integrity while avoiding degradation in a hydrophilic environment [Waghule et al., 2019] [Figure 2.5].



h.

PEGDA sample	Force (N) failure	Sigma (MPa)	Epsilon (µm)	Young modulus (MPa)
MW 250	60	10.2	0.4	25.5
MW 700	12	2.0	0.32	6.25

Figure 2.5: Stiffness and swelling properties of PEGDA differ according to molecular weight. (a) Representative load vs compression graph of PEGDA Mw 250 composed MN base. (b) Representative needles drop failure graph of PEGDA Mw 250 composed MN base. (c) The % change in mass (mg) of PEGDA MW 250 (with and without additive) MN base to assess swelling properties. N=1 D) Load vs compression graph of PEGDA Mw 700 composed MN base. (e) and (f) Needle drop failure graphs of PEGDA Mw 700 composed MN base of base break and needle array break respectively. G) The % change in mass (mg) of PEGDA Mw 700 (with and without additive) MN base to assess swelling properties. N=1. (h) Table of reported force (N) across whole array, sigma, strain and young modulus of MNs made from 250 MW vs 700 MW. (values taken from graphs (a) and (d) respectively.

A variety of polymeric crosslinkers of different molecular weights and structures were used to develop the optimal composition for the MN based with sufficient mechanical properties to enable skin penetration. Firstly, to optimize the mechanical properties and stiffness of the MN array, the crosslinking density was fine-tuned by varying the molecular weight of the hydrophilic crosslinker, PEGDA [Du et al., 2021]. PEGDA, with an average Mn of 250, was first used as the MN base. The chemical composition of this material contains 4-6 repeating (CH_3) units. After crosslinking, the MN formed a curved base, due to the viscosity and compact nature of the material [Figure 2.5a, b]. This curved base confers inconsistencies in compression testing with the force being applied to the needles at different rates. The needles partially failed

at around 60 N, with a normalized failure point of 0.60 N, corresponding to a 40% peak drop at 650 µm conferring a sigma (MPa) of 10.2 MPa and young modulus of 25.5 MPa [Figure 2.5a, b, and h]. Regarding swelling ratio, this formulation led to a less than 2% (w/v) change in hydrophilic environments. While this formulation resulted in geometrical inconsistencies, it offered limited swelling and high mechanical strength, failing at 3.3x the pressure required to penetrate human skin (3.18 MPa) [MCallister et al., 1988; Strambini et al. 2012].

I next investigated the mechanical properties of a larger PEGDA molecular weight (MW=700) which resulted in MN arrays with drastically different properties compared to the same material with low molecular weight [Figure 2.5]. This crosslinker, with up to 7-9 repeating units, resulted in significantly weaker mechanical properties compared to the PEGDA MW 250, with the array breaking at 12 N [Figure 2.5d], equivalent to a normalised failure point of 0.12 N, with base breaking below 1 kg [Figure 2.5e] and corresponding peak drop of individual needles at 425 µm and a sigma (MPa) of 2.0 and young modulus of 6.25 MPa [Figure 2.5f and h]. This highlights the weaker yet consistent mechanical properties of the larger molecular weight PEGDA material to 250 MW and insufficient mechanical force needed to penetrate human skin at 3.18 MPa. This PEGDA crosslinker also generated a greater swelling ratio of over 35% w/v [Figure 2.5g], indicative of the lower crosslinking density or larger mesh size. While the properties were not optimal, this formulation resulted in a more uniform array with a flat base. To maintain the mechanical properties of the 250 MW PEGDA, while achieving the structural integrity of the Mn 700 PEGDA, crosslinking additives were introduced to the MW 700 PEGDA-based formulation. Therefore, I next evaluated the effect of different cross-linking additives on altering the mechanical and swelling properties of the PEGDA MN base.

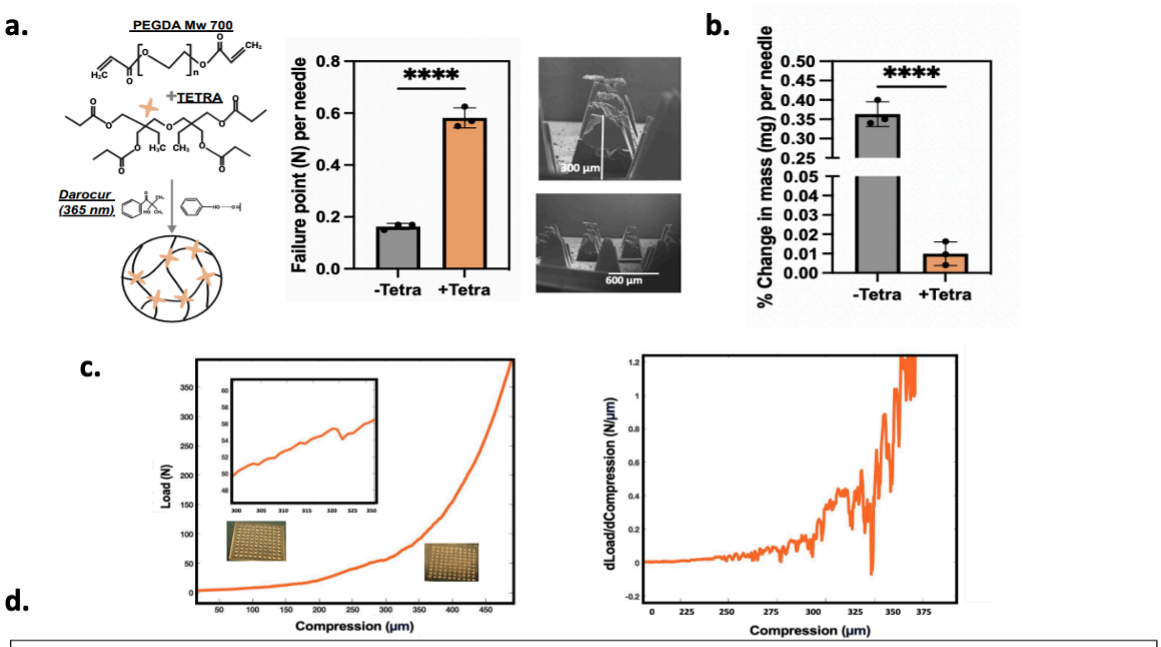
2.3.3 Di(trimethylolpropane) tetra acrylate additive enhances the mechanical properties of Polyethylene Glycol diacrylate (PEGDA)

A Tetra crosslinker additive enhances compression failure point and reduces swelling threshold of PEGDA MN 700

To enhance the mechanical properties of the MN arrays, different crosslinkers were added to the MN 700 PEGDA-based prepolymer mixtures at various concentrations. Using an axial compression test, the failure points of the various MNs were characterized as shown in Figure 2.5. Initially, the crosslinker 1,4-Butanediol diacrylate crosslinker (short crosslinker), with 1-2 repeating methyl (CH_3) units was used; however, a 5% addition of this material was insufficient in improving stiffness or swelling properties of PEGDA MW 700 (Figure 2.5g). A 47.5%

addition of the short cross linker to 47.5% PEGDA 700 raised the stiffness properties of the MN µm base up to >40 N failure point [Appendix 2.1] however the breaking of the needles comparable to figure 2.5a were inconsistent, fracturing at different failure points.

Alternatively, I explored the effect of introducing a more hydrophilic material, namely polypropylene glycol (PPG), to the PEGDA-based formulation. [Kearney et al., 2019]. This was done to compare the swelling properties of a MN PPG base material with a MN PEGDA 700 base material. The MN composed of PPG resulted in poor mechanical strength of the MN array, with needles inconsistently breaking between 10-20 N [Appendix 2.2]. Next, I investigated the impact of the crosslinker with four arms, namely di(trimethylolpropane) tetra acrylate (TETRA) additive on modifying stiffness properties of the PEGDA MN [National center for Biotechnology Information. 2023]. Initially, the crosslinker was used to holistically form the whole MN base to identify the maximum failure point of the array that could be achieved [Appendix 2.3]. Impressively, the MN array failed at 63 N (normalized failure point = 0.63 N/needle), which is comparable to the PEGDA MW 250 formulation [Appendix 2.3]. However, the addition of 47.5% Tetra cross linker to PEGDA 250 MW resulted in the MN base shattering at 20 N due to the brittle nonflexible properties of the combined materials [Appendix 3]. I next investigated the effect of this TETRA cross-linker with PEGDA 700 MW to enhance the mechanical properties and reduce the percentage swelling of the MN array [Figure 2.6].



PEGDA 700 rate (µm/secs)	Force (N) failure	Sigma (MPa)	Epsilon (µm)	Young modulus (MPa)
10	58	9.86	0.4	24.65
25	120	20.40	0.4	51
30	50	8.50	0.4	21.25

Figure 2.6: The addition of di(trimethylolpropane) tetra acrylate to PEGDA Mw 700, enhances mechanical and reduces swelling properties of MN base. (a) Schematic of chemical structure of materials chosen to construct MN base (i.e. PEGDA, and TETRA crosslinker). The normalised failure point per needle before and after addition of the cross-linker additive (47.5%) to PEGDA Mw 700 47.5%) base material (with 5% Darocur photo-initiator). N=3 unpaired T-test. (b) The % change in mass (mg) before and after addition of the cross-linker additive to the PEGDA Mw 700. N=3 (c) Representative load vs compression graph of the MN with Tetra additive. N=3 unpaired T-test. (d) Reported values of force (N) failure, sigma (MPa) Epsilon (µm) and young modulus of MN base formed of 700 MW with TETRA additive failed at different rates (µm/seconds).

Strikingly, the addition of the TETRA crosslinker at the following concentration: 47.5% TETRA, 47.5% PEGDA Mn 700, 5% Darocur® 1173, significantly enhanced the mechanical properties of the MN ($p<0.0001$, unpaired t-test, N=3) [Figure 2.6a]. The MN array had a normalized failure point of >0.58 N at 10 µm/secs [Figure 2.6c Appendix 2.4] >1.2 N at 25 µm/secs [Appendix 2.5], >0.50 at 30 µm/secs [Appendix 2.5]. Different extension rates of the force applied to the MN array were explored to identify the optimal extension rate and the effect of higher insertion speeds on the MN failure point. I postulate that at 25 µm/secs, the polymer chains were unable to realign swiftly enough against the direction of the force, explaining why the failure point at this rate was optimum for high failure point [Bukhamsin et al., 2021; Moussi et al., 2020]. At the 30 µm/secs extension rate, the speed of the force applied

to the MN array is too fast for the needles to withstand. Moreover, across all rates (µm/secs) of force (N) applied to skin, the sigma (MPa) was >2x higher than that needed to pierce human skin, in fact 6x higher at a rate of 25 µm/secs. The young modulus (MPa) also varied from 21-51 MPa across all values, highlighting that the MNs possessed mechanically strong enough properties more than what is required to penetrate human skin.

To assess stability in an aqueous environment, the swelling threshold compared to the standard PEGDA 700 formulation, revealed that the addition of the TETRA cross linker significantly reduced the MN swelling threshold by 95% from >40% to <1.5% wt across the array ($p<0.0001$, unpaired T test, N=3) [Figure 2.6b]. This means that the device is stable in a hydrophilic environment. Overall, with the optimized formulation, my engineered MN array was able to achieve superior mechanical properties compared to previous reports in the literature, where MNs for transdermal drug delivery typically fail at 0.3-0.4 N per needle [Park et al., 2005; Park et al., 2008; Demir et al., 2013; Du et al., 2021]. Having established that my device, once composed of the PEGDA MW 700 material cross linked with TETRA additive, is mechanically strong and stable and thus should be able to effectively withstand force once applied to skin, I next wanted to evaluate its penetration efficacy on human skin.

2.3.4 Optimization: A variable height microneedle device designed to elude the 'bed of nails' effect.

A variable height MN device overcomes the 'bed of nails' phenomenon.

Having selected and characterised optimal materials for my MN base I next wanted to assess its penetration efficacy on human skin. I aimed to establish a MN array that successfully and easily penetrates the basement membrane of human skin that would thus result in specific drug delivery to the epidermal basal layer and/or papillary dermis. My 100 MN array base was designed to have individual needles that are 500 µm and 700 µm in height to enable successful penetration of the drug to the skin basement membrane (20-50 nm thickness) surpassing epidermis (up to 100 µm thickness) [Crowther et al., 2008; Lademann et al., 2008; Richter et al., 2004; Moussi et al., 2020; Kafa].Variable heights of short and tall needles, with an equal spacing of 600 µm between each individual needle base, and 300 µm width of needle base can overcome the 'bed-of-nails' effect [Gomaa et al., 2012]. This effect restricts skin penetration

efficacy as the pressure exerted by each individual needle tip is reduced to an insufficient level to penetrate the skin [Gomaa et al., 2012; Makvandi et al., 2021].

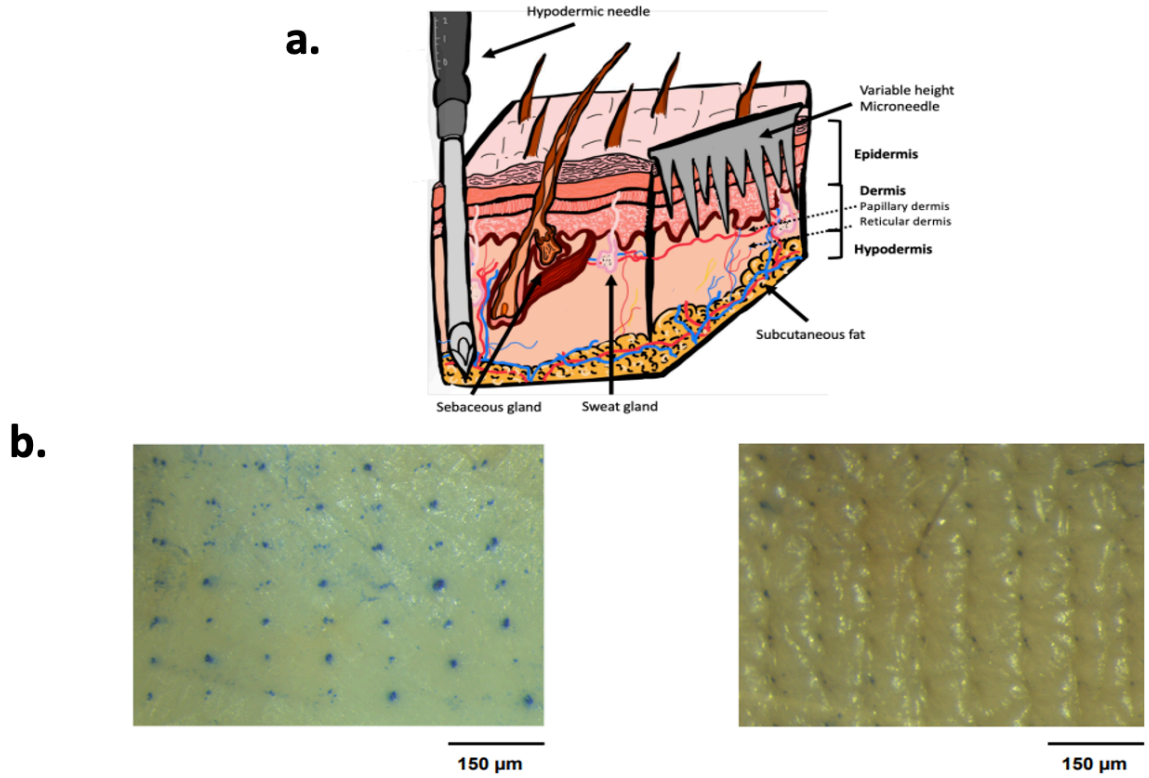


Figure 2.7: The variable design of the MN array enables penetrates of human skin. (a) Schematic of MNor hypodermis needle to skin. (b) Penetration test of variable height MN array (left) and consistent needle MN array (right) on ex vivo human skin using trypan blue N=1.

To elude the bed of nails effect that polymeric MNs commonly present [Gittard et al., 2013], I designed and constructed a 10x10 array of 100 alternating short (500 µm) and tall (700 µm) MNs. The choice of 500 µm needle shank dimensions was designed with the intention to deliver compounds specifically and locally to the epidermal layer which topically applied compounds cannot penetrate down to. Comparatively, the dimensions of the 700 µm tall needles were designed to penetrate the basement membrane to reach the papillary (upper) dermis, which is surpassed during hypodermic needle delivery [Figure 2.7a]. Using trypan blue which stains collagen within the skin dermis, I observed darker blue spots every other spacing likely induced by the 700 µm needles, with light blue staining induced by 500 µm needles, highlighting that my designed geometry ensured that the bed of nails phenomenon was eluded. This suggests that my alternating needle height design was successful not only in surpassing the stratum corneum (via 500 µm needles) but also in penetrating the basement membrane with alternating 700 µm needles [Figure 2.7b (left)]. In contrast, a consistent 10x10 array of 700 µm

needles was unable to penetrate the basement membrane [Figure 2.7b (right)] instead compressing the skin surface. My needles are thus characterized as minimally invasive as they elude deep penetration unlike 2-3 mm microneedles that pierce blood vessels and nerves within the reticular dermis [Ita. 2014].

Following the successful establishment of my MN base in eliciting strong mechanical properties and importantly eluding the bed of nails effect to effectively penetrate the stratum corneum and basement membrane, I next investigated the suitability of PAA as a coating material to encapsulate and deliver drugs of interest to skin.

2.3.5 Design of Coating, and validation of release using artificial skin model, and pH-sensitivity

A PAA hydrogel coating on the MN base alters the morphology of the microneedle device.

Coated MNs offer a versatile and promising strategy for drug delivery MNs, as they ensure the bioactivity of the desired drug is conserved with minimal or no interference from the materials used in the MN base construction [Gill et al., 2007; Migdadi et al., 2018]. With a coated design, the same MN patch can be utilized for the delivery of a wide range of materials from peptides and proteins to DNA, viruses and other microparticles, depending on the desired application [Gill and Prausnitz, 2007].

Importantly, coated MNs can be designed to enable controlled release of the therapeutic cargo under certain conditions and release rates [Makvandi et al., 2021]. There are several types of polymers in the literature with stimuli responsive activity, meaning they can change their intrinsic properties in response to a variety of extrinsic physicochemical factors such as light, pH, temperature, chemical environment, electrical potential, magnetic field, mechanical stress, and ionic strength [Cao et al., 2015]. Given that the uppermost layer of the skin, the stratum corneum, has a slightly acidic pH (pH > 4) and deeper skin layers have more basic pH levels (pH>7-9) [Oliva and Almquist. 2020] I designed my MN to be pH responsive that irrespective of the drug size or charge [Swift et al., 2016] [Figure 2.8a] drugs would diffuse down into the skin upon MN application.

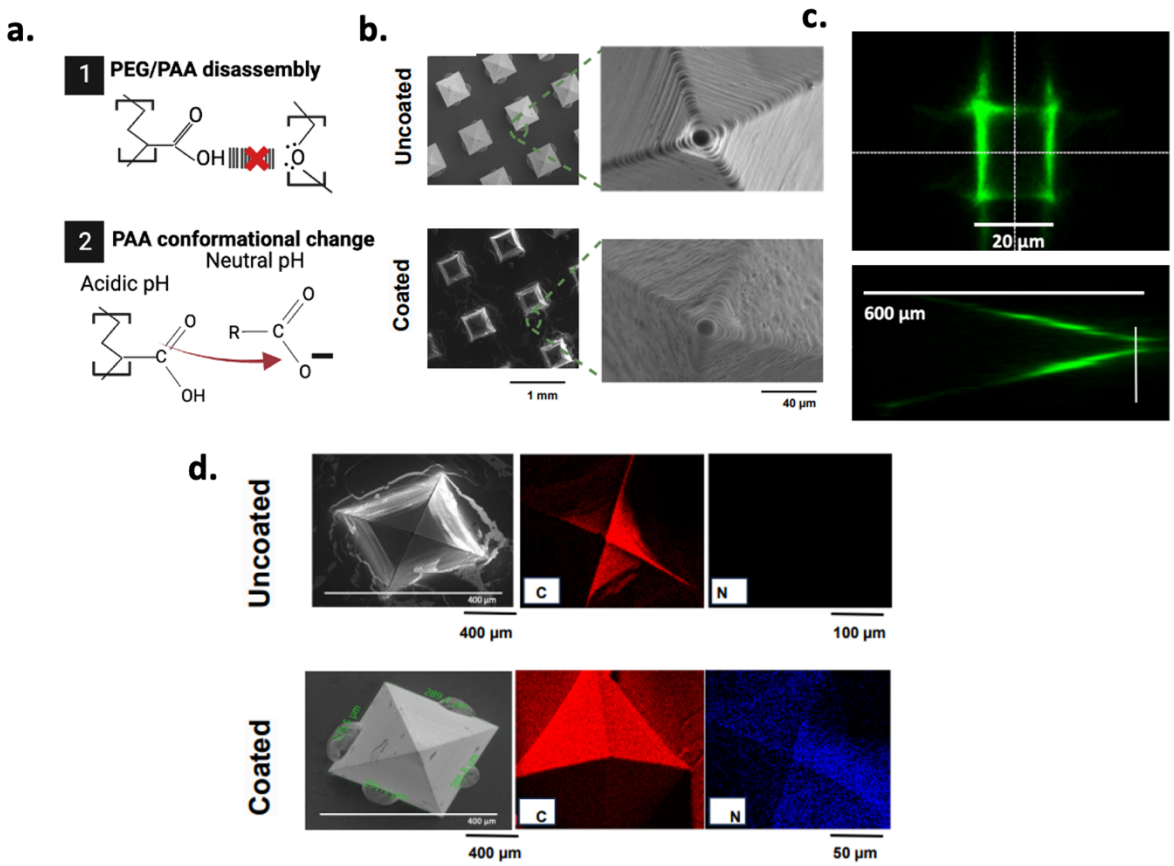


Figure 2.8: PAA forms a filmy layer over needle tips to encapsulate drug of interest. (a) Chemical interaction between PEGDA and PAA, (b) SEM images of coated (BSA-FITC, PAA) (N=1 and uncoated MNs, (c) coated needle shank and tip (700 µm needle) (N=1) (d) EDX representative images of elemental composition of PAA BSA FITC coated MN and uncoated MN (N=1)

I designed the MN coating to be responsive to pH such that drug delivery is only triggered after penetration of human skin. Another important consideration is that the coating remains stable on the MN base prior to skin insertion. Herein, my design is based on a pH-responsive polymer coating material made of the pH-responsive polyelectrolyte, PAA, which alters the morphology of the constructed needles. PAA was also chosen as a stable coating layer on the PEGDA-based MN base as these two materials can form a stable hydrogen bonding complex [Buron et a., 2020; Swift et al., 2016]. This hydrogen bonding interaction is pH-responsive in that under a neutral to high pH environment (pH 7-9, for epidermis and dermis,) the hydrogen bonds are disrupted and the PAA coating is disassembled from the base [Buron et al., 2020] [Figure 2.8a]. A pH-responsive coating material also ensures the cargo is released in a specific environment in response to a specific stimulus. The stratum corneum is acidic (pH<4-6) while the denser under-layers, epidermis, and dermis, are within neutral to high pH (pH<7-9) [Ali and Yosipovitch., 2013]. A PAA coating is adopted in my MN construction as it contains a carboxylic acid group (COOH) that is de-protonated to (COO-) under neutral or more basic conditions. This de-protonation triggers electrostatic repulsion between adjacent polymers

causing conformational changes of the PAA from a compact (globule) to an extended conformation, leading to the release of drug cargo [Swift et al., 2016; Zaibudeen et al., 2018]. The drug is thus expected to be released from the polymeric matrix and diffuse through the epidermis to the dermis down its concentration gradient.

I firstly validated the coating of the MN with PAA.The dip-coating technique is considered the simplest method for MN coating as it is cost-effective and can easily adjust for layer thickness of the coating [Zhang et al., 2012; Ingrole et al., 2019; Liang et al., 2020; Haj-Ahmad et al., 2015]. Fundamentally, this process allows for fine tuning of the coating specifically to the tip of the needle rather than the shank [Liang et al., 2020]. To study the effectiveness and stability of the coating strategy, fluorescence and electron microscopy was used. Initially, fluorescein isothiocyanate (FITC) was diluted in 0.035% wt PAA, and the dye-embedded polymer was applied to the tip of MNs via a dip coating method. Fluorescently labelled bovine serum albumin (BSA), namely BSA-FITC, was used in preliminary studies as a model protein-based drug. 1 μM of PAA BSA-FITC was diluted in 0.035% wt of PAA and coated on the array as previously described in methodology.

SEM images showed the efficacy of the PAA-based coating and demonstrated the differences in the needle tip morphology when uncoated, and coated with BSA-FITC in PAA [Figure 2.8b]. There appeared to be a bumpy and coarse texture at the needle tip with the coating of BSA-FITC PAA (coated MN) compared with smooth texture of uncoated needles [Figure 2.8b]. Confocal microscopy images across the shank of the needle, (700 μM), illustrate the coated exterior of the needle, and an increase in fluorescence intensity transcending further toward the tip of the needle [Figure 2.7c]. The z-stack imaging of a coated 700 μM needle indicated that the PAA-BSA FITC coating was up to >350 μM along the shank of the needle which is sufficient for drug delivery to the epidermis and the subdermis. Energy dispersive x-ray (EDX) was also used to qualitatively confirm the presence of the BSA-FITC within the PAA coating on the MN array [Figure 2.7d]. EDX scans of the BSA-FITC PAA coated needles displayed the presence of nitrogen homogeneously distributed on the needles, which is attributed to nitrogen from the peptide bonds in BSA which overlapped with carbon from the PAA coating itself.

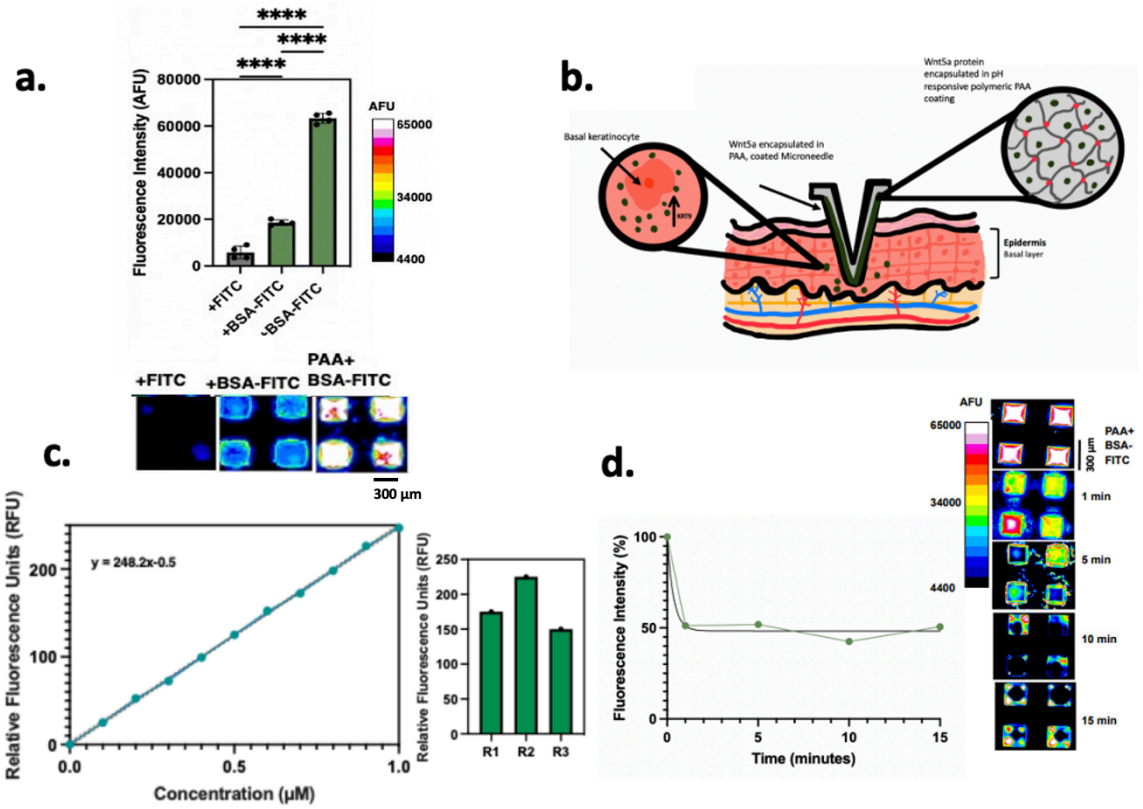


Figure 2.9: PAA can deposit drug of interest in neutral pH environment. (a) The AFU of three individual MNs coated with FITC, BSA-FITC or PAA-BSA FITC (d) Schematic of coated MN application to skin. (one way ANOVA) (N=3) (b) Standard curve (line of best fit) of concentration of BSA in PAA FITC, green lines indicate RFU readout after 2-hour PBS wash corresponding to graph on right (N=1) (c) % Fluorescence intensity of MN and with representative images before and after PBS washing (in descending order) 1 minute, 5 minutes, 10 minutes, and 15 minutes (N=3) (one phase decay fit).

Efficient dip-coating of PAA-BSA FITC was confirmed by studying the fluorescence intensity of FITC coated only, BSA-FITC and PAA-BSA FITC coated MN arrays. This analysis showed limited intensity in FITC-only coated needles, variable staining of BSA-FITC coated needles due to the adhesive properties of BSA [Berchane et al., 2008], and a high statistically significant fluorescence intensity increase only in the BSA-FITC in PAA coated needles ($p<0.0001$, $n=4$) [Figure 2.9a]. Having confirmed that PAA is successful in encapsulating BSA and adhering it to the needle exterior I next wanted to investigate the pH responsiveness of the PAA in neutral pH, and thus its ability to release BSA under specific conditions for localised drug delivery.

The shift in pH which occurs upon skin penetration affects the charge on polymer chains, leading to network swelling and thereby triggering drug release [Schmalijohann. 2006; Seeli et al., 2016; Hezaveh]. If BSA could successfully be released by PAA upon change in pH in the surrounding environment I could then utilize this material to deliver Wnt5a to the human skin for KRT9 upregulation [Figure 2.9b]. To identify release of BSA-FITC from PAA I dip-coated 3 different MNs in PBS for 2 hours and measured the RFU readout before and after wash. This revealed an average of >80% BSA protein release (0.8 μM of protein) across the standard curve, as >175 RFU corresponds to >0.7 μM BSA-FITC [Figure 2.9c]. A PAA BSA-FITC coated needle washed with PBS in timed intervals, 1-, 5-, 10-, 15- minutes revealed >50% reduction in fluorescence intensity (corresponding representative images before and after wash from top to bottom), suggesting these timed intervals in neutral buffer are sufficient to release drug of interest from the needle array [Figure 2.9d].

The absorbance of three independent PAA BSA FITC coated MNs presented comparable findings [Appendix 2.7]. This result is attributed to the deprotonation of the carboxylic acid group (COO^-) in a neutral pH which induces severe electrostatic repulsion between neighbouring groups, subsequently resulting in extensive swelling and cargo release. Having successfully evaluated that PAA forms a coating material on MN tips that encapsulates proteins, I next wanted to assess the extent of pH responsiveness of the coating material at varying pH, to ensure the encapsulated drug can be released in specific pH environments (>neutral) but not in low pH environments [Figure 2.10].

2.3.6. Investigating the pH responsiveness of the MN Coating

PAA hydrogel is pH responsive ensuring encapsulated protein is only released in neutral and high pH environments.

To confirm extent of pH responsiveness of the PAA hydrogel, the effect of various pH buffers on PAA coated needles were explored using the % fluorescent intensity readout [Figure 2.10].

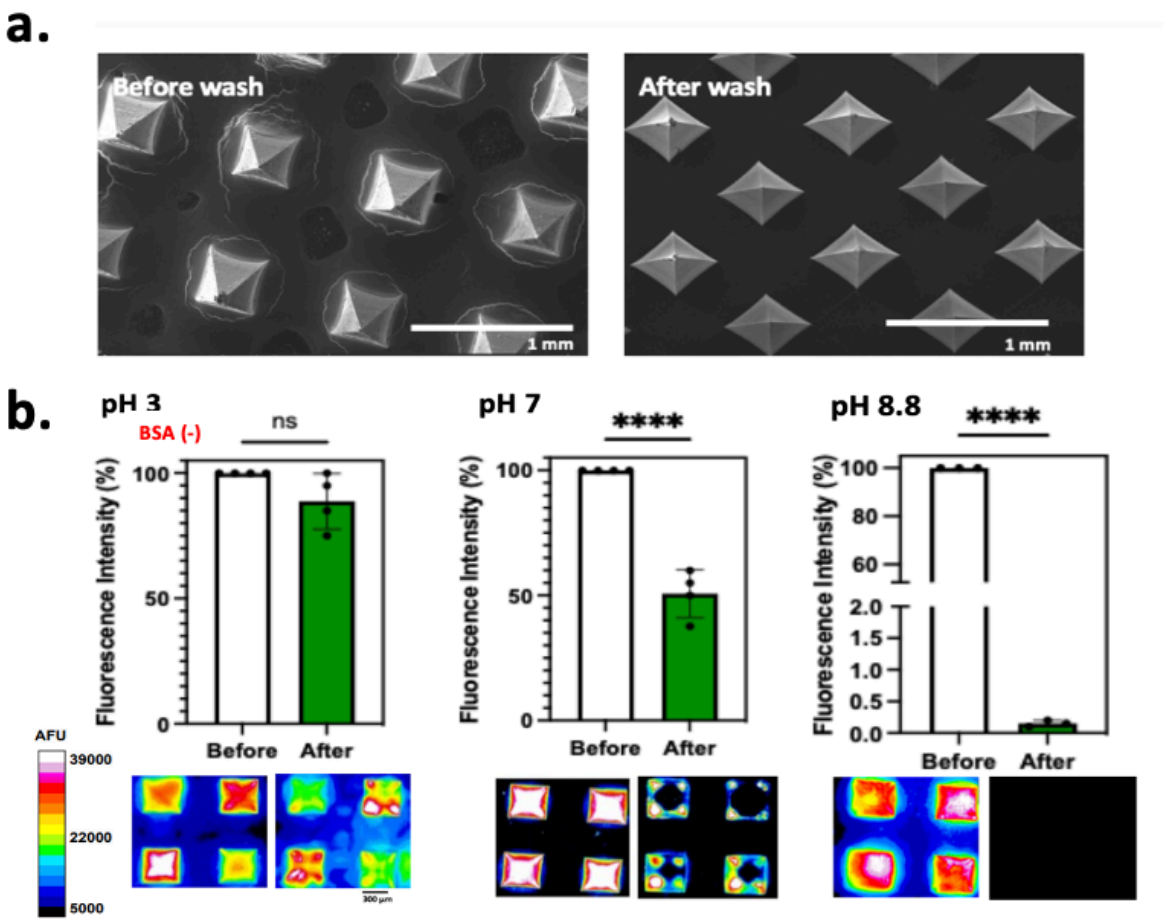


Figure 2.10: PAA is pH responsive, and drug of interest only diffuses out of hydrogel in slightly neutral or alkaline conditions. (a) SEM of coated MN (PAA BSA FITC) before and after 2-hour PBS washing. N=1 (b) % fluorescence intensity of three independently BSA-FITC in PAA coated MNs before after pH specific buffer, from left to right: 1x MES, 1xPBS and 1x TE buffer with representative images before and after wash. N=3 unpaired t-test.

The extent of the pH responsiveness of the PAA coated needles was assessed by dip-coating the MN array in different buffers of varying pH, including 2-(N-morpholino)ethansulfonic acid (MES) with pH=3, 1xPBS with pH=7, and 1xTris EDTA (TE) with pH=8.8. Interestingly, the morphology of PAA BSA FITC coated needles was altered in response to 2-hour PBS wash, revealing a smooth texture [Figure 2.10a]. This highlights the effectiveness of PBS (neutral pH) in washing away PAA. I found that at pH 3, in MES buffer, there is no significant difference in the fluorescence intensity (%) before and after dip coating in the wash [Figure 2.10b]. Only around 10-20% of BSA protein is released after a 15-minute MES wash [Figure 2.10b]. Notably, after a 15-minute PBS (with HCL to achieve pH 7) wash, at least >50% of fluorescence intensity (%) is lost from the array [Figure 2.10b]. However, a statistically significant difference ($p>0.0001$) was observed before and after a 15-minute wash 1xTE buffer, revealing 90% decrease in fluorescence intensity [Figure 2.10b].

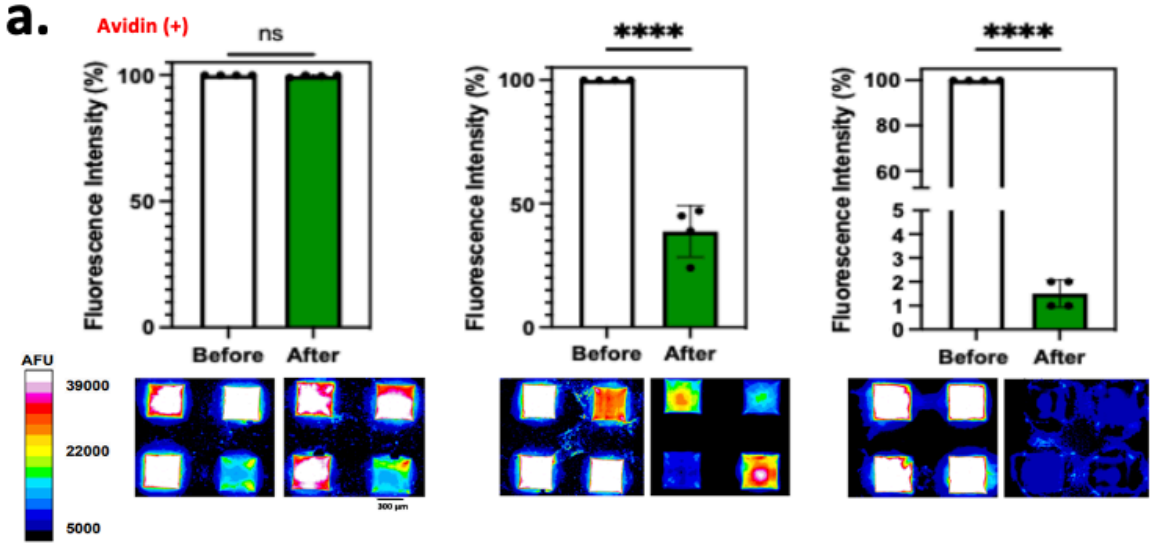


Figure 2.11: PAA is pH responsive, and can deposit positively charged drugs in neutral to high pH environments.. (a) % fluorescence intensity of three independently Avidin-FITC in PAA coated MNs before after pH specific buffer, from left to right: 1x MES, 1xPBS and 1x TE buffer with representative images before and after wash. N=3 unpaired t-test.

To confirm that the drug release mechanism is not affected by electrostatic effects or the cargo's net charge, a protein of similar molecular weight but different net charge was investigated. Experiments of protein coated needles were repeated with Avidin-FITC PAA, as avidin has a comparable size (MW = 66Kda) to BSA but a net positive charge. The results confirmed that the drug is released in a pH-responsive manner, irrespective of the overall charge of the protein drug as shown in Figure 2.11a, and 80-90% of drug was deposited off the needle tip in neutral to alkaline pH environments. The viscosity of both differing charged proteins in 0.035% wt PAA was assessed which showed comparable mPa*S to water or PBS [Appendix 2.7] suggesting it to be low in toxicity and is expected to preserve bioactivity of proteins. Therefore, having successfully evaluated that PAA is a suitable carrier material for drug delivery in pH specific environment I wanted to next access drug diffusion of the coated needles into skin like material.

2.3.7. Designing a GelMA artificial skin mode for drug diffusion validation studies. Gelatin methacryolate exhibits compatible properties to human skin and thus is an effective model for drug diffusion studies.

Optimization of the MN platform requires extensive characterization and validation studies using skin samples yet sourcing enough of such clinical samples can pose a major hurdle to timely development. It was therefore necessary to develop an artificial model to emulate human skin in its mechanical and physicochemical properties [Figure 2.12].

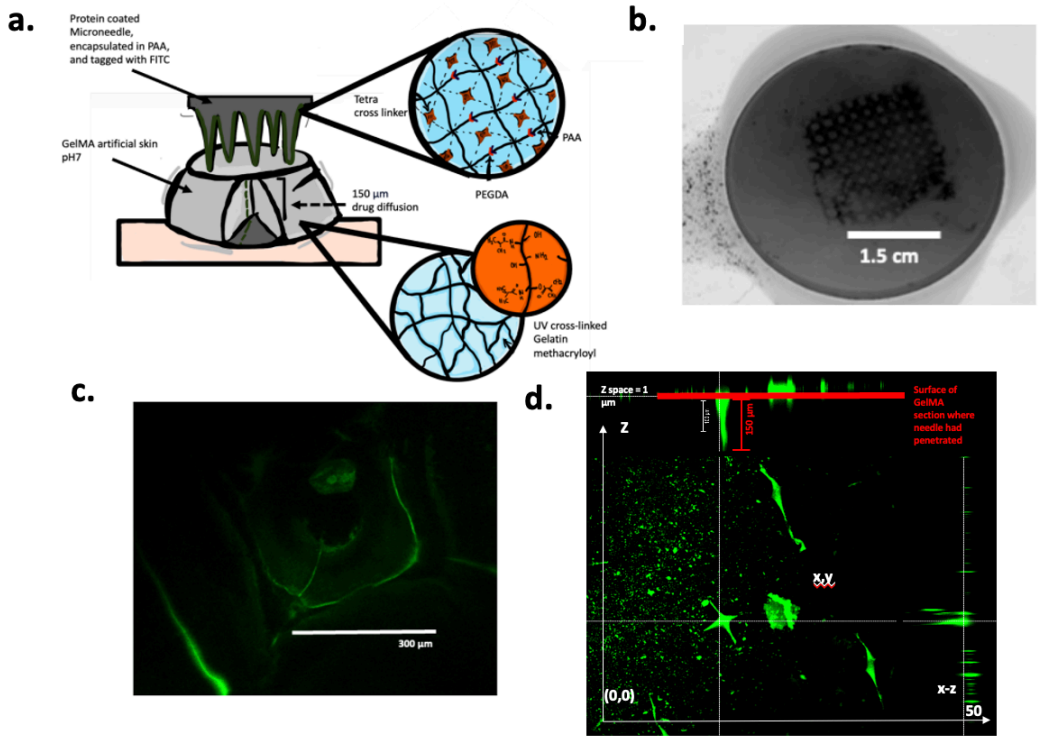


Figure 2.12: MNs successfully pierce GelMA model. (a) Schematic illustration of the material composition, and application of MN onto synthetic GelMA model. (b) Representative image of coated MN stain on GelMA surface generated from gel image scanner. (c) Confocal image of PAA-BSA FITC compression print on GelMA surface after 15-minute application. (d) Z stack graph of measured fluorescent release from needle tip into GelMA after 15-minute application of MN on surface (red line indicates surface of GelMA which needle penetrates 150 µm deep (10 microns per stack of 50-micron stack).

To assess needle penetration properties [Figure 2.12a], drug diffusion properties [Figure 2.12d] I developed a multilayer artificial model for skin based on gelatin methacryolate (GelMA) coated with a layer of parafilm [Figure 2.12a, b, and c]. GelMA was chosen to mimic human skin epidermis and dermis due to its optically transparent nature, biocompatible properties, and similar mechanical properties compared to human skin as well as being neutral in pH [Figure 2.12a] thus equitable to human skin [Piao et al., 2021]. Indeed, GelMA has been used for skin regeneration as it possesses similar mechanical and degradation properties to the dermal extracellular matrix, even acting as a more promising substitute to other hydrogels [Figure 2.12a], i.e., collagen [Zhao et al., 2016]. Applying my established MN device to GelMA successfully pierced the optically transparent material, as visualized by the stained (from FITC coated needle array) print left and imaged on the surface of GelMA (after MN array had been applied for 15 minutes on surface then removed) [Figure 2.12b], and confocal imaging of the print of a FITC tagged Wnt5a in PAA coated MN on the GelMA [Figure 2.12c]. Notably, a

FITC-tagged Wnt5a in PAA coated MN array administered to the synthetic material (for 15 minutes) resulted in drug diffusion of >150 µm [Figure 2.12d].

GelMa model confirms pH responsive release of drug of interest from PAA coated microneedle array.

Using my GelMA synthetic material, I applied a FITC tagged Wnt5a coated MN to the material, for 15 minutes before removing, as Figure 2.10b, and Figure 2.11a,confirmed that 15 minutes is sufficient in depositing majority of the drug material) to evaluate the penetration efficacy of my MNs and the drug diffusion of Wnt5a into the neutral pH synthetic skin like material.

Timed administration of the Wnt5a, tagged with FITC and dissolved in PAA, coated MN array to the synthetic skin demonstrated increasing release of the cargo with longer timed MN application to the model, of up to >90% drug release after 15 minutes [Figure 2.13a].

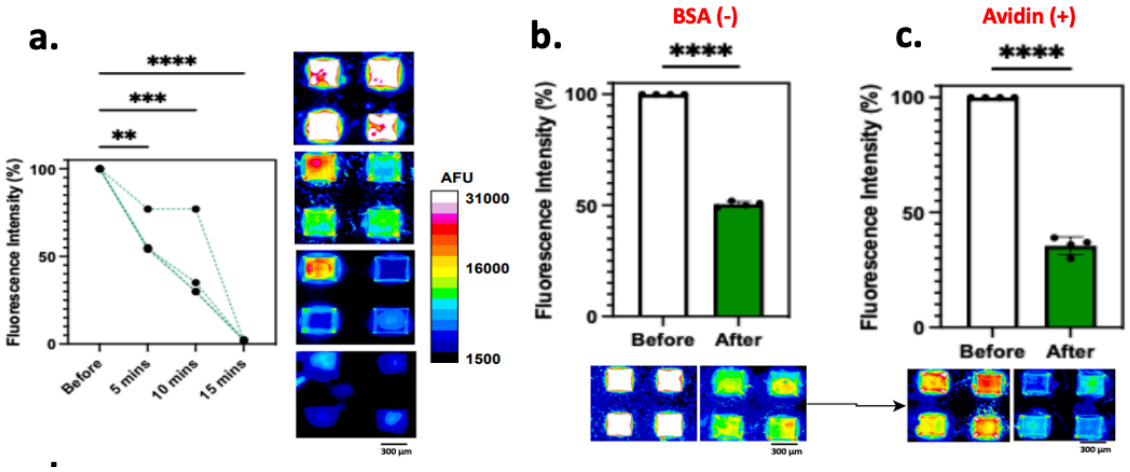


Figure 2.13: Drug coated MNs successfully deposited into GelMA. (a) % Fluorescence intensity of three different Wnt5a-FITC tagged, in PAA coated MNs before and after timed application onto GelMA surface, 5, 10 or 15 minutes with representative images in descending order. (b, c) % Fluorescence intensity before and after 15-minute application of (b) BSA-FITC, in PAA coated MN, (c) Avidin-FITC in PAA coated MN.

To investigate if these findings are equitable to other drugs and thus my device could be used for the delivery of other compounds outside of Wnt5a, I also delivered BSA (-) and Avidin (+) to GelMA using my MN. I assessed the biowaste of BSA and Avidin coated MN on GelMA (applied for 15 minutes) synthetic material by observing % change in fluorescence intensity [Fig 2.13band c]. This identified equitable findings to drug release of coated needles in PBS, with >50% decrease in fluorescence intensity irrespective of protein charge. This indicates that regardless of charge and size of the compound, the cargo should diffuse down the concentration gradient from the MN needle tip into the 3D material. Overall these findings confirm that not only that my constructed design is composed of pH responsive materials, but also reduces the potential of bio-waste of my drug of interest in handling of the device, as the drug of interest will only be diffused out of the encapsulated PAA coated when encountering a high pH (>pH 7) environment within the deeper layers of the skin (epidermis and dermis) not the surface of the skin (pH 4).

Insertion point results of microneedle on parafilm to emulate stratum corneum.

Having established that my device can successfully penetrate the GelMA material (emulating epidermis and dermis) I next investigated the penetration efficacy of my needles to the stratum corneum. The stiffness properties of the stratum corneum is much greater than deeper tissues, therefore skin physical resistance is expected to drop significantly when needles penetrate to the skin underlayers (Young modulus of epidermis including stratum corneum is 4 MPa, (stratum corneum alone is 1.993-4 MPa, dermis is 40 kPa and hypodermis is 15 kPa) [Feng et al., 2022; Hara et al., 2013; Prausnitz. 1996; Boyle et al., 2019].

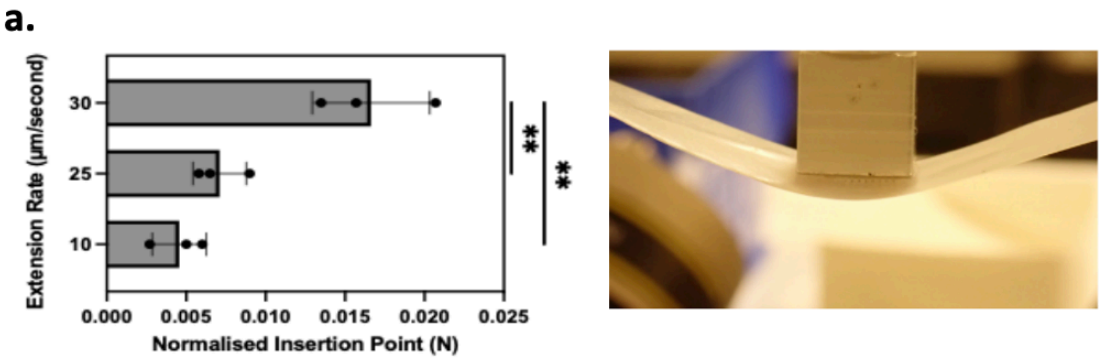


Figure 2.14: Parafilm insertion point.. (a) The normalised insertion points of MN arrays on parafilm at 10, 25 and 30 µm/second. (right) Representative image of needle insertion point (N) testing of MN array on parafilm to emulate stratum corneum.

Parafilm was used to mimic the stratum corneum as it has previously been used to assess insertion forces of MNs as an alternative to human skin [Larrañeta et al., 2014] [Figure 2.14a]. Parafilm possesses a stiffer Young's modulus (E) compared to human skin, with E=30-60 MPa (thickness of 120 µm) [Qu et al., 2016] compared to 4 MPa (thickness of 10-30 µm) for the stratum corneum [Feng et al., 2022]. I performed a tensile compression test which involved applying my MN to parafilm (held by two stands) using a loading cell, to identify the insertion forces of the needles to the stratum corneum-like material.

In my study, the normalized insertion force of the needles was <0.01 N (10 µm/second) to <0.01.5 – 0.02 N with increased extension rate (25 µm/second) with needles being able to withstand the force, remaining unbreakable [Figure 2.14a]. A failure force of a 700 µm needle penetrating the stratum corneum is reported to be 0.22 N per needle [Park et al., 2008]. Additional studies have shown that a MN with a shank height of 720 µm had a normalized insertion force of 0.08 to 3.04 N in the stratum corneum of three human skin subjects [Davis et al., 2004], and around 0.345 N insertion force when applied to the stratum corneum at a thickness of 30 µm [Kong et al., 2011]. This indicates that my constructed needles should easily be able to penetrate the stratum corneum, which is the primary barrier to intradermal drug delivery, without the needles breaking. Having successfully validated that my device is mechanically strong, my coating material is pH responsive and an appropriate carrier for Wnt5a and that Wnt5a should readily diffuse into the skin, having used GelMA as a model, and should surpass the stratum corneum having used parafilm, I next needed to evaluate the biological efficacy of Wnt5a. I validated this on human keratinocyte cells, followed by human skin to thus assess the suitability of the MN as a translatable therapeutic device.

2.3.8 Assessing the biological efficacy of my microneedle on *ex vivo* human skin.

Validating biological activity of Wnt5a on keratinocytes in upregulating KRT9

It has previously been reported that Wnt5a can switch on KRT9 gene expression within skin keratinocytes thus transforming them from a nonplantar to a plantar identity [Alam et al., 2011; Kozera and Rapacz, 2013; Kim et al., 2016; Rinn et al., 2008; Popp et al., 2014; Garza et al., 2011; Wang et al., 2018]. I investigated via qPCR and immunostaining whether Wnt5a induces expression of KRT9 in keratinocytes [Figure 2.10], to confirm what is previously published [Rinn et al., 2008; Garza et al., 2011].

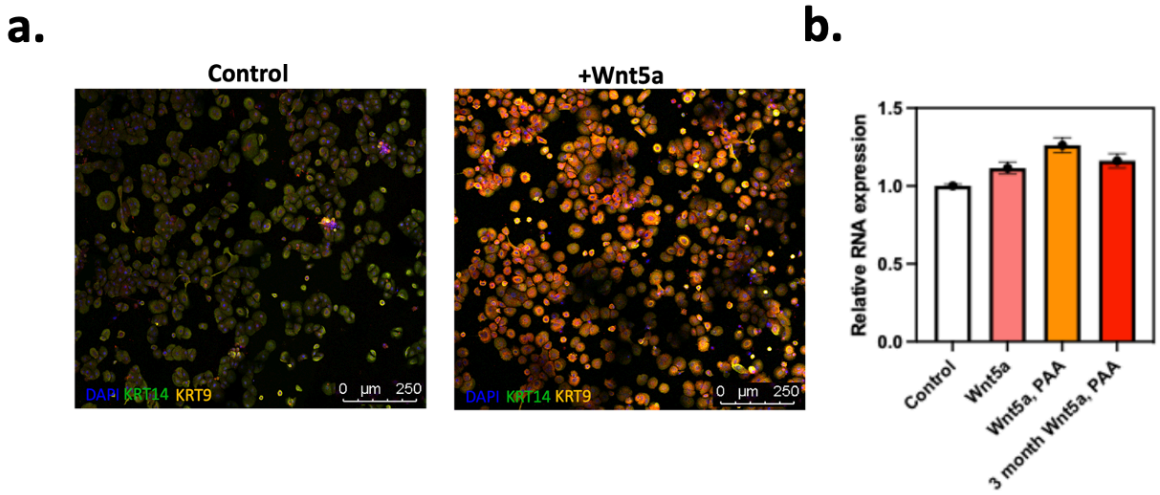


Figure 2.15: KRT9 expression immunostaining with Wnt5a protein administration to cells (a) Keratinocyte immunostaining (green KRT14 and orange KRT9) with upregulation of KRT9 in response to Wnt5a treatment to cells against untreated control (N=1). (b) Relative RNA expression of KRT9 in untreated, Wnt5a (in medium), Wnt5a in PAA, and 3 months stored Wnt5a in PAA treated keratinocytes (in PAA) keratinocytes *in vitro* (N=1).

Immunostaining revealed that KRT9 expression is enhanced in response to Wnt5a in human keratinocyte cells, as is absent in the control [Figure 2.15a]. This confirms previous reports that Wnt5a can switch on the expression of KRT9 in basal keratinocyte cells.

I next evaluated whether Wnt5a remains biologically active in the coating carrier material, PAA. To assess the stability of Wnt5a in freeze dried PAA, I cultured primary basal layer keratinocytes in Wnt5a (KSFM only), Wnt5a in fresh PAA, and Wnt5a in PAA that had been stored for 3 months at 4°C [Fig 2.15b]. I found that Wnt5a encapsulated PAA that had been stored at 4°C degrees for 3 months induced KRT9 expression in keratinocytes to a comparable level to Wnt5a stored in fresh PAA, or Wnt5a in medium only, against untreated control (fold changes varying from 1.1-1.3 compared to control). Notably, Wnt5a in fresh PAA induced

higher KRT9 expression than Wnt5a in medium only (1.1 against 1.3-fold change). This confirmed that PAA the bioactivity of the Wnt5a protein which has a natural short half-life of 140 minutes in the circulatory system [Abedini et al., 2020). The small fold changes in KRT9 expression in response to Wnt5a are expected as the keratinocytes are isolated from non-plantar tissue (confluent keratinocytes were treated with Wnt5a and left to incubate for 24 hours before RNA extraction was performed) and majority of the cells are not suprabasal. Next, I applied my Wnt5a coated MN device to human skin to assess its delivery and biological function.

2.3.9 The constructed microneedle is significantly more effective in delivering a drug of interest to epidermis than topical treatment.

To assess biological activity of my Wnt5a coated MN device I used *ex vivo* human skin as an application [Figure 2.16].

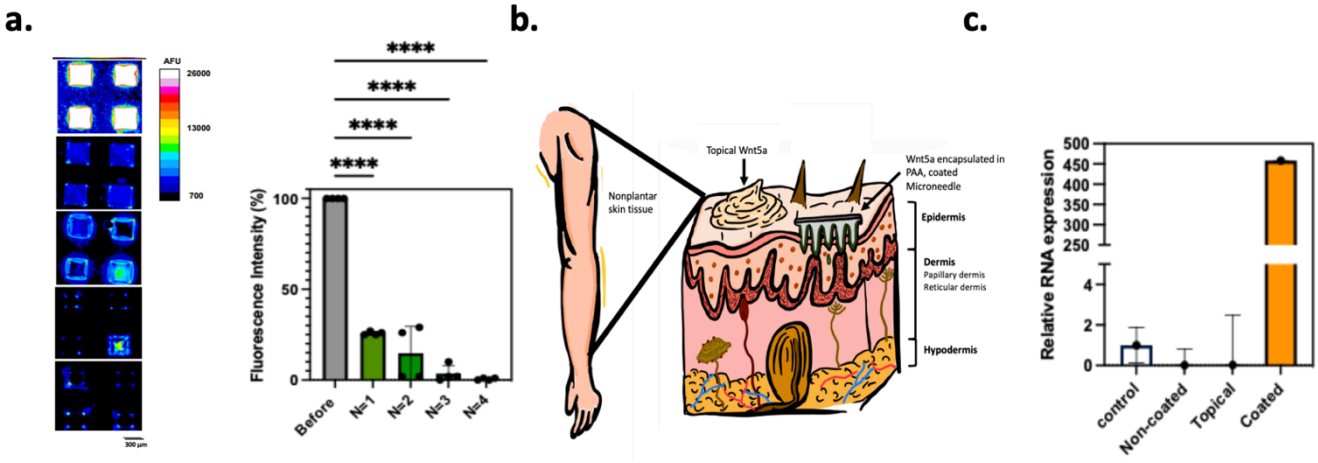


Figure 2.16: Biological function of Wnt5a coated MN on ex vivo human skin was observed. (a) % fluorescence intensity of Wnt5a coated MN to N=4 of *ex vivo* skin sample with representative fluorescent images on left in descending order: before application, and after 15 minutes. (N=4 one-way ANOVA). (b) Schematic of topical vs coated MN application to ex vivo skin. (c) Relative RNA expression of KRT9 expression in response to topical Wnt5a or Wnt5a-PAA coated MN application to *ex vivo* skin (N=1).

I firstly investigated the drug diffusion properties of my Wnt5a coated MN on several human *ex vivo* skin samples (4 biological replicates) to assess whether Wnt5a would be able to diffuse into the model. I found that PAA FITC-tagged Wnt5a could be successfully released after MN application on *ex vivo* skin (N=4) [Figure 2.16a]. Fluorescence intensity % measurements of coated MN before and after application to the skin (15 minutes), showed >90% release (<0.0001, N=4 skin samples) [Figure 2.16a] from the MN. This highlights that

that Wnt5a is successfully deposited from the needle array into the skin however whether it is eliciting its biological function (in enhancing KRT9 expression) was not yet known.

I next explored whether MN Wnt5a-coated delivery is more effective than topical delivery of the protein, in enhancing KRT9 expression [Figure 2.16b]. Wnt5a coated MN or topical Wnt5a was left on *ex vivo* skin pieces for 15 minutes only, and skin was left to incubate for 24 hours before homogenised, and RNA extraction was performed. My qPCR analysis of skin shows that KRT9 expression is upregulated ($p>0.0001$, $n=3$, $N=1$) in MN coated Wnt5a treated skin, following 24-incubation compared to both the Wnt5a topical application, uncoated MN control and untreated control (>450-fold change) [Figure 2.16c]. Overall, these findings suggest that my MN is more effective in delivering the drug of interest to *ex vivo* human epidermis than topical treatment, illustrating its biological efficacy and capacity to surpass the stratum corneum and induce localized gene expression within the epidermis.

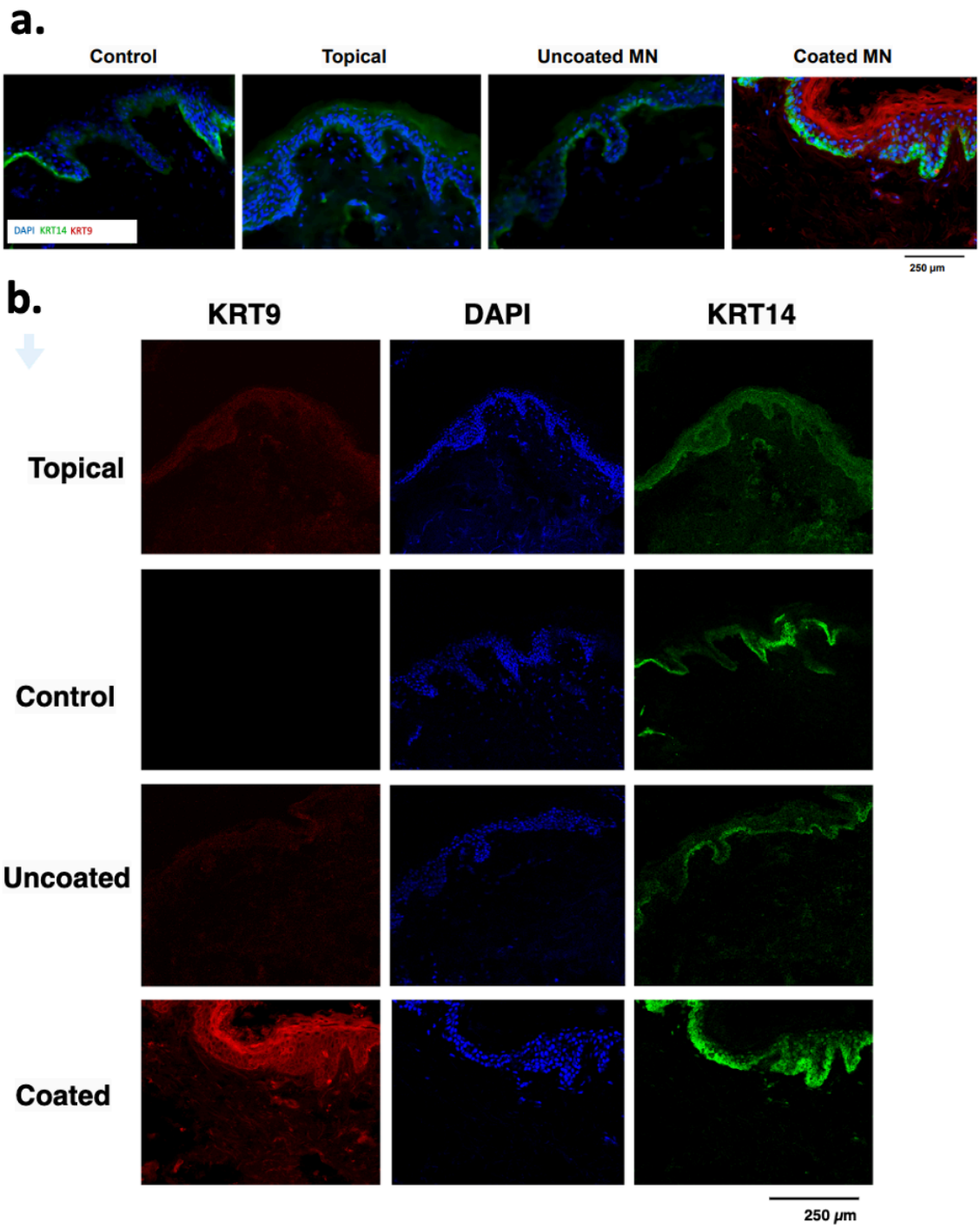


Figure 2.17: Immunostaining of KRT9 from Wnt5a coated MN on ex vivo human skin was observed. (a) Immunostained representative images of *ex vivo* skin treated with MN coated (Uncoated MN) Wnt5a against untreated control and topical Wnt5a (green KRT14, red KRT9) N=1. (b) Overlay channels of *ex vivo* skin treated with MN coated (Uncoated MN) Wnt5a against untreated control and topical Wnt5a (green KRT14, red KRT9) (N=1).

Subsequently, I next explored the effect of Wnt5a on switching gene expression on *ex vivo* skin. A Wnt5a coated MN was applied to *ex vivo* skin for 15 minutes (against untreated control), skin was left to incubate in medium for 5 days before skin samples were embedded, sectioned and immunostained with KRT9, KRT14 and DAPI [Figure 2.17a]. The increase in KRT9 (red) expression within the suprabasal epidermis, (absent in the untreated control, topical and uncoated MN skin samples) in the Wnt5a coated MN treated skin, indicates reprogramming of skin identity to a plantar phenotype [Figure 2.17b].

My MN device which was designed to surpass epidermal barriers for efficient drug delivery has been optimized with superior and sustainable polymeric material choice, a variable needle height array to successfully elude the 'bed of nails' effect, and enhanced mechanical and swelling properties than what has previously been reported. Moreover, the polymeric coating properties have been evaluated to ensure the material is pH-responsive and thus can specifically deposit cargo into the neutral layers of the skin which will prevent bio-waste of drug compounds. The constructed MN device has been applied to an artificial skin model highlighting insertion forces comparable to literature. Finally, the Wnt5a coated therapeutic patch was applied to *ex vivo* human skin and successfully delivered the drug of interest to the epidermis as well as switched on KRT9 expression indicating changes in skin identity.

Lastly, following my finding that my MN shows differing penetration of human skin according to tall or short needles, I explored the prospect of controlled spatio-temporal drug deposition across the MN array [Figure 2.18].

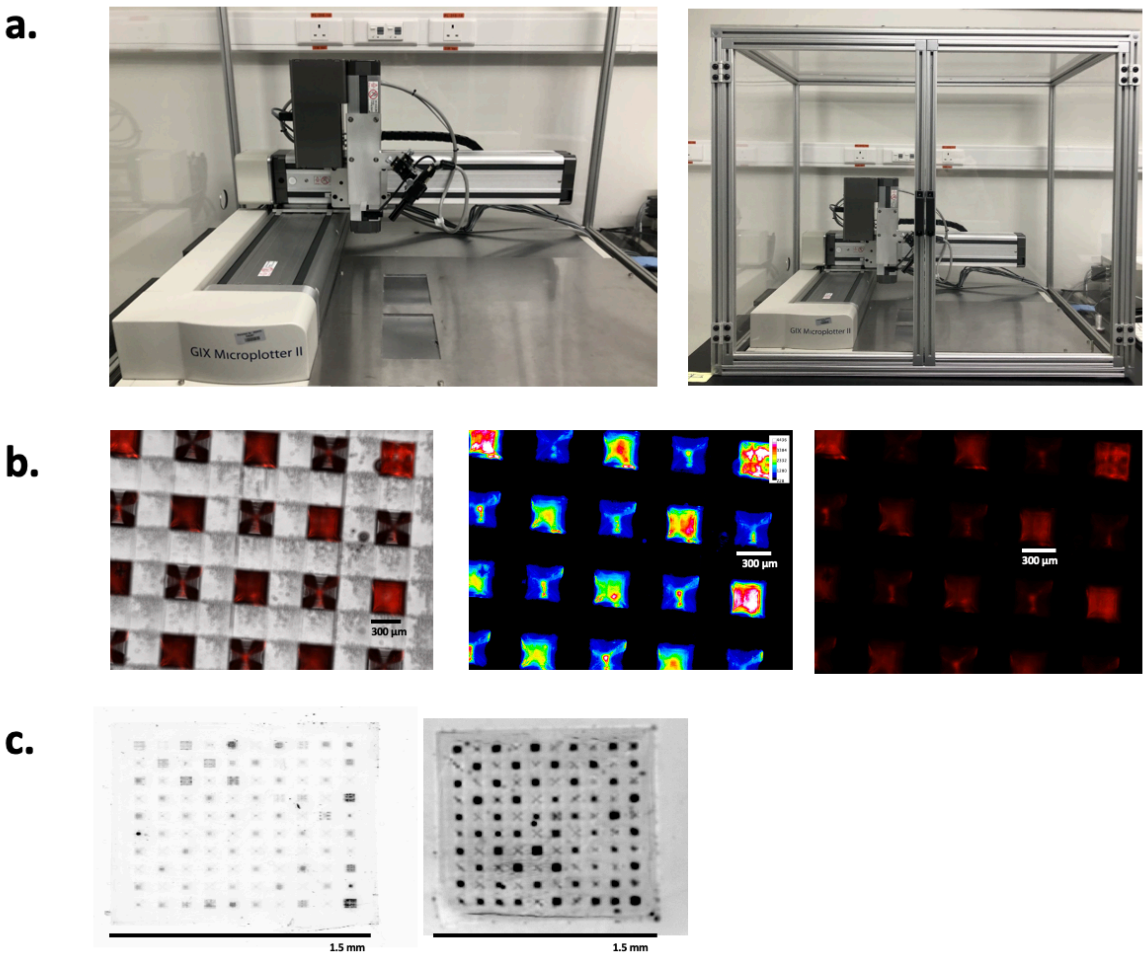


Figure 2.18: Fine-tuning coating of MN array using GIX Microplotter SonoPlot®. (a) images of the GIX Microplotter SonoPlot® instrument showcasing dispenser that is loaded with 30 µL of 1 µm Cy3. (b) Fluorescent microscopic images taken on Zeica Microscope (10x magnification) and analysed on ImageJ. (c) Shows images of uncoated (left) and coated (right) 700 µm microneedles captured on Typhoon Gel Image Scanner at 3 nm scale.

As previously shown the 700 µm needles appear to penetrate the sub dermal, papillary dermal layer of the human skin which is the upper layer of the dermis possessing connective tissue and blood vessels, highlighted by the trypan staining [Figure 2.7]. However, the 500 µm needles show reduced residual trypan staining left on skin after needle removal (and wash of trypan blue) indicating that these needles do not pierce the basement membrane as deeply [Figure 2.7] and thus confers localized delivery of Wnt5a to epidermal keratinocytes. Therefore, if my design showcases differing levels of penetration of variable skin layers it could potentially be utilized to deliver different compounds to specific regions of the skin, the epidermis and dermis respectively. Using a GIX Microplotter SonoPlot® I attempted to optimize coating of each needle across the MN array which successfully showed Cy3 staining of 700 µm needles only via fluorescence staining and camera images against an uncoated control [Figure 2.18]. This

showcases the strong potential of my device being used to deliver several compounds simultaneously to the skin by fine tuning of the desired cargo to the target needles.

2.4 Discussion

In this presented work, I have illustrated a successfully established epidermal drug delivery device possessing sustainable, mechanically superior, and pH-responsive materials designed with variable needle heights to account for the dimensions of the skin tissue and eluding the 'bed of nails' phenomenon [Figure 2.7]. These attractable qualities overcome the limitations of commercially available designs [Bariya et al., 2011; Aldawood et al., 2021; Sabri et al., 2019; Avcil and Çelik. 2021].

My presented work showcases the promise and potential of my established device, with a myriad of benefits, in being an optimal drug delivery tool for the skin. Firstly, my designed device presents a variable needle height design that is effective in piercing the basement membrane. Most polymeric MNs fail to penetrate human skin [Sabri et al., 2019], and even a device constructed with the same material properties as my presented MN but with equal needle heights (700 μm) [Figure 2.7] fails to penetrate human skin. Therefore, my device not only with optimum material choices but also variable needle geometry prevents 'bouncing off' of the array on the surface, eluding the 'bed of nails' phenomenon [Makvandi et al., 2021; Loizidou et al., 2016]. This indicates higher efficiency in epidermal drug delivery than conventional MN designed with consistent needle heights that compress skin.

The hydrophilic MN base material cross-linked with Di(trimethylolpropane) tetra-acrylate formed a compact and stiff polymeric structure, eliciting higher mechanical properties than what has previously been reported (0.3-0.4 N normalised failure point vs 0.6-1.0 N) [Park et al., 2005; Lee et al., 2008; Demir et al., 2013; Du et al., 2019], [Figure 2.6]. I have illustrated a low swelling threshold of my polymeric system to ensure stability in an aqueous environment [Figure 2.6]. My MN device presents strong cohesive bonding between the PEGDA base material and PAA hydrophilic coating that is only disrupted in a high pH (>pH 7) environment, allowing the drug of interest to escape the encapsulation of the polymer, once (<pH 7) globular now linear (>pH7) coating material in the neutral-alkaline pH surrounding. The device possesses facile fine-tuning [Mandal et al., 2018; Samant et al., 2018] in coating material to prevent bio-waste of the drug of interest. I observed 90% deposition of Wnt5a to synthetic skin as well as human skin within 15 minutes of MN application [Figure 2.13,16]. Notably, I

investigated the drug diffusion capacity of three distinct proteins of variable size and charge (BSA (Mw: 66.43 KDa, PI -18e), Avidin (Mw 66 KDa, PI +10.5) and Wnt5a (Mw: 42.3 KDa, PI +8.45) on a synthetically constructed model which demonstrates that irrespective of charge and size, the compound of interest can successfully diffuse down the concentration gradient into the skin upon application of the MN [Figure 2.13].

I have explored the biological application and function of my MN patch on human skin by selecting a known compound that can switch on specific gene expression i.e. Wnt5a induces the expression of KRT9 [Figure 2.15]. I initially validated via *in vitro* qPCR and immunostaining experiments what previous reports have illustrated that Wnt5a can selectively enhance the expression of KRT9 in non-plantar keratinocytes therefore switching the phenotype of these cells to plantar [Figure 2.15]. Next, I assessed the biological function of Wnt5a in elevating KRT9 expression within human skin (*ex vivo* model) both topically and via a MN device, which had not previously been assessed [Figure 2.16, 17]. I have demonstrated an enhancement in KRT9 expression of Wnt5a coated MN patches comparatively to controls, with 450-fold change in KRT9 expression to topical delivery of Wnt5a on human skin. This was further validated with immunostaining (against untreated controls, topical and uncoated) [Figure 2.17]. This suggests that my drug delivery device is not only successful in inducing an appropriate biological function to human skin but is in fact more effective than the conventional route of drug delivery to skin.

Going forward, my presented device possesses the potential to accommodate the delivery of a versatile group of drugs, from larger or smaller proteins to peptides [Jeong et al., 2018; Liu et al., 2017; Bora et al., 2008; Ganeson et al., 2023], or to viruses [Chen et al., 2016; Noh et al., 2022; Feng et al., 2023]. Notably, pH responsive coated MNs have been utilised to deliver engineered nucleic acid to induce expression of proteins into cells to treat cancer disease for example [Jiang et al., 2023; Sayed et al., 2022; Moreira et al., 2019]. Moreover, MNs can be utilised to deliver vaccines to the skin as it can surpass the stratum corneum layer and deliver the loaded cargo upon contact with the interstitial fluid [Kim et al., 2018; Ye et al., 2016]. Notably, constructed MNs have also been used to delivery mRNA-encapsulating extracellular vesicles to mice with photoaged skin which has been shown to restore collagen [You et al., 2023]. This highlights that MNs can be utilised for the delivery of a variety of compounds to skin for different purposes.

In addition, I have found that encapsulating the cargo in dried solid form such as PAA, will not only conserve the bioactivity of the biological material but also enhance its thermostability and bioavailability thereby facilitating its application to the skin target site [Chen et al., 2016; Arya et al., 2016]. Moreover, as I have successfully shown that my constructed MN device can successfully deliver highly specific compounds of interest to the keratinocytes within the basal layer, attached to the basal lamina, it can be utilised to target basal stem cells. The device could essentially deliver compounds to and target stem cells that reside in the basal layer, that once differentiated stop dividing and move toward the surface of the skin [Alberts et al., 2002].

Moreover, many microneedles, typically dissolving or coated, have been utilised to deliver engineered nucleic acid to the immune-cell rich (Langerhans cells and dendritic epidermal T cells) epidermis (having surpassed the stratum corneum) with consistent needle arrays of 700-750 μm in height [Pearton et al., 2012; Weldon et al., 2011; Hiraishi et al., 2011], which my design should also achieve as indicated by my findings. Importantly, examples such as the influenza, tetanus, diphtheria, pertussis, or hepatitis b containing vaccines could be locally and directly delivered to the papillary dermis with my engineered device avoiding issues of invasiveness, pain, irritation, swelling, biomaterial accumulation, and systemic effects that are associated with intradermal vaccine delivery [Niyomnaitham et al., 2022].

I project that my tailored MN patch possesses the capacity to be used to deliver compounds of interest to the sub-dermis due to the pH responsive array possessing 700 μm needles designed to pierce into the slightly alkaline subdermal layers of human skin. This was highlighted in Figure 2.7 which shows clear penetration of the 700 μm needles to the papillary dermis. However, if localised delivery to the reticular dermis was required, I would modify the needle heights from 700 μm to 1-2 mm. Coated MNs commonly present issues of coating thickness that perturbs the needle sharpness and thus minimises skin penetration, however, my coated needles are shown to effectively penetrate human skin and deliver drugs of interest to the epidermal basal layer [Gill et al., 2006]. A further concern with coated MNs is the possibility of needles breaking within the human skin giving rise to symptoms, however, my device is mechanically superior to existing devices so is unlikely to break upon contact with skin tissue and in addition the device is composed of completely biocompatible materials [Browe et al., 2017].

Moreover, a major obstacle with many available coated MN arrays is the issue of selectively coating each needle precisely as to prevent biowaste and to ensure that each needle tip and shank is efficiently coated with the desired amount of drug without touching the base substrate of the device. The method I used in this work was the dip-coating technique which ensured that only a thin film of material was used to cover the array thus ensuring reduced coating thickness that would not reduce sharpness of each needle tip [Cormier et al., 2004; Ameri et al., 2010; Nair et al., 2015; Baek et al., 2017]. However, further optimisation of this coating method would prevent loss of cargo and enable more precise coating of the cargo to each needle to facilitate spatial drug distribution.

In future studies where depositing different drugs to sub sections of the skin for personalised skin therapy becomes necessary, I theorise that my design holds the capacity for spatiotemporal deposition of drugs of interest with further optimisation [Figure 2.18] using a GIX Microplotter SonoPlot® for pL coating [Simonenko et al., 2023; Zhao et al., 2021; Zhou et al., 2022]. This was experimentally trialled [Figure 2.18] which successfully showed specific Cy3 coating of the 700 μm needles as highlighted in red. Therefore, the alternating needle height array accounting for the epidermis and dermis respectively and the ease of fine-tuning drug deposition to the tip of the needle would accommodate special temporal delivery of drugs of interest to skin tissue.

Chapter 3

UVA age acceleration of human skin and impeded wound healing is protected by the klotho protein

3.1 Introduction; Chapter Aims

In this chapter, I characterise a UVA age acclerated *ex vivo* human skin model for the purpose of exploring the effect of this physiologically relevant environment stimuli on skin ageing and regeneration. I assess the link between UVA exposure, biological ageing and wound healing both *ex vivo* and *in vitro.* I introduce the anti-ageing compound, klotho, as a preventative therapeutic to not only regress UVA age acceleration of the skin but also rescue UV impaired skin regeneration. Lastly, I utilise the engineered MN from chapter 2 as a therapeutic device for the delivery of klotho to the skin.

Aims:

1. Identity the effect of UVA (variable doses) on wound healing and assess baseline differences in UV injury in young, and old skin in terms of senescence, wound healing and biological age (epigenetic methylation) to efficiently develop a UVA age acccelerated *ex vivo* skin model.
2. Explore the impact of UVA on epidermal keratinocytes in terms of senescence induction, biological age (epigenetic methylation), and migration patterns and how that may influence skin healing.
3. Assess the effect of UVA on dermal fibroblasts and explore the impact of UVA on the epithelial mesenchymal signalling between keratinocytes and fibroblasts.
4. Evaluate the effect of the anti-ageing compound, klotho, on UV exposed skin and cells in preventing UVA impaired skin regeneration.
5. Utilise the pH responsive MN device to deliver the klotho protein to the human skin *ex vivo* model.

*Parts of this chapter are taken verbatim from this manuscript in preparation:

Abbas A., Tan D., Akhavani M., Limbu S., Lee JJ., Muttagabani K., Alsulaiman D., and Higgins CA. “UVA age acceleration of human skin and impeded wound healing is protected by the klotho protein”

3.1.1 An Ageing population

Across the globe, many regions are undergoing a demographic shift toward an ageing population. This will transform the socioeconomic strategy and public health policies of a country's infrastructure. Human ageing is a nuanced concept that has only recently become more convoluted in respect to health. The total world population surpassed 8 billion in late 2022, bringing attention to not only rapid population growth but the biggest demographic challenge at present, population ageing [Adam. 2022] [Figure 3.1].

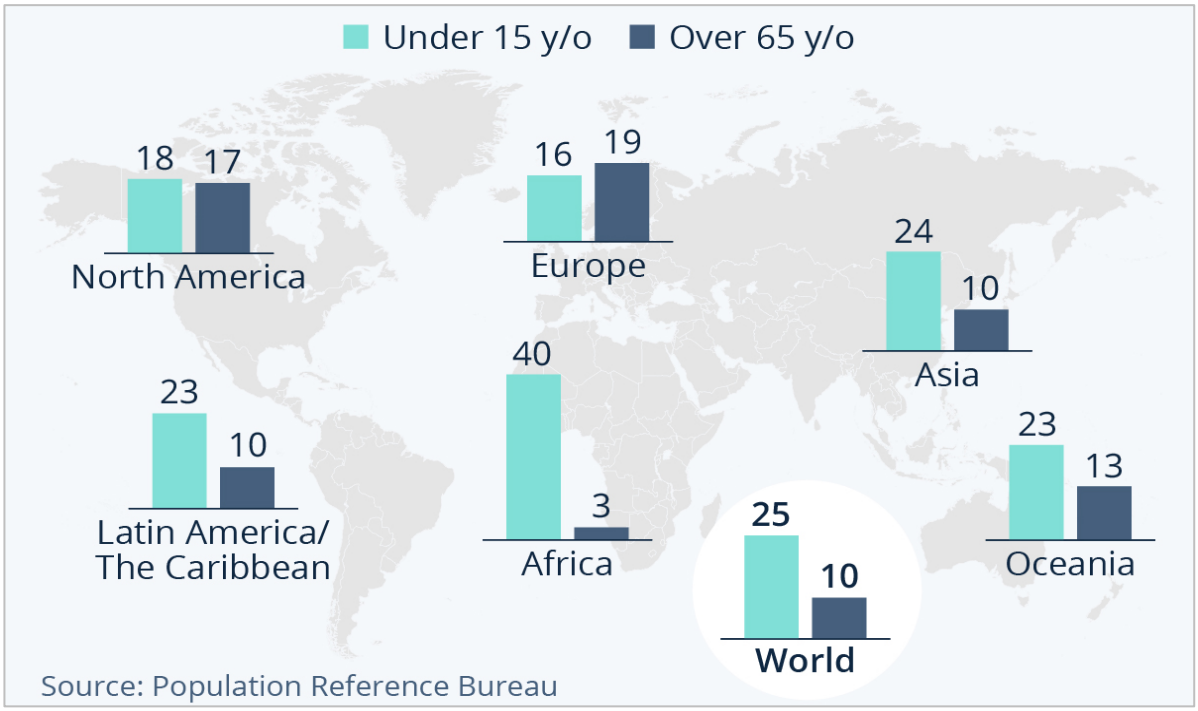


Figure 3.1: The ageing population across the world. The estimated share of the population in stated age groups, reported in 2022, by world region (in %). The cohort aged over 65 years old is higher than the under 15 years of age cohort in Europe.

A rapidly ageing world confers an overwhelming burden of disease and disability.

The global life expectancy is swiftly rising, with a projection of over 2 billion people living >65 years old by the year 2050 [World Health Organization. 2023; Atguna et al., 2021] and an increasing ageing population across Europe [Population Reference Bereau]. With this increased life expectancy, time spent in poor health is also rising [Later Life in the United Kingdom. 2019; Barton and Cooke. 2021].

Ageing is largely defined as growing old. However, this simple definition is becoming more convoluted as researchers redefine what ageing means for the human body and one's health, and how this once perceived natural process can alter one's predisposition to disease [Diehl et al., 2021]. Individuals age differently, meaning that there is a chronological age and a

biological age. Chronological age refers to the number of years one has lived since birth, while the biological age is characterized by the epigenetic changes one undergoes, specifically in the upregulation of DNA methylation; signifying how functional one's cells are and their subsequent susceptibility to age-related disease [Ahadi et al., 2020]. This highlights the need for further tailored preventative therapy which has previously been outbalanced by reactionary medicines [Kaeberlein et al., 2015; Waldman and Terzic. 2019; Da-hye Son et al., 2019; Walker. 2009].

Traditionally skin ageing has held a focal point in the cosmetic industry but in the context of skin disease and healing has progressively become a topic of interest, propelled by the pandemic, and the rise in life-threatening diseases that are more prevalent in old age. The repair of human skin diminishes with ageing, driven in part by the habitual exposure of UVA radiation, which stimulates skin photoaging. The consequences of photoaging on healing have not extensively been explored at both the tissue and cellular level. Here, I sought to investigate if photoaging and diminished skin repair observed with age are correlative or there is a causative impact of photoaging. Herein, I show that UVA-induced photoaging of human skin stimulates senescence and increased epigenetic age of skin cells and tissue, which in turn impairs *ex vivo* wound healing function and *in vitro* migration of cells to injury site. I used a widely recognised anti-ageing compound, klotho, and found that it could ameliorate and prevent UVA-impaired wound healing.

3.1.2 UVA acccelerates skin ageing



A physiologically relevant environmental stimulus for ageing, that is difficult to avoid, is UVA radiation [wavelength of 315-365 nm] forming 95% of solar UVR (ultraviolet radiation); [Figure 3.2] [Maeda. 2018; World Health Organization (WHO), 2016; Maverakis et al., 2010] unfiltered by the Earth's stratospheric ozone layer [Ghissassi et al., 2009; Valerio et al., 2021]. UVA or UVB age acceleration of human skin, *in vivo* models, *in vitro*, and *ex vivo via* changes in epigenetic factors has extensively been explored [de Oliviera and Souza. 2020].

Figure 3.2: Illustration of the many ways UVA and B may impact human health (clockwise) brain, skin structure, skin cancer, skin ageing, eye vision, immune system, and hair growth.

For example, a recent study exposed *ex vivo* skin explants to 16 J/cm^2 of UVA which resulted in enhancement of oxidative damage, inflammatory cells, MMP expression and reduction in elastin expression [Saguie et al., 2021]. This photoaged model was able to assess the myriads of UVA-ageing effects on human skin. Moreover, *in vitro*, repeated UVA exposure (3 or 6 J/cm2 5 repeated doses per day) of human skin fibroblasts resulted in persistent changes in DNA methylation [Tilburg et al., 2020].

A phenotypic way to observe ageing is through changes in the largest organ of the body and barrier to UVR, human skin. With ageing skin undergoes epidermal thinning, wrinkling, and increasing translucency [Blanpain et al., 2006], which UVA accelerates [Gilaberte et al., 2016; Jenson. 2009]. As a result of the high penetration properties of UVA, which is 20 times more abundant than UVB [Karran et al., 2016], UVA exposure also vastly affects the dermal construction of the skin [Battie et al., 2014] leading to premature ageing, or 'photoaging' of skin [Pandel et al., 2013; Rittié and Fisher. 2015] in turn it can, influence one's susceptibility to skin disease [Bosch et al., 2015] and wound healing [Khalid et al., 2022] though the mechanism is not well understood.

3.1.3 CpG methylation

CpG dinucleotides, also known as CpG sites, are characterised as regions in DNA where a cytosine nucleotide is followed by a guanine nucleotide, separated by a phosphodiester bond, in a linear sequence of bases across the DNA strand [Lim et al., 2019; Hughes et al., 2020; Moore et al., 2013]. Up to 70% of human genes possess CpG dinucleotide-rich regions, known CpG islands, in their promoters.

CpG islands are defined as DNA methylation regions in promoters which can regulate gene expression via transcriptional silencing of the corresponding gene [Lim et al., 2019]. DNA methylation at the CpG islands is essential for gene expression and tissue-specific processes. Generally, a CpG island is a region of over 200 bp with a CG dinucleotide percentage of over 50%.

At the single gene level, hypermethylation of CpG sites within promotors is commonly associated with transcriptional repression whereas hypomethylation of CpG sites is associated with transcriptional activation [Moore et al., 2013]. There are various primary mechanisms by which DNA methylation of promoters and distal regulatory regions regulate gene expression. The hypermethylation of CpG sites in regulatory sequences and regions can also destabilise nucleosomes and recruit related proteins, remodel chromatin structure and lead to subsequent blockage of transcription [Vavouri and Lehner. 2012].

Importantly, methylation of CpG sites can occur globally across the genome or at specific CpG sites of the genome [Lim et al., 2019]. Global methylation will give a holistic insight into the overall epigenetic landscape of the genome, whereas specific CpG methylation gives an overview into the regulation of gene expression and its effect on cellular function.

Methylation clocks to assess age in response to UVA

UVA exposure can induce DNA damage and epigenetic methylation which has been recognised as a crucial indicator for biological ageing [Figure 3.3]. The cytosine-5 methylation within the CpG dinucleotides is subject to age-related hypermethylation [Horvath. 2013].

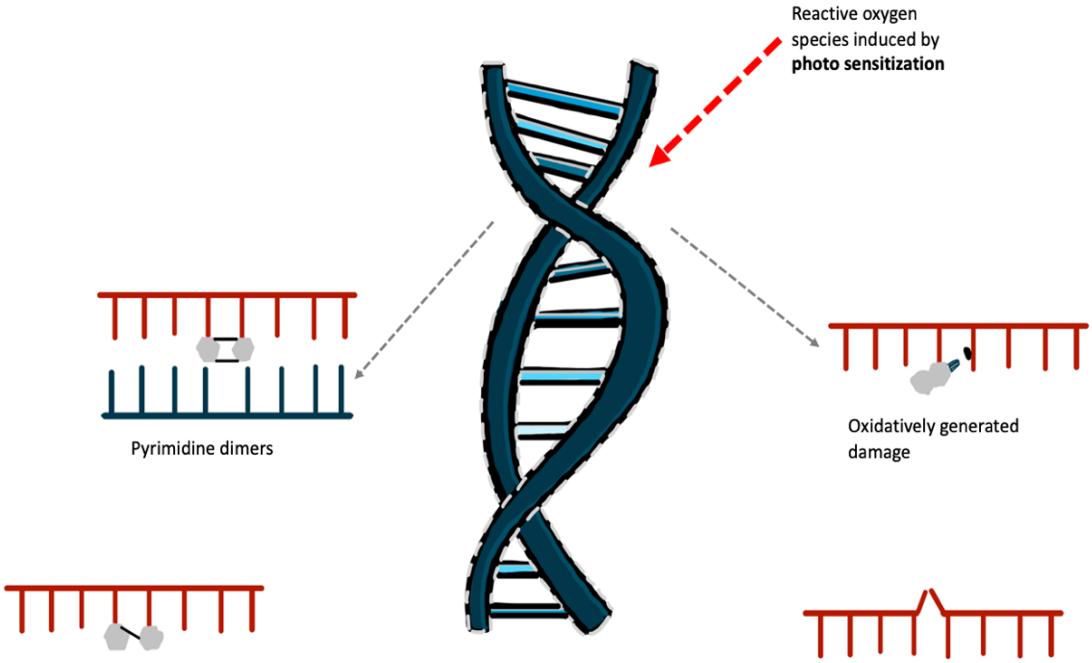


Figure 3.3: Illustration of DNA damage and epigenetic changes. UVA can produce reactive oxygen species which can induce DNA damage breaks across the genome.

The relationship between UV and changes in site-specific methylation patterns has previously been explored with a recent review on UV driven hypermethylation in specific CpG sites of epidermal skin cells [Oliviera et al., 2020]. This discovery could potentially be used to identify biomarkers of skin photoaging. The effect of UVA on p16, MGMT, DAP Kinase and GSTP1 methylation site in HaCAT cells have shown hypermethylation of the tumour suppressor gene p16, thus silencing its function and inducing uncontrolled cell growth [Gupta et al., 2006; Chen et al., 2012]. In addition, UVA exposure *in vivo* has been found to induce hypermethylation in 4140 genes and hypomethylation in 1863 genes [Yang et al., 2014; Mittal et al., 2003]. The Horvath clock, developed in 2013, is an age estimated method based on 353 epigenetic markers on the DNA. These markers measure DNA methylation of CpG dinucleotides. The Horvath clock has been characterised as a highly versatile and accurate age prediction tool which has been utilised in multiple studies [Simpson and Chandra. 2021; He et al., 2021l Fitzgerald et al., 2021; Bell et al., 2019; Palmer. 2022]. As the clock compares chronological age to biological age, it can be used to predict an individual's life expectancy.

This epigenetic clock was initially based on data from whole blood but has shown to correlate strongly with age in some tissues and cells i.e. T cells, B cells, monocytes [Levine et al., 2018]. For 5% of the population, there is a faster biological rate, resulting in a shorter life expectancy and accelerated ageing has been found to increase these adult's risk of death by up to 50% [Levine et al., 2018].

As Horvath clocks are an array-based platform for epigenome-wide analysis, it requires large amounts of high-quality DNA and complex bioinformatics, which can significantly restrict its usage. Moreover, Horvath clocks are proprietary in that the specific 353 CpG sites are not publicly disclosed. This means that individuals interested in determining their biological age using the Horvath clock must send their samples to a company that offers this service. The company then analyses the DNA methylation patterns at the undisclosed CpG sites and provides an estimate of the individual's biological age based on the Horvath clock algorithm. Therefore, in this chapter I have utilised a simpler model than the Horvath clock to predict biological age, specifically identifying methylation changes of the age-related CpG sites of ELOVL fatty acid elongase 2 (ELOVL2) gene.

ELOVL2 is highly correlated with age prediction by regulating lipid metabolism [Li et al., 2020; Chao et al., 2020]. In fact, within the top ten markers predictive of human epigenetic age, four are localised in the CpG islands in the regulatory element of ELOVL2 gene, constituting for over 70% of the single "methylation clock" model [Hannum et al., 2012]. The hypermethylation of this ELOVL2 regulatory region has been presented in multiple studies to strongly correlate with the biological age of humans [Bacalini et al., 2016; Garagnani et al., 2005; Mansego et al., 2015; Rönn et al., 2015; Obeid et al., 2023] and rodents [Spiers et al., 2016]. Notably, ELOVL2 methylation with ageing does not correlate with gene expression changes, emphasising the importance of focusing on methylation status rather gene expression.

3.1.4 UVA induces senescence

Senescence is often referred to as a double-edged sword in the sense that it can act as a deterrent against cancer onset but also a driver of chronic inflammation or 'inflammaging' [Figure 3.4]. Inflammaging is largely attributed to senescence associated secretory phenotype (SASP), a symptom of age-related and skin diseases, and has been reported to drive cancer metastasis in some cases [Huang et al., 2022; Ohtani et al., 2012; Dodig et al., 2019; Pajak et al., 2023; Alimirah et al., 2020; Shin et al., 2019; Liu et al., 2022]. These intrinsic changes observed

within ageing skin occur in part, to increasing age of the tissue [Griffiths et al., 2023] which is contributed by UVA-induced photoaging of skin. Typical senescent markers include the DNA damage protein markers p21, p53 and p16 [Figure 3.4] and typical SASP markers include IL6 and IL8 [Kudlova et al., 2022]. LaminB1 is a nuclear membrane marker, and its loss is indicative of senescence [Fruend et al., 2012].

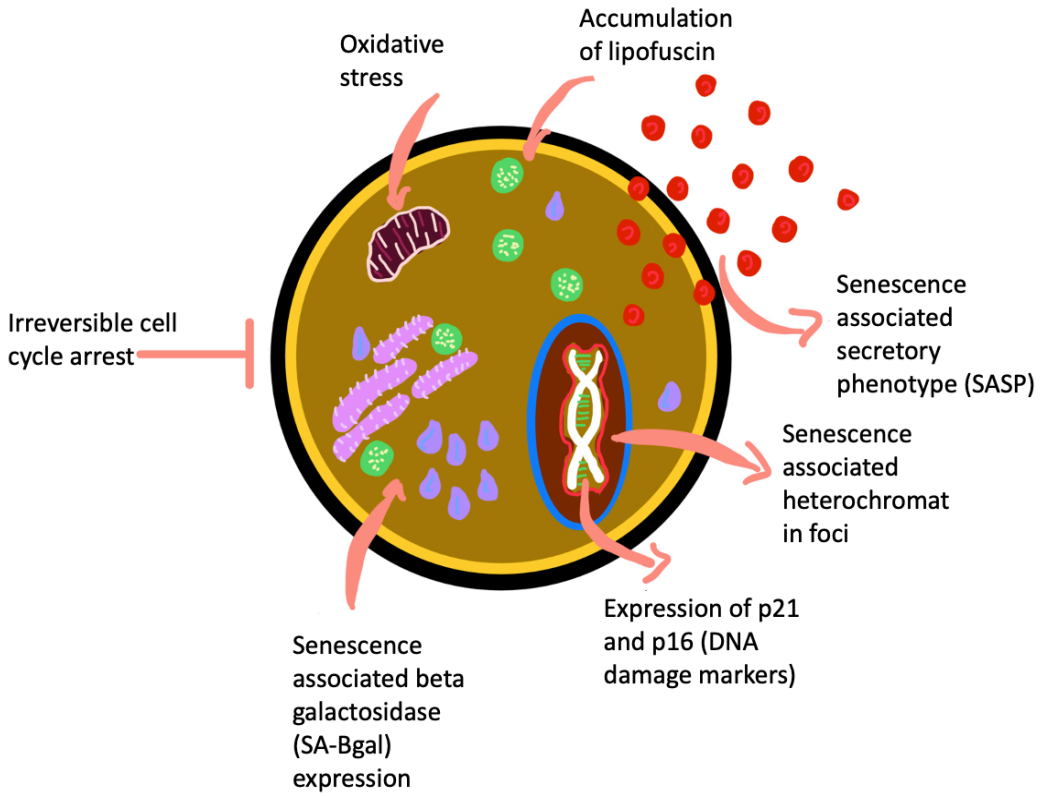


Figure 3.4 Illustration of Senescent cell. Senescent cells express specific DNA damage markers p21, and p16 and SA-Bgal These cells exhibit irreversible cell cycle arrest, a release of SASP factors and oxidative stress.

The detrimental effect of UVA on dermal fibroblasts (*in vivo* and *in vitro*) has heavily been studied [Wlaschek et al., 2001; Scharfetter et al., 1991; Chang et al., 2021; Tilburg et al., 2005; Liu et al., 2012; Nakyai et al., 2018; Zhang et al., 2020; Fitsiou et al., 2020; Fagot et al., 2004] but more recently UVA has been reported to induce senescence in human dermal fibroblasts isolated from young patients (9–12-year-olds), by inducing demethylation of CpG sites in the promoter region of p53, the cell cycle arrest gene, which in turn promotes its tumor suppressive function and thus promotes senescence [Yi et al., 2018]. A notable study has highlighted that UVA appears to induce proteome remodeling and senescence in primary *in vitro* keratinocytes which elicits a pro-inflammatory response to neighboring cells [Valerio et al., 2021]. This illustrates the impact of UVA on cellular morphology and potentially suggests an influence on wound healing.

3.1.5 Ageing and Wound healing,

There is an inverse correlation between ageing and the regenerative capacity of tissues and organs. After injury to the skin, there are subsequent and overlapping phases of wound healing; hemostasis, inflammation, proliferation, remodeling, which ease closure of the wound. 3.8 million patients were inflicted with wound injuries in 2017/2018 in the UK (United Kingdom), with only 45% of chronic wounds, with suspected infection, healing [Guest et al., 2020; Guest et al., 2015]. The annual level or resource required on the NHS (National Healthcare System) to manage wound injuries entails the support of 54.4 million district nurses, 53.6 million healthcare assistants and 28.1 million practice nurse visits. The yearly NHS cost of wound management amounts to £8.3 billion, £5.7 billion contributing to the management of unhealed wounds. Majority of skin wounds are prevalent in older individuals [Gould et al., 2015; Gerstein et al., 1993; Barbenel et al., 1977], internationally presenting in 20.8% of cohorts in long-term aged care homes [LeBlack et al., 2020; Koyano et al., 2016; Skiveren 2017; Rayner et al., 2018; Van Tiggelen et al., 2019].

Further still, elderly skin, >70-year-olds, heals up to 4x slower than younger skin [Gerstein et al., 1993; Standring. 2021; Tobin et al., 2017; Walston. 2020]; this heightens the risk of infection, and the likelihood of a wound becoming chronic [Keyes et al., 2016; Espaulella-Ferrer et al., 2021; Gould et al., 2015]. Ageing significantly affects the skin's healing activity by extending the inflammatory phase and enhancing the production of reactive oxygen species (ROS) [Khalid et al., 2022; Sgonc et al., 2013]. Reduced basal cell density with age leads to impaired proliferation and consequently epithelial migration across the wound bed. Dermal fibroblasts are essential to the wound repair process in that they mediate regulatory molecules, such as growth factors, to cross talk with other cell types, namely keratinocytes to stimulate the latter's proliferation in a double paracrine manner, to in turn allow the keratinocytes to heal the injured site via re-epithelization [Lopez-Ojeda et al., 2022; Brown et al., 2022; Werner et al., 2007; Piipponen et al., 2020; Pastar et al., 2014]. As the UK is shifting toward an ageing population, it is considerably likely that the burden of skin wounds on public health, and the economy will only rise, becoming more of a critical issue, comparable to what is presented in Japan, a region possessing a 'super-aged' society [Nakashima et al., 2018; Sanada et al., 2015; Koyano et al., 2014].

3.1.6 UVA impairs wound healing

A previously published study found that high dosage of UVA or UVB for four months on a hairless Guinea pig model significantly reduced wound tensile strength [Davidson et al., 1991]; similarly, UVB has been shown to impact healing function in murine models [Liu et al., 2015]. Mice are typically used as preclinical models to assess wound healing, as they are cost-effective and amenable to genetic manipulation [Dunn et al., et al., 2013]. However, human skin is much thicker than murine and rodent skin with more epidermal layers and defensins which makes it difficult to translate *in vivo* wound healing studies to humans [Zomer and Trenton. 2018]. Moreover, mice heal via contraction [Dunn et al., 2013] whereas human skin heals via secondary intention where keratinocytes migrate over the wound bed [McCaughan et al., 2018]. In addition, murine models present denser hair than human skin which would affect observational changes to skin. Moreover, differences in the rate of wound closure, the inflammatory responses and composition of ECM proteins between murine and human skin can restrict the applicability of observations concluded from murine studies to human wound healing. Porcine models is another popular model used in clinical studies, exhibiting a tight skin system that resembles human skin, with a thick epidermis, distinct rete pegs, dense collagen fibres, elastic fibres within the dermis and accessory hair and sweat glands [Kuo et al., 2022]. However, porcine models confer high costs for purchase and maintenance, exhibit difficulty in handling due to their large size and are difficult to mimic a nonhealing wound model. Nonetheless, a key limitation with assessing healing function in humans is overcoming the variability observed in in terms of thickness and elasticity that is altered significantly with age [Wei et al., 2017]. Therefore, it is important to consider a range of human skin samples especially of different ages when exploring the effect of UVA radiation [Figure 3.5].

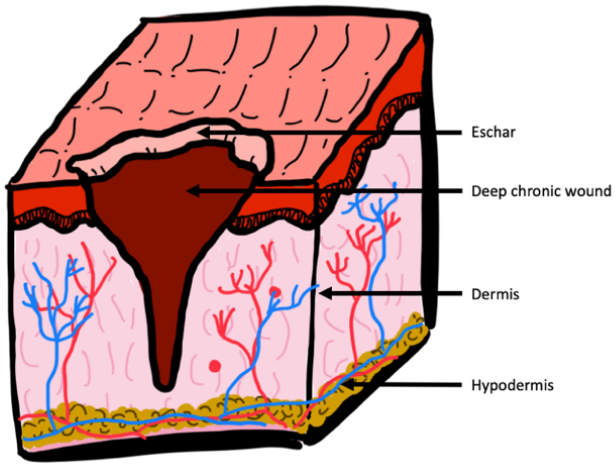


Figure 3.5 Illustration of wounded skin model.

The use of 3D human *ex vivo* models has contributed to generating crucial data that more reliably reflects the workings of the human system compared to 2D skin cell cultures. In 2D skin cell cultures, scratch "wound healing" assays are often utilised to assess for changes in migration, for example, UVA has been shown to disrupt the motility of keratinocyte (via scratch assay readout) and delay the ability to cover a non-cell area suggesting an effect of injury of UV on cells, potentially in wound healing [Niculite et al., 2018]. Notably, 3D models can illustrate drug efficacy more effectively than *in vivo* models [Eberlin et al., 2020]. The impact of UVA on human skin wound healing and whether there is a causative rather than correlative association between that and biological age, and in turn impaired regenerative capacity, has not previously been explored.

3.1.7 Anti-Ageing compounds

Dana Goldman, an economist predicted that the cost of developing a medicine to treat cancer (for one human life) would amount to $489,909 and to treat diabetes would be $147,190 [Goldman et al., 2008; Sinclair. 2019]. However, the cost for developing an anti-ageing compound to extend human life by decades would amount to $8,790. This suggests that from an economical perspective, there appears to be no more cost-effective approach to treating severe conditions than tackling the root of the issue itself, ageing. Anti-ageing compounds possess a range of benefits in that they exhibit potent antioxidant activity, anti-inflammatory, and anti-cancer properties, which make them valuable in preventing age-related disease [Gao et al., 2023; Bjørklund et al., 2022; Ganceviciene et al., 2012]. Bioactive compounds are believed to extend human lifespan via cellular or skin regeneration, or epigenetic modulation of gene activity for example [Liu. 2022]. As the world is continuing to age, exploration into new targets or uses for anti-ageing agents is being investigated, and these findings can then be transferred to healthcare and pharmaceutical industries [Vaiserman et al., 2016].

Senolytics

Senolytics are a class of drugs that selectively eliminate senescent cells. Examples include Dasatinib, Quercetin, Fisetin and Navitoclax [Kirkland and Tchkonia. 2020]. In preclinical models, senolytics can delay, prevent, or alleviate cancers, cardiovascular, neuropsychiatric, kidney, liver, lung, multiskeletal, eye, haematological, metabolic, and skin disorders. Although, senolytics can enhance longevity and reduce the impact of severe chronic diseases, there are

several challenges associated with this form of therapy. The main limitation is identifying a biomarker that is exclusively expressed by senescent cells and not healthy cells, to prevent the off-target effects of the drug and thus improve safety [Pils et al., 2021]. In addition, most senolytics lack potency, universality, or present significant off-target effects. Therefore, a senolytic agent targeting the fundamental mechanisms of senescent cells remains largely inconclusive [Pils et al., Kang. 2019; Chaib et al., 2022; Rad et al., 2024; Morsli et al., 2022; Garrido et al., 2022].

The klotho protein

Klotho is a well-regarded type I single-pass membrane anti-ageing protein that regulates the phosphate homeostasis and activity of the fibroblast growth factor (FGF) family. The KL gene encodes the α-klotho subtype protein, which is the receptor for fibroblast growth factor-23 (FGF23) and is responsible for regulating the phosphate homeostasis and vitamin D metabolism [Buchanan et al., 2020]. The protein is typically positively charged (6.4 isoelectric point), and 55 KDa in size [Brobey et al., 2015]. The compound can be found in the kidney, gut, spleen and skin [Ito et al., 2002; Ito et al., 2000; Kuro-o et al., 2016; Hu et al., 2013]. In this chapter, I have used α-klotho, a soluble secretory subunit of klotho, generated via alternative splicing of the klotho gene which acts on FGF23. The soluble level of klotho is known to decline with ageing across the body [Kim et al., 2015]. Klotho has been recognised as a tumour-suppressor which is downregulated in expression in melanoma, pancreatic and cervical cancer [Wolf et al., 2008]. Interestingly, in hair, klotho expression in human hair follicles also declines with ageing [Jin et al., 2019]. Silencing of the klotho protein induces premature catagen, the transition phase of the hair growth cycle, which blocks human hair growth [Jin et al., 2019]. In terms of senescence, klotho has been found to attenuate kidney epithelial senescence and fibrosis [Maique et al., 2020]. The klotho protein has in fact been reported to protect cells and tissues from oxidative stress [Kuro-o. 2008] as well as elicit anti-senescence effects on vascular endothelial cells through the reduction of H(2)O(2)-induced apoptosis of cells [Ikushima et al., 2005].

Klotho-deficient mice have exhibited accelerated ageing phenotypes, whereas mice model overexpressing klotho present extended lifespans [Kim et al., 2015]. In human skin, an α-klotho deficiency results in skin atrophy, a hallmark of the ageing process however the exact mechanism as to how α-klotho conserves healthy skin have not been previously explored [Lim

et al., 2015]. In recent years published work has concluded that klotho elicits protective effects in human keratinocytes against UVB-induced damage via the reduction of expression in NF-kB pathway [Zhang et al., 2018]. Moreover, defects in the klotho gene expression in mice have shown delayed wound closure, indicative of premature ageing, in contrast to wildtype mice [Yamashita et al., 2014].

3.1.8 Studying the role of UVA in impaired regeneration with ageing

In this chapter, I sought to investigate the direct role and relationship of UVA to biological age, and thus influencing wound healing of human skin *in vitro* and *ex vivo*. To do this I utilized a model of human skin wound healing [Stojadinovic et al., 2013] and assessed the influence of varying doses of UVA on wound closure rate [Figure 3.6].

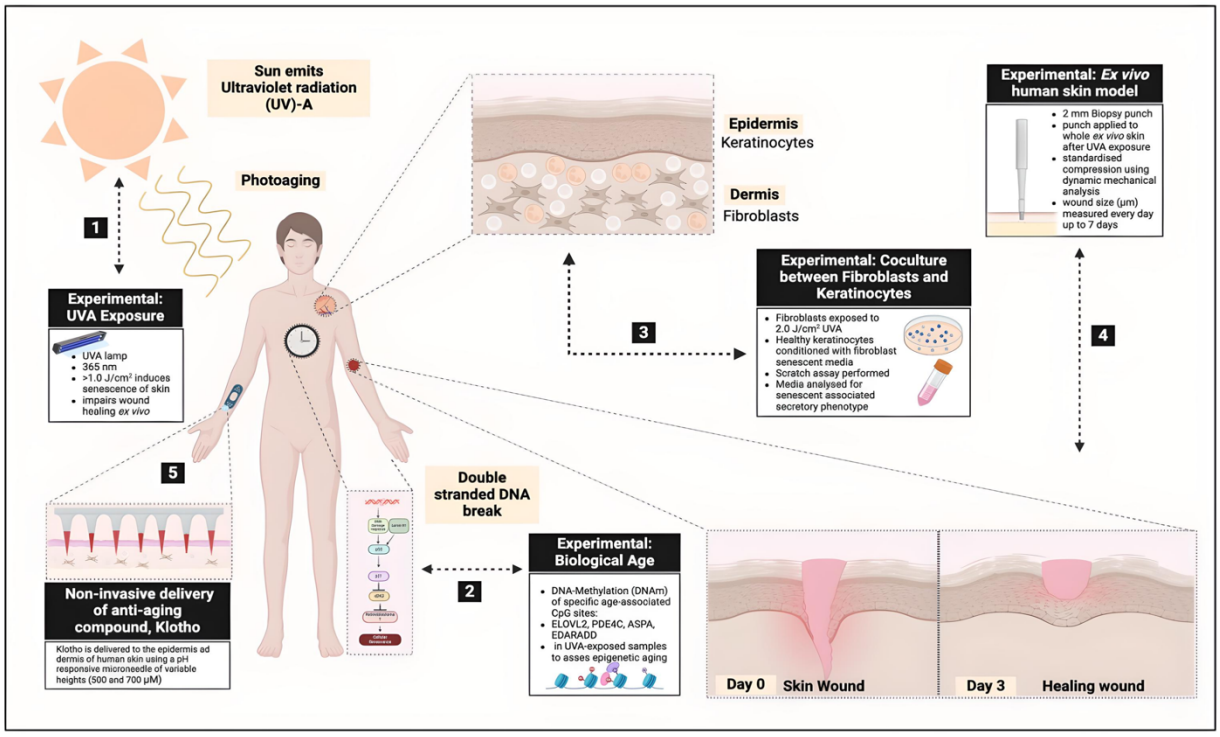


Figure 3.6: Schematic of Project Experimental plan. (1) To emulate physiological environmental stimuli UVA, a UVA chamber at 365 nm was used to expose cells and skin tissue to UV radiation. (2) To assess for changes in tissue and cellular phenotype in response to UVA I observed changes in epigenetic methylation patterns in age-related CpG sites across the genome. (3) To assess for changes in the phenotype of cells and skin in response to UV, several assays were performed, i.e. viability, metabolic activity, SA-bgal assay, and protein and gene expression changes as well as keratinocyte migration changes were observed. (4) To study the changes in wound healing capacity of ex vivo skin a 2 mm biopsy punch was administered to the skin surface immediately after UV dosage. (5) A pH responsive MN device was coated with the anti-aging compound, Klotho protein, and applied to human skin to ameliorate UVA-induced changes of *ex vivo* human skin.

I assessed the intrinsic differences of skin wound healing on skin of different ages; <20-30, <40-50, <80 years old as I hypothesized that the lifetime detrimental exposure of UVA would result in impaired healing with age as skin becomes progressively thinner and thus more vulnerable to UVA-induced DNA damage and impaired healing. I observed UVA induced senescence on young human skin as well as on primary fibroblasts and keratinocytes. I also studied the impaired wound healing ability of keratinocytes in scratch wound assays, and *ex vivo* skin with punch wounds. I also explored how UVA may impact the intrinsic epithelial mesenchymal signaling between both cell types and how this then influences wound healing.

I validated whether these observed effects of UVA are a result of UVA accelerated ageing of skin, by administering the known anti-ageing compound, α-klotho, to UVA exposed human skin and primary cells [Corréa et al., 2022; Jung et al., 2017; Prud'homee et al., 2022; Buchanan et al., 2020; Abraham et al., 2022; Mencke et al., 2017; Dërmaku-Sopjani et al., 2013; Xu et al., 2015; Oishi et al., 2021; Zhou et al., 2023; Zhang et al., 2018; Sadr et al., 2023; Niu et al., 2023; Lim et al., 2015].

I have observed improvement in the healing of UVA-age accelerated skin, UVA-induced senescence, and UVA impaired wound healing upon application of α-klotho. This supports the hypothesis that ageing, and wound healing are intricately linked and driven in part by UV exposure. Finally, to aid translation of my findings for application in the clinic I used my engineered pH-responsive microneedle, introduced in chapter 2, as a preventative delivery device for α-alpha-klotho to human skin.

3.2 Materials and methods

3.2.1 Skin and cell samples

Preparation and processing of human skin samples

To establish an *ex vivo* human skin model, abdominoplasty skin was acquired from patients after receiving their written informed consent, using Imperial College Research Ethics committee (ethics approval number: 21IC6723), or KAUST Institutional Biosafety and Bioethics Committee (IBEC) approved consent forms (ethics approval number: 23IBEC024), depending on where the tissue was collected. Patient samples from 20-30 years old were categorized as young, from 32 to 50 years old as middle aged, and >60 years old as elderly skin samples, classifications based on published paper examining signs of wrinkling on different

chronologically aged skin [Wang et al., 2009]. To sterilize the human skin samples and prevent risk of contamination, the skin samples were trimmed of fat, and the epidermis was septically cleaned using betaiodine, then with cutiwipes (70% ethanol) repeated thrice [Nasir et al., 2019].

Isolation and culture of keratinocytes and fibroblasts from whole skin

To extract primary keratinocytes and fibroblast from human skin, the skin was submerged in Dulbecco's Modified Eagle Medium (DMEM) + Glutamax (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 3x Gibco™ Antibiotic Antimycotic (ABAM) for 10 minutes (disinfection), followed by submersion in DMEM + 2x ABAM for 10 minutes and lastly submersion in DMEM + 1xABAM for 10 minutes. Skin was cut into 1x1 cm pieces and placed in a petri dish containing dispase at 4 degrees overnight. The next day, the epidermis was separated from the dermis using forceps with the former placed in a falcon tube of 10-15 mL 1x Trypsin. The dermis was placed in a falcon tube of 10-15 mL collagenase. Both tubes were incubated in the water bath (37°C) - 30 minutes for the epidermis, and 40 minutes for the dermis - each tube is gently shaken every 5 minutes. Following incubation, to neutralize the trypsin, a volume of Gibco™ defined trypsin inhibitor (DTI) that is equal to the initial trypsin volume, was added to the epidermal pieces - these were then filtered through a 70 μM cell filter to remove tissue debris. The filtered supernatant was centrifuged at 300g for 10 minutes, then the pellet resuspended in keratinocyte serum free media (KSFM) containing bovine pituitary extract (BPE) and epidermal growth factor (EGF) supplements, 1x ABAM and 10 μM Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor (Y-27632 dihydrochloride) (St. Louis, Missouri, United States, Sigma Aldrich). These cells were seeded into flasks precoated for 1 hour with 1 mg/mL fibronectin in PBS. For extraction of fibroblasts from the dermis, 20 mL of fibroblast media (DMEM + 20% Fetal Bovine Serum (FBS) with 1x ABAM was added to the falcon tube with collagenase and passed through a 70 μM cell filter to remove tissue debris. The supernatant was centrifuged at 300g for 10 minutes to pellet the fibroblasts. The supernatant was carefully aspirated, and the fibroblast pellet was resuspended in an

appropriate volume of DMEM containing 10% FBS and 1x ABAM and seeded in a flask to be cultured until passaging[1].

3.2.2 UVA exposure regimen

Optimization of UV doses

To perform UVA irradiation of primary keratinocyte, fibroblast, and skin tissue samples, a UVA (365 nm and 80 Voltage in watts) Irradiation Bio-Link instrument (Vilber, Collégien, France) was used [Battie et al., 2014]. The instrument encompasses a myriad of tools. Firstly, a programmable microprocessor that permanently controls the UV light emission, with irridation terminating automatically once received energy corresponds to the programmed energy. A UV sensor to ensure that irradiation cycles are reproducible irrespective of fluctuations in intensity of UV source. A UV light sensor placed above the irradiation chamber to enable collection of UV measurements for optimal consistency. Lastly, the UV sensor cells is protected qith a quartz protection disc to ensure long lifetime of the UV sensor. Cell samples were rinsed twice in PBS before fresh media was added (for keratinocyte or fibroblast), before plates were placed inside the irradiation instrument. The dosage (J/cm^2) was altered on the instrument according to the sample. Fibroblasts were exposed to 0.5, 1.0, or 2.0 J/cm^2. Keratinocytes were exposed to 0.05, 0.1, or 0.15 J/cm^2 while skin samples dry (no media) were exposed to 0.2, 0.5, 1.0, 2.0, 2.5, 5.0, and 10.0 J/cm^2 [Table 3.1]. Specifically low-dose UVA doses were selected, and effect was assessed via trial and error as published studies use high dose via in vivo and in vitro models [Ju et al., 2012; Valerio et al., 2021; Scharffetter et al., 1991] whilst my aim was to investigate short-term acute UVA effect on primary skin cells.

---

[1] Some keratinocyte cell extractions from whole skin were performed by Dr Summik Limbu, and Mr Jia Jun Lee.

Table 3.1: Information on the time exposure and intensity of UVA doses used on skin tissue and cells calculated using the formula UV Dose = UV Intensity (μW/cm²) x Exposure Time 00(hours):00(minutes):00(seconds):00(milliseconds)

Cell type	UVA dose (J/cm^2)	Exposure (hours/min/secs/milliseconds)	UVA Intensity (uW/cm^2)
Keratinocytes	0.05	00:00:08:71	0.0057
Keratinocytes	0.1	00:00:18:37	0.0054
Keratinocytes	0.15	00:00:26:01	0.0057
Fibroblasts	0.5	00:01:19:96	0.0062
Fibroblasts	1.0	00:02:35:41	0.0064
Fibroblasts	2.0	00:05:16:88	0.0063
Skin Punch	5.0	00:13:25:15	0.3846
Skin Punch	10.0	00:26:50:30	0.4000

3.2.3 Wound healing assays *ex vivo* and *in vitro*

Establishment of whole *ex vivo* skin wound healing assay

To explore the impact UVA-age acceleration has on skin regenerative capacity, a wound healing ex vivo human model was performed. Once fresh abdomen human skin had been processed, (trimmed of fat and antiseptically cleaned), the skin was cut into 1x1 cm pieces. A 2 mm biopsy punch was used to damage the epidermal layer and subdermal layer, creating a skin wound. The depth of the punch on human skin was standardized using a dynamic mechanical analysis (DMA) instrument, whereby the skin sample was adhered to the loading cell via double sided sticky tape, and the biopsy punch was attached to the probe. The punch was performed at a rate of 11 μm/s, with a force of 3-5 N, depending on thickness of human skin as this can vary from sample to sample. Microscopy was used to confirm depth of puncture. Each skin sample was stored in media in a 6-well plate (85% DMEM, 10% FBS, and 1x ABAM).

To assess changes in wound area, size the skin puncture site of each skin sample was imaged once every day for up to 7 days, using a stereo microscope at 2.0x magnification. Using Image J, the wound area size in μM was measured over time, and the % change in area size against the first day value was measured and inverted to identify % of skin healed, with standard deviation. To visually assess differences in skin healing between samples, 100 μL of aniline blue solution which stains for collagen was pipetted onto the surface of the wound area 7 days

after wound induction. After 1 minute, the aniline blue stain was washed off with 1xPBS and skin punctures were imaged using stereo microscope at 2.0x magnification. Light blue aniline stain represented low collagen expression and hence re-epithelisation of the wounded site, whereas dark blue aniline blue stain presented high collagen expression and hence suggested an unhealed wounded site.

***In vitro* wound healing assay**

To identify changes in wound repopulation of keratinocytes a scratch assay was performed. Briefly, the keratinocyte monolayer was scraped across the surface in a cross pattern using a 200 uL pipette tip. The cell media was then aspirated, and cells were washed in PBS once, before fresh cell media was put onto the keratinocytes. To observe changes in wound repopulation over time, the cells were imaged once every hour, using an optical microscope with 5 x magnification, for up to 10 hours and then once more at 24 hours. In this thesis I present and compare changes in wound repopulation (in all scratch experiments) from hour 0 to hour 24 as cells significantly moved in a day (with no considerable movement across ten hours). The % of cells migrated from hour 0 to hour 24 was measured using image J. The cell free area within the cross scratch was measured in μM and the % change in cell free area against the first hour value was measured and inverted to identify the % of cell covered area with standard deviation.

Culturing keratinocytes with UV conditioned fibroblast medium

To prepare conditioned fibroblast media, confluent fibroblasts at passage 3-6 were seeded at 50,000/mL (1 mL per well) in a 12 well plate. Once confluent, the cells in DMEM medium were exposed to 2.0 J/cm^2 of UVA (excluding control wells) and left in a 37°C incubator with 5% CO_2 for 24 hours Media that had been 'conditioned' by the fibroblasts was collected and stored in falcon tubes (stored in -20 for long term). After collection and filtration (using 0.2 μm filter) of conditioned media, this was used on keratinocyte cultures. Keratinocytes were seeded (50,000 per well in 12 well-plate) and maintained in KSFM (1 mL per well) (with growth supplements) until cells were confluent. Keratinocytes were left to incubate for 24 hours (37°C incubator with 5% CO_2). Following 24-hour incubation, keratinocytes were rinsed in PBS, a scratch was made on the cell mono layer using a 200 μL pipettes tip, the KSFM media was aspirated and replaced with the media 'conditioned' by fibroblasts. The wound repopulation was monitored over time, >10 hours and independently at 24 with change between hour 0 vs hour 24 analyzed and presented in this thesis. Analysis was performed using ImageJ

[Schneider et al., 2012], where % cell covered area in μM (within the cross scratch) was measured over time.

Effect of IL-12 on keratinocyte wound repopulation.

To explore effect of IL12 cytokine on the repopulation of healthy confluent keratinocytes, HumaKine® recombinant human IL12 was administered to confluent keratinocytes immediately after scratching the monolayer with 200 μL pipette tip, at a concentration of 2 μg/mL (as per recommended concentration according to supplier, Proteintech Group, Illinois, USA) diluted in KSFM (n=3, N=2). The cells were imaged (5.0 x magnification) hour 0 and 24 hours later to assess for any changes in cell repopulation. Hour 0 and hour 24 are compared in analysis and represented in this thesis. Analysis was performed using ImageJ [Schneider et al., 2012].

Cell and Tissue Analysis

Quantitative polymerase chain reaction (qPCR)

To assess the effect of UVA on keratinocytes and fibroblast *in vitro*, the expression of several senescent markers was assessed in response to UVA exposure of cells. RNA was collected from keratinocytes and fibroblasts 24-hours after UVA exposure and from homogenized skin according to the instructions RNeasy® Plus mini kit (Qiagen, Hilden, Germany) and converted into cDNA using TaqMan™ Reverse Transcription Reagents kit (ThermoFisher, Waltham, Massachusetts, United States).

cDNA conversion

A total of 200 ng RNA (diluted in H_2O) per sample was converted to cDNA using Oligo and dNTP mix (Master Mix (MM) I), DTT, 5 x superscript IV Reverse transcriptase (RT) buffer, DTT, RNase out and Superscript IV RT (MM II). Up to 2 μL of MMI (for 1 reaction: 1 μL of OligoDT, and 1 μL of dNTP mix) was pipetted into each PCR tube followed by 11 μL of the RNA and H_2O mix and placed into a PCR block. During the cycle the mixture was heated at 65°C for 5 minutes and colled down to 4°C for 1 minute. After the has ended, 7 μL of MMII (for 1 reaction: 4 μL of 5x SuperScript IV RT buffer, 1 μL of DTT, 1 μL of RNase OUT and 1 μL of Superscript IV RT) was pipetted into each sample tube and incubated at 52.5°C for 10 minutes, then the reaction was inactivated by heating to 80°C for 10 minutes before finally cooled down to 4°C.

qPCR reaction

The qPCR (QuantStudio® Real-time PCR (Polymerase Chain Reaction)) was next performed. To perform qPCR, per sample a master mix of 10 µL SYBR green, 1 µL primer of interest, 0.5 µL DMSO and 7.5 µL RNase free water was made up, and 19 µL of the master mix solution was pipetted into each well in a 96 well plate along with 1 µL of the corresponding cDNA for a total volume of 20 µL. Negative controls with negative 1 µL cDNA or RNase water were added to the 19 µL master mix solution on the 96 well plate. The qPCR parameters were set up with SYBR green reagent with a standard cycling of : holding stage (50°C for 2 minutes, followed by 95°C for 2 minutes) and cycling stage (95C for 15 seconds and 60°C for 1 minute).

Genes analysed on cells included the DNA damage marker, p21 [Shtutman et al., 2017], the nuclear membrane marker, Lamin B1 [Freund et al., 2012] the photoaging markers, matrix metallopeptidase (MMP-)1 [Quan et al., 2010], the wound healing markers, THBS1, THBS2 and THBS4 [Kyriakides and MacLauchlan. 2009], the senescent associated secretory phenotype (SASP) markers: IL-6, and IL-8 [Dimri et al., 1995; Fitsiou et al., 2020] [Sa et al., 2015]. Primers of a final concentration of 0.8 uM were used [Table 3.2].

Table 3.2: Primer sequencing list of Senescent, wound healing makers, and SASP markers.

Gene	Sequence	Product size (base pairs)
P21 (designed in lab)	FW ACCTGTCACTGTCTTGTACC RV TCCTCTTGGAGAAGATCAGC	252
LaminB1 (designed in lab)	FW ATC TCT CAT TCC GCC TCA GC RV CTT CAC ATC TTC GCC AGT GC	291
IL8 (Origene)	FWGAGAGTGATTGAGAGTGGACCAC RVCACAACCCTCTGCACCCAGTTT	383
IL6 (Origene)	FW AGACAGCCACTCACCTCTTCAG RV TTCTGCCAGTGCCTCTTTGCTG	132

Gene	Sequence	Product size (base pairs)
GAPDH (Housekeeping gene, Origene)	FW GTCTCCTCTGACTTCAACAGCG RV ACCACCCTGTTGCTGTAGCCAA	235
MMP1 (designed in lab)	FW ATGAAGCAGCCCAGATGTGGAG RV TGGTCCACATCTGCTCTTGGCA	230
THBS1 (Origene)	FW GCTGGAAATGTGGTGCTTGTCC RV CTCCATTGTGGTTGAAGCAGGC	223
THBS4 (in lab)	FW TGATGGTATCCCAGACCTGG RV GTACAATCTCCATGCCCTGG	275
THBS2 (Origene)	FW CAGTCTGAGCAAGTGTGACACC RV TTGCAGAGACGGATGCGTGTGA	398
IL12 p70 (Origene)	FW GACATTCTGCGTTCAGGTCCAG RV CATTTTTGCGGCAGATGACCGTG	98

Genes analysed on fibroblasts, young and elderly skin included the following: COL1A1, COL3A1, COL5A2, HMGCR, FASN, FADS1, FBLN2, FBLN5, and ELN which are altered with photoaging or biological ageing [McGrath et al., 2012] [Table 3.3].

Table 3.3: Primer sequencing list of photoaging and biological ageing genes.

Gene	**Sequence** (Origene, Rockville, Maryland, United States)	Product size (base pairs)
COL1A1	FW GATTCCCTGGACCTAAAGGTGC RV AGCCTCTCCATCTTTGCCAGCA	107
COL3A1	FW TGGTCTGCAAGGAATGCCTGGA RV TCTTTCCCTGGGACACCATCAG	372
COL5A2	FW CAGGCTCCATAGGAATCAGAGG RV CCAGCATTTCCTGCTTCTCCAG	211
HMGCR	FW GACGTGAACCTATGCTGGTCAG RV GGTATCTGTTTCAGCCACTAAGG	227
FASN	FW TTCTACGGCTCCACGCTCTTCC	212

Gene	**Sequence** (Origene, Rockville, Maryland, United States)	**Product size (base pairs)**
	RV GAAGAGTCTTCGTCAGCCAGGA	
FADS1	FW CTGTCGGTCTTCAGCACCTCAA	236
	RV CTGGGTCTTTGCGGAAGCAGTT	
FBLN2		
	FW CTGCTACAAGGCACTCACCTGT	136
FBLN5	RV GTAGAAGGAGCCCTTGGTGTTC	
	FW CTGCTGGATGACAACCGAAGCT	139
ELN	RV GATAAGGCTCCTCACAGCGGAT	
	FWGGTTGTGTCACCAGAAGCAGCT	260
	RVCCGTAAGTAGGAATGCCTCCAAC	

Immunostaining

To detect the expression of senescent markers, p21 in the nuclei, and Lamin B1 in the cell membrane immunostaining was performed on skin, keratinocytes, and fibroblasts (both UVA exposed and controls).

Preparing cells for immunostaining

The *in vitro* samples (in a 12 well plate, cells seeded on coverslip per well) were collected 24-hours after UVA exposure. 24 hours following UV exposure of the cells, the plate was removed from incubation (37°C and 5% CO2) and aspirated of media. Each sample well was washed with 1x PBS and kept on shaker for 3 minutes, which was repeated thrice. The samples were then fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature in the fume hood. The samples were washed once more with PBS for 3 minutes, before 1 mL of 0.3% Triton in 5%. Goat serum (diluted in PBS) was added to each sample which were then left for 30 minutes at room temperature. Following incubation, 1:1000 Lamin B1 (Clone: L-5, Catalogue No: 33-2000), and 1:50 p21 (Clone: R229-6 Catalogue No: MA5-14949), (ThermoFisher, Waltham, Massachusetts, United States,) were diluted in 5% goat to each sample which were then left overnight at 4°C [Table 4]. Following overnight incubation, the wells were washed in 1x PBS and left on shaker for 3 minutes, which was repeated thrice. The secondary antibodies: 1:1000 goat anti-mouse Alexa Fluor 488, and 1:50 goat anti-rabbit Alexa Fluor 568, (ThermoFisher, Waltham, Massachusetts, United States) were diluted in PBS

with 5% goat serum and added to samples before incubation for 1 hour at room temperature in the dark. The samples were then washed with 1xPBS three times for three minutes each shaking cycle.

Preparing skin for immunostaining

The *ex vivo* human skin tissue samples were collected 7 days after UVA exposure and embedded in OCT embedding matrix (Newton, Banbridge, United Kingdom) then sectioned at 10 μm using a Cryostat instrument. All samples were washed in 1xPBS for 3 minutes before fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature in the fume hood. The samples were washed once more with PBS for 3 minutes, before 1 mL of 0.3% Triton in 5%. Goat serum (diluted in PBS) was added to each sample and left for 30 minutes at room temperature.

The primary monoclonal antibodies, 1:1000 Lamin B1 (Clone: L-5, Catalogue No: 33-2000), and 1:50 p21 (Clone: R229-6 Catalogue No: MA5-14949), (ThermoFisher, Waltham, Massachusetts, United States,) (were diluted in 5% goat serum and added onto each sample and left overnight at 4°C [Table 4]. Following overnight incubation, the cells were washed in 1xPBS three times for three minutes each cycle.

Table 3.4: List of Primary and Secondary Antibodies

Target	Host	Company	Dilution
Primary			
LaminB1	Mouse	Thermofisher	1:1000
P21	Rabbit	Thermofisher	1:50
Secondary			
Alexa Fluor 488 Mouse	Goat	Thermofisher	1:1000
Alexa Fluor 568 Rabbit	Goat	Thermofisher	1:50

The secondary antibodies: 1:1000 goat anti-mouse Alexa Fluor 488, and 1:50 goat anti-rabbit Alexa Fluor 568, (ThermoFisher, Waltham, Massachusetts, United States) were diluted in PBS and added to samples, before incubation for 1 hour at room temperature in the dark. The samples were then washed with 1xPBS three times for three minutes each shaking cycle. Finally, coverslips were mounted to slides with Fluoroshield Mounting Medium with 4',6'-diamidino-2-phenylindole (DAPI) (ab104139) (ABCAM, Cambridge, United Kingdom). The samples were imaged on the Leica SP8 Confocal Microscope instrument, at 10x or 40x magnification for fibroblasts and keratinocytes, and 20x magnification for skin tissue sections.

Biological age

To calculate the biological age of human skin or keratinocytes and fibroblasts *in vitro*, the CpG methylation of ELOVL2 [Moore et al., 2012] was determined using bisulfite converted DNA (bsfDNA) [Frommer et al., 1992; Li and Tollefsboi. 2011]. Following 24-hour incubation (37°C and 5% CO_2) of UVA exposed keratinocytes and fibroblasts, DNA was extracted according to the instructions of the Qiagen DNA extraction kit (Qiagen, Hilden, Germany). Bisulfide conversion was performed on 100 ng of genomic DNA samples as per the instructions of the EZ-96 DNA Methylation-lightning bisulfide conversion kit (Zymo Research, Irvine, California, United States). In bsfDNA, the non-methylated cytosines residues were converted into uracil. The bsfDNA (2 uL) was amplified by Hot-start PCR using Platinum™ II Hot-Start PCR master Mix (2X) (ThermoFisher, Waltham, Massachusetts, United States) in 25 uL of the reaction. A final concentration of 0.8 uM ELOV2 primer was used for PCR amplification [Table 3.4]. The PCR procedure started with initial hold at 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Then a final extension step at 72°C for 6 mins. The PCR products were ligated and transformed using the Topo™ TA Cloning™ Kit sequencing, with One Shot™ TOP10 Chemically Competent E. Coli (ThermoFisher, Waltham, Massachusetts, United States), using LB broth and LB plates with ampicillin and X-Gal. Following transformation, 10 colonies per LB plates (grown overnight) were selected and cultured in LB broth overnight. The plasmid DNA was extracted as per instructions of QIAamp DNA mini kit (Qiagen, Hilden, Germany), and sent for sanger sequencing to Source BioScience (Nottingham, England).

10 independent clones were sequenced per condition, and the number of T (YG sites) points across this specific sequence (below with 9 highlighted CpG sites) of the ELOVL2 whole amplified region (30 CpG sites) of the genome were measured.
(TAGGTTTAGTYGGYGTYGGTTTYGYGYGGYGGTTTAAYGTTTAYG):
Using CodonCode Aligner (CodonCode Corporation, Centerville, Massachusetts, USA), methylation of 2 sites (in bold) of these 9 highlighted CpG sites across this specific sequence were measured. The sequence per n sample received from Soruce BioScience was inputted into CodonCode Aligner and using the search function for the above ELOVL2 primer sequence, the number of highlighted methylated regions were measured.
The % methylation of CpG sites 5 (YG in red bold) and 7 (YG in green bold) of 10 colonies per sample were calculated and inserted into the equation:
-42.8393176902677+(0.63266203860581*CpG5) +(0.877474742612866*CpG7)
[Daunay et al., 2019].
For example, if n=2 out of n=10 sequences per sample underwent methylated changes at the CpG 5 site then the value 0.2 was multiplied by 0.63266203860581in that bracket etc.

Table 3.5: Primer sequencing of ELOVL2 age-related marker.

Gene	Sequence [Bekaert et al., 2015]	Product Size (base pairs)
ELOVL2	**FW**AGGGGCGTAGGGTAAGTGA **RV**AAACCCAACTATAAACAAAACCA	267

Cytokine assay

To identify cytokines secreted by fibroblasts that may be signalling to keratinocytes upstream, a cytokine assay was performed on UVA conditioned fibroblast media and untreated control media according to the instructions of the RayBio® C-Series Human Cytokine Antibody Array C1000 kit (RayBiotech, Norcross, Georgia, United States). To analyze, the Protein Analyzer Plugin for ImageJ was used to examine the cytokine 6 and cytokine 7 antibody membranes. Analysis began by using the 'Analysis Array Menu', followed by the 'Array Analysis' function. This action induced background subtraction and built a graphical interface for the dot matrix analysis. The visualisation was optimised by activating options available from the graphical interface. Three dots were selected forming angle (top-left, top-right, and low-left) to set a template for the interface of the membrane. A grid was subsequently formed, and the matrix was measured automatically as a table of values. Once the arrays were individually

analysed using the "Array Analysis" tools, the "Group Pattern" menu constructs a global view of a set of arrays. The parent folder was set which contained the analysed arrays. This folder was selected containing the array analyses by the "Masterize from Analysis Repertories" function. This function searched for result tables acquired from the "Array Analysis" functions, in the parent folder. The tool explored any sublevels, and built a master image, or pattern, associated to a master table presenting all the results. The values generated from this analysis were used to measure the Log2FoldChange and -Log10(p-value) difference between control and experimental conditions for each cytokine, to thus generate a volcano plot using GraphPad Prism (GraphPad Software, San Diego, California USA, www.graphpad.com).

Anti-Ageing therapeutic strategy

Administration of Klotho in media

To investigate the preventative effect of the anti-ageing compound, klotho, on the UVA-treated accelerated biological age of cells (keratinocytes and fibroblasts) and skin tissue samples, the protein was administered to the samples (at low dose of 0.1 μg/mL as per recommended dose of supplier Proteintech Group, Illinois, United States). Once cells were confluent, the media of control cells was changed to fresh media, and samples to be treated with klotho were treated with 1 mL of diluted 0.1 μg/mL of klotho protein in cell medium (KSFM or DMEM+10% FBS). The cells were left for 24 hours in incubation (37°C and 5% CO_2) before they were washed in PBS, trypsinised, pelleted, then extracted of DNA following the steps of the Qiagen DNA extraction kit (Qiagen, Hilden, Germany). For skin samples, klotho was administered, at 0.1 μg/mL in DMEM media, topically to human skin (100 μL volume), 24 hours before punch was applied. The experiment was closed at 7 days (following incubation: 37°C and 5% CO_2), and a piece of the skin was sliced and lysed, before DNA was extracted according to the steps of the Qiagen DNA extraction kit (Qiagen, Hilden, Germany). For both cell and tissue samples, qPCR, immunostaining, bisulfite conversation, as well as wound healing studies were performed (scratch assay for keratinocytes, biopsy punch assay for skin tissue samples).

Klotho in downregulating senescence of keratinocytes and fibroblasts

To assess the effect of klotho as a preventative to UVA-induced senescence of keratinocytes and fibroblast cells, confluent cells were treated with klotho (1 mL of 0.1 μg/mL diluted in KSFM) and incubated for 24 hours (37°C and 5% CO_2). Following 24-hour incubation of the respective cells, they were UVA exposed (1.0 J/cm^2), then following further 24-hour incubation, RNA was extracted which was then converted to cDNA and qPCR or

immunostaining was performed to assess the expression of senescent markers: p21 and Lamin B1, at the RNA and protein level.

Klotho in scratch assay

To explore the impact of the anti-ageing compound, klotho, on the repopulation of UVA exposed keratinocytes, klotho was administered to confluent keratinocytes (initially seeded at 50,000/mL, 1 mL per well in 12 well plate) 24 hours before UVA exposure, at a concentration of 0.1 µg/mL diluted in KSFM. The KSFM medium was changed 24-hour after incubation (37°C and 5% CO_2), the cells were then UVA exposed (1.0 J/cm^2), and following a further 24-hour incubation, medium was changed once again, and the keratinocyte cell monolayer was scraped in a cross-scratch pattern using a 200 µL pipette tip. The cells were imaged (5.0x magnification) once every hour for up to 10-15 hours and then once more at 24 hours to assess for any changes in cell migration. Hour 0 and hour 24 are compared in analysis and represented in this thesis as they present considerable change in % cell covered area. Analysis was performed using ImageJ [Schneider et al., 2012] [Figure 3.7].

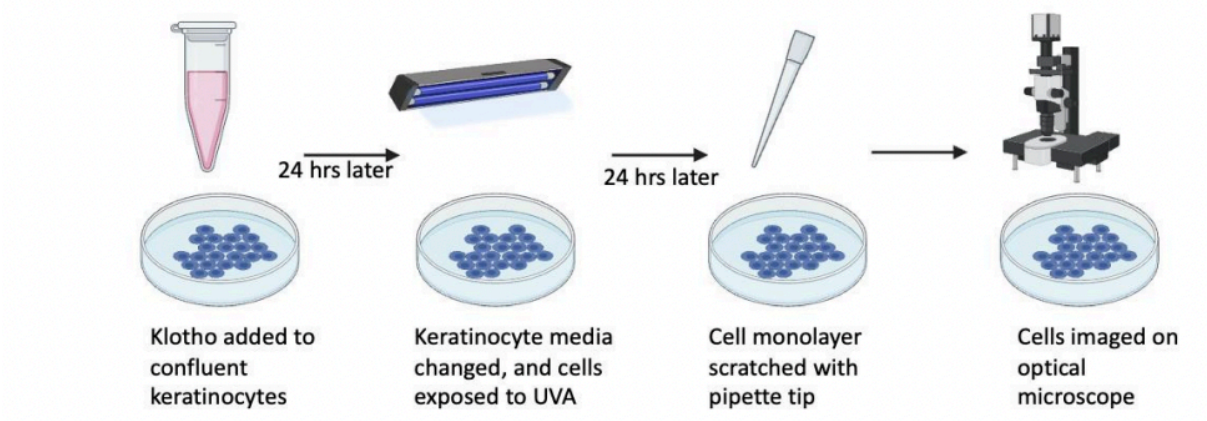


Figure 3.7: Schematic of klotho administration to keratinocytes methodology.

Klotho in epithelial mesenchymal signaling.

To prepare conditioned fibroblast media, confluent fibroblasts at passage 3-6 were seeded at 50,000/mL (1 mL per well) in a 12 well plate. Once confluent, the cells in DMEM medium were first treated with the klotho protein (0.1 µg/mL) diluted in DMEM medium and incubated for 24 hours at 37°C and 5% CO_2. Next, the cells were washed in 1 mL of 1xPBS, and cultured with fresh DMEM medium (no klotho) then exposed to 2.0 J/cm^2 of UVA (excluding control wells i.e. control only, and control and klotho only) and left in a 37°C incubator with 5% CO_2 for 24 hours medium that had been 'conditioned' by the fibroblasts was collected and stored in falcon tubes (stored in 20°C long term) [Figure 3.8].

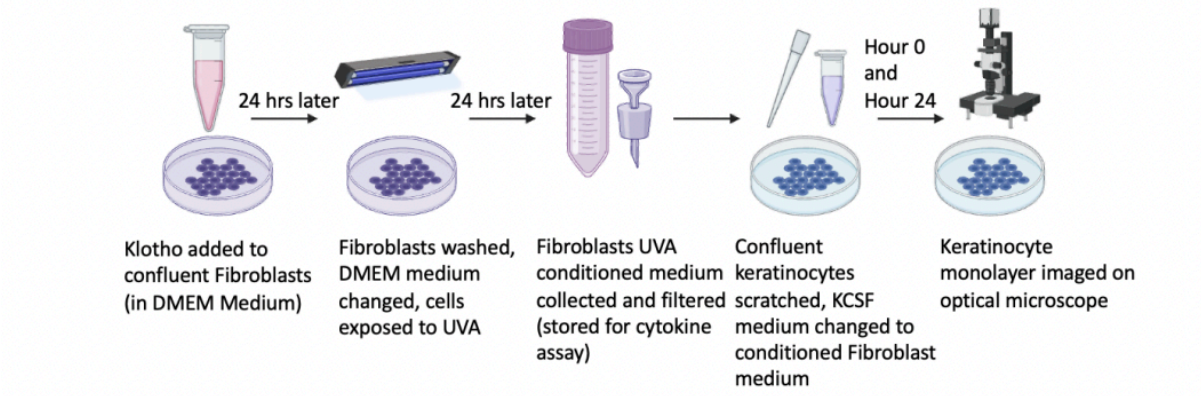


Figure 3.8*:* Experimental schematic of assessing klotho effect on epithelial mesenchymal signalling.

This encompassed 4 conditioned samples: control, control and klotho treated medium, UVA only, and UVA and klotho treated medium. After collection and filtration (using 0.2 µm filter) of conditioned media, this was used on keratinocyte cultures. Keratinocytes were seeded (50,000 per well in 12 well-plate) and maintained in KSFM (1 mL per well) (with growth supplements) until cells were confluent. Keratinocytes were rinsed in PBS; a scratch was made on the cell mono layer using a 200 µL pipettes tip. At this point the media was aspirated and replaced with the media 'conditioned' by fibroblasts, then left to incubate for 24 hours. Cell repopulation was monitored over time, >10 hours and independently at 24 hours. N=3 for fibroblast conditioned medium, N=1 of keratinocytes, and n=3 technical repeats of fibroblast medium and keratinocyte cells.

Klotho effect on wound healing

To assess for changes in UVA impaired wound healing 30 µL of diluted Klotho protein (0.1 µg/mL in DMEM media) was topically or via coated MN administered (15 minutes only before topical treatment aspirated off or MN was removed) to the surface of processed skin pieces in media, in a 6-well plate. Following 24-hour incubation (37°C and 5% CO_2) samples excluding controls were exposed to 1.0 J/cm^2 of UVA, and immediately after a 2 mm biopsy punch was administered. The skin samples were imaged once every day for up to 7 days using a stereo microscope (2.0 x magnification) and the wound area size in µM was measured using ImageJ [Schneider et al., 2012] comparing day 0 to day 7 [Figure 3.9].

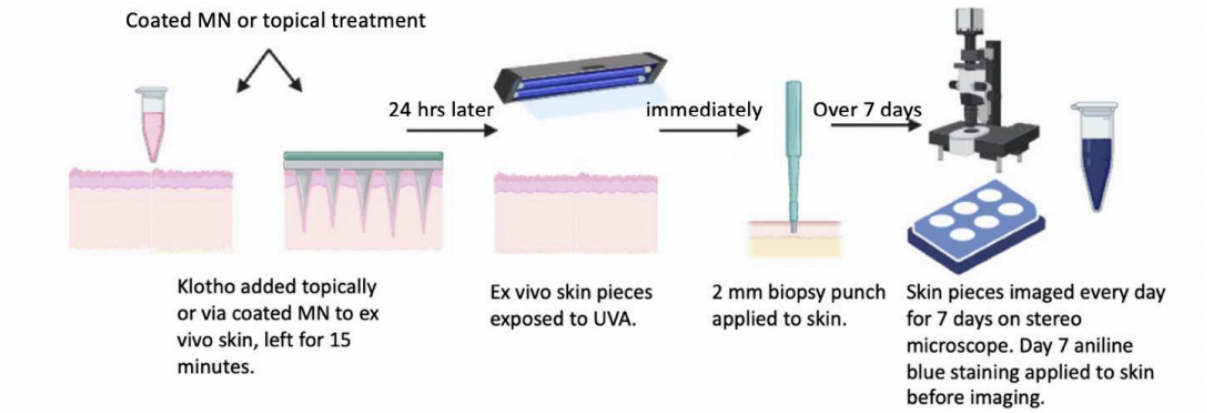


Figure 3.9: Experimental schematic of klotho effect on wound healing.

Bioactivity of Klotho in PAA

To assess the bioactivity of the klotho protein in polyacrylic acid (PAA) (Sigma, St Louis, Missouri, United States), a hydrogel coating for a microneedle device, the protein was diluted (0.1 µg/mL) in high MW (100,000 MW) 0.035% wt. PAA: and freeze-dried high MW (100,000 MW) 0.035% wt. PAA, as well as low MW (2000 MW) PAA for 3 days. To assess bioactivity of long term stored freeze dried klotho in high MW PAA, freeze dried klotho in PAA that had been stored at 4°C for 9 months (long term) was administered to confluent keratinocytes. Following 24-hour incubation (37°C and 5% CO_2) of the cells, they were exposed to UVA (0.15 J/cm^2), against untreated controls, klotho treated controls, and UVA only treated cells. After a subsequent 24-hour incubation of the cells, the cell monolayer was scraped across the surface in a cross pattern and the repopulation across after 24 hours was assessed. The % of cells migrated over time was measured using ImageJ [Schneider et al., 2012]. The same method was applied using klotho dissolved in KCSF medium stored for 3 months at 4°C to assess bioactivity of non-PAA encapsulated long-term stored klotho on keratinocyte repopulation.

Klotho coated MN penetration of GelMA® synthetic model

To confirm penetration and diffusion of FITC-tagged klotho coated microneedles to synthetic skin samples, the coated microneedle device was pressed onto the surface of Photogel®-IRG, Methacrylate Gelatin Hydrogel kit (Sigma Aldrich, St. Louis, MO, USA) [Bedell et al., 2020; Piao et al., 2021; Bupphathong et al., 2022; Sun et al., 2018] GelMA® for 15 minutes, (method of manufacturing GelMA® outlined in chapter 2). Once needles had been removed, the area of the synthetic skin where the microneedle had been applied was imaged on a Leica SP8 TCS STED 3X Confocal microscope (20 x magnification). A Z stack (10 µm per stack) was imaged

to assess the diffusion penetration of FITC-tagged Klotho in PAA on the GelMA skin. Images were analysed using ImageJ [Schneider et al., 2012].

Klotho coated Microneedle application on *ex vivo* skin.

To evaluate the effect of klotho coated MN on dermal genes, the coated device was applied to *ex vivo* skin (no wounding). Klotho at a dose of 0.1 µg/mL was diluted in 0.035% wt PAA, and the engineered MN (from chapter 2) was dip-coated in 30 µL of solution and desiccated for 30-40 minutes. The coated device was then applied to processed 1x1 cm skin pieces for 15 minutes compared with topical appointment of klotho (in PAA, 30 µL) which was aspirated off for 15 minutes. All samples stored in DMEM+10% FBS in a 6 well plates (24 hours after 37ºC, 5% CO_2 incubation) were exposed to 1.0 J/cm^2 UVA (except for control). Skin pieces (in a 6 well plate, n=6, N=3) were maintained for 7 days in 37ºC and 5% CO_2 before skin was homogenized, RNA extracted, and cDNA converted. A qPCR on the extracted RNA was performed to assess effect of topical, klotho coated MN, on UV exposed skin against UV only and untreated controls, on modulating dermal gene expression. Genes evaluated are outlined in table 3.3, with presented primer sequences.

Statistics and Reproducibility

Cell and skin experiments were performed with n=3 (cells) or 6 (skin) technical replicates for each independent condition, as well as N=3 biological replicates. All graphs were generated using graph pad prism version 9 or 10. Statistical analysis testing including one way ANOVA, or two-way ANOVA (for N=3 biological replicate samples) or unpaired T test were performed using GraphPad Prism version 10 (GraphPad Software, San Diego, California USA, www.graphpad.com). (*P < 0.05, **P < 0.01, ***P < 0.001; ****P < 0.0001). Statistical details for each experiment can be found in the corresponding figure legends.

3.3 Results

Human skin undergoes intrinsic changes with age. These changes are driven by a variety of factors but the most physiologically relevant is UVR. UVA is well known to induce photoaging of human skin however, the mechanism behind age acceleration of the skin has not been extensively explored. Moreover, the part that UVA exposure serves in contributing to impeded regeneration of human skin with ageing, is also poorly understood.

3.3.1 Optimisation of UV doses and wound healing on *Ex vivo* skin.

In the preliminary optimisation of UVA doses, I selected UVA doses to assess; 0.2, 1.0, 5.0, and 10.0 J/cm^2. From these early experiments I identified that >1.0 J/cm^2 was sufficient in delaying the healing capacity of *ex vivo* skin [Figure 3.10a, bi, bii].

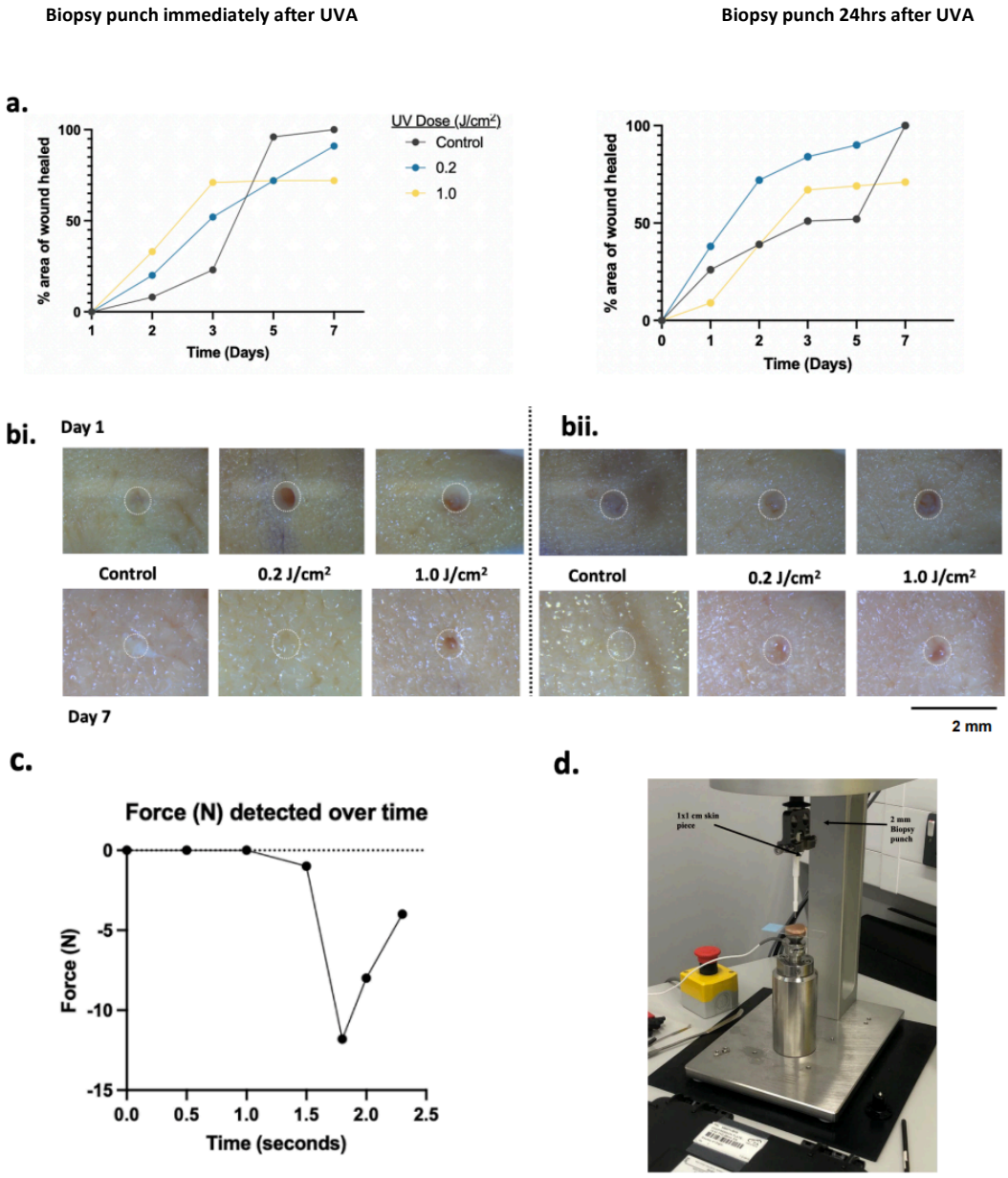


Figure 3.10: UVA optimisation. (a) Graphical representation of % area of wound healed over 7 days, on unexposed skin, 0.2 and 1.0 exposed skin, (left) biopsy punch immediately administered to UVA exposed skin, (right) biopsy punch administered 24 hours after UVA exposure n=1, N=1. (bi) Optimal timing of biopsy punch application following UVA exposure was compared between immediate exposure vs (bii) 24 hours after UVA exposure and biopsy administration. The images were compared from day 0 and day 7 (1.75 x magnification) N=1. (c) The force (N) required for the 2 mm biopsy punch to pierce the sub dermal layer of the skin was to be 11.8 N at a rate of 1.7 seconds and 0.55 mm depth N=1. (d) diagram of the DMA instrument set up with 2 mm biopsy punch and 1x1 cm skin piece.

To identify the optimal time point of administering a biopsy wound on skin following UVA exposure, I compared healing capacity of skin between skin that was wounded immediately following UVA, and skin wounded 24 hours after UVA exposure. The control samples differed between both conditions, healing at a faster rate (between 3-5 days) in the experiment where biopsy punch was applied immediately after UV exposure vs 24 hours after. However, I found that irrespective of when the wound was created relative to the UVA exposure, 1.0 J/cm^2 was sufficient in delaying wound healing compared to controls [Figure 3.10a, bi, bii]. Therefore, in future experiments I applied the biopsy punch to *ex vivo* (1x1 cm) skin pieces immediately after the samples had been exposed to UVA.

Next, to standardise all 2 mm biopsy punches on the surface of *ex vivo* skin, I began using a DMA instrument [Figure 3.10c, d]. Initially, forces ranging from 10-12 N [Conci et al., 2017; were trialled on 1x1 cm skin pieces to identify the optimum force required to puncture the skin using a 2 mm biopsy punch. Forces from 10 N were tested on skin because material testing systems have previously reported that forces required to puncture the skin range from 9.8 to 56.7 N depending on material choice [Nolan et al., 2012]. Therefore, the 2 mm biopsy punch was plastered to the header of the machine and 10, 11 or 12 (N) was applied on skin over time (seconds) to identify the time needed to induce penetration of skin piece. I identified from the force displacement curve that an application of 11.8 N on the skin piece for 2 seconds was sufficient to puncture the 1x1 cm *ex vivo* skin piece [Figure 3.10c, d]. The skin was compressed at a depth of 0.55 mm that resulted in sub dermal skin breakage. These wounds were thus standardised in depth but inconsistent in area wound size, however, this was always accounted for in subsequent analysis.

3.3.2 Young and Old skin heal differently.

First to investigate if chronological age influences wound healing and the detrimental impact UVA exposure has on human skin, I looked for differences in the biological age, senescent marker expression and wound healing capacity between young vs old skin. Initially, I explored the baseline differences in wound healing between young (29 years old), middle aged (48 years old) and old (80 years old) *ex vivo* skin [Figure 3.11]. The intrinsic differences in wound healing capacity between different aged skin was assessed to explore the relationship that rising chronological age may have on wound healing function.

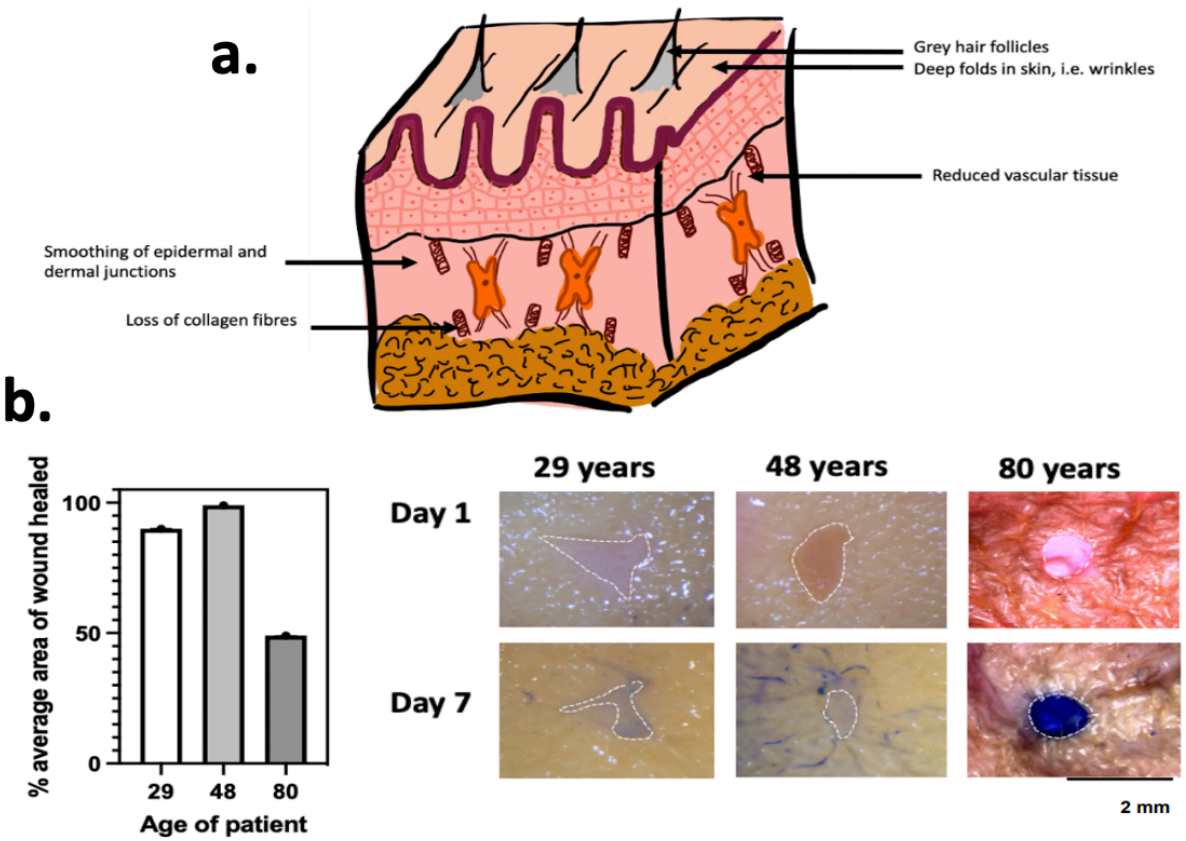


Figure 3.11: Baseline differences in wound healing in young vs elderly skin. (a) Schematic of the phenotype of elderly skin. (b) Differences in healing capacity between young (29 years old), middle-aged (48 years old) and the elderly (80 years old) skin (left) graph n-6 N=1.(Right) The skin is stained at day 7 using aniline blue staining.

Elderly skin undergoes various phenotypic and intrinsic ages because of time, largely attributed to prolonged UVA/B exposure [Figure 3.11a]. These changes include loss in collagen expression, smoothing of the DEJ, wrinkling and translucency of the skin, and reduced vascular tissue [Figure 3.11a]. These changes in skin integrity will influence the healing capacity and so I wanted to assess how considerably wound healing of elderly skin varies from young skin.

Firstly, punctured skin (young, middle aged or elderly) was imaged (day 0) and incubated for 7 days at 37°C. At day 7 the skin samples were stained with aniline blue and imaged [Figure 3.11b]. Young and middle-aged skin was found to heal completely by day 7 as aniline blue staining was a very light blue hue whereas the elderly skin sample presented a dark blue stain. Aniline blue stains collagen, which would only be exposed if reepithelization has not occurred. This indicates that the wound area of the old skin had not re-epithelised whereas young and middle-aged skin had at 7 days following skin puncture. Wound healing is in fact delayed in

80-year-old skin vs 29 and 48 years old, highlighted by a statistically significant reduction of 50% in average wound area size at 7 days following puncture, compared with young and middle-aged skin, which is close to if not at 100% healed [Figure 3.11b]. This suggests that with chronological ageing, wound healing is impacted, supporting previous reports that old skin presents delayed healing compared to young skin [Khalid et al., 2022; Gerstein et al., 1993].

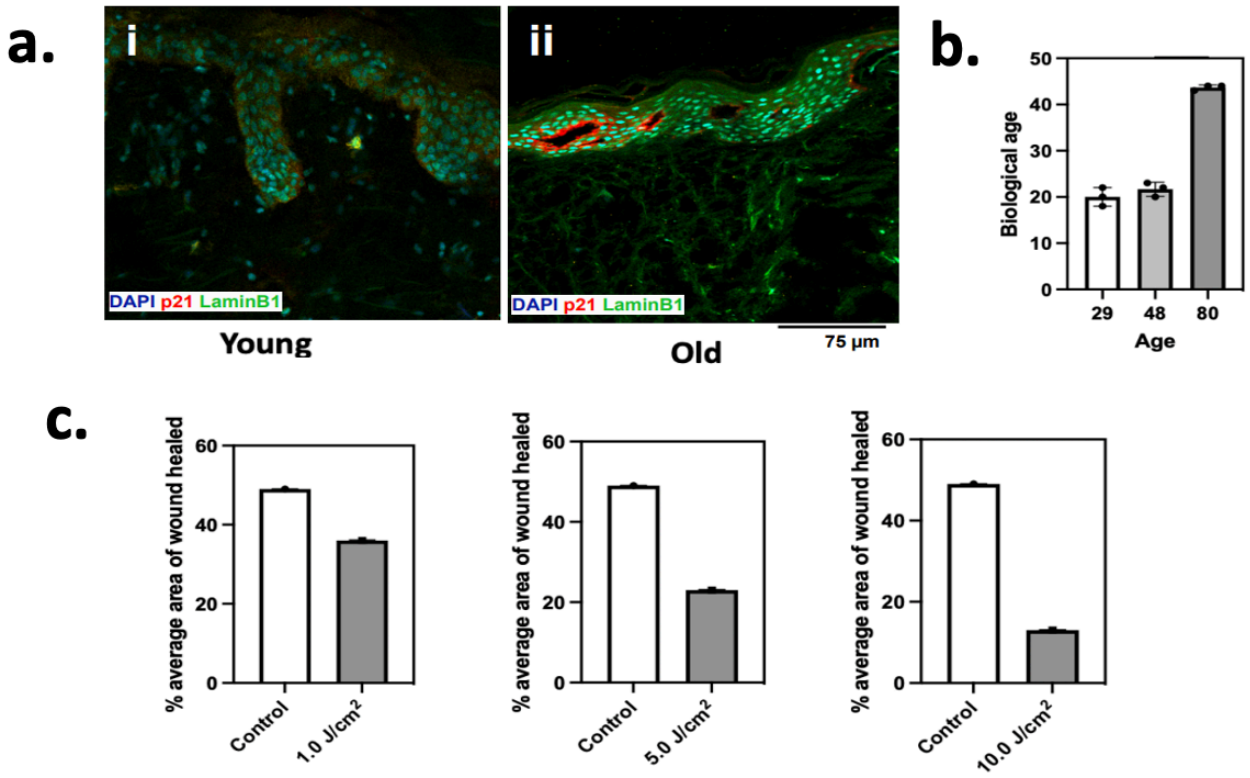


Figure 3.12: Changes in wound healing of elderly skin in response to UV. (a) Immunostaining images of young (i) vs old (ii) *ex vivo* skin samples with p21 (red), and LaminB1 (green) and DAPI (blue). (b) Biological age in young, middle aged and old skin n=3, N=1 (e) Effects of UVA exposure on wound healing of old skin n=6 N-1. Images were captured at 2.0x magnification.

Moreover, I assessed for any differences in senescent marker expression, p21 and laminB1 with chronological ageing [Figure 3.12a]. It has previously been reported that human skin exposed to UVA and UVB present upregulation of senescent markers p21 and p53 (4 hours after irradiation) [Pontén et al., 1995]. Notably, my immunostaining of young vs old skin revealed enhanced p21 expression in the old skin, while LaminB1 expression was largely unchanged [Figure 3.12a[.. This indicates that old skin presents greater DNA damage than young skin likely driven by increased vulnerability of old skin to UV damage (compared with young skin) due to thinner epidermis caused by the long-term exposure to UVA/B. DNA damage has been

reported to drive aberrant DNA methylation which can thus enhance one's biological age [Fernandez et al., 2021; Morano et al., 2014]. I explored the differences in biological age in young, middle aged and old skin by assessing changes in methylation of age-related CpG sites of the ELOVL2 gene. I identified that old skin presented consistent hypermethylation of age related CpG sites resulting 44 years of age [Figure 3.12b, Appendix 3.1], double the biological age calculated in young and middle-aged skin. Overall, these findings showcase that with chronological ageing, regenerative capacity is dwindled, and biological ageing is at its peak (using ELOVL2 equation) however the mechanism behind these intrinsic differences is not heavily understood and is likely attributed to a variety of internal and external factors i.e. genetic factors, lifestyle, UVA exposure etc [Lovell et al., 1987; Chaudhary et al., 2020].

UVA exposure is a physiologically relevant stimuli that every individual is habitually exposed to, and it is well acknowledged that this stimulus drives photoaging of the skin which can alter skin identity and thus drive skin to become more susceptible to subsequent UVA damage. Notably, old skin exhibits further significantly slowed wound healing following pre-emptive UVA exposure, with a 30% reduction in wound area healed at 1.0 J/cm^2, to 60% at 5.0 J/cm^2 and 80% at 10.0 J/cm^2 all after 7 days (relative to the control) following UVA exposure [Figure 3.12c]. As these findings highlight that UVA exposure leads to greater decline in wound healing of elderly skin, I chose to next explore the effect of this stimuli on the healing capacity of young skin. I investigated whether UVA could enhance the biological age of young skin as well as transform the skin to exhibit the impaired healing capacity that old skin exhibits.

3.3.3 UVA age accelerates human skin and impairs wound healing *via* DNA damage induction.

Following the observation of baseline differences in wound healing between young and old skin, I next investigated whether UVA exposure could impede the healing capacity of young skin (33 to 37 years old), to heal comparably to old skin [Figure 3.13].

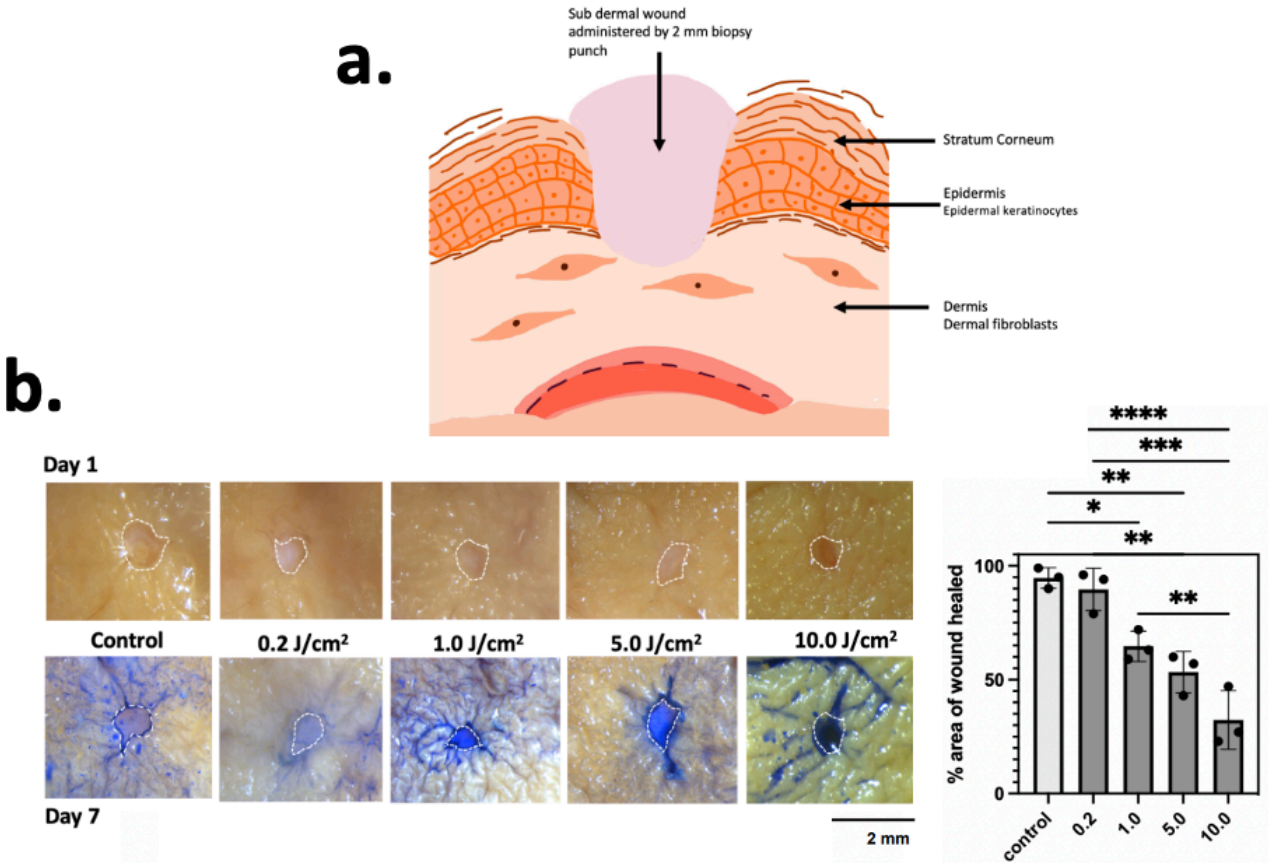


Figure 3.13: UV injury accelerates ageing of young skin impairing wound healing. (a) Schematic of dermal biopsy punch administered to all skin samples. (b) (left) Visual representation of punctured young skin stained with aniline blue 7 days after UV exposure. (right) % wound healed of N=3 biological replicates, with n=6 technical repeats of *ex vivo* human skin in response to variable doses of UVA (two-way ANOVA). (

A subdermal wound was applied to all skin samples (control and UVA exposed alike) using the DMA compression instrument. Reepithelization of the wound was visualised at day 7 using aniline blue staining [Figure 3.13a, and b]]. Interestingly, >1.0 J/cm^2 UVA exposure is shown to significantly impair wound healing with a 30% reduction in wound closure 7 days following puncture compared with the un-exposed control [Figure 3.13b]. Moreover, enhanced aniline blue staining (light to dark blue gradient) of UVA exposed (>1.0 J/cm^2) skin 7 days following puncture highlights that increasing UVA dosage correlates with declining wound healing [Figure 3.12c]. Old skin heals at 50% the speed of young skin [Figure 3.11b], and young skin exposed to 10 J/cm^2 of UVA heals at 28% of the speed of nonexposed skin [Figure 3.13b]. As aniline blue staining switches from a light to dark hue from non-UVA to increasing UVA exposed skin samples, this suggests that acute UVA exposure is sufficient in impairing the regenerative capacity of young skin.

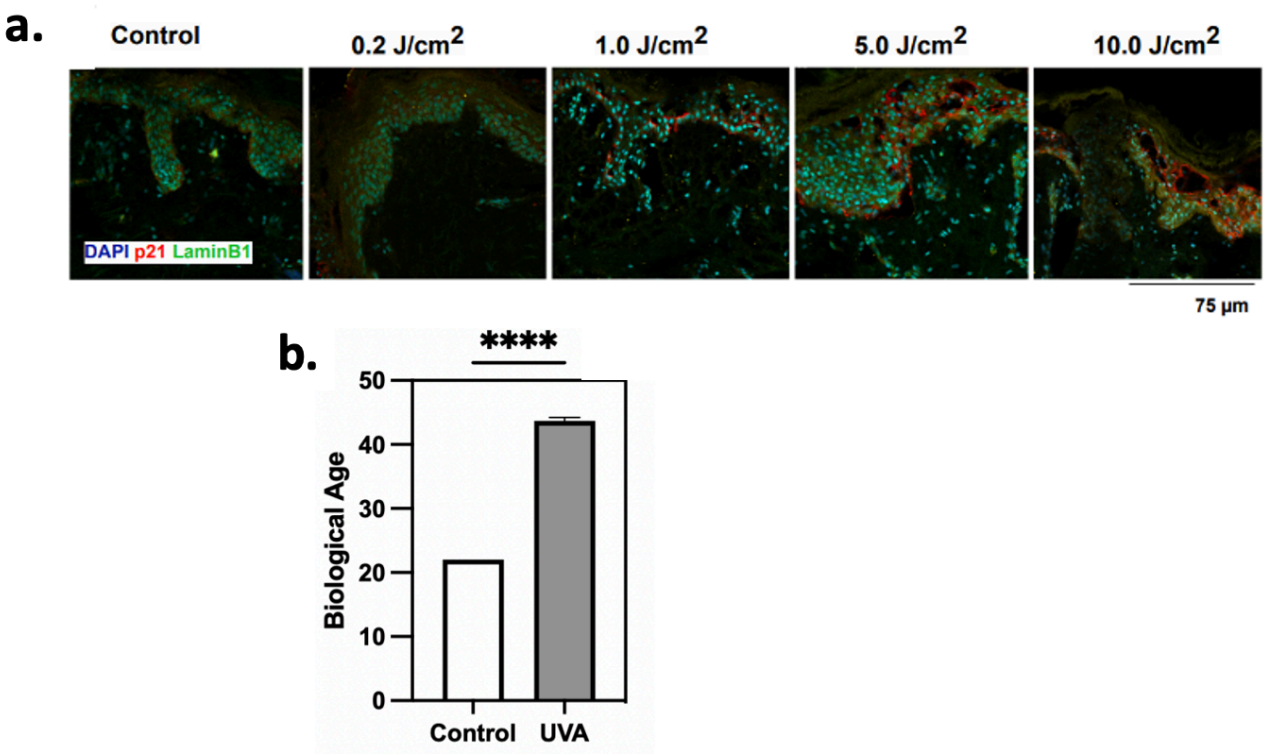


Figure 3.14: UV injury induces DNA damage in young skin. (a) Presents immunostaining of young skin in response to increasing UVA dose (0.2, 1.0, 5.0 and 10.0 J/cm^2), (blue DAPI, green LaminB1, and red p21), images captured at 20x magnification. (b) Biological age of young skin in response to UVA (n=10 N=3 unpaired t-test).

In response to a dose of UVA (>1.0 J/cm^2) there is an increasing trend of p21 DNA damage expression in young skin while LaminB1 expression remains consistent across samples [Figure 3.14a]. This is comparable to old skin which presented strong DNA damage expression [Figure 3.12a

To explore whether UVA exposure also drives biological ageing of the skin, I assessed the effect of this stimuli on age-related CpG sites. I found that CpG methylation was significantly elevated in young skin in response to UV injury (1.0 J/cm^2), with a doubling of the biological age (22 to 44 years old between control and UVA (patient matched) exposed samples) [Figure 3.14b, Appendix 3.2], thus highlighting that UV exposure biologically ages human skin in parallel with impeding the intrinsic wound healing capacity. These findings show that UVA exposure is sufficient in not only biologically ageing young skin via hypermethylation but also in delaying the intrinsic healing capacity, a phenotype presented in elderly skin. To understand the mechanism behind this UVA correlative relationship with skin ageing and in turn diminished healing I next assessed the effect of this stimuli on epidermal and dermal primary cells.

3.3.4 UVA exposure modifies the phenotype of keratinocytes and fibroblasts in young skin.

To understand the mechanism behind UVA impaired healing of young skin, I explored the effect of this physiological stimuli at the cellular level on epidermal keratinocytes and dermal fibroblasts, respectively [Figure 3.15].

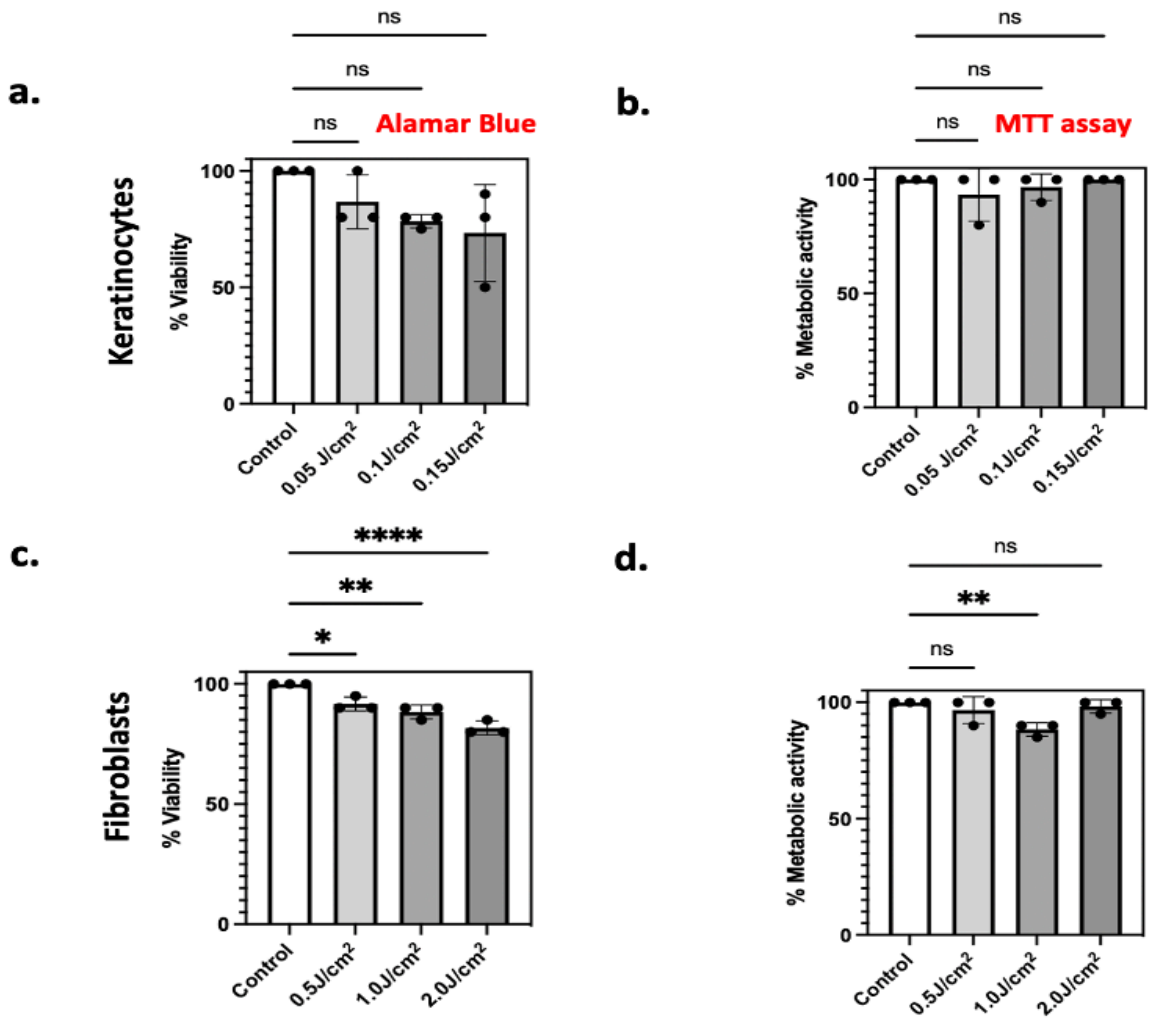


Figure 3.15: Assessing viability and metabolic activity of UV exposed Keratinocytes and Fibroblasts. (a, c) presents the % viability of keratinocytes (control vs 0.05 J/cm^2: p-value: 0.5535, control vs 0.1 J/cm^2: p-value: 0.1992, and control vs 0.15 J/cm^2: p-value: 0.0982) and fibroblasts (control vs 0.5 J/cm^2: p-value 0.0149, control vs 1.0 J/cm^2 p-value 00020, and control vs 2.0 J/cm^2: p-value <0.0001), respectively using alamar blue staining of cells in response to UVA dose after 24-hour incubation of cells. (b, d) % metabolic activity of keratinocytes (control vs 0.05 J/cm^2: p-value 0.4843, control vs 0.1 J/cm^2: p-value 0.8624, and control vs 0.15 J/cm^2: >0.9999) and fibroblasts (control vs 0.5 J/cm^2: p-value 0.5485 , control vs 1.0 J/cm^2: p-value 0.0095, and control vs 2.0 J/cm^2: p-value 0.8894), respectively in response to UVA dose following 24-hour incubation of cells using MTT assay. (statistical test with two-way ANOVA, n=3, N=3).

Firstly, in preliminary experiments I assessed the effect of varying UV doses on the metabolic and proliferative activity of keratinocytes and fibroblasts, to establish a suitable threshold of UVA that modified the phenotype of the skin cells without inducing cell death [Figure 3.15]. I identified that 24 hours after UVA exposure, <0.15 J/cm^2 on keratinocytes and <2.0 J/cm^2 on fibroblasts, there was no significant impact on % cell viability nor metabolic activity for keratinocytes but significant difference in viability in fibroblasts with increasing UV but % viability nor % metabolic activity did not fall under 80% with any UV doses. This suggests that cells remained healthy 24 hours following UVA exposure and so were suitable for subsequent study on UVA effect on cellular functionality. I also assessed the effect of repeated UV dose on keratinocytes and fibroblasts over a 4-day period [Appendix 3.3 and 3.4] to identify the time of UVA exposure that would preserve this healthy proliferative state until UVA induced cell death was reached. However, repeated UVA exposure on keratinocytes and fibroblasts resulted in increasing cell death with every additional UVA dose, thus I decided to only assess single UVA dose on cells from that point forward.

After identifying the optimal UVA dose that would elude cell death, I next investigated the impact this stimulus has on cellular morphology to further understand the mechanism behind UVA impaired healing of *ex vivo* skin. Epidermal keratinocytes are essential skin cells necessary for mediating the recruitment of immune cells, neutrophils, and macrophages, to an injury site, and in signalling to fibroblasts for the development of the granulation in the wound bed [Kelly. 2016]. Notably, during injury keratinocytes, as primary cells that form the skin epidermis, migrate over the wounded site, and proliferate to cover the wound site to prevent infection and subsequent damage. I assessed for any changes in keratinocyte repopulation *in vitro* in response to UVA exposure using a scratch assay model [Figure 3.16].

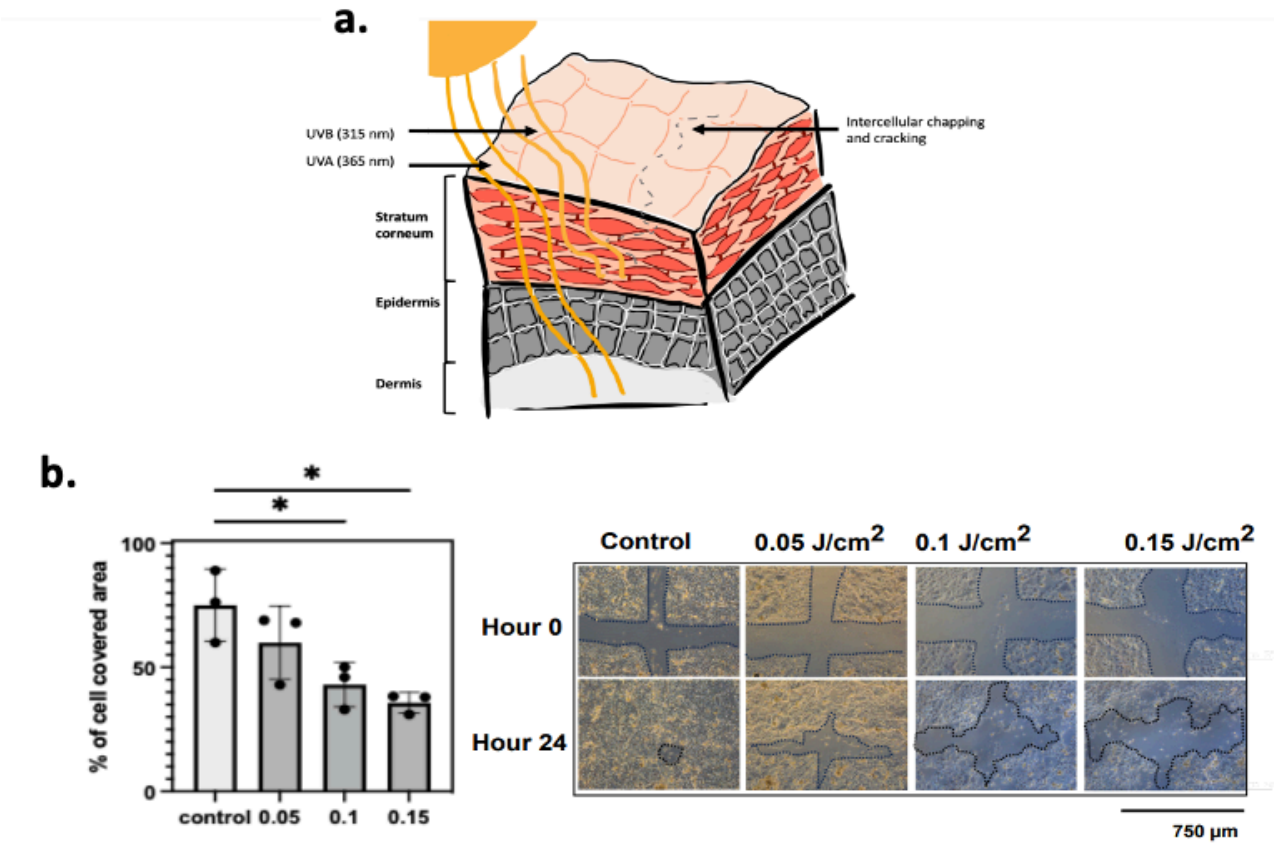


Figure 3.16: Effect of UVA dose (0.05, 0.1 and 0.15 J/cm^2) on keratinocyte cells. (a) Schematic of UVA/B penetration of human skin. (b) (left) Graph of N=3 keratinocyte cells and n=3 repeats showing % change in cell covered area of Keratinocyte monolayer 24 hours after scratch induction, (N=3 two-way ANOVA). (Right) Representative images of scratched keratinocyte monolayer, UVA exposed vs control samples (Hour 0 vs Hour 24

UVA exposure (0.05, 0.1, and 0.15 J/cm^2) 24 hours before scratch induction on the keratinocyte monolayer resulted in significantly delayed repopulation of keratinocytes, contrary to non-UVA treated cells [3.16a], which almost covered the cell monolayer 24 hours after the scratch was made [Figure 3.16b]. Notably, 0.1 J/cm^2 injury on keratinocytes culminated in only 50% of a cell covered monolayer 24 hours following scratch induction indicating impaired repopulation of the cells in response to the UVA stimuli [Figure 3.16b]. Senescent keratinocytes have been found to contribute to the declining regenerative capacity of the skin [Wilkinson et al., 2020; Velarde et al., 2012; Zouboulis et al., 2008; Gosain et al., 2004] therefore, to further understand how UVA may be driving the delay in keratinocyte repopulation following scratch induction, I evaluated changes in senescent marker expression.

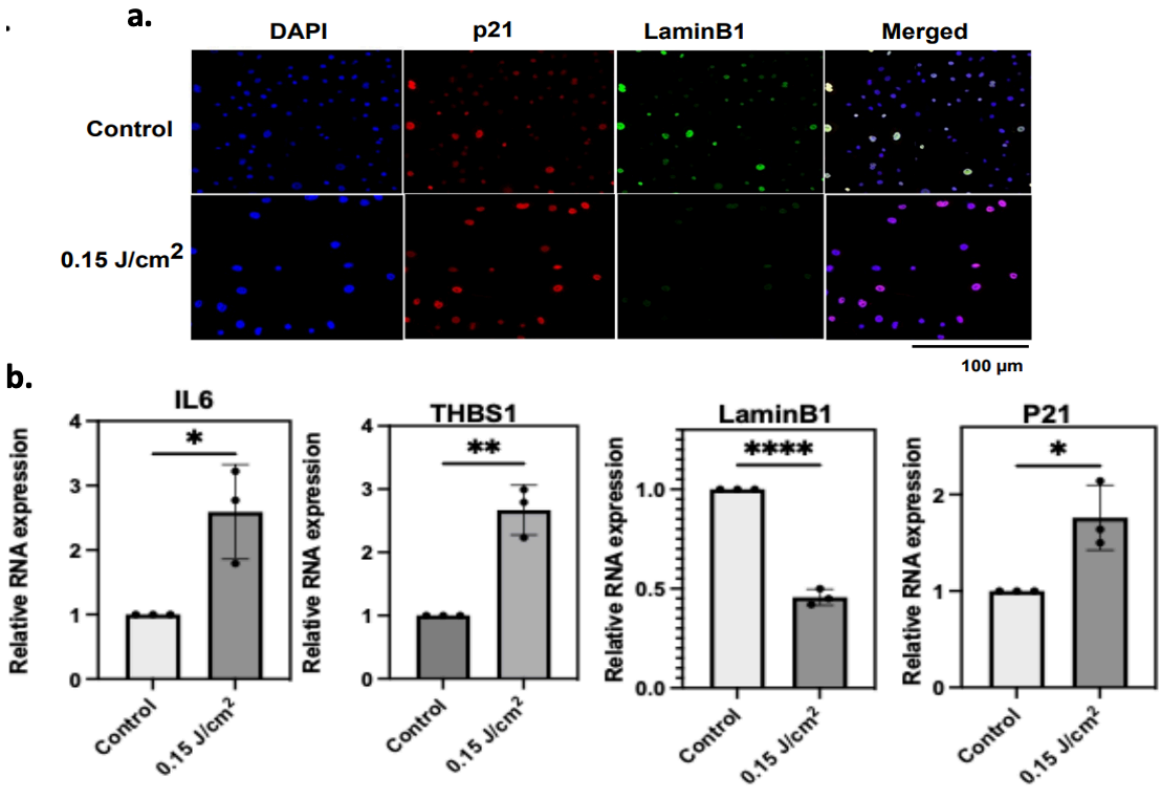


Figure 3.17: Effect of UVA dose (0.15 J/cm^2) on gene expression of keratinocyte cells. (a) Representative images of immunostained keratinocyte cells at 40 x magnification (blue DAPI, red p21, green LaminB1, and overlayed image) N=1. (b) Gene expression changes in response to UVA in keratinocytes acquired from qPCR, (n=3, N=3 unpaired t-test).

To expand on this, I explored whether the effect of UVA causes a short term or immediate release of SASP factors that may be contributing to the delayed repopulation of the cells. UVB has been reported to induce the expression of the SASP factor IL6 in human keratinocytes [Petit-Frère et al., 1998], which may impair the repopulation of the cells and thus contribute to delayed skin wound healing, as has been reported in diabetic skin patients [Lee et al., 2019]. Notably, 0.15 J/cm^2 of UVA induced senescence of keratinocytes *in vitro* via p21^{high} and LaminB1^{low} protein expression [Figure 3.17a]. In addition, in human skin, the secretion of the SASP factor, THBS1, by epidermal keratinocytes is found to suppress skin wound healing [Streit et al., 2000]. Notably, UVB radiation has been found to induce the expression of THBS1 in human skin [Seo et al., 2010]. However, UV (specifically UVA) effect on expression levels of THBS1 in keratinocytes and whether it correlates with impaired keratinocyte repopulation has not previously been explored. To assess this, I evaluated for changes in THBS1, as well as IL6 gene expression in epidermal keratinocytes in response to UVA.

Notably, UVA exposed (0.15 J/cm^2) keratinocytes significantly enhanced the gene expression of not only the senescent marker p21, but also the SASP factors IL6, and THBS1 and significantly reduced the gene expression of the nuclear membrane marker of LaminB1 [Figure 3.14d]. Therefore, these UVA exposed keratinocytes appear to adopt a senescent p21^{high}, laminb1^{low} phenotype, releasing factors IL6 and THBS1, which in turn contributes to impaired repopulation of these cells *in vitro* and this ultimately may contribute to delayed healing of human skin as observed in Figure 3.13b.

However, UVA, due to its long wavelength of 365 nm, not only penetrates the epidermis but also the dermis (unlike UVB) so I next chose to explore the effect of this stimuli on primary dermal cells, fibroblasts, to further understand how UVA is driving impaired healing of skin [Figure 3.18].

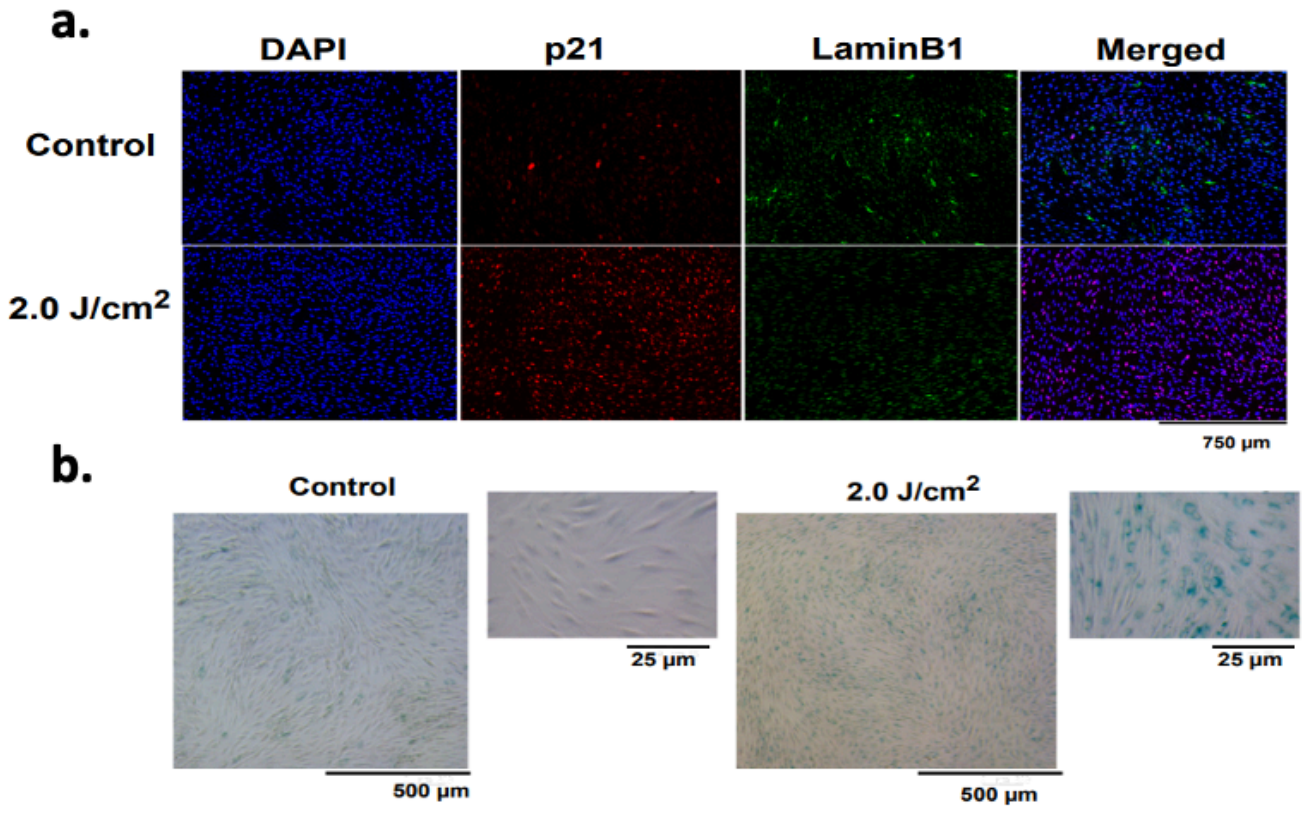


Figure 3.18: The effect of UVA dose (2.0 J/cm^2) on fibroblasts. (a) Representative images of immunostained fibroblast cells at 20 x magnification (blue DAPI, red p21, green LaminB1, and overlayed image) n=3 N=1) (b) SA-bgal stained fibroblasts (5 (left) and 20x magnification (right)), against UVA exposed fibroblasts. N=1.

To explore the effect of UVA on fibroblasts, I looked for changes in phenotype via senescent marker expression. Similarly to the senescent phenotype observed in UVA exposed keratinocytes, fibroblasts also presented $p21^{high}$ and $laminB1^{low}$ expression in response to 2.0 J/cm^2 [Figure 3.18a]. To validate the senescent phenotype of UVA exposed fibroblasts I performed a SA-bgal assay on these cells (against untreated control) which resulted in upregulation of the senescent marker, SA-bgal expression in the UVA exposed cells [Figure 3.18b]. This confirms that UVA is inducing a senescent phenotype via $p21^{high}$, $laminB1^{low}$, Sa-$bgal^{high}$ expression and SASP release. I next investigated for changes in expression of SASP and wound healing factors in dermal fibroblasts 24 hours following UVA exposure, to identify whether UVA is photoaging these dermal cells and how that may alter fibroblast morphology and function [3.19].

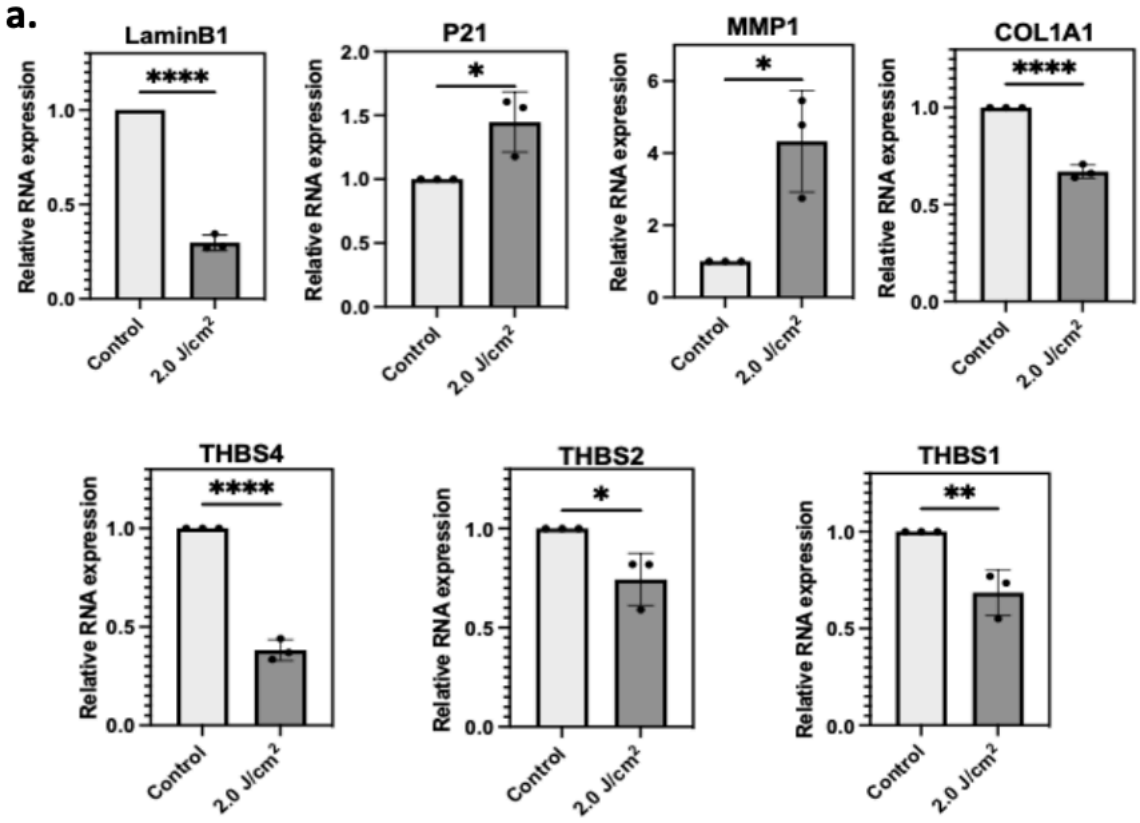


Figure 3.19: The effect of UVA dose (2.0 J/cm^2) on gene expression of fibroblasts. (a) Gene expression changes of UVA exposed fibroblasts (N=3) generated from qPCR in response to UVA exposure (n=3 N=6 unpaired T-test).

I selected several SASP factors detected from RNA-sequencing to be conserved senescence associated genes in primary human senescent fibroblasts [Marthandan et al., 2016; Foote et al., 2019]. MMP1 is reported to be responsible for inducing collagen fragmentation and is typically enhanced in expression in photoaged skin [Pittayapruek et al., 2016; Fisher et al., 2009; Marthandan et al., 2016]. THBS2 and THBS4 are responsible for modulating fibroblast-ECM interactions in early wounds, [Kyriakides et al., 1999] as well as in mediating fibroblast repopulation for skin regeneration [Klaas et al., 2021; Foote et al., 2019]. UVA exposure significantly upregulated the expression of p21 and MMP1, and significantly downregulated the expression of LaminB1, COL1A1, THBS1, THBS2 and THBS4 [Figure 3.19a]. Previous findings have reported the UV induced damage of dermal collagen [Budden et al., 2021; Jariashvili et al., 2012], and with ageing, it is known that dermal collagen undergoes gradual loss and fragmentation which culminates in weakened skin [Quan and Fisher. 2015]. Therefore, the UVA driven decrease in expression of these genes suggests that UV not only impairs epidermal keratinocytes and their role in healing but the function of dermal fibroblasts also.

Overall, the UVA induced upregulation of senescent markers and the downregulation of collagen and wound healing genes suggests that UV modifies the conventional phenotype of fibroblasts, alike to keratinocytes, to a senescent one, in turn altering their wound healing functionality. My findings showcase that UVA alters the dermal tissue by modifying the functionality and phenotype of dermal fibroblasts which are integral to the re-epithelisation of the wound site via mesenchymal signalling with epidermal keratinocytes [Koivisto et al., 2015; Zeltz et al., 2016; Boraschi-Diaz et al., 2017].

3.3.5 UVA exposed fibroblasts signal to keratinocytes via IL12, to reduce repopulation to injury site.

As UVA exposure modifies the dermal fibroblasts via dermal collagen reduction, modulating wound healing gene expression, and senescent markers, and dermal fibroblasts have known roles signalling to keratinocytes, I next explored the impact of UVA exposure on the mesenchymal signalling network between dermal and epidermal cells [Figure 3.20].

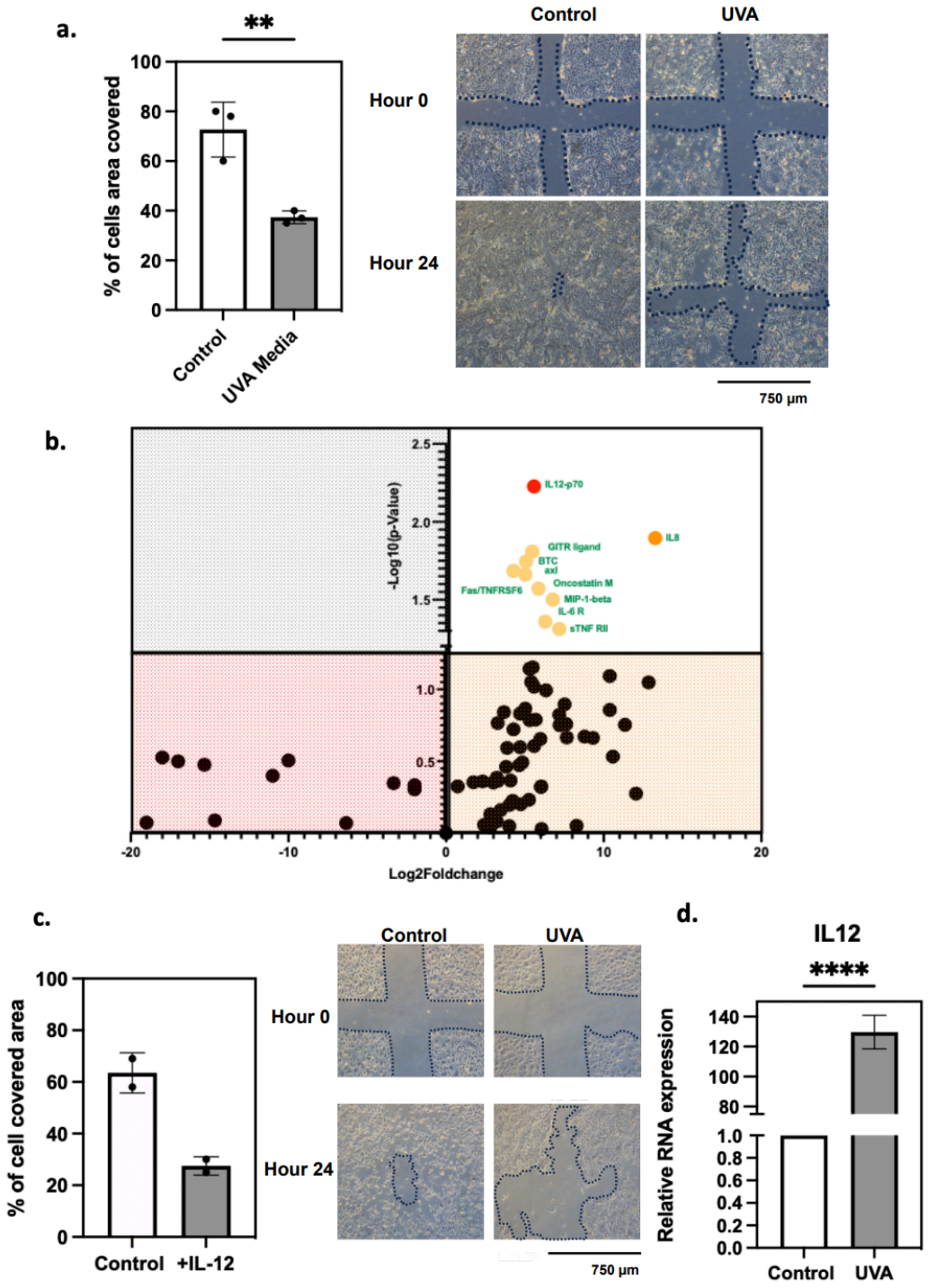


Figure 3.20: The mechanism behind UVA impaired migration of keratinocyte cells. (a) (left) Graph of n=3 N=3 % cell covered area of keratinocytes conditioned with UVA exposed fibroblast medium vs UV unexposed fibroblast medium (unpaired t-test). (right) Representative images captured at 5x magnification were taken hour 0 of scratch induction and 24 hours following scratch induction. (b) Volcano plot of cytokine changes in UVA exposed fibroblast medium compared to untreated fibroblast medium.n=3 N=2 (c) (left) Graph presenting % cell covered area of keratinocytes treated with IL-12 (against untreated control) after scratch induction (n=3 N=2 unpaired t-test) (right) representative images of IL12 treated keratinocyte monolayer hour 0 vs hour 24 (against control). (d) Il-12 gene expression changes in fibroblasts treated with and without UVA (n=3 N=3 unpaired t-test).

As UVA has been shown to induce photoaging of the dermis and alter the phenotype of fibroblasts I chose to explore the effect of a modified fibroblast phenotype on epidermal keratinocytes. To do this, I first investigated whether culturing healthy keratinocytes (immediately after scratch induction) with UVA conditioned fibroblast medium could influence keratinocyte repopulation *in vitro*. Interestingly, UVA conditioned (2.0 J/cm^2) fibroblast medium strikingly delayed repopulation of the keratinocytes, resulting in a significant reduction in % cell covered area by >50%, against untreated control [Figure 3.20a]. This suggests that UVA exposure of fibroblasts disrupts the mesenchymal signalling network between dermal fibroblasts and epidermal keratinocytes.

Furthermore, to identify any notable factors secreted from the UVA exposed fibroblast conditioned medium that may be signalling upstream to epidermal keratinocytes; to fuel their impeded migration, a cytokine release assay was performed on the conditioned fibroblast medium. The cytokine release assay on the UVA conditioned fibroblast medium revealed significantly enhanced expression of several factors, notably SASP factor IL8 had the highest fold change, and IL12 p70 had the lowest p-value [Figure 3.20b]. Although, IL8 is a reported SASP factor and contributes to inflammaging [Wolf et al., 2012] multiple reports have noted that IL8 in fact promotes the repopulation of human keratinocytes [Jiang et al., 2012; Rennekampff et al., 2000]. However, in the literature, the knockout of IL12 in murine models has resulted in enhanced healing [Matias et al., 2011], suggesting that IL12 elevation may potentially contribute to delayed wound healing though this has not been explored in human skin models. Therefore, although UVA may induce the release of a myriad of cytokines, not all of them will negatively influence keratinocyte repopulation. In this case it appears that IL12, not IL8, according to the literature may have some effect on the epithelial signalling network. Moreover, from my cytokine data, although IL8 presented higher fold change, the IL12 expression in control vs UV conditioned medium exhibited the most significant change.

Therefore, I next chose to explore the influence of IL12 on keratinocyte repopulation to identify how UVA disrupts the epithelial mesenchymal signalling network and if its damaging effect could be ameliorated [Figure 3.20c]. To assess if IL12 elicits a reductive effect on keratinocyte cell repopulation, comparable to the effect of UVA exposed fibroblast medium, I administered the IL12 protein to healthy keratinocytes immediately after scratch induction (in fresh KSFM medium). I observed a significantly reduced % cell covered area, against untreated control, by

>50% [Figure 3.20c]. This is comparable to the effect of keratinocytes conditioned with UVA treated fibroblast medium, which resulted in >50% reduced cell covered area against control [Figure 3.20c]. This suggests that UVA disrupts the epithelial mesenchymal network, via induced secretion of IL-12 by fibroblasts, which in turn influences the repopulation of keratinocyte cell via upstream signalling. Moreover, the relative gene expression of IL-12 is significantly upregulated by >100 fold in UVA (2.0 J/cm^2) exposed fibroblast cells [Figure 3.20d], reaffirming that this cytokine is stimulated in response to UVA, by senescent fibroblasts, supporting the findings presented in Figure 3.16b.

3.3.6 The klotho protein prevents UVA-age acceleration of primary epidermal and dermal cells.

To validate that UVA is enhancing the biological ageing of human skin and primary epidermal and dermal cells that is in turn disrupting wound healing capacity I investigated the effect of a well-known anti-ageing compound, klotho, in potentially ameliorating UVA damage on wound healing, keratinocyte repopulation, biological ageing, and cellular morphology [Figure 3.21].

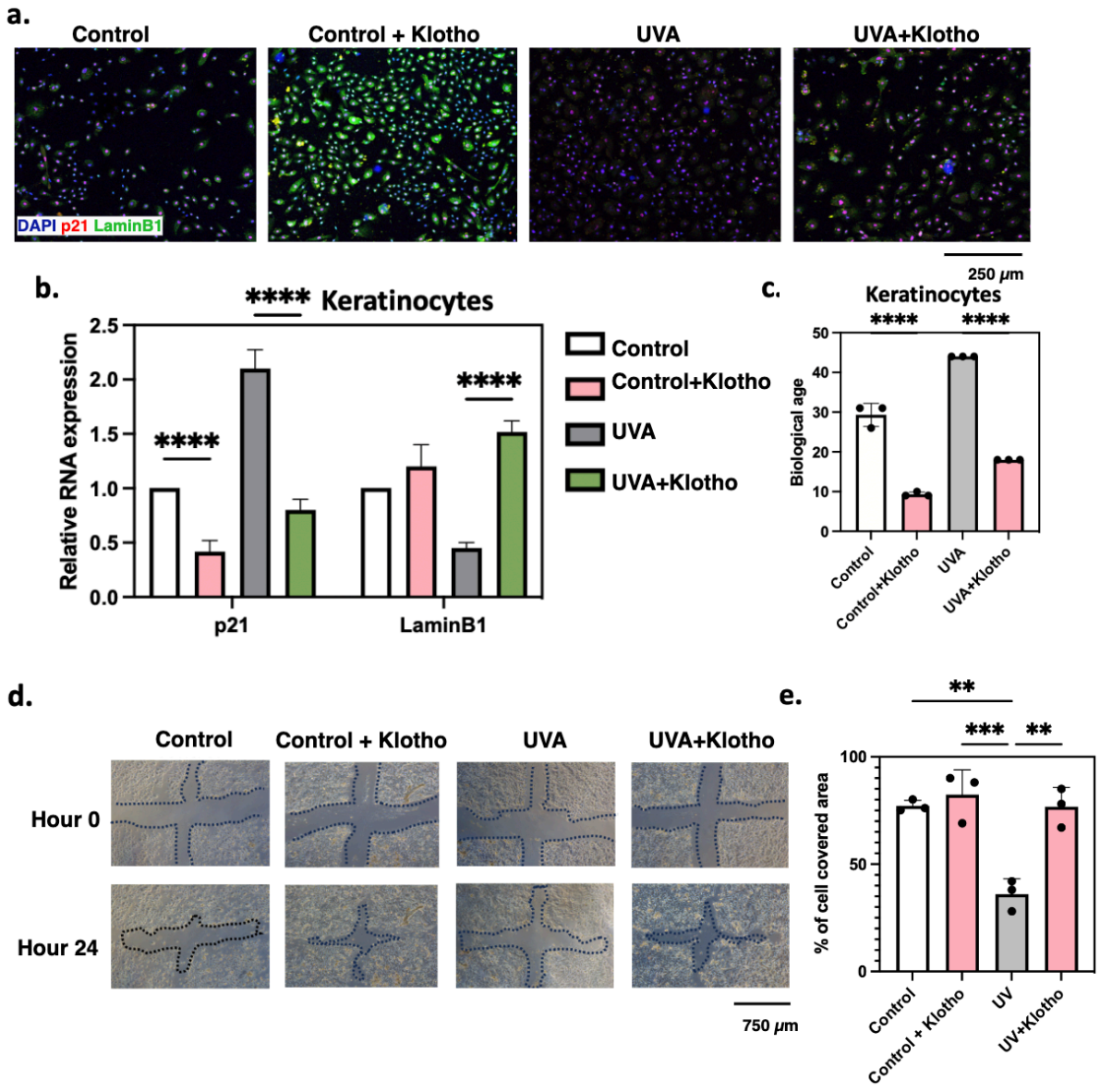


Figure 3.21: Effect of klotho on UVA exposed keratinocytes. (a) Immunostaining of Klotho treated UVA exposed keratinocytes against controls (Blue DAPI, Red p21, Green LaminB1) N=1. (b) Expression changes in p21 and LaminB1 genes in klotho treated UVA exposed keratinocytes (n=3 N=3 two way ANOVA). (c) Calculated biological age (using ELOVL2 gene CpG sites 5 and 7) of klotho treated UVA exposed keratinocytes (n=10 N=3 two way ANOVA). (d) Representative Scratch Assay images of Klotho treated UVA exposed keratinocytes and (e) (right) Graph of N=3 (two-way ANOVA).

I next wanted to assess whether the klotho protein could influence UVA modified keratinocyte phenotype i.e. induced senescent expression or impaired repopulation of cells. The klotho protein was administered to keratinocytes in culture, 24 hours before they were UVA exposed, to identify if the protein could prevent UVA driven damage. To evaluate whether klotho treatment to the primary epidermal skin cells could prevent UVA altered phenotype of such cells, changes in the expression of senescence markers of klotho treated UVA exposed (against UV only treated cells) was first assessed [Figure 3.21a].

To evaluate if klotho may elicit any protective effect against DNA damage, I performed immunostaining on confluent keratinocytes, that had been treated with Klotho, then 24 hours later exposed to UVA. The expression changes in laminB1 and p21 markers were observed in response to klotho pre-treated UVA exposed cells. The expression of laminB1 was found to visually increase in the klotho treated keratinocytes (no UVA) and less so in pre-treated UVA exposed keratinocytes, while p21 expression appeared to not as strikingly differ in expression [Figure 3.21b]. The data generated from the qPCR revealed a significant upregulation in the expression of LaminB1 gene and significant decrease in the expression of the p21 gene in keratinocytes [Figure 3.21b]. This indicates that the anti-ageing compound may protect keratinocytes against the phenotypical changes induced by UVA exposure.

Comparable to how UVA was found to enhance the biological age of skin, I observed a significant upregulation in biological age of UVA exposed keratinocytes by >10 years in UVA [Figure 3.21c Appendix 3.5]. UVA enhanced the age-related CpG site methylation (CpG sites 5 and 7) across the genome of epidermal keratinocytes, which suggests that UVA may be impairing cell repopulation by altering the conventional phenotype of the cells to a senescent SASP releasing phenotype as a result of age acceleration of the cells. Holistically, these findings suggest that UVA exposure modifies the conventional nature of keratinocytes by accelerating the ageing of these cells via epigenetic changes. To validate the effect of the anti-ageing protein klotho on aged cells, I pre-treated UVA exposed keratinocytes to klotho 24 hours before UVA exposure. I found that klotho significantly reduced the biological age of UV exposed keratinocytes by >20 years [Figure 3.21c].

Lastly, I assessed if the protein had any influence on the migration of UVA exposed keratinocytes. Klotho was found to protect against impairment in healing induced by UV as UV impaired repopulation of keratinocytes (50% reduction in cell covered area 24 hours after scratch induction) was protected against by pre- treatment of klotho to cells [Figure 3.17d, e]. This suggests that klotho is effective in directly improving keratinocyte UVA impaired repopulation.

Next, I evaluated the effect of the anti-ageing compound, klotho, on UVA altered phenotype of fibroblasts [Figure 3.22].

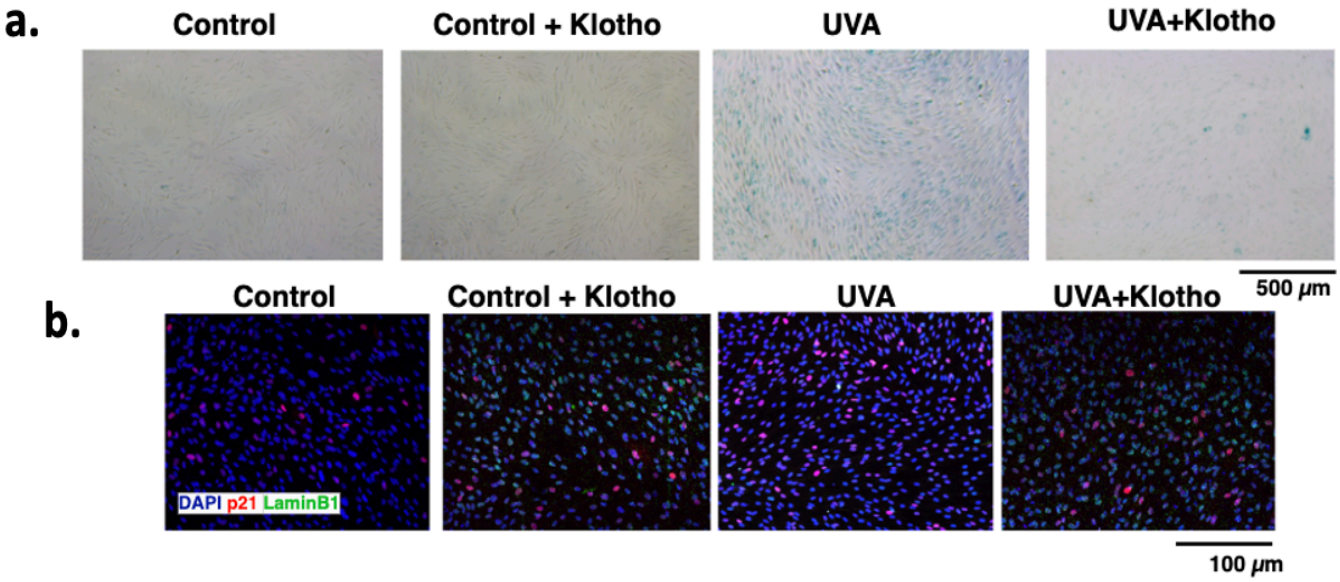


Figure 3.22: Effect of klotho on UVA exposed fibroblasts. (a) Sa-bgal staining of klotho treated UVA exposed fibroblasts against controls N=1 (b) Immunostaining of Klotho treated UVA exposed fibroblasts against controls (Green laminB1, Red p21, Blue DAPI) N=1.

Notably, klotho pre-treatment of| UVA exposed fibroblasts appeared to reduce senescent marker, Sa-Bgal, expression [Figure 3.22a]. Moreover, laminB1 protein expression was visually enhanced in control klotho treated fibroblasts, and in klotho UVA treated fibroblasts [Figure 3.22b].

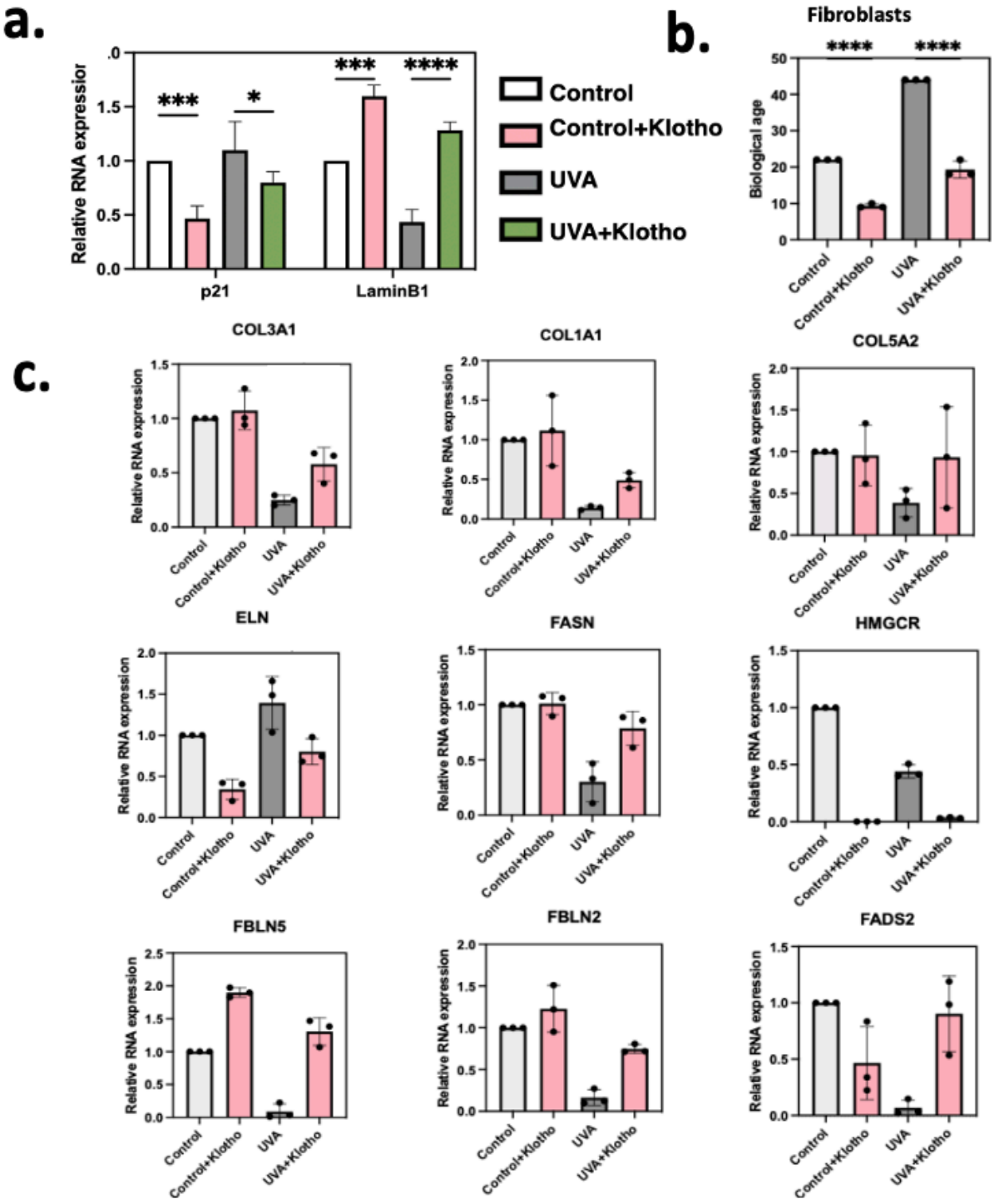


Figure 3.23: Effect of klotho on UVA exposed fibroblasts. (a) Gene expression of p21 and Lamin B1of klotho pre-treated UVA exposed fibroblasts against controls generated from qPCR (n=3 N=3 two-way ANOVA). (b) Calculated biological age (from ELOVL2 gene) of klotho treated UVA exposed fibroblast cells against controls (n=10 N=3 two-way ANOVA). (c) expression changes in dermal genes in klotho treated UVA exposed fibroblasts (N=1 one way ANOVA),

LaminB1 expression, comparable to klotho treated UVA exposed keratinocytes, was statistically upregulated in the klotho treated UVA exposed fibroblasts compared to UVA only treated fibroblasts [Figure 3.23a]. The p21 expression statistically declined in response to klotho pre-treatment to UVA exposed fibroblasts. Similarly to klotho treated UVA exposed keratinocytes, klotho pre-treatment also reduced the calculated biological age of UVA exposed fibroblasts by over 20 years [Figure 3.23b].

Lastly, I wanted to evaluate if the anti-ageing compound could improve the expression of genes commonly associated with biologically aged (DNA damage induced) or photoaged skin (UVA induced) skin. Therefore, I assessed for any changes in dermal gene expression in klotho-pre-treated UVA exposed fibroblasts [Figure 3.23c]. The chosen genes were collagen 1 (COL1A1), collagen III (COL3A1), collagen V (COL5A1), fibulin 2 (FBLN2), fibulin 5 (FBLN5), elastin (ELN), fatty acid desaturase 2 (FADS2), 3-hydroxy-3-methyl-3-glutaryl-coenzyme A reductase (HMGCR), and fatty acid synthase (FASN). These genes were selected from the McGrath et al., 2012 paper. The authors sought to identify the differences in gene expression between young and old, sun-exposed and sun-protected skin, to help identify new biomarkers for aged skin for therapeutic prevention in the future. They generated transcriptomic data from young and old skin from sun-exposed and sun-protected skin (10 samples of each) and analysed the data set using hierarchal clustering for theme analysis and interaction mapping.

As reported in this paper, ELN, and FBLN2/5, were upregulated in photoaged skin, COL1A1/3A1/5A1 were downregulated in biologically aged skin and HMGCR, FASN, and FADS2 were downregulated in both cases. I found that klotho significantly enhanced the expression of COL3A1, FASN, FBLN2, FBLN5 and FADS2, as well as significantly reduced expression of ELN in fibroblasts. The upregulation of COL3A1, FASN, and FADS2 supports the findings of the McGrath et al., in that these genes are downregulated with biological age, which I observe in UVA exposed fibroblasts (UVA biologically ages the cells as presented in Figure 3.23c) but is rescued by the anti-ageing compound. Notably, the beta-klotho protein when reportedly bound to FGFR4, to trigger FGF15 signalling, in turn induces the expression of FASN [Cui et al., 2018], thus the alpha-klotho protein used in my study may act on the same pathway. Specifically, UVB has been reported to reduce the expression of COL3A1 in human fibroblasts [Lago et al., 2019], and ageing fibroblasts present reduced COL1A1/3A1 expression in old mice vs young mice [Kanazawa et al., 2023]. In this case UVA shows the same effect. Notably, klotho deficiency is associated with deficient COL1A1 and COL3A1 expression in

the hearts of mice [Navarro-Garcia et al., 2020], indicating that klotho may directly act on the same pathway as these genes. Moreover, the downregulation of ELN by klotho, an ECM gene responsible for extensibility, which was upregulated by UVA, supports the paper, as elastin can be stimulated in response to UVA [Bernstein et al., 1995; Philips et al., 2018; Pageon et al., 2021], typically presenting as solar elastosis in skin. Notably, one paper reported that klotho deficiency in mice drives increased collagen but reduced elastin expression in the aorta [Chen et al., 2015], contrary to my presented findings on the dermal fibroblasts, highlighting the potential myriads of functions of this protein in various tissues and cell types. Moreover, I observed a significant downregulation in HMGCR in response to klotho (in UVA exposed fibroblasts), Although McGrath et al., found a stark decrease in this gene in response to UVA (as my data also shows) the further decrease by klotho may in fact be positive, as this gene has been found to be highly stimulated in elderly skin [Trapani et al., 2009]. Notably, the blocking of HMGCR boosts klotho expression [Kuwahara et al., 2008] hence they may act on the same pathway.

However, the downregulation of FBLN2/5 in response to UVA (and upregulation in response to klotho) is contrary to the findings of McGrath et al., which showed the reverse in response to UVA. However, other studies have reported that these age-associated ECM genes, responsible for skin development and remodelling [Ngassie et al., 2013], are degraded by UVA and B [Phillips et al., 2018; Charoenchon et al., 2022; Kadoya et al., 2005]. Overall, these presented findings indicate that UVA hugely transforms dermal composition via gene expression changes, but this is rescued by the anti-ageing klotho protein. Holistically, these promising findings illustrate that the anti-ageing compound, klotho successfully prevents UVA induced damage, i.e. DNA damage phenotype, elevated biological age, and impaired repopulation, on epidermal keratinocytes and dermal fibroblasts respectively. Therefore, I subsequently wanted to explore the preventative effect of klotho on UVA impaired epithelial mesenchymal signalling between these primary skin cells.

3.3.7 The klotho protein reduces p21 expression and enhances LaminB1 expression in keratinocytes treated with UVA conditioned Fibroblast medium.

Having observed that klotho successfully prevents UVA altered phenotype of fibroblasts I chose to observe whether klotho could improve the UVA disrupted epithelial mesenchymal cell

network. As I have found that UVA conditioned fibroblast medium induces the release of IL-12 which impairs keratinocyte repopulation, I assessed the influence of Klotho on IL12 gene expression changes in fibroblasts [Figure 3.24].

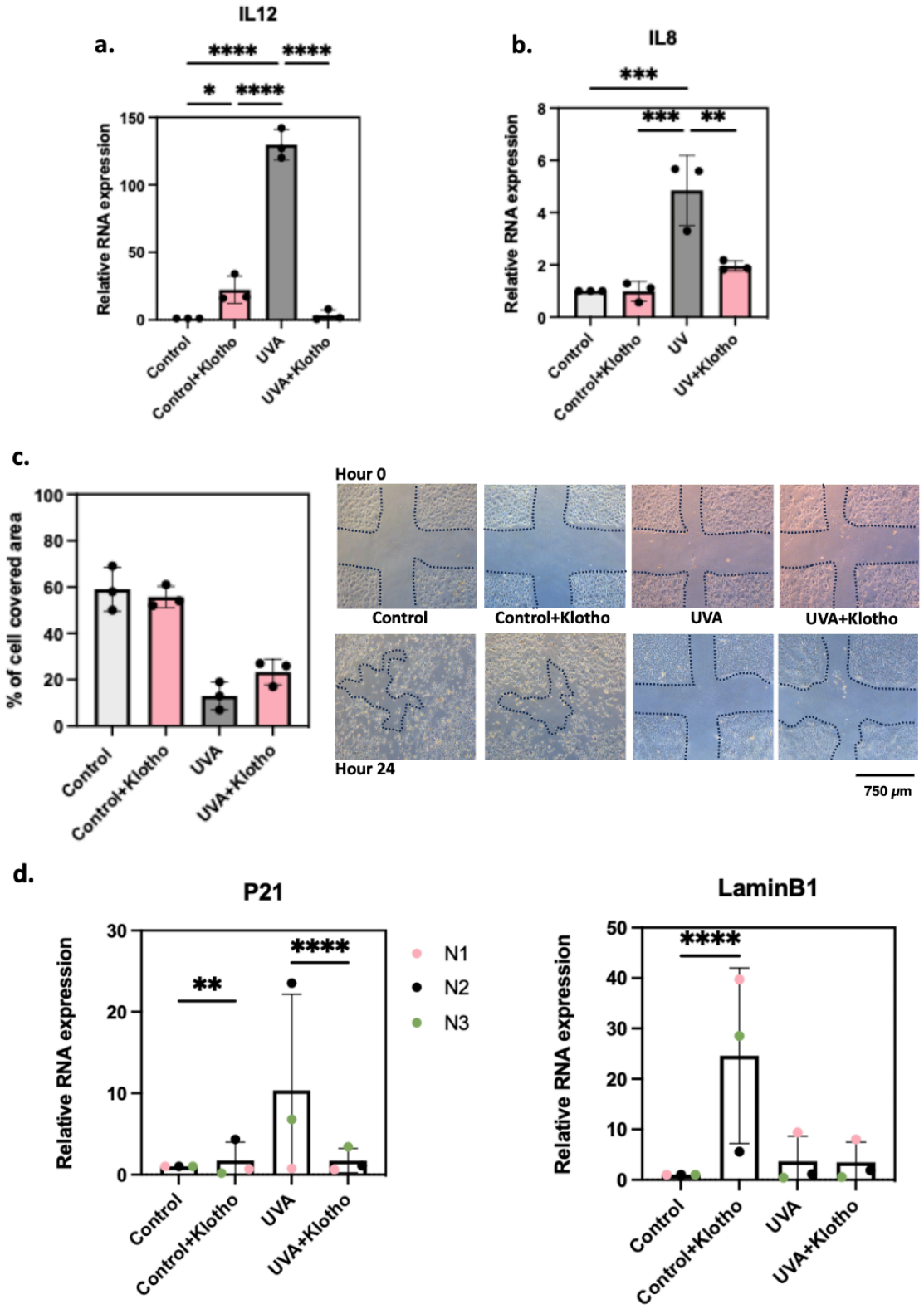


Figure 3.24: Effect of klotho on the epithelial mesenchymal network. (a) Relative RNA expression levels of IL12 in klotho pre-treated UVA exposed keratinocytes (against controls) (n=3 N=3) (b) of IL-8 (N=3 two-way ANOVA). (c) % cell covered area of keratinocytes cultured with UVA conditioned fibroblast medium (and pre-treated with klotho) 24 hours after scratch, (n=3 N=3 two-way ANOVA) (right) representative images of scratch at hour 0 vs hour 24 after scratch. (d) Relative RNA expression levels of p21 and LaminB1 of keratinocytes cultured with UVA conditioned fibroblast medium (pre-treated with klotho) (n=3 N=3 two-way ANOVA).

Firstly, as discovered in figure 3.20 treating healthy confluent keratinocytes with IL-12 (immediately after scratching induced) resulted in around 40% of cell covered area of the monolayer, 24 hours after scratch induced [Figure 3.20]. This highlights that comparable to the effect of UVA on confluent keratinocytes, the pre-treatment of IL-12 is sufficient in impairing repopulation of the epidermal cells. Therefore, I subsequently investigated the effect of klotho on modulating the expression levels of IL-12. Fibroblasts were pre-treated with klotho, then exposed to UVA (24 hours later) and changes in IL12 gene expression were assessed. Klotho pre-treatment of UVA exposed fibroblasts resulted in a significantly reduced expression of IL12 by >100-fold (N=3) against UVA only treated fibroblasts [Figure 3.24a]. This suggests that klotho can target and downregulate the expression of IL-12 in UVA treated fibroblasts. Similarly, klotho was also found to target and downregulate the expression of the SASP factor IL-8 in UVA treated fibroblast cells, which was found to be upregulated in expression in UV conditioned fibroblast medium [Figure 3.24b].

Subsequently, I wanted to assess if klotho could prevent keratinocyte repopulation following treatment with UVA conditioned fibroblast medium. Klotho was administered to fibroblast cells (in DMEM medium). Following 24-hour incubation, the fibroblast medium was changed to fresh DMEM, and the cells (in medium) and exposed UVA. Healthy keratinocytes were cultured with the filtered conditioned medium immediately after scratch induction. Klotho failed to significantly improve the impaired migration of these epidermal keratinocytes (N=3) [Figure 3.24c], suggesting that this protein is ineffective in pre-emptively improving UVA conditioned fibroblast medium induced damage on keratinocyte repopulation. However, klotho treated fibroblast conditioned media was found to significantly reduce p21 gene expression in UVA exposed keratinocytes in three biological samples (N=3) [Figure 3.24d]. Moreover, klotho significantly enhanced laminB1 expression in control only (not UVA cell samples) keratinocytes [Figure 3.24d]. This suggests that the anti-ageing protein improves DNA damage marker expression in some cases but not entirely, having a more significant direct impact on UVA exposed keratinocytes in improving their repopulation but not indirectly on keratinocytes cultured with UVA conditioned fibroblast medium. I subsequently chose to investigate the effect of this compound in potentially preventing UVA damaged *ex vivo* skin.

3.3.8 Klotho prevents UVA induced damage on young skin.

My findings have shown that UVA age accelerates human skin by accelerated biological ageing and induces DNA damage expression at the skin surface and cellular level, in parallel impairing wound healing function *ex vivo* and *in vitro* which is overturned at the cellular level by klotho pre-treatment. Therefore, I wanted to assess if klotho could also ameliorate the UVA damaging effects, observed in epidermal and dermal primary cells, in *ex vivo* skin [Figure 3.25].

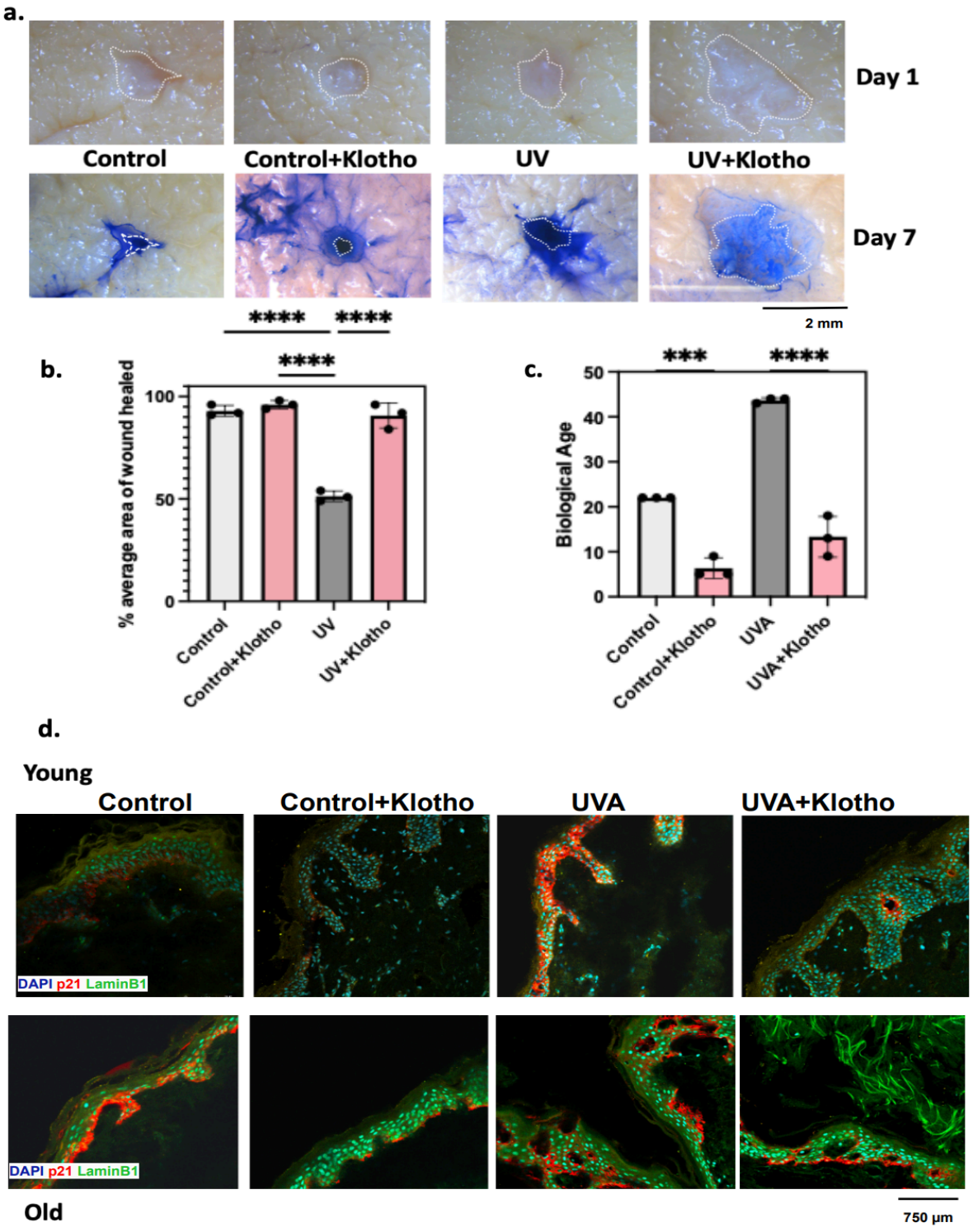


Figure 3.25: Effect of klotho on *ex vivo* human skin. (a) Representative images of ex vivo skin treated with klotho protein 24 hours before UVA exposure (day 0 vs day 7), day 7 stained with aniline blue. (imaged at 2x magnification). (b) Analysis of n=6, N=3, per sample of the % average of the area of the wound healed (N=3 two-way ANOVA). (c) Biological age of klotho pre-treated skin (24 hours prior to UVA exposure), (n=10, N=3 two-way ANOVA) (d) Immunostained klotho treated UV exposed young skin (top) and old skin (below) with (red) p21, (green) laminB1, and (blue) DAPI N=1.

To investigate the impact of klotho on skin wound healing and phenotype I explored the capacity of klotho in ameliorating the detrimental effect of UVA in age accelerating *ex vivo* skin, by topically administering a single low dose (0.1 μg/mL) of the klotho protein (diluted in 0.035% wt PAA and medium) 24 hours prior to UVA injury. I found that klotho pre-treatment to UVA exposed *ex vivo* skin significantly improved UVA impaired wound healing, as highlighted by a gradient of aniline blue staining from dark to light hue in UV only treated (dark blue) vs UV and klotho treated skin (light blue) [Figure 3.25a]. This suggests that there is enhanced reepithelization of the skin wound site with klotho pre-treatment 7 days following UVA exposure. The topical application of klotho to UVA exposed skin resulted in a recovery of wound closure back to no UVA exposure levels. [Figure 3.25b].

In young skin klotho-pre-treatment blocks the UVA driven hypermethylation of age-related CpG sites as the protein stimulates reduced biological age by >20 years to an average of 9-10 years, as calculated using the ELOVL2 CpG sites [Figure 3.25c]. Notably, in young skin klotho pre-treatment visually downregulated p21 expression, while laminB1 expression remained visually unchanged, suggesting that UVA induced DNA damage is prevented with the administration of the anti-ageing compound [Figure 3.20d]. Moreover, in elderly skin although laminB1 and p21 expression remained consistent in klotho treated UVA exposed skin, there was a visually reduced p21 staining in the klotho treated control sample. I observed no visual change in UVA impaired wound healing of elderly skin in response to the anti-ageing protein [Appendix 3.8].

I chose next to evaluate expression changes in photoaging and biological age-related genes, forementioned in figure 3.23, in UV exposed unwounded elderly skin pre-treated with klotho. I identified a upregulation of Elastin in klotho treated unexposed skin but not klotho treated UVA exposed skin [Figure 3.26]. The loss of elastin in the skin with ageing causes the skin to hang loosely [Baumann et al., 2021]. The fact that elastin expression is improved in elderly skin in response to klotho treatment highlights the potential reversible and restoring effects of this anti-aging compound on elderly skin.

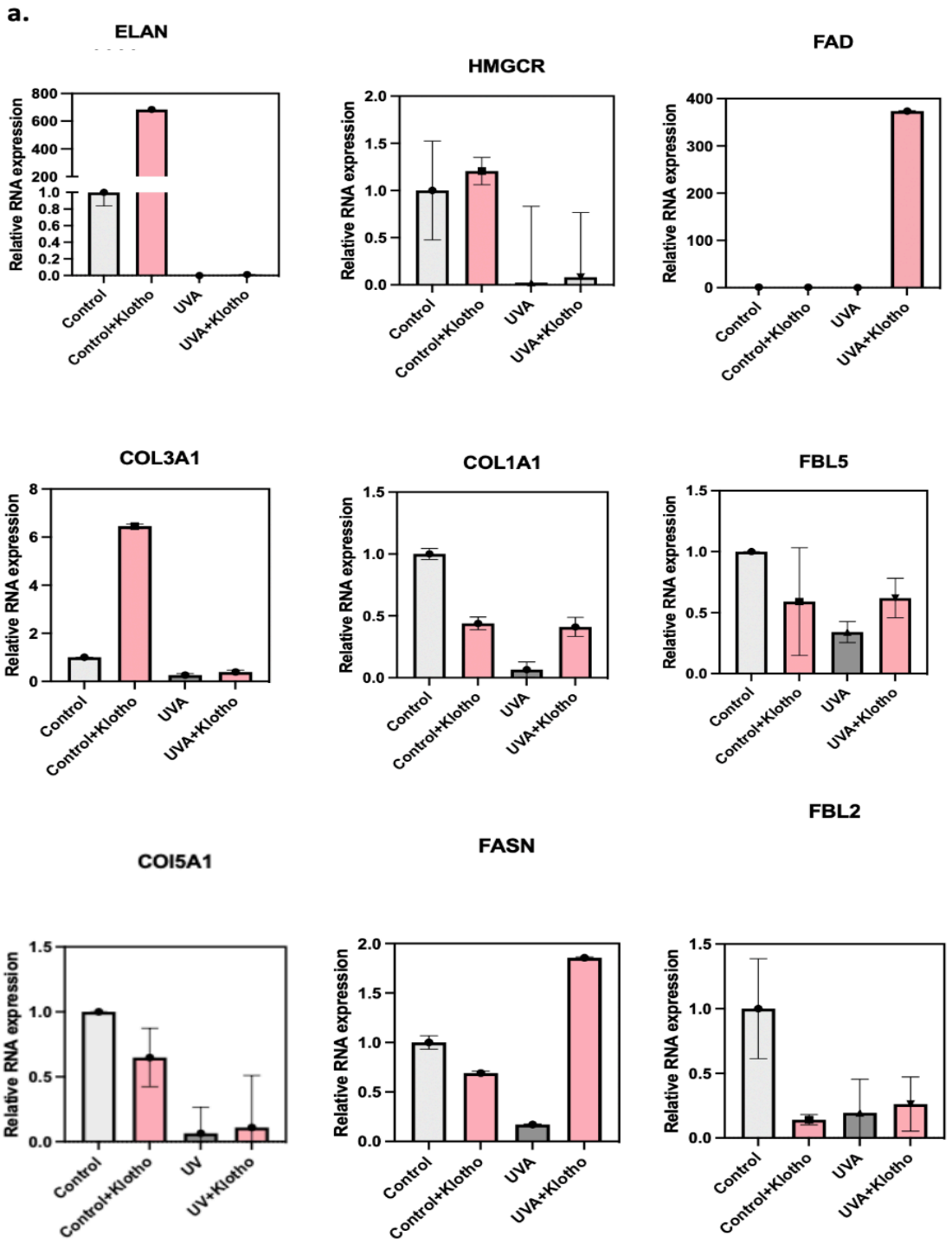


Figure 3.26: Changes in gene expression (photoaging and biological ageing genes) in elderly skin in response to klotho protein acquired from qPCR n=3, N=1. Statistical analysis performed using one-way ANOVA test.

The elastin ECM protein is downregulated in sun-protected elderly skin, [Baumann et al., 2021; Zhang et al., 2018] causing skin to become stiffer, therefore the upregulation of this protein in response to klotho is promising, however, klotho treated UVA damaged elderly skin does not present a rise in the protein. Notably, topical Klotho treated UVA exposed elderly skin induces a statistically significant enhancement in FADS2 [Figure 3.26a] which is commonly downregulated in inflammatory conditions i.e. dermatitis, psoriasis, and in adult vs neonatal keratinocytes [Ye et al., 2002; Pararasa et al., 2022; McGrath et al., 2012]. This suggests that klotho may have some capacity for reversing ageing in elderly skin however whether it can reverse photoaging, or UV induced damage would require further study.

3.3.9 A pH responsive microneedle efficiently delivers klotho to human skin as a preventative therapeutic.

Lastly following the promising effects of the α-klotho protein notably in protecting against UVA impaired regeneration in *ex vivo* young skin, I utilised the pH responsive MN device, developed and constructed in chapter 2, for the localised delivery of the compound to human skin [Figure 3.27]. However, firstly I had to assess if klotho remained bioactive in the PAA carrier material. Holistically, the previously presented promising findings of klotho strongly validate that not only does UVA stimuli accelerates ageing of primary skin cells that is prevented by the anti-ageing compound but also highlights the potential therapeutic use of this compound as a preventative treatment to impaired wound healing.

To further explore the possibility of the latter, I assessed the capacity of this compound as a translational therapeutic option. The klotho protein possesses a relatively short half-life of 7.5 hours [Leon et al., 2017] which poses an immense clinical challenge of rapid degradation which would thus impact protein efficacy [Zaman et al., 2019]. Therefore, to be effective as a potential therapeutic the bioactivity of the compound must be extended and conserved for long time storage and use [Figure 3.27].

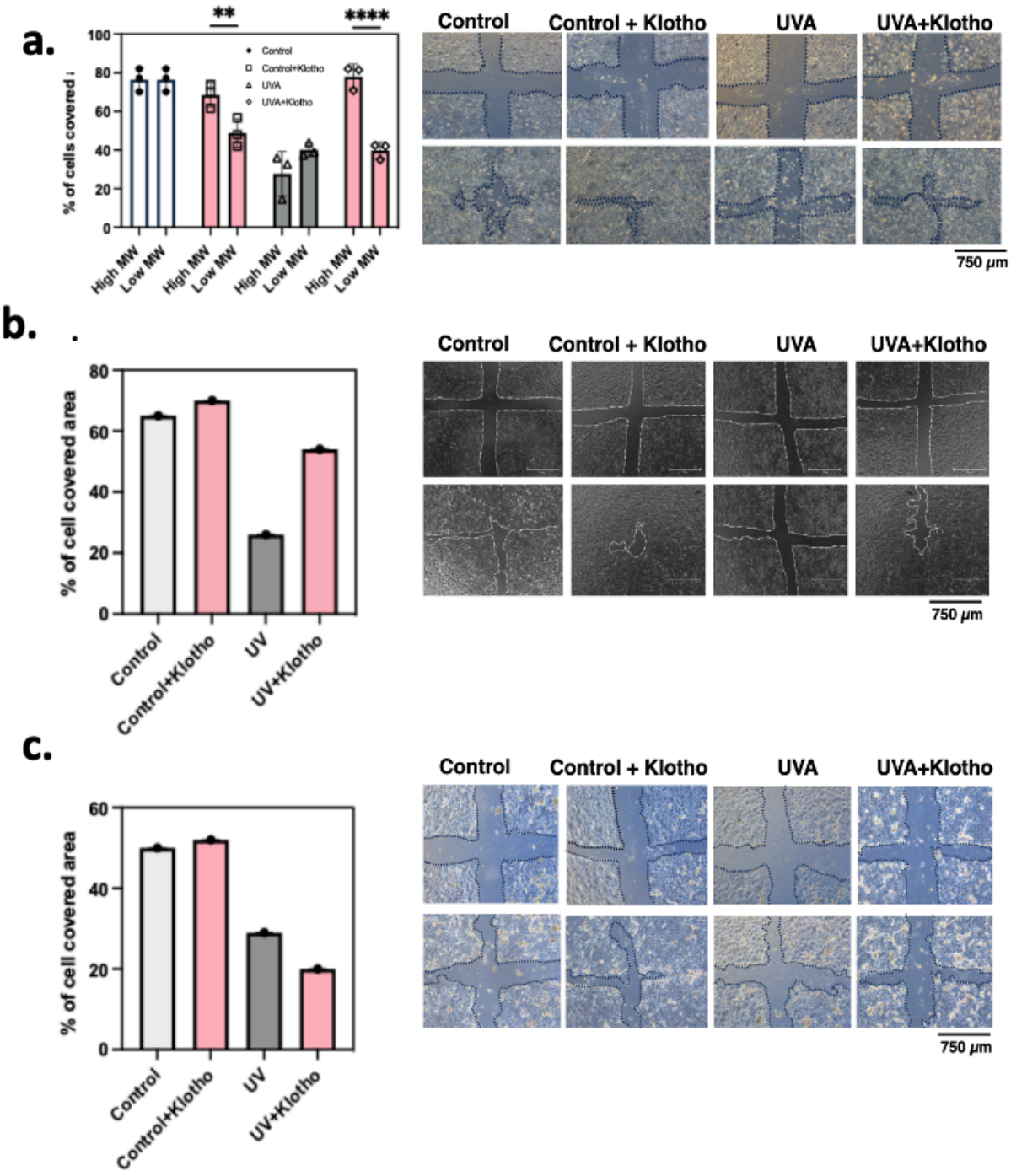


Figure 3.27: Bioactivity of klotho in polyacrylic acid material. (a) The % cell covered area of UVA exposed keratinocytes treated with klotho (diluted in polyacrylic acid (PAA)) and representative images of keratinocytes treated with high MW PAA (top hour 0, bottom hour 24). (all analysis performed with two-way ANOVA, N=3). (b) Representative images with adjacent graph (statistical analysis performed using one way ANOVA N=1 n=3) of keratinocytes treated with freeze-dried klotho encapsulated in PAA to assess their migration from hour 0 (top) and hour 24 (bottom images). (c) Representative images with adjacent graph (statistical analysis performed using one way ANOVA N=1 n=3) of keratinocytes treated with long-term stored klotho without PAA to assess their migration from hour 0 (top) and hour 24 (bottom images).

Therefore, the bioactivity of klotho was assessed using high (100, 000 MW) or low (2000 MW) MW PAA which klotho was dissolved in the polymeric material and subsequently, administered to epidermal keratinocytes 24 hours prior to UVA injury (against untreated controls) [Figure 3.27a]. The repopulation of keratinocytes was observed, and klotho diluted in PAA (high MW) resulted in improved % cell coverage after 24 hours whereas klotho diluted in PAA (low MW) with a 40% higher cell covered monolayer in high MW diluted klotho compared to low MW pre-treated keratinocytes prior to UVA injury. This highlights the enhanced effectiveness of the high MW PAA, compared with low MW PAA, in conserving the biological function of klotho. This is comparable to that presented in Figure 3.21e where pre-treated klotho cells prior to UV injury resulted in 80% cell covered area 24 hours following scratch induction. A plausible explanation for this is that the high MW of PAA possesses enhanced loading capacity and stability with increased protein uptake [You et al., 2023].

Moreover, PAA is a recognised pH responsive material that undergoes a conformational change from globular to linear at >pH 7 due to the deprotonation of its COO group [Pourmadadi et al., 2023]. Therefore, treatment of PAA encapsulated klotho to keratinocytes should result in carrier disassembly and drug diffusion as KSFM exhibits a pH of 7. Interestingly, the long term (9 months) and short term (3 days) stored klotho diluted in high MW PAA was sufficient in conserving the bioactivity of the compound in preventing UV impaired keratinocyte repopulation [Figure 3.27b]. The long-term storage of klotho without PAA (3 months) appeared to diminish its biological function indicated by lack of keratinocyte repopulation pre-treated with klotho and in response to UVA injury [Figure 3.27c]. This successfully exhibits the retained bioactivity of this short-half-life compound over time using the PAA polymeric material that would otherwise be attenuated. Therefore, in subsequent experiments I diluted klotho in PAA, (then medium) to retain its biological function in application to skin or cells.

Having verified that klotho successfully remains biologically active in PAA carrier material, whether my engineered MN from chapter 2 appeared to be an effective and efficient delivery method of the protein to the dermal layer of the skin was next assessed [Figure 3.28].This was done to identify whether MN delivery of the compound may improve biological application of the drug in skin specifically within the dermal tissue compared to topical appointment.

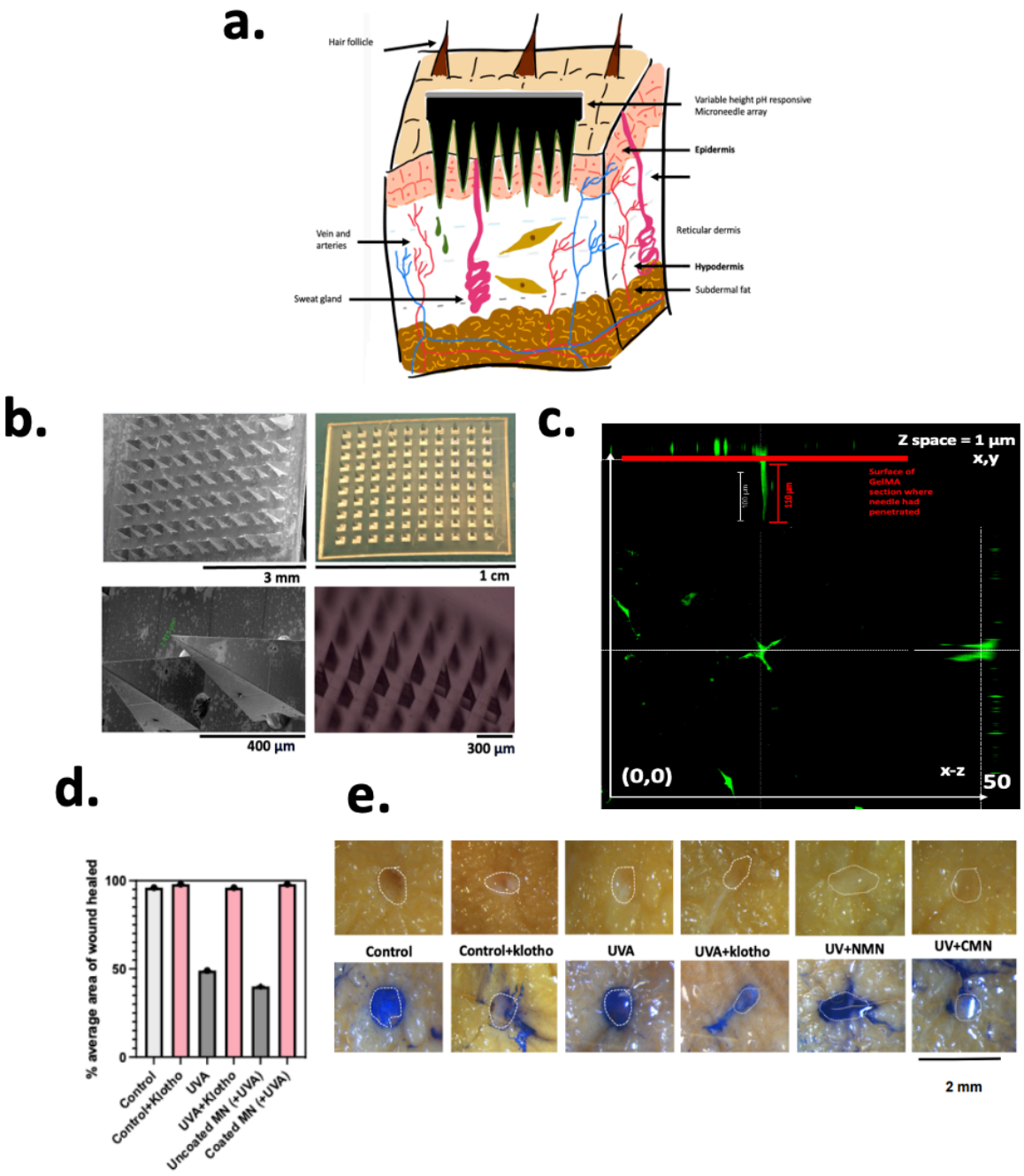


Figure 3.28*:* Effect of Klotho coated MN on *ex vivo* human skin. (a) Schematic of MN application to human skin. (b) Images of engineered MN device highlighting dimensions and the entire MN array. (c) Confocal z-stack image of klotho-FITC tagged, (encapsulated in PAA) coated MNs penetrated GelMA. Z-stack (10 micron per stack, 50 stacks total) of the tagged protein diffused into the artificial skin is used to assess the depth of drug diffusion N=1. (d) (left) Graph of topically klotho treated ex vivo UV exposed skin, and klotho coated MN to UVA exposed skin (CMN), against the untreated control and NMN (uncoated MN in UVA exposed skin) after 7 days (n=6, N=1 one-way ANOVA) (e) (right) representative images (stained with aniline blue at day 7 vs day 0).

Microneedles have emerged as an attractive therapeutic device for the non-invasive delivery of drugs to human skin [Figure 3.28a]. I present images of my engineered microneedle dimensions with an array of 10x10 polymeric constructed needles alternating in heights from 700 μm to 500 μm [Figure 3.28b], which were described in more detail in Chapter 2. Having established that klotho is effective in ameliorating UVA induced damage on *ex vivo* human skin and fibroblasts and keratinocytes respectively, I next wanted to evaluate if my engineered MN device could improve the delivery of the compound to the skin.

To identify the depth of drug diffusion of the klotho protein upon MN application, the device coated with klotho-FITC tagged, encapsulated in PAA, was applied for 15 minutes to a synthetic skin GelMA model (established in chapter 2). Application of the coated MN device on the synthetic skin surface identified drug diffusion of >100 μm [Figure 3.28c] which is beyond the epidermis which has a thickness of 50-100 μm [Crowther et al., 2008; Lademann et al., 2008; Richter et al., 2004]. Next, klotho pre-treatment 24 hours before UVA exposure and injury of *ex vivo* skin (topically or via a MN device) resulted in prevention of the deleterious effect of UVA on wound healing. This ameliorated effect of UV driven by Klotho, was visualised through a light blue aniline blue staining [Figure 3.28d].

The MN device possesses 700 μm needles that were shown in chapter 2 to penetrate the basement membrane of *ex vivo* skin, therefore, I next assessed not only if klotho could alter dermal gene expression (as observed in fibroblasts) but whether the MN device could alter the expression of dermal specific genes via Klotho, more effectively than topical appointment of the protein. As illustrated in previous figures 3.23 and 3.26, I selected COL1A1, COL3A1 and COL5A1 genes as their loss in expression is strongly associated with enhanced biological ageing [McGrath et al., 2012]. Upregulation of the genes Elastin, FBLN2 and FBLN5 are strongly associated with photoaging (in response to UVB) [McGrath et al., 2012]. Lastly, reduction in the expression of the genes HMGCR, FASN, and FADS2 have been strongly associated with both biological and photoaging of the human skin [McGrath et al., 2012] Therefore, I chose to investigate the expression of these genes in response to klotho pre-treated (topically or via MN) UVA exposed *ex vivo* skin samples (against UV only, control and klotho, or control only controls) [Figure 3.29]. Klotho was either topically or via a MN device applied to *ex vivo* skin samples for 15 minutes before removal (aspirated off or MN detached from

skin), then 24 hours following 37°C incubation, samples were exposed to UVA. No injury to the skin was performed in this experiment.

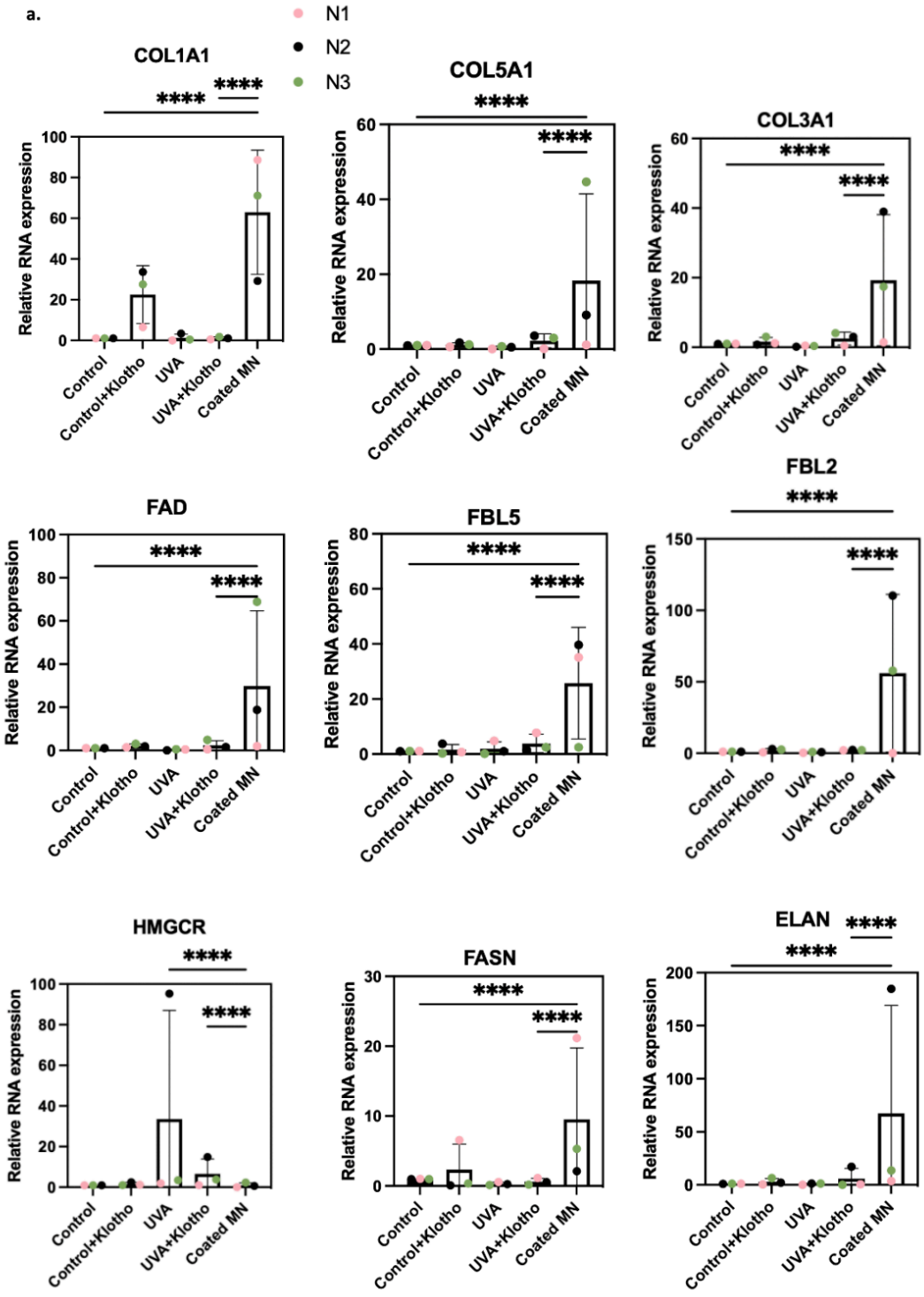


Figure 3.29: The effect of klotho coated MN on photoaging and biological ageing gene expression. (a) Gene expression changes in klotho treated ex vivo UVA exposed young skin topically and via MN (CMN). Data generated from qPCR n=3 N=3. (two-way ANOVA).

Several dermal skin genes were significantly upregulated in expression in response to klotho, coated MN array (N=3) which was applied for 15 minutes, compared to topically delivered klotho including: COL1A1, COL3A1, COL5A1 FADS2 and FASN, FBLN2/5 and ELAN [Figure 3.29] with their loss in expression associated with biological ageing of skin [McGrath et al., 2012; Bernstein et al., 1995; Kadoya et al., 2005; Charoenchon et al., 2022; Lago et al., 2019; Navarro-Garcia et al., 2020]. As presented, in klotho treated dermal fibroblasts, UVA reduces COL1A1/3A1/5A1 expression, but this is restored by the klotho protein. Elastin is downregulated with ageing [Baumann et al., 2021] but can also be induced in response to UVA photoaging. Therefore, in fibroblasts UVA will heighten elastin expression due to photoaging [Weihermann et al., 2017] which klotho counteracts however, in skin (young and old) klotho appears to enhance the expression of the dermal protein elastin by rejuvenating the skin to some capacity it appears.

Notably, the data presented in figure 3.26 is contrary to the findings presented in figure 3.29, in showing the varied effect of klotho on young vs old skin. In elderly skin, klotho appears to upregulate the expression of ELN, COL3A1 and HMGCR in unexposed skin, all of which downregulate with ageing. The effect of klotho here may be attributed to its ability to counteract the age-related decline in gene expression. Although N=1 for the elderly skin, the data suggests that klotho has a rejuvenating effect on gene expression in elderly skin. Moreover, klotho enhanced the expression of FASN and FAD in UV exposed elderly skin (not in unexposed skin), which UV has been shown to decrease the expression of in skin *in vivo* [Kim et al., 2010]. This highlights the protective effect of klotho against UV damaged skin which is further illustrated in young skin.

In young skin (N=3), the upregulation of several genes (typically downregulated with age) appears to specifically be triggered by UV exposure. This suggests that klotho serves a role in the skin's response to UV damage and helps to repair the effects of UV exposure. Moreover, the upregulation of such genes is specifically observed only in MN delivery of klotho not topical in young skin, and as these are dermal specific genes, it is highly likely that the microneedle enhances the penetration of klotho into the dermis. If the klotho coated microneedle was applied to elderly skin it is plausible that the observed upregulation in the photoaging and biological age-related genes would be heightened, perhaps comparable to levels presented in young skin. In addition, study on further biological replicates of elderly skin

would provide a more holistic overview of the effect of klotho on old skin, and whether it is comparable to the effect observed in young skin.

In chapter 3, I have found there to be penetration of my designed and constructed needles to the basement membrane of the skin indicating that the 700 μm needle tips reach the papillary dermis which explains the upregulation in dermal genes with the MN delivery of klotho but not in the topical delivery of klotho that would not be able to diffuse to the dermal layer and only elicit superficial effect on the skin surface. This suggests that topical delivery of klotho is successful in preventing and improving UVA impaired wound healing. However, in conserving young skin integrity it is not effective, but the MN delivery of klotho is.

3.4 Discussion

In this chapter I set out to establish a UVA age accelerated *ex vivo* human skin model that mimics the physiological photoaging mechanisms of UVA exposure at the tissue and cellular levels. My established model illustrates the significant damaging impact that UVA elicits on the protective barrier of the body. As skin exists as the crucial interface to environmental stimuli it is the phenotypic window to biological ageing, i.e. skin translucency, wrinkling, and thinning. Striking increases in UVA radiation due to climate change is an immense global health concern. Rising UVA primarily because of ozone depletion will most likely cause an increase in UV driven disease, beyond skin cancer onset. In this chapter, I have explored the age accelerated effect UVA elicits on *ex vivo* human and further investigated DNA damage induction, and wound healing. I have shown that UVA exposure significantly enhances the biological age of human skin tissue, epidermal keratinocytes, and dermal fibroblasts. In parallel with UVA age acceleration, I observed a significant change in $p21^{high}$ and $laminb1^{low}$ expressing keratinocytes and fibroblasts, highlighted by qPCR data. I confirm that UVA elicits damage on young skin, assessed via epigenetic hypermethylation, DNA damage, and wound healing delay in elderly vs young skin. This can explain why old vs young skin exhibits delayed healing and damage due to long-term exposure of UVA of aged skin resulting in reduced collagen, flexibility, and thinner epidermis and DEJ.

Notably, after optimisation I found that a single dose of UVA (1.0 J/cm^2) significantly impairs wound healing of *ex vivo* human skin and significantly delays the repopulation of keratinocytes across scratch wounds *in vitro.* Upon exploration of the effect of UVA on the intricate mesenchymal signalling between keratinocytes and fibroblasts I identified a statistically significant upregulation in the SASP factors IL12 and IL8 from UVA exposed fibroblast senescent conditioned media. In turn, this conditioned media when cultured with healthy keratinocytes caused significantly delay in repopulation to injury site *in vitro.*

The increase in IL8 in response to UVA exposure is comparable to previous research which found that UVB significantly heightens IL8 in the skin epidermis, fibroblasts, and keratinocytes [Strickland et al., 1997; Storey et al., 2005]. However, the indication that IL8 secretion in senescent fibroblast media may signal upstream and impair keratinocyte repopulation challenges previous publications which have concluded that IL8 significantly improves the rate of keratinocyte repopulation [Jiang et al., 2011; Rennekampff et al., 2000; Sivamani et al., 2007]. Therefore, I evaluated the effect of IL12 on epidermal keratinocyte repopulation as this protein was also significantly enhanced in expression in UVA conditioned fibroblast medium and has been reported to influence *in vivo* wound healing when knocked out in mice [Matias et al., 2021].

I observed a significant reduction in the % cell covered area of keratinocytes (hour 24 vs 0) of the IL12 treated cells by over 40% against the untreated control. This suggests that UVA is disrupting repopulation of the epidermal keratinocytes in human skin by stimulating the release of IL12 by dermal fibroblasts which influences the epithelial mesenchymal signalling network. The anti-aging compound klotho was ineffective in improving UV disruption of the epithelial mesenchymal network. This highlights that klotho is not entirely sufficient as a preventative to UVA damaged fibroblasts. This is likely due to other factors (i.e. growth factors) being at play in influencing the UV disrupted epithelial mesenchymal network that the sole application of klotho would not resolve. However, klotho exhibited a myriad of other benefits on UVA damaged fibroblasts and notably more so on epidermal keratinocytes.

I found that the pre-emptive administration of α-klotho protein prior to UVA exposure, significantly ameliorated UV impaired wound healing of *ex vivo* young skin and significantly improved keratinocyte repopulation *in vitro*. In addition, I observed upregulation of laminB1

in keratinocytes and fibroblasts in response to klotho administration *in vitro*. The enhancement of laminB1 in response to klotho treatment has not previously been observed but the correlative increase of klotho and laminB1 expression in aged fibroblasts in response to an antioxidant centcyamine has [Abadie et al., 2021]. These findings along with improved skin elastin and collagen expression indicated slowed skin ageing onset and suggests that laminB1 expression may be linked to α-klotho signalling which is crucial to maintaining skin homeostasis.

I translated my promising klotho findings toward a translational device. I utilised my designed and engineered pH-responsive MN device (presented in chapter 2) for the dermal delivery of the α-klotho protein to *ex vivo* skin in young skin. My translational pH responsive microneedle device has previously shown to be effective in the delivery of multiple compounds irrespective of charge or size to a synthetic skin model and *ex vivo* human tissue. In this chapter, my MN patch has proven successful in efficiently delivering the α-klotho protein to my established UVA age accelerated skin model by upregulating expression of some dermal genes. I successfully presented a statistically significant upregulation of COL1A1 and COL3A1 using the klotho coated MN patch compared with topical klotho treatment suggesting increased α-klotho efficiency in delivery with the MN device.

Holistically, my presented findings have expanded on previous studies exploring UVA effect on human skin, in accelerating the biological age, impacting wound healing, and altering phenotype of epidermal/dermal primary cells. The contribution of UVA in delaying wound healing had been theorised but not extensively studied prior to this publication. I have successfully shown that UVA exposure exerts significant damage to skin regeneration. My findings suggest that the mechanism behind UV impaired wound healing is the senescent induction of fibroblasts which secrete SASP factors that signal upstream to keratinocytes and impair their repopulation to injury site. Klotho may proactively ameliorate UVA impaired wound healing by blocking the p53/p21 pathway in dermal fibroblasts, a mechanism which has been previously reported [de Oliveira. 2006]. This in turn may elevate laminB1 expression, thus preventing UVA induced DNA damage of fibroblasts which prevents the secretion of SASP factors from signalling upstream to keratinocytes to delay their repopulation to injury site.

In summary, in this chapter, I have explored the intrinsic changes in human skin that occur in response to a habitual environmental stimulus that is UVA. My work began as a biological discovery that was successfully translated to a preventative therapy. I have emphasised the physiological relevance of UVA as an environmental stimulus that humans are habitually exposed to daily irrespective of where they reside across the world, and this factor will only become more relevant with the depleting ozone layer. As population age advances toward an elderly demographic, this pool of people will be particularly vulnerable to UVA damage due to epidermal thinning and photoaged skin, therefore UVA driven disease will likely rise. The skin is an imperative protective barrier between the internal organs and environmental stimuli.

As the interface to the outside world, skin is phenotypic indicator of human ageing. Considerable study on UVB induced damage skin has previously been done, however, less so has been performed with regards to UVA induced skin damage. During this chapter, I investigated the effect of UVA exposure on *ex vivo* skin, focusing on the mechanism behind UVA age-acceleration and impeded wound healing of young skin. My findings identified UV enhanced ageing of *ex vivo* skin and cells that was protected by the klotho protein and translated using a klotho coated MN device.

Chapter 4

The impact of skin colour on skin health.

4.1 Introduction; Chapter Aims

In this chapter I discuss the influence skin colour can have on skin disease susceptibility. Notably, I focus on the role of melanocytes within the epidermis and the changes in their phenotype and function depending on location of the body, with ageing, and between people of variable skin colour. I assess how the UVA/B protective capacity of melanocytes differs from person to person depending on skin colour and how that in turn may influence skin cancer, wound healing or dermatoligic disease risk or prevalance. Lastly, I highlight the need for further research and education into skin diseases that present on medium to darker skin types to imrpove latent diagnosis within these pool of people.

1. To explore and understand the pivotal role of melanocytes in giving rise to skin pigementation.
2. To assess how melanocyte function and morphology may be altered depending on location of the body or with ageing or with UVA/B exposure.
3. To investigate the pathology and mechanism behind skin cancer, dermatologic disease, and wound healing across people of variable skin colour.
4. To highlight discrepencies in diagnosis of dermatological disease in dark skin and urge the need for further study and advanced knowledge of how skin diseases may present differently on dark skin types compared to light skin.

*Parts of this chapter are taken verbaitm from this manuscript in preparation:
Abbas AA., and Higgins CA. “The Impact of skin colour on skin health”

4.1.1 The disrepency in diagnosis of skin diseases in different ethnic cohorts

A striking physiological variance between human beings is skin colour which is evident across demographic populations. Skin colour is determined by the amount of eumelanin, a subtype of melanin, in the skin. This difference impacts and shapes individuals in immense ways psychosocially and notably in skin health. Pigment-producing melanocytes are fundamental drivers in causing variance in complexions across ethnic cohorts, with a key role in skin disease. Their behaviour is influenced by intrinsic and extrinsic factors including ultraviolet (UV) radiation and chronological ageing. An ongoing concern within the national health care (NHS) system in the United Kingdom (UK) is the lack of skin disease diagnosis algorithms across ethnic groups, with many symptoms being defined according to type I skin (according to the Fitzpatrick Skin Type (FST) scale). Across the pond, the US stands as the only developed country without a universal health care system which one can argue drives a division in health care access based on socioeconomic status. Black (constituting 20.1% of poverty) and Hispanic cohorts (28.4% of poverty) overrepresent in poverty with white and Asian underrepresenting [United States® Census Bureau].

This suggests that specific ethnic groups may have less available access to privatized health care than other groups due to low income, which can lead to lack of diagnosis of skin conditions in such cohorts, as well as lack of education on how skin diseases may present on skin of specific groups that vary in colour, due to less medical encounters with such groups. Ultimately, this marginalisation and exclusivity in healthcare toward underrepresented groups also stems from an insufficiency in readily available medical sources and a limited understanding of how individuals from different ethnic cohorts, possessing variable skin tones, differ both in their predisposition for certain skin diseases and how they present corresponding symptoms. It is imperative that this variance in skin tone and how it impacts one’s skin health is acknowledged to improve early-stage diagnosis of critical skin conditions among underrepresented groups. In this chapter, I discuss how pigmentation impacts patients with skin disease, from diagnosis and treatment, through to disease susceptibility.

4.1.2 Melanocytes in Skin Homeostasis

Melanocytes are neural crest derived dendritic cells responsible for producing a protective, skin darkening pigment known as melanin [Brenner and Hearing. 2008]. These specialised cells are derived from melanoblasts which migrate to various locations around the body during development, including skin [Erickson and Goins 1995]. Throughout this process, these cells

proliferate and differentiate into melanocytes which are found in the inner ear [Beelen et al., 2020], nervous system [Yaar & Park, 2012], heart [Yajima & Larue, 2008], vaginal epithelium [Pankaj et al., 2016], meninges [Gudjohnsen et al., 2015] and bone [Hirano, 1990]. They are abundantly present in the bulb of the hair follicle where they produce melanin giving colour to the hair fibre [Slominski et al., 2005], and notably in the basal layer of the human epidermis giving colour to the skin [Joly-Tonetti et al., 2016]. In this body of work, I discuss the role of melanocytes in the skin specifically and discuss the intra and interpersonal variability in the phenotype and function of these cells and how that impacts skin health.

Melanocytes in human skin

In human skin, each melanocyte forms a functional and structural unit with 30-40 keratinocytes known as the epidermal melanin unit [Fitzpatrick, 1963]. This unit is dynamic and reactive to intrinsic and extrinsic stimuli [Brenner and Hearing Hearing, 2008]. After producing melanin, melanocytes package this into melanosomes. These melanosomes are then transferred to keratinocytes in the epidermal melanin unit, via the keratinocyte proteinase-activated receptor (PAR)-2 which stimulates uptake of melanosomes through phagocytosis in a Rho-dependent manner [Seiberg et al., 2000; Van Den Bossche et al., 2006; Scott et al., 2004]. Filopodia serve a key role in the mechanism of melanosome transfer from melanocytes to keratinocytes, as well as between keratinocytes [Singh et al., 2010].–Once in the keratinocyte, the melanosome degranulates releasing its' cargo, melanin [Ando et al., 2012]. This melanin accumulates in the supranuclear region where it confers a protective effect to DNA against ultraviolet (UV) radiation-induced damage [Del Bio et al., 2018].

4.1.3 Melanin production and phenotypic diversity in skin colour

Spectrum Scales to classify skin colour from light to dark.

Variation in skin colour across the global population is one of, if not the most prominent example of human phenotypic diversity. The term 'Skin of colour' is typically used to encompass individuals of brown or black skin. However, this term is race-based and exclusionary, thus not truly reflective of the range in human phenotypic diversity [Dadzie et al., 2022]. Therefore, this calls for a novel nomenclature to appropriately classify the spectrum of skin colour across humans, irrespective of race or ethnicity, based on objective scientific data. Herein, I have used the term 'eumelanin-rich skin' (ERC) to classify those with moderately or darkly pigmented skin because of eumelanin abundance. However, in this

chapter I will use typically used skin pigmentation nomenclatures to refer to what has been used in publication.

The Fitzpatrick skin type (FST) phototype scale [Figure 4.1] is commonly used to classify skin Types I (White) to VI (Black) [Charlton et al., 2020; Goon et al., 2021]. This is a graded scale measured by sensitivity of the skin to tan with UV exposure and is not representative of actual skin colour [Charlton et al., 2020]. The FST is classified using the individual typology angle (ITA) which separates skin into six groups on how easily it burns/tans, ranging from very light to dark: very light>55°>light>41°>intermediate>28°>tan>10°>brown> -30°>dark [Wilkes et al., 2015].

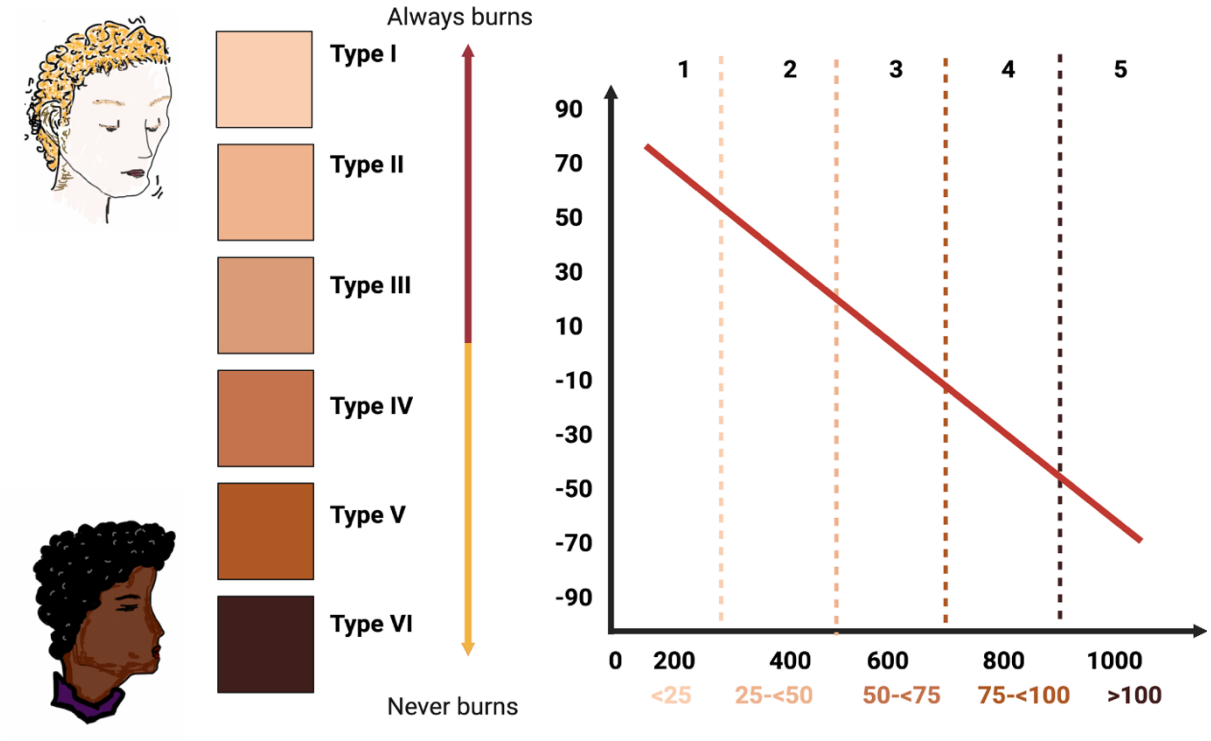


Figure 4.1: Schematic of the FST (left) and Eumelanin skin score (right) scales.

A key discrepancy within the FST photo-typing scale is the ambiguity in specifically identifying non-white (Type I-III) and non-black (>Type VI) skin tones [Figure 4.1], as Middle Eastern, South Asian, East Asian, South-East Asian, and South American skin tones can vary greatly from light to dark, across the spectrum, depending on demographics and genetics.

However, the FST score does not account for melanin content despite the main mechanism that drives these differences in skin colour is melanin production by pigment-producing melanocytes [Deng and Xu. 2017]. Melanin exists in two forms: soluble sulfur-containing pheomelanin, a yellow-reddish hue, or insoluble eumelanin, a black-brown shade. While all skin tends to possess more eumelanin than pheomelanin the relative proportions can vary.

Pheomelanin is more prevalent in the skin of light-complexioned (<Type III) individuals [Zanetti et al., 2001], produced in the presence of L-tyrosine [van Nieuwpoort et al., 2004] whereas eumelanin is more abundant in ratio to pheomelanin in the skin of dark-complexioned individuals, ranging from 4 or 3 to 1 (in FST >Type III) [Naoi et al., 2010; Ito et al., 2003; Hennessy et al., 2005; Thody et al., 1991; Riley 1997; Barsh. 2003]. There are also two types of eumelanin; 5,6-dihydroxyindole (DHI)-eumelanin and 5,6-dihydroxyindole-2-carboxylic acid (DHICA)-eumelanin [Alaluf et al., 2001]. In Type V and VI skin, (DHI)-eumelanin makes up 60-70% of epidermal melanin content, (DHICA)-eumelanin makes up 25-35% while pheomelanin constitutes just 2-8% [Alaluf et al., 2001]. In Type I-III eumelanin content accounts for 74% of total melanin content and pheomelanin constitutes 26% of total melanin content [Del Bino et al., 2015]. Moreover, in FST Type I-III skin, the epidermis produces pheomelanin with a higher ratio of benzothiazine (4-AHP) to benzothiazole (TTCA) moiety compared to Type V-VI skin [Del Bino et al., 2015].

More recently, the eumelanin human skin colour scale (EHSCS) [Figure 4.1] has been proposed to assess skin colour than FST. This classification spectrum is based on the melanin index, ranging from eumelanin low (EML), <25; eumelanin intermediate low (EMIL), 25 to <50; eumelanin intermediate (EMI), 50 to <75; eumelanin intermediate high (EMIH), 75 to < 100; and eumelanin high (EH), >100 [Dadzie et al., 2022]. While the eumelanin skin score is a more accurate measurement of skin colour as it accounts for melanin content, many older studies typically use the Fitzpatrick scale as a determinant of skin colour. Within this chapter I have reported with what previous studies have used, which is typically the FST scale.

Genetic polymorphisms can drive pigmentation.

Numerous variations or polymorphisms in genes associated with pigmentation has been shown to contribute to some of the diversity in skin colour observed around the world. For example, a non-synonymous single nucleotide polymorphism (SNP) in *SLC24A5* (rs1426654) has been found to account for 25-38% of the variation between Africans and European ancestries with a G allele predominant in African populations and the A allele predominant in people of European ancestry. Notably, East Asians though can exhibit type I-III skin are in fact reported to be 20x less susceptible to melanoma than Europeans [Farage et al., 2008]. One study has tried to explore the aetiology behind why east Asians of light skin present lower skin cancer rates than Europeans and identified the presence of mutations in the SLC24A5 and SLC45A2 genes, which has been linked to cancer susceptibility, in Europeans but not East Asians [Ang

et al., 2012; Gorbatenko et al., 2014]. This finding poses one explanation as to why both Europeans and East Asians with lighter skin evolved to allow for improved synthesis of vitamin D in northern climates but variability in skin cancer susceptibility.

Interestingly, a genetic analysis of 1570 Africans with extensive variation in skin pigmentation revealed that the A allele was introduced into East Africa by migration of Europeans to the African continent [Crawford et al., 2017; Lamason et al., 2005]. Variants in other genes, including *MFSD12, DDB1, TMEM138, OCA2* and *HERC2*A have also been associated with skin pigmentation [Crawford et al., 2017]. The damage-specific DNA binding protein 1 (DDB1) gene is reported to be associated with light pigmentation and serve a role in UV response and melanoma risk, and functions in DNA repair and genomic stability in developing the epidermis which melanocytes reside [Crawford et al., 2017]. Genetic variants near DDB1 have been linked to lighter pigmentation, with mutations in this gene having migrated out of Africa to Eurasia [Crawford et al., 2017]. Interestingly, silencing of the major facilitator superfamily domain containing 12 (MFSD12) gene in skin melanocytes, has been found to increase eumelanin levels within the skin, leading to darker pigmentation [Feng et al., 2021; Crawford et al., 2017]. Moreover, dark skinned (type VI) people residing in Eastern Africa have been found to possess variants of the MFSD12 gene [Feng et al., 2021; Crawford et al., 2017]. This suggests the influence of genetic evolution in skin pigmentation in Type VI skin and thus their intrinsic defence against habitual UV exposure in strong sun exposed regions.

Variants in the melanocortin 1 receptor (MC1R), which is important for eumelanin synthesis, leads to a red hair/pale skin phenotype that is typically seen in northern Europe [Valverde et al., 1995; Box et al., 1997; Smith et al., 1998]. Notably possessing this gene confers an increased risk for the development of cutaneous melanoma, (irrespective of phenotypic characteristics) [Kennedy et al., 2001] with up to 40% increased risk with a single MC1R variant [Manganelli et al., 2021].

The influence of the environment on skin pigmentation

Across the world, skin colour is highly associated with latitude levels, specifically the dispersal of UV radiation. Exposure to UV can be beneficial for human skin as it facilitates the synthesis of vitamin D, which possesses many health benefits, notably acting as an additional protective barrier to subsequent sun damage by contributing to the formation of the epidermal layer and restoring melanocyte integrity [Raymond-Lezman et al., 2024; Mostafa and Hegazy. 2015;

Bocheva et al., 2021]. However, an overexposure of UV can also lead to many defects for example a reduction in folic acid levels [D'Orazio et al., 2013; Juzeniene et al., 2010] and exists as a risk factor for skin cancer [Armstrong et al., 2001; De Gruijl et al., 2001; Brenner and Hearing. 2008; Greaves. 2014].

Melanin in the skin confers a protect effect against UV radiation by absorbing it and preventing epidermal penetration [Brenner et al., 2007]. Specifically, eumelanin elicits a more protective function against UV radiation than pheomelanin, which is prone to photodegradation [Corani et al., 2014; Solano. 2020]. Eumelanin is effective in shielding against UV by acting as a physical barrier; scattering, and absorbing UV light [Kaidbey et al., 1979]. The photodegredation of pheomelanin leads to an increase in reactive oxygen species (ROS) which can be damaging to skin [Ito et al., 2018]. In Type I-III, 24% UVB and 55% UVA passes through the epidermis, while in dark Type VI skin, only 7.4% of UVB and 17.5% of UVA can penetrate the epidermis [Halder et al., 1995]. The melanin present within Type IV skin confers a natural SPF of 13.4, while the melanin present in Type I skin confers only 4-5 SPF [Kaidbey et al., 1979]. Unlike UVA however, UVB is readily able to induce melanin production, enabling the skin to protect itself against further UV effect by scattering UV radiation to reduce penetration of it to the epidermis [Grant. 2008; Atillasoy et al., 1998; Wendt et al.,2011; Flament et al., 2013; Gloster et al., 2006]. Populations residing close to the equator, where there is greater UV exposure, often present with dark skin tones which acts as a protective defence against UV [Brenner and Hearing. 2008].

In fact, in Type VI photoexposed skin, eumelanin levels increase by 1.5x from 4668 ng/mg to 7212 ng/mg, whilst eumelanin levels in Type I photoexposed skin rise by 1.25x from 1774 ng/mg to 2229 ng/mg [Hennesy et al., 2005; Alaluf et al., 2001]. The pheomelanin levels in Type VI photoexposed skin rise by 1.5x from 290 ng/mg to 462 ng/mg and in Type I skin by 1.17x from 123 ng/mg to 145 ng/mg [Hennesy et al., 2005]. A previous study exploring UV-induced DNA damage and melanin content in human skin across various racial origins found that increasing eumelanin content in the skin is inversely correlated with significant DNA damage [Tadokoro et al., 2003]. Skin types III to VI, often present dyspigmentation (abnormal skin colouring) as a result of photoaging, driven by UVA, however, ERS is reported to be less susceptible to sun-induced damage as it is less likely to present lentigines, rhytides (fine lines), telangiectasias (widened blood vessels on skin), dark spots, and loss of elasticity, all indicators of photoaging, driven by DNA damage, observed in light skin (type I) [Alam et al., 2009].

A recent study exposed 31 healthy subjects with Type I-III and Type V-VI skin types to daily doses of visible light (VL) (400-700 m, dose 120 J/cm^2) for four consecutive days in the buttock region of the body. [Kim et al., 2022] Repeated low-dose VL irradiation stimulated immediate pigment darkening and delayed tanning in dark-skinned individuals. In contrast in light skinned individuals no phenotypic pigmentation was observed and instead erythema was presented [Kim et al., 2022].Transcriptomics analysis showed that several melanogenic genes were upregulated in all skin types following repeated VL exposure, including tyrosinase (TYR), premelanosome protein (PMEL), tyrosinase-related protein-1 (TYRP1), melan-A (MILANA), dopachrome tautomerase (DCT), and solute carrier family 24, member 5 (SLC24A5) [Kim et al., 2022].

This reflects the double-edged sword of UV radiation, in that short term UVB radiation promotes melanogenesis which further elicits protection to the skin, against photoaging, however long-term persistent exposure to UVA radiation drives the eventual effect of photoaging, earlier in Type I skin (has less intrinsic natural melanin) than type VI skin (more intrinsic natural melanin) [Wang et al., 2019].

The double-edged sword of UV Exposure

In high latitude regions of the northern European people tend to have Type I skin. These pools of people are more vulnerable to UV-induced damage due to less eumelanin, they have the enhanced capacity to photosynthesise vitamin D (23-hydroxyvitamin D (25(OH)D) by basal keratinocytes, a UV-dependent process. Here the reduction in UV in reaching the skin keratinocytes in Type V-VI skin is detrimental [Mendes et al., 2019; Jablonski et al., 2017; Hart et al., 2011]. This may be explained by the fact that Vitamin D synthesis is inversely proportional to the concentration of melanin in the skin as melanin absorbs and scatters UVB leading to limited conversion of 7-dehydrocholesterol to pre-vitamin D3 [Jablonski. 2004; Bonilla et al., 2014]. This means that Type VI skin will experience much slower vitamin D synthesis than Type I skin. In fact, severe vitamin D deficiency accounts for 18.46% (<30 nmol/L of mean 25(OH)D levels indicated deficiency) in the African continent [Mogire et al., 2020; Bouillon. 2019; Harris. 2006] increasing brain and skin related disease [Umar et al., 2017]. This is contrary to the popular held belief that Africa, where there is habitual sunshine, is the optimal region for Vitamin D synthesis.

Therefore, it is important to note that too much UV filtration, due to high eumelanin content, into the skin can lead to vitamin D deficiency. Severe vitamin D deficiency has been linked to a higher prevalence of cardiovascular disease, diabetes, and skin cancers observed among African American people [Mogire et al., 2020; Boilon. 2019; Harris. 2006]. These findings highlight the nuances of UV impact on various skin colours and in being both a benefit and detriment to human health depending on the threshold of exposure [Farrar et al., 2011]. As despite this efficiency in producing vitamin D, Type I-II skin will be vulnerable to elevated sunburn response, reduced tanning ability and greater susceptibility to skin cancers, when exposed to periods of sunlight [Wagner et al., 2002; Sturm. 2002]. However, vitamin D deficiency can be associated with high risk of Alzheimer's Disease (AD) and psoriasis [Umar et al., 2017; Hata et al., 2014].

Melanogenic activity influences skin colour.

Despite different skin tones harbouring melanocytes which produce varying contents of pheomelanin and eumelanin, studies of the human epidermis have revealed that there is no significant difference in the total number of melanocytes between skin Types I through to VI on the FST [Toda et al., 1972; Sun et al., 2021]. What differs between skin types is the number of melanosomes that melanocytes produce, as well as the amount of melanin in which they package. Variations in melanin production pertains to the activity of the melanocyte [Chadwick et al., 2012]. This is considered to serve a more prominent role, than the proportions of either eumelanin or pheomelanin, in determining human skin colour [Barsh. 2003]. Melanogenesis varies across skin types due in part to the activity of melanogenic enzymes within melanosomes. Melanosomes originating from Type VI skin have a neutral to alkaline pH and a higher activity of melanogenic enzymes, whereas melanosomes from Type I skin are more acidic in nature and have lower melanogenic activity [Smith et al.,2004; Fuller at al., 2001]. Total melanin content, produced from melanosomes, in the basal layers of the epidermis is 3.6-fold higher in Type VI, compared to Type I skin [Brenner and Hearing. 2008; Kollias et al., 1991], while in medium toned Type III-V skin it is 2-fold higher than in Type I skin [Brenner and Hearing et al., 2008]. There is also more eumelanin in Type VI skin, as well as larger size and a higher number of melanosomes in this skin type compared to Type I skin [Ito et al., 2003; Szabo et al., 1969].

Type VI skin melanosomes are larger in size and more dispersed within basal keratinocytes whereas Type I skin melanosomes are smaller and clustered together, forming melanosome complexes which bind to the keratinocyte cell membrane [Hurbain et al., 2018; Szabo et al., 1969; Konrad and Wolff. 1973; Alaluf et al., 2002]. Melanosomes in Type VI skin have an elliptical shape compared to the membrane bound cluster melanosomes present in Type I skin (800 vs 400 nm). These long elliptical melanosomes are present as single entities and it has been theorized that they are transferred to keratinocytes individually via a shedding vesicle system, which contributes to their efficient absorption of light [Ando et al., 2011]. In contrast, the clusters of spherical melanocytes found in Type I skin are thought to be packaged and transferred to the keratinocytes as complexes [Kollias et al., 1991]. Notably the mechanism behind melanosome loss in skin is varied, as keratinocytes from Type I-III skin presents higher autophagy activity than Type V-VI skin keratinocytes [Szabo et al., 1969; Murase et al., 2013]. This means that Type I-II derived keratinocytes play a fundamental role in leading to melanosome loss and thus in the pigmentation of skin.

Melanocytes present regional diversity across the human body.

Within body sites, skin colour in young, healthy individuals is remarkably uniform. This is largely attributed to the vastly homogenous distribution of melanocytes throughout the basal layer of the epidermis [Thingnes. 2012; Tang et al., 2021]. However, factors such as age and body location influence melanocyte density. Interestingly, none of the described skin scores for skin colour take into account regional diversity across body sites or the effects of age [Figure 4.2].

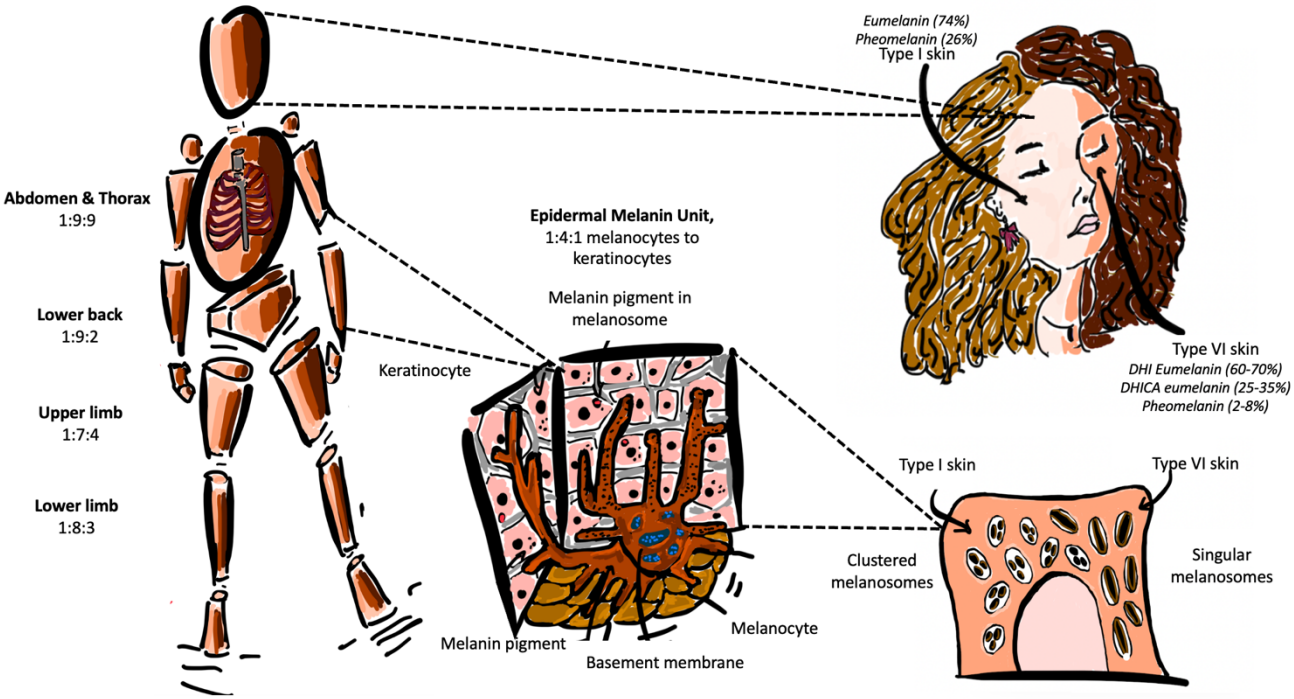


Figure 4.2: The distribution of melanocytes across the body.

A recent study explored the ratio of melanocytes to basal layer keratinocytes in the basal layer to identify the relationships of this ratio with sex, age, body location, and epidermal thickness, within the Chinese Han population [Sun et al., 2021]. The average ratio of melanocytes to basal keratinocytes was found to be 1:6.5 in males and 1:7.4 in females (aged 16 to 87 tears old) with no significant difference between sexes ($P<0.127$). [Sun et al., 2021]. In decreasing order, the ratio of melanocytes to keratinocytes in the basal layer of the skin are found to be as follows: face (1:4.0) >neck (1:5.1) >hip (1:5.7) >upper limb (1:7.4) >lower limb (1:8.3) >lower back (1:9.2) >thorax and abdomen (1:9.9); notably melanocyte: keratinocyte ratio was not related to epidermal thickness [Sun et al., 2021; McKee. 2019; Fitzpatrick. 1963]. This validates previous reports that melanocyte density is higher in the face, neck and hip than in the limbs and torso; [Thingnes. 2012; Sun et al., 2021; Yamaguchi. 2007; Yamaguchi. 2004; Szabò. 1954;

Yamaguchi and Hearing. 2008]. Sun et al also found that within each body site analysed, melanocyte distribution remained uniform [Sun et al., 2021]. Averaged across the whole basal layer of the adult human epidermis, the average melanocyte density is 1,500 cells/mm^2. Evidently, melanocyte densities in sun exposed body areas are higher than those in sun protected areas. The forehead has the highest density, at 2,310 mm^2 while the abdomen and back have 800 mm^2 and 1,100 mm^2 respectively [Whiteman et al., 1999]. However, when comparing the same body site in individuals (aged between 16 to 87 years old) with different skin colour according to FST, melanocyte density is virtually identical irrespective of gestational age, birth weight and season of birth [Thingnes. 2012; Sun et al., 2021; Yamaguchi. 2007; Park et al., 2005]. This suggests that melanocyte prevalence at the forehead region of the body is determined genetically as appose to in response UV exposure [Park et al., 2005]. The fact that melanocyte density is equal within the body sites of different skin colours supports the idea that this is genetically driven and not in response to the environment.

The lowest melanocyte density in the body is found in palmoplantar skin. Melanocyte density in palmoplantar skin is five times lower than in non-palmoplantar sites across all skin types according to the Fitzpatrick scale [Yamaguchi. 2007; Yamaguchi et al., 2004, Brenner and Hearing et al., 2008]. One of the reasons as to why there is less melanocytes present in nonplantar skin is due to the fact that melanocyte migration ends at the palms and soles of the feet during embryogenesis leading to hypopigmentation. Moreover, the-expression of the Wnt inhibitor, Dickkopf 1 (DKK1) in palmoplantar fibroblasts, limits melanocyte growth, proliferation and pigmentation, via downregulation of microphthalmia-associated transcription factor (MITF) and β-catenin [Yamaguchi et al., 2010]. DKK1 also reduces expression levels of PAR-2 [Yamaguchi et al., 2010] which is important for the transfer of melanin to keratinocytes.

Melanocytes in ageing.

A significant decrease in melanocyte density in the skin has been associated with increasing chronological age. The mean melanocyte naevi on hands, in 50–59-year-olds is reported to be 25.9%, down to 20.8% by 60-69 years of age, 19.4% by 70-79 years of age and 14.6% in <80-year-olds [Whiteman et al., 1999]. It is important to note that scientists Gilchrest, Mitchell and Whiteman studied 8, 15 and 123 skin samples respectively and showed melanocyte density decreased as age increased [Whiteman et al., 1999; Gilchrest et al., 1979; Mitchell. 1963; Stierner et al., 1989]. Moreover, a more recent study discovered that the average ratio of

melanocytes to keratinocytes to be 1:7, however this gradually declines with increasing age (with statistically significant difference), at 1:5:8 before 50 years of age, to 1:7:9 at 51-65 years of age, and 1:8:5 at >65 years of age [Sun et al., 2021]. This may be attributed by epidermal thinning that occurs with increasing age, as keratinocyte loss will in turn influence melanocyte density [Quevedo et al., 1969; Fitzpatrick et al., 1964; Snell and Bischitz et al., 1963]. With advancing age, melanogenic activity not only declines, but tyrosinase activity also decreases, contributing to the pale pallor presented in the elderly [Lavker et al., 1979]. Moreover, due to the abundance of eumelanin in skin of colour, even with declining levels seen with increasing age, means that Type VI skin often will often present clinical manifestations of photoaging 10 to 20 years later than those of age-matched Type I-III counterparts, meaning there is a decline in age-related sun-induced damage (i.e. skin cancer) in dark skin types compared to fair [Kaidbey et al., 1979; Vashi et al., 2016]. However, Type V-VI skin will be more prone to hyperpigmentation disorders than Type I-III skin.

This is because the remaining melanocytes within the skin increase in size with age and produce more melanin due to persistent sun exposure over time, leading to pigment age spots known as lentigos. These are characterized by overproduction of melanin by single melanocytes, with no localized increase in the total number of melanocytes [Blume-Peytavi et al., 2016; Imokawa et al., 2019]. As Type V-VI skin possesses more total melanin content than Type I-III skin, they will retain more melanocytes with increasing age than Type I-III skin. Therefore, pigmentary disorders such as melasma, a skin conditioned characterised by freckle-like brown spots, are commonly presented in aged skin and is more common in Type III-VI skin than Type I skin [Mpofana et al., 2023].

4.1.4 Melanocytes and Skin Disease

Several pigmentary skin diseases, including vitiligo, Piebaldism, café-au-lait spots and progressive acromelanosis, are associated with abnormalities in melanocyte density or activity. [Sun et al., 2021]. For example, vitiligo is an inherited pigmentary trait that can cause localised decreases in melanocyte density [Sun et al., 2021; Yamaguchi. 2007]. In the sub-sections below, I discuss disease caused directly as a result of mutations in genes responsible for melanocyte formation, diseases that vary in incidence in skin of different colours, and diseases caused by external factors that impact melanocytes.

Diseases caused by variations in melanogenic activity.

Vitiligo is more evident in dark-skinned individuals.

Vitiligo is a commonly inherited pigmentary condition that arises from a lack of melanin pigmentation on a person's skin [Figure 4.3].

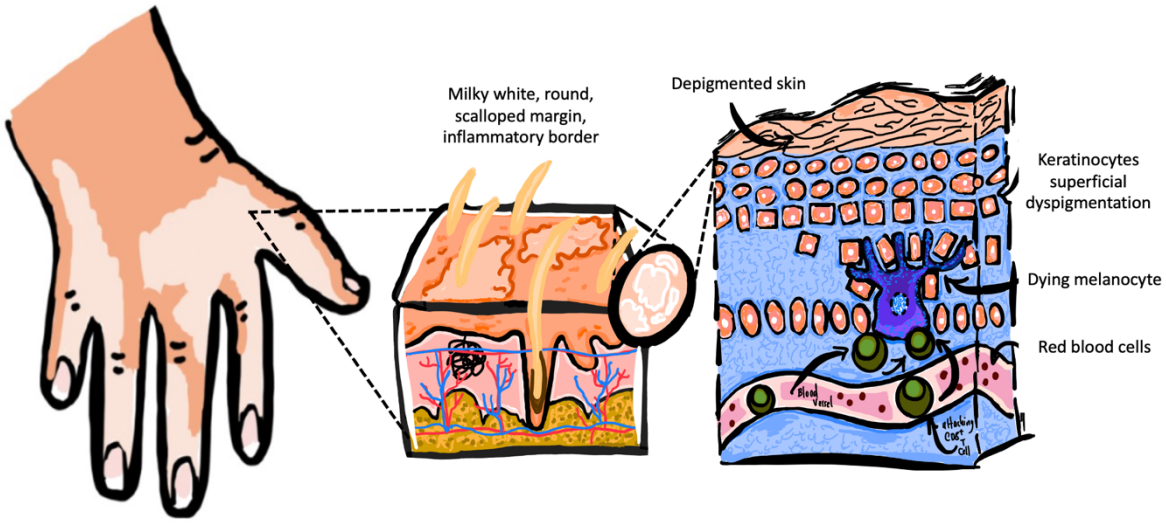


Figure 4.3: Illustration of vitiligo presentation on skin.

It is an autoimmune disease caused by cytotoxic T cell elimination of melanocytes leaving patches of unpigmented skin [Dina et al., 2019]. Vitiligo IFNγ driven inflammation breaks down melanocytes within lesions, causing long-lasting depigmentation [Stassner et al., 2016]. The disease arises as a result of genetic mutations, or environmental UV exposure. Notably, other autoimmune diseases i.e. Addison's disease, psoriasis, rheumatoid arthritis or lupus can increase risk for vitiligo. Interestingly, secreted frizzled-related protein 5 (SFRP5), an inhibitor of Wnt signaling, is overexpressed in skin lesions of patients with vitiligo, and specifically in vitiligo melanocytes (PIG3V) [Zou et al., 2020], where it is thought to block melanin synthesis via downregulation of MITF and Wnt/β-catenin signaling [Zou et al., 2020]. Notably, previous studies on patients with vitiligo discovered that melanocytes are not wholly absent in depigmented epidermis, and in fact the vitiligo melanocytes could recover their function both *in vivo* and *in vitro* with Demamethasone-cyclophosphamide pulse (DCP) therapy [Kanwar et al., 2013; Dogra et al., 2005].

Vitiligo can negatively influence an individual's quality of life by triggering psychological stigmatization and social discrimination. It affects up to 2.28% of the global population with up to 80% of patients acquiring the condition before the age of 30 [Ezzedine et al., 2016]. This disease can occur among people of any racial background but has higher prevalence among

Hispanic/Latino patients (ranging from 0.04% - 0.57% in Latin America) and those of Asian descent (prevalence of 0.27%) [Kruger et al., 2012; de Cesar Silva Castro et al., 2018; Tolentino Junior et al., 2019; Mastacouris et al., 2023]. Despite this, it is more evident on people with Type VI skin due to the contrast of the unpigmented patches against the skin with higher levels of eumelanin, presenting in 0.22% of type V-VI patients [Mastacouris et al., 2023; Ezzedine et al., 2016]. However, it is important to note that though vitiligo appearance in ERS (Eumelanin rich skin) is more apparent due to sharp contrasts against darker skin phenotypes, vitiligo does not have a predilection for ERS populations [Sangha. 2022], in fact it appears that those with mixed or medium skin tones are most affected by this disease.

Piebaldism susceptibility is not influenced by ethnic diversity.

Piebaldism is a rare genetic disorder affecting approximately 1 in 20 000 individuals worldwide [Shah et al., 2023; Agarwal et al., 2012] whereby both female and male individuals are equally affected [Figure 4.4].

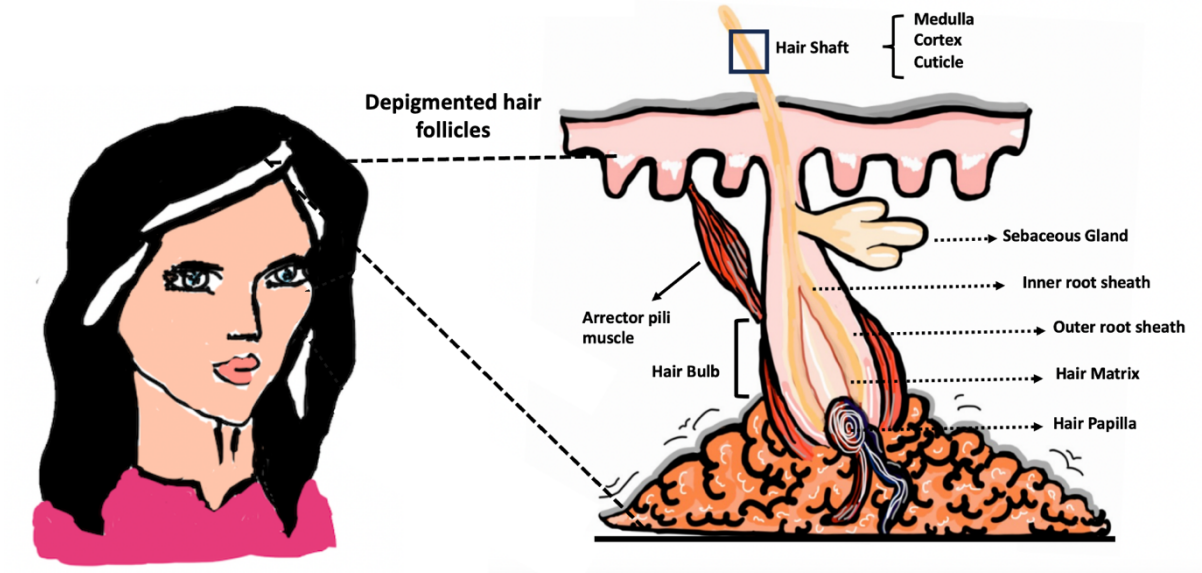


Figure 4.4: Illustration of piebalidsm presentation on skin.

At present, there is no known racial predilection of this benign disorder that is stable and non-progressive [Shah et al., 2023; Agarwal et al., 2012]. The condition is characterised by depigmentation of the skin and hair whereby melanocytes are either absent or greatly reduced in numbers, resulting in white patches or streaks [Janjua et al., 2007; López et al., 2002; Saleem et al., 2019; Spritz et al., 1994]; These hypopigmented areas are described as well-circumscribed, irregular areas lacking convex borders and may vary in size and shape [Janjua et al., 2007; López et al., 2002; Saleem et al., 2019; Spritz et al., 1994]. The symptoms of

piebaldism varies widely depending on the individual and can range from small white patches to extensive depigmentation of the skin and hair. Typically, hyperpigmentation can occur irregularly or uniformly distributed [Zalaudek et al., 2009]. These clinical manifestations are usually present from birth and likely to persist into adulthood [Shah et al., 2023; Grob et al., 2016; Agarwal et al., 2012]. However, unlike vitiligo, the hypopigmented areas do not progress after birth [Shah et al., 2023; Agarwal et al.,2012].

Piebaldism is an autosomal dominant condition, meaning that a single copy of the mutated KIT gene is sufficient to cause the disease. The KIT gene is a proto-oncogene located within chromosome 4q12 and encodes a receptor tyrosine kinase that is expressed on the surface of certain cells, including melanocytes as a result of mosaicism [Sorlin et al., 2017; Hoist et al., 1999]. Mutations in the KIT gene can disrupt differentiation, migration, proliferation, and survival of melanoblasts from the neural crest during the embryonic life, leading to abnormal melanocyte migration and the absence of melanocyte in the skin and hair. This results in the depigmentation of the skin and hair in affected individuals [Agarwal et al., 2012; Janjua et al., 2007; Spritz. 1994]. The phenotypic severity of piebaldism correlates with the site and type of mutation within the KIT gene [Agarwal et al., 2012; Spriz. 1994]. Diagnosis of this condition is based on the characteristic appearance of the skin and hair, along with genetic testing [Shah et al., 2023]. While there is currently no cure for piebaldism, various methods such as dermabrasion and split skin grafting, autologous melanocyte transplantation, epidermal sheet grafting, and autologous cell suspension transplantation following superficial full surface ablation have been effective in treating the hypopigmented areas [Shah et al., 2023]. Additionally, recent studies on vitiligo showing promising results with the use of stem cell therapy to restore pigmentation in affected individuals may also offer a potential treatment for piebaldism [Esquivel et al., 2020].

Albinism prevalence varies worldwide.

Oculocutaneous Albinism (OCA) is characterized as a heritable condition with reduced or absent melanin in the ectoderm-derived tissues, notably the skin, hair, and eyes [Federico et al., 2022]. Lack of melanin in the eyes can lead to reduced vision driven by foveal hypoplasia and diversions in the optic nerve fibres [Grønskov et al., 2007]. Around 1 in 70 people have variants in the genes, TYR, OCA2, TYRP1, and MATP1, for OCA, however, the prevalence of albinism varies greatly worldwide [Grønskov et al., 2007]. Epidemiologic data on albinism has revealed the high prevalence of this skin condition in South Africa, Zimbabwe, Tanzania, and Nigeria

(1 in 1000, Type VI skin)) [Hong et al., 2006]. The prevalence in OCA in European countries range from 1 in 12,000, 1 in 10,000 to 1 in 15,000, however there is likelihood this may be underestimated or misdiagnosed in Type I skin as OCA or visual impairment [Kromberg et al., 2023]. OCA is a genetically inherited autosomal recessive condition, and OCA2, tyrosine-positive albinism, is the most prevalent type found throughout Africa. As a result of the lack of melanin, people with albinism are more vulnerable to the detrimental effects of UV radiation [Hong et al., 2006] and are directly associated with hereditary conditions to which albinism presents as a symptom. Types of albinism include Hermanksy-Pudlak syndrome (HPS), a rare, hereditary disorder that is characterized by OCA, visual impairment, blood platelet dysfunction with persistent bleeding [Hurford and Sebastiano. 2008; Power et al., 2019]. In addition, the Chediak-Higashi syndrome is an autosomal recessive disorder that is depicted by OCA, easy bruising, and recurrent pyogenic infections [Ajitkumar et al., 2023]. This highlights the intrinsic connection between genetic disease and loss of pigmentation.

Societally, those with albinism often face a series of myths and stereotypes regarding their condition, and are often seen as disabled to others, leading them to feel excluded from their communities and facing discrimination [Phatoli et al., 2015; Tambala-Kaliati et al., 2021].

Skin melanin content and disease susceptibility

A recent study revealed that the average percentage of holistic publications relevant to ERS is low, at 16.3% [Lim et al., 2021]. Despite this skin conditions commonly occur in darker skin tones (>type III) such as eczema, or notably Hidradenitis Suppurativa (HS).

Hidradenitis Suppurativa (HS)

HS is a long-term skin disease that is prevalent in Type VI skin giving rise to abscesses and skin scarring, however the aetiology remains poorly understood. HS typically occurs in areas near hair follicles and sweat glands such as the groin, bottom, breasts and armpits [Choudhary et al., 2013]. Histopathology from the pigmented lesions of HS samples presented irregular epidermis, acanthosis, and extended rete ridges with accumulated basal melanin pigmentation [Choudhary et al., 2013]. The estimated global prevalence of HS is 0.00033-4.1% with a prevalence of 0.7-1.2% in Type I-III skin [Alotaibi. 2023]. HS incidence rates are highest in Type VI skin, at 1.3%, highlighting the possibility of genetic aetiology in HS progression [Sachdeva et al., 2021; Ulschmid et al., 2022; Vaidya et al., 2017]. A longitudinal study of HS-related hospitalisations in the US over 11 years (2008-2018) revealed that the incidence of

hospitalisations with HS diagnosis rose from 3.5 to 6.9 per 100 000 persons, and HS diagnosis in Black patients rose from 49.7% in 2008 to 62.9% in 2018 [Edigin et al., 2021]. However, despite the prevalence of HS in Type VI skin, it has been reported that in phase II and III trials from 2000 to 2019, 68% of participants were Caucasian, and only 14% were of African descent [Price et al., 2021] thus highlighting an exclusivity in the public health sector against people with ERS.

Psoriasis presents differently depending on skin colour.

Psoriasis is a heterogenous lifelong immune-mediated inflammatory skin disease that can present as a plaque, flexural or inverse (body folds), Pustules (white bumps filled with pus), guttate (small round papules) or as an erythroderma (red rash resembling a burn) [Raharja et al., 2021]. Psoriasis is characterised by the excessive proliferation and abnormal differentiation of keratinocytes [Zhou et al., 2022; Ippagunta et al., 2016]. Interestingly, psoriatic immune cell secreted IL-17 stimulated inflammation can activate keratinocytes driving chronic inflammation which temporarily inactivates melanocytes causing hypopigmentation [Stassner et al., 2016; Cengiz et al., 2016]. The primary psoriasis risk gene, HLA-C*06:02 is recognized by a T cell receptor (TCR) of CD8 T cells [Arakawa et al., 2015]. This TCR, Vα3S1/Vβ13S1 in psoriasis lesions attack melanocytes, by detecting the HLA-C*06:02-presented melanocytic autoantigen ADAMTS-like protein 5 (ADAMTSL5) of Vα3S1/Vβ13S1. This suggests that in psoriasis, HLA-C*06:02 initiates an autoimmune response against melanocytes through the autoantigen ADAMTSL5 [Arakawa et al., 2015; Bonifacio et al., 2016]. Typically, psoriasis is treated with vitamin D analogues and notably, phototherapy which alters the pro inflammatory cytokine profile, and ultimately slows the production of keratinocytes. This can also enhance migration of melanocytes and repigmentation of affected skin areas [Raharja et al., 2021; Wong et al., 2013; Yardman et al., 2020].

Globally, psoriasis affects 1-3% of people [Lowes et al., 2014]. Psoriasis prevalence across the world varies from 0.025% to 0.9% in Western Africa, 0.14% in East Asia, 1.99% in Australia, and up to 3.46% in Western Europe, 5.32% in Central Europe, and 3.60% in North America [Parisi et al.,2020; Chandran et al., 2010; Morrone et al., 2020]. In the USA, the prevalence of psoriasis in African Americans is 1.3%, and 2.5% in European-Americans–[Gelfand et al., 2005]. As Type VI skin, has a higher melanin content than Type I skin this can impact the way that psoriasis appears and is diagnosed [Stüttgen et al., 1979; Alexis et al., 2014]. On Type I skin, psoriasis presents as ink or red patches with silvery-white scales. Comparatively, on Type

VI skin, psoriasis presents as violaceous patches with grey scales and these phenotypical features can overlap with other papulosquamous disorders and less conspicuous erythema [Alexis et al., 2014]. Notably, recently published survey results from The National Psoriasis Expert identified key differences in psoriasis presentation between Type I and Type VI skin, with darker skin types exhibiting a higher degree of dyspigmentation (abnormalities in pigmentation) but a lower amount of erythema [Alexis et al., 2014; McMicheal et al., 2012]. Type VI skin can present with less conspicuous erythema presenting as hyper-pigmented, therefore making it difficult to visualise areas of active inflammation compared to Type I skin. Notably, Type III skin has a greater body surface area involvement at initial psoriasis appearance than Type I skin [Abrouk et al., 2017] and has been reported to present with significantly more severe psoriasis than Type I skin.

Recently, it was reported that most subjects in phase II psoriasis clinical trials are Caucasian, with only 14.2% being non-white participants, and only 3.09% of Black participants [Reddy et al., 2020]. Moreover, it has been projected that up to $1/5^{th}$ of newly developed medications to treat psoriasis present interracial variability in terms of pharmokinetics, safety, and efficacy profiles, dosing, and pharmacogenetics [Reddy et al., 2020]. For example, the BCL2 gene exhibits genetic variants among ethnic groups and shows differential responses to adalimumab treatment [Ramamoorthy et al., 2015; Liu et al., 2020]. This reinforces the stringent need for underrepresented groups to be included in clinical trials toward tackling skin diseases such as this that are often misdiagnosed or present more severely in darker skin types.

Atopic Dermatitis exhibits highest incidence in Type VI skin.

Atopic Dermatitis (AD), more commonly known as Eczema, is a chronic inflammatory skin condition present in skin of all colours [Figure 4.5] [Kaufman et al., 2018]. In AD, disease flareups are characterized by an increase in inflammation and breakdown of the skin barrier, leading to severe itching and erythema, or skin redness. Inflammation-associated pigmentation changes are also common in AD [Eichenfield et al., 2017; Givler et al., 2020; Fenner et al., 2018]. In a recent study, spontaneously healed atopic AD samples were compared to healthy control samples using single cell sequencing revealing melanocytes possessed the largest numbers of differentially expressed genes in comparison to healthy controls, including anti-inflammatory markers such as PLA2G7 [Rindler et al., 2021]. The genes of keratinocytes were

indistinguishable to that of healthy skin samples. Therefore, melanocytes may contribute a specific regulatory milieu in spontaneously healed AD skin [Rindler et al., 2021].

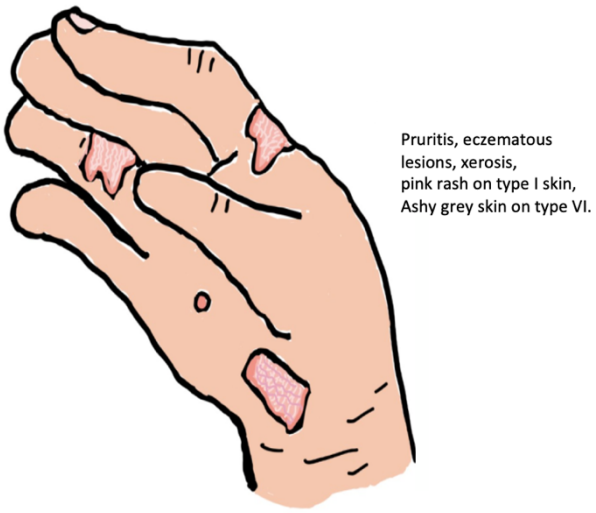


Figure 4.5: Illustration of dermatitis on skin.

In 2017, the global incidence rate of AD was 274 million [James et al., 2018]. The incidence rate of AD has dropped from 24% between 1990—2007 to 13% between 2007-2017 [James et al., 2018]. AD occurs most frequently in Black and Asian ethnic cohorts, with eczema prevalence in Africa ranging from 4.7% to 23% [Ait-Khaled et al., 2007].

Population-based studies in the USA have identified a higher prevalence of eczema in African American children, whom are 1.7 times more likely to develop AD than their European American counterparts [Shaw et al., 2011; Weiland. 2004; Fu et al., 2013; Silverberg et al., 2013; Wegienka et al., 2011; Odhiambo et al., 2009; Janumpally et al., 2002]. In the UK, London born Black children have twice the number of AD diagnoses as London born White children [Williams et al., 1995].

AD is often diagnosed based on an erythema scoring system (such as oSCORAD) [Ben-Gashir and Hay. 2002]. A study examined the effectiveness of the oSCORAD on 18 patients of various levels of skin darkness by five trained clinicians and found the reliability and validity of this classification system to be poor for highly pigmented patients (Type IV-VI). This suggests that the use of these scoring systems can lead to underreported diagnosis in darker skin types and thus severity in cases. This, together with decreased healthcare utilization within black communities means that Black children often do not visit a dermatologist until their symptoms become severe. In the USA, black children are 30% less likely to visit a hospital due to an eczema flareup, meaning they are 6 times more likely to present with severe AD than their white counterparts [Silverberg and Simpson. 2014; Jameson et al., 2022]. This highlights an existing barrier to health care for Black patients with eczema despite their increased likelihood to present severe cases of eczema. Therefore, it is imperative that these disparities in healthcare utilization are addressed to support marginalized groups who also tend to be more at risk of obtaining an exacerbated form of eczema.

The pathogenesis of rare cutaneous skin conditions is understudied in ERS.

Erythrokeratodermia variabilis (EKV) encompasses a rare heterogenous group of skin diseases known as erythrokeratodermi [Karadag et al., 2013; Scott et al., 2012] that presents from infancy and is characterised by erythematous lesions and plaques [Chouk et al., 2022; Neema et al., 2022]. EKV is estimated to occur in 1 in 2,000,000 people [Mzereeuw-Hautier and Tournier. 2015]. EKV lesions are hyperkeratotic (increased thickness of stratum corneum), and typically present on the exterior surface of the buttocks and face as silvery and red pigmented scaly patches but these lesions vary with stress and heat [Papadavid et al., 1998; Neema et al., 2022]. The aetiology and pathogenesis of this skin disease remains unclear, especially in terms of presentation and prevalence across different skin colours with only clinical small studies (<6 people, 3 of European descent and Type I-III skin and 3 of Indian descent and Type-Type III skin) having been performed; no specific therapy is currently available [Vandersteen et al., 1971; Karadag et al., 2013; Neema et al., 2022].

Griscelli Syndrome (GS) is also a rare cutaneous disease (only 160 cases reported worldwide to date with most cases in the Turkish and Mediterranean population) with a prevalence of less than 1 per million [Nejad et al., 2014; Sheela et al., 2004; Perugu et al., 2023; Shah et al., 2016]. GS is characterised by a grey sheen of the hair and hypopigmentation of the skin that is linked to neurological or immunological impairment but critically in most cases leads to death in the first decade of life [Nejad et al., 2014].

The Waardenburg syndrome (WS), estimated to affect 1 in 40,000 people, is also a genetic disease characterised by hypopigmentation but is also symptomized by deafness and broadening of the base of the nose [Read and Newton. 1997]. Although WS is typically identified in Type I-III skin, the highest incidence is reported among Kenyan Africans, with sporadic cases of the syndrome reported in Japanese, Taiwanese, and Middle Eastern populations, typically in >type III skin [Nayak et al., 2003].

Moreover, as these are characterised as rare skin conditions in general that do not typically occur in Type I-III whether they are in fact slightly more presented in >type III skin remains unclear. The incidence is unidentified and thus prevalence among various ethnic groups has not been comprehensively studied. Therefore, the lack of understanding in the aetiology leads to a lack of development in holistic and personalised treatments.

4.1.5 Wound repair and scarring differ across racial groups.

Lack of identification of Pressure ulcers in ERS

Vast discrepancies in healthcare of racially underrepresented groups are an ongoing social and health concern, highlighting the desperate need for improved diagnosis and treatment options for all demographics. Pressure ulcers (PUs) are characterized as localized skin injuries that are driven by pressure or pressure with shear and typically occur in elderly people [NPUAP. 2016]. PUs is depicted via 4 grades, encompassing: Type 1) a superficial type of ulcer (intact skin that is itchy), Type 2) damage to epidermal and dermal layers creating an open wound (skin loss), Type 3) full thickness skin wound where the underlying subcutaneous tissue is affected, and Type 4) deep tissue injury with muscle damage (tissue necrosis) [Bhattacharya and Mishra. 2015]. Pressure injury progression has been widely studied in Type I-III skin but there has been a lack of exploration in dark skin tones [Gunowa et al., 2018]. In 2017 only 11 peer-reviewed papers that discussed PUs in >Type III skin were published (35 articles published on PUs in Type I-III skin) [Gunowa et al., 2017; Borojeny et al., 2020].

People with >Type IV skin are more likely to develop higher stage pressure injuries, with African Americans possessing a 1.7x higher chance of developing PUs than White Americans [Harms et al., 2014]. It has been reported that across 59,740 long term residents (LTRs) of nursing homes, PU prevalence is 18.2% in Type V-VI skin and 13.8% in Type I-III skin [Cai et al., 2012]. Reportedly, among 142 racially diverse nursing home residents, more Black individuals have presented persistent trunk and severe stage 4 pressure injuries compared to other ethnic counterparts [Bliss et al., 2017]. These injuries were characterized as necrotic, unstageable, with little granulation [Bates-Jenson et al., 2021]. However, Black people are 26% less likely than White people to access a nursing home [Akamigbo et al., 2007]. This disparity is attributed to a lack of accurate assessment protocols of PUs in >Type III skin [Gunowa et al., 2017]. Early-stage PUs are indicated by red or purple colouring of the skin, characterised as erythema, however this is difficult to visually see on dark skin types (>Type III) [Zaidi et al., 2022]. >Type III skin PU sites are typically diagnosed by other indicators including warmth, swelling or skin induration. However, these other symptoms are not always assessed for if the phenotypic erythema is not initially visible. The ineffectual skin assessment protocols available for dark skin mean that PUs is not appropriately recognized. This thus enables further damage and disease progression to occur as the ulcers remain untreated thus leading to stage diagnosis and typically poor outcome [Bates-Jenson et al., 2009; Gunowa et al., 2018]].

Keloids are typically presented in Type VI skin.

Keloids are characterised as progressing scars that continue to grow from an original wound [Figure 4.6] [Chiki-Obi et al., 2009]. The formation of pathological scarring i.e., hypertrophic scars or keloids, is a result of adverse skin wound healing [Chike-Obi et al., 2009]. Keloids occur in 5-15% of wounds, equally affecting both genders [Jansen et al., 2021]. Geographically, the incidence of keloids ranges from 0.09% in England to 16% in Zaire [Huang et al., 2020; Kelly. 2004; Kelly. 1988; Louw et al., 2000]. There is a reported association between pathological scar formation and skin colour, as incidences of keloid formation are up to 15x higher in Type VI than in Type I skin [Tan et al., 2019]. Although, keloids are reported in 5-16% of Africans, they are rarely seen in patients with albinism [Breytenbach and Segwapa. 2019], suggesting that melanocytes influence incidence. In fact, a recent study revealed that melanocytes activate the TGF-B signalling pathway in fibroblasts which contributes to keloid formation [Rakar et al., 2020].

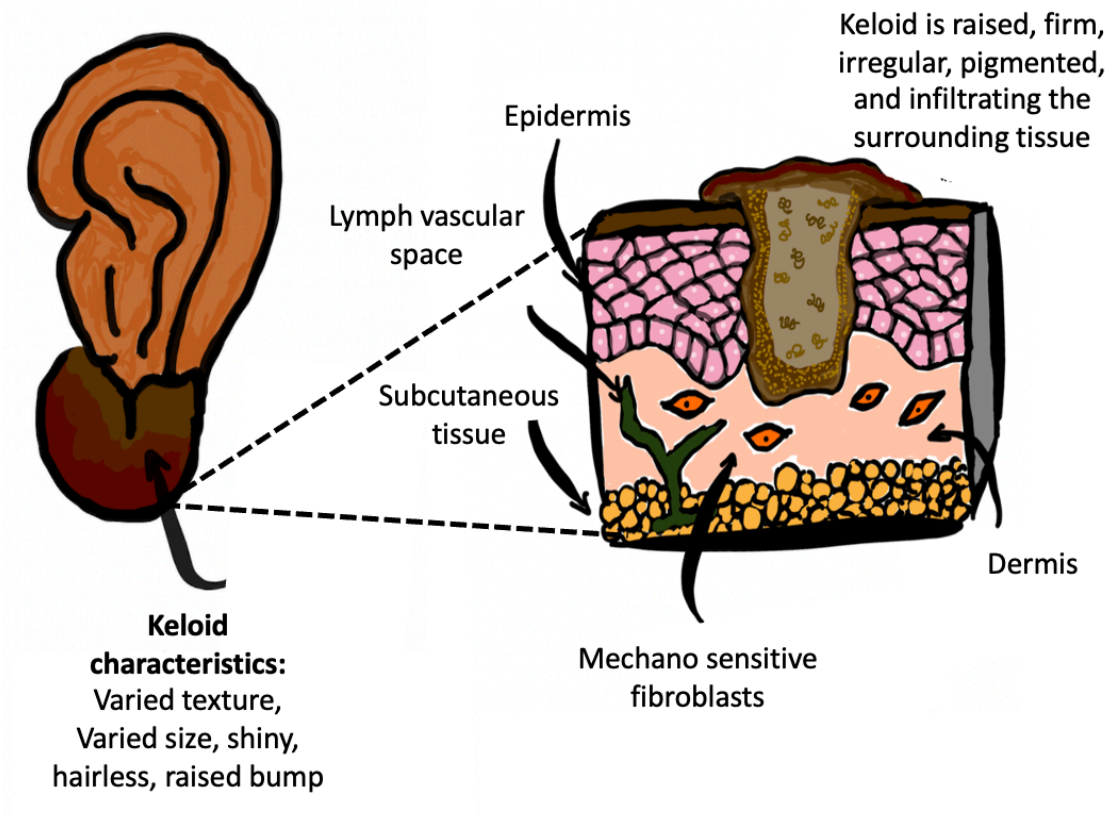


Figure 4.6: Illustration of keloid presentation on skin.

Areas of the body surface possessing high concentrations of melanocytes are more susceptible to keloid formation such as the chest (34% of cases), shoulders (17%), upper limbs (13%), lower limbs (10%) and earlobes (9%), and notably, a significant difference between melanin

concentration and collagen deposition in the skin surrounding keloid tissue has been identified [Chike-Obi et al., 2009; Ramakrishnan et al., 1974; Ketchum et al., 1974; Andrews et al., 2016]. Melanin concentration surrounding keloid tissue also has a positive association with fibrocyte number [Wihastyoko et al., 2021]. When wounds occur, the damaged epidermal tissue can induce the proliferation of melanocytes and thus the formation of melanin [Zhang et al., 2000]. Therefore, keloids will typically present on hyperpigmented areas of the body [Gao et al., 2013] but whether this is causative, or correlative remains unclear. Further observations of keloid samples have revealed that keloids occur in areas exposed to UV light, (up to 69% of cases), meaning in areas with high melanin synthesis, reflecting the double-edged sword role of UV in skin health [Chiarelli et al., 2014].

Skin cancers are most prevalent in Type 1 skin but severe in Type VI skin.

UV radiation is a fundamental environmental factor that largely drives the onset of skin cancer disease. Most skin cancers (occurring 70x more in Type I-III skin than Type V-VI skin) are caused by the repeated exposure to UVA or B [D'Orazio et al., 2013]: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm), in order of descending wavelength [D'Orazio et al., 2013] are the subtypes of UV radiation. Up to 95% of solar UV rays that reach the earth's surface are UVA, with 5% from UVB [van Weelden et al., 1988]. Due to the long wavelengths of UVA and UVB, these rays can both penetrate the human skin but to different degrees. UVA unlike UVB can not only penetrates the uppermost layer of the human skin, the epidermis, but penetrates through to the deeper layer of the human skin, the dermis [Koh et al., 1996]. Typically, UVB is associated with skin burning because of DNA damage, due to possessing higher energy levels than UVA, however, UVA due to its longer wavelength, is commonly associated with skin ageing [Wang et al., 2019]. UV driven skin cancers encompass melanoma, squamous cell carcinoma (SCC), basal cell carcinoma (BCC), as well as rare skin cancers entailing merkel cell carcinoma (MCC), and dermatofibrosarcoma (DFSP).

Melanoma: Late-stage melanoma is prevalent in Type III-Type VI skin

Melanoma is characterised as a tumour established by the malignant transformation of melanocytes [Figure 4.7] [Davis et al., 2019; Heistein et al., 2023]. Up to 70% of cutaneous malignant melanomas are believed to be caused by UVR [Koh et al., 1996; Sample and He. 2018]. Both UVB and UVA can drive the pathogenesis of melanoma [Sample and He. 2018] which was not always known as up until the 1980s before UVA-blocking sunscreen became available [Drissi et al., 2022]. UVA exposure accounts for up to 80% of phenotypic signs of

skin ageing such as dry texture, wrinkling, epidermal thinning leading to reduced number of melanocytes, and scalping [Grant. 2008]. A study published in 2012, using central European subjects found that those with early signs of wrinkling on the neck were more than four times susceptible to melanoma than the general population, indicating the role of persistent sun exposure, hence UVA induced photoaging, can serve a pivotal role in stimulating skin cancer

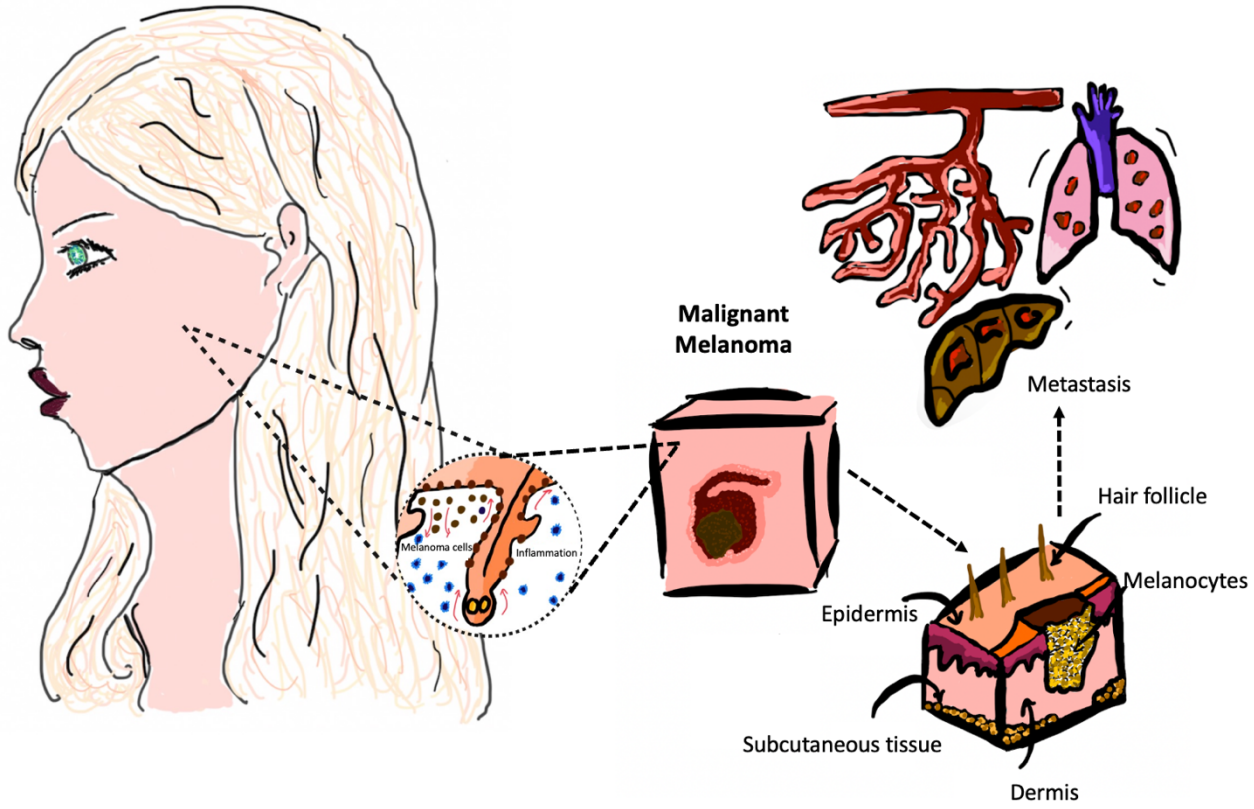


Figure 4.7: Illustration of pathogenesis of melanoma.

Cutaneous melanoma, the most aggressive form of cancer, is typically driven by modified melanin [Cabaço et al., 2022]. Melanocytes for the most part of one's life can absorb UV, and thus scavenge ROS which are produced from radiation. However, with time, because of persistent chronic UV exposure, and due to pheomelanin and eumelanin degradation ROS levels can strikingly rise [Ito et al., 2018; Cabaço et al., 2022]. In turn, ROS can alter melanin and induce DNA damage in cutaneous melanocyte cells, via cyclobutene pyrimidine dimers. The melanin secreted from cutaneous melanocyte cells can immunomodulate the tumor microenvironment, by transforming dermal fibroblasts to cancer associated fibroblasts, driving melanoma metastasis [Cabaço et al., 2022]. This highlights how the protective role of melanocytes is modified with age, to become detrimental because of persistent UV exposure

with time, leading to prevalence of many skin diseases across various racial groups in later life.

Incidence of melanoma is highest in Caucasians, with 91.6% of neoplasms, in the UK, being presented in this ethnic group. The remaining neoplasms are presented in Indian and Black (0.2%) people, Chinese (0.02%) and in other ethnicities (7.3%) [Ascott et al., 2023; Halder and Bridgeman-Shah. 1995; Gloster and Neal. 2006]. The low incidence of skin cancers in darker skinned individuals is a result of photo-protection elicited by eumelanin, which filters twice as much UV as pheomelanin [Montagna.1991]. This can explain why melanoma, a cutaneous malignancy arising in melanocytes is predominant in Caucasians, who possess less eumelanin. Pale skin, e.g. type I, with high pheomelanin levels present increased melanoma risk [Rhodes et al., 1987].

Melanocortin-1 receptor (MC1-R) variants are abundant in 50% of Caucasian populations, with loss of function in MC1-R mutations having been shown to account for pheomelanin-red hair phenotype in humans, an absence of eumelanin and an increased melanoma risk [Schaffer et al., 2001]. Evidence has shown that there is a higher pheomelanin content in melanomas compared to normal melanocytes from the same patient [Pavel et al., 2004]. In fact, albinos that do not produce melanin are more prone to non-melanoma skin cancers than malignant melanoma [Streutker et al., 2000]. The loss of pheomelanin production in MC1R abrogates melanoma susceptibility despite the presence of the inactivating MC1R mutation, highlighting the strong indication of pheomelanin in melanoma pathogenesis and the potential of the pheomelanin synthetic pathway as a carcinogenic independent of UV radiation [Mitra et al., 2012].

Despite the higher incidence of total melanoma in Type I-III skin, the proportion of late-stage cutaneous melanoma (stages II-IV) is higher in Type VI skin accounting for 11.07% in stage IV compared with 4.4% late-stage cases in Type I skin [Dawes et al., 2016]. Melanoma atypically presents in >Type III skin which makes survival in Type VI individuals significantly lower. African Americans have a 1.48-fold higher rate of risk-adjusted, stage-specific mortality from melanoma, compared with European-Americans [Cormier et al., 2006]. The five-year melanoma survival rate is 70% of Type VI skin patients compared to 94% in patients with Type I skin [Gupta et al., 2016]. Notably, lower extremity and acral lentiginous melanomas (ALM) are found to be more prevalent among Black patients compared to White (27.6% to 24.4%).

However, in a recent investigation on ALM, Black patients constituted only 0.58% of the participant pool (251,864 patients) [Carter et al., 2021]. Moreover, although, in South Africa the melanoma incidence in White people is 23.2 and 0.5 in Black people per 100,000 individuals, the South African demographic is predominantly Black (80.2% Black, 8.4% White). meaning annually there are an estimated 238 new cases of melanoma in Black individuals (17%) [Alicea and Rebecca. 2023]. Recently, in South Africa an expanded access program was established to evaluate the efficacy of immune checkpoint inhibitors on the African ALM patient population [Rapoport et al., 2017]. However, of the 108 patients only 2 were Black (1.85%), while 93 were White (86.1%).

The increasing morbidity and mortality rate correlated with skin cancers in non-White skin groups is fueled by limited awareness, latent diagnosis, socioeconomic barriers hindering access to health care and exclusivity in clinical trials [Alicea and Rebecca. 2023; Cormier et al., 2006].

Squamous cell carcinoma: Type III-IV skin is not immune to skin cancer susceptibility.
SCC is a rapidly growing severe tumor derived from the squamous cells of the skin epidermis, developing in sun exposed areas or from a scar or burn [Gordon. 2013]. A common misconception regarding skin cancer is that dark skin types, especially Type VI, possess complete protection against this disease because of their abundant melanin pigmentation, but this is false [Gordon et al., 2016]. While skin cancer is rare in ERS skin, [Agbai et al., 2014; Kailas et al., 2016], SCC is reported to be the most common type of skin cancer presented in African Americans, accounting for up to 30-65% of all skin cancers that can present in dark skinned people (>Type III), although concrete incidence statistics fail to be reported or made accessible. A study performed in 2022 on 540 African Albino cancer patients found 64% prevalence of SCC among the pool, and 31% of BCC [Onyishi et al., 2022].

SCC accounts for 15-25% of all skin cancers in white skin (>Type I), indicating that it is the least presented common skin cancer in light pigmented skin [Gloster and Neal. 2006]. In Type VI skin, SCC presents as rolled edges and brown or black bumps, but as reddish patches in Type I skin [McCall et al., 2002]. Interestingly, sun exposure over a lifetime confers increased risk of SCC in women, however, sun exposure at an early age increases risk of SCC in men which is theorized to be driven by the variable lifestyles exhibited by men and women rather than as a result of genetic differences [Chen et al., 2010]. Notably, case studies on Black

Americans with SCC, identified that most patients had associated predisposing conditions and lesions on non-sun exposed skin [Bang et al., 1987; McCall et al., 2002]. This suggests that holistically the prevalence of SCC in Type V-VI skin may be driven by genetic processes outside of skin pigmentation [van der Pols. 2011].

Basal Cell Carcinoma: is prevalent in sun exposed countries.

Basal Cell Carcinoma (BCC) is a malignant skin neoplasm originating from basal cells of the epidermis [Gupta et al., 2016]. It is the most common cutaneous malignancy in Caucasians Type I-III skin accounting for 65-75% skin cancers, while it accounts for 20-30% of skin cancers in non-White racial groups [Gloster and Neal. 2006]. BCC is often linked to UV light exposure and typically presents on sun-exposed areas, notably the face [Steding-Jessen et al., 2010] with 1 million new cases reported each year [Bradford et al., 2009; Gupta et al., 2016]. BCC presents as translucent skin growth in Type I-III skin and as a 'pearly' brown or black appearance in >Type III skin [Bradford. 2009]. BCC incidence rates per 100,000 of the populations across the last fifty decades include 0.7-1.4 in Asian Indians, 1-2 in African Americans and varies considerably in East Asians from 0.31-6.4 [Gupta et al., 2016; Lukiowak et al., 2020]. The incidence of BCC in White skin varies depending on location, for example in Australia it exceeds 1,000 per 100, 000 of the population, in the USA it is around 450 per 1000, 0000 and in Europe 100 per 1000, 000 [Apalla et al., 2017]. BCC is 1.6x more prevalent than SCC for Asians and 1.69x for white people [Lukiowak et al., 2020].

Incidence rates of BCC have been rising between 3-8% annually since the 1980s in Asian skin [Kim et al., 2009]. The strong association between UV exposure and BCC in dark skin types (>Type III) can explain the higher incidence of this skin malignancy among darker-skinned cohorts living in sunny climates [Chang et al., 2009; Leiter et al., 2014]. This is why mostly Latinos, Chinese, and Japanese Asians are more susceptible to BCC, as they reside in s typically sunny countries. Most BCC cases present in skin after the age of 50, and concerningly, in recent years, the number of skin cancer cases in people >65 years old, has increased rapidly [Sinikumpu et al., 2022; Rubin et al., 2005]. The worldwide surge in skin cancer incidence within the last two decades has reached an 'epidemic' scale, because of long, lifetime sun exposure in a rapidly ageing population in parallel with a globally UV intensifying climate [Donaldson et al., 2011].

Rare Skin cancers: lack of diagnostic understanding in type III-type VI skin types.

Merkel cell carcinoma (MCC) is a rare cancer type with 2,000 cases annually diagnosed in the US, often presenting as a flesh colored or bluish-red nodule on sun-exposed regions such as the face, head or neck [Brady et al., 2022; Siquiera et al.,2023]. MCC is driven by lifetime UV exposure, advancing age, and immunosuppression [Trofymenko et al., 2018; Brady et al., 2022; Paulson et al., 2018]. MCC majorly affects old White males, as highlighted in a study of 3780 patients (all were White) the disease predominantly affected men over the age of 49 years (61.5%) compared to women (38.5%) [Albores-Saavedra et al., 2010]. In fact, MCC incidence exponentially rises with age, from 0.1, to 1.0 to 9.8 (per 100,000 person-years) among age groups 40-44 years, 6-0-64 years and >85 years old [Paulson et al., 2018].

The estimated incidence of MCC in Black and Hispanic patients is 0.1 and 0.3 cases per 100,000 compared to 0.8 in non-Hispanic white people. [Paulson et al., 2018]. MCC is typically characterized by poor outcome. In best cases with early detection and treatment only 75% of people have 5-year survival, but 50% of patients exhibit recurrence within that time [Nayak et al., 2023]. Notably, Type VI skin has been associated with advanced stage disease at the time of initial diagnosis and has been shown to exhibit the lowest survival rates across racial groups which may be driven by later detection [Trofymenko et al., 2018; Freeman et al., 2019]. For example, a previously published case report on a middle-aged black woman with MCC detailed the poor outcome of the patient despite radical management with the authors emphasizing the need for comparative studies to be performed on the prognosis of MCC tumors between Type I-II and Type IV-VI skin types [Kadiri et al., 2017].

Another rare type of skin cancer is dermatofibrosarcoma protuberans (DFSP) which occurs in the connective tissue cells of the dermis then slowly metastasizes to other areas of the skin [Hao et al., 2020]. Incidence is 0.8-4.5 cases per million persons each year worldwide [Brooks and Ramsey. 2023]. Interestingly, DFSP incidence is twice as high in Black patients than Type I skin, and Black patients present 1.7 times higher risk for all cause death from DFSP than White patients [Halder and Bridgeman-Shah. 1995; Criscito et al., 2016; Trofymenko et al., 2017; Kreicher et al., 2016]. This is partially attributed to the increase in tumor size, as black patients are 1.8-fold more likely to exhibit large tumors [Criscito et al., 2016; Trofymenko et al., 2017], with Black men being 1.6x more likely to present than black women [Ogun et al., 2020]. It is unclear whether the severity of this rare disease presented on Black skin is a result

of tumours developing faster than on White skin, or due to significant disparities in diagnosis of this skin condition between skin colour types.

Ultimately, one if not the major critical explanation for the elevated morbidity and mortality rate associated with all skin cancers in Type V-Type VI skin types is due to the lack in understanding of how such malignancies present on ERS, as well as the socioeconomic obstacles limiting access to health care, in turn leading to delayed diagnosis, advanced stages of disease with poor outcome [Gupta et al., 2016].

4.2 Discussion

The primary phenotypic classifier used to separate human beings is based on variance is skin colour. Irrespective of ethnicity and race, human beings range from Type I to Type VI skin colour, exhibiting elevating levels of eumelanin content across the EHSCS [Dadzie et al., 2022]. The colour of one's skin is influenced by several physiological factors including melanin abundance and the quality of melanosomes in skin, the amount of UVA or B exposure to skin, and one's own genetic disposition [Nail et al., 2021]. Remarkably, this difference in skin tone has an immense impact on one's susceptibility to skin disease, diagnosis of skin disease and thus their lifestyle. A critical gap in the dermatology field is the limited understanding in type III-Type VI skin characteristics and health compared to Type I-II, which thus effects the efficient ability to diagnose and treat diseases that present in dark skin types. Here, I have discussed the baseline differences in melanosome structure and eumelanin content between people of ERS, presented the ways in which ERS may age differently, may be affected by UVA or UVB differently, and may exhibit clinical symptoms differently, highlighting nuances in skin health behind variable skin colour.

In this chapter I have highlighted that contrary to the common belief that melanocytes may differ in number between individuals of different pigmentation they are in fact the same number, irrespective of colour. It is the epidermal melanin content, specifically the eumelanin content which varies between people of different skin colour. The level of eumelanin that one possesses within their melanosomes confers the level of defence that the individual will confer against UV damage. UV is largely regarded as the pivotal driver for the evolution of human skin complexions. Determining the exact relationship UV has with melanocytes, whether protective or defective, is crucial in understanding why and how humans contrarily respond to ageing

stimuli as well as the differing susceptibility to skin diseases. Importantly, it is necessary to consider the threshold at which UV exposure becomes detrimental as I have discussed that too little exposure can lead to vitamin D deficiency which is typically prevalent in >Type III skin, and too much UV exposure can give rise to sun burn or skin cancer which is prevalent in <type III skin. I have exhibited the significant discrepancies in skin disease diagnosis in dark skin types, notably in identifying AD in >Type III skin with the exclusivity of the SCORAD system. Moreover, keloids tend to occur in >Type III skin but are commonly identified at late stage. This reinforces the need for further education on treating skin conditions that present on ERS.

In 2021, The American Academy of Dermatology (AAD) announced a 3-year plan to broaden inclusion and diversity within the dermatology field [Diversity in Dermatology. 2021). Similarly, the British Association of Dermatologists (BAD) are exploring issues related to ethnicity in dermatology, within their minority ethnic group subcommittee, by training and educating health care specialists in this area [British Association of Dermatologists. 2020] In addition, UK university medical students are also attempting to understand how skin diseases may present differently in ERS, as medical textbooks often present skin diseases on Type I-III skin only [Salihu et al., 2023]. Many researchers have previously called for comprehensive exploration of skin diseases on skin tones darker than Type II to be performed, especially on skin types within the middle region from Type III-Type IV where there is a stark lack of inclusion in clinical studies [Dodd et al., 2023; Diao et al., 2022; Buster et al., 2012; Narla et al., 2022]. The variation in skin tones and how this can influence skin disease has not been extensively addressed and studied and is only harming those with ERS in the long term. This lack of research in the nuances of skin colour in impacting skin health also continues to make it difficult to understand if the prevalence and progression of some skin conditions is exacerbated in darker skin tones or is ultimately a result of lack of diagnosis and education available to understand the pathology of diseases on darker skin. Moreover, it is highly important that clinical trials become more inclusive to help facilitate the development of therapeutics for a broad range of people.

As we live in a racially diverse world, it is highly important that people of all colours can access as well as receive tailored medical care to prevent latent diagnosis as well as fuel the development of necessary therapies and cures.

Chapter 5

5.1 Discussion

During my PhD I have overarchingly explored skin heterogeneity with focus on how ageing may impact skin regeneration and how body site influences skin thickness. My experimental focus has been centred on using protein therapy to alter skin identity i.e. to rejuvenate or transform skin respectively. In chapter 2, I designed a MN device with a variable needle height array, composed of pH responsive polymeric materials for drug delivery to the epidermal basal layer. My device was successful in locally targeting basal keratinocytes and transforming their phenotype by upregulating KRT9 expression. In chapter 3, I developed a UVA age accelerated ex vivo skin model and investigated the effect of this physiological stimuli on biological age, cellular function and repopulation, and skin wound healing function. I utilised my MN device, established in chapter 2, to deliver an anti-ageing compound to UVA age accelerated skin, specifically to dermal tissue. My delivery of the klotho coated MN to human skin upregulated collagen genes which highlighted skin rejuvenation. Overall, my findings highlight the potential for my device to be utilised for drug delivery to both layers of the human skin, epidermis and dermis.

5.1.1 Chapter 2

MNs are regarded as non-invasive modes of delivery that can penetrate skin tissue but create much smaller pores compared to that of a hypodermic needle which would in turn require less healing time and confer reduced microbial penetration which is further minimized if the MN is designed with polymeric materials [Waghule et al., 2019; Khan et al., 2021]. Moreover, MNs ensure a targeted drug delivery approach and in fact MNs are shown to possess a rapid drug delivery action due to the direct release of the cargo for absorption in systemic circulation compared with topical routes of drug delivery [Bilal et al., 2021]. A common phenomenon of MN penetration of human skin is the “bed of nails” effect. Skin undergoes stretching and bending when pressed against by the MN patch leading to an incomplete insertion of the MNs. The incomplete insertion of the needles into the skin, will result in an indentation or compression mark and from this inefficient drug delivery there is also a wastage of drug biomaterial [Chen et al., 2015; Min et al., 2022]. However, the “bed of nails” effect can be eluded if the MN device is designed with tall needles that can effectively penetrate deep into the skin, with each needle designed with equal spacing at above 150 μm. Moreover, the

constructed MN device should be constructed with mechanically strong yet highly flexible materials therefore polymeric vs metallic materials proves beneficial in this regard [Min et al., 2022].

In chapter 2, I showcased my established MN device. MNs are a highly efficient drug delivery device possessing numerous benefits. MN patches confer very few risks or faults. However, a major obstacle with MN devices is being able to surpass the stratum corneum layer of the skin epidermis, as typically polymeric MN patches cannot overcome the "bed of nails" phenomenon. My work is a step forward as I have successfully shown that my designed needles penetrate the stratum corneum unlike MN device with consistent needle heights that compress the skin. My device is also minimally invasive compared to hypodermic needles which penetrate deeper to the reticular dermal layer. My established device was successful in delivering Wnt5a to the epidermal skin layer and eliciting a biological function on keratinocytes by upregulating plantar KRT9 which is not typically expressed in keratinocyte cells, in nonplantar skin. This shows immense potential in the utilisation of my device in transforming human skin by effectively delivering a compound with the biological function to do so, that would not otherwise be delivered to the epidermal basal layer, either topically (due to the barrier of stratum corneum) or intravenously (hypodermic needles reach the dermis not the epidermis).

5.1.2 Chapter 3

In chapter 3, I discussed the establishment of my physiologically relevant UVA age accelerated *ex vivo* human skin model. I generated this model for the purpose of studying the effect of a highly physiologically relevant environmental stimuli, which is intensifying due to climate change, on human skin, specifically in impacting skin healing. My model was designed to further understand the mechanism behind UVA age acceleration of human skin, and how that contributes to impeded regeneration that is known to occur with chronological ageing.

I optimised all UVA doses for inducing DNA damage at the tissue and cellular level. For keratinocytes I found that 0.15 J/cm^2 induced DNA damage, 2.0 J/cm^2 for fibroblasts, and >1.0 J/cm^2 for human skin. I found that UVA injury immediately impaired wound healing of *ex vivo* skin with no difference in healing if the biopsy punch was administered instantly after UVA or 24 hours after exposure. I identified a significant upregulation in photoaging and biological age-related genes in response to UVA injury in young and old *ex vivo* human skin. Similarly, I observed the epigenetic methylation of age related CpG sites in young and old ex vivo skin,

keratinocytes, and fibroblasts in response to UVA injury. My findings confirm that UVA exposure can delay the healing of young skin to heal to a comparable level presented in elderly skin. Overall, my findings suggest that UVA exposure increases the epigenetic age of *ex vivo* human skin and increases expression of cellular DNA damage in tandem with impaired skin regeneration. My presented work indicates that senescent fibroblasts secrete the cytokine IL12, which signals upstream to keratinocytes to impair repopulation of these epidermal cells to site of injury. Therefore, UVA injury impairs the mesenchymal signalling network between dermal fibroblasts and epidermal keratinocytes due to the long wavelength that penetrates the dense skin dermis unlike UVB.

I have identified from extensive literature review, the anti-ageing compound, klotho, that when administered to skin and cells to assess for improvement in UV age acceleration of the skin, specifically for any effect on UV induced cellular DNA damage expression, and UV impaired wound healing of the skin. The pre-treatment of anti-ageing compound, klotho, to UVA injured *ex vivo* skin was found to significantly reduce the biological age of skin and cells which confirms that UVA exposure drives age acceleration. Klotho not only enhanced the expression of the nuclear membrane marker LaminB1 in senescent skin tissue and cells, but also prevented wound healing delay. Notably, klotho pre-treatment to skin and keratinocytes enhanced wound healing after 7 days and prevented UV impaired keratinocyte repopulation to injury site, resulting in % cell covered area levels comparable to the control. The mechanism behind this improvement is believed to involve the p53 pathway as klotho blocks p53 expression [Machado de Oliviera. 2006; Takegahara et al., 2021]. The activation of the tumor suppressor p53 in response to DNA damage (which can be attributed to UV exposure) leads to cell growth arrest to allow for DNA repair but can when overstimulated direct cellular DNA damage and disrupt keratinocyte repopulation [Mijit et al., 2020; Freije et al., 2014]. As the klotho protein can block p53 expression this will thus protect keratinocytes from UV induced DNA damage, thus enhanced biological age, senescent induction and notably protect against the disruption of keratinocyte repopulation to the injury site.

I translated this biological model and discovery to a preventative therapeutic device that is stimuli-responsive to ensure controlled release of the drug of interest to dermal portions of human skin. The device I established as shown in this piece of work is multi-purpose, not solely for the delivery of an anti-ageing compound but for the delivery of skin transforming proteins and peptides, irrespective of charge or size. Moreover, due to the variable needle height of the

array, not only can the MN deliver locally to the epidermis (as presented in chapter 2) but also to the dermal tissue indicated by selectively switching on of collagen dermal genes (in UVA treated skin) with the klotho coated MN device that topical klotho treatment on skin could not achieve.

Fundamentally, in chapter 3 my purpose was to assess the effects of an anti-ageing compound on human skin. It is well received in published studies that chronological ageing impairs wound healing, which is driven by a myriad of factors. I specifically chose to establish a UVA age accelerated model to emulate an existing relevant stimulus that all humans irrespective of location, skin colour, and age are habitually exposed to. I chose to delve into the potential mechanism behind UVA impeded skin healing, focusing on biological age acceleration via epigenetic changes on age related CpG sites, and DNA damage marker expression. With my established model I then evaluated the potential of klotho protein therapy as a preventative to UVA induced damage on skin. My chosen therapeutic has shown great promise in ameliorating the detrimental effects of UVA on skin and on primary epidermal and dermal cells. My work may contribute to the biology of ageing, photobiology, and tissue regeneration fields in showcasing the new roles of the klotho protein in not only improving UVA age acceleration of skin tissue and primary skin cells but in also protecting cells from DNA damage induced by UVA, and importantly in preventing UVA impeded skin healing.

5.1.3 Chapter 4

In chapter 4 I discussed the impact that skin pigmentation, specifically melanin expression which varies from person to person, can have on skin health. I focused on how depending on eumelanin levels in one's body, the susceptibility to UVA/B driven damage may vary, and how biological ageing will vary, and skin disease susceptibility will also fluctuate. My purpose for writing this review of the literature was to fundamentally highlight the lack of inclusion in the dermatology field of the study of dark skin types which effects diagnosis of skin disease and is heavily based on limited understanding of the immense impact eumelanin levels (irrespective of race and ethnicity) will have on skin disease risk, and how skin disease symptoms present.
The skin work presented in both chapters 2 and 3 were all type I-III on the FST scale, as dark skin types is typically difficult to access for study and thus can be a variable that can be difficult to account. Therefore, although I did not experimentally evaluate how UVA age acceleration of skin may fluctuate depending on skin colour, I wanted to explore the impact it could have

via literature view analysis and bring attention to a concerning issue for the clinical health and dermatology sector.

My review of the literature on the critical role of melanocyte cells in giving rise to variable skin colours and how that in turn impacts skin disease provided a holistic overview of this field. The purpose of accounting for the variation of skin colour in skin disease susceptibility, especially UV driven skin disease susceptibility and in wound healing was to highlight the significance and impact that skin colour has on skin disease that I was not able to experimentally account for in my model in chapter 3. Moreover, a crucial discrepancy in many health care systems, notably in the UK and USA is the latent diagnosis of skin disease on dark skin tones (>type III). Therefore, my overview of the literature outlining the prevalence of skin cancers in type I compared with type VI skin tones showcases the varied protective response individuals will have to UV driven disease because of skin colour. Moreover, my review of the literature gives insight into the varied efficiency in vitamin D synthesis between type I and type VI skin colour.

5.2 Future work

5.2.1 Chapter 2

In terms of skin transformation, in this body of work I have utilised Wnt5a as a proof-of-concept delivery drug for my designed and constructed MN device. To expand on my work, I would utilise the MN patch as a personalised therapeutic that could potentially transform nonplantar tissue to plantar tissue. Palmoplantar skin has enhanced tolerance to mechanical load compared to non-plantar tissue [Boyle et al., 2019]. The thick stratum corneum of plantar tissue provides defence against stress-based injuries i.e. skin tears and blisters. The dense epidermal and dermal composition of the plantar tissue exerts defence against deformation-based injuries i.e. pressure ulcers. Limb amputation is characterised by the partial or complete removal of the upper or lower limbs [Choo et al., 2022]. This can lead to physical trauma, notably predisposition to skin injury due to the nonplantar tissue of the stump site. It has been shown that KRT9 is the most uniquely enriched transcript in plantar skin, and that its expression is selectively stimulated by plantar fibroblasts [Kim et al., 2016]. However, interestingly, gain of function and loss of function experiments revealed that KRT9 expression is also induced through overlapping paracrine or autocrine canonical Wnt-β-catenin signalling. It has been reported that WNT5α induces the expression of plantar KRT9 [Kim et al., 2016; Yamaguchi et

al., 2009; Yamaguchi et al., 1999; Rinn et al., 2008]. However, it isnot known if Wnt5a can change the mechanical properties to that of nonplantar skin which is what I would next evaluate.

Translating Wnt5a coated MNs as a personalised form of therapy for limb patients.

To uncover the possibilities of this I would progress from my work using Wnt5a *in vitro* and *ex vivo,* to identify the effect this protein has on the young modulus of human skin via its upregulation of KRT9. This would allow me to identify if delivery of Wnt5a to human skin via the MN device is sufficient in transforming human skin from non-plantar to plantar tissue and thus act as a viable therapeutic option for amputee patients in protecting stump site tissue from skin injury. If this experiment proved successful, I would then progress in optimising the dosage of Wnt5a and optimum time delivery of the therapeutic to human skin whether it be several times a day or once a day, for example and for what amount of time would the therapeutic need to be consecutively administered to the stump site to transform skin i.e. a few weeks or a few months or a few years.

Delivering peptides to skin via pH responsive microneedle

The microneedle I designed and presented in this thesis is shown to be multi-purpose for the delivery of several proteins of variable charge and size. However, to progress from these results I would next assess the suitability of this device in delivering smaller compounds such as peptides and viruses to human skin.

Peptides are a specific class of pharmaceutical agents formed of a series of amino acids, typically with a molecular weight of 500-5000 Da [Wang et al., 2022]. These therapeutic agents have illustrated several benefits over proteins or antibodies, in that they are small, confer low production costs, and possess the ability to penetrate cell membranes [Fosgerau et al., 2014; Wang et al., 2022]. In addition, therapeutic peptides exhibit high functional activity, specificity and affinity, present minimal drug-drug interaction as well biological and chemical diversity [Ali et al., 2013; Marqus et al., 2017]. A key advantage of delivering peptides for treatment is that they do not amass in specific organs such as the kidney or liver, thereby reducing risk of toxic side effects [Ali et al., 2013]. Lastly, peptides can be easily and swiftly synthesized as well as straightforward to modify [Wang et al., 2022].

Foxy-5 is a reported Wnt5a-mimicking peptide that has been found to reduce the metastatic spread of Wnt5a low prostate cancer cells in an orthotopic mouse model [Canesin et al., 2017;

Säfholm et al., 2008; Kelsey. 2017., Rekair et al., 2019]. Moreover, Foxy-5 comparable to Wnt5a also elevates the expression of a tumor suppressor, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) which is downregulated in colorectal cancer (CRC). However, whether Foxy-5 also enhances the expression of KRT9 like Wnt5a has not previously been studied. Currently, in the lab, the team are exploring this hypothesis that Foxy-5 like Wnt5a upregulates KRT9 expression *in vitro* and *ex vivo* and can be utilised as a skin transforming therapeutic for non-plantar to plantar tissue for amputee patients. If this hypothesis is experimentally proven successful, it could be delivered to the stump site using my engineered MN device.

The oral bioavailability of peptides is restricted by degradation in the GI tract and the inability to pass the epithelial barrier due to possessing a size of >500 Da. Moreover, many proteins and peptides tend to not only have high MWs, but low lipophilicity, as well as possess charged functional groups that disrupt their absorption into the skin [Aungust et al., 1996]. Therefore, leading to low bioavailability of majority of orally administered peptides (<2%) and short half-lives (<30 minutes) [Borchardt et al., 1997]. The intravenous of subcutaneous delivery of peptides and proteins will overcome the obstacle of absorption but systemic proteases, non-covalent complexation with blood products rapid metabolism, opsonization, conformational changes, dissociation of subunit proteins, and the destruction of labile side groups all limit the bioavailability of peptide and protein therapeutics [Craik et al., 2013; Torchilin et al., 2009]. Microneedles have largely been used for the delivery of small, hydrophobic drugs but not extensively on peptides [Yu et al., 2015]. Therefore, the MN device I engineered would present as a multipurpose device for not only the delivery of proteins but even smaller compounds, peptides.

Toxicity effects of microneedles, i.e. skin irritation or allergy.

MN patches are considered safe therapeutic devices with no associated serious side effects [Jung et al., 2021; Zhang et al., 2021]. However, allergic reactions, skin inflammation or skin irritation is a possible occurrence from MN drug delivery if needles were broken inside the skin [Jiang et al., 2016; Ita. 2018; Valhov et al., 2018; Ganeson et al., 2023]. My engineered MN device was purposely constructed with biocompatible polymeric materials to elude the possibility of skin irritation if needles were to break inside the skin.

However, in my mechanical testing and administration of the MN patch to *ex vivo* skin I found that needles do not break upon application (with typical young modulus of <5 MPa) and if they happened to, I used biocompatible materials that should not cause irritation to the skin [Pailler-Mattei et al., 2008; Agache et al., 1980; Manschot et al., 1986; Pawlaczyk et al., 2013]. It has been reported however, that allergy or skin inflammation can occur from the build-up of MN delivered drugs in the skin [Waghule et al., 2019; Bellefroid et al., 2019; Seetharam et al., 2020]. Moreover, the skin can present slight swelling around the application site as the stratum corneum is disrupted with a foreign material being inserted into the skin [Ganeson et al., 2023]. Optimising the drug coating of each needle across the MN array and designing the device to thus have spatiotemporal controlled release into the skin would elude the issue of accumulation of the drug which could lead to toxicity or skin irritation. Moreover, refinement of the drug coating per needle would also prevent biowaste of the compounds.

Spatiotemporal microneedles.

I would investigate the spatiotemporal efficacy of delivering several different drugs using one microneedle array, to different layers of the human skin, epidermis, and dermis. Spatiotemporal drug delivery ensures controlled and complete release of the drug of interest to deep specific regions of the skin. Previously, a microneedle system has been utilised for the multi-functional delivery of nanoparticles to melanoma sites *in vivo* [Zhao et al., 2021]. The authors successfully showed that their multifunctional nanoparticle-integrated dissolving microneedles (DMNs) could co-load multi-modal drugs, improve specific uptake and release compounds spatiotemporally. The MNs enhanced drug accumulation in the tumour site, which proved to elicit high therapeutic efficacy and limited side effects. This DMN system emerged as a minimally invasive, specialised, and efficient system for cancer therapy. An additional previous study presented strongly similar findings of a DMN system that successfully conferred uniform distribution of compounds of interest to the tumour site with stable zone accumulation [Qin et al., 2020]. Spatiotemporal MN systems have more recently been designed and utilised for delivery of melatonin to deep regions of the cortical region of the brain *in vivo* [Wang et al., 2024]. Notably, a spatiotemporal MN patches integrated with nanoparticles have been administered in vivo for wound management [Shan et al., 2023].

To enhance my designed and engineered MN device for personalised epidermal and dermal drug delivery I would fine tune coating of compounds of interest to the individual needles across the array. To achieve this, I would use a nano plotter device to coat each individual

needle at the nanolitre [Summers et al., 2022]. This would ensure reduced biowaste compared to dip coating the entire MN array in a reservoir of material. Moreover, using such an instrument would give capacity to coat each needle with a different compound. Therefore, this refined approach give capacity to have the shorter 500 μm needles coated with a drug localised to the epidermis and to coat the taller 700 μm needles with a drug localised to the subdermal region. This would allow for example for Wnt5a to be delivered to the epidermis to transform skin from non-plantar to plantar via upregulation of KRT9 expression but also the simultaneous delivery of other compounds to the dermis for rejuvenation i.e klotho. This device could be useful for amputee patients in transforming and preventing age acceleration of skin. I would finally scale up the microneedle patch from a proof-of-concept model to a sheet that could be utilised as a wearable face mask for skin rejuvenation or patch for amputee patients for skin transformation [Pruettijarai et al., 2022].

5.2.2 Chapter 3

Other indicators of ageing i.e. telomere shortening, DNA damage, metabolism

The hallmarks of ageing include several factors such as genomic instability, telomere shortening, epigenetic changes, dysregulated nutrient-sensing, loss of proteolysis cellular senescence, mitochondrial dysfunction, stem cell exhaustion, and modified intercellular signalling [López-Otín et al., 2013; Dog et al., 2019]. To expand on the findings presented in this thesis, it would be beneficial to study other ageing factors other than cellular senescence and epigenetic changes, as all are indicators of accelerated ageing.

Telomeres are specialised DNA-protein structures of human chromosomes. The length of telomeres is characterised as an accurate measurement of the replicative ability of cells. The fundamental function of telomeres is to protect the chromosomes from degradation rearrangements, end-to-end fusions, and chromosome loss [Siderakis et al., 2007]. At each cellular division, telomere attrition occurs but telomerase, an enzyme complex, maintains the telomere length by protecting the chromosome ends from exposure to the DNA damage response [Bernal et al., 2018]. Telomere progressively shortens with the replication of nuclear DNA during mitosis, as a result of oxidative stress or with senescence and ageing [Sanders et al., 2013]. By exploring all these factors, it will provide a holistic representation of age-acceleration at the biological level in tissue and cells in response to UVA exposure.

Assessing the systemic effects of UV beyond the human skin.

UV and the Brain

To progress from the findings presented in this thesis, it would be interesting to explore the systemic effects of UV damage on other parts of the body, beyond the human skin. Limited research has been performed on the potential role of UV in neurodegeneration via DNA damage, however theoretical hypothesis on this area have been published. A recent paper discovered that UVA exposure on the cortical surface of rodents can induce a focal brain lesion [Nakata et al., 2021]. Notably, a single dose of UVA exposure was sufficient to drive neurodegeneration within 24 hours and after 5 days all neurons had been destroyed [Nakata et al., 2021]. This study orchestrated that UVA can effectively penetrate through the reticular dermis of the scalp tissue but whether it can directly penetrate the skull is not completely understood and is an ongoing area of research and debate. One study has highlighted that small amount, around 1000 less of UVA that reaches the reticular dermis would surpass the skull and as the brain is highly sensitive to radiation exposure the small amounts of UVA could have adverse effects on brain function and health [Gonzalez-Lima and Barrett. 2014; Nakata et al., 2021]. The mechanism behind the UV-induced neuronal cell death is also poorly understood and thus is an interesting area to explore considering neurodegenerative cases are significantly rising across the world [Schepdendom and D'haeseleer. 2023; Feigin et al., 2019] and so perhaps UVR may contribute to this. In addition, a more recent study on the aetiology of Alzheimer's disease (AD) explored the potential connection between dysfunctional mitochondrial DNA (driven by oxidation of ROS which UVR exposure generates) and AD [Draganova-Filipova et al., 2022; Welch and Tsai. 2022]. The study highlighted that UV exposure could potentially contribute to AD pathogenesis through ROS generation or DNA damage induction however more substantial research would have to be performed to understand how significant a role UV plays in brain ageing, and neurodegeneration.

Interestingly, the klotho protein has been found to significantly protect dopaminergic neurons against oxidative damage by partly regulating the p38 MAPK pathway [Brobey et al., 2015]. My findings have added to the photobiology and anti-ageing field with new functions of klotho in terms of eliciting protective effects against senescence and impaired wound healing as a result of UVA exposure. Therefore, as klotho has presented defence against UVA, and has presented defence against oxidative neuronal damages perhaps if UVA exposure facilitates

brain ageing which requires further study, whether klotho pre-treatment could act as a preventative to UV driven neuronal damage would be fascinating to explore.

Epigenetic ageing and Skin cancer

It is widely acknowledged that UVR induces DNA damage which results in specific mutations which can lead to the emergence of skin cancer often after long chronic exposure of UVR [Pfeifer. 2020]. Epigenetic age acceleration is associated with higher cancer and notably cardiovascular disease (CVD) mortality in Cox models using Horvath's predictor [Perna et al., 2016]. In fact, the Horvath clock has revealed that cancers (15 cancer types i.e. breast and cervical cancer) can induce an epigenetic age approximately 36 years older than healthy tissue in the same person [Yu et al., 2020; Yang et al., 2016]. However, it is not completely understood whether cancer is the driving force for enhanced epigenetic ageing or if epigenetic ageing is the root stimulant for cancer risk. One could theorize that as DNA methylation has been characterised as an age predictor of all-cause and cause-specific mortality and a holistic marker of healthy ageing, it would then be interesting to explore the effect if UVA age acceleration of skin and if it also predisposes the skin to increased cancer risk.

UV and Immunosenescence

Immunosenescence is the process of immune deterioration that is a result of ageing, and entails the remodelling of lymphoid organs, and disruptions in immune functions of the elderly. This is highly associated with induction of infectious diseases, autoimmune diseases, as well as malignant tumours [Lian et al., 2020]. It is known that exposure to UVR modifies the functional activity and distribution of cells required for triggering immune response in the skin [Alshishtawy et al., 2011]. However, UVR can also directly modify the conventional network of the immune system which can have beneficial and deleterious impact [Patra et al., 2016]. UVR is found to trigger the production and secretion of anti-microbial peptides, which influence the innate immune system and essentially blocks the adaptive cellular immune response. UVR induced immune suppression is mediated via T cells notably regulatory T cells [Elmets et al., 1983; Schwarz. 2008; Schweintzger et al., 2015; Schweintzger et al., 2016]. It has been theorised that since UVR suppresses the immune system in response to infectious diseases that UVR exposure could enhance susceptibility to microbial infections and could amplify infectious disease but the clinical evidence for increased infections following UVR exposure remains low and requires further study.

With ageing, the innate and highly specific adaptive immune system weakens. Notably, the phagocytic function declines as macrophage precursors are decreased and neutrophil dysfunction occurs [Ventura et al., 2017]. Moreover, the number of senescence CD8+ T cells that exhibit NK like activity increases, but naïve T cells produce less IL-2 [Abbas et al., 2021]. With the observed immunological changes in the elderly, there is reduced resistance to infections, increased presence of neoplasia, and enhanced secretion of autoantibodies associated with autoimmune disease occurrence [Aw et al., 2007].

A study published in 2021 discovered that needle injury in elderly skin causes an increased recruitment of cutaneous senescent fibroblasts expressing the potent chemokine for monocytes and immune cells, CCL2, which in turn led to the recruitment of inflammatory monocytes [Chambers et al., 2021]. The infiltrating monocytes secreted prostaglandin E2, which blocked the stimulation and proliferation of resident memory T cells. The findings presented in this thesis have shown UVA exposure leads to an increase in senescent fibroblasts but whether this in turn influences monocytes and immune cells would be interesting to explore to understand the holistic impact UVA has on skin cells and important the impact this stimulus has on cell signalling. The paper also states that pre-treating elderly participants with a p38 mitogen-activated protein kinase inhibitor *in vivo* reduced CCL2 expression and blocked monocyte recruitment and the release of prostaglandin E2 [Chambers et al., 2021]. Klotho protein has been found to protect human monocytes from immunosenescence (induced by long-term *Lipopolysaccharide* (LPS) bacteria stimulation), therefore not only indicating the protective role klotho has against impaired immune activity but also in protecting against pathogenic exposure [Mytych et al., 2018]. Therefore, I would like to expand on these findings by exploring the broader myriad of benefits of klotho on age deterioration of cells beyond the keratinocytes and fibroblasts and beyond the skin.

UV and the Microbiome

UVR exposure has been shown to injure human skin and the skin microbiome which encompasses bacteria and viruses. UVR alters the skin microbiome by directly and indirectly damaging DNA [Nelson et al., 2018]. The primary mechanism behind UV driven disruption of the microbiome is through the generation of ROS. Recently, the microbiome has been found to elicit a layer of protection against UVR i.e. cyanobacteria produce the pigment *scytonemin*,

a photo-stable UV shield in prokaryotes [Sinha et al., 2008; Proteau et al., 1993]. Notably, bacterial bioactive pigments have been used in cosmetic products to protect against oxidants and sun exposure [Choksi et al., 2020]. A recent publication with a study population of ten lifeguards exposed for 8 hours daily to the sun discovered that the skin microbiome bacteria is enriched following the summer sun exposure [Harel et al., 2023]. Two bacterial families, *sphingomonas* and *Enterobacteriaceae*, were found to potentially possess the capacity to protect against UVR as they generated photoprotective compounds. The *sphingomonas* strain was also found to be highly resistant to UVB and notably decreased ROS levels in human keratinocytes. Additionally, UVB has been shown to stimulate a local pro-inflammatory environment in the skin in presence of microbes, but notably stimulates an immunosuppressive environment in the skin in the absence of the microbiomes, highlighting the significance of the microbiome in the skin against UVR damage [Nakatsuji et al., 2018; Patra et al., 2019].

UVB exposure to the skin has been shown to modulate the gut microbiome, with increased biodiversity of *Firmicutes* and *Proteobacteria*, but reduced *Bacteroidetes* highlighting the skin-gut axis that would influence human health [Conteville and Vicente. 2020; Bosman et al., 2019]. Notably UVA has been shown to result in different observable effects on the skin microbiome to UVB [Burns et al., 2019]. In this study, six male participants were exposed to doses of UVA (22-47 J/cm^2) or UVB (100-350 mJ/cm^2) to assess alterations in the cutaneous microbiome. The isolated DNA from the cutaneous samples results revealed variation between UVA and UVB on their respective effects on the skin microbiome. Namely, *Pseudomonadaceae* decreased instantly following UVR exposure but declined to a greater extent following UVA exposure compared with UVB. *Pseudomonadaceae* is commonly expressed on human skin but its reduction due to UVR exposure (specifically the *Pseudonoma* genera due to its antimicrobial properties), especially in response to UVA, was theorised to be due to its intolerance to oxidative damage as it is strain dependent on aerobic respiration. Additionally. UVR exposure enhanced cyanobacteria which has previously been shown to synthesise UV-absorbing molecules including *scytonemin*, to defence against solar radiation [Cockell et al., 1999; Kumari et al., 2021; Gupta et al., 2023].

To expand on the findings presented in this study I would account for the role of the microbiome in eliciting photoprotective function against UV within the skin. I would assess

more extensively for the differences in effect between UVA and UVB on the skin microbiome as perhaps photoaging compared with skin burn variably modifies the microbiome. I would also specifically try to explore the mechanism behind UV disruption or enhancement of specific strains existing on the skin microbiome i.e. if there is correlative changes in ROS levels in isolated DNA from UV exposed skin samples as particular bacterial strains are altered.

Comparing the effects of UVA and UVB on *ex vivo* skin.

Multiple studies have explored the effect of UVB damage on human skin and cells and my work provides a contribution to the photobiology field as I assess the effect of UVA on cells and tissues. However, it would be interesting to compare photoaging damage from UVB and UVA damage as both differ in potency and wavelength. UVB is considered more potent than UVA, but UVA has a longer wavelength that penetrates the dermal as well as the epidermal layer whereas UVB penetrates the epidermal layer of human skin only.

UVA has been reported to significantly enhanced oxidative damage in DNA, the destruction of the collagen fibre within the dermis, and the apoptosis of fibroblasts, leading to skin inflammation [Wang et al., 2019]. Whereas UVB exposure has been shown to enhance the carbonylation of several proteins and to induce proliferation of keratinocytes. The mechanisms behind UVA and UVB exposure has been shown to differ. UVA culminates in cellular death and inflammation regulated by caspase-3 and activator protein 1/nuclear factor kappa-light-chain-enhancer of activated B cells via the NF-κB pathway. Whereas UVB primarily enhances skin carcinogenesis risk entailing the oncogenes p53 and c-Myc [Wang et al., 2019]. To further the findings presented in this piece of work I would repeat all *ex vivo* and *in vitro* experiments but substitute UVA with UVB and compare the detrimental effects of both namely comparing UVA induced photoaging compared with UVB driven skin burning. I would additionally investigate whether the α-klotho protein elicits protective defence against UVB induced damage as it does to UVA.

Examining the role of endothelial cells in wound healing.

Impaired wound healing is a result of a several disrupted pathophysiological processes, including not only impaired keratinocyte proliferation, but because of microbial invasion, reduced vascularization, oxygenation supply, epithelial breakdown, enhanced oxidative stress and disrupted immune activity. Endothelial cells serve a fundamental role in inflammation

regulation at the early stages of wound healing. In future work I would account for the role of endothelial cells in my UVA age accelerated ex vivo skin model to assess how UVA injury may influence these cells also.

As cell-cell contact detaches, the expression of the adhesion molecules allows endothelial cells to facilitate the movement of circulating inflammatory cells into the tissue ay the injury site [Johnson and Wilgus. 2014]. Endothelial cells are critical in forming newly formed tissue, as all tissues depend on a blood supply which thus depend on endothelial cells [Glat et al., 1997; Diegelmann et al., 2004; Velnar et 2009]. Angiogenesis is an important stage of the wound healing process. It is typically this stage that is impaired during many chronic non-healing wounds. The promotion of angiogenesis is a promising stage for the healing of chronic wounds [Shaabani et al., 2022]. Endothelial cells undergo a series of morphological changes regulated by biochemicals during capillary growth [Velnar and Gradisnik. 2018]. Chemotactic agents act on cell surface receptors to regulate the endothelial cell movement during the morphological process [Wilkinson et al., 1982; Augustin et al., 1992].

Chemotactic activity ensures migration of the endothelial cells which is critical for vascular remodelling, integral to angiogenesis. Haptotaxis (migration in response to the gradient of immobilised ligands) and mechanotaxis (migration mediated by mechanical factors) follow chemotaxis. Endothelial cells motility encompasses 1) protrusion at the cellular front, adhesion, 2) attaching the actin cytoskeleton to the substratum, and lastly 3) traction which drives the trailing cytoplasm forward. There are several factors that mediate the vessel wall repair and neovascularization at the injury site, including endothelial cell growth factor (ECGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-α, fibrin, angiopoietin 1, heparin, and lipid growth factors [Terranova et al., 1985]. VEGF is a potent pro-angiogenic growth factor (VEGF) that is present at the wound site and significant influence wound healing. VEGF is restricted to endothelial cells but also expressed by keratinocytes and macrophages. The extra-angiogenic effects of VEGF on wound repair is not well known but it is theorised to play an important role [Johnson and Wilgus. 2014]. It is necessary to consider how endothelial cells, like fibroblast cells, interact with keratinocytes to facilitate wound healing, but importantly how this network is perturbed in response to UVA exposure on skin, and how that also contributes to delayed skin healing.

Notably, one study explored the effect of autocrine and paracrine functions of vascular endothelial cells on wound healing [Lu et al., 2021]. The authors found that human umbilical vascular endothelial cell-conditioned growth medium (HUVEC-CM) stimulates the proliferation and migration of epidermal stem cells, skin fibroblasts, bone marrow mesenchymal stem cells and the HUVEC cells. In addition, HUVEC-CM was found to induce angiogenesis in mouse skin wounds and granulation tissue formation and can accelerate the wound surface epithelisation and collagen synthesis, thus stimulating wound healing. These findings suggested that cytokines secreted by the vascular endothelial cells can activate wound healing therefore to progress with my work I would account for the important role endothelial cells serve in wound healing and similarly to how I assessed the effect of UVA fibroblast conditioned media on keratinocyte migration I would do the same with the effect of UVA exposed endothelial cells on keratinocytes. I would evaluate for any secreted factors from UVA conditioned endothelial cells that may also be influencing keratinocyte functionality in parallel with IL12 secreted from UVA exposed fibroblast cells. Notably, klotho has been shown to elicit anti-senescence effects on endothelial cells [Ikushima et al., 2006]. Therefore, in the future I would investigate the effect of klotho on UVA exposed endothelial cells and whether it could preserve or protect the functionality or phenotype of these cells against UVA, as the protein does with keratinocytes and fibroblasts.

Keratinocytes are also one of the primary sources of VEGF during wound healing as cells that form the epidermal barrier and thus serve to reseal the barrier upon skin damage. It is believed that VEGF secreted by epidermal keratinocytes function in a paracrine fashion, thus stimulating endothelial cells in blood vessels within the dermis. However, functional VEGFRs identified on keratinocytes, indicates autocrine VEGF signalling in keratinocytes [Wilgus et al., 2005; Lichtenberger et al., 2010; Tiese et al., 2012; Johnson and Wilgus. 2014]. I would evaluate whether VEGF may elicit any protective function against UVA impeded wound healing.

Klotho and UV prevention

In the future, I would assess the preventative effectiveness of sun protection factor (SPF) against UV induced photoaging in comparison to the klotho protein. Most SPF ratings refer to UVB only. SPF 15 sunscreen blocks 93% of UVB radiation, SPF 30 blocks 97%, SPF 50 blocks 98%, and SPF 100 blocks up to 99% of rays from reaching the skin [Sander et al., 2020]. Emerging evidence has indicated that some chemical sunscreen ingredients are systemically absorbed, however, the clinical significance behind this remains unclear. In addition, ultraviolet

filters present within chemical sunscreens may be harmful to the environment. Sunscreens with existing chemical filters, include oxybenzone, avobenzone, octocrylene and ecamsule, are aromatic compounds that absorb high-intensity UV, leading to the excitation of high-energy states. Once these molecules return to the ground states, this triggers the conversion of absorbed energy into lower-energy wavelengths, i.e. infrared radiation [Sander et al., 2020]. Physical sunscreen filters, including titanium dioxide and zinc oxide, reflect or refract UV away from the skin. Notably, experimental studies have shown that when particles sizes are very small, for example, micronized sunscreens, the mechanism of action is like that of chemical filters [Geoffrey et al., 2019; Boyd et al., 1995]. These potential toxic effects demand for further research on the efficacy and side effects of commercially available sunscreens. In recent years there has been a paradigm shift in the cosmetic field toward the use of anti-ageing compounds in addition to or instead of sunscreens as UVA results in photoaging [Shanbhag et al., 2019].

Furthermore, to progress from the promising results of the α-klotho protein, as a preventative to UV age acceleration and impaired wound healing in human skin, and to UVA driven senescence induction in keratinocytes and fibroblasts I would study the effect of this compound in protecting UV age acceleration and impaired wound healing in vivo. 2D in vitro models face issues of immense variability and lack of control in standardisation which I have tried to overcome by performing at least 3 technical repeats and 3 biological replicates with cells, and with 3D ex vivo models I have performed all experiments with 6 technical repeats per condition with N=3 biological replicates. To expand on my findings, I would explore the effect of α-klotho in *in vivo* mice models. Previously, the defect in the klotho gene has shown to emulate premature ageing [Kuro-o et al., 1997]. Moreover, the overexpression of klotho has been shown to extend the lifespan of mice [Kurosu et al., 2008]. Moreover, klotho deficient mice (kl/kl) mice have shown delayed wound healing, presenting a phenotype comparable to aged humans [Yamauchi et al., 2016].

Notably, utilising *in vivo* models would allow me to assess the safety, toxicity, and efficacy of α-klotho protein in living organism in real-time [Xu et al., 2021]. In addition, an *in vivo* model would allow the for me to mimic specific diseases in animals with high precision i.e. chronic wounds [Brackman and Coenye. 2016], biological age accelerated mice [Yang et al., 2023]. It is widely known that the human skin is homogenous in nature. With ageing, skin becomes thinner, less tense, less flexible and notably less stiff. In fact, the biomechanical skin parameters

are reported to change with time. Moreover, it has been reported that the skin tension assessed in vivo uniaxial load and elasticity modulus are elevated in children than in elderly adults [Pawlaczyk et al., 2012]. I would explore whether a single dose of UVA can significantly disrupt the mechanical properties of *ex vivo* skin, by enhancing the young modulus, and reducing the elasticity. In turn I would assess whether klotho could potentially improve the mechanical properties of elderly skin or prevent the UV induced disruption of the mechanical properties of human skin.

Anti-Ageing Compounds for Skin therapy

Anti-ageing compounds exhibit many benefits, namely the ability to delay biological ageing onset and ameliorate age-related diseases thus prolonging the healthy lifespan [Di Micco et al., 2021; Herranz et al., 2018; Lee et al., 2019; Song et al., 2023]. In the future I would evaluate the efficacy of other anti-ageing compounds on UVA impaired healing of skin or utilise my MN device to deliver such compounds to the skin tissue. Anti-ageing compounds function by elicit an antioxidant activity, anti-inflammatory response, immune, telomere generation mitochondrial repair and anti-apoptosis function [Chuang et al., 2014; Tundis et al., 2015; Song et al., 2023]. Anti-ageing compounds can enhance or slow down the decline of collagen and elastin of the skin, as loss of both occurs with age. The premature photoaging of human skin results in thinner epidermis, deep wrinkling, discolouration, dullness, and roughness [Makrantonaki et al., 2007]. This in turn culminates in reduced desquamation and delayed wound healing in elderly skin.

Hyaluronic acid is a widely used compound in many anti-ageing cosmetic products as it preserves the hydration and elasticity of the skin [Papakonstantinou et al., 2012]. Sericin gel has been shown to elicit a mositurizing effect when topically applied to the skin likely attributed by the hydration of the epidermal cells and elevated hydroxyproline levels [Padamwar et al., 2005]. Polyphenols have emerged as an attractive anti-aging compound that confers antioxidant properties against UVR exposure [Poljsak et al., 2012]. Anti-ageing compounds that have progressed to clinical studies on human subjects include ceramide, retinol, hyaluronic acid, astaxanthin, collagen tripeptide, acetylzinc ketone, epigallocatechin gallate, vitamin C, melatonin, protcatechuic acid, rhamnose, timosaponin, and glycolic acid [He et al., 2023].

5.2.3 Chapter 4

There is a critical concern in the NHS in latent diagnosis of skin conditions of darker skin complexion that type III on the Fitzpatrick spectrum. In this body of work, I have discussed the variation in skin disease susceptibility between skin of different colours as well the varied response different skin colours will have to UVR. However, to progress in the future would experimentally explore how UVA exposure impacts wound healing capacity of skin of dark complexions compared with light complexions, and how UVA impacts melanocyte functional activity and how that may then influence keratinocyte function and migration to injury site.

One study has extensively explored in the literature the range of photobiological responses across various skin colours including DNA damage and immunosuppression. Notably, the study concluded that DNA photodamage initiates non-melanoma skin cancer which is reduced by a factor of 3 in pigmented skin vs white (type I) skin [Fajuyigbe and Young. 2016]. Thus, suggesting that a modest reduction in DNA damage can lead to a significantly lower skin cancer incidence in black (type VI) skin, the use of sun protection may be beneficial to a susceptible population. To progress from the findings presented in this thesis I would study the variable protective mechanism skin colours across the Fitzpatrick spectrum or eumelanin score would have against UVA impeded wound healing.

5.3 Concluding remarks

During my PhD I have explored the heterogeneity of human skin specifically focusing on change in skin phenotype depending on body location, with biological ageing, and skin pigmentation. I believe my work will elicit an impact on research falling under the umbrella of dermatology, particularly bringing new insights to the photobiology, translational therapy, senescence, and biology of ageing fields. As presented in this thesis, the human skin is a delicate yet complex piece of tissue that is tremendously imperative to protecting one’s internal organs and maintaining overall health.

6.0 References

Abadie S., Leduc C., Linter K., and Bedos P. (2021) "The alkaloid centcyamine increases expression of klotho and lamin B1, slowing the onset of skin ageing in vitro and in vivo" Int J Cosmet Sci. **43**(5), pp. 561-572.

Abbas, A. A. and A. N. Akbar (2021). "Induction of T Cell Senescence by Cytokine Induced Bystander Activation." Frontiers in Aging **2**.

Abdo JM., Spoko NA., and Milner SM. (2020) "The applied anatomy of human skin: A model for regeneration" Wound Medicine.

Abedini A., Sayed C., Carter LE., Boerboom D., and Vanderhyden BC. (2020) "Non-canonical WNT5a regulates Epithelial-to-Mesenchymal Transition in the mouse ovarian surface epithelium" Scientific Reports. 10

Abraham CR., and Li A. (2022) "Aging-suppressor Klotho: Prospects in diagnostics and therapeutics" Ageing Research Reviews. **82.**

Abrouk M., Nakamura M., Zhu TH., Farahnik B., Koo J., and Bhutani T. (2017) "The impact of PASI 75 and PASI 90 on quality of life in moderate to severe psoriasis patients" J Dermatolog Treat. **28**(6), pp. 488-491.

Abud T., Kounidas G., Martin KR., Werth M., Cooper K., and Myint PK. (2022) "Determinants of health ageing: a systematic review of contemporary literature": Aging Clin Exp Res. 24(6), pp 1215-1223.

Adam D. (2022) "World Population hits eight billion, here's how researchers predict it will grow" Nature.

Agache PG., Monneur C., Leveque JL., and de Rigal J. (1980) "Mechanical properties and Young's modulus of human skin in vivo". Arch Dermatol Res. **269**, pp.127–33

Agarwal S. and Ojha A. (2012) "Piebaldism: A brief report and review of the literature" Indian Dermatol Online J. **3**(2), pp.144-147

Agbai ON., Buster K., Sanchez M., Hernandez C., Kundu RV., Chiu M et al., (2014) "Skin cancer and photoprotection in people of color: a review and recommendations for physicians and the public" J Am Acad Dermatol. **70**(4), pp. 748-762.

Agrawal M, Biswas S, Vilet KV. (2017) "Vacuum Technology Helps Develop Microneedles for Life-saving Drug Delivery Systems" Vacuum Advances in Biotechnology

Ahadi S., W. Zhou, SM. Schüssler-Fiorenza Rose, MR. Sailani, K. Contrepois, M. Avina, M. Ashland, A. Brunet and M. Snyder (2020). "Personal aging markers and ageotypes revealed by deep longitudinal profiling." Nature Medicine **26**(1): 83-90.

Ahmad N, Thomas GN, Gill P and Torella F (2016). "The prevalence of major lower limb amputation in the diabetic and non-diabetic population of England 2003–2013." Diabetes and Vascular Disease Research **13**(5): 348-353.

Ahmad N, Thomas GN, Gill P, Chan C and Torella F (2014). "Lower limb amputation in England: prevalence, regional variation and relationship with revascularisation, deprivation and risk factors. A retrospective review of hospital data." Journal of the Royal Society of Medicine **107**(12): 483-489.

Ahmadi-Ashtiani H-R., Bishe P., Baldisserotto A., Buso P., Manfredini S., and Vertiani. (202) "Stem cells as a target for the Dleivery of Active Molecules to Skin by Topical Administration" Int J Mol Sci. **21**(6), pp. 2251.

Ahuja V., Thapa D., and Ghai B. (2018) "Strategies for prevention of lower limb post-amputation pain" A clinical narrative review". J Anaesthesiol Clin Pharmacol. **34**(4), pp. 439-449.

Ait-Khaled N., Odhiambo J., Pearce N., Adjoh KS., Maesano IA., Benhabyles B et al., (2007). "Prevalence of symptoms of asthma, rhinitis and eczema in 13- to 14-year-old children in

Africa: the International Study of Asthma and Allergies in Childhood Phase III." Allergy **62**(3), pp.247-258.
Ajitkumar A., Naga S., Yarrarapu S., and Ramphul K. (2023) "Chediak-Higashi Syndrome" StatPearls.
Akamigbo AB., and Wolinsky FD. (2007) "New evidence of racial differences in access and their effects on the use of nursing homes among older adults" Med Care. **45**(7), pp. 672-9.
Alafnan, A., A. A. Seetharam, T. Hussain, M. S. Gupta, S. M. D. Rizvi, A. Moin, A. Alamri, A. Unnisa, A. M. Awadelkareem, A. O. Elkhalifa, P. Jayahanumaiah, M. Khalid and N. Balashanmugam (2022). "Development and Characterization of PEGDA Microneedles for Localized Drug Delivery of Gemcitabine to Treat Inflammatory Breast Cancer." Materials **15**(21): 7693.
Alaluf, S., et al. (2002). "The Impact of Epidermal Melanin on Objective Measurements of Human Skin Colour." Pigment Cell Research **15**(2): 119-126.
Alam H., Sehgal L., Kundu ST., Dalal SN., and Vaidya MM. (2011) "Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells" Mol Biol Cell. **22**(21), pp. 4068-4078.
Alberts B., Johnson A., and Lewis J. (2002) "Molecular Biology of the Cell" Garland Science.
Albores-Saavedra J., Batich K., Chable-Montero F., Sagy N., Schwartz AM., and Henson DE. (2010) "Merkel cell carcinoma demographics, morphology, and survival based on 3870 cases: a population-based study" J Cutan Pathol. **37**(1), pp. 20-7.
Aldawood FK., Andar A., and Desai S. (2021) "A Comprehensive Review of Microneedles: Types, Materials, Prpcesses, Characterisations and Applications" Polymers (Basel). **13**(16), pp. 2815.
Ali R, Rani R, Kumar S. (2013) "New Peptide Based Therapeutic Approaches. In: Ghulam Md" A, Ishfaq Ahmed S, editors. Advances in Protein Chemistry. Jeddah: OMICS Group eBooks.
Ali SM., and Yosipovitch G. (2013) "Skin pH: From Basic Science to Basic Skin Care" Advances in Dermatology and Venerology. **93**(3).
Alicea GM., and Rebecca VW. (2023) "Un-Fair Skin: racial disparities in acral melanoma research" Nat Rev Cancer. **22**(3), pp. 127-128.
Alimirah F., Pulido T., Valdovinos A., Alptekin S., Chang E., Jones E., et al. (2020) "Cellular senescence promotes skim carcinogenesis through p38MAPK and p44/p42 MAPK signaling" Cancer Res. **80**(17), pp. 3606-3619.
Allanurov AM., Zdrok AY., Loschilov AG., and Malyutin ND. (2014) "Problem of Ink Evaporation while Using Plotter Systems to Manufacture Printed Electronic Products" Procedia Technology. **18**, pp. 19-24.
Almassi F, Mousavi B, Masumi M, Souroush MR and Honari G (2009). "Skin Disorders Associated with Bilateral Lower Extremity Amputation." Pakistan Journal of Biological Sciences **12**(20): 1381-1384.
Almohammadi, A.A. Alnashri, M.M. Harun, R.A.T. Alsamiri, S.A. Alkhatieb, M.T. (2022) "Pattern and type of amputation and mortality rate associated with diabetic foot in Jeddah, Saudi Arabia: A retrospective Cohort Study. Ann Med Surg (Lond). **73:** 103174
Alonso L., and Fuchs E. (2003) "Stem cells of the skin epithelium" PNAS. **100**(suppl_1), pp. 11830-11835.
Alotaibi HM. (2023) "Incidence, Rish Factors, and Prognosis of Hidradenitis Suppurativa Across the Globe: Insights from the Literature" Clin Cosmet Investig Dermatol. **16**, pp. 545-552.
Alshishtawy MM. (2011) "To be or not to be exposed to direct sunlight: vitamin d deficiency in oman" Sultan Qaboos Univ Med J. **11**(2), pp. 196-200.

AlSulaiman D., Chang JYH., Bennett NR., Topouzi H., Higgins C., Irvine DJ., and Ladame S. (2019). "Hydrogel-Coated Microneedle Arrays for Minimally Invasive Sampling and Sensing of Specific Circulating Nucleic Acids from Skin Interstitial Fluid." ACS Nano **13**(8): 9620-9628.
Alzahrani, H. A. (2012). "Diabetes-Related Lower Extremities Amputations in Saudi Arabia: The Magnitude of the Problem." Annals of Vascular Diseases.
Amani H., Shahbazi M-A., D'Amico C., Fontana F., Abbaszadeh S., and Santos HA. (2021) "Microneedles for painless transdermal immunotherapeutic applications" Journal of Controlled Release. **330**, pp. 185-217.
Amarnani, R. and Shende, P. (2022). "Microneedles in diagnostic, treatment and theragnostic: An advancement in minimally invasive delivery system." Biomedical Microdevices **24**(1).
Amaro-Ortiz A., Yan B., and D'Orazio JA. (2014). "Ultraviolet Radiation, Aging and the Skin: Prevention of Damage by Topical cAMP Manipulation." Molecules **19**(5): 6202-6219.
Ameri M., Fan SC., and Maa YF. (2010) "Parathyroid hormone PTH(1-34) formulation that enables uniform coating on a novel trandermalmicroprojection delivery system" Pharm Res. **27**, pp. 303-313.
Amiri, N., A. P. Golin, R. B. Jalili and A. Ghahary (2022). "Roles of cutaneous cell-cell communication in wound healing outcome: An emphasis on keratinocyte-fibroblast crosstalk." Experimental Dermatology **31**(4): 475-484.
Amrstrong DG., Swerdlow MA., Armstrong AA., Conte MS., Padula WV., and Bus SA. (2020) "Five-year mortality and direct costs of care for people with diabetic foot comlications are comparable to cancer" J Foot Ankle Res. **13**, 16.
Ando H., Niki Y., Ito M., Akiyama K., Matsui MS., Yarosh DB., et al. (2012). "Melanosomes Are Transferred from Melanocytes to Keratinocytes through the Processes of Packaging, Release, Uptake, and Dispersion." Journal of Investigative Dermatology **132**(4): 1222-1229.
Andrade, A. M., M. Sun, N. S. Gasek, G. R. Hargis, R. Sharafieh and M. Xu (2022). "Role of Senescent Cells in Cutaneous Wound Healing." Biology **11**(12): 1731.
Andrews JP., Marttala J., Macarak E., Rosenbloom J., and Uitto J. (2016) "Keloids" The paradigm of skin fibrosis – path0mechanisms and treatment" Matrix Biol. **41**, pp. 37-46.
Ang KC., Ngu MS., Reid KP., Teh MS., Aida ZS., Koh DX., et al., (2012) "Skin color variation in Orang Asli tribes of Peninsula Malaysia" PLoS One. **7**(8).
Anselmo AC., Mitragotri S. (2014) "An Overview of Clinical and Commercial Impact of Drug Delivery Systems". J. Control. Release **190**, pp.15–28.
Apalla Z., Lallas A., Sotiriou E., Lazaridou E., and Ioannides D. (2017) "Epidemiological trends in skin cancer" Dermatology Practical & Conceptual. **7**(2), pp. 1-6.
Arakawa, A., K. Siewert, J. Stöhr, P. Besgen, S.-M. Kim, G. Rühl, J. et al., (2015). "Melanocyte antigen triggers autoimmunity in human psoriasis." Journal of Experimental Medicine **212**(13): 2203-2212.
Armstrong BK, Kricker A. (2001) "The epidemiology of UV induced skin cancer" J Photochem Photobiol B. **63**(1-3), pp. 8-18:doi:10.1016/s1011-1344(01)00198-1
Armstrong CA, Murray N, Kennedy M, Koppula SV, Tara D, and Ansel JC. (1994) "Melanoma-derived interleukin 6 inhibits in vivo melanoma growth". Journal of investigative Dermatology, **102**(3):doi:10.1111/1523-1747.ep12371782.
Arya J., and Praunitz (2016) "Microneedle patches for vaccination in developing countries" J. Control. Release. **240**, pp. 135-141.
Ascott A., van Bodegraven B., Ahmed S., Harwood C., Proby C., Sommerlad M., Vernon S., Welsh C., and Venables Z. (2023) "P49 Epidemiology of skin cancer by ethnicity in England, 2013-2020: a cohort study" British Journal of Dermatology. **188**(supplement 4).

Ashcroft GS, Dodsworth J, Van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, et al. (1997) "Estrogen accelerates cutaneous wound healing associated with an increase in TGF-b1 levels". Nat Med **3**.1209e15.
Ashcroft GS, Horan MA, and Ferguson MW. (1995) "The effects of ageing on cutaneous wound healing in mammals". J Anat. **187**, pp.1–26
Ashcroft GS, Mills SJ, and Ashworth JJ. (2002) Ageing and wound healing". Biogerontology 3, pp. 337-345.
Ataguba JE, Bloom DE, Scott AJ. (2021) "A Global Institution for an Aging World" Project Syndicate. October 3.
Atillasoy ES, Seykora JT, Soballe PW, Elenitsas R, Nesbit M, Elder DE, et al., (1998) "UVB induces atypical melanocytic lesions and melanoma in human skin". Am J Pathol.**152**(5), pp.1179-86
Aubert G., and Lansdorp PM. (2008) "Telomeres and aging" Physiol Rev. **88**(2), pp. 557-579.
Augustin HG, Kozian DH, and Johnson RC. (1994) Differentiation of endothelial cells: analysis of the constitutive and activated endothelial cell phenotypes. Bioassays. **16**(12), pp. 901–906.
Aumailley M., Bruckner-Tuderman L., Carter WG., Deutzmann R., Edgar D., Ekblom P et al., (2005) "A simplified laminin nomenclature". Matrix Biol 24, pp. 326–332.
Aungst B., Saitoh H., Burcham D., Huand S., Mousa S., and Hussain M. (1996) "Enhancement of the intestinal absorption of peptides and nonpeptides". J. Control. Release. **41**(1), pp. 19–31.
Avcil M., and Çelik A. (2021) "Microneedles in Drug Delivery: Progress and Challenges" Micromachines (Basel). **12**(11), pp. 1321.
Aw D., Silva AB., and Palmer DB. (2007) "Immunosenescence: emerging challenges for an ageing population" Immunology. **120**, pp. 435-46.
Awad F, Assrawi E, Louvrier C, Jumeau C, Giurgea I, Amselem S, Karabina SA. (2018) "Photoaging and skin cancer: Is the inflammosome the missing link?" Mech Ageing Dev. **172**, pp.131-137:doi:10.1016/j.mad.2018.03.003.
Bacalini MG., Deelen J., Pirazzini C., Cecco MD., Giuliani C., Lanzarini C et al., (2017) "Systemic Age-Associated DNA Hypermethylation of ELOVL2 Gene: In Vivo and In Vitro Evidences of a Cell Replication Process" The Journals of Gerontology. **72**(8), pp. 1015-1023.
Badimon L., Padro T., and Vilahur G. (2023) "Chapter 27 – Moving from reactive to preventative medicine" Aging: From Fundamental Biology to Societal Impact, pp. 663-681.
Badran M., Kuntsche, J., and Fahr, A. (2009) "Skin penetration enhancement by a microneedle device (Dermaroller®) in vitro: Dependency on needle size and applied formulation". Eur. J. Pharm. Sci. **36**, pp. 511–523.
Badylak SF, Freytes DO, Gilbert TW. (2009) "Extracellular matrix as a biological scaffold material: structure and function". Acta Biomater 5, pp. 1–13.
Baek SH., Shin JH., and Kim YC. (2017) "Drug-coated microneedles for rapid and painless local anesthesia" Biomed Microdevices. **19**, pp. 2.
Baker H., and Kligman AM. (1967) "Measurement of transepidermal water loss by electrical hygrometry. Instrumentation and responses to physical and chemical insults" Arch Dermatol. **96**, pp. 441-452.
Bal, SM., Kruithof, AC., Zwier, R., Dietz, E., Bouwstra, JA., Lademann, J., and Meinke, MC. (2010) "Influence of microneedle shape on the transport of a fluorescent dye into human skin in vivo". J. Control. Release **147**, pp. 218–224.
Banerjee J. and Sen CK. (2013) "MicroRNAs in Skin and Wound Healing" Methods Mol Biol. **936**, pp. 343-356.
Bang KM, Halder RM, White JE, and Sampson CC. (1987) "Skin Cancer in Black Americans: A Review of 126 Cases" J Natl Med Assoc. **79(**1), pp. 51-58

Barbenel JC, Jordan MM, Nicol SM, Clark MO. (2009) “Dilemmas in measuring and using pressure ulcer prevalence and incidence: an international consensus”. Int Wound J **6**, pp.97–104.
Barbieri JS., Wanat K., and Seykora J. (2014) “Skin: Basic Structure and Function” Pathobiology of Human Disease, pp. 1134-1144.
Bariya SH., Gohel MC., Mehta TA., and Sharma OP. (2011) “Microneedles: an emerging transdermal drug delivery system” Journal of Pharmacy and Pharmacology. **64**, pp. 11-29.
Barsh, G. S. (2003). "What Controls Variation in Human Skin Color?" PLoS Biology **1**(1): e27.
Barton F and Cooke A. (2021) “Deaths registered in England and Wales: 2021” Office for National Statistics. Census 2021.
Bäsler K., and Brandner JM. (2017) “Tight Junctions in skin inflammation” Pflugers Arch. **469**(1), pp. 3-14.
Basu, S. and V. Shukla (2012). Complications of Wound Healing, Springer London**:** pp**.** 109-144.
Bates-Jensen BM., Anber K., Chen MM., Collins S., Esparza AN., Gieschen K et al., (2021) “Natural History of Pressure Injury Among Ethnically/Racially Diverse Nursing Home Residents: The pressure ulcer detection study” J Gerontol Nurs. 1;**47**(3), pp. 37-46.
Battie, C., S. Jitsukawa, F. Bernerd, S. Del Bino, C. Marionnet and M. Verschoore (2014). "New insights in photoaging, UVA induced damage and skin types." Experimental Dermatology **23**: 7-12.
Bauleth-Ramos, T., El-Sayed, N., Fontana, F., Lobita, M., Shahbazi, M.-A., and Santos, HA. (2023) “Recent approaches for enhancing the performance of dissolving microneedles in drug delivery applications”. Mater. Today **63**, pp.239–287.
Baum J., and Duffy HS. (2012) “Fibroblasts and Myofibroblasts: what are we talking about?” J Cardiovasc Pharmacol. **57**(4), pp. 376-379.
Baumann L., Bernstein EF., Weiss AS., Bates D., Humphrey S., Silberberg M., and Dnaiels R. (2021) “Clinical Relevace of Elastin in the Structure and Function of Skin” Aesthet Surg J Open Forum. **3**(3).
Bedell ML, Melchiorri A, Aleman J, Skardal A, Mikos A. (2020) “A high-throughput approach to compare the biocompatibility of candidate bioink formulations”. Bioprinting, **17**
Beelen, ESA., van der Valk WH., de Groot JCMJ., Hensen EF., Locher H., and van Benthem PPG. (2020). "Migration and fate of vestibular melanocytes during the development of the human inner ear." Developmental Neurobiology **80**(11-12): 411-432.
Bekaert B, Kamalandua A, Zapico SC, Van De Voorde W, Decorte R. (2015) “Improved age
Bell CG., Lowe R., Adams PD., Baccarelli AA., Beck S., Bell JT., Christensen BC., et al., (2019) “DNA methylation aging clocks: challenges and recommendations” Genome Biology. **20**
Bellefroid C., Lechanteur A., Evrard B., Mottet D., Debacq-Chainiaux F., and Piel G. (2019) “In vitro skin penetration enhancement techniques: A combined approach of ethosomes and microneedles”. Int. J. Pharm. **572**, pp. 118793. doi: 10.1016/j.ijpharm.2019.118793.
Belmadani, A., Jung H., Ren D., and Miller RJ. (2009). "The chemokine SDF-1/CXCL12 regulates the migration of melanocyte progenitors in mouse hair follicles." Differentiation **77**(4): 395-411.
Ben-Gashir B, and Hay R. (2002) “Reliance on erythema scores may mask severe atopic dermatitis in black children compared with their white counterparts”. British Journal of Dermatology. **147**, pp.920-925.
Berchane, N.S. Andrews, M.J. Kerr, S. Slater, N.K.H. and Jebrail, F.F. (2008) “On the mechanical properties of bovine serum albumin (BSA) adhesives” J Mater Sci Mater Med. **19**(4), 1831-8: doi:10.1007/s10856-007-3360-7

Bernal A, and Tusell L. (2018) “Telomeres: Implications for cancer development”. Int J Mol Sci. **19**, pp.E294. 10.3390/ijms19010294.
Bernstein EF., Brown DB., Urbach F., Forbes D., Monaco MD., Wu M., Katchman SD., and Uitto J. (1995) “Ultraviolet Radiation Activates the Human Elastin Promoter in Transgenic Mice” A Novel In Vivo and In Vitro Model of Cutaneous Photoaging” The Society for Investigative Dermatology.
Bhatt PM., and Malgor R. (2014) “Wnt5a: A player in the pathogenesis of Atherosclerosis and other Inflammatory Disorders” Atherosclerosis. **237**(1), pp. 155-162.
Bhattacharya S, and Mishra RK (2015). "Pressure ulcers: Current understanding and newer modalities of treatment." Indian Journal of Plastic Surgery **48**(01): 004-016.
Bichakjian CK, Lowe L, Lao CD, Sandler HM, Bradford CR, Johnson TM et al., (2007) “Merkel cell carcinoma: critical review with guidelines for multidisciplinary management” Cancer. **110**(1), pp.1-12: doi: 10.1002/cncr.22765.
Bilal M., Mehmood S., Raza A., Hayat U., Rasheed T., and Iqbal HM. (2021) “Microneedles in smart drug delivery”. Adv. Wound Care. **10**, pp. 204–219. doi: 10.1089/wound.2019.1122.
Bjørklund, G., M. Shanaida, R. Lysiuk, M. Butnariu, M. Peana, I. Sarac, O. Strus, K. Smetanina and S. Chirumbolo (2022). "Natural Compounds and Products from an Anti-Aging Perspective." Molecules **27**(20): 7084.
Blanpain C and Fuchs E. (2006) “Epidermal Stem Cells of the Skin” Annu Rev Cell Dev Biol. **22**, pp. 339-373.
Bliss, D. Z., et al. (2017). "Incidence and Predictors of Incontinence-Associated Skin Damage in Nursing Home Residents With New-Onset Incontinence." Journal of Wound, Ostomy & Continence Nursing **44**(2): 165-171.
Blume-Peytavi, U., J. Kottner, W. Sterry, M. W. Hodin, T. W. Griffiths, R. E. B. Watson, R. J. Hay and C. E. M. Griffiths (2016). "Age-Associated Skin Conditions and Diseases: Current Perspectives and Future Options." The Gerontologist **56**(Suppl 2): S230-S242.
Bocheva G., Slominski RM., and Slominski AT. (2021) “The Impact of Vitamin D on Skin Aging” Int J Mol Sci. **22**(16), pp. 9097.
Boniface K, Seneschal J, Picardo M, Taïeb A. (2018) “Vitiligo: focus on clinical aspects, immunopathogenesis, and therapy”. Clin Rev Allergy Immunol, **54**(1), pp.52-67:doi:10.1007/s12016-017-8622-7
Bonifacio KM., Kunjravia N., Krueger JG., and Fuenter-Duculan J. (2016) “Cutaenous Expression of a disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5 (ADAMTSL5) in Psoriasis goes beyond Melanocytes” J Pigment DIsord. **3**(3), pp. 244.
Bonilla C, Ness AR, Wills AK, Lawlor DA, Lewis SJ, Smith GD. (2014) “Skin pigmentation, sun exposure and vitamin D levels in in children of the Avon Longitudinal Study of Parents and Children” BMC Public Health. **14**, pp.597
Bora P., Kumar L., and Bansal A. (2008) “Microneedle Technology for Advanced Drug Delivery: Evolving Vistas” Current Res. Inf. Pharm. Ci.
Boraschi-Diaz I., Wang J., Mort JS., and Komarova SV. (2017) “Collagen Type I as a Ligand for Receptor-Mediated Signaling”. Front. Phys. **5**(12).
Borchardt T., Jeffrey A., Siahaan TJ., Gangwar S., and Pauletti GM. (1997) “Improvement of oral peptide bioavailability: peptidomimetics and prodrug strategies”. Adv. Drug Deliv. Rev. **27**(2–3), pp.235–256.
Borojeny LA., Albatineh AN., Dehkordi AH., and Gheshlagh RG. (2020) “The Incidence of Pressure Ulcers and its Associations in Different Wards of the Hopsitcal Systenatic Review and Meta-Analysis” Int J Prev Med. **11**, pp. 171.

Bos JD, Meinardi MM. (2000) “The 500 Dalton rule for the skin penetration of chemical compounds and drugs”. Exp. Dermatol. **9**, pp. 165–169. doi: 10.1034/j.1600-0625.2000.009003165.x
Bosch, R., N. Philips, J. Suárez-Pérez, A. Juarranz, A. Devmurari, J. Chalensouk-Khaosaat and S. González (2015). "Mechanisms of Photoaging and Cutaneous Photocarcinogenesis, and Photoprotective Strategies with Phytochemicals." Antioxidants **4**(2), pp. 248-268.
Bosman ES., Albert AY., Lui H., Dutz JP., and Vallance BA. (2019) “Skin exposure to narrow band ultraviolet (UVB) light modulates the human intestinal microbiome”. Front Microbiol. 10, pp. 2410. doi: 10.3389/fmicb.2019.02410.
Bosman FT, Stamenkovic I. (2003) “Functional structure and composition of the extracellular matrix”. J Pathol 200, pp. 423–428.
Bouillon, R., F. Schuit, L. Antonio and F. Rastinejad (2020). "Vitamin D Binding Protein: A Historic Overview." Frontiers in Endocrinology **10**.
Bouten CV., Oomens CW., Baaijens FP., and Bader DL. (2003) “The etiology of pressure ulcers: Skin deep or muscle bound?” Arch. Phys. Med. Rehabil. **84**, pp. 616-619.
Bowers S., and Franco E. (2020) “Chronic wounds: Evaluation and management” Am Fam Physician. **101**(3), pp. 159-166.
Box NF., Wyeth JR., O’Gorman LE., Martin NG., Sturm RA. (1997) “Characterization of melanocyte stimulating hormone variant alleles In twins with red hair” Hum Mol Genet. **6**, pp.1891-1897
Boyce S, Supp D. (2016) “Chapter 11 – Biologic Skin Substitutes” Skin Tissue Engineering and Regenerative Medicine, pp. 211-238.
Boyd AS., Naylor M., Cameron GS., Pearse AD., Gaskell SA. And Neldner KH. (1995) “The effects of chronic sunscreen use on the histologic changes of dermatoheloisis” J Am Acad Dermatol. **33**(6), pp. 941-946.
Boyle, C. J., M. Plotczyk, S. F. Villalta, S. Patel, S. Hettiaratchy, S. D. Masouros, M. A. Masen and C. A. Higgins (2019). "Morphology and composition play distinct and complementary roles in the tolerance of plantar skin to mechanical load." Science Advances **5**(10): eaay0244.
Boyle, C.J. and Higgins, C.A. (2021). "Can plantar fibroblast implantation protect amputees from skin injury? A recipe for skin augmentation." Experimental Dermatology **30**(12): 1829-1833.
Brackman, G. and T. Coenye (2015). In Vitro and In Vivo Biofilm Wound Models and Their Application, Springer International Publishing**:** 15-32.
Bradford PT. (2009) “Skin Cancer in Skin of Color” Dermatol Nurs. **21**(4), pp. 170-178.
Brady M, Spiker AM. (2022) “Merkel Cell Carcinoma of the skin” StatPearls.
Brambilla D., Luciani P., and Leroux J. (2014) “Breakthrough Discoveries in Drug Delivery Technologies: The Next 30 years”. J. Control. Release 190 pp. 9–14
Brenner, M. and V. J. Hearing (2008). "The Protective Role of Melanin Against UV Damage in Human Skin." Photochemistry and Photobiology **84**(3): 539-549.
Breytenbach JM., and Segwapa K. (2019) “Keloid in a patient with albinism” Keloid Research. **3**(1).
British Association of Dermatology. (2020) “BAD and minority ethnic group inclusivity”
Brito J., Moon J., Hlushko R., Aliakseyeu A., Andrianov AK., and Sukhishvili S. (2024) “Engineering Degradation Rate of Polyphosphaene-Based Layer-by-Layer Polymer Coatings” Journal of Functional Biomaterials. **15**(2), pp. 26.
Brobey RK., Dheghani M., Foster PP., Kuro-o M., and Rosenblatt KP. (2015) “Klotho Regulates 14-3-2 Monomerization and Binding to the ASK1 signaling Complex in Response to Oxidative Stress” PLoS One. **10**(10) pp. e0141968.

Brobey, R. K., D. German, P. K. Sonsalla, P. Gurnani, J. Pastor, C. C. Hsieh, J. Papaconstantinou, P. P. Foster, M. Kuro-O and K. P. Rosenblatt (2015). "Klotho Protects Dopaminergic Neuron Oxidant-Induced Degeneration by Modulating ASK1 and p38 MAPK Signaling Pathways." PLOS ONE **10**(10): e0139914.
Bronaugh RL. and Maibach HI. (2005) "Percutaneous Absorption". Strategies to Protect the Health of Deployed U.S. Forces: Force Protection and Decontamination.
Brooks J., and Ramsey ML. (2023). "Dermatofibrosarcoma Protuberans" StatPearls.
Browe DP., Wood C., Sze MT., White KA., Scott T., Olabisi RM., and Freeman JW. (2017) "Characterization and optimization of actuating poly(ethylene glycol) diacrylate/acrylic acid hydrogels as artificial muscles" Polymer. **117**, pp. 331-341.
Brown TM, Krishnamurthy K. (2022) "Histology, Dermis" StatPearls.
Brown TM., and Krishnamurthy K. (2022) "Histology, Dermis" StatPearls.
Buchanan, S., E. Combet, P. Stenvinkel and P. G. Shiels (2020). "Klotho, Aging, and the Failing Kidney." Frontiers in Endocrinology **11**.
Budden T., Gaudy-Marqueste C., Porter A., Kay E., Gurung S., Earnshaw CH., et al. (2021) "Ultraviolet light-induced collagen degradation inhibits melanoma invasion" Nature communications. **12**.
Buikema KES, Meyerele JH. (2014) "Amputation stump: Privileged harbor for infections, tumors, and immune disorders" Clinics in Dermatology **32**(5), pp. 670-677.
Bukhamsin, A., K. Moussi, R. Tao, G. Lubineau, I. Blilou, K. N. Salama and J. Kosel (2021). "Robust, Long-Term, and Exceptionally Sensitive Microneedle-Based Bioimpedance Sensor for Precision Farming." Advanced Science **8**(16): 2101261.
Bupphathong, S., Quiroz C., Huang W., Chung P-F., Tao H-Y., and Lon C-H.et al. (2022). "Gelatin Methacrylate Hydrogel for Tissue Engineering Applications—A Review on Material Modifications." Pharmaceuticals **15**(2): 171.
Burns EM., Ahmed H., Isedeh PN., Kohli I., Van Der Pol W., Shaheen A. et al. (2019) "Ultraviolet radiation, both UVA and UVB, influences the composition of the skin microbiome" Exp Dermatol. **28**(2), pp. 136-141.
Buron, C. C., T. Vrlinic, T. Le Gallou, S. Lakard, A. Bolopion, P. Rougeot and B. Lakard (2020). "pH-Responsive PEG/PAA Multilayer Assemblies for Reversible Adhesion of Micro-Objects." ACS Applied Polymer Materials **2**(12): 5646-5653.
Buster KJ., Stevens EI., and Elmets CA. (2013) "Dermatologic Health disparities" Dermatol. Clin. **30**(1), 53-viii.
Bytheway. B (1995) "Ageism"
Cabaço LC, Tomás A, Pojo M and Barral DC. (2022) "The Dark Side of Melanin Secretion in Cutaenous Melanoma Aggressiveness" Frontiers in Oncology. **12**
Cabral-Pacheco G., Garza-Veloz I., De la Rosa CC., Ramirez-Acuña JM., Peres-Romero BA., Guerrero-Rodriguez JF et al., (2020) "The roles of matrix metalloproteinases and their inhibitors in Human diseases" Int J Mol Sci. **21**(24), pp. 9739.
Cai SCS., Chen H., Koh W-P., Common JEA., van Bever HP., McLean WHI et al., (2012) "Filaggrin mutations are associated with recurrent skin infection in Singaporean Chinese patients with atopic dermatitis" Br J Dermatol. **166**(1), pp. 200-3.
Campos AC., Groth AK., and Branco AB (2008) "Assessment and nutritional aspects of wound healing" Curr Opin Clin Nutr Metab Care. **11**, pp. 281-288
Canesin, G., S. Evans-Axelsson, R. Hellsten, A. Krzyzanowska, C. P. Prasad, A. Bjartell and T. Andersson (2017). "Treatment with the WNT5A-mimicking peptide Foxy-5 effectively reduces the metastatic spread of WNT5A-low prostate cancer cells in an orthotopic mouse model." PLOS ONE **12**(9): e0184418.
Cao PF, Mangadlao JD, Advincula RC. (2015). "Stimuli-Responsive Polymers and their Potential Applications in Oil-Gas Industry." Polymer Reviews **55**(4): 706-733.

Carrasco, E., M. M. Gómez De Las Heras, E. Gabandé-Rodríguez, G. Desdín-Micó, J. F. Aranda and M. Mittelbrunn (2022). "The role of T cells in age-related diseases." Nature Reviews Immunology **22**(2): 97-111.
Carter TM., Strassale PD., Ollila DW., Stitzenberg KB., Meyers MO., and Maduekwe UN. (2021) "Does acral lentiginous melanoma subtype account for differences in patterns of care in Black patients?" Am J Surg. **221**(4), pp. 706-711.
Cavallo, A. Magadhiele, M. Masullo, U. Lionetto, M.G. Sannino, A. (2017). "Photo-crosslinked poly (ethylene glycol) diacrylate (PEGDA) hydrogels from low molecular weight prepolymer: Swelling and permeation studies." Journal of Applied Polymer Science **134**(2).
Cengiz FP., Emiroglu N., Bahali AG., Ozkaya DB., Su O., and Onsun N. (2016) "The Relationship of Psoriasis and Melanocytic Nevi" Indian J Dermatol. **61**(6), pp. 664-667
Chadwick, S., R. Heath and M. Shah (2012). "Abnormal pigmentation within cutaneous scars: A complication of wound healing." Indian Journal of Plastic Surgery **45**(02): 403-411.
Chaib, S., T. Tchkonia and J. L. Kirkland (2022). "Cellular senescence and senolytics: the path to the clinic." Nature Medicine **28**(8): 1556-1568.
Chalermphanchai N., Arunothong W., Jettavan N., Chanta S., Boonpisitsakul T., Mayoon M. et al. (2022) "Safety, tolerability, and antibody response after intradermal vaccination of PFE-BNT in adults who have completed two-doses of Verocell (inactivated vaccine)" *Vaccine X.*
Chambers, E. S., M. Vukmanovic-Stejic, B. B. Shih, H. Trahair, P. Subramanian, O. P. Devine, J. Glanville, D. Gilroy, M. H. A. Rustin, T. C. Freeman, N. A. Mabbott and A. N. Akbar (2021). "Recruitment of inflammatory monocytes by senescent fibroblasts inhibits antigen-specific tissue immunity during human aging." Nature Aging **1**(1): 101-113.
Chan LS. (2023) "Keratinocytes" Atopic Dermatitis" Inside Out Or Outside in.
Chandran V., and Raychaudhuri SP. (2010) "Geoepidemiology and environmental factors of psoriasis and psoriatic arthritis" J Autoimmune. **34**(3), pp. 314-21.
Chandran, R. Tohit, E.R.M. Stanslas, J. Mahmood, T.M.T. (2019) "Chapter 14 – Recent Advances and Challenges in Microneedle-Mediated Transdermal Protein and Peptide Drug Delivery" Biomaterials and Bionanotechnology **14**, pp. 495-525
Chang L., Fan W., Pan X., and X Zhu. (2022) "Stem cells to reverse aging" Chin Med J (Engl) **135**(8), pp. 901-910.
Chang Y, Barrett JH, Bishop DT, Armstrong BK, Bataille V, Bergman W et al., (2009) "Sun exposure and melanoma risk at different latitudes: A pooled analysis of 5700 cases and 7216 controls". Int J Epidemiol.**38**(3)pp.814–30.
Chang YS., Ahn SY., Yoo HY., Sung SI., Choi SJ., Oh WI et al., (2014) "Mesenchymal stem cells for bronopulmonary dysplasia: phase 1 dose-escalation clinical trial" J Pediatr. **164**(5), pp. 966-972.
Chang, H-H., H-H. Ko, T-M. Lu, J-Y. Lin, D-C. Chang, T. W. Chu and C.-F. Hung (2021). "Inhibition of UVA Damage on Human Skin Dermis Fibroblasts by the Isoflavonoid Intermediate Deoxybenzoin-3A." Chemical Research in Toxicology **34**(4), pp.1133-1139.
Chang. M. (2023) "Matrix metalloproteinase profiling and their roles in disease" RSC Advances. (9).
Chao DL., and Skowaronska-Krawcyk D. (2020) "ELOVL2: Not just a biomarker of aging" Trans Med Aging. **4**, pp. 78-80.
Charles, S. T. and L. L. Carstensen (2010). "Social and Emotional Aging." Annual Review of Psychology **61**(1): 383-409.
Charlton M, Stanley SA, Whitman Z, Wenn V, Coats TJ, Sims M et al., (2020), "The effect of constitutive pigmentation on the measured emissivity of human skin. PLOS ONE, **15**(11): https://doi.org/10.1371/journal. pone.0241843

Charoenchon N., Rhodes LE., Nicolaou A., Williamson G., Watson REB., and Farrar MD. (2022) "Ultraviolet radiation-induced degradation of dermal extracellular matrix and protection by green tea catechins: a randomised controlled trial" Clinical and Experimental Dermatology.
Chaudhary M, Khan A, Gupta M. (2020) "Skin Ageing: Pathophysiology and Current Market Treatment Approaches" Current Aging Science. **13**(1), pp.22-30.
Chaudhary M., Khan A., and Gupta M. (2020) "Skin Ageing" Pathophysiology and Current Market Treatment Approaches" Curr Aging Sci. **13**(1), pp. 22-30.
Chen I.P., Henning S., Faust A., Boukamp P., Volkmer B., and Greinert R. (2012) "UVA-induced epigenetic regulation of P16(INK4a) in human epidermal keratinocytes and skin tumor derived cells". Photochem. Photobiol. Sci. **11**, pp.180–190. doi: 10.1039/C1PP05197K.
Chen K., Zhou X., and Sun Z. (2015) "Haplodeficiency of Klotho Gene Caucses Arterial Stiffening via Upregulation of Scleraxis Expression and Induction of Autophagy" Hypertension. **66**(5), pp. 1006-13.
Chen MC., Ling MH., and Kusuma SJ. (2015) "Poly-γ-glutamic acid microneedles with a supporting structure design as a potential tool for transdermal delivery of insulin". Acta Biomaterials. **24**, pp.106–116. doi: 10.1016/j.actbio.2015.06.021.
Chen W., Li H., Shi D., Liu Z., and Yuan W. (2016) "Microneedles as a Delivery System for Gene Therapy" Front. Pharmacol. **7.**
Chen Y.E., Fischbach M.A., Belkaid Y. Skin microbiota-host interactions. *Nature.* 2018;553:427–436. doi: 10.1038/nature25177.
Chen, P., P. Hu, D. Xie, Y. Qin, F. Wang and H. Wang (2010). "Meta-analysis of vitamin D, calcium and the prevention of breast cancer." Breast Cancer Research and Treatment **121**(2): 469-477.
Chen, Y.-C., Christiani DC., Su H-JJ., Hsueh Y-M., Smith TJ., Ryan LM et al., (2010). "Early-life or lifetime sun exposure, sun reaction, and the risk of squamous cell carcinoma in an Asian population." Cancer Causes & Control **21**(5): 771-776.
Chiarelli-Neto O, Ferreira AS, Martins WK, Pavani C, Severino D, Faião-Flores F, et al. (2014) "Melanin photosensitization and the effect of visible light on epithelial cells". PLoS One., **9**(11):e113266. https://doi.org/10.1371/journal.pone.0113266
Chien LH., Qi J., Grandhi R., Harris T., Kim N., Jang MS., et al (2018) "Chronological Aging in African-American Skin: A Reliable Photonumeric Scale Demonstrates Age and Body Mass Index as Contributing Factors" Journal of the National Medical Association. **110**(6),
Chike-Obi, C., P. Cole and A. Brissett (2009). "Keloids: Pathogenesis, Clinical Features, and Management." Seminars in Plastic Surgery **23**(03): 178-184.
Childs, B. G., M. Gluscevic, D. J. Baker, R.-M. Laberge, D. Marquess, J. Dananberg and J. M. Van Deursen (2017). "Senescent cells: an emerging target for diseases of ageing." Nature Reviews Drug Discovery **16**(10): 718-735.
Chiquet M, Renedo AS, Huber F., and Flũck M (2003) "How do fibroblasts translate mechanical signals into changes in extracellular matrix production?" Matrix Biol 22, pp. 73–80.
Chocksi J., Vora J., and Shrivastava N. (2020) "Bioactive pigments from isolated bacteria and its antibacterial, antioxidant and sun protective application useful for cosmetic products" Indian J Microbiol. **60**, pp. 379-382.
Choo YJ., Kim DH., and Chang MC. (2022) "Amputation stump management: A narrative review" World J Clin Cases. **10**(13), pp. 3981-3988.
Chopra, K. Calva, D. Sosin, M. Tadisina, K.K. Banda, A. Cruz, C.D.L. et al., (2015) "A comprehensive examination of topographic thickness of skin in the human face" Aesthet Surg J. **35**(8), pp. 1007-13:doi:10.1093/asj/sjv079.

Choudhary SV., Jain D., Agrawal P., and Singh A. (2013) “Downling-Degos disease and hidradenitis suppurativa: Co-occurrence or association?” Indian Dermatol Online J. **4**(3), pp. 191-194.
Chouk C., and Litaiem N. (2023) “Erythrokeratodermia Variabilis” StatPearls.
Chu, D.H. (2008). “Overview of biology, development, and structure of skin”. Fitzpatrick’s dermatology in general medicine **7**, pp. 57–73.
Chuang SY., Lin CH., and Fang JY. (2014) “Natural compounds and aging: Between autophagy and inflammasome”. Biomed. Res. Int. pp. e297293.
Cochran AJ. (1069) “Malignant melanoma. A review of 10 years’ experience in Glasgow, Scotland” Cancer, **23,** pp. 1190.
Cochran AJ. (1970) “The incidence of melanocytes in normal human skin” The Journal of Investigative Dermatology. **55**(1).
Cockell CS., and Knowland J. (1999) “Ultraviolet radiation screening compounds”. Biol. Rev. **74**, pp. 311–345. doi: 10.1017/S0006323199005356.
Conteville, L. C. and A. C. P. Vicente (2020). "Skin exposure to sunlight: a factor modulating the human gut microbiome composition." Gut Microbes **11**(5): 1135-1138.
Corani, A., et al. (2014). "Superior Photoprotective Motifs and Mechanisms in Eumelanins Uncovered." Journal of the American Chemical Society **136**(33): 11626-11635.
Cormier M., Johnson B., Ameri M., Nyam K., Libiran I., Zhang DD., and Daddona P. (2004) “Transdermal delivery of desmopressin using a coated microneedle array patch system” J Control Release. **97**, pp. 503-511.
Cormier, J. N., et al. (2006). "Ethnic Differences Among Patients With Cutaneous Melanoma." Archives of Internal Medicine **166**(17): 1907.
Corrêa, HDL., ATO. Raab, TM. Araújo, LA. Deus, AL. Reis, FS. Honorato, et al., (2022). "A systematic review and meta-analysis demonstrating Klotho as an emerging exerkine." Scientific Reports **12**(1).
Cotta-Pereira G, Guerra Rodrigo F, Bittencourt-Sampaio S.(1976) “ Oxytalan, elaunin, and elastic fibers in the human skin”. J Invest Dermatol. 66(3), pp.143-8.
Craik DJ., Fairlie DP., Liras S., and Price D. (2013) “The future of peptide-based drugs”. Chem. Biol. Drug Des. **81**(1), pp.136–147.
Crawford, NGDE., Kelly, MEB., Hansen, MH., Beltrame, S. Fan, SL., Bowman, E. et al., (2017). "Loci associated with skin pigmentation identified in African populations." Science **358**(6365): eaan8433.
Criscito MC., Martires KJ., and Stein JA. (2016) “Prognostic Factors, Treatment,and Survival in Dermatofibrosarcoma Protuberans” JAMA Dermatol. **152**(12), pp. 1365-1371.
Crossland SR., Sairally F., Edwards J., Culmer P., and Brackett CL. (2024) “Mechanical characteristics of diabetic and non-diabetic plantar skin” Journal of the Mechanical Behaviour of Biomedical Materials. **150**, pp. 106279.
Crowther JM., Sieg A., Blenkiron P., Marcott C., Matts PJ., Kaczvinsky JR et al. (2008) “Measuring the effects of topical mosturisers on changes in stratum cornuem thickness, water gradients and hydration in vivo” Br J Dermatol. **159**(3), pp. 567-77.
Crowther JM., Sieg A., Blenkiron P., Margot C., Matts PJ., Kaczvinsky JR., et al. (2008) “Measuring the effects of topical moisturisers on changes in stratum corneum thickness, water gradients and hydration in vivo” Br J Dermatol. **159**(3), pp. 567-77
Cui G., Martin RC., Jin H., Liu X., Pandit H., Zhao H et al. (2018) “Up-regulation of FGF15/19 signaling promotes hepatocellular carcinoma in the background of liver” J Exp Clin Cancer Res. 37(1), pp. 136.
Cylus J. and Tayara LA. (2021) “Health, an ageing labour force, and the economy: Does health moderate the relationship between population age-structure and economic growth?” Soc Sci Med. **287**.

Czekalla C., Schönborn KH., Ladermann J., and Meinke MC. (2019) “Noninvasive Determination of Epidermal and Stratum Corneum Thickness In vivo Using Two-Photon Microscopy and Optical Coherence Tomography: Impact of Body Area, Age, and Gender” Skin Pharmacology and Physiology. **32**(3), pp. 142-150.
D'Orazio, J., et al. (2013). "UV Radiation and the Skin." International Journal of Molecular Sciences **14**(6): 12222-12248.
Dadzie OE., Sturm RA., Fajuyigbe D., Petit A, and Jablonski NG. (2022) “The Eumleanin Human Skin Colour Scale: a proof-of concept study” Global Health and Equity.
Dardano, P., et al. (2015). "A Photolithographic Approach to Polymeric Microneedles Array Fabrication." Materials **8**(12): 8661-8673.
Datta, S., R. Stocek and K. Naskar (2021). "Influence of Ultraviolet Radiation on Mechanical Properties of a Photoinitiator Compounded High Vinyl Styrene–Butadiene–Styrene Block Copolymer." Polymers **13**(8): 1287.
Daunay A., Baudrin LG., Deleuze JF., and How-Kit A. (2019) “Evaluation of six blood-based age prediction models using DNA methylation analysis by pyrosequencing” Scientific Reports. 2019.
Davidson A., Al-Qallaf B., and Das, DB. (2008) “Transdermal drug delivery by coated microneedles: Geometry effects on effective skin thickness and drug permeability”. Chem. Eng. Res. Des. **86**, pp. 1196–1206.
Davies KC., McGrath M., Savage Z., Moser D., and Zahedi. (2020) “Using perforated liners to combat the detrimental effects of excessive sweating in lower limb prosthesis users” Can Propesthet Orthot J. **3**(2), pp. 34610.
Davis, SP., Landis, BJ., Adams, ZH., Allen, MG., and Prausnitz, MR. (2004) “Insertion of microneedles into skin: Measurement and prediction of insertion force and needle fracture force”. J. Biomech. **37**, pp. 1155–1163.
Dawes, S. M., et al. (2016). "Racial disparities in melanoma survival." Journal of the American Academy of Dermatology **75**(5): 983-991.
De Cesar Silva Castro C., and Miot HA. (2018) “Prevalence of citiligo in Brazil – a population survey. Pigment cell Melanoma Res. **31**(3), pp. 448-450.
De Gruijl FR. And van der Leun JC. (2000) “Environment and health: 3. Ozone depletion and ultraviolet radiation” CMAJ. 163(7), pp. 851-855.
De Oliveira, R. M. (2006). "Klotho RNAi induces premature senescence of human cells via a p53/p21 dependent pathway." FEBS Letters **580**(24): 5753-5758.
De-Souza, I. M. F., G. L. N. Vitral and Z. S. N. Reis (2019). "Skin thickness dimensions in histological section measurement during late-fetal and neonatal developmental period: A systematic review." Skin Research and Technology **25**(6): 793-800.
Del Bino S., Ito S., Sok J., Nakaishi Y., Bastien P., Wakamatsu K., and Bernerd F. (2015) “Chemical analysis of constitutive pigmentation of human epidermis reveals constant eumelanin to pheomelanin ratio” Pigment Cell & Melanoma.
Demir, Y. K., Z. Akan and O. Kerimoglu (2013). "Characterization of Polymeric Microneedle Arrays for Transdermal Drug Delivery." PLoS ONE **8**(10): e77289.
Deng, L. and S. Xu (2018). "Adaptation of human skin color in various populations." Hereditas **155**(1).
Dërmaky-Sopjani M., Kolgeci S., Abazi S., and Sopjani M. (2013) “Significance of the anti-aging protein Klotho” Mol Membr Biol. **30**(8), pp. 369-385.
Desai H. (1997) “Ageing and wounds. Part 2: healing in old age”. J Wound Care. 6(5), pp. 237239. https://doi.org/10.12968/jowc.1997.6.5.237
determination of blood and teeth samples using a selected set of DNA methylation markers.

Di Micco R., Krizhanovsky V., Baker D., and d'Adda di Fagagna F. (2021) "Cellular senescence in ageing: From mechanisms to therapeutic opportunities". Nat. Rev. Mol. Cell Biol. 22, pp. 75–95.
Diao JA., and Adamson A., (2022) "Representation and misdiagnosis of dark skin in a large-scale visual diagnostic challenge" Journal of the American Academy of Dermatology. **86**(4), pp.950-951.
Dibbs, R. Grome, L. and Pederson, W. (2019). "Free Tissue Transfer for Upper Extremity Reconstruction." Seminars in Plastic Surgery **33**(01): 017-023.
Diegelmann RF, and Evans MC. (2004) "Wound healing: an overview of acute, fibrotyc and delayed healing". Front Biosci. **1**(9), pp. 283–289.
Diehl M, Wettstein M, Spuling SM, Wurm S. (2021) "Age-Related Change in Self-Perceptions of Aging: Longitudinal Trajectories and Predictors of Change" Psychol Aging, **36**(3), pp.344-359.
Diehm C, Schuster A, Allenberg JR, Darious J, Haberl R, Lange S, et al. (2004) "High prevalence of peripheral arterial disease and co-morbidity in 6880 primary care patients: cross sectional study". Atherosclerosis, 172, pp.95–105.
Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj and O. Pereira-Smith (1995). "A biomarker that identifies senescent human cells in culture and in aging skin in vivo." Proceedings of the National Academy of Sciences **92**(20): 9363-9367.
Dina Y, McKesey J, Pandya AG. (2019) 'Disorders of hypopigmentationn'. J Drugs Dermatol, **18**(3):s115-s116.
Ding X., Kakanj P., Leptin M., and Eming SA. (2021) 'Regulation of the Wound Healing Response during Aging" Skin Repair and Aging.
Diversity In Dermatology: Diversity Committee Approved Plan (2021) 2021–2023.
Dodd RV., Ravi D., Stackhouse AA., Brown CA., Westacott RJ., Meeran K., et al. (2023) "The impact of patient skin colour on diagnostic ability and confidence of medical students" Adv Health Sci Educ Theory Prac. **28**(4), pp. 1171-1189.
Dodig, S., I. Čepelak and I. Pavić (2019). "Hallmarks of senescence and aging." Biochemia medica **29**(3), pp.483-497.
Dogra S, and Bushan K. (2005) "Repigmentatin in vitiligo universalis: Role of melanocytes density, disease duraction, and melanocytic reservoir" Dermatology Online Journal. **11**(3) doi:10.1016/j.ijbiomac.2015.12.002
Donaldson MR, Coldiron BM (2011). "No end in sight: the skin cancer epidemic continues". Semin Cutan Med Surg, **30**(1): 3-5.
Donnelly RF., Garland MJ., Morrow, DI., Migalska, K., Singh, TRR., Majithiya, R., and Woolfson, AD. (2010) "Optical coherence tomography is a valuable tool in the study of the effects of microneedle geometry on skin penetration characteristics and in-skin dissolution". J. Control. Release **147**, pp. 333–341.
Donnelly RF., Morrow DI., Singh TR., Migalska K., McCarron PA., O'Mahony C. and Woolfson AD. (2009) "Processing difficulties and instability of carbohydrate microneedle arrays". Drug Dev. Ind. Pharm. **35**(10), pp. 1242–1254.
Donnelly, R. F., T. R. R. Singh and A. D. Woolfson (2010). "Microneedle-based drug delivery systems: Microfabrication, drug delivery, and safety." Drug Delivery **17**(4): 187-207.
Draganova-Filipova, M., V. Bojilova and P. Zagorchev (2022). "Alzheimer's disease: the hypotheses, known and unknown connections between UV-radiation, mtDNA haplotypes and life span – a review." Folia Medica **64**(6): 878-883.
Drissi M., Carr E., and Housewright C. (2022) "Sunscreen: a brief walk-through history" Proc(Bayl Univ Med Cent), **35**(1), pp. 121-122

Drury, J.L. and Mooney, D.J. (2003) “Hydrogels for tissue engineering: scaffold design variables and applications”. Biomaterials, **24** , pp. 4337 —4351
Du H., Liu P., Zhu J., LAN J., Li Y., Zhang L., et al. (2019) “Hyaluronic Acid-Based Dissolving Patch Loaded with Methotrexate for Impoved Treatment of Psoriasis” ACS Appl Mater Interfaces. **11**(46), pp.43588-43598.
Du Nou¨y PL. (1916) “Cicatrisation of wounds : III. The relation between the age of the patient, the area of the wound, and the index of cicatrisation”. J Exp Med **24**, pp. 461e70
Du, G., Z. Zhang, P. He, Z. Zhang and X. Sun (2021). "Determination of the mechanical properties of polymeric microneedles by micromanipulation." Journal of the Mechanical Behavior of Biomedical Materials **117**: 104384.
Dunn L., Prosser HCG., Tan JTM., Vanags LZ., Ng MKC., and Bursil CA. (2013) “Murine Model of Wound Healing” J Vis Exp. (75), pp. 50265.
Eberlin S., da Silva MS., Facchini G., Da Silva GH., Pinheiro ALTA., Eberlin S., da Silva Pinheiro A. (2020) “The Ex Vivo Skin Model as an Alyernative Tool for the Efficacy and Safety Evaluation of Topical Products” SageJournals. **48**(1),
Eckes B, Nischt R, Krieg T. (2010) “Cell-matrix interactions in dermal repair and scarring”. Fibrogenesis Tissue Repair 3, pp.4.
Edigin, E., S. Kaul, P. O. Eseaton and J. Albrecht (2021). "Racial and Ethnic Distribution of Patients Hospitalized With Hidradenitis Suppurativa, 2008-2018." JAMA Dermatology **157**(9): 1125.
EER Training Modules, *Layers of the skin*. U. S. National Institutes of Health, National Cancer Institute. Day Month Year (2024)
Egelrud T., and Stigbrand T. (1989) “Regional variations in cytokeratin expression in palmo-plantar epidermis” Acta Derm Venereol. **69**, pp. 373-379.
Eichenfield, L. and L. Stein Gold (2017). "Practical Strategies for the Diagnosis and Assessment of Atopic Dermatitis." Seminars in Cutaneous Medicine and Surgery **36**(2S): S36-S38.
Elmets CA., Bergstresser PR., Tigelaar RE., Wood PJ., and Streilein JW. (1983). Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. J. Exp. Med. **158**, pp. 781–794. doi: 10.1084/jem.158.3.781
Eming SA., Martin P., and Tomic-Canic M. (2014) “Wound repair and regeneration: Mechanisms, signaling, and translation” Sci. Transl. Med. **6**(265).
Erem AS. And Razzaque MS. (2021) “Vitamin D-independent benefits of safe sunlight exposure” The Journal of Steroid Biochemistry and Molecular Biology. **213**.
Erickson, C. A. and T. L. Goins (1995). "Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes." Development **121**(3): 915-924.
Espaulella-Ferrer M, Espaulella-Panicot J, Noell-Boix R, Casals-Zorita M, Ferrer-Sola M, Puigoriol-Juvanteny E, Cullel-Dalmau M, Otero-Viñas M. (2021). "Assessment of frailty in elderly patients attending a multidisciplinary wound care centre: a cohort study." BMC Geriatrics **21**(1).
Espaulella-Ferrer M., Espaulella-Panicot J., Noell-Boix R., Casals-Zortia M., Ferrer-Sola M., Puigoriol-Juvanteny E., Cullell-Dalamu M., and Otero-Viñas M. (2021) “Assessment of frailty in elderly patients attending a multidisciplinary wound care centre: a cohort study” BMC Geriatrics. **21**.
Espaulella-Ferrer, M., et al. (2021). "Assessment of frailty in elderly patients attending a multidisciplinary wound care centre: a cohort study." BMC Geriatrics **21**(1).
Esquivel, D., Mishra, R., Srivastava, A. (2020) “Stem Cell Therapy Offers a Possible Safe and Promising Alternative Approach for Treating Vitiligo: A Review” Curr Pharm Des. **26**(37), pp. 4815-4821, doi:10.2174/1381612826666200730221446.

Ezzedine K, Silverberg N. (2016) “A practical approach to the diagnosis and treatment of vitiligo in children”. Pediatrics, **138**(1):e20154126. doi:10.1542/peds.2015-4126
Fagot D, Asselineau D, Bernerd F. (2004) “Matrix metalloproteinase-1 production observed after solar-simulated radiation exposure is assumed by dermal fibroblasts but involves a paracrine activation through epidermal keratinocytes”. Photochem. Photobiol. **79**, pp.499–506.
Fajuyigbe, D. and A. R. Young (2016). "The impact of skin colour on human photobiological responses." Pigment Cell & Melanoma Research **29**(6): 607-618.
Fakeih E., Aguirre-Pablo AA., Thoroddsen ST., and Slama KN. (2023) “Fabrication and Characterisation of Porous Microneedles for Enhanced Fluid Injection and Suction: A Two-Photo Polymerization Approach” Advanced Engineering Materials.
Falanga V., Faria K., and Bollenbach T. (2014) “Chapter 77- Bioengineered Skin Constructs” Principles of Tissue Engineering (Fourth Edition), pp. 1619-1643.
Farage MA., Miller KW., Elsner P. and Maibach HI. (2008) “Intrinsic and extrinsic factors in skin ageing: a review” International Journal of Cosmetic Science. **30**(2), pp. 87-95
Farage MA., Miller KW., Elsner P., and Maibach HW. (2013) “Characteristics of the Aging Skin” Adv Wound Care (New Rochelle). **2**(1), pp. 5-10.
Farrar MD., Kift R., Felton SJ., Berry JL., Durkin MT., Allan D., et al., (2011). “Recommended summer sunlight exposure levels fail to produce sufficient vitamin D status in UK adults of South Asian origin”. *Am. J. Clin. Nutr.* **94**, pp.1219–1224. doi: 10.3945/ajcn.111.019976.
Federico JR., and Krishnamurthy K. (2023) “Albinism” StatPearls.
Feigin VL., Vos T., Nichols E., O Owolabi M., Carroll WM., Dichgans M., Deuschl G., Parmar P., Brainin M., and Murray C. (2019) “The global burden of neurological disorders: Translating evidence into policy”. Lancet Neurol. **19**, pp. 255–265. doi: 10.1016/S1474-4422(19)30411-9.
Feng Y-X., Hu H., Wong Y-Y., Yao X., and He M-L. (2023) “Microneedles: An emerging Vaccine Delivery Tool and a Prospective Solution to the Challenges of SARS-CoV-2 Mass Vaccination” Pharamceutics. **15**(5), pp.1349.
Feng, X., G.-Y. Li, A. Ramier, A. M. Eltony and S.-H. Yun (2022). "In vivo stiffness measurement of epidermis, dermis, and hypodermis using broadband Rayleigh-wave optical coherence elastography." Acta Biomaterialia **146**: 295-305.
Feng, Y. and Y. Lu (2022). "Advances in vitiligo: Update on therapeutic targets." Frontiers in Immunology **13**.
Fenner J, Silverberg NB. (2018) “Skin diseases associated with atopic dermatitis” Clinics in Dermatology. **36**(5)doi: https://doi.org/10.1016/j.clindermatol.2018.05.004.
Fernandez A., O’Leary C., O’Byrne KJ., Burgess J., Richard DJ., and Suraweera A. (2021) “Epigentic Mechanisms in DNA Double Strand Break Repair: A Clinical Review” Front. Mol. Biosci. **8**.
Fischer ER., Hansen BT., Nair V., Hoyt FH., and Dorward DW. (2012). "Scanning Electron Microscopy." Current Protocols in Microbiology **25**(1).
Fisher GJ., Quan T., Purohit T., Shao Y., Cho MK., He T., Varani J., Kang S., and Voorhees JJ. (2009) “Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase_1 in fibroblasts in aged human skin” Am. J. Pathol. **174**, pp. 101-114.
Fitsiou, E., T. Pulido, J. Campisi, F. Alimirah and M. Demaria (2021). "Cellular Senescence and the Senescence-Associated Secretory Phenotype as Drivers of Skin Photoaging." Journal of Investigative Dermatology **141**(4): 1119-1126.
Fitzgerald KN., Hodges R., Hanes D., Stack E., Cheishvil D., Szyf M., et al. (2021) “Potential reversal of epigenetic age using a diet and lifestyle intervention: a pilot randomized clinical trial” Aging (Albany NY). **13**(7), pp. 9419-9432.
Fitzpatric TB. (1988) “The validity and practicality of sun-reactive skin types I through VI” Arch Dermarol. **124**(6), pp. 869-71:doi:10.1001/archderm.124.6.869.

Fitzpatrick TB, Breathnach AS. (1963) 'The Epidermal Melanin Unit System" Dermatol Wochenschr. **18**(147), pp. 481-9.
Fitzpatrick TB, Breathnach AS. (1963) "The epidermal melanin unit system". Dermatol Wochenschr. **147**, pp. 481–9.
Fitzpatrick THB, Szabo G, and Mitchell RE. (1965) "Age changes in the human melanocyte system." Advances in biology of skin **6**: pp.35-50.
Flament, F., et al. (2013). "Effect of the sun on visible clinical signs of aging in Caucasian skin." Clinical, Cosmetic and Investigational Dermatology: 221.
Flores I., Cayuela ML., and Blasco MA. "Effects of telomerase and telomere length on epidermal stem cell behavior" Science. **309**, pp. 1253-1256.
Foote AQ., Wang Z., Kendziorski C., and Thibeault SL. (2019) "Tissue specific human fibroblast differential expression based on RNA sequencing analysis" BMC Genomics**. 20.**
Fosgerau K, Hoffmann T. (2014) "Peptide therapeutics: current status and future directions" Drug Discovery Today, **20**(1), pp. 122-128:doi: https://doi.org/10.1016/j.drudis.2014.10.003
Fowkes FGR, Housley E, Cawood EHH, Macintyre CCA, Ruckley CV, Prescott RJ. (1991) "Edinburgh Artery Study: prevalence of asymptomatic and symptomatic peripheral arterial disease in the general population". Int J Epidemiol 326, pp.381–386.
Frantz C, Stewart KM, Weaver VM. (2010) "The extracellular matrix at a glance". J Cell Sci **123**, pp.4195–4200.
Frasca D, Saada YB, Garcia D, Friguet B. (2021) "Effects of cellulcar senescence on metabolic pathways in non-immune and immune cells" Mechanisms of Ageing and Development. 194, pp. 1111428, doi: https://doi.org/10.1016/j.mad.2020.111428
Freeman, MB., DM. Holman, J. Qin and NB. Lunsford (2019). "Merkel cell carcinoma incidence, trends, and survival rates among adults aged ≥50 years from United States Cancer Statistics." Journal of the American Academy of Dermatology **80**(4): 1154-1156.
Freije A., Molineuvo R., Ceballos L., Cagigas M., Alonso-Lecue P., Rodriguez R. (2014) "Inactivation of p53 in Human Keratinocytes Leads to Squamous Differentiation and Shedding via Replication Stress and Mitotic Slippage" Cell Rep. **9**(4) pp. 1349-60.
Freund A., Laberge R-M., Demaria M., and Campisi. (2012) "Lamin B1 loss is a senescence-associated biomarker" Mol Biol Cell. **23**(11), pp. 2066-2075.
Freund, A., R.-M. Laberge, M. Demaria and J. Campisi (2012). "Lamin B1 loss is a senescence-associated biomarker." Molecular Biology of the Cell **23**(11): 2066-2075.
Frommer, M., L. E. McDonald, D. S. Millar, C. M. Collis, F. Watt, G. W. Grigg, P. L. Molloy and C. L. Paul (1992). "A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands." Proceedings of the National Academy of Sciences **89**(5): 1827-1831.
Fu DJ., Thomson C., Lunny DP., Dopping-Hepenstal PJ., McGrath JA., Smith FJD., Irawin McLean WH., and Pedrioli L. (2014) "Keratin 9 is required for the structural integrity and terminal differentiation of the palmoplantar epidermis" J. Invest. Dermatol. **134**, pp. 754-763.
Fu, T., Keiser E., Linos E., Rotatori RM., Sainani K., Lingala B et al., (2014). "Eczema and Sensitization to Common Allergens in the United States: A Multiethnic, Population-Based Study." Pediatric Dermatology **31**(1): 21-26.
Fu, TE. Keiser, E. Linos, RM. Rotatori, K. Sainani, B. Lingala, AT. et al., (2014). "Eczema and Sensitization to Common Allergens in the United States: A Multiethnic, Population-Based Study." Pediatric Dermatology **31**(1): 21-26.
Fuchs C., Stalnaker KJ., Dalgard CL., Sukumar G., Hupalo D., Dreyfuss JM et al. (2024) "Plantar skin exhibits altered physiology, constitutive activation of wound-associated phenotypes, and inherently delayed healing" J Invest Dermatol. **16**(24).

Fuller BB, Spaulding DT, and Smith DR (2001). “Regulation of the catalytic activity of preexisting tyrosinase in black and Caucasian human melanocyte cell cultures”. Experimental Cell Research, **262**, pp. 197–208. 10.1006/excr.2000.509
Gallo, R. L. (2017). "Human Skin Is the Largest Epithelial Surface for Interaction with Microbes." Journal of Investigative Dermatology **137**(6): 1213-1214.
Ganceviciene, R., A. I. Liakou, A. Theodoridis, E. Makrantonaki and C. C. Zouboulis (2012). "Skin anti-aging strategies." Dermato-Endocrinology **4**(3): 308-319.
Ganeson K., Alias AH., Murugaiyah V., Amirul A-AA., Ramakrishna S., and Vigneswari S. (2023) “Microneedles for Efficient and Precise Drug Delivery in Cance Therapy” Pharmaceutics. **15**(3), pp. 744.
Gao FL, Jin R, Zhang L, Zhang YG. (2013) “The contribution of melanocytes to pathological scar formation during wound healing” Int J Clin Exp Med, **6**(7), pp.609-13.
Gao, L., X. Liu, X. Luo, X. Lou, P. Li, X. Li and X. Liu (2023). "Antiaging effects of dietary supplements and natural products." Frontiers in Pharmacology **14**.
Gao, Y., M. Hou, R. Yang, L. Zhang, Z. Xu, Y. Kang and P. Xue (2018). "PEGDA/PVP Microneedles with Tailorable Matrix Constitutions for Controllable Transdermal Drug Delivery." Macromolecular Materials and Engineering **303**(12): 1800233.
Garagnani P., Bacalini MG., Pirazzini C., Gori D, Giuliani C., Mari D et al., (2012) “Methylation of ELOVL2 gene as a new epigenetic marker of age” Aging Cell. **11**(6), pp. 1132-1134.
Garrido, A. M., A. Kaistha, A. K. Uryga, S. Oc, K. Foote, A. Shah, A. Finigan, N. Figg, L. Dobnikar, H. Jørgensen and M. Bennett (2021). "Efficacy and limitations of senolysis in atherosclerosis." Cardiovascular Research.
Garza LA., Yang CC., Zhao T., Blatt HB., Lee M., He H., et al., (2011) “Bald scalp in men with androgenetic slope is retains hair follicle stem cells but lacks CD200-rich and CD34-positive hair follicle progenitor cells” J Clin Invest. **121**, pp. 613-622.
Gelfand JM., Weinstein R., Porter SB., Neimann AL., Berlin JA., and Margolis DJ. (2005) “Prevalence and treatment of psoriasis in the United Kingdom: a population-based study” Arch Dermatol. **141**(12), pp. 1537-41.
Geoffrey K., Mwangi AN., Maru SM. (2019) “Sunscreen products: rationale for use, formulation development and regulatory considerations” Saudi Pharm J. **27**, pp. 1009-1018.
Gerstein AD, Phillips TJ, Rogers GS, Gilchrest BA. (1993) “Wound healing and aging” Dermatol Clin. **11**(4), pp. 749-57.
Ghazarian A., Garner WL., and Ehrlich PH. (2000) “Memory of Past Exposure to the Chemokine IL-8 Inhibits the Contraction of Fibroblast-Populated Collagen Lattices” Experimental and Molecular Pathology. **69**, pp. 242-247.
Ghissassi F.E, Baan R, Straif K, Grosse Y, Secretan B, Bouvard V. (2009) “A review of human carcinogens – Part D: Radiation” The Lancet. **10**(8), pp. 751-752.
Giangreco A., Goldie SJ., Faila V., Saintigny G., and FM Watt. (2010) “Human Skin Aging is Associated with Reduced Expression of the Stem Cell markers B1 Integrin and MCSP” JID. **130**(2), pp. 604-608.
Giannakopoulos AE., and Panagiotopoulos DI. (2009) “Conical indentation of incompressible rubber-like material” International Journal of solids and structures. **46**(6), pp. 1436-1447
Gilaberte Y, Prieto-Torres L, Pastushenko I, Juarranz Á. (2016) “Anatomy and Function of the Skin”. Nanoscience in Dermatology, pp. 1–14.
Gilchrest BA. (1983) “In vitro assessment of keratinocyte aging”. J Invest Dermatol, 81(1), S184–S189. https://doi.org/10.1111/1523-1747.ep12541084
Gill, H.S. and Prausnitz, M.R. (2006) “Coated microneedles for transdermal delivery” J Control Release. **117**(2), pp.227-37, doi: 10.1016/j.jconrel.2006.10.017

Gill, HS., Denson, DD., Burris, BAB., and Prausnitz, MR. (2008) “Effect of Microneedle Design on Pain in Human Volunteers”. Clin. J. Pain **24**, pp. 585–594
Gittard SD., Chen B., Xu H., Ovsianikov A., Chichkov BN., Monteiro-Riviere NA., and Narayan RJ. (2013) “The Effects of Geomtry on Skin Penetration and Failure of Polymer Microneedles” J Adhes Sci Technol. **27**(3), pp. 227-243.
Gittard, SD., Chen, B., Xu, H., Ovsianikov, A., Chichkov, BN., Monteiro-Riviere, NA., and Narayan, RJ. (2013) “The effects of geometry on skin penetration and failure of polymer microneedles”. J. Adhes. Sci. Technol. **27**, pp. 227–243.
Givler DN, Basit H, Givler A. (2020) “Pityriasis Alba” StatPearls.
Glat PM, and Longaker MT. (1997) “Wound healing. In: Aston SJ, Beasley RW, Thorne CH, editors”. Grabb and Smith’s plastic surgery. 5th. Lippencot; pp. 3–12.
Gloster HM, Neal K. (2006) “Skin cancer in skin of color” Journal of the American Academy of Dermatology. **55**(5), pp.741-760
Goldman DP., Cutler DM., Shekelle PG., Bhattacharya J., Shang B., Joyce GF. Et al., (2008) “Modeling the Health and Medical Care Spending of the Future Elderly” RAND Corporation.
Goldman, D. P., C. Chen, J. Zissimopoulos, J. W. Rowe, J. W. Rowe, T. Antonucci, L. Berkman, A. Borsch Supan, L. Carstensen, D. P. Goldman, L. Fried, F. Furstenberg, J. Jackson, M. Kohli, J. Olshansky, D. Rehkopf, J. Rother and J. Zissimopoulos (2018). "Measuring how countries adapt to societal aging." Proceedings of the National Academy of Sciences **115**(3): 435-437.
Goldschmidt H., and Kligman AM. (1963) “Quantitative Estimation of Kerain Production by the Epidermis” Arch Dermatol. **88**, pp. 709-712.
Gomaa, Y.A. El-Khordagui, L.K. Garland, M.J. Donnelly, R.F. McInnes, F. and Meidan, V.M. (2012). "Effect of microneedle treatment on the skin permeation of a nanoencapsulated dye." Journal of Pharmacy and Pharmacology **64**(11): 1592-1602.
Gonzalez-Lima F and Barrett DW. (2014) “Augmentation of cognitive brain functions with transcranial lasers” Front. Syst. Neurosci. **8**.
Goodson WH 3rd., and Hunt TK. (1979) “Wound healing and aging”. J Invest Dermatol **73** pp. 88-91.
Goon, P., et al. (2021). "Skin cancers in skin types IV–VI: Does the Fitzpatrick scale give a false sense of security?" Skin Health and Disease **1**(3).
Gorbatenko A., Olesen CW., Boedtkjer E., and Pederson SF. (2014) “Regulation and roles of bicarbonate transporters in cancer” Front Physiol. **5**(130**)**.
Gordon L., Olsen C., Whiteman DC., Elliott TM., Janda M., and Green A. (2019) “Prevention versus early detection for long-term control of melanoma and keratinocyte carcinomas: a cost-effectiveness modelling study” BMJ Open. **10.**
Gordon LG, Elliott TM, Wright CY, Deghaye N, Visser W. (2016) “Modelling the healthcare costs of skin cancer in South Africa”. BMC Health Serv Res. **16**:113. doi:10.1186/s12913-016-1364-z
Gosain A., and DiPetro LA. (2004) “Aging and Wound Healing” World J. Surg. **28**, pp. 321-326.
Gosain A., and DiPietro LA (2004) “Aging and wound healing” World J Surg. **28**, pp. 321-326.
Gould, L., P. Abadir, H. Brem, M. Carter, T. Conner-Kerr, J. Davidson, et al., (2015). "Chronic Wound Repair and Healing in Older Adults: Current Status and Future Research." Journal of the American Geriatrics Society **63**(3): 427-438.
Grant WB. (2008) “The effect of solar UVB doses and vitamin D production, skin cancer action spectra, and smoking I explaining links between skin cancers and solid tumours” Eur. J Cancer. **44**(1), pp.12-5:doi:10.1016/j.ejca.2007.09.009

Greaves, M. (2014). "Was skin cancer a selective force for black pigmentation in early hominin evolution?" Proceedings of the Royal Society B: Biological Sciences **281**(1781): 20132955.
Green EM, Mansfield JC, Bell JS, Winlove CP. (2014) "The structure and micromechanics of elastic tissue". Interface Focus. 4(2), pp. 20130058.
Griffiths TW., Watson REB., and Langton AK. (2023) "Skin ageing and topical rejuvenation strategies" British Journal of Dermatology. **189**, pp. 17-23.
Grimes PE. (2009) "Management of hyperpigmentation in darker racial ethnic groups" Semin Cutan Med Surg. **28**(2), pp.77-85:doi: 10.1016/j.sder.2009.04.001
Grob A, and Grekin S. (2016) "Piebaldism in children". Paediatric Dermatology. **97**(2).
Gromkowska-Kępka, K. J., A. Puścion-Jakubik, R. Markiewicz-Żukowska and K. Socha (2021). "The impact of ultraviolet radiation on skin photoaging — review of in vitro studies." Journal of Cosmetic Dermatology **20**(11): 3427-3431.
Grønskov, K., J. Ek and K. Brondum-Nielsen (2007). "Oculocutaneous albinism." Orphanet Journal of Rare Diseases **2**(1): 43.
Grove GL., and Kligman AM. (1983) "Age-associated changes in human epidermal cell renewal" J Gerontol. **38**(2), pp. 137-42.
Groves RB., Coulman SA., Birchall JC., and Evans SL. (2013) "An anisotropic, hyperelastic model for skin: Experimental measurements, finite element modelling and identification of parameters for human and murine skin" Journal of mechanical behaviour of biomedical materials. **18**, pp.167-180.
Gruijl FRD, Kranen HJV, Mullenders LH. (2001) "UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer" J Photocem Photobiol B. **63**(1-3), pp. 19-27:doi: 10.1016/s1011-1344(01)00199-3.
Gu L-H., and Coulombe PA. (2007) "Keratin function in skin eptihelia: a broadening palette with surprising shades" Curr Opin Cell Biol. **19**(1), pp. 13-23.
Gudjohnsen SA, Atacho DA, Gesbert F, Raposo G, Hurbain I, Larue L, et al., (2015) "Meningeal Melanocytes in the Mouse: Distribution and Dependence on Mitf". Front Neuroanat. **25**(9), pp.49. doi:10.3389/fnana.2015.00149.
Guest JF., Ayoub N., McIlwraith T., Uchtegbu I., Gerrish A., Weidlich D., Vowden K., and Vowden P. (2015) "Health economic burden that wounds impose on the National Health Service in the UK". BMJ Open. **5**(12):e009283. https:// doi.org/10.1136/bmjopen-2015-009283
Guest JF., Vowden K., and Vowden P. (2017) "The health economic burden that acute and chronic wounds impose on an average clinical commissioning group/health board in the UK". J Wound Care. **26**(6), pp.292–303. https://doi.org/10.12968/ jowc.2017.26.6.292
Guest, J. F., G. W. Fuller and P. Vowden (2020). "Cohort study evaluating the burden of wounds to the UK's National Health Service in 2017/2018: update from 2012/2013." BMJ Open **10**(12): e045253.
Guillot AJ, Cordeiro AS, Donnelly RF, Montesinos C. (2020) "Microneedle-Based Delivery: An Overview of Current Applications and Trends" Pharmacuetics. **12**(6), pp.569:doi: 10.3390/pharmaceutics12060569
Gunowa NO., Hutchinson M., Brooke J., and Jackson D. (2018). "Pressure injuries in people with darker skin tones: A literature review." Journal of Clinical Nursing **27**(17-18), pp. 3266-3275.
Guo S., and DiPietro LA. (2010) "Factors Affecting Wound Healing" J Dent Res. **89**(3), pp. 219-229.
Gupta A., Singh AP., Singh VK., Singh PR., Jaiswal J., Kumari N., Upadhye V., Singh SC., and Sinha RP. (2023) "Natural sun-screening compounds and DNA-repair enzymes: Photoprotection and photoaging". Catalysts. **13**, pp. 745. doi: 10.3390/catal13040745.]

Gupta AK., Bharadwaj M., and Mehrotra R. (2016) "Skin Cancer Concerns in People of Colour" Risk Factors and Prevention" Asian Pac J Cancer Prev. **17**(12), pp. 5257-5264
Gupta N., and Ruiz ES. (2022) "Current Perspectives in the Treatment of Locally Advanced Basal Cell Carcinoma" Dovepress, pp.183-190.
Gupta N., Chakrobarty A., Raman G., and Banerjee G. (2006) "Cloning and identification of EDD gene from ultraviolet-irradiated HaCaT cells". Photodermatol. Photoimmunol. Photomed. **22**, pp.278–284
Guy RH. and Hadgraft, J. (2003) "Transdermal Drug Delivery" Nat. Biotechnol. **26**(11), pp. 1261-1268.
Gwak H., Cho S., Song Y-J., Park J-H., and Seo S. (2023) "A study on the fabrication of metal microneedle array electrodies for ECG detection based on low melting point Bi-In_Sn alloys" Scientific Reports. 13
Gyi DE and Porter JM. (1999) "Interface pressure and the prediction of car seat discomfort" Appl. Ergon. 30, pp. 99-107.
H D V. MCallister, MG. Allen and MR. Prausnitz , (1988) J. Pharm. Sci., **87**(922)
Hahnel E, Lichterfeld A, Blume-Peytavi U and Kottner J. (2016) "The epidemiology of skin conditions in the aged systematic review" J Tissue Viability. **26**(1), pp:20-38:doi: 10.1016/j.jtv.2016.04.001.
Haj-Ahmad, R., H. Khan, M. Arshad, M. Rasekh, A. Hussain, S. Walsh, X. Li, M.-W. Chang and Z. Ahmad (2015). "Microneedle Coating Techniques for Transdermal Drug Delivery." Pharmaceutics **7**(4): 486-502.
Halaweh, H., S. Dahlin-Ivanoff, U. Svantesson and C. Willén (2018). "Perspectives of Older Adults on Aging Well: A Focus Group Study." Journal of Aging Research **2018**: 1-9.
Halder RM, Bridgeman-Shah S. (1995) "Skin Cancer in African Americans", Cancer, **75,** pp. 667-673.
Halder RM, Nootheti PK. (2003) "Ethnic skin disorders overview" J Am Acad Dermatol. **48**(6),pp.143-8:doi:10.1067/mjd.2003.274
Halder, RM., Bridgerman. Skin cancer in African Americans.1995(75) pp. 667-673
Han C, Yang H, Wang Z. (2020) "Exogenous Pyruvate facilitates ultraviolet induced DNA damage repair by H3K9 acetylation in keratinocyte melanocytes" Biomedicine & Pharmacotherapy. **126,** doi: https://doi.org/10.1016/j.biopha.2020.110082
Han T., and Das DB. (2015) "Potential of Combined Ultrasound and Microneedles for Enhanced Transdermal Drug Permeation: A Review". Eur. J. Pharm. Biopharm. **89**, pp. 312–328.
Hannum G., Guinney J., Zhao L., Zhang L., Hudges G., Sadda S., et al. (2013) "Genome-wide Methylation Profiles Reveal Quantitiative Views of Human Aging Rates" Molecular Cell Resource.
Hao X, Billings SD, Wu F, Stultz TW, Procop GW, Mirkin G, and Vidimos AT. (2020) "Dermatofibrosarcoma Protuberans: Update on the Diagnosis and Treatment" J Clin Med. **9**(6), pp.1752: doi: 10.3390/jcm9061752
Hara Y., Masuda Y., Hirao T., and Yoshikawa N. (2013) "The relationship between the Young's modulus of the stratum corneum and age: a pilot study" Skin Res Technol. **19**(3), pp. 339-45.
Harel N., Ogen-Shtern N., Reshef L., Biran D., Ron EZ., and Gophna U. (2023) "Skin microbiome bacteria enriched following long sun exposure can reduce oxidative damage" Research in Microbiology. **174**(8), pp. 104138.
Harms S, Bliss DZ, Garrard J, Cunanan K, Savik K, Gurvich O, et al.,. (2014). "Prevalence of pressure ulcers by race and ethnicity for older adults admitted to nursing homes". Journal of Gerontological Nursing, **40**, pp.20– 26.
Harris SS. (2006) "Vitamin D and African Americans" J Nutr. **136**, pp. 1126-1129.

Hart PH, Gorman S. (2013) “Exposure to UV Wavelengths in Sunlight Suppresses Immunity. To What Extent is UV-induced Vitamin D3 the Mediator Responsible?” Clin Biochem Rev. **34**(1), pp.3-13.
Hartl, F. U., A. Bracher and M. Hayer-Hartl (2011). "Molecular chaperones in protein folding and proteostasis." Nature **475**(7356): 324-332.
Hautier-Mazereeuw and Tournier E. (2015) “Erythrokeratoderma variabilis progressiva” The portal for rare diseases and orphan drugs.
Hayflick L., and Moorhead PS. (1961) “The Serial Cultivation of Human Diploid Cell Strains”. Experimental Cell Research, 25, pp. 585-621.https://doi.org/10.1016/0014-4827(61)90192-6
He X., Liu J., Liu B., and Shi J. (2021) “The use of DNA methylation clock in aging research” Exp Biol Med (Maywood), **246**(4), pp. 436-446.
He, X., F. Wan, W. Su and W. Xie (2023). "Research Progress on Skin Aging and Active Ingredients." Molecules **28**(14): 5556.
Hearing VJ. (2005) “Biogenesis of pigment granules: a sensitive way to regulate melanocyte function”. Journal of Dermatology Science, **37**(1):doi:10.1016/j.jdermsci.2004.08.014.
Heistein JB., Acharya U., and Mukkamalia SKR. (2023) “Malignant Melanoma” StatPearls.
Hennessy, A., Oh C., Diffey B., Wakamatsu K., Ito S., and Rees J. (2005). "Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation." Pigment Cell Research **18**(3): 220-223.
Herman, J. R. (2010). "Global increase in UV irradiance during the past 30 years (1979–2008) estimated from satellite data." Journal of Geophysical Research: Atmospheres **115**(D4).
Herranz N., and Gil J. (2018) “Mechanisms and functions of cellular senescence”. J. Clin. Investig. 128, pp. 1238–1246.
Herter F, Ninkovic M, Ninkovic M. (2007) “Rational flap selection and timing for coverage of complex upper extremity trauma”. J Plast Reconstr Aesthet Surg, 60(07), pp.760–768.
Hezaveh, H. and Muhamad, I.I. (2013) “Controlled drug release via minimization of burst release in pH-responsive kappa-carrageenan/polyvinyl alcohol hydrogels. Chem. Eng. Res. Des. **91**, pp. 508-519, doi:10.1016/j.cherd.2012.08.014.
Hien, L. X., D. M. Thanh and N. H. Tai (2018). "Influence of Darocur 1173 photoinitiator on the photocrosslinking and properties of UV-cured coatings based on an epoxidiacrylate oligomer and hexanediol diacrylate monomer." Vietnam Journal of Chemistry **56**(6): 761-766.
Highsmith, M.J. Kahle, J.T. Klenow, T.D. Andrews, C.R. Lewis, K.L. Bradley, R.C. et al. (2016). "Interventions to Manage Residual Limb Ulceration Due To Prosthetic Use in Individuals with Lower Extremity Amputation: A Systematic Review of the Literature." Technology & Innovation **18**(2): 115-123.
Hinz B., and Lagares D. (2019) “Evasion of apoptisis by myofibroblasts: a hallmark of fibrotic diseases” nature reviews rheumatology. **16**, pp. 11-31.
Hiraishi, Y., S. Nandakumar, S.-O. Choi, JW. Lee, Y.-C. Kim, JE. Posey, SB. Sable and MR. Prausnitz (2011). "Bacillus Calmette-Guérin vaccination using a microneedle patch." Vaccine **29**(14): 2626-2636.
Hirano, S. (1990). "Observations on pigment granules in the bones of silky fowls." Archives of Histology and Cytology **53**(1): 89-93.
Hoang DM., Pham PT., Bach TQ., Ngo ATL., Nguyen QT., Phan TTK et al., (2022) “Stem cell-based therapy for human diseases: Signal Transduction and Targeted Therap”> 7, 272.
Hoist VA., marshall CE., Moskaluk CA., Frierson HF. (1999) “KIT protein expression and analysis of c-kit gene mutation in adenoid cystic carcinoma” Mod Pathol. **12**(10), pp. 956-60

Hong, E. S., H. Zeeb and M. H. Repacholi (2006). "Albinism in Africa as a public health issue." BMC Public Health **6**(1).
Hu MC., Shiizaki K., Kuro-o M., Moe OW. (2013) "Fibroblast growth factor 23 and klotho: physiology and pathophysiology of an endocrine network of mineral metabolism". Annu Rev Physiol. **75**, pp. 503-533.
Huang C., Murphy GF., Akaishi S., and Ogawa R. (2013) "Keloids and hypertrophic scars: Update and Future Directions" Plas Reconstr Surg Glob Open. **1**(4), pp. e25.
Huang, C., Z. Wu, Y. Du and R. Ogawa (2020). "The Epidemiology of Keloids", Springer International Publishing: pp.29-35.
Huang, W., L. J. Hickson, A. Eirin, J. L. Kirkland and L. O. Lerman (2022). "Cellular senescence: the good, the bad and the unknown." Nature Reviews Nephrology **18**(10): 611-627.
Hughes AL., Kelley J., and Klose RJ. (2020) "Understanding the interplay between CpG island-associated gene promoters and H3K4 methylation" Biochim Biphys Acta Gene Regul Mech. **1863**(8), pp. 194567.
Hughes, M. L. and D. R. Touron (2021). "Aging in Context: Incorporating Everyday Experiences Into the Study of Subjective Age." Frontiers in Psychiatry **12**.
Hurbain, IM. Romao, P. Sextius, E. Bourreau, C. Marchal, F. Bernerd, C et al., (2018). "Melanosome Distribution in Keratinocytes in Different Skin Types: Melanosome Clusters Are Not Degradative Organelles." Journal of Investigative Dermatology **138**(3): 647-656.
Hurford MT., and Sebastiano C. (2008) "Hermansky-Pudlak Syndrome: Report of a Case and Review of the Literature" Int J Clin Exp Pathol. **1**(6), pp. 550-554
Hussain, M., S. Krishnamurthy, J. Patel, E. Kim, B. A. Baptiste, D. L. Croteau and V. A. Bohr (2021). "Skin Abnormalities in Disorders with DNA Repair Defects, Premature Aging, and Mitochondrial Dysfunction." Journal of Investigative Dermatology **141**(4): 968-975.
Ikushima M., Rakugi H., Ishikawa K., Maekawa Y., Yamamoto K., Ohta J., et al. (2006) "Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells" Biochem Biphys Res Commun. **339**(3), pp. 827-32.
Ikushima, M., H. Rakugi, K. Ishikawa, Y. Maekawa, K. Yamamoto, J. Ohta, Y. Chihara, I. Imokawa, G. (2019). "Melanocyte Activation Mechanisms and Rational Therapeutic Treatments of Solar Lentigos." International Journal of Molecular Sciences **20**(15): 3666.
Ingrole, R.S.J. and Gill, H.S. (2019) "Microneedle Coating Methods: A Review with a Perspective" The Journal of Pharmacoloy and Experimental Therapeutics. **370**(3), pp. 555-569.
Iocono JA., Colleran KR., Remick DG., Gillespie BW., Ehrlich HP., and Garner WL. (2000) "Interleukin-8 levels and activity in delayed-healing human thermal wounds" Wound Repair Regen. **8**(3), pp. 216-25.
Ippagunta SK., Gangwar R., Finkelstein D., and Hacker H. (2016) "Keratinocytes contribute intrinsically to psorasis upon loss of Tnip1 function" Biological sciences.
Ita K. (2014) "Transdermal Drug Delivery: Progress and Challenges". J. Drug Deliv. Sci. Technol. **24** pp. 245–250
Ita K. (2018) "Ceramic microneedles and hollow microneedles for transdermal drug delivery: Two decades of research". J. Drug Deliv. Sci. Technol. 44, pp.314–322.
doi: 10.1016/j.jddst.2018.01.004.
Itahana, K., Y. Itahana and G. P. Dimri (2013). Colorimetric Detection of Senescence-Associated β Galactosidase, Humana Press**:** 143-156.
Ito S., Fujimori T., Hayashizaki Y., Nabeshima Y. (2002) "Identification of a novel mouse membrane-bound family 1 glycosidase-like protein, which carries an atypical active site structure" Biochim Biophys Acta BBA Gene Struct Expr. **1576**. Pp. 341-345.

Ito S., Kinoshita S., Shiraishi N., Nakagawa S., Sekine S., Fujimori T., et al. (2000) "Molecular cloning and expression analyses of mouse Bklotho, which encodes a novel klotho family protein. Mech Dev. **98**, pp. 115-119.
Ito S., Wakamatsu K., and Sarna T. (2017) "Photodegradation of Eumelanin and Pheomelanin and its Pathophysiological Implications" Photochemistry and Photobiology.
Ito, S. and K. Wakamatsu (2003). "Quantitative Analysis of Eumelanin and Pheomelanin in Humans, Mice, and Other Animals: a Comparative Review." Pigment Cell Research **16**(5): 523-531.
Itoh Y. (2015) "Membrane-type matrix metalloproteinases: Their functions and regulations" Matrix Biology. **44-46**, pp. 207-223.
Jablonski NG. The evolution of human skin and skin color. *Annu Rev Anthropol.* 2004;**33**:585–623. doi: 10.1146/annurev.anthro.33.070203.143955.
Jablonski, D., et al. (2017). "Shaping the Latitudinal Diversity Gradient: New Perspectives from a Synthesis of Paleobiology and Biogeography." The American Naturalist **189**(1): 1-12.
James, SL., Abate, D., Abate, KH., Abay, SM., Abbafati, C., Abbasi, N., et al. (2018). "Global, Regional, and National Incidence, Prevalence, and Years Lived With Disability for 354 Diseases and Injuries for 195 Countries and Territories, 1990–2017: A Systematic Analysis for the Global Burden of Disease Study 2017". Lancet **392** (10159), pp. 1789–1858. doi: 10.1016/S0140-6736(18)32279-7
Jameson, CK. A. Boulton, N. Silove and AJ. Guastella (2022). "Eczema and related atopic diseases are associated with increased symptom severity in children with autism spectrum disorder." Translational Psychiatry **12**(1).
Janjua SA, Khachemoune A, and Guldbakke KK. (2007) "Piebaldism: A Case Report and a Concise Review of the Literature". Indian Dermatol. Online J. **80**(2): doi: 10.4103/2229-5178.96722
Jansen D. (2021) "Keloids" Drugs & Diseases.
Janson DG., Saintigny G., van A Adrichem., Mahé C., and Ghalbzouri AE. (2012) "Different Gene Expression Patterns in Human Papillary and Reticular Fibroblasts" Journal of Investigative Dermatology. **132**(11), pp. 2565-2572.
Janumpally, SR., SR. Feldman, AK. Gupta and AB. Fleischer (2002). "In the United States, Blacks and Asian/Pacific Islanders Are More Likely Than Whites to Seek Medical Care for Atopic Dermatitis." Archives of Dermatology **138**(5).
Jariashvili K., Madhan B., Brodsky B., Kuchava A., Namicheishvili L., and Metreveli N. (2012) "UV Damage of Collagen: Insights from Model Collagen Peptides" **97**(3), pp. 189-198.
Jeggy C. (2004) "Micro-injection Moudling: From Process to Modelling". Presses Univ, de Louvain, Louvain-la-Neuve, Belgium.
Jensen JM, Proksch E. (2009) "The skin's barrier" G Ital Dermatol Venereol. **144**(6), pp. 689-700.
Jeong H-R., Kim J-Y., Kim S-N., and Park J-H. (2018) "Local dermal delivery of cyclosporin A, a hydrophobic and high molecular weight drug, using dissolving microneedles." European Journal of Pharmaceutics and Biopharmceutics. **127**, pp. 237-243.
Jiang X., Xia W., Pan J., Yang W., Zhang S., Li C et al., (2023) "Engineered microneedle systems for topical cancer therapy" Applied Materialstoday. **31**.
Jiang, W. G., A. J. Sanders, F. Ruge and K. G. Harding (2012). "Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC-γ and potential clinical implications." Experimental and Therapeutic Medicine **3**(2): 231-236.
Jiang, Y., L. C. Tsoi, A. C. Billi, N. L. Ward, P. W. Harms, C. Zeng, E. Maverakis, J. M. Kahlenberg and J. E. Gudjonsson (2020). "Cytokinocytes: the diverse contribution of keratinocytes to immune responses in skin." JCI Insight **5**(20).

Jin, X.-H., L.-Q. Pi and W.-S. Lee (2019). "Expression Pattern and Role of Klotho in Human Hair Follicles." Annals of Dermatology **31**(5): 511.
Johnson, K. E. and T. A. Wilgus (2014). "Vascular Endothelial Growth Factor and Angiogenesis in the Regulation of Cutaneous Wound Repair." Advances in Wound Care **3**(10): 647-661.
Joly-Tonetti, N., et al. (2016). "Melanin fate in the human epidermis: a reassessment of how best to detect and analyse histologically." Experimental Dermatology **25**(7): 501-504.
Ju M., Chen K., Chang B., and Gu H. (2012) "UVA1 irradiation inhibits fibroblast proliferation and alleviates pathological changes of scleroderma in a mouse model" J Biomed Res. **26**(2), pp. 135-142.
Jung, D., Y. Xu and Z. Sun (2017). "Induction of anti-aging gene klotho with a small chemical compound that demethylates CpG islands." Oncotarget **8**(29), pp.46745-46755.
Jung, J.H. and Jin, S.G. (2021) "Microneedle for transdermal drug delivery: current trends and fabrication" Journal of Pharmaceutical investigation **51,** pp.503-517.
Juntongjin, P., et al. (2022). "Awareness of vitiligo among multi-ethnic populations." Journal of Cosmetic Dermatology **21**(11): 5922-5930.
Juzeniene, A., et al. (2010). "The seasonality of pandemic and non-pandemic influenzas: the roles of solar radiation and vitamin D." International Journal of Infectious Diseases **14**(12): e1099-e1105.
Kadiri S, Aissa A, Berhili S, Khmou M, Elmajjaoui S, Kebdani T et al., (2017) "Skin Cancer Conerns in People of Color: Risk Factors and Prevention" Asian Pac J Cancer Prev. 17(12), pp. 5257-5264.
Kadoya K., Sasaki T., Kostka R., Timpl K., Matsuzaki N., Kumagi N., (2005) "Fibulin-5 deposition in human skin: decrease with ageing and ultraviolet B exposure and increase in solar elastosis" BJD.
Kaeberlein, M., P. S. Rabinovitch and G. M. Martin (2015). "Healthy aging: The ultimate preventative medicine." Science **350**(6265), pp. 1191-1193.
Kaidbey KH, Agin PP, Sayre RM, Kligman AM. (1979) "Photoprotection by melanin-a comparison of Black and Caucasian skin" J Am Acad Dermatol. **1,** pp. 249-260.
Kailas, A., J. A. Solomon, E. N. Mostow, D. S. Rigel, R. Kittles and S. C. Taylor (2016). "Gaps in the understanding and treatment of skin cancer in people of color." Journal of the American Academy of Dermatology **74**(5): 1020-1021.
Kang C. (2019) "Senolytics and Senostatics: A Two-Pronged approach to Target Cellular Senescence for Delaying Aging and Age-related diseases". Molecules and Cells.
Kanitakis, J. (2002). "Anatomy, histology and immunohistochemistry of normal human skin". European Journal of Dermatology, **12**(4), pp. 390–401.
Kanwar AJ, Mahajan R, and Parsad D. (2013) "Low-dose oral mini-pulse dexamethasone therapy in progressive unstable vitiligo. J Cutan Med Surg. **17**(4), pp:259-68: doi: 10.2310/7750.2013.12053.
Karadag AS., Bilhili SG., Calka O., and Bayram I. (2013) "Eryhrokeratodermia variabilis" Two case reports" Indian Dermatol Online J. **4**(4), pp. 340-343.
Karamanos NK., Theocharis AD., Piperigkou Z., Manou D., Passi A.,Skandalis SS., et al. (2021) "A guide to the composition and functions of the extracellular matrix" The FEEBs Journal. **288**(24), pp. 6850-6912.
Karran P and Brem R. (2016) "Protein Oxidation, UVA and human DNA repair" DNA Repair (Amst), **44**, pp. 178-185: doi: 10.1016/j.dnarep.2016.05.024
Kaufman, B. P., et al. (2018). "Atopic dermatitis in diverse racial and ethnic groups-Variations in epidemiology, genetics, clinical presentation and treatment." Experimental Dermatology **27**(4): 340-357.

Kefalides NA., and Borel JP. (2005) “Morphology and Ultrastructure of Basement Membranes” Current Topics in Membranes. **56**, pp.19-42.
Kelly AP. (1988) “Keloids”. Dermatol Clin. **6**(3), pp.413–24.
Kelly AP. (2004) “Medical and surgical therapies for keloids” Dermatol Ther. **17**(2), pp. 212-8.
Kelly R. (2016) “17-Keratins in wound healing” Wound Healing Biomaterials. **2,** pp. 353-365.
Kelsey, R. (2017). "Foxy–5 in prostate cancer model." Nature Reviews Urology **14**(11): 638-638.
Kennedy C., ter Huurne J., Berkhout M., Gruis M., Bastiaens M., Bergman W., Willemze R., Bavinck JN. (2001) “Melanocortin 1 receptor (MC1R) gene variants are associated with an increased risk for cutaneous melanoma which is largely independent of skin type and hair color” J Invest Dermatol. **117**, pp. 294-300.
Ketchum LD, Cohen IK, Masters FW. (1974) “Hypertrophic scars and keloids. A collective review”. Plast Reconstr Surg. **53**(2), pp.140-54:doi:10.1097/00006534-197402000-00004.
Keyes BE, Lio S, Asare A, Naik S, Levorse J, Polak L et al. (2016). "Impaired Epidermal to Dendritic T Cell Signaling Slows Wound Repair in Aged Skin." Cell **167**(5): 1323-1338.e1314.
Keyes WM., Wu Y., Vogel H., Guo X., Lowe SW and Mills AA. (2005) “p63 deficiency activates a program of cellular senescence and leads to accelerated aging” Genes & Development. **19**, pp. 1986-1999.
Khalid KA., AFM Nawi., N Zulkifli., MA Barkat., and H. Hadi (2022). "Aging and Wound Healing of the Skin: A Review of Clinical and Pathophysiological Hallmarks." Life **12**(12): 2142.
Khan S., Hasan A., Attar F., Babadaei MMN., Zeinabad HA., Salehi M., Alizadeh M., Hassan M., Derakhshankhah H., Hamblin MR., et al. (2021) “Diagnostic and drug release systems based on microneedle arrays in breast cancer therapy”. J. Control. Release. **338**, pp. 341–357. doi: 10.1016/j.jconrel.2021.08.036.
Kida and T. Ogihara (2006). "Anti-apoptotic and anti-senescence effects of Klotho on vascular endothelial cells." Biochemical and Biophysical Research Communications **339**(3): 827-832.
Kim D., Hossain MZ., Nieves A., Gu L., Ratliff TS., Oh SM., et al., (2016) “To Control Site-Specific Skin Gene Expression, Autocrine Mimics Paracrine Canonical Wnt Signaling and Is Activated Ectopically in Skin Disease” Am J Pathol. **186**(4), pp. 1140-1150
Kim E, Kim D, Yoo S, Hong YH, Han SY, Jeong S, et al. (2018) “The skin protective effects of compound K, a metabolite of ginsenoside Rb1 from Panax ginseng”. J Ginseng Res, **42**(2), pp.218-24. https://doi.org/10.1016/j.jgr.2017.03.007 PMid:29719469
Kim EJ., Jin X-J., Kim YK., Oh IK., Kim JE., Park C-H et al., (2010) “UV decreases the synthesis of free fatty acids and triglycerides in the epidermis of human skin in vivo, contributing to development of skin photoaging" J Dermatol Sci. **57**(1), pp. 19-26.
Kim HS., Min J-A., Kim H-O., and Park YM. (2009) “Basal cell carcinoma of the finger resembling a pyogenic granuloma” J Dermatol. **36**(3), pp. 174-5.
Kim JH., Shin JU., Kim SH., Noh JY., Kim HR., Lee J., et al. (2018) “Successful transdermal allergen delivery and allergen-specific immunotherapy using biodegradable microneedle patches” Biomaterials. 150, pp. 38-48.
Kim JY and Dao H. (2023) “Physiology, Integument” StatPearls.
Kim, D., M. Z. Hossain, A. Nieves, L. Gu, T. S. Ratliff, S. Mi Oh, A. Park, S. Han, N. B. Yang, J. Qi, J. M. Taube, S. Kang and L. A. Garza (2016). "To Control Site-Specific Skin Gene Expression, Autocrine Mimics Paracrine Canonical Wnt Signaling and Is Activated Ectopically in Skin Disease." The American Journal of Pathology **186**(5): 1140-1150.

Kim, J.-H., K.-H. Hwang, K.-S. Park, I. D. Kong and S.-K. Cha (2015). "Biological Role of Anti-aging Protein Klotho." Journal of Lifestyle Medicine **5**(1): 1-6.
Kim, S., et al. (2022). "Clinical and molecular change induced by repeated low-dose visible light exposure in both light-skinned and dark-skinned individuals." Photodermatology, Photoimmunology & Photomedicine.
Kirkland, J. L. and T. Tchkonia (2020). "Senolytic drugs: from discovery to translation." Journal of Internal Medicine **288**(5): 518-536.
Kiu Y., Wang H., Taylor M., Cook C., Martínez-Berdeja A., North JP et al., (2022) "Classification of human chronic inflammatory skin disease based on single-cell immune profiling" Sci Immunol. **7**(70).
KJ. Krieger, N. Bertollo, M. Dangol, JT. Sheridan, MM. Lowery, ED. O'Cearbhaill, (2019) "Simple and customizable method for fabrication of high-aspect ratio microneedle molds using low-cost 3D printing" Microsyst Nanoeng. **9**(5), pp. 42.
Klaas M., Mämets-Allas K., Heinmäe E., Lagus H., Cárdenas-León CG., Arak T., et al., (2021) "Thrombospondin-4 Is a Soluble Dermal Inflammatory Signal That Selectively Promotes Fibroblast Migration and Keratinocyte Proliferation for Skin Regeneration and Wound Healing" Front Cell Biol. **9**: 745637.
Knapp, A.C. Franke, W.W. Heid, H. Hatzfield, M. Jorcano, J.L. Moll, R. (1986) "Cytokeratin No. 9, an epidermal type I keratin characteristic of a special program of keratinocyte differentiation displaying body site specificity". J Cell Biol, **103**, pp,657–667.
Kochhar, JS., Quek, TC., Soon, WJ., Choi, J., Zou S., and Kang, L. (2013) "Effect of Microneedle Geometry and Supporting Substrate on Microneedle Array Penetration into Skin". J. Pharm. Sci. **102**, pp. 4100–4108.
Koh HK, Geller AC, Miller DR, Grossbart TA, Lew RA. Prevention and early detection strategies for melanoma and skin cancer - Current status. *Arch Dermatol.* 1996;132(4):436–443. doi: 10.1001/archderm.1996.03890280098014.
Koike, Y., M. Yozaki, A. Utani and H. Murota (2020). "Fibroblast growth factor 2 accelerates the epithelial–mesenchymal transition in keratinocytes during wound healing process." Scientific Reports **10**(1).
Koivisto L., Heino J., Häkkinen L., and Larjava H. (2014) "Integrins in Wound Healing" Adv. Wound Care. **3**, pp. 762-783.
Kollias N, Sayre RM, Zeise L, and Chedekel MR. (1991) "Photoprotection by melanin" J Photochem Photobiol B. **9**(2), pp:135-60:doi: 10.1016/1011-1344(91)80147-a.
Kong, X. Q., P. Zhou and C. W. Wu (2011). "Numerical simulation of microneedles' insertion into skin." Computer Methods in Biomechanics and Biomedical Engineering **14**(9): 827-835.
Konrad K, Wolff K. (1973) "Hyperpigmentation, melanosome size, and distribution patterns of melanosomes". Arch Dermatol, **107**(6), pp.853-60. PMID: 4711116.
Koul, A.R. Patil, R.K. and Nahar, S. (2013). "Unfavourable results in free tissue transfer." Indian Journal of Plastic Surgery **46**(02): 247-255.
Koyano, Y., G. Nakagami, S. Iizaka, T. Minematsu, H. Noguchi, N. Tamai, et al., (2016). "Exploring the prevalence of skin tears and skin properties related to skin tears in elderly patients at a long-term medical facility in Japan." International Wound Journal, **13**(2), pp.189-197.
Kozera B and Rapacz M. (2013) "Reference genes in real-time PCR" J Appl Genet. **54**(4), pp.391-406
Kreicher KL, Kurlander DE, Gittleman HR, Barnholtz-Sloan JS, Bordeaux JS. (2016) "Incidence and Survival of Primary Dermatofibrosarcoma Protuberans in the United States" Dermatol Surg. **42**(1), pp. 24-31:doi:10.1097/DSS.0000000000000300

Kretsos K., and Kasting GB. (2007) “A Geometrical Model of Dermal Capillary Clearance”. Math. Biosci. **208**, pp. 430-453.
Kretzschmar K., and Watt FM. (2014) “Markers of Epidermal stem cell subpopulations in adult mammalian skin” Cold Spring Harb Perspect Med. **4**(10), pp. a013631.
Kromberg JGR., Flynn KA., and Kerr RA. (2023) “Determining a Worldwide Prevalence of Oculocutaneous Albinism: A Systematic Review” Invest Ophthalmol Vis Sci. **64**(10) pp.14
Kromberg, JGR., KA. Flynn and RA. Kerr (2023). "Determining a Worldwide Prevalence of Oculocutaneous Albinism: A Systematic Review." Investigative Opthalmology & Visual Science **64**(10):14.
Kruger C., and Schallreuter KU. (2012) “A review of the worldwide prevalence of vitiligo in children/adolescents and adults” Int J Dermatol. **51**(10) pp. 1206-1212.
Kudlova N., Sanctis JBD., and Hajduch M. (2022) “Cellular Senescence: Molecular Targets, Biomarkers, Senolytic Drugs” Int J Mol Sci. 23(8), pp. 4168.
Kular JK., Basu S., and Sharma RI. (2014) “The extracellular matrix: structure, composition, age-related differences, tools for analysis and applications for tissue engineering” J Tissue Eng. **5**,
Kumari N., Pathak J., Dwivedy AK., and Sinha RP. (2021) “Bioprospection of UV-screening compounds from lichens inhabiting the Indian state of Sikkim”. Plant Arch. **21**, pp. 1168–1177. doi: 10.51470/PLANTARCHIVES.2021.v21.no1.155.
Kurban RS, and Bhawan J. (1990) “Histologic changes in skin associated with aging”. J Dermatol Surg Oncol.**16**(10), pp.908–914. https://doi.org/10.1111/j.1524-4725.1990.tb01554.x
Kuo T-Y., Huang C-C., Shieh S-J., Wang Y-B., Lin M-J., and Huang LLH. (2022) “Skin wound healing assessment via an optimized wound array model in miniature pigs” Nature. **12**, pp. 445.
Kuro-o M., Moe OW. (2017) “FGF23-αKlotho as a paradigm for a kidney-bone network” Bone. **100**, pp. 4-18.
Kuro-O, M., Y. Matsumura, H. Aizawa, H. Kawaguchi, T. Suga, T. Utsugi, Y. Ohyama, M. Kurabayashi, T. Kaname, E. Kume, H. Iwasaki, A. Iida, T. Shiraki-Iida, S. Nishikawa, R. Nagai and Y.-I. Nabeshima (1997). "Mutation of the mouse klotho gene leads to a syndrome resembling ageing." Nature **390**(6655): 45-51.
Kurosu, H., M. Yamamoto, J. D. Clark, J. V. Pastor, A. Nandi, P. Gurnani, O. P. McGuinness, H. Chikuda, M. Yamaguchi, H. Kawaguchi, I. Shimomura, Y. Takayama, J. Herz, C. R. Kahn, Kurosu H., Yamamoto M., Clark JD., Pastor JV., Nandi A., Gurnani P et al., (2005). "Suppression of Aging in Mice by the Hormone Klotho." Science **309**(5742): 1829-1833.
Kuwahara N., Sasaki S., Kobara M., Nakata T., Tatsumi T., Irie H., et al. (2008) “HMG-CoA reductase inhibition improves anti-aging klotho protein expression and arterioscelerosis in rats with chronic inhibition of nitric oxide synthesis” Int J Cardiol. **123**(2), 84-90.
Kwasnieski M., and Mitchel D. (2022) “Post Amputation Skin and Wound Care” Phys Med Rehabil Clin N Am. **33**(4), pp. 857-870.
Kwon KM., Lim S-M., Choi S., Kim D-H., Jin H-E., Jee G., Hong K-J., and Kim J-Y. (2017) “Microneedles” quick and easy delivery methods of vaccines” Clin Exp Vaccine Res. **6**(2), pp. 156-159.
Kyriakides TR and MacLauchlan S. (2009) “The role of thrombospondins in wound healing, ischemia, and the foreign body reaction” 3(3-4), pp. 215-225.
Kyriakides TR., Tam JWY., and Bornstein P. (1999) “Accelerated Wound Healing in Mice With a Disruption of the Thrombospondin 2 Gene 1,2,3” Journal of Investigative Dermatology. **113**(5), pp. 782-787.

Lademann J., Richter H., Astner S., Patzelt A., Knorr F., Sterry W et al., (2008) "Determination of the thickness and structure of the skin barrier by in vivo laser scanning microscopy" Laser Phys Lett. **5**(4), pp. 311-5.
Lademann J., Richter H., Astner S., Patzelt A., Knorr F., Sterry W et al. (2008) "Determination of the thickness and stricture of the skin barrier byin vivolaser scanning microscopy" Laser Phys. Lett. **5**(4), pp. 311-5.
Lago JC., and Puzi MB. (2019) "The effect of aging in primary human dermal fibroblasts" PLoS One. **14**(7).
Land KC., and Lamb VL. (2008) "Demography of Aging: Population Aging". International Encyclopaedia of Public Health.
Landen NX., Li D and Stahle M. (2016) "Transition from inflammation to proliferation: a critical step during wound" Cell Mol. Life Sci. **73**, pp. 3861-3885.
Lang, F. R. and F. S. Rupprecht (2019). "Motivation for Longevity Across the Life Span: An Emerging Issue." Innovation in Aging **3**(2).
Langton AK., Graham HK.m Griffiths CEM., and Watson REB. (2019) "Ageing significantly impacts the biomechanical function and structural composition of skin" Exp Dermatol. **28**(8), pp. 981-984.
Lannan FM., Powell J., Kim GM., Hansen CR., Pasquina PF and Smith DG. (2021) "Hypderhidrosis of the residual limb: a narrative review of the measurement and treatment of excess perspiration affecting individuals with amputation" Prosthetics and Orthotics International. **45**(6), pp. 477-486.
Larouche J., Sheoran S., Maruyama K., and Martino MM. (2018) "Immune Regylation of Skin Wound Healing: Mechanisms and Novel Therapeutic Targets" Adv. Wound Care. **7**, pp. 209-231.
Larrañeta E, Moore J, Vicente-Pérez EM, González-Vázques P, Lutton R, Lutton R et al., (2014) "A proposed model membrane and test method for microneedle insertion studies". International Journal of Pharmaceutics. **472**(1-2), pp. 65-73.
Lavker RM. (1979) "Structural alterations in exposed and unexposed aged skin" J Investig Dermatol. **73**, pp. 59-66.
LeBlanc K, Woo KY, VanDenKerkhof E., and Woodbury MG. (2020) "Skin tear prevalence and incidence in the long-term care population: a prospective study". J Wound Care. **29**(7), pp.S16-S22.
LeBlanc K., Alam T., and Langemo D. (2016) "Clinical challenges of differentiating skin tears from pressure ulcers. Eur Wound Manag Acad J. **16**(1) pp. 17–23
LeBlanc K., Campbell K., Wood E., and Beeckman D. (2018) "Best practice recommendations for the prevention and management of skin tears in aged skin". https://tinyurl.com/ yd7u7kso.
Lebre, M. C., A. M. G. Van Der Aar, L. Van Baarsen, T. M. M. Van Capel, J. H. N. Schuitemaker, M. L. Kapsenberg and E. C. De Jong (2007). "Human Keratinocytes Express Functional Toll-Like Receptor 3, 4, 5, and 9." Journal of Investigative Dermatology **127**(2): 331-341.
Lee EG., Luckett-Chastain LR., Calhoun KN., Frempah B., Bastian A., and Galluci RM. (2019) "Interleukin 6 Function in the Skin and Isolated Keratinocytes IS Modeulated by Hperglycemia" J Immunol Res. 3.
Lee JW., Park J-H., and Prausnitz MR. (2008) "Dissolving microneedles for transdermal drug delivery" Biomaterials. **29**(13) pp. 2113-24.
Lee S, Tong X, Yang F. (2016). "Effects of the poly(ethylene glycol) hydrogel crosslinking mechanism on protein release." Biomaterials Science **4**(3): 405-411.
Lee S., and Schmitt CA. (2019) "The dynamic nature of senescence in cancer". Nat. Cell Biol. 21, pp. 94–101.

Lee WJ., Park KH., Cha HW., Sohn MY., Park KD., Lee S-J et al., (2014) “The expression of involucrin, loricrin, and filaggrin in cultured sebocytes” Ann Dermatol. **26**(1), pp. 134-137.
Lee, J.W. Han, M.-R. and Park, J.-H. (2013) “Polymer microneedles for transdermal drug delivery” J Drug Target. **21**(3), pp.211-223
Lee, S. Tong, X. Yang, F. (2016). "Effects of the poly(ethylene glycol) hydrogel crosslinking mechanism on protein release." Biomaterials Science **4**(3): 405-411.
Leikam, C., et al. (2015). "In vitro evidence for senescent multinucleated melanocytes as a source for tumor-initiating cells." Cell Death & Disease **6**(4): e1711-e1711.
Leiter, U., et al. (2014). Epidemiology of Skin Cancer, Springer New York**:** 120-140.
Leon J., Moreno AJ., Garay B., Chalkley R., Burlingame AL., Wang D., and Dubal DB. (2017) “Peripheral Elevation of a Klotho Fragment Enhances Brain Function and Resilience in Young, Aging, and α=Synuclein Transgenic Mice” Cell Rep. **20**, pp. 1360-1371.
Levine ME., Lu AT., Quach A., Chen BH., Assimes TL., Bandielli S., et al. (2018) “An epigenetic biomarker of aging for lifespan and healthspan” Aging. **10**(4), pp. 573-591.
Li QY, Jia Nan Z, Bo Zhi C, Qi Lei W, Xin Dong G. (2017) “A solid polymer microneedle patch pretreatment enhances the permeation of drug molecules into the skin”. RSC Adv. **7**(25), pp. 15408–15415.
Li X., Wang J., Wang L., Gao Y., Feng G., Li G. et al. (2022) “Lipid metabolism dysfunction induced by age-dependent DNA methylation accelerates aging” Nature: Signal transduction and targeted therapy. **7**
Li, Y. and T. O. Tollefsbol (2011). DNA Methylation Detection: Bisulfite Genomic Sequencing Analysis, Humana Press**:** 11-21.
Lian, J., Y. Yue, W. Yu and Y. Zhang (2020). "Immunosenescence: a key player in cancer development." Journal of Hematology & Oncology **13**(1).
Liang, L. Chen, Y. Zhang, B.L. Zhang, X.P. Liu, J.L. Shen, C.B. et al., (2020) “Optimization of dip-coating methods for the fabrication of coated microneedles for drug delivery” Journal of Drug Delivery Science and Technology **55**
Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, and Sibilia M. (2010) “Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development”. Cell. **140**, PP.268**.**
Lim W-J., Kim KH., Kim J-Y., Jeong S., and Kim N. (2019) “Identifcation of DNA-Methylated CpG Islands Associated with Gene Silencing in the Adult Body Tissues of the Ogye Chicken Using RNA-Seq and Reduced Representation Bisulfite Sequencing” Front. Genet. **10**.
Lim, K., A. Groen, G. Molostvov, T. Lu, K. S. Lilley, D. Snead, S. James, I. B. Wilkinson, S. Ting, L.-L. Hsiao, T. F. Hiemstra and D. Zehnder (2015). "α-Klotho Expression in Human Tissues." The Journal of Clinical Endocrinology & Metabolism **100**(10): E1308-E1318.
Lim, W., F. Parel, S. De Hoog, A. Verbon and WWJ. Van De Sande (2021). "Melanin production in coelomycetous agents of black grain eumycetoma." Transactions of The Royal Society of Tropical Medicine and Hygiene **115**(4): 324-327.
Lin Y.-H., Lee I-C., Hsu W-C., and Hsu C-H. (2016). "Rapid fabrication method of a microneedle mold with controllable needle height and width." Biomedical Microdevices **18**(5).
Lin, C.C. and Anseth, K.S. (2009) “PEG Hydrogels for the Controlled Release of Biomolecules in Regnerative Medicine. *Pharm. Res.*, **26** pp. 631 —643
Lintzeri DA., Karimian N., Blume-Peytavi U., and Kottner J. (2022) “Epidermal thickness in healthy humans: a systematic review and meta-analysis” Journal of the European Academy of Dermatology and Venereology. **36**(8), pp. 1191-1200.

Liu H., Yue J., Lei Q., Gou X., Chen S-Y., He Y-Y., and Wu X. (2015) "Ultraviolet B (UVB) Inhibits Skin Wound Healing by Affecting Focal Adhesion Dynamics" Photochem Photobiol. **91**(4), pp. 909-916.
Liu L., and Rando TA. (2011) "Manifestations and mechanisms of stem cell aging" J Cell Biol. 193(2), pp. 257-266.
Liu M., Wang W., Wang J., Fang C., and Liu T. (2022) "Z-Guggulsterone alleviates renal fibrosis by mitigating G2/M cycle arrest through Klotho/p53 signaling" Chemico-Biological Interactions. **354**.
Liu S., Yeo DC., Wiraja C., Tey HL., Mrksich M., and Xu C. (2017) "Peptide delivery with poly(ethylene glycol) diacrylate microneedles through swelling effect" Bioengineering & Translational Medicine.
Liu Z., H. Chen., H. Yang., J. Liang., and X. Li (2012). "Low-Dose UVA Radiation-Induced Adaptive Response in Cultured Human Dermal Fibroblasts." International Journal of Photoenergy pp. 1-11.
Liu, H., RS. Kang, K. Bagnowski, JM. Yu, S. Radecki, WL. Daniel, et al., (2020). "Targeting the IL-17 Receptor Using Liposomal Spherical Nucleic Acids as Topical Therapy for Psoriasis." Journal of Investigative Dermatology **140**(2): 435-444.e434.
Liu, J.-K. (2022). "Antiaging agents: safe interventions to slow aging and healthy life span extension." Natural Products and Bioprospecting **12**(1).
Loizidou EZ., Inoue NT., Ashton-Barnett J., Barrow DA., and Allender CJ. (2016) "Evaluation of geometrical effects of microneedles on skin penetration by CT scan and finite element analysis" European Journal of Pharmaceutics and Biopharmaceutics. **107**, pp. 1-6.
Loizidou, EZ., Inoue, NT., Ashton-Barnett, J., Barrow, DA., and Allender, CJ. (2016) "Evaluation of geometrical effects of microneedles on skin penetration by CT scan and finite element analysis". Eur. J. Pharm. Biopharm. **107**, pp. 1–6.
Lòpez V, and Jordà E. (2011) "Piebaldism In a 2-year-old girl". Dermatology online Journal, **17**(2).
Lopez-Ojeda W., Pandey A., Alhajj M. and Oakley AM. (2022) "Anatomy, Skin" StatPearls.
López-Otín, C., M. A. Blasco, L. Partridge, M. Serrano and G. Kroemer (2013). "The Hallmarks of Aging." Cell **153**(6): 1194-1217.
Louw L. (2000) "Keloids in rural black south Africans. Part 1: general overview and essential fatty acid hypotheses for keloid formation and prevention". Prostaglandins Leukot Essent Fatty Acids. **63**(5), pp.237–45.
Lovell CR., Smolenski KA., Duance VC., Light ND., Young S., and Dyson M. (1987) "Type I and III collagen content and fibre distribution in normal human skin during ageing". Br. J. Dermatol. **117**(4), pp. 419–428. doi: 10.1111/j.1365-2133.1987.tb04921.x.
Lowes, M. A., M. Suárez-Fariñas and J. G. Krueger (2014). "Immunology of Psoriasis." Annual Review of Immunology **32**(1): 227-255.
Lu, Y., Y. Yang, L. Xiao, S. Li, X. Liao and H. Liu (2021). "Autocrine and Paracrine Effects of Vascular Endothelial Cells Promote Cutaneous Wound Healing." BioMed Research International **2021**: 1-10.
Luigi AJD., and Cooper RA. (2014) "Adaptive sports technology and Biomechanic: Prosthetics" Paralympic Sports Medicine and Science. **6**(85), pp. S40-S57.
Lukiowiak TM., Aizman L., Perz A., Miller CJ., Sobanko JF., Shin TM., et al. (2020) "Association of Age, Sex, Race, and Geographic Region With Variation of the Ratio of Basal Cell to Cutaneous Squamous Cell Carcinomas in the United States" JAMA Dermatology, **156**(11), pp. 1192-1198.
Lyengar B and Misra RS. (1987) "Reaction of dendritic melanocytes in vitiligo to the substrates of tyrosine metabolism". Acta Anat, **129***(3):* doi: 10.1159/000146400.

Lyon C.C, Kulkarni J, Zimerson E, Ross E.V. Beck M.H. (2000) “Skin disorders in amputees” J Am Acad Dermatol. **42**(3), pp. 501-7:doi: 10.1016/s0190-9622(00)90227-5
Machado de Oliveira R. (2006) “Klotho RNAi induces premature senescence of human cells via a p53/p31 dependent pathway” FEBS Lett. **580**(24), pp. 5753-8.
Maeda, K. (2018). "Analysis of Ultraviolet Radiation Wavelengths Causing Hardening and Reduced Elasticity of Collagen Gels In Vitro." Cosmetics, **5**(1): 14.
Maique, J., B. Flores, M. Shi, S. Shepard, Z. Zhou, S. Yan, O. W. Moe and M. C. Hu (2020). "High Phosphate Induces and Klotho Attenuates Kidney Epithelial Senescence and Fibrosis." Frontiers in Pharmacology **11**.
Makrantonaki E., Zouboulis CC., and William J. (2007) “Cunliffe Scientific Awards Characteristics and pathomechanisms of endogenously aged skin”. Dermatology. **214**(4), pp. 352–60. doi: 10.1159/000100890.
Makvandi, P. Kirkby, M. Hutton, A.R.J. Shabani, M. Yiu, C.K.Y. Baghbantaraghdari, Z. et al., (2021). "Engineering Microneedle Patches for Improved Penetration: Analysis, Skin Models and Factors Affecting Needle Insertion." Nano-Micro Letters **13**(1).
Mandai, M., Watanabe A., Kurimoto Y., Hirami Y., Morinaga C., Daimon T et al., (2017) Autologous induced stem-cell-derived retinal cells for macular degeneration. N. Engl. J. Med. **376**, pp. 1038–1046
Mandal, A. Boopathy, A.V. Lam, L.K.W. Moynihan, K.D. Welch, M.E. Bennett, N.R. et al., (2018) “Cell and fluid sampling microneedle patches for monitoring skin-resident immunity” Science Translational Medicine **10**(467), doi: 10.1126/scitranslmed.aar2227
Manganelli, M., S. Guida, A. Ferretta, G. Pellacani, L. Porcelli, A. Azzariti and G. Guida (2021). "Behind the Scene: Exploiting MC1R in Skin Cancer Risk and Prevention." Genes **12**(7): 1093.
Mann DM, Yates PO, and Barton CM. (1977) “Variations in melanin content with age in the human substantia nigra” Biochem Exp Biol. **13**(2), pp. 137-9.
Manou D, Caon I, Bouris P, Triantaphyllidou IE, Giaroni C, Passi A, Karamanos NK, Vigetti D & Theocharis AD (2019) “The complex interplay between extracellular matrix and cells in tissues”. Methods Mol Biol.**1952**, pp.1–20.
Manschot JF., and Brakkee AJ. (1986) “The measurement and modelling of the mechanical properties of human skin in vivo - I. The measurement”. J Biomech. **19**, pp. 511–5.
Mansego ML., Milagro FM., Zulet MA., Moreno-Aliaga MJ., and Martinez JA. (2015) “Differential DNA methylation in Relation to Age and Health Risks of Obesity” Int J. Mol. Sci. **16**(8), pp. 16816-16832.
Mansoor I., Lai J., Ranamukhaarachchi S., and Schmitt V (2015). "A microneedle-based method for the characterization of diffusion in skin tissue using doxorubicin as a model drug." Biomedical Microdevices **17**(3).
Mansouri-Nejad SE., Yazdan Panah MJ., Tayyebi Meibodi N., Zadeh FA., Akhondian J., Beiraghi M., et al. (2014) “Griscelli syndrome: A Case Report” Iran J Child Neurol. **8**(4), pp. 72-75.
Mao Y, Schwarzbauer JE. (2005) “Fibronectin fibrillogenesis, a cell-mediated matrix assembly process”. Matrix Biol 24, pp.389–399.
Marqus, S. Pirogova, E. Piva, T.J. (2017). "Evaluation of the use of therapeutic peptides for cancer treatment." Journal of Biomedical Science **24**(1).
Marthandan S., Baumgart M., Priebe S., Groth M., Schaer J., Kaether C et al., (2016) “Conserved Senescence Asscoiated Genes and Pathways in Primary Human Fibriblasts Detected by RNA-Seq” PLoS One. 11(5): e0154531.
Martin MR., Mohsin N., Vilasi S., Reed and Brownell I. (2022) “Challenges documenting racial disaprities in Merkell cell Carcinoma” Cancer Biol Ther. **23**(1), pp. 1-3.

Martini F. (2004) “Fundamentals of Anatomy and Physiology” Benjamin-Cummings. San Francisco.
Mastacouris, N., A. Strunk and A. Garg (2023). "Incidence and Prevalence of Diagnosed Vitiligo According to Race and Ethnicity, Age, and Sex in the US." JAMA Dermatology **159**(9): 986.
Matias MAT., Saunus JM., Ivanovski S., Walsh LJ., and Farah CS. (2011) “Accelerated wound healing phenotype in Interleukin 12/23 deficient mice”| J Inflamm. **8**, pp.39.
Maverakis E., Miyamura Y., Bowen MP., Correa G., Ono Y., and Goodarzi H. (2010) “Light, including Ultraviolet” J. Autoimmune. 34(3), pp. J247-57.
McCall CO, Chen SC. (2002) “Squamous cell carcinoma of the legs in African Americans” J Am Acad Dermatol. **47**(4), pp.524-9; doi: 10.1067/mjd.2002.124083.
McCaughan D., Sheard L., Cullum N., Dumville J., and Chetter I. (2018) “Patient;s perceptions and experiences of living with surgical wound healing y secondary intention: A qualitative study” Int J Nurs Stud. **77**, pp. 29-38.
McCrudden MT., Singh TRR., Migalska K., and Donnelly RF. (2013) “Strategies for Enhanced Peptide and Protein Delivery”. Ther. Deliv. **4**, pp. 593–614.
McDonald CL, Westcott-McCoy S, Weaver MR, Haagsma J, Kartin D. (2020) “Global prevalence of traumatic non-fatal limb amputation” Prosthetics and Orthotics International. **45**(2), pp. 105-114, doi: 10.1177/0309364620972258
McEwen E. (1990) “Age: The unrecognised Discrimination, Age Concern England, London”
McGrath JA., Robinson MK., and Binder RL. (2012) “Skin differences based on age and chronicity of ultraviolet exposure: results from a gene expression profiling study” British Journal of Dermatology. **166**(2), p. 9-15.
McKee PH. (2019) “McKee’s pathology of the Skin”. Fifth Edition. 2019. Elsevier Limited, pp. 11-13
McMichael AJ, Vachiramon V, Guzman-Sanchez DA, Camacho F. (2012) “Psoriasis in African-Americans: a caregivers’ survey”. J Drugs Dermatol.**11**:478–482.
MedlinePlus [Internet]. Brodkey, F. (MD): National Library of Medicine (US); [updated 2020 Jun 24]. Aging changes in skin; [updated 2022 July 23; reviewed 2022 July 21. Available from:https://medlineplus.gov/ency/article/004014.htm#:~:text=Aging%20skin%20repairs%20itself%20more,other%20factors%20also%20affect%20healing.
Meijer WT, Hoes AW, Rutgers D, Bots ML, Hofman A, Grobbee DE. (1998) “Peripheral arterial disease in the elderly: the Rotterdam Study”. Arterioscler Throm Vasc Biol, 18, pp.185–192.
Mellott MB, Searcy K, Pishko MV. (2001) “Release of protein from highly cross-linked hydrogels of poly(ethylene glycol) diacrylate fabricated by UV polymerization” Biomaterials **22**(9), pp. 929-941:doi:https://doi.org/10.1016/S0142-9612(00)00258-1
Menasché, P. Vanneauz V., Hagège A., Bel A., Cholley B., Parouchev A et al., (2018) “Transplantation of human embryonic stem cell-derived cardiovascular progenitors for severe ischemic left ventricular dysfunction”. J. Am. Coll. Cardiol. **71**, 429–438
Menassa M., Stronks K., Khatami F., Díaz ZMR., Espinola OP., Gamba M et al., (2023) “Concepts and definitions of healthy ageing: a systematic review and synthesis of theoretical models” eClinicalMedicine. **56**, pp. 101821.
Mencke R., and Hillebrands J-L (2017) “The role of the anti-ageing protein Klotho in vascular physiology and pathophysiology” Ageing Research Reviews. **35**, pp. 124-146
Mendes, M. M., et al. (2019). "Impact of high latitude, urban living and ethnicity on 25-hydroxyvitamin D status: A need for multidisciplinary action?" The Journal of Steroid Biochemistry and Molecular Biology **188**: 95-102.

Meulenbelt, H.E.J. Geertzen, J.H.B. Dijkstra, P.U. and Jonkman, M.F. (2007). "Skin problems in lower limb amputees: an overview by case reports." Journal of the European Academy of Dermatology and Venereology **21**(2).
Meunier, M., et al. (2022). "Targeting SDF-1 as an efficient strategy to resolve skin hyperpigmentation issues with Himanthalia elongate extract." Journal of Cosmetic Dermatology.
Migdadi, E.M. Courtenay, A.J. Tekko, I.A. McCrudden, M.T.C. Kearney, M.C. McAlister, E. et al., (2018) "Hydrogel-forming microneedles enhance transdermal delivery of metformin hydrochloride"Journal of Controlled Release, **285**, pp.142-151, ISSN 0168-3659,
Mikit M., Caracciolo V., Melillo A., Amicarelli F., and Giordano A. (2020) "Role of p53 in the Regulation of Cellular Senescence" Biomolecules. **10**(3), pp. 430.
Min HS., Kim Y., Kang G., Jang M., Yang H., and Jung H. (2022) "Shape of dissolving microneedles determines skin penetration ability and efficacy of drug delivery". Biomater. Advances. **145**, pp. 213248. doi: 10.1016/j.bioadv.2022.213248.
Mitchell RE (1963) The effect of prolonged solar radiation on melanocytes of the human epidermis. J Invest Dermatol 41:199– 212
Mitra, D., et al. (2012). "An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background." Nature **491**(7424): 449-453.
Mittal A., Piyathilake C., Hara Y., and Katiyar S.K. (2003) "Exceptionally high protection of photocarcinogenesis by topical application of (-)-epigallocatechin-3-gallate in hydrophilic cream in SKH-1 hairless mouse model: Relationship to inhibition of UVB-induced global DNA hypomethylation". Neoplasia. **5**, pp. 555–565. doi: 10.1016/S1476-5586(03)80039-8.
Mogire RM, Mutua A, Kimita W, Kamau A, Bejon P, Pettifor JM. (2020) "Prevalence of vitamin D deficiency in Africa: a systematic review and meta-analysis" The Lancet, Global Health. **8**(1), E134-142,
Montagna W. (1991) "The architecture of black and white skin" Journal of the American Academy of Dermatology. **24**, pp.29-37.
Moore, L. D., T. Le and G. Fan (2013). "DNA Methylation and Its Basic Function." Neuropsychopharmacology **38**(1): 23-38.
Morano A., Angrisano T., Russo G., Landi R., Pezone A., Bartollino S. (2014) "Targeted DNA methylation by homology-directed repair in mammalian cells. Transcription reshapes methylation on the repaired gene" Nucleic Acids Res. **42**, pp. 804-821.
Moreira AF., Rodrigues CF., Jacinto TA., Miguel SP., Costa ES., and Correia IJ. (2019) "Microneedle-based delivery devices for cancer therapy: A review" Pharmacological Research. 148, pp. 104438.
Morrone, A., M. L. Dell'Anna, A. Cristaudo, T. Wubayehu, H. Godefay, G. A. Barnabas, F. Dassoni, V. Padovese, O. Latini, T. Lotti, P. Saraceni, E. G. Di Domenico and V. Lora (2022). "Psoriasis in Tigray, Ethiopia: Focusing on available treatments." Dermatologic Therapy **35**(3).
Morsli S., Doherty GJ., and Muñoz-Espín D. (2022) "Activatable senoprobes and senolytics: Novel strategies to detect and target senescence cells" Mechanisms of Ageing and Development**. 202.**
Mostafa WZ., and Hegazy RA. (2015) "Vitamin D and the Skin: Focus on a complex relationship: A review" J Adv Res. **6**(6), pp. 793-804.
Mottolini N. (2022) "Diabetes and lower limb complications: A thematic review of clinical negligence claims" NHS Resolution.
Mousavi BF, Mirsalimi MR, Soroush M., Masumi and Montazeri A, (2009). "Quality of life in war related bilateral lower limb amputees: Results of a national project from Iran". J. Iranian Instit. Health Sci. Res., 8: 303-310.

Moussi, J.and Kosel, J. (2018) "3-D printed biocompatible micro-bellow membranes" Microelectromechanical Systems. Syst. **27**(3), pp. 472-478
Moussi, K., A. Bukhamsin, T. Hidalgo and J. Kosel (2020). "Biocompatible 3D Printed Microneedles for Transdermal, Intradermal, and Percutaneous Applications." Advanced Engineering Materials **22**(2): 2070005.
Mpofana, N., B. Chibi, N. Gqaleni, A. Hussein, A. J. Finlayson, K. Kgarosi and N. C. Dlova (2023). "Melasma in people with darker skin types: a scoping review protocol on prevalence, treatment options for melasma and impact on quality of life." Systematic Reviews **12**(1).
Murai K., Skrupskelyte G., Piedrafita G., Hall M., Kostiou V., Ong SW et al., (2018) "Epidermal Tissue Adapts to Restrain Progenitors Carrying Clonal p53 Mutations" Cell Stem Cell. **23**(5), pp. 687-699.
Murase, D., A. Hachiya, K. Takano, R. Hicks, M. O. Visscher, T. Kitahara, et al., (2013). "Autophagy Has a Significant Role in Determining Skin Color by Regulating Melanosome Degradation in Keratinocytes." Journal of Investigative Dermatology **133**(10): 2416-2424.
Murphrey MB., Miao JH and Zito PM. (2022) "Histology, Stratum Corneum" StatPearls.
Mytych J., Romerowicz-Misielak M., and Koziorowski M. (2018) "Klotho protects human monocytes from LPS-induced immune impairment associated with immunisebescent-like phenotype" Mol Cell Endocrinol. 15(470), pp. 1-13.
Naik PP and Farrukh SN. (2022) "Influence of Ethncities and Skin Colour Variance in Different Populations: A Review" Dermatology **35**(2), pp.65-76:doi: https://doi.org/10.1159/000518826
Nair K., Whiteside B., Grant C., Patel R., Tuinea-Bobe C., Norris K., and Paradkar A. (2015) "Investigation of plasma treatment on micro-injection moulded microneedle for drug delivery" Pharmaceutics. **7**, pp. 471-485.
Nakashima, S., H. Yamanashi, S. Komiya, K. Tanaka and T. Maeda (2018). "Prevalence of pressure injuries in Japanese older people: A population-based cross-sectional study." PLOS ONE **13**(6): e0198073.
Nakata, M., M. Shimoda and S. Yamamoto (2021). "UV-Induced Neuronal Degeneration in the Rat Cerebral Cortex." Cerebral Cortex Communications **2**(1).
Nakatsuji T., Chen TH., Butcher AM., Trzoss LL., Nam SJ., Shirakawa KT., Zhou W., Oh J., Otto M., and Fenical W.A (2018) "Commensal strain of Staphylococcus epidermidis protects against skin neoplasia". Sci. Adv. **4**, pp. eaao4502.
Nakyai W., M. Tissot, P. Humbert, F. Grandmottet, J. Viyoch and C. Viennet (2018). "Effects of Repeated UVA Irradiation on Human Skin Fibroblasts Embedded in 3D Tense Collagen Matrix." Photochemistry and Photobiology **94**(4): 715-724.
Naoi M., Maruyama W., and Riederer P. (2010) "Melanin" Encyclopedia of Movement Disorders, pp. 169-171.
Napolitano, M., et al. (2019). "Fewer common melanocytic nevi in atopic vs. non-atopic dermatitis children in a birth cohort study." International Journal of Dermatology **58**(12).
Narla S., Heath CR., Alexis A., and Silverberg JI. (2023) "Racial disparities in Dermatology" Arch Dermatol Res. **315**(5), pp. 1215-1223.
Nasir NAM., Paus R., and Ansell DM. (2019) "Fluorescent cell tracer dye permits real-time assessment of re-epithelization in a serum-free ex vivo human skin wound assay" Wound Repair Regen. **21**(1), pp. 126-133.
National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 175585, Ditrimethylolpropane tetraacrylate. Retrieved March 28, 2023, from https://pubchem.ncbi.nlm.nih.gov/compound/Ditrimethylolpropane-tetraacrylate.
National Pressure Ulcer Advisory Panel (2008). "National Pressure Ulcer Advisory Panel (NPUAP) pressure ulcer stages/categories". Retrieved from http://www.npuap.org/wp-content/uploads/2012/01/NPUAP-Pressure-Ulcer-Stages-Categories.pdf

Navarro-Garcia JA., Rueda A., Romero-Garcia T., Aceves-Ripoli J., Rodriguez-Sánchez E., González-Lafuente L et al., (2020) "Enhanced Klotho availability protects against cardiac dysnfunction induced by uraemic cardiomyopathy by regulating Ca^{2+} handling" Br J Pharmacol. **177**(20).
Nayak AL., Pickett AT., Delisle M., Dingley B., Mallick R., Hamilton T., et al. (2023) "Surivival of Patienys with Head and Neck Merkel Cell Cancer; Findings from the Pan-Canadain Merkel Cell Cancer Collaborative" Otolaryngology. **6**(11),
Nayak CS., and Isaacson G. (2003) "Worldwide Distribution of Waardenbury Syndrome" Annals of Otology, Rhinology & Laryngology. **112**(9).
Neema S., Sandhu S., and Kinra P. (2022) "Dermoscopy of Erythrokeratoderma Variabilis" Indian Dermatol. Online. J. **13**(4), pp. 556-557.
Negri VA., and Watt FM. (2022) "Understanding Human Epidermal Stem Cells at Single-Cell Resolution" Journal of Investigate Dermatology. **142**(8), pp. 2061-2067.
Nelson KL., Boehm AB., Davies-Colley RJ., Dodd MC., Kohn T., Linden KG et al. (2018) "Sunlight-mediated inactivation of health-relevant microorganisms in water: a review of mechanisms and modelling approaches" Environ. Sci. J. Integr. Environ. Res: Process Impacts. **20**(8), pp. 1089-1122.
Ngassie MLK., Vries MD., Borghuis T., Timens W., Sin DD., Nickle D et al., (2023) "Age-associated differences in the human lung extracellular matrix" Am J physiol Lung Cell Mol Physiol. **324**(6), pp. L799-L814.
Nicolai, S., A. Rossi, N. Di Daniele, G. Melino, M. Annicchiarico-Petruzzelli and G. Raschellà (2015). "DNA repair and aging: the impact of the p53 family." Aging **7**(12): 1050-1065.
Niculite CM., Nechifor MT., Urs AO., Olariu L., Ceafalan LC., and Leabu M.(2018) "Keratinocyte motility is Affected by UVA radiation – A comparison between Normal and Dysplastic Cells" Int J Mol Sci. **19**(6), pp.1700
Nishiguchi MA., Spencer CA., Leung DH., and Leung TH. (2018). "Aging Suppresses Skin-Derived Circulating SDF1 to Promote Full-Thickness Tissue Regeneration." Cell Reports **24**(13), pp.3383-3392.e3385.
Nishimura EK. (2011) "Melanocyte stem cells: a melanocyte reservoir in hair follicles for hair and skin pigmentation" Pigment Cell Melanoma Res. **24**, pp. 401-410.
Niu, Q., H. Chen, Q. Ou, S. Yang, Y. Peng, Y. Xie, L. (2023). "Klotho enhances bone regenerative function of hPDLSCs via modulating immunoregulatory function and cell autophagy." Journal of Orthopaedic Surgery and Research **18**(1).
Niyomnaitham S., Chatsiricharoenkul S., Toh ZQ., Senawong S., Pheerapanyawaranun C., Phumiamorn S., Licciardi PV., and Chokephaibulkit K. (2022) "Evaluation of the Safety and Immunogenicity of Fractional Inradermal COVID-19 Vaccines as Booster: A Pilot Study" Vaccines (Basel). **10**(9), pp. 1497.
Noh I., Lee K., and Rhee Y-S. (2022) "Microneedle systems for delivering nuclei acid drugs" J Pharm Investig. **52**(3), pp. 273-292.
Nolan G., Lawes S., Hainsworth S., and Rutty G. (2012) "A study considering the force required for broken glass bottles to penetrate a skin simulant" Int J Legal Med. **126**(1), pp. 19-25.
Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG, et al. (2007) "Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II)". Eur J Vasc Endovasc Surg, 33(1), pp.S1–S75.
Obeid R., Rickens P., Heine GH., Emrich IE., Fliser D., Zawada AM., Bodis M., and Geisel J. (2023) "ELOVL2-methylation and renal and cardiovascular event in patients with chronic kidney disease" Eur. J Clin Invest. **53**(12), pp. e14068.

Odhiambo JA., Williams HE., Clayton TO., Robertson CF., and Asher MI. (2009). "Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three." Journal of Allergy and Clinical Immunology **124**(6): 1251-1258.e1223.
Office for National Statistics. Living longer—how our population is changing and why it matters. (2018). Accessed from https://tinyurl.com/yd369so8 (accessed 5 November 2018)
Ogun GO., Ezenkwa US., and Ayandipo OO. (2020) "Dermatofibrosarcoma protuberance in a black African cohort – a clinicopathologic study" E Cancer Medical Science. **14**, pp. 1086.
Ogura S, and Jakovljevic M. (2014) "Health financing constrained by population aging - an opportunity to learn from Japanese experience". Serbian J Exp Clin Res.**15**, pp. 175–81. doi: 10.2478/SJECR-2014-0022
Ogura, Seiritsu and Jakovlijevic MM. (2018). "Editorial: Global Population Aging - Health Care, Social and Economic Consequences". Frontiers in Public Health. 6(305) doi: 10.3389/fpubh.2018.00335
Ohtani, N., A. Takahashi, D. J. Mann and E. Hara (2012). "Cellular senescence: a double-edged sword in the fight against cancer." Experimental Dermatology **21**: 1-4.
Oishi H., Doi S., Nakashima A., Ike T., Maeoka Y., Sasaki K. et al., (2021) "Klotho overexpression protects against renal aging along with suppression of transforming growth factor-B1 signaling pathways" American Journal of Physiology, Renal Physiology.
Oiso, N., K. Fukai, A. Kawada and T. Suzuki (2013). "Piebaldism." The Journal of Dermatology **40**(5): 330-335.
Okazaki M., Yoshimura k., Uchida G., and Harri K. (2005). "Correlation between age and the secretions of melanocyte-stimulating cytokines in cultured keratinocytes and fibroblasts." British Journal of Dermatology **153**(s2), pp.23-29.
Olatunji O., Das DB., Garland MJ., Belaid L., and Donnelly RF. (2013) "Influence of array interspacing on the force required for successful microneedle skin penetration: theoretical and practical approaches" J. Pharm. Sci. **102**(4), pp. 1209-1221.
Oliva, N. and BD. Almquist (2020). "Spatiotemporal delivery of bioactive molecules for wound healing using stimuli-responsive biomaterials." Advanced Drug Delivery Reviews **161-162**: 22-41.
Onyishi NT., and Ohayi SR. (2022) "Carcinomas in African Albino Skin Cancer Lesions: A Systematic Review and Meta-Analysis of proportion" Journal of Skin Cancer.
Ortonne JP. (1990) Pigmentary changes of the ageing skin" Br J Dermatol. (35), pp.21-8:doi:10.1111/j.1365-2133.1990.tb16121.x.
Ouali, S., Y. Louis, P. Germain, R. Gourdon and V. Desjardin (2018). "Leaching and Biodegradation of Darocur 1173 used as a Photo-Initiator in the Production of Photocrosslinked Silicone Acrylates." Journal of Polymers and the Environment **26**(1): 244-253.
Owolabi, E.O. Adeloye, D. Ajayi, A.I. McCaul, M. Davies, J. and Chu, K.M. (2022). "Lower limb amputations among individuals living with diabetes mellitus in low- and middle-income countries: A systematic review protocol." PLOS ONE **17**(4): e0266907.
Padamwar MN, Pawar AP, Daithankar AV, Mahadik KR. "Silk sericin as a moisturizer: an in vivo study". J Cosmet Dermatol. **4**(4), pp. 250–7. doi: 10.1111/j.1473-2165.2005.00200.x
Pageon H., Zucchi H., Ricois S., Bastien P., and Asselineau D. (2021) "UVA Exposure Combined with Glycation of the Dermis Are Two Catalysts for Skin Aging and Promotes a Favorable Environment to the Appearance of Elastosis" J Aging Res.
Pailler-Mattei C., Beca S., and Zahouani H. (2008) "In vivo measurements of the elastic mechanical properties of human skin by indentation tests". Med Engin Phys. **30**, pp.599–606.
Pajak J., Nowicka D., and Szepietowski JC. (2023) "Inflammaging and Immunosenescence as Part of Skin Aging – A Narrative Review" Int. J. Mol. Sci. **24**(9), pp. 7784.

Pakshir P, Alizadehgiashi M, Wong B, Coelho NM, Chen X, Gong Z, Shenoy VB, McCulloch CA, and Hinz B (2019). “Dynamic fibroblast contractions attract remote macrophages in fibrillar collagen matrix”. Nat Commun **10**, pp. 1850.
Palmer RD. (2022) “Aging clocks & mortality timers, methylation glycomic telomeric and more. A window to measuring biological age” Aging Med (Milton). **5**(2), pp. 12-125.
Pandel, R., B. Poljšak, A. Godic and R. Dahmane (2013). "Skin Photoaging and the Role of Antioxidants in Its Prevention." ISRN Dermatology **2013**, pp.1-11.
Pankaj, S., et al. (2016). "Malignant Melanoma of Vagina: A Report and Review of Literature." The Journal of Obstetrics and Gynecology of India **66**(5): 394-396.
Papadavid E, Koumantaki E, and Dawber RP. (1998) “Erythrokeratoderma variabilis: Case report and review of the literature.” J Eur Acad Dermatol Venereol. **11**, pp.180–3.
Papakonstantinou, E., M. Roth and G. Karakiulakis (2012). "Hyaluronic acid: A key molecule in skin aging." Dermato-Endocrinology **4**(3): 253-258.
Pararasa C., Messenger DJ., Barrett KE., Hyliands D., Talbot D., Fowler MI., et al. (2022) “Lower polyunsaturated fatty acid levels and FADS2 expression in adult compared to neonatal keratinocytes are associated with FADS2 promoter hypermethylation” Biochem Biophys Res Commun.**23**(601), pp. 9-15.
Parisi R., Kontopantellis E., and Ashcroft DM. (2020) “National, regiona, worldwide epidemiology of psoriasis: systematic analysis and modelling study” thebmj. **369**
Park, J.-H. and M. Prausnitz (2010). "Analysis of mechanical failure of polymer microneedles by axial force." Journal of the Korean Physical Society **56**(4): 1223-1227.
Park, J.-H. and M.-H. Lee (2005). "A Study of Skin Color by Melanin Index According to Site, Gestational Age, Birth Weight and Season of Birth in Korean Neonates." Journal of Korean Medical Science **20**(1): 105.
Park, J.-H., Yoon, Y.-K., Choi, S.-O., Prausnitz, MR., and Allen, MG. (2007) “Tapered Conical Polymer Microneedles Fabricated Using an Integrated Lens Technique for Transdermal Drug Delivery”. IEEE Trans. Biomed. Eng. **54**, pp. 903–913.
Park, J.H. Allen, M.G. Prausnitz, M.R. (2005) “Biodegradable and polymer microneedles: fabrication, mechanics, and transdermal drug delivery” J Control Release **104**(1), pp.51-66:doi 10.1016/j.jconrel.2005.02.002
Park, J.H. Yoon, Y.K. Choi, S.O. Prausnitz, M.R. and Allen, M.G. (2008) “Tapered Conical Polymer Microneedles Fabricated Using an Integrated Lens Technique for Transdermal Drug Delivery” IEEE Transactions on Biomedical Engineeering **54**(5)
Parrado, C., S. Mercado-Saenz, A. Perez-Davo, Y. Gilaberte, S. Gonzalez and A. Juarranz (2019). "Environmental Stressors on Skin Aging. Mechanistic Insights." Frontiers in Pharmacology **10**.
Pastar I, Stojadinovic O, Yin NC, Ramirez H, Nusbaum AG, Sawaya A, Patel SB, Khalid L., Isseroff RR, Tomic-Canic M. (2014) “Epithelialization in Wound Healing: A Comprehensive Review”. Adv. Wound Care, pp.445–464. doi: 10.1089/wound.2013.0473
Pastar I., Stojadinovic O., Yin NC., Ramirez H., Nusbaum AG., Sawaya A., et al. (2014) “Epithelisation in Wound Healing: A Comprehensive Review” Advances in Wound Care. **3**(7), pp. 445-464.
Patra VK., Byrne SN., and Wolf P. (2016) “The Skin Microbiome: Is It Affected by UV-induced Immune Suppression” Frontiers in Microbiology.
Patra, V., K. Wagner, V. Arulampalam and P. Wolf (2019). "Skin Microbiome Modulates the Effect of Ultraviolet Radiation on Cellular Response and Immune Function." iScience **15**: 211-222.
Paulson KG., Park SY., Vandeven NA., Lachance K., Thomas H., Chapuis AG., Harms KL et al. (2018) “Merkel cell carcinoma: Current US incidence and projected increases based on changing demographics” J Am Acad Dermatol. **3** pp. 457-463.

Pavel SF, van Nieuwpoort H, van der Meulen C, Out K, Pizinger P, Cetkovska N, et al., (2004) 'Disturbed melanin synthesis and chronic oxidative stress in dysplastic naevi" Eur. J. Cancer, **40**, pp.1423-1439
Pawlaczyk, M., M. Lelonkiewicz and M. Wieczorowski (2013). "Age-dependent biomechanical properties of the skin." Advances in Dermatology and Allergology **5**: 302-306.
Pearton M., Saller V., Coulman SA., Gately C., Antsey AV., Zarnitsyn V., and Birchall JC. (2012) "Microneedle delivery of plasmid DNA to living human skin: Formulation coating. Skin insertion and gene expression" Journal of Controlled Release. 160(3), pp. 561-569.
Peppas, N.A. Keys, K.B. Torres-Lugo, M. and Lowman, A.M. (1999) Poly(ethylene glycol)-containing hydrogels in drug delivery. *J. Controlled Release,* **62** , pp.81 —87
Perna, L., Y. Zhang, U. Mons, B. Holleczek, K.-U. Saum and H. Brenner (2016). "Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort." Clinical Epigenetics **8**(1).
Perugu RKT., Karra N., Shaik SS>, Venigalla WC., Preeti G., and Maram MR. (2023) "Griscelli Syndrome With Hemophagocytic Lymphohistiocytosis: A Rare Case Reeport" Cereus. **15**(8).
Petit-Frère C., Clingen PH., Grewe M., Krutmann J., Roza L., Arlett CF., and Green MH. (1998) "Induction of interleukin-6 production by ultraviolet radiation in normal human epidermal keratinocytes and in a human keratinocyte cell line is mediated by DNA damage" J Invest Dermatol. **111**(3), pp. 354-9.
Pfeifer GP. "Mechanisms of UV-induced mutations and skin cancer" Genome Instability & Disease. **1**, pp. 99-113.
Phatoli, R., N. Bila and E. Ross (2015). "Being black in a white skin: Beliefs and stereotypes around albinism at a South African university." African Journal of Disability **4**(1).
Philips N., Chalensouk-Khaosaat J., and Gonzalez S. (2018) "Simulation of the Elastin and Fibrillin in Non-Irradiated or UVA Radiated Fibroblasts, and Direct Inhibition of Elastase or Matrix Metalloproteinases Activitiy by Nicotinamide or Its Derivatives" J Cosmet Sci. **69**(1), pp. 47-56.
Piao Y., You H., Xu T., Bei HP., Piwko IZ., Kwan YY., and Zhao X. (2021) "Biomedical applications of gelatin methacryloyl hydrogels" Engineered Regeneration **2**, pp.47-56:doi:https://doi.org/10.1016/j.engreg.2021.03.002
Piedrafita G., Kostiou V., Wabik A., Colom B., Fernandez-Antoran D., Herms A et al., (2020) "A single propgenitor model as the unifying paradigm of epidermal and esophageal epithelial maintenance in mice" Nature communications. **11**, pp. 1429.
Piipponen, M., D. Li and N. X. Landén (2020). "The Immune Functions of Keratinocytes in Skin Wound Healing." International Journal of Molecular Sciences **21**(22): 8790.
Pils V, Ring N, Valdivieso K, Lämmermann I, Gruber F, Schosserer M et al., (2021) "Promises and challenges of senolytics in skin regeneration, pathology and ageing" Mechanisms of Ageing and Development. **200**.
Pittayapruek P., Meephansan J., Prapapan O., Komine M., and Ohtsuki M. (2016) "Role of Matrix Metalloproteinases in Photoaging and Photocarcinogenesis" Int J Mol Sci. **17**(6), pp. 868.
Plikus, M. V., X. Wang, S. Sinha, E. Forte, S. M. Thompson, E. L. Herzog, R. R. Driskell, N. Rosenthal, J. Biernaskie and V. Horsley (2021). "Fibroblasts: Origins, definitions, and functions in health and disease." Cell **184**(15): 3852-3872.
Poljšak, B. and R. Dahmane (2012). "Free Radicals and Extrinsic Skin Aging." Dermatology Research and Practice pp. 1-4.
Pontén F., Berne B., Ren ZP., Nistér M., and Pontén J. (1995) "Ultraviolet light induces expression of p53 and p21 in human skin: effect of sunscreen and constitutive p21 expression in skin appendages" J Invest Dermatol. **105**(3), pp. 402-6.

Pop T., Steinritz D., Brett A., Deppe J., Egea V., Schmidt A., et al., (2014) “Wnt5a/[beta]-catenin signaling drives calcium-induced differentiation of human primary keratinocytes” J Invest Dermatol. **134**, pp. 2183-2191
Popp, T., D. Steinritz, A. Breit, J. Deppe, V. Egea, A. Schmidt, T. Gudermann, C. Weber and C. Ries (2014). "Wnt5a/β-Catenin Signaling Drives Calcium-Induced Differentiation of Human Primary Keratinocytes." Journal of Investigative Dermatology **134**(8): 2183-2191.
Population Reference Bereau. (2022) “World Population Data Sheet” Accessed: https://www.prb.org/wp-content/uploads/2022/09/2022-World-Population-Data-Sheet-Booklet.pdf
Posnett J. (2007) “The costs of skin breakdown and ulceration in the UK” Skin Breakdown.
Pourmadadi M.m Darvishan S., Abdouss M., Yazdian F., Rahdar A., and Díez-Pascual AM. (2023) “pH-responsive polyacrylic acid (PAA)-carboxymethyl cellulose (SMS) hydrogel incorporating halloysite nanotuves (HNT) for controlled curcumin delivery” Industrial Crops and Products. **197**.
Power B., CR. Ferreira, D. Chen, WM. Zein, KJ. O’Brien, WJ. Introne, et al., (2019). "Hermansky-Pudlak syndrome and oculocutaneous albinism in Chinese children with pigmentation defects and easy bruising." Orphanet Journal of Rare Diseases **14**(1).
Pozzi A., Yurchenco PD., and Iozzo RV. (2017) “The nature and biology of basement membranes” Matrix Biol. 57-58, 1-11.
Prausnitz MR (1996) “The effects of electric current applied to skin: A review for transdermal drug delivery” Advanced Drug Delivery Reviews **18**(3), pp. 395-425.
Prausnitz MR., Mitragotri S. and Langer R. (2004) “Current status and future potential of transdermal drug delivery”. Nat. Rev. Drug Discov. **3**, pp. 115–124
Prausnitz, MR. (2004) “Microneedles for transdermal drug delivery”. Adv. Drug Deliv. Rev. **56**, pp. 581–587.
Price KN., Hsiao JL., and Shi VY. (2021). "Race and Ethnicity Gaps in Global Hidradenitis Suppurativa Clinical Trials." Dermatology **237**(1): 97-102.
Prost-Squarcioni C, Fraitag S, Heller M, Boehm N. (2008) “Functional histology of dermis”. Ann Dermatol Venereol. **135**(1 Pt 2), pp.1S5-20.
Proteau P., Gerwick W., Garcia-Pichel F and Castenholz R. (1993) “The structure of scytonemin an ultraviolet sunscreen pigment from the sheaths of cyanobacteria” Experientia. **49**, pp. 825-829.
Prud’homme GJ., Kurt M., and Wang Q. (2022) “Pathobiology of the Klotho Antiaging Protein and Therapuetic Considerations” Front. Aging. **V**
Pruettijarai, U., J. Meephansan, O. Prapapan, P. Pureesrisak, P. Sirithanabadeekul, K. Tantisantisom, S. Thongma, Y. Rayanasukha, P. Adulyaritthikul and P. Khanchaitit (2022). "Efficacy of a novel microneedle patch for rejuvenation of the nasolabial fold." Skin Research and Technology **28**(6): 786-791.
Qin, W., G. Quan, Y. Sun, M. Chen, P. Yang, D. Feng, T. Wen, X. Hu, X. Pan and C. Wu (2020). "Dissolving Microneedles with Spatiotemporally controlled pulsatile release Nanosystem for Synergistic Chemo-photothermal Therapy of Melanoma." Theranostics **10**(18): 8179-8196.
Qu, J., L. Ouyang, C.-C. Kuo and D. C. Martin (2016). "Stiffness, strength and adhesion characterization of electrochemically deposited conjugated polymer films." Acta Biomaterialia **31**: 114-121.
Quan T., and Fisher GJ. (2015) “Role of Age-Associated Alterations of the Dermal Extraellular Matrix Microenvironment in Human Skin Aging” Gerontology. **61**(5) pp. 427-434.

Quan, T., Z. Qin, W. Xia, Y. Shao, J. J. Voorhees and G. J. Fisher (2009). "Matrix-Degrading Metalloproteinases in Photoaging." Journal of Investigative Dermatology Symposium Proceedings **14**(1): 20-24.
Quevedo WC. (1973) "Genetic Control of Melanin Metabolism within the Melanin Unit of Mammalian Epidermis". Journal of Investigative Dermatology. **60**(6), pp.407-417:doi https://doi.org/10.1111/1523-1747.ep12702604
Rabheru K., Byles JE., and Kalache A., (2022) "How "old age" was withdrawn as a diagnosis from ICD-11". The Lancet. Healthy Longevity. 3(7)
Rad AN., and Grillari J. (2024) "Current senolytics: Mode of action, efficacy and limitations, and their future" Mechanisms of Ageing and Development **217**.
Raharja, A., S. K. Mahil and J. N. Barker (2021). "Psoriasis: a brief overview." Clinical Medicine **21**(3): 170-173.
Rakar J., Krammer MP., and Kratz G. (2015) "Human melanocytes mitigate keratinocyte-dependent contraction in vitro collagen contraction assay" Burns. **41**(5), pp. 1035-1042.
Rakshit, S. McGough, M. Amin, K. and Cox, C. (2022) "How does U.S. life expectancy compare to other countries?" Health & Wellbeing. 2022.
Ramakrishnan KM., Thomas KP and Sundararajan CR. (1974) "Study of 1,000 patients with keloids in South India" Plast Reconstr Surg. **53**(3), pp. 276-80.
Ramamoorthy, A., M. Pacanowski, J. Bull and L. Zhang (2015). "Racial/ethnic differences in drug disposition and response: Review of recently approved drugs." Clinical Pharmacology & Therapeutics **97**(3): 263-273.
Rampersad, S. N. (2012). "Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays." Sensors **12**(9): 12347-12360.
Rando TA. (2006) "Stem cells, ageing and the quest for immortality" Nature. **441**, pp. 1080-1086.
Rapoport, BL., D. A. Vorobiof, LM. Dreosti, A. Nosworthy, G. McAdam, J. P. Jordaan, H. Miller-Jansön, et al., (2017). "Ipilimumab in Pretreated Patients With Advanced Malignant Melanoma: Results of the South African Expanded-Access Program." Journal of Global Oncology **3**(5): 515-523.
Raymond-Lehman JR., and Riskin SI. (2023) "Benefits and Risks of Skin Exposure to Maintain Adequate Vitamin D LeveLs"Cureus. **15**(5).
Raymond-Lezman, J. R. and S. I. Riskin (2024). "Sunscreen Safety and Efficacy for the Prevention of Cutaneous Neoplasm." Cureus.
Rayner R, Carville K, Leslie G, Dhaliwal SS. (2018) "Skin tear risks associated with aged care residents: a cross-sectional study". Wound Pract Res. **6**(3), pp.127-135.
Read AP and Newton VE. (1997) "Waardenburg syndrome" Journal of Medical Genetics. **34**(8), pp.656-665
Reddy VD., Myers BA., Chan SY., Thibodeaux QG., Brownstone ND., Bhutani T et al. (2021) "A review of current phase III clinical trials of plaque psoriasis: under-representation of nonwhite participants and need for reform" Br J Dermatol. **184**(2), pp. 348-350.
Rees JL. (2004) "The genetics of sun sensitivity in humans" Am. J. Hum. Genet. **75**, pp. 739-751
Rekai, K. Belkharoubi, K. and Larbaoui, B. (2019). "Paclitaxel for the treatment of anal cancer after cisplatin and 5-fluorouracil." Annals of Oncology **30**(Supplement_4): iv1.
Rennekampff H-O., Hansbrough JF., Kiessig V., Doré C., Sticherling M., and Schröder J-M. (2000) "Bioactive Interleukin-8 Is Expressed in Wounds and Enhances Wound Healing" Journal of Surgical Research. **93**(1), pp. 41-54.
Rhinn, M., B. Ritschka and W. M. Keyes (2019). "Cellular senescence in development, regeneration and disease." Development **146**(20): dev151837.

Rhodes AR, Weinstock MA, Fitzpatrick TB, Mihm MC, Jr, and Sober AJ. (1987) 'Risk factors for cutaneous melanoma: a practical method of recognizing predisposed individuals. JAMA: the journal of the American Medical Association. **258**, pp. 3146-54.
Richardson M. (2003) "Understanding the structure and function of the skin" Nurs Times. **99**(31), pp. 46-48.
Richter T., Peuckert C., Sattler M., Koenig K., Riemann I., Hintze U. Et al., (2004) "Dead but highly dynamic – the stratum corneum is divided into three hydration zones" Skin Pharmacol Physiol. **17**(5), pp. 246-57
Richter T., Peuckert C., Sattler M., Koenig K., Riemann I., Hintze U et al. (2004) "Dead but highly dynamic – the stratum cornuem is divided into three hydration zones" Skin Pharmcol Physiol. **17**(5), pp. 246-57.
Ridky, T. W. (2007). "Nonmelanoma skin cancer." Journal of the American Academy of Dermatology **57**(3): 484-501.
Riese A., Eilert Y., Meyer Y., Arin M., Baron JM., Eming S et al. (2012) "Epidermal expression of neuropilin 1 protects murine keratinocytes from UVB-induced apoptosis". PLoS One 7, PP.e50944.
Riley PA. (1997) "Melanin". Int J Biochem Cell Biol, **29**(11), pp. 1235-9: doi: 10.1016/s1357-2725(97)00013-7. PMID: 9451820.
Rindler K, Krausgruber T, Thaler FM, Alkon N, Bangert C, Kurz H et al., (2021). "Spontaneously Resolved Atopic Dermatitis Shows Melanocyte and Immune Cell Activation Distinct from Healthy Control Skin" Front. Immunol. **12**.
Rinn, J.L. Wang, J.K. Allen, N. Brugmann, S.A. Mikels, A.J. Liu, H. et al. (2008). "A dermal HOX transcriptional program regulates site-specific epidermal fate." Genes & Development **22**(3): 303-307.
Ritchie H., and Roser M. (2019) "Age Structure" Our World in Data. Accessed: https://ourworldindata.org/age-structure
Ritchie H., Rodés-Guirao L., Mathieu E., Gerber M., Ortiz-Ospina E., Hasell J., and Roser M. (2023) "Population Growth" Published online at OurWorldInData.org. Retrieved from: 'https://ourworldindata.org/population-growth' [Online Resource]
Rittie, L. and G. J. Fisher (2015). "Natural and Sun-Induced Aging of Human Skin." Cold Spring Harbor Perspectives in Medicine **5**(1), pp. a015370-a015370.
Rizwan M, Yahya R, Hassan A, Yar M, Azzahari AD, Selvanathan V et al. (2017). "pH Sensitive Hydrogels in Drug Delivery: Brief History, Properties, Swelling, and Release Mechanism, Material Selection and Applications." Polymers **9**(12): 137
Robinson MK. (1999) "Population differences in skin structure and physiology and the susceptibility to irritant and allergic contact dermatitis: implications for skin safety testing and risk assessment" Contact Derm. **41**, pp. 65-79.
Römgens, A., Bader, D., Bouwstra, J., Baaijens, F., and Oomens, C. (2014) "Monitoring the penetration process of single microneedles with varying tip diameters". J. Mech. Behav. Biomed. Mater. **40**, pp. 397–405.
Rönn T., Volkov P., Gillberg L., Kokosar M., Perfilyev A., Jacobsen AL. et al. (2015) "Impact of age, BMI, HBA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood" Human Molecular Genetics. **24**(13), pp. 3792-3813.
Roos, W. P., A. D. Thomas and B. Kaina (2016). "DNA damage and the balance between survival and death in cancer biology." Nature Reviews Cancer **16**(1): 20-33.
Rossi DJ., Jamieson CH., and Weissman IL. (2008) "Stem cells and the pathways to aging and cancer" Cell. **132**, pp. 681-696.
Rotty JD., and Coulombe PA. (2012) "A wound-induced keratin inhibits Src activity during keratinocyte migration and tissue repair". J Cell Biol. **197**, pp. 381-389.

Roussou E., Fasoi G., Stavropoulou A., Kelesi M., Vasilopoulos G., Gerogianni G., and Alikari V. (2023) “Quality of life of patients with pressure ulcers: a systematic review” Med Pharm Rep. **96**(2), pp. 123-130.
Roxhed, N., Gasser TC., Griss P., and Holzapel G. et al. (2007). "Penetration-Enhanced Ultrasharp Microneedles and Prediction on Skin Interaction for Efficient Transdermal Drug Delivery." Journal of Microelectromechanical Systems **16**(6): 1429-1440.
Rubin AI., Chen EH., and Ratner D. (2005) “Basal-cell carcinoma” N Engl J Med. **353**(21), pp. 2262-9.
Rünger, T. M. (2007). "How Different Wavelengths of the Ultraviolet Spectrum Contribute to Skin Carcinogenesis: The Role of Cellular Damage Responses." Journal of Investigative Dermatology **127**(9): 2103-2105.
Ruocco, V. Brunetti, G. Puca, R. and Ruocco, E. (2009). "The immunocompromised district: a unifying concept for lymphoedematous, herpes-infected and otherwise damaged sites." Journal of the European Academy of Dermatology and Venereology **23**(12): 1364-1373.
Russo B., Brembilla NC., and Chizzolini C. (2020) “Interplay Between Keratinocytes and Fibroblasts: A systematic Review Providing Angle for Understanding Skin Fibrotic Disorders” **11**, pp. 648.
Russo, B., N. C. Brembilla and C. Chizzolini (2020). "Interplay Between Keratinocytes and Fibroblasts: A Systematic Review Providing a New Angle for Understanding Skin Fibrotic Disorders." Frontiers in Immunology **11**.
Sa, Y., C. Li, H. Li and H. Guo (2015). "TIMP-1 Induces α-Smooth Muscle Actin in Fibroblasts to Promote Urethral Scar Formation." Cellular Physiology and Biochemistry **35**(6): 2233-2243.
Sabri AH., Olivier J., Abdulhamid K., Shpadaruk V., McKenna J., Segal J., et al. (2019) “Expanding the applications of microneedles in dermatology” European Journal of Pharmaceutics and Biopharmceutics. **140**, pp. 121-140.
Sachdeva M., Shah M., and Alavi A. (2021) “Race-specific Prevalence of Hidradenitis Suppurativa” **25**(2), pp. 177-187.
Sadr N., and Qayyum R. (2023) “Association between Klotho protein and melanoma and nonmelanoma skin cancer in the US adult population: A cross-sectional study” JAAD Int. **28**(13), pp. 95-97
Säfholm A, Tuomela J, Rosenkvist J, Dejmek J, Härkönen P, Andersson T. (2008) “The Wnt-5a-Derived Hexapeptide Foxy-5 Inhibits Breast Cancer Metastasis In Vivo by Targeting Cell Motility” Cancer Therapy: Preclinical. **14**(20), pp. 6556-6563.
Saguie BO., Martins RL., de Souza de Fonseca A., Romana-Souza B., and Monte-Alto-Costa A. (2021) “An ex vivo model of human skin photoaging induced by UVA radiation compatible with summer exposure in Brazil” J Photochem Photobiol B. **221**, pp. 112255.
Salam, A. A. (2023). "Ageing in Saudi Arabia: new dimensions and intervention strategies." Scientific Reports **13**(1).
Salawu, A. Middleton, C. Gilbertson, A. Kodavali, K. and Neumann, V. (2006). "Stump Ulcers and Continued Prosthetic Limb Use." Prosthetics & Orthotics International **30**(3): 279-285.
Saleem, MD. (2019) “Biology of human melanocyte development, Piebaldism, and Waardenburg syndrome” Pediatr Dermatol. **36**(1), pp. 72-84, doi: 10.1111 /pde.13713. Epub 2018 Dec 18
Salihu S., Hart H., Wort C., and Reza MM. (2023) “Students bridging the diversity gap in dermatology-A mixed methods study of medical education” JEADV Clinical Practice.
Samant P., and Prausnitz MR. (2018) “Mechanisms of sampling Interstitial Fluid from Skin Using a Microneedle Patch.” Proceedings of the National Academy of Sciences. **115**.

Samouei R.and Kayvanara M. (2022) “Identifying strategies for dealing with the aging population from the perspective of health system experts: A qualitative study” J Educ Health Promot. **11**(21)
Sample H and He Y-Y. (2018) “Mechanisms and prevention of UV-induced melanoma” Photodermal Photoimmunol Photomed. 34(1), pp.13-24, doi: 10.1111/phpp.12329
Sanada, H., G. Nakagami, Y. Koyano, S. Iizaka and J. Sugama (2015). "Incidence of skin tears in the extremities among elderly patients at a long-term medical facility in Japan: A prospective cohort study." Geriatrics Gerontology International **15**(8), pp. 1058-1063.
Sander M., Sander M., Burbidge T., and Beecker J. (2020) “The efficacy and safety of sunscreen use for the prevention of skin cancer” CMAJ. **192**(50), pp. E1802-1808.
Sanders JL, and Newman AB. (2013) “Telomere length in epidemiology: A biomarker of aging, age-related disease, both, or neither?” Epidemiol Rev. **35**, pp.112–31. 10.1093/epirev/mxs008
Sandy-Møller J., Poulsen T., and Wulf HC. (2003) “Epidermal thickness at different body sites” relationship to age, gender, pigmentation, blood content, skin type and smoking habits” Acta Derm Venereol. **83**(6) pp. 410-3.
Sangha AM. (2022) “Dermatoligcal Conditions in SKIN OF COLOR-: Debunking” J Clin Aesthet Dermatol. **15**(9 Suppl 1), S10-S11.
Sargioti N., Levingstone TJ., O’Cearbhaill ED., McCarthy HO., and Dunne NJ. (2023) “Metallic Microneedles for Transdermal Drug Delivery: Applications, Fabrication Techniques and the Effect of Geometrical Characteristics” Bioengineering. **10**(1), 24.
Sayed N., Allawadhi P., Khurana A., Singh V., Navik U., Pasmurathi SK., et al., (2022) “Gene therapy: comprehensive overview and therapeutic applications” Life Sci. **294**.
Scharffetter K., M. Wlaschek, A. Hogg, K. Bolsen, A. Schothorst, G. Goerz, T. Krieg and G. Plewig (1991). "UVA irradiation induces collagenase in human dermal fibroblasts in vitro and in vivo." Archives of Dermatological Research **283**(8), pp. 506-511.
Schmaljiohann, D. (2006) “Thermo- and pH- responsive polymers in drug delivery” Adv Drug Deliv Rev. **58**(15), pp.1655-70:doi:10.1016/j.addr.2006.09.020.
Schneider CA., Rasband WS., and Eliceiri KW. (2012). "NIH Image to ImageJ: 25 years of image analysis." Nature Methods **9**(7): 671-675.
Schoellhammer CM., Blankschtein D., and Langer R. (2014) “Skin Permeabilization for Transdermal Drug Delivery: Recent Advances and Future Prospects”. Expert Opin. Drug Deliv. **11**, pp. 393–407
Schwartz RA, Bagley MP, Janniger CK, Lambert WC. (1991) “Verrucous Carcinoma of a Leg Amputation Stump” Dermatology **182**(3), pp. 193-195.
Schwartz, SD., Regillo CD., Lam BL., Eliott D., Rosenfeld PJ., Gregori NZ et al., (2015) “Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt’s macular dystrophy: follow-up of two open-label phase 1/2 studies”. Lancet **385**, pp. 509–516.
Schwarz, T. (2008). “25 years of UV-induced immunosuppression mediated by T cells-from disregarded T suppressor cells to highly respected regulatory T cells”. Photochem. Photobiol. **84**, pp. 10–18. doi: 10.1111/j.1751-1097.2007.00223.x
Schweintzger N., Gruber-Wackernagel A., Reginato E., Bambach I., Quehenberger F., Byrne SN., et al. (2015). “Levels and function of regulatory T cells in patients with polymorphic light eruption: relation to photohardening”. Br. J. Dermatol. **173**, pp. 519–526. doi: 10.1111/bjd.13930
Schweintzger NA., Gruber-Wackernagel A., Shirsath N., Quehenberger F., Obermayer-Pietsch B., and Wolf P. (2016). “Influence of the season on vitamin D levels and regulatory T cells in patients with polymorphic light eruption”. Photochem. Photobiol. Sci. **15**, pp. 440–446. doi: 10.1039/C5PP00398A

Schweizer J., Baust I., and Winter H. (1989) “Identification of murine type I keratin 9 (73 KDa) and its immunolocalization in neonatal and adult mouse foot sole epidermis” Exp Cell Res. **184**, pp. 193-206.
Scimecar M., Bischetti S., Lamsira HP., Bonfiglio R., and Bonanno E. (2018) “Energy dispersive x-ray (EDX) microanalysis: A powerful tool in biomedical research and diagnosis” Eur J Histochem. **62**(1), pp. 2841.
Scott, G., Leopardi, S., Printup, S., Malhi, N., Seiberg, M., and Lapoint, R. (2004). “Proteinase-activated receptor-2 stimulates prostaglandin production in keratinocytes: analysis of prostaglandin receptors on human melanocytes and effects of PGE2 and PGF2α on melanocyte dendricity”. Journal of investigative dermatology, **122**(5), pp.1214-1224.
Seeli DS, Prabaharan M. (2016) “Guar gum succinate as a carrier for colon-specific drug delivery. Int. J. Biol. Macromol. **84,** pp.10-15, doi:10.1016/j.ijbiomac.2015.12.002
Seetharam AA., Choudhry H., Bakhrebah MA., Abdulaal WH., Gupta MS., Rizvi SMD., Alam Q., Gowda DV., and Moin A. (2020) “Microneedles drug delivery systems for treatment of cancer: A recent update”. Pharmaceutics. **12**, pp. 1101. doi: 10.3390/pharmaceutics12111101.
Seiberg M, Paine C, Sharlow E, Andrade-Gordon P, Costanzo M, Eisinger M et al., (2000) “The Protease-Activated Receptor 2 Regulates Pigmentation via Keratinocyte-Melanocyte Interactions” Experimental Cell Research. **254**(1), pp. 25-32,
Seo J-E., Kim S., Shin M-H., Kim M-S., Eun HC., Park C-H., and Chung JH. (2010) “Ultraviolet irradiation induces thrombospondin-1 which attenuates type I procollagen downregulation in human dermal fibroblasts” Journal of Dermatological Science. **59**(1), pp. 16-24.
Sgonc, R. and J. Gruber (2013). "Age-Related Aspects of Cutaneous Wound Healing: A Mini-Review." Gerontology **59**(2), pp. 159-164.
Shaabani, E., M. Sharifiaghdam, R. Faridi-Majidi, S. C. De Smedt, K. Braeckmans and J. C. Fraire (2022). "Gene therapy to enhance angiogenesis in chronic wounds." Molecular Therapy - Nucleic Acids **29**: 871-899.
Shah BJ., Jagati AK>, Katrodiya NK., and Patel SM. (2016) “Griscelli syndrome type 3” Indian Dermatol Online J. **7**(6), pp. 506-508.
Shah M, Patton E, and Zedak D. (2022) Piebaldism. StatPearls.
Shah SK, Arthur A, Yang YC, Stevens S, Alexis AF. (20111A retrospective study to investigate racial and ethnic variations in the treatment of psoriasis with etanercept. *J Drugs Dermatol.* 2011;10:866–872.
Shan J., Zhang X., Wang L., and Zhao Y. (2023) “Spatiotemporal Catalytic Nanozymes Microneedle Patches with Opposite Properties for Wound Management” Nano Micro Small.
Shanbhag, S., A. Nayak, R. Narayan and U. Y. Nayak (2019). "Anti-aging and Sunscreens: Paradigm Shift in Cosmetics." Advanced Pharmaceutical Bulletin **9**(3): 348-359.
Sharpless NE and DePinho RA (2007) “How stem cells age and why this makes us grow old” Nat Rev. **8**, pp. 703-713.
Shaw, T. E., et al. (2011). "Eczema Prevalence in the United States: Data from the 2003 National Survey of Children's Health." Journal of Investigative Dermatology **131**(1): 67-73.
Sheela SR., Latha M., and Susy JL. (2004) “Griscelli syndrome: Rab 27a mutation” Indian Paediatrics. **41**, pp. 944-947.
Shin J-W., Kwon S-H., Choi J-Y., Na J-I., Huh C-H., Choi H-R., et al. (2019) “Molecular Mechanisms of Dermal Aging and Anti-aging Approaches” Int J Mol Sci. **20**(9), pp. 2126.
Shirai, M. (2014). "Photocrosslinkable polymers with degradable properties." Polymer Journal **46**(12): 859-865.

Shojaei H, Baghbani M, Reyegani M, Masoumi M and Mousavi B (2009). “Effective factors and approach to musculoskeletal pain 20 years after bilateral lower limb amputation”. 5th world congress of the international society of physical and rehabilitation medicine. pp: 306.
Shravanth SH., Osmani RAM., Jyothi SL., Anupama VP., Rahamathulla M., and Gangadharappa HV. (2021) “Microneedles-based drug delivery for the treatment of psoriasis” Journal of Drug Delivery Science and Technology. **64**.
Shtutman, M., B.-D. Chang, G. P. Schools and E. V. Broude (2017). “Cellular Model of p21-Induced Senescence” Springer New York**,** pp**.** 31-39.
Siderakis M, and Tarsounas M. (2007) “Telomere regulation and function during meiosis”. Chromosome Res. **15**, pp. 667–79. 10.1007/s10577-007-1149-7
Silverberg, J. I., et al. (2013). "Climatic Factors Are Associated with Childhood Eczema Prevalence in the United States." Journal of Investigative Dermatology **133**(7): 1752-1759.
Silverberg, JI. and EL. Simpson (2014). "Associations of Childhood Eczema Severity." Dermatitis **25**(3): 107-114.
Simonenko TL., Simonenko NP., Gorobstov PY., Simonenko EP., and Kuznetsov NT. (2023) “Microplotter printing of a Miniature Flexible Supercapacitor Electrode Based on Hierarchically Organised $NiCo_2O_4$ Nanostructures” Materials. **16**(12), pp. 4202
Simpson DJ. And Chandra T. (2021) “Epigenetic age prediction” Aging Cell. **20**(9), pp. e13452.
Sinclair D. (2019) “Lifespan: why we age, and why we don’t have to” Atria Books (US).
Sinha JK., Ghosh S., and Raghunath M. (2014) “Progeria” A rare genetic prematureageing disorder” Indian J Med Res. **139**(5), pp. 667-674.
Sinha RP., and Häder DP. (2008) “UV-protectants in cyanobacteria” Plant Sci. **174**(3), pp. 278-289.
Sinikumpu, S.-P., et al. (2022). "Skin cancers and their risk factors in older persons: a population-based study." BMC Geriatrics **22**(1).
Siquieria SOM., Campos-do-Carmo G., Santos ALSD., Martins C., and de Melo AC. (2023) “Merkel cell carcinoma: epidemiology, clinical features, diagnosis and treatment of a rare disease” An Bras Dermatol. **98**(3), pp. 277-286.
Sivamani RK., Garcia MS., and Isseroff RR. (2007) “Wound re-epithelialization: modulating keratinocyte migration in wound healing”. Front Biosci. **12**, pp. 2849–2868. doi: 10.2741/2277.
Skiveren J, Wahlers B, Bermark S.(2017) “Prevalence of skin tears in the extremities among elderly residents at a nursing home in Denmark”. J Wound Care. **26**(Sup2), pp. S32-S36.
Sloan DD., Nicholson B., Urguidl V., and Goodison S. (2004) “Detection of Differntially Expressed Genes in an Isogenic Breast Metastasis Model using RNA arbitrarily Primed-Polymerase Chain Reaction Coupled with Array Hybridzation (RAP-Array)” Am J Pathol. **154**(1), pp. 315-323.
Slominski, A., et al. (2005). "Hair Follicle Pigmentation." Journal of Investigative Dermatology **124**(1): 13-21.
Smith DR., Spaulding DT., Glenn HM., and Fuller BB. (2004) “The relationship between Na(+)/H(+) exchange expression and tyrosinase activity in human melanocytes” Exp Cell Res. **298**(2), pp. 521-34.
Smith R., Healy E., Siddiqui S., Flanagan N., Steijlen PM., Roshdal I., Jacques JP, et al. (1998) “Melanocortin 1 receptor variants in an Irish population” J Invest Dermatol. **111**, pp.119-122.
Snell RS, and Bischitz PG. (1963) "The melanocytes and melanin in human abdominal wall skin: a survey made at different ages in both sexes and during pregnancy." Journal of Anatomy **97**:pp.361.
Solano, F. (2020). "Photoprotection and Skin Pigmentation: Melanin-Related Molecules and Some Other New Agents Obtained from Natural Sources." Molecules **25**(7): 1537.

Son, DH., et al. (2019). "Recent Advances in Anti-Aging Medicine." Korean Journal of Family Medicine **40**(5), pp. 289-296.
Song L., and Zhang S. (2023) “Anti-Aging Activity and Modes of Action of Compounds from Natural Food Sources” Biomolecules. **13**(11), pp. 1600.
Sorlin, A., A. Maruani, M.-H. Aubriot-Lorton, P. Kuentz, Y. Duffourd, S. Teysseire, et al., (2017). "Mosaicism for a KITLG Mutation in Linear and Whorled Nevoid Hypermelanosis." Journal of Investigative Dermatology **137**(7): 1575-1578.
Spiers H., Hannon E., Wells S., Williams B., Fernandes C., and Mil J. (2016) “Age-associated changes in DNA methylation across multiple tissues in an inbred mouse model” Mechanisms of Ageing and Development. **154**. pp. 20-23.
Spritz RA. (1994) “Molecular basis of human piebaldism”. Journal of Investigative Dermatology. **103**:doi:10.1111/1523-1747.ep12399455.
Sriraman, A., Turja, B. Xhemalce and Kyle (2020). "Making it or breaking it: DNA methylation and genome integrity." Essays in Biochemistry **64**(5): 687-703.
Standring S. (2021) “Skin and its appendages”. Gray's Anatomy. 42nd ed. Philadelphia, PA: Elsevier; chap 7.
Starrico J, Pinkus H. (1957) “Quantitative and qualitative data on the pigment cells of adult human epidermis. J Invest Dermatol. **28**(1), pp.33-45: doi: 10.1038/jid.1957.4.
Steding-Jessen M, Birch-Johansen F, Jensen A, Schüz J, Kjaer SK and Dalton SO. (2010) “Socioeconomic status and non-melanoma skin cancer: a nationwide cohort study of incidence and survival in Denmark”. Cancer Epidemiol. **34**(6),pp.689–95.
Stierner U, Rosdahl I, Augustsson A, Kagedal B (1989) UVB irradiation induces melanocyte increase in both exposed and shielded human skin. J Invest Dermatol 92 :561–564
Stoddard-Bennett, T. & Reijo Pera, R. (2019) “Treatment of Parkinsonas disease through personalized medicine and induced pluripotent stem cells”. Cells **8**, 26
Stojadinovic O., and Tomic-Canic M. (2013) “Human ex vivo wound healing model” Methods Mol. Bio. **1037**, pp. 255-64.
Storey, A., F. McArdle, P. S. Friedmann, M. J. Jackson and L. E. Rhodes (2005). "Eicosapentaenoic Acid and Docosahexaenoic Acid Reduce UVB- and TNF-α-induced IL-8 Secretion in Keratinocytes and UVB-induced IL-8 in Fibroblasts." Journal of Investigative Dermatology **124**(1): 248-255.
Strambini, L. M., A. Longo, A. Diligenti and G. Barillaro (2012). "A minimally invasive microchip for transdermal injection/sampling applications." Lab on a Chip **12**(18): 3370.
Strassner, J. P., M. Rashighi and J. E. Harris (2016). "Melanocytes in psoriasis: convicted culprit or bullied bystander?" Pigment Cell & Melanoma Research **29**(3): 261-263.
Streit M., Velasco P., Brown LF., Skoke M., Richard L., Riccardi L.,et al., (1999) “Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas” Am J Pathol. **155**(2), pp. 441-52.
Streutker CJ., McCready D., Jimbow K., From L. (2000) “Malignant melanoma in a patient with oculocutaneous albinism” J Cutan Med Surg. **4**, pp.149-152
Strickland I., Rhodes LE., Flanagan BF., and Friedmann PS. (1997) “TNF-alpha and IL-8 are upregulated in epidermis of normal human skin after UVB exposure” correlation with neutrophil accumulation and E-selectin expression” J Invest Dermatol. **108**(5), pp. 763-768.
Sturm RA. (2002) “Skin colour and skin cancer - MC1R, the genetic link”. Melanoma Res. 2002;**12**:405–16. doi: 10.1097/00008390-200209000-00001.
Stüttgen, G., I. Brinkmann-Raestrupp, L. Haller, V. Kentsch and W. Schalla (1979). "Melanin granula distribution and phagocytosis in psoriasis vulgaris after PUVA therapy." Archives of Dermatological Research **264**(1): 29-35.

Su C-Y, Wang H-V, Hughes MW, Liu T-Y, Chuong C-M Yang W-C (2022). "Successful Repigmentation of Full-Thickness Wound Healing in Fraser's Dolphins (Lagenodelphis hosei)." Animals **12**(12): 1482.
Sudel KM., Venzke K., Mielke H. Breitenbach U., Mundt C., Jaspers S. et al., (2005) "Novel aspects of intrinsic and extrinsic aging of human skin: beneficial effects of soy extract" Photochem Photobiol. **81**(3), pp. 581-587.
Summers, A. J., J. P. Devadhasan, J. Gu, D. C. Montgomery, B. Fischer, M. A. Gates-Hollingsworth, K. J. Pflughoeft, T. Vo-Dinh, D. P. Aucoin and F. Zenhausern (2022). "Optimization of an Antibody Microarray Printing Process Using a Designed Experiment." ACS Omega **7**(36): 32262-32271.
Sun KL, Liu W, Gao XM, Yang M, C JM. (2021) "A Study of Normal Epidermal Melanocyte Distribution" International Journal of Dermatology and Venereology, **4**(1), pp. 32-35:doi:10.1097/JD9.0000000000000125
Sun M., Sun X., Wang Z., Guo S., Yu G., and Yang H. (2018) "Synthesis and Properties of Gelatin Methacryloyl (GelMA) Hydrogels and Their Recent Applications in Load-Bearing Tissue" Polymers. **10**(11), pp. 1290.
Sun Q. Rabbani P, Takeo M, Lee S-H, Lim CH, Noel E-NS et al., (2018). "Dissecting Wnt Signaling for Melanocyte Regulation during Wound Healing." Journal of Investigative Dermatology **138**(7): 1591-1600.
Sundberg, M. Bogetofte H., Lawson T., Jansson J., Smith G., Astradsson A et al., (2013) "Improved cell therapy protocols for Parkinson's disease based on differentiation efficiency and safety of hESC-, hiPSC-, and non-human primate iPSC-derived dopaminergic neurons". Stem Cells **31**, pp. 1548–1562.
Swensoon O., and Eady RA. (1996) "Morphology of the keratin filament network in palm and sole skin: evidence for site-dependent features based on stereological analysis" Arch Dermatol Res. **288**, pp. 55-62.
Swensson O., Langbein L., McMillan JR., Stevens HP., Leigh IM., McLean WH et al. (1998) "Specialized keratin expression pattern in human ridged skin as an adaptation to high physical stress" Br J Dermatol. **139**, pp. 767-775.
Swensson, O. Langbein, L. McMillan, J.R. Stevens, H.P. Leigh, I.M. McLean, W.H. et al. (1998) Specialized keratin expression pattern in human ridged skin as an adaptation to high physical stress. Br J Dermatol, **139**(5), pp.767-75
Swift, T. Swanson, L. Geoghegan, M. Rimmer, S. (2016). "The pH-responsive behaviour of poly(acrylic acid) in aqueous solution is dependent on molar mass." Soft Matter **12**(9): 2542-2549.
Szabó G, Gerald AB, Pathak MA, Fitzpatric TB. (1969). "Racial Differences in the Fate of Melanosomes in Human Epidermis." Nature **222**(5198): 1081-1082.
Tadokoro T, Kobayashi N, Zmudzka BZ, Ito S, Wakamatsu K, Yamaguchi Y et al., (2003). "UV-induced DNA damage and melanin content in human skin differing in racial/ethnic origin." The FASEB Journal **17**(9): 1177-1179.
Tagliabue, E., S. Gandini, R. Bellocco, P. Maisonneuve, J. Newton-Bishop, D. Polsky, et al., (2018). "MC1R variants as melanoma risk factors independent of at-risk phenotypic characteristics: a pooled analysis from the M-SKIP project." Cancer Management and Research **10**: 1143-1154.
Takegahara K., Usuda J., Inoue T., Sonokawa T., Matsui T., and Matsumoto M. (2021) "Antiaging gene Klotho regulates epithelial-mesenchymal transition and increases sensitivity to pemetrexed by inducing lipocalin-2 expression" Oncology letters. **418**.
Tambala-Kaliati, T., Adomako, EB., Frimpong-Manso, K. (2021) "Living with albinism in an African community: exploring the challenges of persons with albinism Lilongwe District, Malawi" Heliyon. **7**(5),

Tan, S., N. Khumalo and A. Bayat (2019). "Understanding Keloid Pathobiology From a Quasi-Neoplastic Perspective: Less of a Scar and More of a Chronic Inflammatory Disease With Cancer-Like Tendencies." Frontiers in Immunology **10**.
Tang J, Fewings E, Chang D, Zeng H, Liu S, Jorapur A et al., (2020). "The genomic landscapes of individual melanocytes from human skin." Nature **586**(7830): 600-605.
Tani H., Morris RJ., and Kaur P. (2000) "Enrichment for murine keratinocyte stem cells based on cell surface phenotype" National Acad Sciences. **97**(20), pp. 10960-10965
Taylor SC. (2003) "Epidemiology of skin diseases in ethnic populations" Dermatol Clin. **21**(4):doi: 10.1016/s0733-8635(03)00075-5.
Taylor SC. and Cook-Bolden F. (2002) "Defining Skin of Color" Highlighting Skin of Colour. **69**
Terranova VP, DiFlorio R, Lyall RM, Hic S, Friesel R, Maciag T. Human endothelial cells are chemotactic to endothelial cell growth factor and heparin. (1985) J Cel Biol. **101**(6), pp. 1330–1334.
Theocharis AD, Gialeli C, Bouris P, Giannopoulou E, Skandalis SS, Aletras AJ, Iozzo RV & Karamanos NK (2014) "Cell-matrix interactions: focus on proteoglycan-proteinase interplay and pharmacological targeting in cancer". FEBS J 281,pp. 5023–5042.
Theocharis AD, Gialeli C, Hascall VC & Karamanos NK (2012) "Extracellular matrix: a functional scaffold. In Extracellular Matrix: Pathobiology and signaling" Walter de Gruyter
Theocharis AD, Skandalis SS, Gialeli C & Karamanos NK (2016) "Extracellular matrix structure". Adv Drug Deliv Rev **97**,pp. 4–27.
Thingnes J Lavelle TJ, Hoving E, Omholt SW. (2012). "Understanding the Melanocyte Distribution in Human Epidermis: An Agent-Based Computational Model Approach." PLoS ONE **7**(7): e40377.
Thody AJ, Higgins EM, Wakamatsu K, Ito S, Burchill SA, and Marks JM. (1991), "Pheomelanin as well as Eumelanin Is Present in Human Epidermis". Journal of Investigative Dermatology, **97**(2), pp.340-344:https://doi.org/10.1111/1523-1747.ep12480680.
Tilburg, J., RC. Slieker, A. Heath, DV. Heemst, FR. De Gruijl, DA. Gunn and BT. Heijmans (2020). "Repeat UVA exposure of human skin fibroblasts induces both a transitionary and recovery DNA methylation response." Epigenomics **12**(7), pp. 563-573.
Tinker A. (2002) "The social implications of an ageing population" Mechanisms of Ageing and Development. **123**(7), pp. 729-735.
Tobin DJ, Veysey EC, Finlay AY. (2017) "Aging and the skin". Brocklehurst's Textbook of Geriatric Medicine and Gerontology. 8th ed. Philadelphia, PA: Elsevier; chap 25.
Toda, K., et al. (1972). "Alteration of Racial Differences in Melanosome Distribution in Human Epidermis after Exposure to Ultraviolet Light." Nature New Biology **236**(66): 143-145.
Tolentino Junior DS., and de Oliveira CM. (2019) "Population-based study of 24 autoimmune diseases carried out in a Brazilian microregion" J Epidemiology Global Health. **9**(4), pp. 243-251.
Topouzi H., Boyle CJ., Williams G., and Higgins, CA. (2020). "Harnessing the secretome of hair follicle fibroblasts to accelerate ex vivo healing of human skin wounds". J. Invest. Dermatol. 140, pp. 1075–1084.e11. https://doi.org/10.1016/j. jid.2019.09.019
Torchilin V. (2009) "Intracellular delivery of protein and peptide therapeutics". Drug Discov. Today Technol. **5**(2–3), pp. e95–e103.
Trapani L., and Pallottini V. (2010) "Age-Related Hypercholesterolemia and HMG-CoA Reductase Dysreugulation: Sex Does Matter (A Gender Perspective)" Curr Gerontol Geriatr Res.
Trofymenko O., Zeitouni NC., and Kurtzman DJB. (2018) "Factors associated with advanced-stage Merkel cell carcinoma at initial diagnosis and the use of radiation therapy: Results from the National Cancer Database" J Am Acad Dermatol. **79**(4), pp. 680-688.

Tundis R., Loizzo MR., Bonesi M., and Menichini F. (2015) “Potential role of natural compounds against skin aging”. Curr. Med. Chem. 22, pp. 1515–1538
Uitto J, Li Q, Urban Z. (2013) “The complexity of elastic fibre biogenesis in the skin--a perspective to the clinical heterogeneity of cutis laxa”. Exp Dermatol. **22**(2), pp.88-92.
Ulschmid, C., L. Serrano, R. Wu, G. M. Roth and O. Sokumbi (2023). "African American race is a risk factor for severe hidradenitis suppurativa." International Journal of Dermatology **62**(5): 657-663.
United Nations, Department of Economic and Social Affairs, Population Division (2017). World Population Prospects: The 2017 Revision. New York: United Nations.
United Nations, Department of Economic and Social Affairs, Population Division (2022). World Population Prospects 2022: Summary of Results. UN DESA/POP/2022/TR/NO. 3.
Unites States® Census of Bureau. (2021) “Black Individuals had Record Low Official Poverty Rate in 2022: Poverty rate for the Black population fell below pre-pandemic levels”.
Vaidya T., Vangipuram R., and Alikhan A. (2017) “Examining the race-specific prevalence of hidradenitis suppurativa at a large academic center; results from a retrospective chart review” Dermatol Online J. **23**(6).
Vaiserman AM., and Marotta F. (2016) “Longevity-promoting pharmaceuticals: is it a time for implementation?” Trends Pharmacol Sci. **37**, pp. 331-333.
Valerio, H. P., F. G. Ravagnani, G. E. Ronsein and P. Di Mascio (2021). "A single dose of Ultraviolet-A induces proteome remodeling and senescence in primary human keratinocytes." Scientific Reports **11**(1).
Vallhov H., Xia W., Engqvist H., and Scheynius A. (2018) “Bioceramic microneedle arrays are able to deliver OVA to dendritic cells in human skin”. J. Mater. Chem. B. 6, pp. 6808–6816. doi: 10.1039/C8TB01476K.
Valverde P., Healy E., Sikkink S., Haldane F., Thody AJ., Carothers A., Jackson JJ et al., (1996) “The Asp84Glu variant of the melancortin 1 receptor (MC1R) is associated with melanoma” Hum Mol Genet. **5**, pp. 1663-1666.
Van Beek, J. H. G. M., T. B. L. Kirkwood and J. B. Bassingthwaighte (2016). "Understanding the physiology of the ageing individual: computational modelling of changes in metabolism and endurance." Interface Focus **6**(2): 20150079.
Van Beelen ESA, van der Valk WH, de Groot JCMJ, Hensen EF, Locher H, and van Benthem PPG. (2020) “Migration and fate of vestibular melanocytes during the development of the human inner ear”. Developmental Neurobiology. **80**(11-12): doi:10.1002/dneu.22786
Van Den Bossche K, Naeyaert J-M, Lambert Jl. (2006). "The Quest for the Mechanism of Melanin Transfer." Traffic **7**(7): 769-778.
Van der Maaden K., Varypataki EM., Yu H., Romeijn HYS., Jiskoot W., and Bouwstra J. (2014) “Parameter optimization toward optimal microneedle-based dermal vaccination” Eur. J. Pharm. Biopharmaceuticals. **64**, pp. 18-25.
Van der Pols, JC. (2011) ‘Epidemiology of basal cell and squamous cell carcinoma of the skin” Skin Cancer – A world-wide Perspective” Drummer, Reinhard G., Pittelkow, Mark R., Iwatsuki, Keiji, Green, Adele, and Elwan, Nagwa M. Berlin: Springer-Verlag.3-12.
Van Nieuwpoort FNP, Smit R, Kolb H, van der Meulen H, Koerten and Patel S. (2004) “Tyrosine-induced melanogenesis shows differences in morphologic and meanogenic preferences of melanosomes from light and dark skin types” J. Invest. Dermatol. **122**, pp.1251-1255
Van Schependom, J. and M. D’Haeseleer (2023). "Advances in Neurodegenerative Diseases." Journal of Clinical Medicine **12**(5): 1709.

Van Smeden J., and Bouwstra JA. (2016) “Stratum Corneum Lipids: Their Role for the Skin Barrier Function in Health Subjects and Atopic Dermatitis Patients” Current Problems in Dermatology.
Van Tiggelen H, Van Damme N, Theys S, Vanheyste E., Verhaeghe S., LeBlanc K. et al. (2019) “The prevalence and associated factors of skin tears in Belgian nursing homes: a cross-sectional observational study”. J Tissue Viability. **28**(2), pp.100-106.
van Weelden H, de Gruijl FR, van der Putte SC, Toonstra J, van der Leun JC. The carcinogenic risks of modern tanning equipment: is UV-A safer than UV-B? Arch Dermatol Res. 1988;280(5):300–307. doi: 10.1007/BF00440604.
Vandersteen PR., Sigfrid A., and Muller MD. (1971) “Erythrokeratodermia Variabilis, An Enzyme Histochemical and Ultrastructural Study” JAMA Dermatology. **103**(4), pp. 362-370.
Vashi NA., Maymone MBDC., and Kundu RV. (2016) “Aging Differences in Ethnic Skin” J Clin Aesthet Dermatol. **9**(1), pp. 31-38.
Vavouri T and Lehner B. (2012) “Human genes with CpG island promoters have a distinct transcription-associated chromatin organization” Genome Biol. **13**(11), pp. R110.
Vela-Romera A., Carriel V., Martin-Piedra MA., Aneiros-Fernandez J., Campos F., Chato-Astrain J et al. (2019) “Characterization of the human ridged and non-ridged skin: a comprehensive histological, histochemical and immunohistochemical analysis” Histochem Cell Biol. **151**, pp. 57-73.
Velarde MC., Flynn JM., Day NY., Melov S., and Campisi J. (2012) “Mitochondrial oxidative stress caused by Sod2 deficiency promotes cellular senescence and aging phenotypes in the skin” Aging. **4**, pp. 3-12.
Velnar, T. and L. Gradisnik (2018). "Tissue Augmentation in Wound Healing: the Role of Endothelial and Epithelial Cells." Medical Archives **72**(6): 444.
Ventura MT., Casciaro M., Gangemi S., and Buquicchio R. (2017) “Immunosenescence in aging: between immune cells depletion and cytokines up-regulation” Clin Mol Allergy. **15**, pp. 21.
Verbaan FJ., Bal SM., van den Berg DJ., Groenink WHH., Verpoorten H., Lüttge R et al., (2007) “Assembled microneedle arrays enhance the transport of compounds varying over a large range of molecular weight across human dermatomed skin” Journal of Controlled Release. **117**(2), pp. 238-245.
Verbaan, FJ., Bal, SM., Van den Berg, DJ., Groenink, WHH., Verpoorten H., Lüttge R., Bouwstra, JA. (2007) “Assembled microneedle arrays enhance the transport of compounds varying over a large range of molecular weight across human dermatomed skin”. J. Control. Release **117**, pp. 238–245
Vermulst, M., J. Wanagat, G. C. Kujoth, J. H. Bielas, P. S. Rabinovitch, T. A. Prolla and L. A. Loeb (2008). "DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice." Nature Genetics **40**(4): 392-394.
Victorelli, S., et al. (2019). "Senescent human melanocytes drive skin ageing via paracrine telomere dysfunction." The EMBO Journal **38**(23).
Vierkötter, A. and J. Krutmann (2012). "Environmental influences on skin aging and ethnic-specific manifestations." Dermato-Endocrinology **4**(3): 227-231.
Vora LK., Courtenay AJ., Tekko IA., Larrañeta E., and Donnelly RF. (2020) “Pullulan-based dissolving microneedle arrays for enhanced transdermal delivery of small and large biomolecules”. Int. J. Biol. Macromol. **146**, pp. 290–298.
Wade A., McKinney A., and Phillipis JJ. (2014) “Matrix regulators in neural stem cell functions” Biochemica et Biophysica Acta (BBA) -General subjects. **1840**(8), pp. 2520-2525
Waghule T., Singhvi G., Dubey S.K., Pandey M.M., Gupta G., Singh M., and Dua K. (2019) “Microneedles: A smart approach and increasing potential for transdermal drug delivery system”. Biomed. Pharmacotherapy. **109**, pp. 1249–1258. doi: 10.1016/j.biopha.2018.10.078.

Waghule, T. Singhvi, G. Dubey, S.K. Pandey, M.M. Gupta, G. Singh, M. et al., (2019) "Microneedles: A smart approach and increasing potential for transdermal drug delivery system" Biomed Pharmacother. **109**(1249-1258), doi:10.1016/j.biopha.2018.10.078.
Wagner JK, Parra EJ, Norton LH, Jovel C, Shriver MD. (2002) "Skin responses to ultraviolet radiation: effects of constitutive pigmentation, sex, and ancestry". Pigment Cell Res. **15**, pp.385–90. doi: 10.1034/j.1600-0749.2002.02046.x.
Waldman, S. A. and A. Terzic (2019). "Health Care Evolves From Reactive to Proactive." Clinical Pharmacology & Therapeutics **105**(1): 10-13.
Waldman, S. A. and A. Terzic (2019). "Health Care Evolves from Reactive to Proactive." Clinical Pharmacology & Therapeutics **105**(1), pp.10-13.
Walker A and Maltby T. (1997) "Ageing Europe, 56Open University Press, Buckingham"
Walker, R. F. (2006). "On the evolution of anti-aging medicine." Clinical Interventions in Aging **1**(3), pp. 201-203.
Walston JD. (2020) "Common clinical sequelae of aging". Goldman-Cecil Medicine. 26th ed. Philadelphia, PA: Elsevier:chap 22.
Wan DC, Rochlin DH. (2019) "Scalp Reconstruction" Global Reconstructive Surgery, pp. 182-189
Wang P-W, Hung Y-C, Lin T-Y, Fang J-Y, Yang P-M, Chen M-H et al., (2019) "Comparison of the Biological Impact of UVA and UVB upon the Skin with Functional Proteomics and Immunohistochemistry" Antioxidants (Basel) **8(**12), pp. 569, doi: 10.3390/antiox8120569
Wang S., Drummon ML., Guerrero-Juarez CF., Tarapore E., MacLean AL., Stabell AR et al., (2020) "single cell transcriptomics of human epidermis identifies basal stem cell transition states" Nature communications. **11**.
Wang Y, Chi J, Wang T, Zhang Y, Li Z, Chen J et al., (2022) "Vitiligo as a potential degenerative disease: from oxidative stress to cellular senescence" Aging Pathobiology and Therapeutics **4**(2).
Wang, A. S. and O. Dreesen (2018). "Biomarkers of Cellular Senescence and Skin Aging." Frontiers in Genetics **9**.
Wang, F., S. Chen, H. B. Liu, C. A. Parent and P. A. Coulombe (2018). "Keratin 6 regulates collective keratinocyte migration by altering cell–cell and cell–matrix adhesion." Journal of Cell Biology **217**(12): 4314-4330.
Wang, L. Wang, N. Zhang, W. Cheng, X. Yan, Z. Shao, G. Wang, X. Wang, R. and Fu, C. (2022). "Therapeutic peptides: current applications and future directions." Signal Transduction and Targeted Therapy **7**(1).
Wang, P.-W., Y.-C. Hung, T.-Y. Lin, J.-Y. Fang, P.-M. Yang, M.-H. Chen and T.-L. Pan (2019). "Comparison of the Biological Impact of UVA and UVB upon the Skin with Functional Proteomics and Immunohistochemistry." Antioxidants **8**(12): 569.
Wang, Y., Z. Chen, B. Davis, W. Lipman, S. Xing, L. Zhang, T. Wang, P. Hafiz, W. Xie, Z. Yan, Z. Huang, J. Song and W. Bai (2024). "Digital automation of transdermal drug delivery with high spatiotemporal resolution." Nature Communications **15**(1).
Wany Y-N., Fang H., and Zhu W-F. (2009) "Survey on skin aging status and related influential factors in Southeast China" J Chejiang Univ Sci B. **10**(1), pp. 57-66.
Wawersik MJ., Mazzalupo S., Nguyen D., and Coulombe PA. (2001) "Increased levels of keratin 16 alter epithelisation potential of mouse skin keratinocytes" Mol Biol Cell. **12**, pp, 3439-3450.
Wegienka, G., et al. (2012). "Racial disparities in allergic outcomes in African Americans emerge as early as age 2 years." Clinical; Experimental Allergy **42**(6), pp. 909-917.
Weihermann AC., Lorencini M., Brohem CA., and Carvalho CM. (2017) "Elasting structure and its involvement in skin photoageing" Int J Cosmet Sci. **39**(3), pp. 241-247.

Weiland, S. K. (2004). "Climate and the prevalence of symptoms of asthma, allergic rhinitis, and atopic eczema in children." Occupational and Environmental Medicine **61**(7), pp. 609-615.
Welch G., and Tsai L-H. (2022) "Mechanisms of DNA damage-medited neurotoxicity in neurodegenerative disease" EMBO Rep. **23**(6), pp. e54217.
Weldon, WC., MP. Martin, V. Zarnitsyn, B. Wang, D. Koutsonanos, I. Skountzou, MR. Prausnitz and RW. Compans (2011). "Microneedle Vaccination with Stabilized Recombinant Influenza Virus Hemagglutinin Induces Improved Protective Immunity." Clinical and Vaccine Immunology **18**(4): 647-654.
Wendt, J., et al. (2011). "Site-dependent actinic skin damage as risk factor for melanoma in a central European population." Pigment Cell; Melanoma Research **25**(2): 234-242.
Werner, S., T. Krieg and H. Smola (2007). "Keratinocyte–Fibroblast Interactions in Wound Healing." Journal of Investigative Dermatology **127**(5), pp. 998-1008.
White DJ, Puranen S, Johnson MS, and Heino J. (2004) "The collagen receptor subfamily of the integrins". Int J Bio
Whiteman DC, Parsons PG, Green AC. (1999) "Determinants of melanocyte density in adult human skin" Arch Dermatol Res. **291**(1), pp. 511-516:doi: 10.1007/s004030050446.
Wiedemann J., Billi AC., Bocci F., Nie Q., Gudjonsson JE., Andersen B. et al. (2023) "Differential cell composition and splot epidermal differentiation in human palm, sole, and hip skin" Cell reports. **42**(1), pp. 111994.
Wihastyoko HYL, Perdanakusuma DS, Soeharto S, Widjajanto E, Handono K, Pardjianto B. (2021) "Correlation of Melanin Content with Collagen Density in Keloid Patients" B-Clinical Science. **9**(B), pp.265-271.
Wilgus TA, Matthies AM, Radek KA, Dovi JV., Burns AL., Shankar R., DiPeitro LA. (2005) "Novel function for vascular endothelial growth factor receptor-1 on epidermal keratinocytes". Am J Pathol **167**, PP. 1257**.**
Wilkes M., Wright CY., du Plessis JL. And Reeder A. (2015) "Fitzpatrick Skin Type, Individual Typology Angle, and Melanin Index in an African Population Steps Toward Universally Applicable Skin Photosensitivity Assessments" JAMA Dermatology. **151**(8), pp. 902-903.
Wilkinson HN., and Hardman MJ. (2020) "Senescence in Wound Repair: Emerging Strategies to Target Chronic Healing Wounds" Front. Cell. Dev. Biol. **8**(773).
Wilkinson PC. (1982) "Chemotaxis & inflammation. Principles of the measurement of leukocyte locomotion and chemotaxis: assay systems*"*. Longman **(2nd)** pp. 35–62.
Williams, A. (2003) "Transdermal and Topical Drug Delivery" Pharmaceutical Press,
Wlaschek M., Tantcheva-Poór I., Naderi L., Ma W., Scheider LA., Razi-Wolf Z. et al., (2001) "Solar UV irradiation and dermal photoaging" Journal of Photochemistry and Photobiology B: Biology. **63**(1-3), pp. 41-51.
Wledon WC., Martin MP., Zarnotsyn V., Wang B., Koutsonanos D., Skoutzou I., Prausnitz MR., and Compans RW. (2011) "Microneedle Vaccination with Stabilised Recombinant Influenza Virus Hemagglutinin Induces Improved Protective Immunity" Clinical and Vaccine Immunology. **18**(4).
Wolf J., Wienberged B, Arnold CR., Maier AD., Westendorp RGJ., and Grubeck-Loebenstein B. (2012) "The effect of chronological age on the inflammatory response of human fibroblasts" Exp Gerontol. **47**(9), pp. 749-753.
Wolf, I., S. Levanon-Cohen, S. Bose, H. Ligumsky, B. Sredni, H. Kanety, M. Kuro-O, B. Karlan, B. Kaufman, H. P. Koeffler and T. Rubinek (2008). "Klotho: a tumor suppressor and a modulator of the IGF-1 and FGF pathways in human breast cancer." Oncogene **27**(56): 7094-7105.

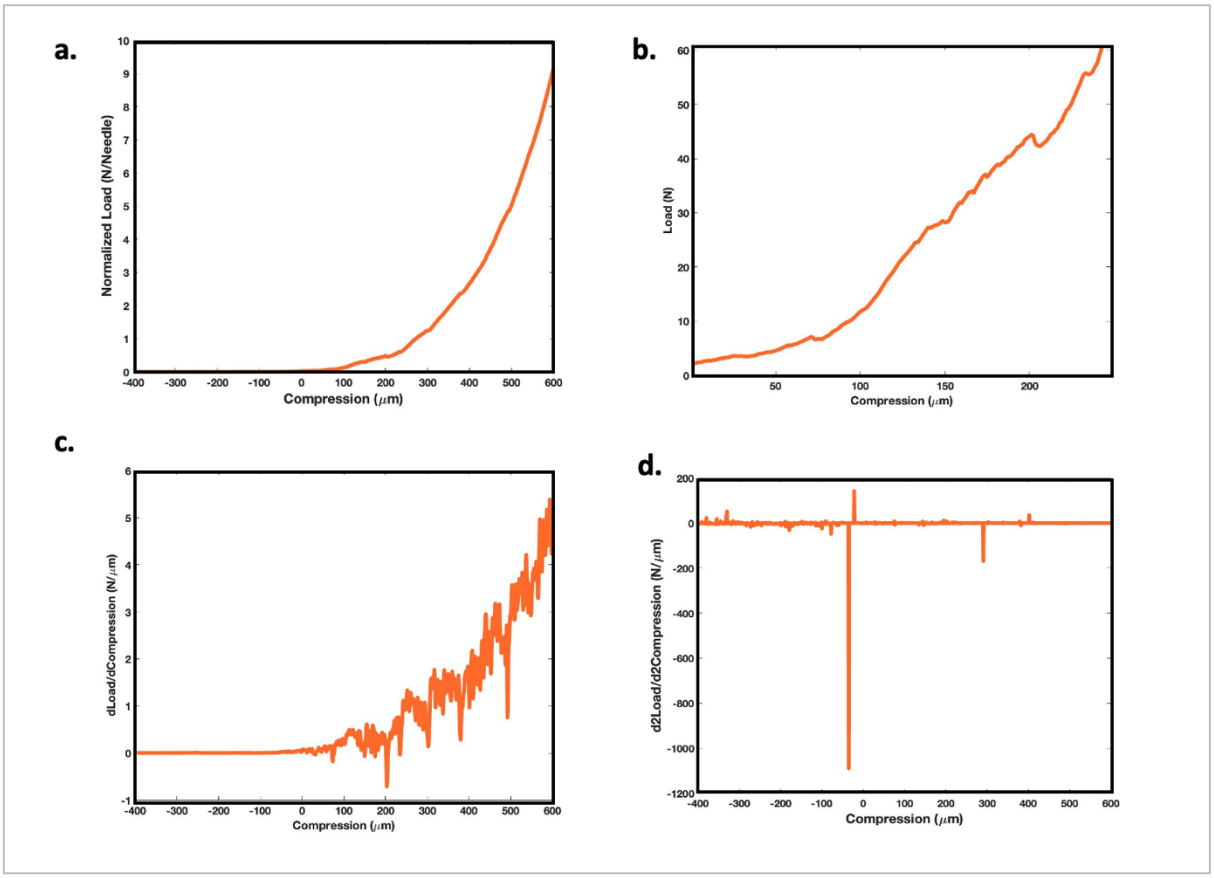
Wong BST., Hsu BAL., and Liao MDW. (2013) "Phototherapy in Psoriasis: A Review of Mechanisms of Action" J Cutan Med Surg. **17**(1), pp. 6-12
Wong P., and Coulombe PA. (2003) "Loss of keratin 6 (K6) proteins reveals a function for intermediate filaments during wound repair" J Cell Biol. **163**, pp. 327-337.
World Health Organisation. (2022). *ICD-11 Implementation.* [https://www.who.int/standards/classifications/frequently-asked-questions/icd-11-implementation]
World Health Organization (2016) "Radiation: Ultraviolet (UV) radiation" https://www.who.int/news-room/questions-and-answers/item/radiation-ultraviolet-(uv)
World Health Organization. "Old age" 2022. https://www.who.int/standards/classifications/frequently-asked-questions/old-age
World Health Organization. (2023) Ageing. https://www.who.int/health-topics/ageing#tab=tab_1
Xu Y., and Sun Z. (2015) "Molecular Basis of Klotho: From Gene to Function in Aging". Endocrine Reviews. **36**(2), pp. 174-193.
Xu Y., Shrestha N., Préat V., and Beloqui A. (2021) "An overview of in vitro, ex vivo, and in vivo models for studying the transport of drugs across intestinal barriers" Advanced Drug Delivery Reviews. **175**, pp. 113795.
Yaar, M. and H.-Y. Park (2012). "Melanocytes: A Window into the Nervous System." Journal of Investigative Dermatology **132**(3): 835-845.
Yajima, I. and L. Larue (2008). "The location of heart melanocytes is specified and the level of pigmentation in the heart may correlate with coat color." Pigment Cell; Melanoma Research **21**(4): 471-476.
Yamaguchi U, Brenner M, and Hearing VJ. (2007) "The Regulation of Skin Pigmentation", The Journal of Biological Chemistry, **282**(38), pp. 27557-27561, doi: 10.1074/jbc.R700026200
Yamaguchi Y, and Hearing VJ. (2006). Melanocyte Distribution and Function in Human Skin. In: Hearing, V.J., Leong, S.P.L. (eds) From Melanocytes to Melanoma. Humana Press. https://doi.org/10.1007/978-1-59259-994-3_6
Yamaguchi Y., Itami S., Tarutani M., Hosokawa., Miura H., and Yoshikawa K. (1999) "Regulation of keratin 9 in nonpamoplantar keratinocytes by palmoplantar fibroblasts through epithelial-mesenchymal interactions". J Invest Dermatol. **112**, pp. 483-488.
Yamaguchi Y., Morita A., Maeda A., and Hearing VJ. (2009) "Regulation of Skin Pigmentation and Thickness by Dickkopf 1 (DKK1)" Journal of Investigative Dermatology Symposium Proceedings. **14**, pp. 73-75.
Yamaguchi, T. P., A. Bradley, A. P. McMahon and S. Jones (1999). "A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo." Development **126**(6): 1211-1223.
Yamaguchi, Y., et al. (2004). "Mesenchymal–epithelial interactions in the skin." Journal of Cell Biology **165**(2): 275-285.
Yamashita K., Yotsuynagi T., Yamauchi M., and Young D. (2014) "Klotho Mice: A Novel Wound Model of Skin". Plastic and Reconstructive Surgery Global Open. **2(**1), p e101.
Yamauchi M., Hirohashi Y., Torigoe T., Matsumato Y., Yamashita K., Kayama M. et al., (2016) "Wound healing delays in an a-klotho- deficient mice that have skin appearance similar to that in aged humans – Study of delayed wound healing mechanism" Biochemical and Biophysical Research Communication. **473**(4), pp. 845-852
Yang AY., Lee JH., Shu L., Zhang C., Su ZY., Lu Y., et al. (2014) "Genome-wide analysis of DNA methylation in UVB-and DMBA/TPA=induced mouse skin cancer models" Life Sci. **113**, pp. 45-54.

Yang CC, Chen CC, and Chen WC. (2013) “Aging and Anti-Aging in Hair and Hair Loss”. Inflammation, Advancing Age and Nutrition, doi:10.1016/B978-0-12-397803-5.00019-8
Yang J-H., Hayano M., Griffin PT., Amorim JA., Bonkowski MS., Apostolides JK et al., (2023) “Loss of epigenetic information as a cause of mammalian aging” Cell. **186**(2), pp. 305-326.
Yang Z., Wong A., Kuh D., Paul DS., Rakyan VK., Leslie DR. et al., (2016) “Correlation of an epigenetic mitotic clock with cancer risk” Genome Biology. **17**(205).
Yang, N.B. Garza, L.A. Foote, C.E. Kang, S. and Meyerle, J.H. (2012). “High Prevalence of Stump Dermatoses 38 Years or More After Amputation.” Archives of Dermatology **148**(11): 1283.
Yanpu H., Hong C., Jiahe L., Howard MT., Li Y., Turvey ME et al., (2018) “Synthetic Charge-Invertible Polymer for Rapid and Complete Impkantation of Layer-by-Layer Microneedle Drug Films for Enhanced Transdermal Vaccination” ACS NANO. **12**(10), pp. 10272-10280.
Yardman-Frank, J. M. and D. E. Fisher (2021). "Skin pigmentation and its control: From ultraviolet radiation to stem cells." Experimental Dermatology **30**(4): 560-571.
Ye J., Garg A., Calhoun C., Geingold KR., Elias PM., and Ghadially R. (2008) “Alterations in cytokine regulation in aged epidermis: Implications for permeability barrier homeostasis and inflammation” Experimental Dermatology. **11**(3), pp. 209-216.
Ye Y., Wang J., Hu Q., Hochu GM., Xin H., Wang C., and Gu Z. (2016) “Synergistic transcutaneous immunotherapy enhances through delivery of checkpoint inhibitors” ACS Nano. **10**(9), pp. 8956-8963.
Yew, C. K., et al. (2022). "Case Series of Neonatal Extravasation Injury: Importance of Early Identification and Management." Cureus.
Yi Y., Xie H., Xiao X., Wang B., Du R., Liu Y., et al. (2018) “Ultraviolet A irradiation induces senescence in human dermal fibroblasts by down-regulating DNMT1 via ZEB1” Aging. **10**(2), pp. 212-228.
Yi Z., Zhu L., Xu Y., Jiang J., and Zhu B. (2011) “Polypropylene Glycol: The Hydrophilic Phenomena in the Modification of Polyethersulfone Membrane” ACSPublications. **50**(1), pp. 11297.
Yoshino N., Kim CJ, and Sirivunnabood. P. (2019) “Aging Population and its Impacts on Fiscal Sustainability”. Aging population and Its Economic Impact + Immigration. T20 Japan.
You X., Wang L., Zhang J., Tang., Dai C., Chen C., and Wu J. (2023) “Effects of polymer molecular weight on in vitro and in vivo performance of nanoparticle drug carriers for lymphoma therapy” Chinese Chemical letters. **34**(4).
You Y., Tian Y., Yang Z., Shi J.,Kwak KJ., Tong Y., Estania AP et al., (2023) “Intradermally delivered mRNA-ecapsulating extracellular vesicles for collagen-replacement therapy” Nature biomedical engineering. **7**, pp. 887-900.
Young AL., Levy S., Nighland M., Grossman R., DN Silvers and Celebi JT. (2010) “Are dark-skinned people really protected from ultraviolet radiation?” Clin Exp Dermatol. **35**(4), pp. 392-6.
Yousef A., Alhajj M. and Sharma S. (2022) “Anatomy, Skin, Epidermis” StatPearls.
Yu M., Hazelton WD., Luebeck GE., and Grady WM. (2020) “Epigenetic aging: more than just a clock when it comes to cancer” Cancer Res. **80**(3), pp. 367-374.
Yu W., Jiang G., Liu D., Li L., Tong Z., Yao J., and Kong X. (2017) “Transdermal delivery of insulin with bioceramic composite microneedles fabricated by gelatin and hydroxyapatite”. *Mater. Sci. Eng. C.* 73, pp. 425–428. doi: 10.1016/j.msec.2016.12.111
Yu, J., Y. Zhang, Y. Ye, R. Disanto, W. Sun, D. Ranson, F. S. Ligler, J. B. Buse and Z. Gu (2015). "Microneedle-array patches loaded with hypoxia-sensitive vesicles provide fast glucose-responsive insulin delivery." Proceedings of the National Academy of Sciences **112**(27): 8260-8265.

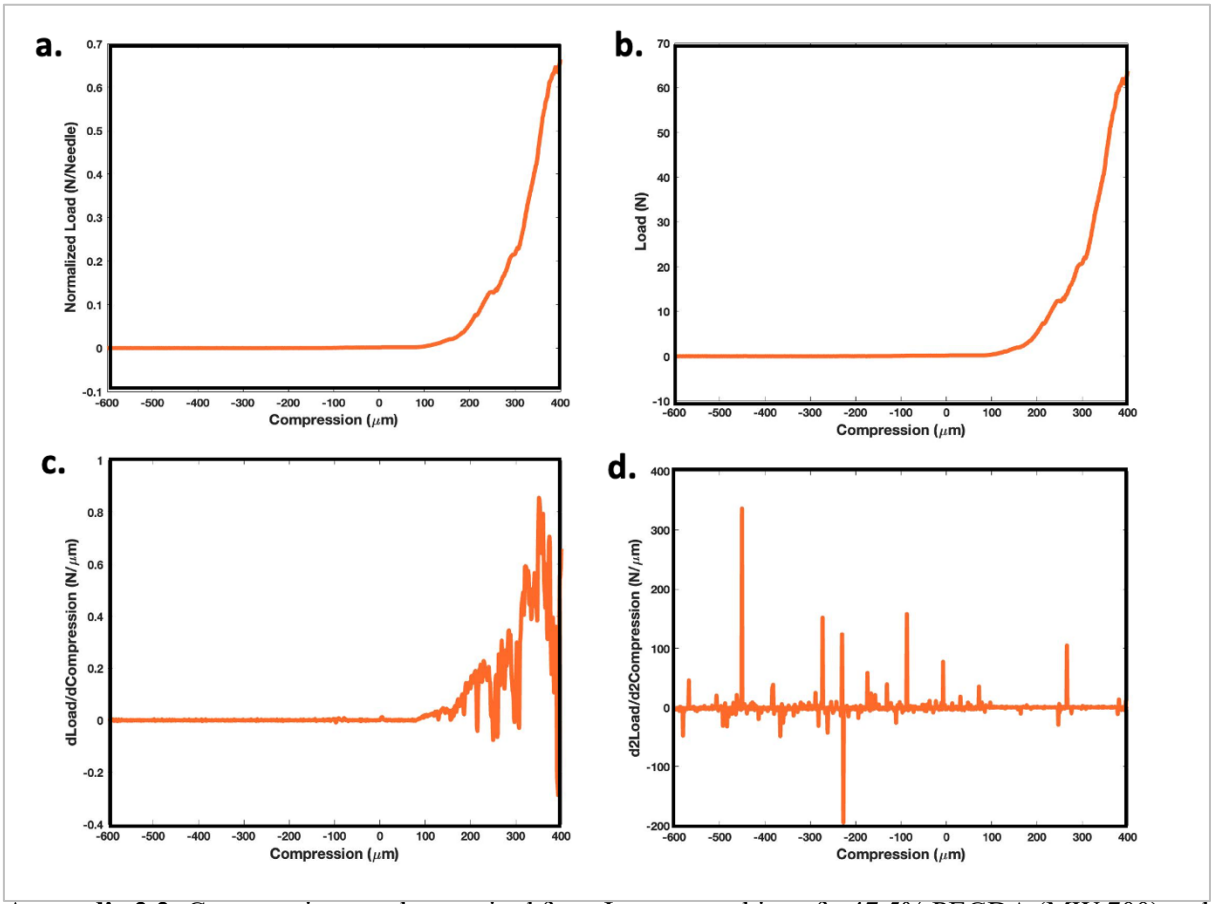
Yuan R., Yang N., Huang Y., Li W., Zeng Y., Liu Z et al., (2023) “Layer-by-Layer Microneedle-Mediated rhEGF Transdermal Delivery for Enhanced Wound Epidermal Regeneration and Angiogenesis” ACS Applied Materials & Interfaces. **15(**18), pp. 21929-21940.
Yun, M. (2015). "Changes in Regenerative Capacity through Lifespan." International Journal of Molecular Sciences **16**(10): 25392-25432
Zaibudeen, AW. and Phillip, J. (2017) “Thermally tunable grating using thermo-responsive magnetic fluid” Optical Materials. **66,** pp.117-121.
Zaidi ARH., and Sharma S. (2024) “Pressure Ulcer” StatPearls.
Zalaudek I., Docimo G., and Argenziano G. (2009) “Using Dermoscopic Criteria and Patient-Related Factors for the Management of Pigmented Melanocytic Nevi” Arch Dermatol. **145**(7), pp. 816-826.
Zaman R., Islam RA., Ibnat N., Othman L., Zaini A., Lee CY., Chwohury EH. (2019) “Current strategies in extending half-lives of therapeutic proteins” J Control Release. **10**(301), pp. 176-189.
Zanetti R, Prota G, Napolitano A, Martinez C, Sancho-Garnier H, Osterlind A, et al., (2001), “Development of an integrated method of skin phenotype measurement using the melanins” Melanoma Res. **11,** pp.551-557
Zargar, M.H. Araghizadeh, H. Soroush, M.R. and Khaji, A. (2007). "Iranian casualties during the eight years of Iraq-Iran conflict." Revista de Saúde Pública **41**(6): 1065-1066.
Zbieć-Pierkarska R., Spólnicka M., Kupiec T., Makowaska Z., Spas A., Parys-Proszek A., Kucharczyk K., Ploski R., and Branicki W. (2015) “Examination of DNA methylation status of the ELOVL2 marker may be useful for human age prediction in forensic science” Forensic Science International: Genetics. **14,** pp. 161-167.
Zeltz C., and Gulberg D. (2016) “The integrin-collagen connection-A glue for tissue repair?” J. Cell Sci. **129**, pp. 653-664.
ZF. Rad, RE. Nordon, CJ. Anthony, L. Bilston, PD. Prewett, J.-Y. Arns, CH. Arns, L. Zhang, GJ. Davies “High-fidelity replication of thermoplastic microneedles with open microfluidic channels” Microsyst. Nanoeng. **9**(3), pp. 17034.
Zhang L. and Liu T. (2018) “Clinical implication of alterations in serum Klotho levels in patients with type 2 diabetes mellitus and its associated complications” J Diabetes Complications. **32**(10), pp. 922-930.
Zhang LX., Guo SZ., and Wang Z. (2000) “Biological effects of supernatant from melanocytes culture on proliferation of hypertrophic scar fibroblasts” J Fourth Mil Med Univ. **21**, pp. 669-670.
Zhang S., and Duan E. (2018) “Fighting against Skin Aging” Cell Transplant. **27**(5), pp. 729-738.
Zhang XP., Wang BB., Hu LF., Fei WM., Cui Y., and Guo XD. (2021) “Safety evaluation of 3-month effects of microneedle patches prepared from hyaluronic acid in mice” Biochemical Engineering Journal. **176**, pp. 108157.
Zhang, B., J. Xu, Z. Quan, M. Qian, W. Liu, W. Zheng, et al., (2018). "Klotho Protein Protects Human Keratinocytes from UVB-Induced Damage Possibly by Reducing Expression and Nuclear Translocation of NF-κB." Medical Science Monitor **24**, pp. 8583-8591.
Zhang, J., et al. (2013). "Wnt5a Inhibits the Proliferation and Melanogenesis of Melanocytes." International Journal of Medical Sciences **10**(6): 699-706.
Zhang, Y. Siebenaler, K. Brown, K. Dohmeier, D. Hansen, K. (2012) “Adjuvants to prolong the local anesthetic effects of coated microneedle products” International Journal of Pharmaceutics. **439**(1-2), pp. 187-192

Zhang, Y., Brown K., Siebenaler K., Determan A., Dohmeier D., Hansen K et al. (2012). "Development of Lidocaine-Coated Microneedle Product for Rapid, Safe, and Prolonged Local Analgesic Action." Pharmaceutical Research **29**(1): 170-177.
Zhao CY., Wijayanti A., Doria MC., Harris AG., Jain SV., Legaspi KN et al., (2015) "The reliability and validity of outcome measures for atopic dermatitis in patients with pigmented skin: A grey area" Int J Womens Dermatol. **1**(3), pp. 150-154.
Zhao Y., Zhou Y., Yang D., Gao X., Wen T., Fu J., et al. (2021) "Intelligent and spatiotemporal drug release based on multifunctional nanoparticle-integrated dissolving microneedle system for synergetic chemo-photothermal therapy to eradicate melanoma" Acta Biomater. **135**, pp. 164-178.
Zhao Z, Chen Y, Shi Y. (2020). "Microneedles: a potential strategy in transdermal delivery and application in the management of psoriasis." RSC Advances **10**(24): 14040-14049.
Zhao, X., X. Sun, L. Yildirimer, Q. Lang, Z. Y. Lin, R. Zheng, Y. Zhang, W. Cui, N. Annabi and A. Khademhosseini (2016). "Cell infiltrative hydrogel fibrous scaffolds for accelerated wound healing." Acta Biomaterialia **49**: 66-77.
Zhou X., Chen Y., Cui L., Shi Y., and Guo C. (2022) "Advances in the pathogenesis of psoriasis: from keratinocyte perspective" Nature, cell death & disease. **81**
Zhou X., Li B., Guo M., Peng W., Wang D., Guo Q., et al., (2022) "Microneedle patch based on molecular motor as a spatiotemporal controllable dosing strategy of L-DOPA for Parkinson's Disease" Chemical Engineering Journal. **427**.
Zhou, S., J. Hum, K. Taskintuna, S. Olaya, J. Steinman, J. Ma and N. Golestaneh (2023). "The Anti-Aging Hormone Klotho Promotes Retinal Pigment Epithelium Cell Viability and Metabolism by Activating the AMPK/PGC-1α Pathway." Antioxidants **12**(2), pp. 385.
Zhu N., Qin L., Luo Z., Guo Q., Yang L., and Liao D. (2014) "Challenging role of Wnt5a and its signaling pathway in cancer metastasis" Exp Ther Med **8**(1), pp. 3-8.
Zhu Z., Ding J., and Tredget EE. (2016) "The molecular basis of hypertrophic scars" Burn trauma. **4**(1), pp. 2
Zomer HD., and Trenton AG. (2018) "Skin wound healing in humans and mice: Challenges in transnational research" Journal of Dermatological Science. **90**(1), pp. 3-12.
Zou DP, Chen YM, Zhang LZ, Yua XH, Zhang YJ, Inggawati A et al., "SFRP5 inhibits melanin synthesis of melanocytes in vitiligo by suppressing the Wnt/β-catenin signaling" Genes Dis. **8**(5), pp. 677-688: doi:10.1016/j.gendis.2020.06.003

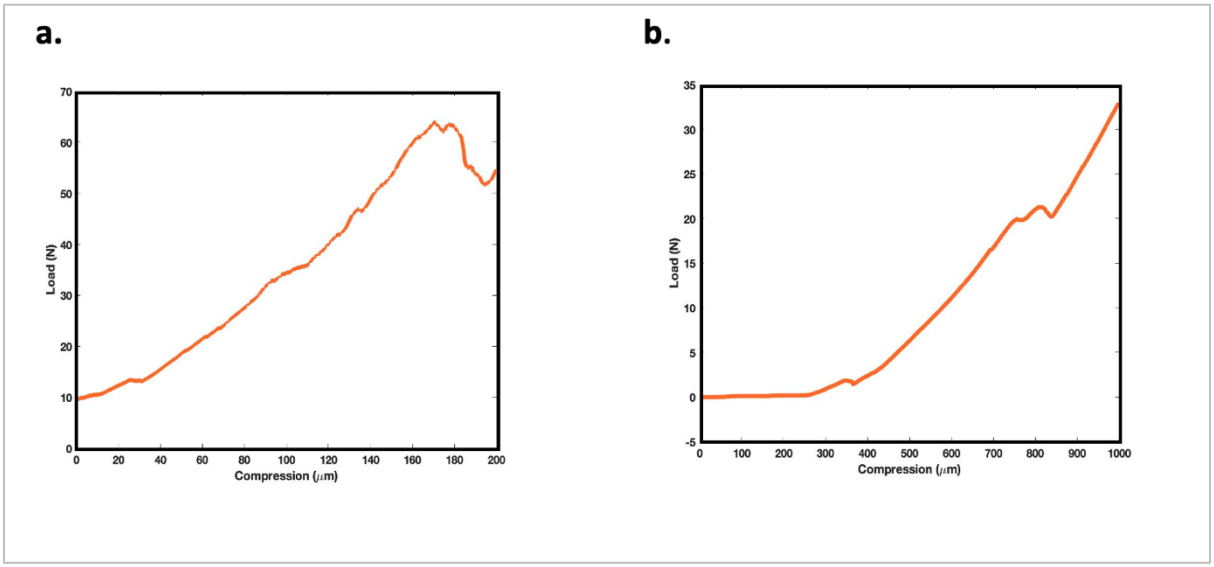
7.0 Appendix



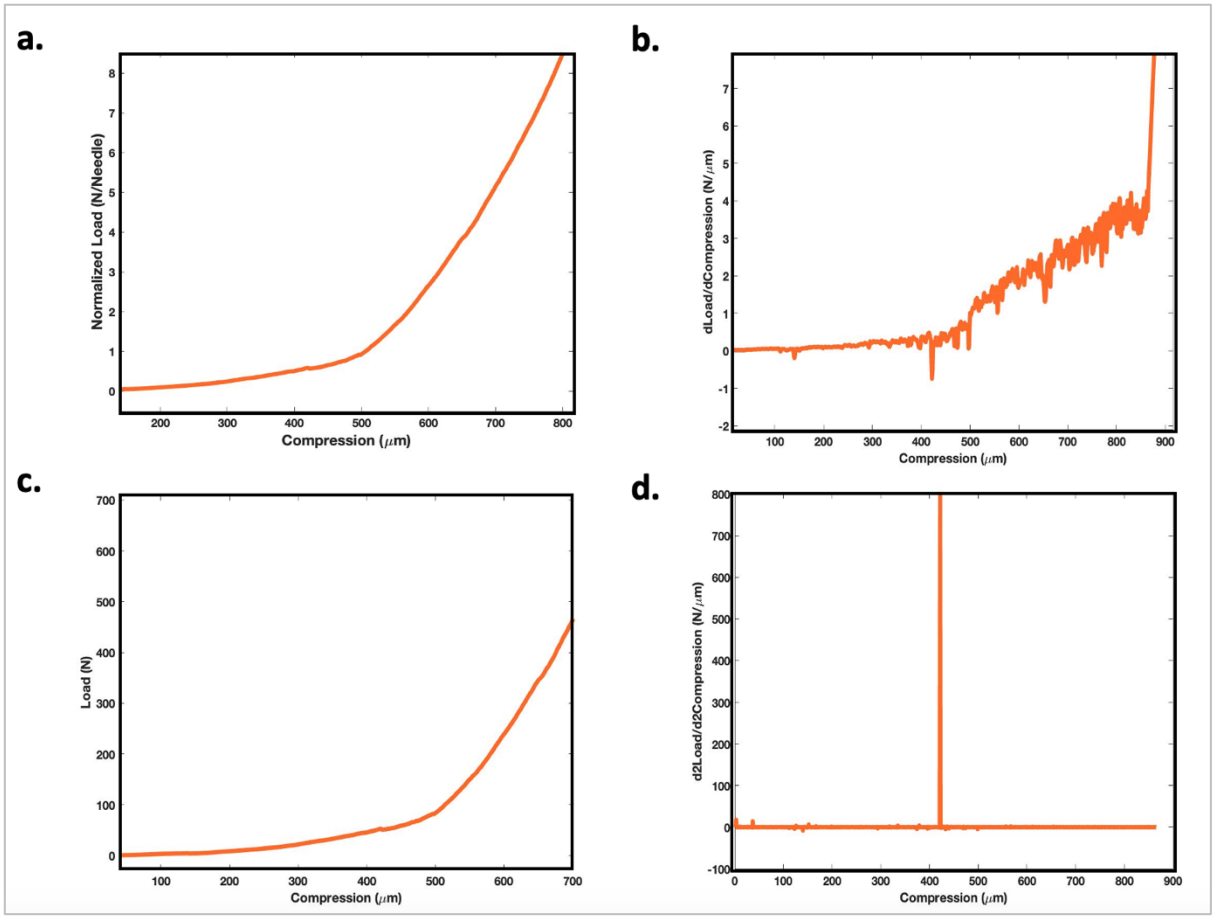
Appendix 2.1: Compression graphs acquired from Instron machine of a 47.5% PEGDA (MW 700) and 47.5% short-cross linker (with 5% Darocur®) (a) shows the normalised needle breakage at 0.3-0.5 N per needle. (b) shows the force at which the MN array breaks to be between 30-50 N due to inconsistencies of holistic breaking across the entire array. (c) exhibits the 1/derivative in compression, indicating the rate of change which a peak drop-in rate of loading at 200 µm. (d) the change in the rate of change in loading between 200-300 µm.



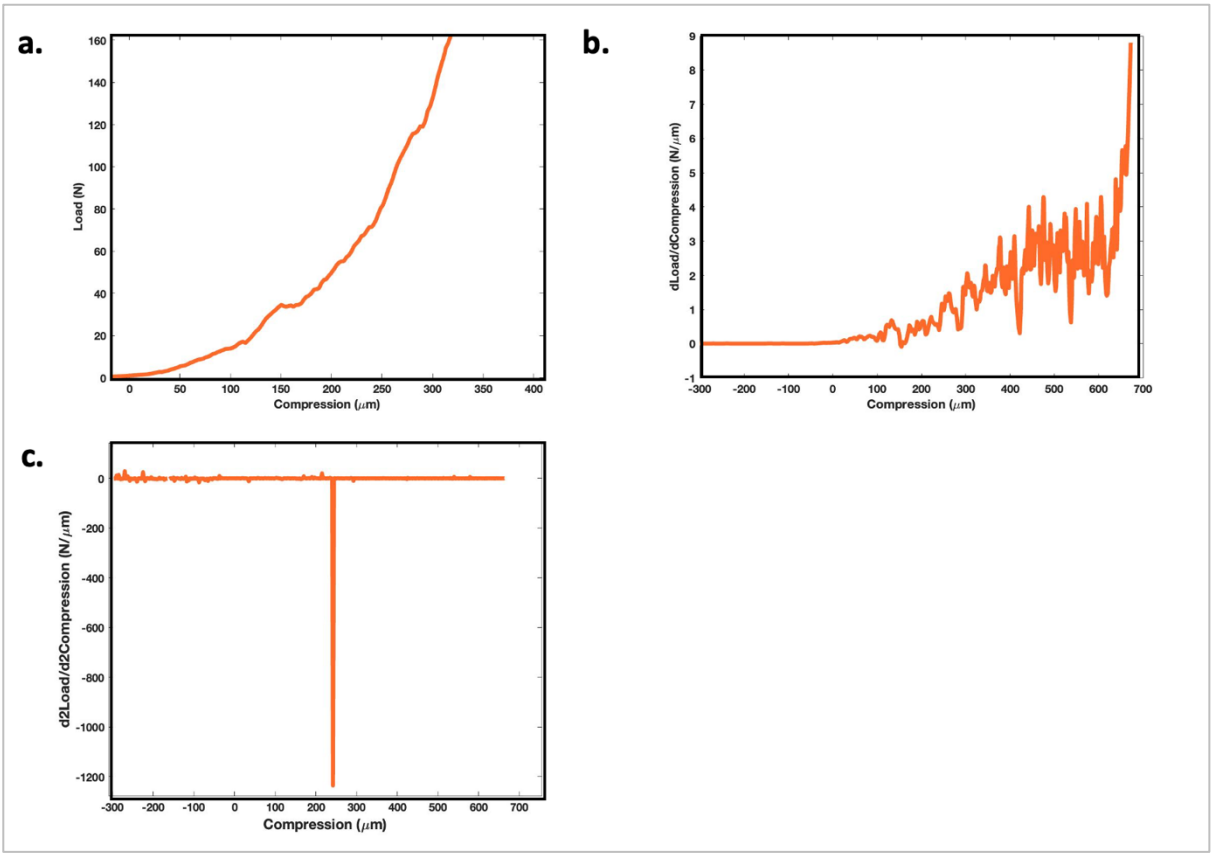
Appendix 2.2: Compression graphs acquired from Instron machine of a 47.5% PEGDA (MW 700) and 47.5% PPG (with 5% Darocur®). (a) shows the normalised needle breakage at 0.1-0.2 N per needle. (b) shows the force at which the MN array breaks to be between 10-20 N. (c) exhibits the 1/derivative in compression, indicating the rate of change which a peak drop-in rate of loading at 200 µm. (d) the change in the rate of change in loading between 200-300 µm.



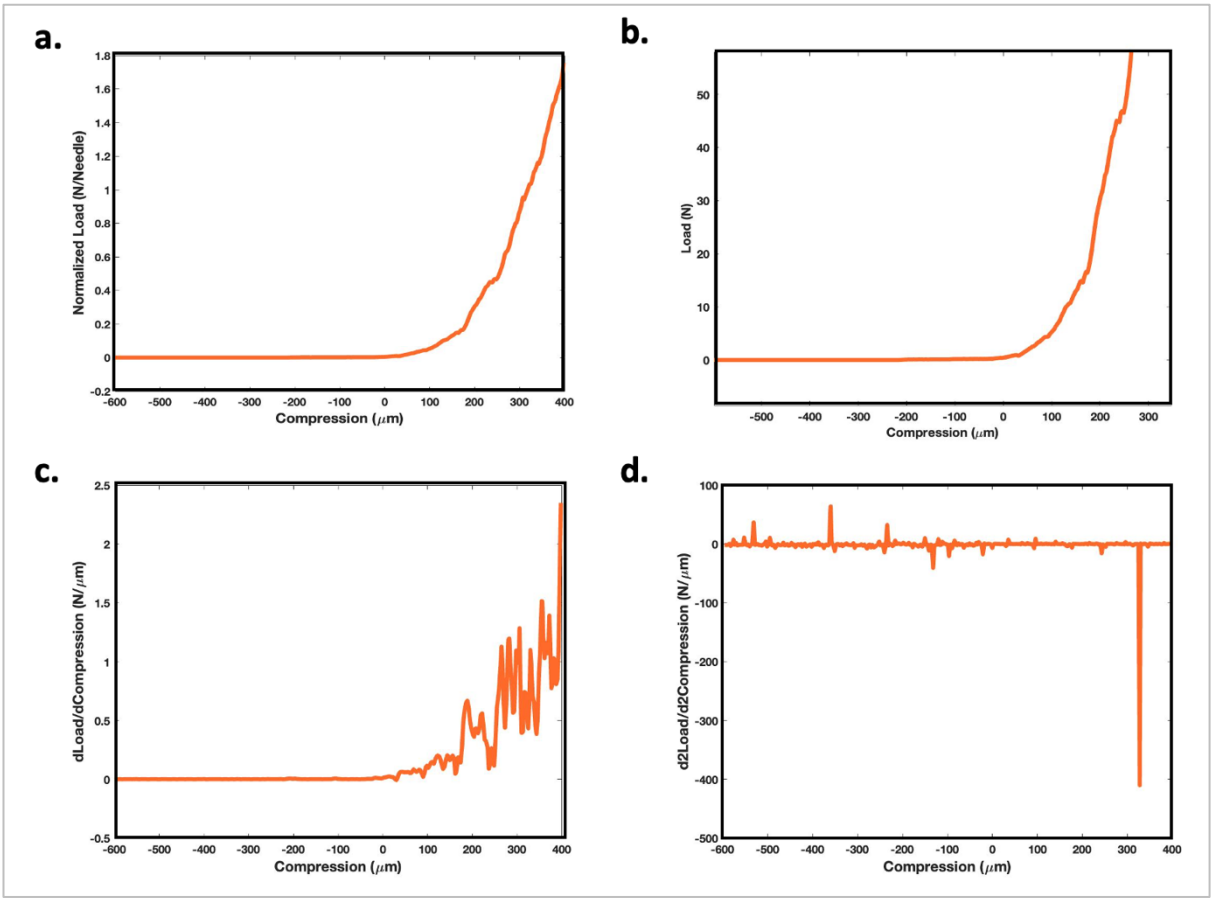
Appendix 2.3: Compression graphs acquired from Instron machine of a Tetra additive MN. (a) shows the force at which the MN array breaks to be between 60-70 N for a 95% Tetra constructed MN (with 5% Darocur®). (b) shows the force at which the MN array breaks to be between 20-25 N for a MW 50% 250 MN with 45% Tetra Additive (with 5% Darocur®).



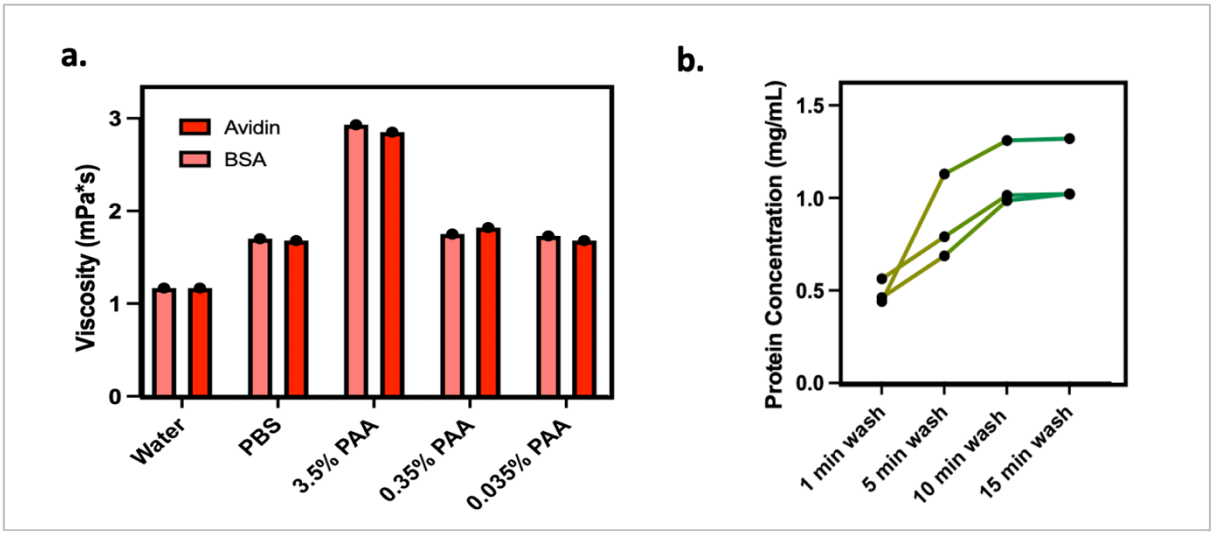
Appendix 2.4: Compression graphs acquired from Instron machine of a 47.5% PEGDA (MW 700) and 47.5% Tetra additive (with 5% Darocur®) at 10 µm/second. (a) shows the normalised needle breakage at 0.58 N per needle. (b) exhibits the 1/derivative in compression, indicating the rate of change which a peak drop-in rate of loading at 400 µm. (c) shows the force at which the MN array breaks to be between 58 N (d) the change in the rate of change in loading at 400 µm.



Appendix 2.5: Compression graphs acquired from Instron machine of a 47.5% PEGDA (MW 700) and 47.5% Tetra additive (with 5% Darocur®) at 25 µm/second. (a) shows the force at which the MN array breaks to be between 120 N. (b) exhibits the 1/derivative in compression, indicating the rate of change which a peak drop-in rate of loading at 200-300 µm. (c) the change in the rate of change in loading between 200-300 µm.

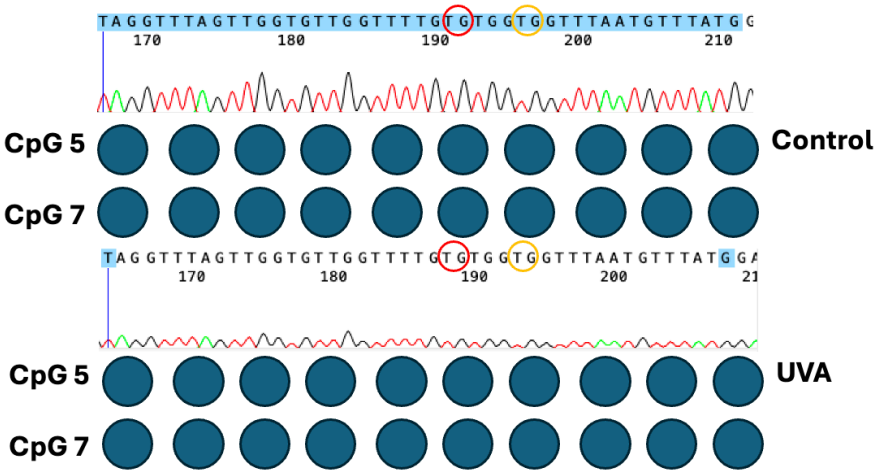


Appendix 2.6: Compression graphs acquired from Instron machine of a 47.5% PEGDA (MW 700) and 47.5% Tetra additive (with 5% Darocur®) at 30 µm/second. (a) shows the normalised needle breakage at 0.5 N per needle. (b) shows the force at which the MN array breaks to be between 50 N. (c) exhibits the 1/derivative in compression, indicating the rate of change which a peak drop-in rate of loading at 200 µm. (d) the change in the rate of change in loading between 200-300 µm.

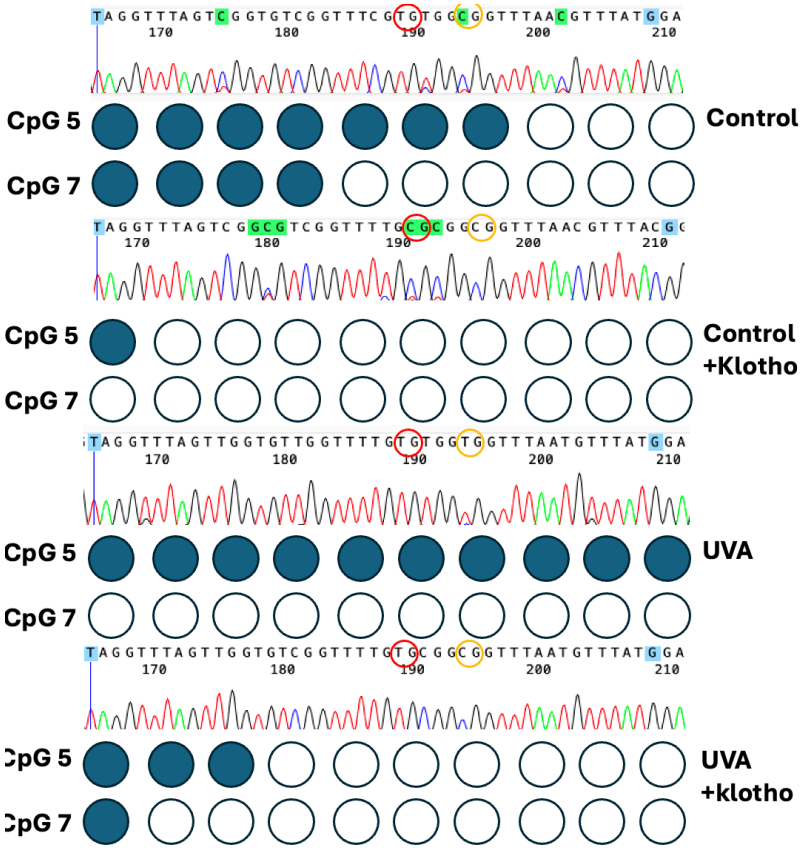


Appendix 2.7: (a) shows the viscosity assessment of BSA and Avidin proteins diluted in varying concentrations of PAA, 3.5%, 0.45% and 0.035% against water and PBS controls. (b) shows protein release of BSA coated MNs after 5-, 19-, or 15-minute PBS wash highlighting maximum released is achieved after 15 minutes of washing.

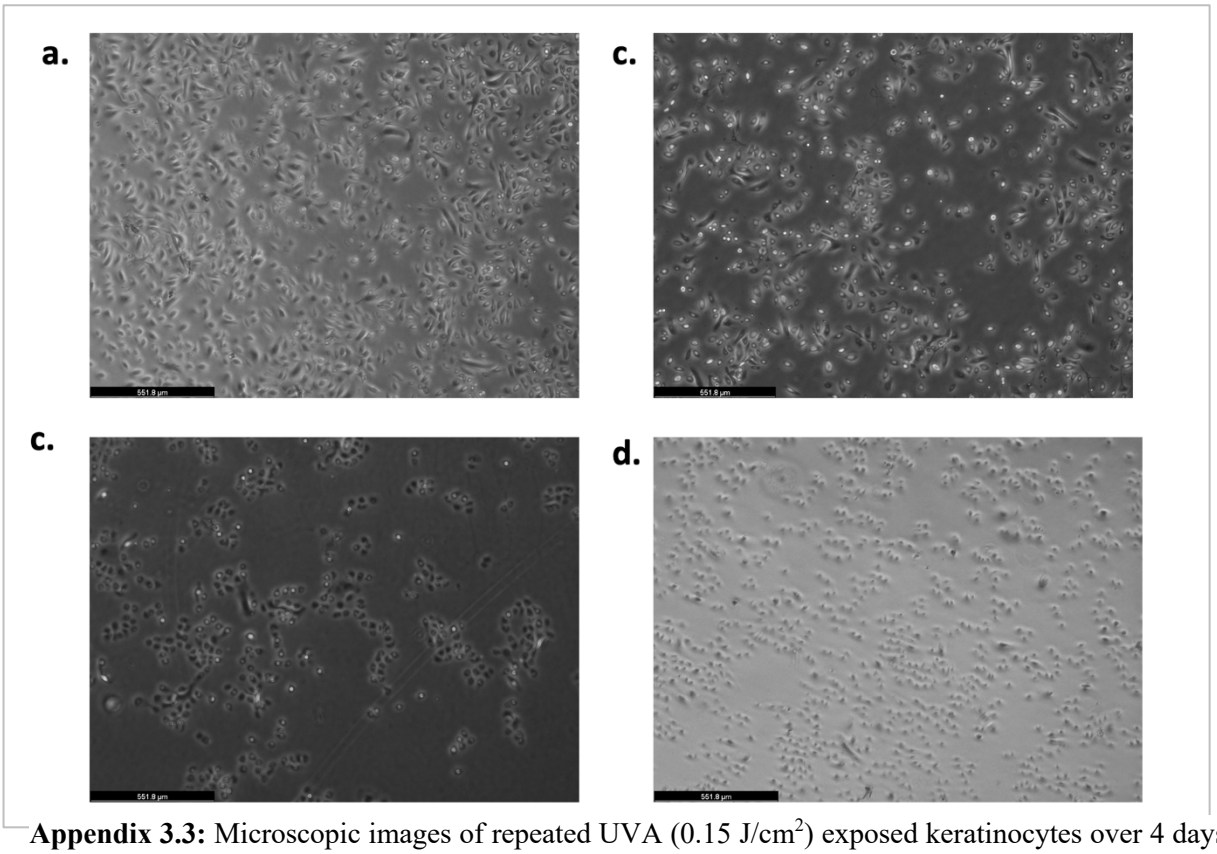
s



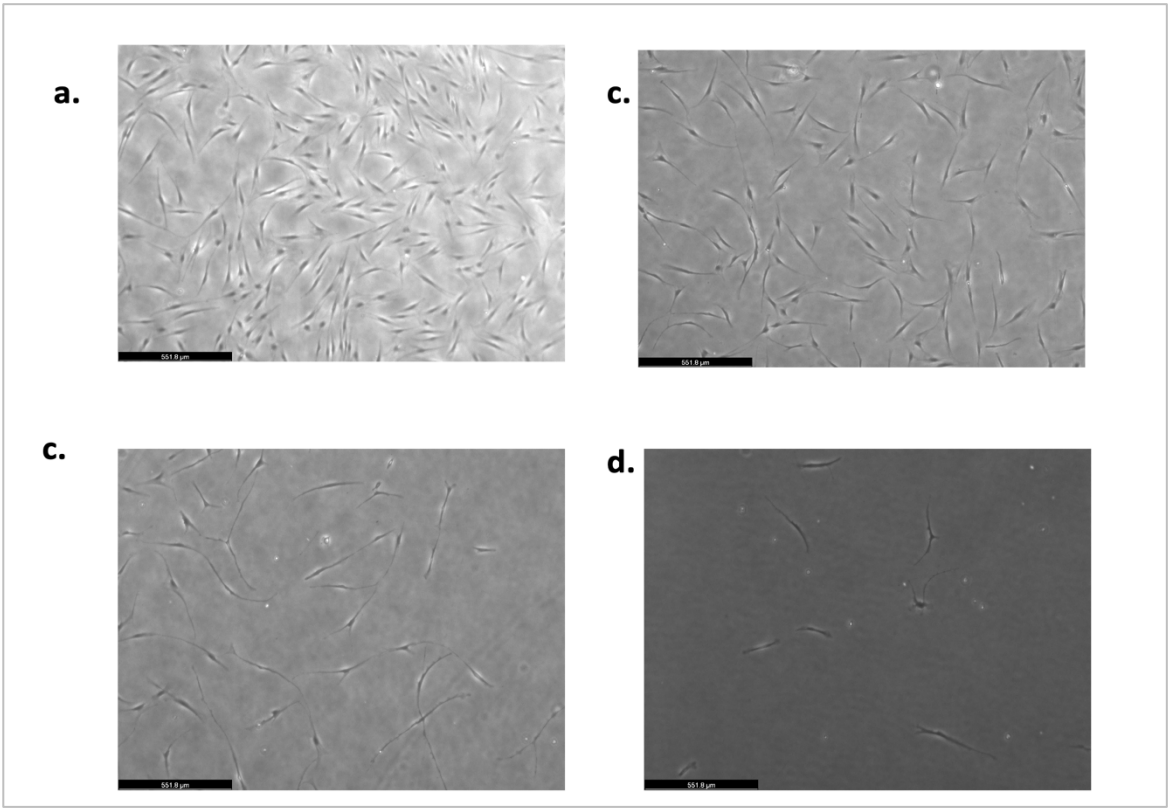
Appendix 3.1: Methylation changes in old skin. Large circles represent technical repeats, (blue methylated, white unmethylated). The Number of methylated colonies from 10 was calculated as a percentage i.e. 2/10 = 0.2% which was inputted into ELOVL2 calculation. Red circles represent CpG site 5. And orange CpG site 7.



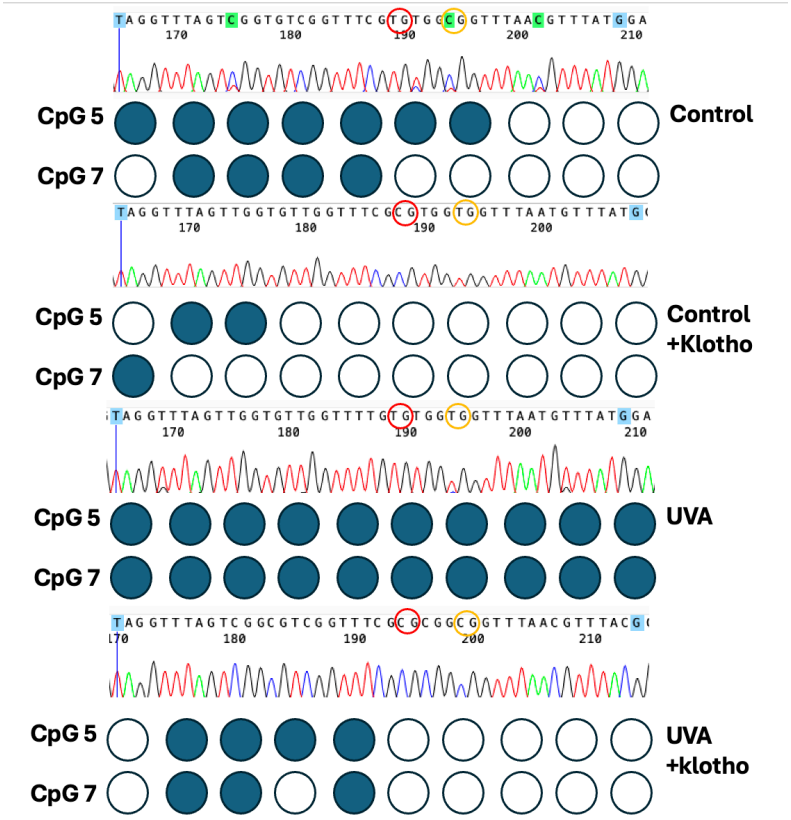
Appendix 3.2: Methylation changes in old skin. Large circles represent technical repeats, (blue methylated, white unmethylated). The Number of methylated colonies from 10 was calculated as a percentage i.e. 2/10 = 0.2% which was inputted into ELOVL2 calculation. Red circles represent CpG site 5. And orange CpG site 7.



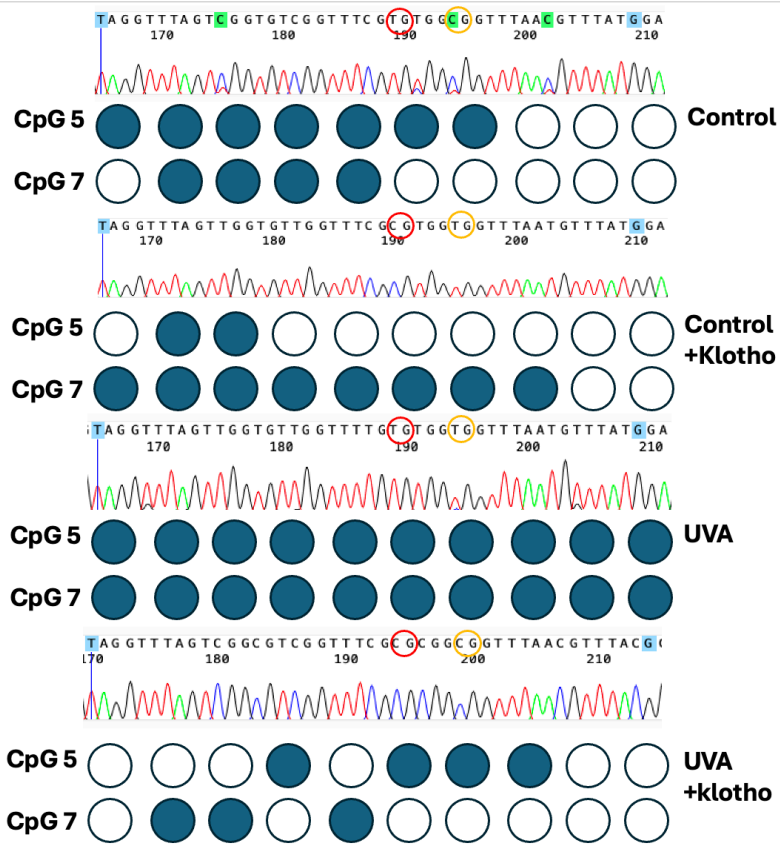
Appendix 3.3: Microscopic images of repeated UVA (0.15 J/cm^2) exposed keratinocytes over 4 days, once every day. (a) Day 1 (24 hours) after single dose UVA. (b) Day 2 (24 hours) after two repeated doses of UVA. (c) Day 3 (24 hours) after three repeated UVA. (d) Day 4 (24 hours) after four repeated doses of UVA.



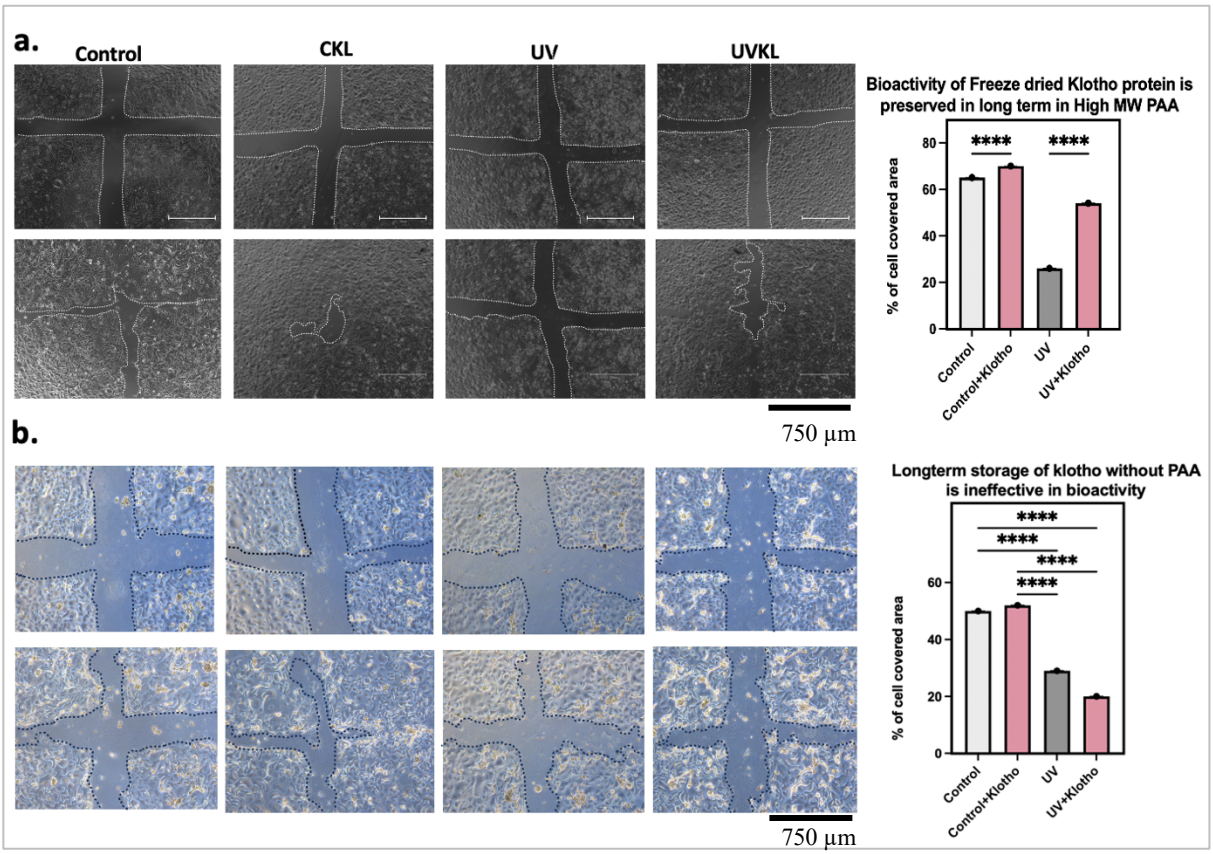
Appendix 3.4: Microscopic images of repeated UVA (2.0 J/cm^2) exposed fibroblasts over 4 days, once every day. (a) Day 1 (24 hours) after single dose UVA. (b) Day 2 (24 hours) after two repeated doses of UVA. (c) Day 3 (24 hours) after three repeated UVA. (d) Day 4 (24 hours) after four repeated doses of UVA.



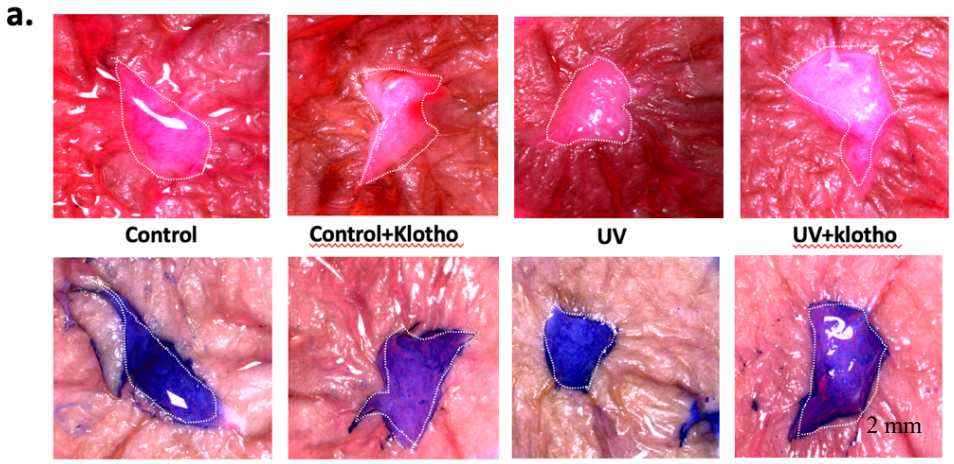
Appendix 3.5: Methylation changes in epidermal keratinocytes. Large circles represent technical repeats, (blue methylated, white unmethylated). The Number of methylated colonies from 10 was calculated as a percentage i.e. 2/10 = 0.2% which was inputted into ELOVL2 calculation. Red circles represent CpG site 5. And orange CpG site 7.



Appendix 3.6: Methylation changes in dermal fibroblasts. Large circles represent technical repeats, (blue methylated, white unmethylated). The Number of methylated colonies from 10 was calculated as a percentage i.e. 2/10 = 0.2% which was inputted into ELOVL2 calculation. Red circles represent CpG site 5. And orange CpG site 7.



Appendix 3.7: Impact of PAA in conserving the bioactivity of the klotho protein long term. (a) representative images with adjacent graph (one way ANOVA) of keratinocytes treated with freeze-dried klotho encapsulated in PAA to assess their migration from hour 0 (top) and hour 24 (bottom images) 20x mag. (b) representative images with adjacent graph (one way ANOVA) of keratinocytes treated with long-term stored klotho without PAA to assess their migration from hour 0 (top) and hour 24 (bottom images) 5x mag.



Append epresentative images of klotho treated skin samples to assess wound healing capacity, top (day 0), bottom (day 7 following 7 days of incubation at 37°C and 5% CO_2) stained with aniline blue. (b) representative graph of technical repeats presenting % average area of wound healed after 7 days.