The effect of intrinsic and extrinsic influences on skin ageing within associated demographics

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Abstract

The skin ages because of intrinsic and extrinsic influences, resulting in damage to mitochondrial DNA (mtDNA). Intrinsic ageing occurs chronologically and cannot be controlled; however, extrinsic skin ageing results from exposure to environmental factors such as ultraviolet radiation (UVR). Although only reported in a small number of publications, previous studies have used a skin swab technique to detect differences in mtDNA damage as a result of UVR exposure. Limited repair mechanisms make mtDNA an effective biomarker of ageing, pioneered predominantly by the Birch-Machin laboratory.

A plasmid containing a mitochondrial region of interest was developed to improve normalisation methodology for mtDNA damage comparison. A VISIA® Skin Analysis system was also used to investigate UV spot variation, which was used alongside the swab technique to determine whether differences in mtDNA damage following recent UVR exposure and lifestyle factors can be detected using a skin swab, including a large seasonal study (n=87). Although forskolin and caffeine are natural compounds frequently used in the cosmetic industry, their combined effect has not been investigated; therefore, their protective effects against complete solar light in human dermal neonatal fibroblasts (HDFn) was investigated using cell viability assays.

We built upon previous research involving the skin swab by investigating differences in mtDNA damage between individuals. Results did not show a consistent increase in damage immediately following high intensity UVR exposure and significant correlations were not observed with sun exposure and protection behaviours. A seasonal study showed the greatest level of mtDNA damage in spring, in comparison to summer and autumn and a significant positive corelation was seen between protection behaviours and mtDNA damage in swabs collected from the left cheek in summer ($p=0.02$). UV spot %Area increased during summer and decreased during winter, and trends were observed with age and skin type. A trending correlation was observed between mtDNA damage and UV spots in samples collected from the right side of the face in spring ($p=0.09$). A combination of caffeine and forskolin was found to have protective effects against 4.32 standard erythemal dose (SED) complete solar light.

Although mtDNA damage observed did not reflect perceived recent UVR exposure under our experimental study protocol, facial imaging analysis showed some correlations; however, further studies are necessary. Future studies would employ an objective measure of sunscreen use which would enable a clearer conclusion. Facial imaging analysis could not only prove

effective at measuring damage, but could also be useful in the screening of protective skincare compounds such as forskolin and caffeine.

Declaration

This thesis is submitted for the degree of Doctor of Philosophy. The research for this submission was performed in the department of Dermatological Sciences, Institute of Translational and Clinical Research, Newcastle University, United Kingdom from September 2018 to February 2022, under the supervision of Professor Mark A. Birch-Machin. All work is original unless acknowledged via reference. None of the material has been submitted previously for a degree or any other qualification at this University or any other institution.

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Abbreviations

acetyl-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
AMP	anti-microbial peptide
α-MSH	alpha-melanocyte-stimulating hormone
ATP	adenosine triphosphate
cAMP	3',5'-cyclic AMP
CO_2	carbon dioxide
CPD	cyclobutene pyrimidine dimer
CREB	cAMP responsive binding element
Ct	cycle threshold number
DHAP	dihydroxyacetone phosphate
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulfoxide
ETC	electron transport chain
$FADH_2$	flavin adenine dinucleotide
FBS	foetal bovine serum
FSPT	fitzpatrick skin phototype
GPX	glutathione peroxidase
G3P	glyceraldehyde-3-phosphate
HA	hyaluronic acid

HDFn	human dermal neonatal fibroblasts
H&E	hematoxylin and eosin
HIF1α	hypoxia-inducible factor 1-alpha
H_2O_2	hydrogen peroxide
MC1R	melanocortin 1 receptor
MITF	microphthalmia transcription factor
MMP	matrix metalloproteinase
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
nDNA	nuclear DNA
NER	nucleotide excision repair
OCT	optimal coherence tomography
$ONOO^{\cdot}$	peroxynitrite
OXPHOS	oxidative phosphorylation
$^1O_2$	singlet oxygen
$O_2^{-\cdot}$	superoxide radical
$^{\cdot}OH$	hydroxyl radical
PKA	protein kinase A
POMC	proopiomelanocortin
PRX	peroxiredoxin
ROS	reactive oxygen species
rRNA	ribosomal RNA

SED	standard erythemal dose
SOD	superoxide dismutase
TCA	tricarboxylic acid
tRNA	transfer RNA
UVR	ultraviolet radiation

Chapter 1. General Introduction

1.1 Skin biology

1.1.1 Skin structure

The skin is considered the largest organ in the human body due to contributing to approximately 16% of the total human body weight (1). It performs multiple vital functions such as protection against external insults and water loss (2). Human skin consists of 3 layers: epidermis, dermis and hypodermis or subcutaneous adipose layer (Figure 1.1). Keratinocytes comprise approximately 95% of the epidermis which is divided into 4 sublayers: stratum corneum, stratum granulosum, stratum spinosum and stratum basale, depending on keratinocyte differentiation status. The remaining 5% of the epidermis is composed of melanocytes, Langerhans and Merkel cells (3, 4). Keratinocytes are responsible for the synthesis of keratin, a protein composed of coiled peptide chains which combine to form supercoils of several polypeptides linked by disulphide bonds, responsible for providing the skins structure (1).

The stratum basale is often one cell thick and contains keratinocyte stem cells which continuously produce new keratinocytes through cell division. Newly generated keratinocytes move progressively upwards through the epidermis to generate the upper layers. Keratinocytes within the stratum basale attach to both the basement membrane and the stratum spinosum layer through desmosomes. Melanocytes make up approximately 5-10% of the cell population in this layer and are responsible for the production of melanin, which is packed into melanosomes and transported to neighbouring basal keratinocytes through dendrites. Each melanosome can supply melanin to approximately 30-40 surrounding keratinocytes. Melanin is responsible for providing protection against ultraviolet radiation (UVR), as well as determining skin and hair colour (1, 4, 5). The skin colour of healthy individuals is consistent due to the even distribution of melanocytes throughout this layer of the epidermis (5).

Basal cells move towards the surface and form the stratum spinosum layer which consists of polyhedral keratinocytes that are unable to proliferate; connected by desmosomes, as well as Langerhans cells. The stratum granulosum sits above the stratum spinosum layer and plays a vital role in the protective barrier function of the skin, as well as the maintenance of skin hydration. Granular cells contain keratohyalin granules; located within the cytoplasm, which are filled with proteins highly cross-linked with keratin filaments and promote dehydration of the cell. The cross-linking of keratin filaments creates a tight barrier in the epidermis. As the

epidermis differentiates, the keratinocytes become flattened as a result of the action of filaggrin, a protein component of keratohyalin granules. The process of cornification by keratohyalin granules is known as keratinisation. Keratin and filaggrin comprise 80-90% of the mass of the epidermis. The stratum granulosum is also responsible for the release of lipid components from lamellar bodies into the intercellular space. This contributes to its role in the protective barrier of the skin and also plays an important role in the intercellular cohesion with the stratum corneum. Loss of function mutations in filaggrin affect approximately 10% of the population and are the major genetic risk factor for atopic eczema and several other allergic disorders (1, 4, 6).

The stratum corneum is the outermost later of the epidermis and is comprised of cells that have migrated from the stratum granulosum. It consists of 15-20 layers of corneocytes which undergo changes such as flattening, as well as the loss of nuclei and cytoplasmic organelles. The stratum corneum is relatively impermeable and is therefore responsible for the barrier function of the skin. Cells are arranged in a scaffold-like lattice bound together by keratohyalin and the intercellular spaces are filled with a lipid-rich matrix, providing a robust waterproofing barrier. A stratum lucidum may also be present between the stratum granulosum and stratum corneum in areas where the skin is thick such as palms and soles (1, 6).

Overall, it takes approximately 28 days from cell division to shedding of the stratum corneum, a process which balances proliferating keratinocytes that form in the stratum basale (1, 6). Thickness of the epidermal layer varies depending on anatomical site, with the hands having a thicker epidermis in comparison to the forearm (7). The epidermal layer does not contain nerves or blood vessels and relies on the dermal layer below for metabolism (8).

The epidermis and dermis are separated by the cutaneous basement membrane which is less than 200nm. It is responsible for linking the keratin intermediate filaments of basal keratinocytes with collagen fibres in the dermis through proteins and glycoproteins (4). In comparison to the epidermis, the dermis is a tough layer that provides protection against mechanical injury, with thickness varying between approximately 1mm and 5mm depending on anatomical site. The dermis is composed of two layers: a thin upper layer known as the papillary dermis, and a deeper layer known as the reticular dermis (Figure 1.1).

The papillary dermis is in contact with the basement membrane and is supplied with blood vessels and sensory nerve endings, whereas the reticular dermis is in contact with the subcutaneous adipose layer. Fibroblasts are the main cell type within the dermal layer and are responsible for the synthesis of collagen and elastin fibres. Mastocytes and macrophages are also present within the dermis, as well as blood vessels, lymphatic channels and sensory nerves.

The connective tissue is the most abundant component of this region which is constituted of collagen fibres. Approximately 70% of the dry weight of the dermis is made up of collagens, of which types I and III are predominant. The properties of collagen have been shown to change both quantitatively and qualitatively with ageing. Elastic fibres account for 5% of the dry weight of the dermis and are responsible for skin elasticity, due to being less tough than collagen fibres (1, 3, 4). Hyaluronic acid (HA) is also found within the skin, accounting for approximately 50% of total body HA. It is a key molecule involved in skin hydration due to its ability to bind and retain water molecules and is therefore significant in skin ageing. The stratum granulosum is responsible for the maintenance of skin hydration due to the inability of aqueous materials to diffuse through due to lipids synthesised by the keratinocytes (9).

Finally, the subcutaneous adipose layer is composed of lipocytes arranged into fat lobules, separated by fibrous septae and provides insulation for thermoregulation, as well as energy storage (4, 10). Approximately 80% of body fat is found in this layer in healthy individuals (4).

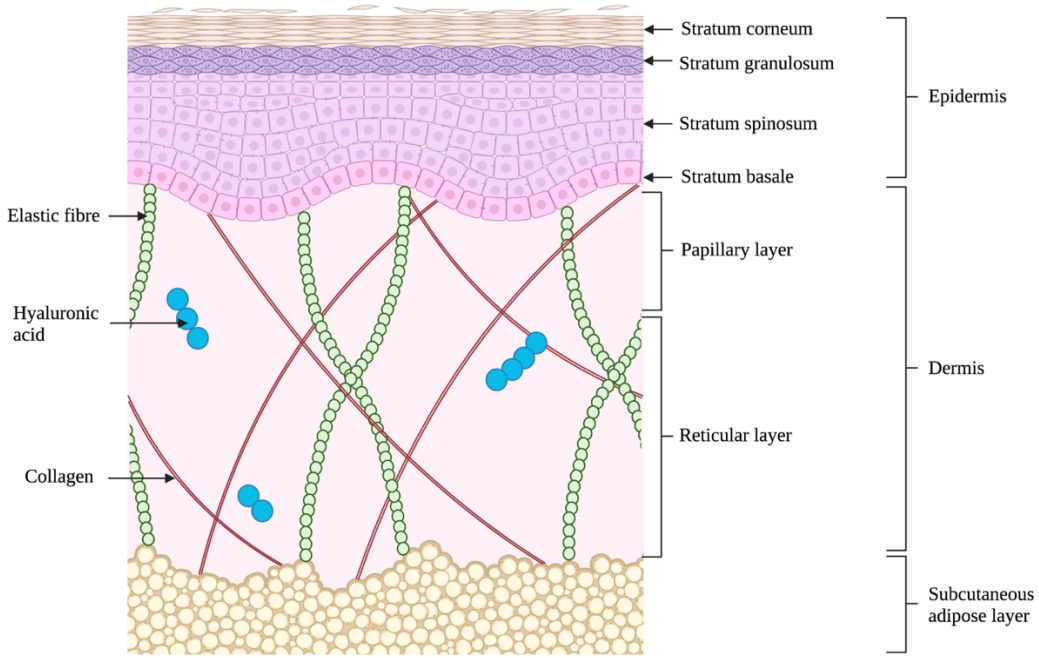


Figure 1.1 The structure of human skin.
The skin comprises three main layers: epidermis, dermis and subcutaneous adipose layer. The epidermis is generally made up of 4 different layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. A stratum lucidum may also be present between the stratum granulosum and stratum corneum in areas of increased skin thickness such as the palms and soles. The basement membrane sits between the epidermis and dermis and is attached to keratinocytes within the stratum basale by desmosomes. The dermis is beneath the basement membrane and consists of a papillary layer that is connected to the basement membrane and a reticular layer that is in contact with the subcutaneous adipose layer. The subcutaneous adipose layer sits underneath the dermis and is composed of lipocytes arranged into fat lobules. Made using Biorender.com.

1.1.2 Skin function

The skin is a vital organ as it is the first line of defence between the body and external environment. It protects against external stressors such as UVR, harmful chemicals and pathogenic microorganisms. It also plays important roles in immunological processes, thermoregulation, metabolic processes and maintaining homeostasis within the body (11).

These functions are mainly carried out by proteins and molecules that are expressed by the epidermis as the dermis is highly permeable, and it is widely accepted that most of the barrier functions of the epidermis localise to the stratum corneum. The scaffold-like lattice of keratinocytes and intercellular lipid-rich matrix provide protection through the generation of a robust waterproof barrier (1, 12-14). The condition of this barrier is dependent on its physical properties, such as sebum production, epidermal hydration, transepidermal water loss and the pH gradient between the skin surface and the inside of the body (11).

The skin functions as a first line of defence against invading microorganisms through the production of anti-microbial peptides (AMPs), epidermal Langerhans cells and epidermal T cells. The dryness of the outer layer of the epidermis also contributes to this defence mechanism as the continual shedding of keratinocytes prevents any sustained growth of unwanted organisms on the skins surface. Epidermal cells produce AMPs which are responsible for killing Gram-positive and Gram-negative organisms, fungi and some viruses. The two main families of AMPs are defensins and cathelicidins, which are synthesised in the stratum spinosum and stratum granulosum and subsequently transported to the stratum corneum. Langerhans cells within the epidermis can migrate to the paracortical areas of lymph nodes and act as antigen-presenting cells and T cells can be found in the dermis and around the skin appendages (1). Skin acidification also plays an important role in its antimicrobial function as it helps to maintain a pH of less than 5.5, which also suppresses growth of pathogenic microorganisms (14).

Exposure to UVR accounts for approximately 90% of visible skin ageing (15); also known as photoageing, and is characterised by deep wrinkles, laxity, leathery appearance and increased skin fragility (16). The skin provides protection against UVR through the reflection by the stratum corneum, upregulation of melanocyte activity following exposure and heat-shock proteins. This reduces absorption of UVR by DNA, which can lead to photoageing and carcinogenesis. As well as absorbing UVR and preventing DNA damage, melanin also acts as an antioxidant and radical scavenger for those generated following UVR exposure (1, 17, 18).

Finally, the skin is a critical organ in aid of survival and reduces the risk of tissue damage as it helps to detect potentially dangerous stimuli such as touch, vibration, itch, pain, temperature and pressure through sensory perception due to the rich supply of free nerve endings. This allows behavioural changes in responses to these potentially harmful stimuli, such as moving away from a cold or hot source to prevent burning or removing a layer of clothing in response to environmental temperature changes (1, 4).

1.1.3 Skin pigmentation

In 1975 Fitzpatrick first described sun-reactive skin types I to IV to classify individuals with white skin which was later modified to include types V and VI for pigmented skin (Figure 1.2). Fitzpatrick skin phototype (FSPT) is the most common method used to assess sunburn risk and is frequently estimated based on an individual's appearance, with a lower FSPT corresponding to skin that is more sensitive to UVR (19, 20).

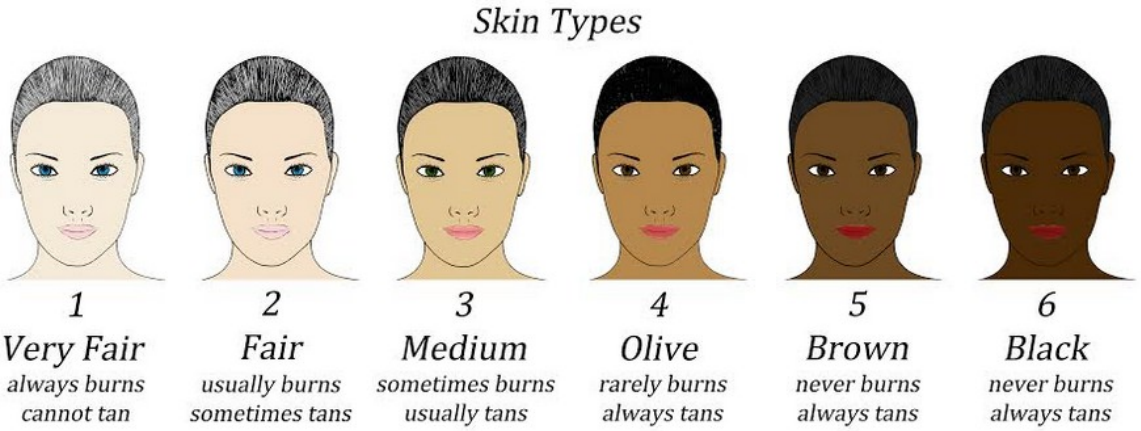


Figure 1.2 Fitzpatrick skin types 1 to 6.
Different skin types vary in sensitivity to UVR due to the level of pigmentation and can be classified using Fitzpatrick skin phototype.

Skin pigmentation involves the cooperation of melanocytes and keratinocytes to produce melanosomes, which are subsequently transferred to keratinocytes within the epidermis and distributed (17). The melanocortin 1 receptor (MC1R) is a G protein-coupled receptor expressed on the surface of melanocytes and is responsible for skin pigmentation, sun sensitivity and melanoma risk through the control of melanin synthesis (21-23). Melanin therefore functions to protect the skin against the damaging effects of UVR (24). There are two types of melanin; pheomelanin and eumelanin, which are responsible for determining skin and

hair colour. MC1R receptor activation in human melanocytes as a result of UVR exposure stimulates skin pigmentation by promoting a switch in synthesis from the red/yellow pheomelanin to the brown/black eumelanin and is therefore also involved in the skin tanning response. If the receptor is not activated or blocked, melanocytes produce pheomelanin rather than eumelanin which does not protect the skin from UVR and has the potential to generate free radicals in response to exposure, resulting in increased risk of sun damage (22, 24). As well as protecting against UVR by limiting penetration into the skin, melanin also enhances nucleotide excision repair (NER), a major type of DNA damage repair. The significance of MC1R in determining human pigmentation was revealed by specific nucleotide polymorphisms present in the gene that result in varying degrees of receptor activity and are associated with a phenotype of red hair and pale skin, as well as the inability to tan (22).

The adaptive pigmentation pathway begins with UVR exposure which results in DNA damage to keratinocytes within the epidermis, subsequently increasing proopiomelanocortin (POMC) expression in a p53-dependent manner (Figure 1.3). POMC is then cleaved, generating the positive agonist alpha-melanocyte-stimulating hormone (α-MSH). The agonist binds to MC1R on the surface of melanocytes, resulting in adenylate cyclase activation 3',5'-cyclic AMP (cAMP) production. This accumulates and promotes the activation of protein kinase A (PKA), resulting in phosphorylation of cAMP responsive binding element (CREB), a transcription factor which leads to the upregulation of microphthalmia transcription factor (MITF). Transcriptional changes of DNA within the nucleus are then able to occur, followed by the upregulation of multiple pigment-dependent enzymes including tyrosinase. Eumelanin is subsequently synthesised and transported to keratinocytes to provide further protection against UVR (25). MC1R variants all have the ability to bind the ligand α-MSH, but show diminished intracellular ability to activate adenylate cyclase in the signalling cascade and synthesise eumelanin (26).

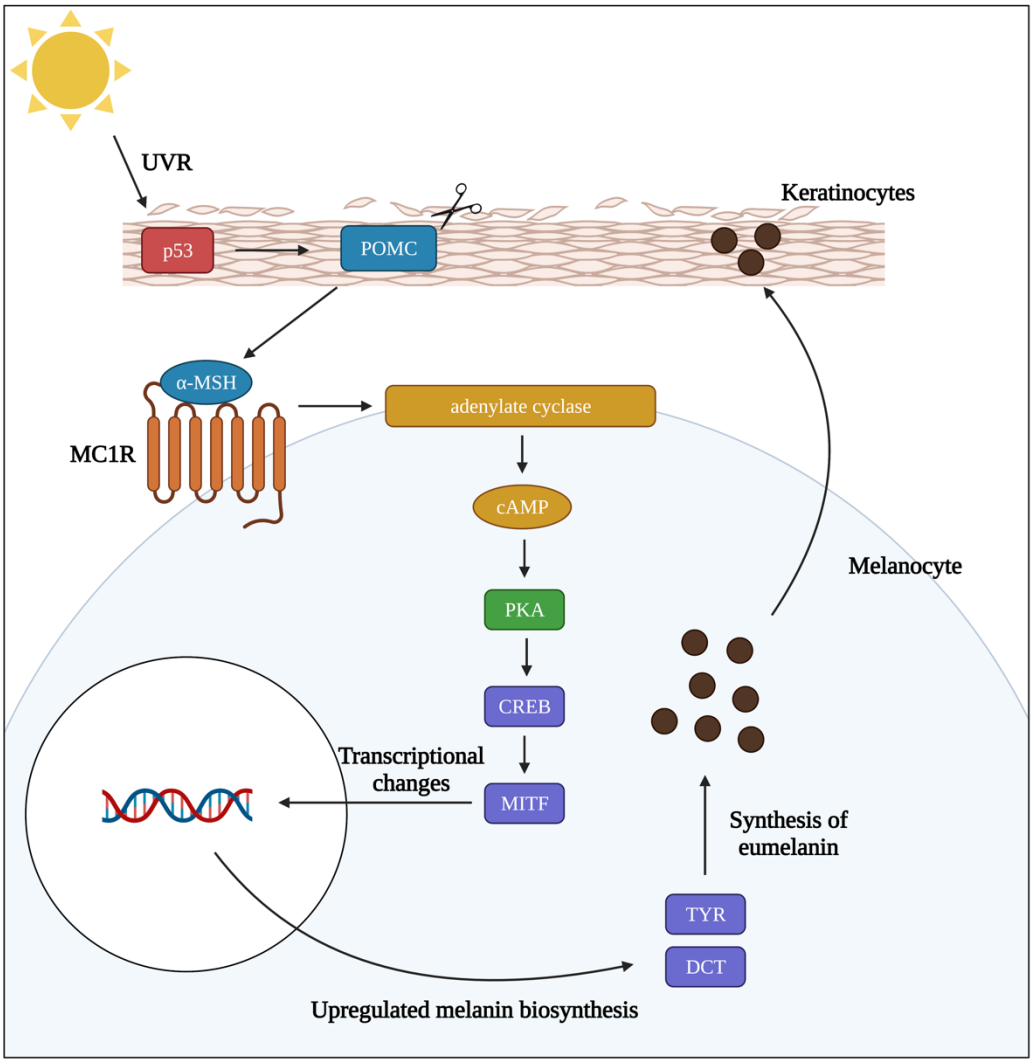


Figure 1.3 Generation of melanin through MC1R and the adaptive pigmentation pathway.
Exposure to UVR activates a cascade of events, including the activation of MC1R through binding of a-MSH, generation of cAMP and transcriptional changes to DNA through the activation of transcription factors. Eumelanin is synthesised within melanocytes and transported to keratinocytes to provide increased protection against UVR and subsequent DNA damage. Made using Biorender.com.

Melanin is an optically dense material which absorbs radiation at both visible and UV wavelengths (27). Individuals differ in susceptibility to skin ageing as a result of environmental stressors, with studies showing that Caucasian skin has earlier onset as well as greater skin wrinkling in comparison to populations with higher levels of pigmentation (28). Research has also shown that winkle onset is delayed approximately 10 years in Chinese women in comparison to French women, similarly thought to be due to differing levels of melanin within the skin (29). Black skin has been shown to be more resistant to UVR than white skin as the average natural SPF for black skin is 13.1 (30, 31).

1.2 Intrinsic skin ageing

1.2.1 Mitochondria

Mitochondria are subcellular organelles known as the "powerhouse of the cell" as they play an important role in cellular energy production and are responsible for generating approximately 90% of total cellular energy (Figure 1.4). There are approximately 100-1000 mitochondria within the cytoplasm of a eukaryotic cell, which varies depending upon metabolic demand. Mitochondria have a length of 1-2µm and a diameter of 0.5-1µm and are composed of an outer membrane and an inner membrane (32). The outer membrane is responsible for separating the mitochondria from the cell cytosol, whereas the inner membrane is folded to form cristae (33). They produce energy in the form of adenosine triphosphate (ATP) through a process known as respiration, which can be both aerobic; oxygen requiring, and anaerobic. The electron transport chain (ETC) occurs at the inner membrane and is responsible for the majority of mitochondrial energy production in higher organisms through oxidative phosphorylation (OXPHOS), a process requiring oxygen. As previously mentioned, the inner membrane is folded to form cristae; therefore, increasing the surface area and ATP-generating capacity (32).

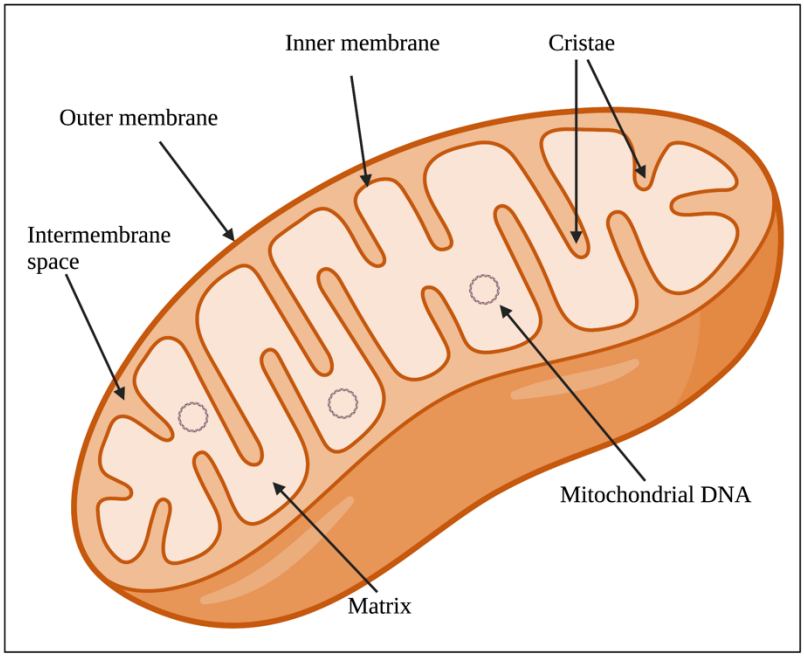


Figure 1.4 Mitochondrial structure.
Structure of the mitochondria including inner membrane folded into cristae to form the site of energy production within the mitochondria. Mitochondrial DNA is located within the matrix. Made using Biorender.com.

Mitochondrial DNA (mtDNA) is located within the mitochondrial matrix; attached to the inner mitochondrial membrane, in close proximity to the site of OXPHOS (32, 33). Human mtDNA

is a closed circular double-stranded molecule composed of 16,569 bases and 37 genes; coding for 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and 13 polypeptides (32). It consists of a 'heavy' and 'light' strand, with the heavy strand carrying the majority of the genetic information, including genes for two rRNAs, 14 tRNAs and 12 polypeptides. In contrast, the light strand codes for 8 tRNAs and a single polypeptide. All 13 protein products are constituents of the enzyme complexes of the OXPHOS system within the ETC (33). The displacement loop; also known as the D-loop, is a region of 1124 bp at position 16024-576. It contains important elements required for replication and transcription and is a non-coding region that acts as a promoter for heavy and light strands. The importance of this region is highlighted by the mtDNA mutation hotspots at hypervariable region I (16024-16383) and hypervariable region II (57-372) within the D-loop (32). There are two major transcription initiation sites located within the D-loop; IT_{H1} and IT_L, situated within 150 bp of one another. A promoter element with a 15 bp consensus sequence motif surrounds the transcription initiation sites and is critical for transcription (33). Replication of mtDNA requires DNA polymerase γ and occurs bidirectionally at two origins of replication, O_H and O_L, for heavy and light strands. Replication of mtDNA is semi-autonomous and is not based on the nuclear genome replication, although both are regulated factors which are currently unknown (32). The genes lack introns and except for one regulatory region, intergenetic sequences are absent or limited to a few bases (33); therefore, more than 95% of the mtDNA is coding (32). Unlike nuclear DNA (nDNA), mtDNA lacks protective histones and certain DNA repair mechanisms are inefficient or absent; therefore, greater damage occurs and at a more rapid rate (32). In mammalian cells, mtDNA is highly conserved and transferred to offspring via the maternal X chromosome. Cells contain many copies of the mitochondrial genome and contain a ratio of damaged mtDNA to undamaged mtDNA, termed heteroplasmy (34). Mutations within mtDNA are functionally recessive as the wild type molecules compensate for any deleterious effects of mutant genomes, known as complementation (35). The threshold of tolerable accumulated damage can be breached overtime, resulting in the disease state manifestation (34). This level is often between 50-60% for mtDNA deletions, but slightly higher at 60-90% for point mutations.

Mitochondria play an important role in the skin by providing energy for various processes such as cell signalling, wound healing, pigmentation, vasculature homeostasis, microbial defence and hair growth. As a result of metabolic stress induced by hypoxia following bacterial infection, ATP production has been found to increase rapidly in the skin. This implements mitochondrial reactive oxygen species (ROS) signalling in the defence against skin infection through hypoxia-inducible factor 1-alpha (HIF1α) and the recruitment of immune cells (8, 36). Multiple skin conditions have been linked to patients with primary mitochondrial disorders and

can be categorised into four groups: acrocyanosis, rashes, pigmentation abnormalities and hair abnormalities (37).

1.2.2 Mitochondrial energy production

Overall, cellular respiration is composed of three pathways: glycolysis, tricarboxylic acid (TCA) cycle; also known as the Krebs cycle, and OXPHOS through the mitochondrial ETC (32).

1.2.2.1 Tricarboxylic acid cycle

The TCA cycle was proposed by Hans Adolf Krebs in 1937 and plays an essential role in OXPHOS (Figure 1.5) (38). It is responsible for the oxidation of molecules such as carbohydrates, lipids and amino acids in order to produce nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), also known as electron donors. The TCA generates approximately 67% of all electron donors which enter the ETC through mitochondrial complexes I and II, resulting in the production of ATP via OXPHOS (39).

In eukaryotic cells, the TCA cycle occurs in the mitochondrial matrix and involves eight enzymes: citrate synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinyl coenzyme A synthetase, succinate dehydrogenase, fumarase and malate dehydrogenase (Figure 1.5) (38). Glycolysis occurs within the cytosol prior to the TCA cycle and is responsible for the generation of pyruvate, which is subsequently converted into acetyl coenzyme A (acetyl-CoA) in order to feed into the TCA cycle (Figure 1.5). The first step of glycolysis is the conversion of glucose to fructose-1,6-bisphosphate, a process which requires two molecules of ATP. Fructose-1,6-bisphosphate is then split into dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P). G3P is subsequently converted to pyruvate, generating two molecules of ATP and one molecule of NADH, which is able to feed into the ETC. This process can occur twice per molecule of glucose as DHAP can be converted to G3P in order to form pyruvate; therefore, one molecule of glucose is able to form two molecules of ATP, NADH and pyruvate. Each molecule of pyruvate is then decarboxylated to produce two molecules of acetyl-CoA; a two-carbon molecule that is able to enter the TCA cycle, resulting in the production of a further two NADH molecules, as well as two carbon dioxide (CO_2) molecules (38, 40).

As acetyl-CoA feeds into the TCA cycle, it is combined with oxaloacetate; a four-carbon molecule, in the presence of citrate synthase, generating the six-carbon molecule citrate.

Aconitase is responsible for converting citrate to its intermediate cis-aconitate, and subsequently isocitrate. Isocitrate is then converted to α-ketoglutarate by isocitrate dehydrogenase, producing one molecule of NADH and one molecule of CO_2. Succinyl-CoA is then generated from α-ketoglutarate by α-ketoglutarate dehydrogenase, producing a second molecule of NADH and CO_2. Succinyl-CoA is converted to succinate by succinyl coenzyme A synthetase generating one molecule of GTP, an important signalling molecule that can also be used to carry energy. Succinate dehydrogenase converts succinate to fumarate, producing one molecule of $FADH_2$. Hydration of the double bond then converts fumarate to malate, catalysed by fumarase. Finally, malate is dehydrogenated by malate dehydrogenase to form oxaloacetate and one molecule of NADH. The TCA cycle is then able to start again following the reaction between oxaloacetate and another molecule of acetyl-CoA. The cycle can occur twice per molecule of glucose due to the generation of 2 molecules of acetyl-CoA. Per molecule of glucose, 6 molecules of NADH, 4 molecules of CO_2, 2 molecules of GTP and 2 molecules of $FADH_2$ are generated by the TCA cycle. Molecules of NADH and $FADH_2$ are able to feed into the ETC through complexes I and II for OXPHOS (38).

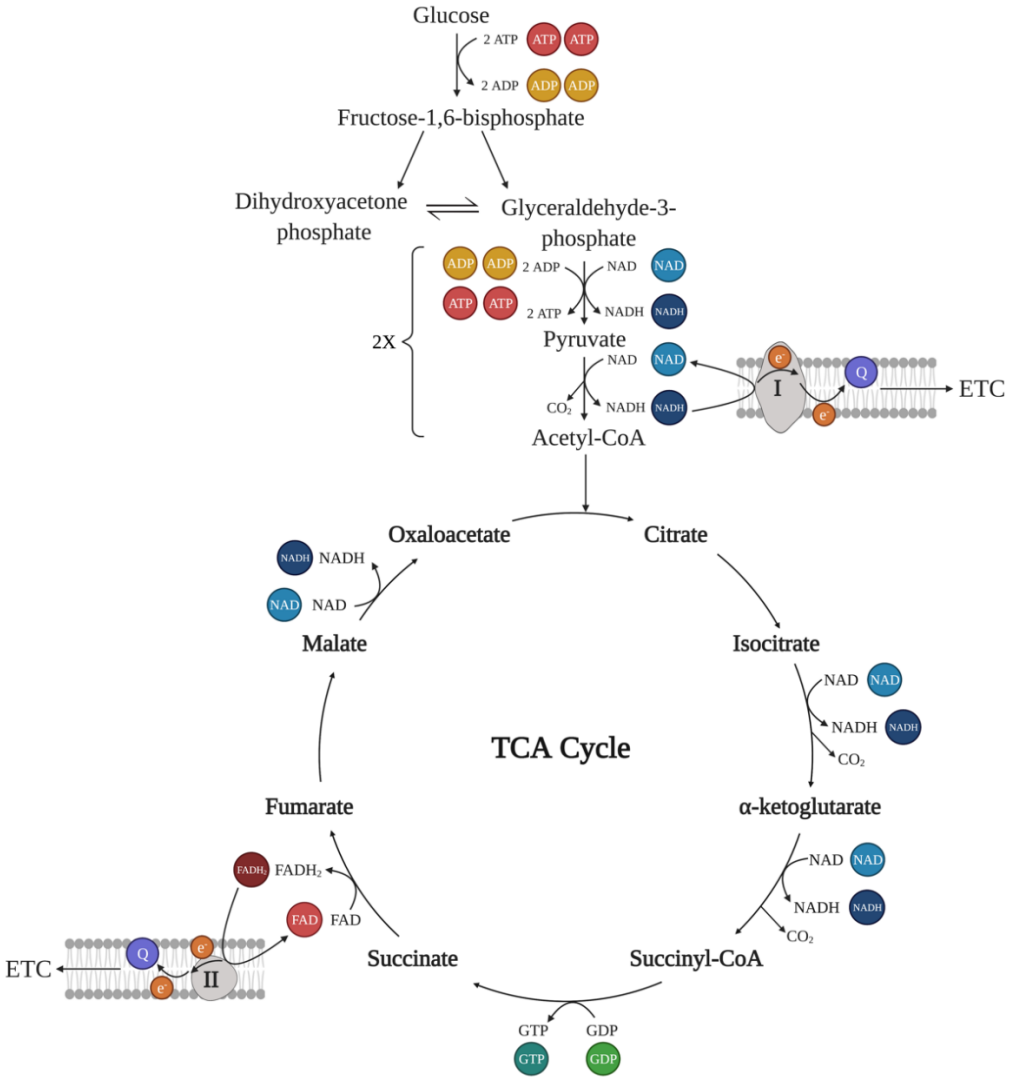


Figure 1.5 Glycolysis, pyruvate decarboxylation and the tricarboxylic acid cycle.
Glucose is converted into two molecules of pyruvate through the process of cytosolic glycolysis. Pyruvate decarboxylation generates two molecules of acetyl-CoA which feed into the TCA cycle. The TCA cycle consists of eight enzymes and is responsible for the production of NADH and $FADH_2$; known as electron donors, which supply electrons to ETC complexes I and II for the generation of ATP (41).

1.2.2.2 Electron transport chain (ETC)

The ETC is responsible for producing cellular energy in the form of ATP through OXPHOS (Figure 1.6). The OXPHOS machinery is made up of the ETC and ATP synthase (complex V) which are located in the inner mitochondrial membrane (32). There are four complexes within the ETC (I-IV) named NADH-ubiquinone oxidoreductase, succinate-quinone oxidoreductase, cytochrome bc_1 complex and cytochrome *c* oxidase, respectively. Ubiquinone; also known as coenzyme Q, and cytochrome *c* are also part of the ETC (42).

The largest enzyme of the OXPHOS system is complex I, which is responsible for catalysing the transfer of two electrons from NADH to ubiquinone. This reaction is coupled to the pumping of four protons across the inner mitochondrial membrane from the matrix into the

intermembrane space to generate an electrochemical proton gradient. Complex I is composed of three structurally distinct modules: N-module which is responsible for NADH oxidation, Q-module which transfers electrons to ubiquinone and the P-module which pumps proteins to form a proton gradient (42). Ubiquinone carries electrons through the membrane to complex III, which are then transferred to cytochrome *c* and subsequently to complex IV, where they are transferred to molecular oxygen (32). Like complex I, complexes III and IV are also involved in pumping protons into the intermembrane space to form a proton gradient (42). Complex II receives electrons from succinate; a derivative of the TCA cycle, which are transferred to $FADH_2$ and then coenzyme Q through complex II, before passing to complexes III and IV as previously described (32). During this process, succinate is oxidised to fumarate (42). Overall, complexes I-IV are responsible for the generation of an electromotive proton gradient across the inner mitochondrial membrane by coupling the transport of electrons with proton pumping, using ubiquinone and cytochrome *c* as electron carriers. Electrons are combined with oxygen by complex IV and the movement of H^+ through complex V down the electrochemical proton gradient is used to provide energy for the conversion of adenosine diphosphate (ADP) to ATP to provide cellular energy, known as chemiosmotic coupling, known as chemiosmotic coupling (32, 43).

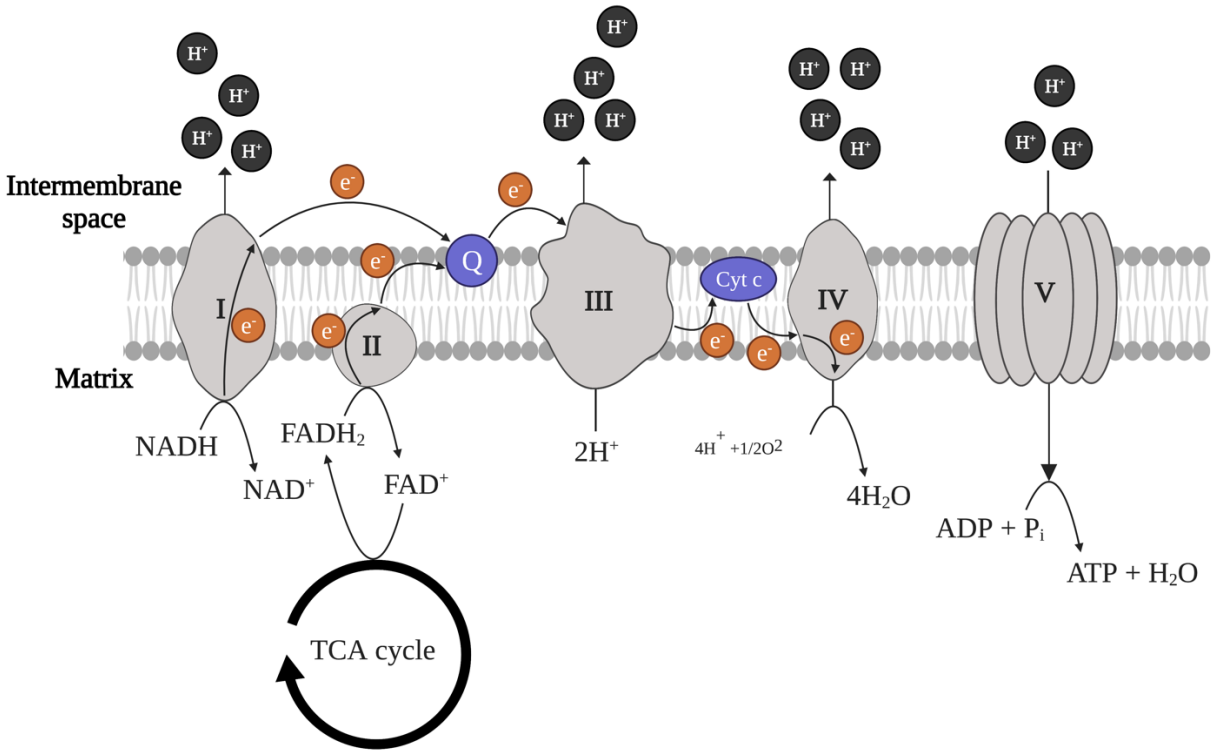


Figure 1.6 Generation of ATP through by the electron transport chain.
Complexes I and II within the ETC receive electrons from donors NADH and $FADH_2$, respectively. Electrons are transported down the ETC, which is coupled with the pumping of H^+ through complexes I, III and IV from the matrix into the intermembrane space, generating a proton gradient. Water is formed from oxygen at complex IV. ATP is generated from ADP and P_i, as well as the movement of H^+ down its proton gradient through complex V (41).

1.2.3 The role of mitochondrial dysfunction in ageing

Approximately 90% of the oxygen consumed within eukaryotes is used in mitochondrial respiration. Under normal physiological conditions up to 5% of this oxygen is converted to ROS, oxygen-containing species with an unpaired electron (35). The majority of ROS are generated from the superoxide radical ($O_2^{-\cdot}$) which is generated when electrons leak from the ETC and subsequently react with oxygen. Electrons mainly leak from complexes I and III within the ETC; however, more recent studies suggest that complex II is also involved (32). ROS are highly reactive and damaging due to their unpaired electron which enables them to readily oxidise other molecules.

As ROS are also involved in cell signalling, tight regulation is required by antioxidant enzymes such as superoxide dismutase (SOD), which can eliminate ROS. SOD is responsible for the conversion of $O_2^{-\cdot}$ to hydrogen peroxide (H_2O_2), which is less reactive. There are 3 different types of SOD: SOD1 is located in the mitochondrial intermembrane space, SOD2 is located in the mitochondrial matrix and SOD3 is attached to the extracellular matrix (44). Although H_2O_2

is less reactive itself, it can cross membranes easily and result in the generation of the highly reactive free hydroxyl radical (·OH), which is known to damage the mitochondrial genome in the form of strand breaks. These oxygen radicals can subsequently initiate other pathways that lead to the generation of further ROS, such as more radicals, aldehydes, epoxides and hydroperoxides (32). There are multiple enzymes that are able to remove H_2O_2; such as, peroxiredoxins (PRX), glutathione peroxidases (GPX) and catalase (44). As well as damage to mtDNA, ROS are also known to damage proteins and membranes by oxidation, particularly $O_2^{-\cdot}$, H_2O_2, ·OH and peroxynitrite ($ONOO^{\cdot}$) (35).

Antioxidants act as natural defence mechanisms to eliminate and protect against ROS by donating an electron without becoming unstable, resulting in the free radical becoming unreactive. High levels of ROS can exceed this natural capacity and result in oxidative stress and cellular damage. Severe oxidative stress has been linked to mechanisms in many diseases associated with ageing such as cardiovascular (e.g. atherosclerosis), cancer and neurological (e.g. Parkinson's and Alzheimer's disease) (32).

The mitochondrial genome is at risk of damage by ROS due to its close proximity to the site of ROS production within the mitochondrial matrix. As well as this, mtDNA lacks protective histones; therefore, the rate of mutation is approximately 10-17 times greater than nDNA (32, 35). Mitochondria are also deficient in NER pathways and are unable to repair UVR-induced photoproducts such as pyrimidine dimers. In addition to this, 95% of mtDNA is encoding in comparison to 3% of nDNA; therefore, any mutagenesis in mtDNA is likely to affect a coding region. Cellular dysfunction occurs when the ratio of mutated mtDNA to wild-type DNA exceeds a threshold level, which is approximately 50-60% for deletions and 60-90% for point mutations (35).

Damage to the mitochondrial genome can have a detrimental effect on energy production. As the damage accumulates, components of the ETC are not transcribed and translated; due to being encoded by the mitochondrial genome, resulting in a decreased proton gradient across the membrane. This therefore results in decreased energy generation as well as increased ROS due to the leaking of electrons, resulting in further damage and deletions (32). Although complexes I, III and IV are encoded by the mitochondrial genome, complex II is exclusively formed of nuclear-encoded proteins (42).

ATP generation is required for cell turnover, which is defined as the balance between cell proliferation and death. The average lifespan of different cell types within the body varies; for example, epidermal cells rely on a high cell turnover and short lifespan, whereas cells within the heart and brain have a low rate of turnover and a much longer lifespan. Studies have shown

that the expression of OXPHOS genes decreases with age in multiple cells and tissues, suggesting that the slowing down of mitochondrial ATP production and cell turnover may be responsible for skin ageing (45). Intrinsic ageing occurs at different rates in all organisms due to the accumulation of ROS, resulting in damage to membranes, enzymes and DNA. As proliferation rates continuously begin to decrease within the epidermis, there is also a steady rate of deterioration of skin function and structure; therefore, resulting in visible signs of skin ageing such as wrinkles. It is suggested that the accumulation of mtDNA damage due to ROS production as well as the slowed cell turnover results in skin ageing (46).

1.2.4 Theories of ageing

The free radical theory of ageing; proposed by Denham Harman, arose in 1954 and remains the most vigorous theory. The theory suggests that organisms age over time due to free radical accumulation; generated during aerobic respiration, resulting in cumulative damage to cells. The concept of endogenous oxidants was initially controversial; however, the identification of SOD which is responsible for the removal of $O_2^{\cdot -}$, provided support for the theory (47-49). In 1978, Harman extended the theory to the mitochondrial theory of ageing, which implicated mitochondria in the production of ROS and remains the most widely accepted. As previously mentioned, the mitochondrial genome is located within the matrix; in close proximity to the site of ROS production, forming the basis of the theory (50). Incomplete oxygen reduction within the ETC can result in the generation of $O_2^{\cdot -}$ which subsequently damages the mitochondrial genome. ROS formation is mainly due to the leaking of electrons from complexes I and III of the ETC, which react with molecules to form free radicals (35). The rapid half-life of these free radicals suggests that the damage they cause is often contained within the mitochondria themselves (32). Finally, the theory of a vicious cycle of events; an extension of the free radical theory, suggests that increased ROS generation leads to mtDNA damage, which subsequently results in mitochondrial dysfunction, resulting in further ROS generation (32). The integrity of mtDNA is essential for mitochondrial function; therefore, mtDNA mutation accumulation is thought to contribute to ROS generation through the leaking of electrons from faulty ETC complexes and lead to further oxidative damage to the mitochondria in a continuous cycle (51). This combined with the lack of protective histones in mtDNA make it vulnerable to damage; including UVR-induced damage, as well as a sensitive biomarker for ageing, pioneered by multiple groups including the Birch-Machin laboratory (50, 52).

1.2.5 Telomere length shortening

Telomeres are repetitive nucleoprotein complexes that protect the terminal regions of eukaryotic chromosomes and are important for a long lifespan. They shorten with each cycle of cell division and provide a counting mechanism to limit the number of times a cell can divide, acting as a protective mechanism against the accumulation of genomically unstable cells and cancer initiation (53-55). Telomere length shortening limits cell proliferation through the activation of checkpoints that induce replicative senescence or apoptosis (54). It is also thought that oxidative stress and the presence of ROS are likely to contribute to telomere shortening, as well as environmental factors and genetic background (55, 56).

Telomerase; a ribonucleoprotein (RNP) enzyme, is required to enhance the replicative capacity of regenerative cells such as stem and progenitor cells through the synthesis of six-nucleotide repeats, and subsequently telomere elongation. This provides a telomere maintenance mechanism for approximately 90% of cancers which activate telomerase to catalyse the synthesis of telomeric repeats (53, 54). Previous studies have associated telomere length with a number of age-related outcomes and it is hypothesised to be a quantitative indicator of ageing; however, the exact relationship between telomere length and ageing is not fully understood. Previous research investigating the link between mtDNA damage, telomere length and ageing has shown a positive correlation between telomere length and mtDNA copy number, suggesting that both are involved in the ageing process and that both theories of ageing are connected (55, 56).

1.3 Extrinsic skin ageing

Although the formation of ROS is a natural process during OXPHOS, environmental and lifestyle factors can also increase ROS levels and cause mtDNA damage, resulting in photooxidative damage and subsequently skin ageing (Figure 1.7). The human skin is constantly exposed to damaging environmental stressors such as UVR and pollution; known as extrinsic stressors, due to being the outermost barrier between the body and the environment. Extrinsic stressors have the ability to form ROS in amounts that overwhelm the skins natural antioxidant defence system, resulting in oxidative damage (16, 32).

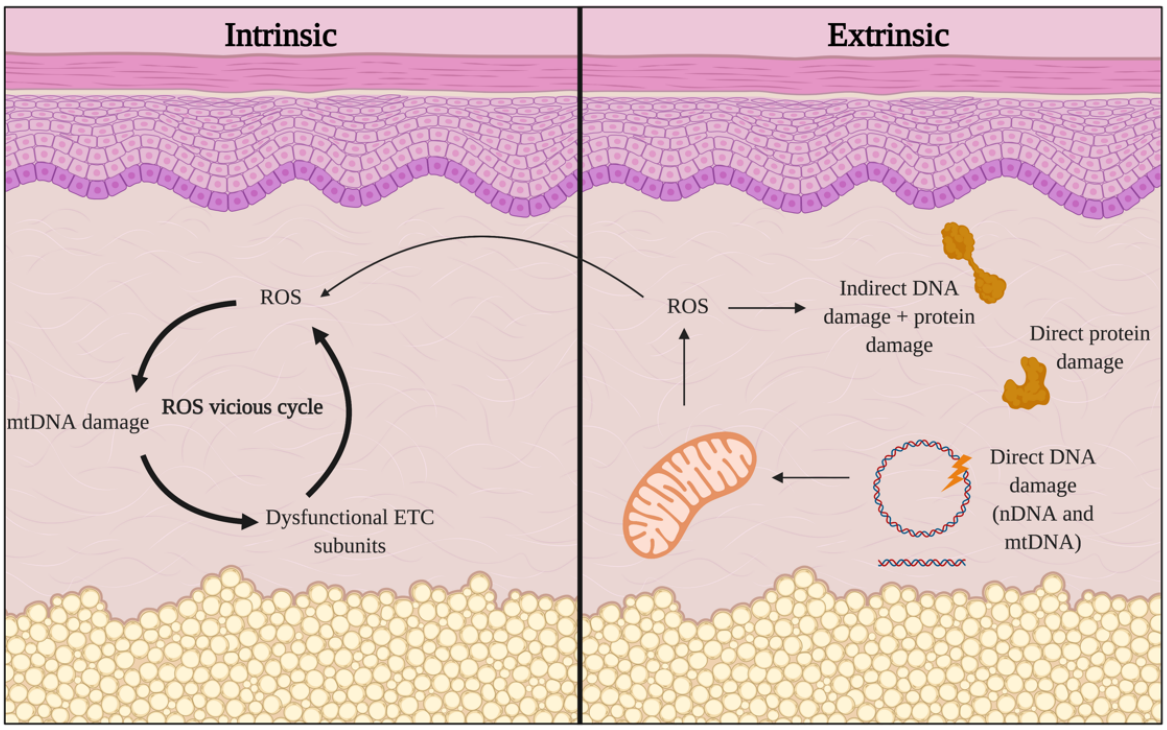


Figure 1.7 Schematic of the contribution of intrinsic and extrinsic factors on skin ageing.
ROS are produced intrinsically within mitochondria by the vicious cycle of events theory. This results in mtDNA damage, dysfunctional ETC subunits and subsequently the production of ROS which leads to further mtDNA damage. Extrinsic factors such as exposure to UVR also contribute to skin ageing through direct damage to DNA. This results in faulty mitochondrial ETC complexes and therefore the production of ROS due to the leaking of electrons, which can result in indirect DNA and protein damage. Direct protein damage can also occur. Made using Biorender.com

1.3.1 Ultraviolet radiation

Although only 5% of the electromagnetic spectrum is UVR, it has been widely reported to affect the skin and is responsible for approximately 90% of visible skin ageing. There are three types of UVR: UVA (320-400nm), UVB (280-320nm) and UVC (110-280nm), with approximately 95% of the UVR reaching the earth's surface UVA and the remainder UVB (15, 32). Studies have linked UVR with DNA damage, oxidative stress, inflammation and suppression of the immune response in exposed skin (32).

UVA is able to penetrate both the epidermis and dermis and can therefore interact with both keratinocytes and fibroblasts and is mainly responsible for photoageing. It causes an upregulation in matrix metalloproteinases (MMP), which results in degradation of the dermal extracellular matrix including collagens and elastin (32). It is thought that UVA causes damage indirectly through the generation of ROS, which has been shown to result in mtDNA damage as well as lipid peroxidation and the activation of transcription factors. As previously described, the generation of ROS and subsequent mtDNA damage will result in reduced OXPHOS

capacity due to the production of faulty mitochondrial ETC complexes; therefore, accelerating ROS production due to electron leakage (15, 57, 58).

Although UVB is only able to penetrate the epidermis, it is more harmful to the skin as it has the most mutagenic and cytotoxic wavelength range which results in erythema, burning and the development of skin cancer. It causes DNA damage in the keratinocytes and melanocytes and activates proteolytic enzymes which results in further damage. The absorption spectrum for DNA has a peak of around 260nm, but it can absorb UVB and to a lesser extent UVA (32). UVB is also able to generate ROS; but to a much lesser extent than UVA, and mainly exerts its effects through direct interaction with DNA (57, 58). ROS generation as a result of UVA and UVB requires the absorption of photons by endogenous photosensitiser molecules. When the photosensitiser becomes excited it is able to react with oxygen, which results in the generation of ROS including $O_2^{-\cdot}$ and singlet oxygen ($^1O_2$). Both are also produced by neutrophils which are increased in photodamaged skin. SOD converts superoxide to H_2O_2 which is able to cross cell membranes easily and drives the generation of the highly toxic $^{\cdot}OH$. Both singlet oxygen and $^{\cdot}OH$; generated from O_2^-, can then cause cellular damage such as lipid peroxidation of cellular membranes (16). Nuclear DNA acts as a chromophore for UVB by directly absorbing the photons, leading to dimeric photoproducts and a wide range of DNA damage including protein-DNA crosslinks, thymine glycol and single strand breaks. The most prevalent photoproducts are cyclobutene pyrimidine dimers (CPD) followed by the pyrimidine pyrimidone (6-4) photoproducts. Other molecules such as urocanic acid act as a chromophore for UVA (32). Finally, UVC is mainly absorbed by the ozone stratosphere and therefore does not reach our skin; however, with ozone depletion, protection against UVC may be required in the future (15, 32). Although UVR results in multiple damaging effects on human skin, it is also responsible for mediating the natural synthesis of vitamin D and endorphins in the skin; therefore, UVR has mixed effects on human skin (20).

1.4 Mitochondrial DNA as a biomarker of exposure to UVR

Multiple studies highlight the damaging effect of sunlight on photoageing, resulting in damage to both mtDNA and nDNA. UVR exposure has been shown to have a negative effect on the mitochondrial genome in the form of the 4799 bp deletion; known as the “common deletion”, 3895 bp deletion and T414G mutation (58-60), which have been proposed as an underlying cause of the ageing process.

The 4977 bp deletion is considered the "common" deletion due to substantial levels of investigation revealing that the deletion is present in several mitochondrial diseases. The deletion is attributed to 12 mitochondrial genes, encoding several subunits of the electron transport chain. This includes 4 genes associated with complex I, 1 gene associated with complex IV, 2 associated with ATP synthase and 5 tRNA genes (61). The existence and relative quantities of the 4977 bp deletion was first examined by Cortopassi *et al.* (1992), showing that mtDNA damage accumulation in ageing individuals is tissue-specific, with a higher level of the deletion present in tissues with low mitotic activity and a high metabolic rate. Muscle and brain tissue were found to have high levels of the deletion, which is thought to be due to their high energy demands (62). A subsequent study by Pang *et al.* (1994) demonstrated the existence of multiple mtDNA deletions in human skin tissues obtained from different body sites. In keeping with previous findings, the results show that the common age-related 4977 bp deletion was present in different proportions in the different tissues examined; therefore, providing evidence that mtDNA damage in ageing individuals is tissue specific and dependent on cell turnover. Slower growing tissue was found to have higher levels of damage in comparison to tissue growing more quickly; assumed to be due to damage accumulation, and later supported by other studies (62-64). It was also found that sun-exposed skin had high levels of the deletion in comparison to chronologically aged tissues (64). Leading on from this, Birch-Machin *et al.* (1998) showed a significantly higher level of the deletion in sun-exposed body sites in comparison to sun-protected sites (52). Furthermore, high levels of the deletion were found exclusively in the dermis, in comparison to the epidermis, which was later supported by other studies, suggesting that it is due to the slow turnover rate of cells in the dermis (52, 62, 65). There also appeared to be no relationship between deletion frequency and age, suggesting that mtDNA deletions reflect photoageing rather than chronological ageing (52).

The presence of the 3895 bp deletion has been shown to increase following UVR-exposure. The deletion is present within the minor arc region of the mitochondrial genome, a region that does not harbour as many deletions as the major arc (60, 66). The regions of the genome deleted are from the mtTF1 binding site in the D-loop to tRNA methionine. Deleted genes also include 12s rRNA, 16s rRNA, ND1 and also the promoters for the transcription of both the heavy and light strands (60). Research has shown a significantly higher level of the deletion in areas that are usually sun-exposed, in comparison to those that are occasionally sun-exposed in both the epidermis and dermis. Furthermore, the deletion was not detected in body sites that are rarely exposed, indicating that deletion variation is a result of photoageing as opposed to chronological ageing. Although the deletion was found to be present in both the epidermis and dermis, the frequency of occurrence was higher in the dermis, which was assumed to be due to the low

turnover rate (60). A later study furthered this work by using quantitative real-time polymerase chain reaction (qPCR) to investigate the actual level; as opposed to the frequency of occurrence, of the deletion in human skin. Findings were in line with previous results, showing a significantly higher level of the deletion in usually sun-exposed skin (66). The two previous studies rely on invasive methods of obtaining skin samples; therefore, a later study by Harbottle *et al.* (2010) aimed to extend previous methodology by developing a less invasive method of obtaining skin samples, which involved the use of a skin swab to collect mtDNA from the epidermis. The results are in line with previous findings and show an increased level of the 3895 bp deletion with increasing sun exposure; however, previous findings were extended as results show an increase in the deletion with sun exposure in the epidermis despite the high turnover rate which would usually keep levels to a minimum. This suggests that the skin swab technique can indicate recent UVR exposure rather than long-term damage. The did not show a trend with age, similarly suggesting that the swabs are measuring recent UVR exposure. Full skin thickness needle biopsies were also used to obtain both epidermis and dermis from the eyebrow and results showed an increasing level of 3895 bp deletion with age, a contradicting result to that shown by the skin swabs which harbour only the epidermis. It was suggested to be due to the low proliferation rate of fibroblasts within the dermis; therefore, resulting in deletion accumulation (50). Furthermore, the concentration of antioxidants has been shown to be higher in the epidermis which may contribute to the results observed (67).

A T414G transversion mutation has also been identified as a mtDNA biomarker for skin ageing. The mutation is considered the most prevalent control region mutation in fibroblasts, occurring in mtDNA promoter regions for RNA primer synthesis of mtDNA heavy strand synthesis and light strand transcription (59). The mutation has been correlated with intrinsic ageing but is also found in Alzheimer's disease and other age-related diseases (8). Studies have shown an accumulation with age within the dermis. As well as this, a significantly greater incidence of the mutation was observed in skin from sun-exposed sites; therefore, indicating that UVR exposure accelerates the accumulation of the ageing-depending mtDNA mutation in human skin. The study also discovered a frequent genetic linkage between the common photoaging-associated 3895 bp deletion and the T414G mutation and found that one of the breakpoints of the 3895 bp deletion lies close to the T414G mutation (59). A later study aimed to investigate the phenotypical importance of the T414G mutation within the control region of mtDNA, as it has been shown to accumulate in both chronologically and photoaged human skin. Overall, the authors proposed that this particular mutation may have little effect on ROS production and the onset of cellular senescence in cultured fibroblasts (68).

Overall, skin ageing is a complex process which results from both intrinsic and extrinsic factors. As previously mentioned, intrinsic ageing is genetically predetermined; whereas, extrinsic ageing is caused by environmental influences and is characterised by deep wrinkles rough texture and irregular pigmentation. Research has shown that mitochondria play a key role in the ageing process which is described by multiple theories; however, the exact role of mitochondria in skin ageing has not yet been determined. Mitochondria are thought to be the main source of ROS production, which are formed as a by-product of OXPHOS. Although ROS have physiological roles within the body, research shows that they are also responsible for causing mtDNA damage due to its close proximity to the site of ROS production within the mitochondria. The integrity of mtDNA is essential for mitochondrial function; therefore, the accumulation of mutations results in faulty mitochondrial subunits, increased electron leak and further ROS production. ROS as a result of extrinsic stressors such as UVR exposure can also contribute mtDNA damage and therefore the production of faulty mitochondrial subunits (51).

1.5 General aims

Overall, this research project aimed to investigate mtDNA damage variation as a result of UVR exposure between individuals, as well as optimise methodology, determine whether sun exposure habits changed during lockdown as a result of COVID-19 and test natural compounds for their protective effects against UVR.

The first aim of this research project was to investigate the biological and basic scientific understanding of mtDNA variation between individuals, and whether this can be accurately measured using a skin swab technique. It is widely accepted that individual behaviours and attitudes will affect biological principles; therefore, we aimed to investigate the effect it has on skin ageing. COVID-19 has had major effects in health care including increases in stroke and myocardial infarction rate, death tolls and skin cancer diagnosis. This led us on to aim II, which investigated the impact of COVID-19 lockdown on sun exposure and behaviours within the UK, due to the heatwave experienced by the UK in March 2020. Finally, the third aim was to investigate natural compounds as protection methods, and therefore explored the protective effects of caffeine and forskolin in the presence of solar simulated light.

Chapter 2. General Materials and Methods

2.1 Skin swabs

2.1.1 Sample collection

In house optimisation was performed previously to determine the most effective swab to use. Sample sites were first cleaned using an ethanol wipe before using a cotton swab (Deltalab, Europe) to collect skin cells from the epidermis of the sample site (Figure 2.1). Swabs were dipped into skin swab buffer (Isohelix, UK) prior to sample collection to help prevent DNA degradation before DNA extraction. Sample sites chosen were inner arm, nose, left cheek and right cheek.

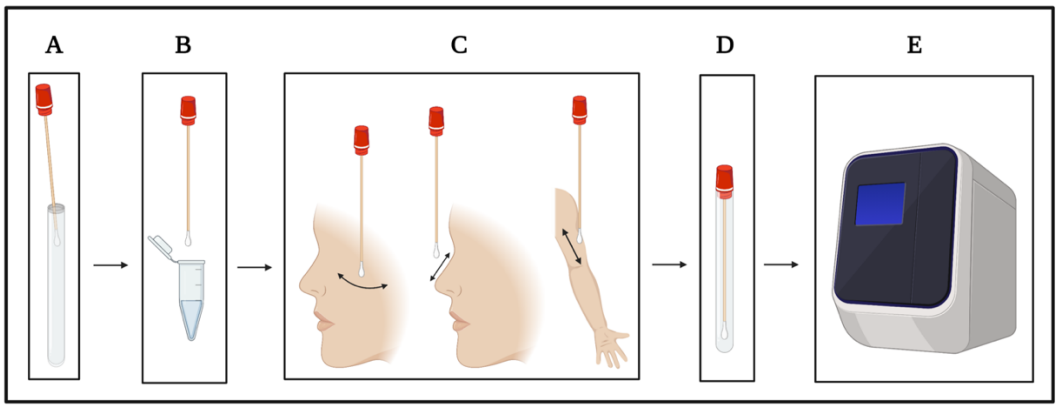


Figure 2.1 Skin swab collection and analysis methodology.
(A) Swab removed from sterile plastic tube. (B) Swab dipped in skin swab buffer. (C) Sample site cleaned with ethanol wipe and swabbed 30 times (each back and forth movement equates to 1 swab). (D) Swab returned to sterile plastic tube. (E) Swab sent to laboratory for DNA extraction and qPCR analysis. Image created using Biorender.com.

2.1.2 DNA extraction from skin swabs

DNA was extracted from cotton swabs using the QIAamp DNA Mini Kit (QIAGEN, Europe) or the BuccalPrep Plus DNA Isolation Kit (Isohelix, UK), as per the manufacturer's instructions with the following modifications:

Modifications made to the QIAamp DNA Mini Kit protocol (DNA purification from buccal swabs) are as follows:

1. Following addition of 400µl PBS, 20µl proteinase K and 400µl buffer AL, samples were vortexed for 60 seconds and incubated at 56°C for 30 minutes.

2. Volume of buffer AE added was reduced to 60µl and samples were incubated in buffer AE at room temperature for 20 minutes.

Modifications made to the BuccalPrep Plus DNA Isolation Kit protocol are as follows:

1. Volume of TE solution was reduced to 60µl and samples were incubated in TE solution at room temperature for 20 minutes.

2.2 DNA quantification and storage

Total cell DNA concentration was quantified using a Nanodrop ND-100 (Nanodrop technologies, UK) by pipetting 1µl of each DNA sample onto the spectrophotometer. A_{260}/A_{280} and A_{260}/A_{230} ratios were assessed according to manufacturer's protocol to ensure that the DNA was pure with no contaminants. DNA obtained from skin swabs was analysed without quantification as the concentration obtained was too low to be accurately determined. DNA was stored at 4°C for same day qPCR analysis or -20°C for long term storage.

2.3 Quantitative real-time PCR

Throughout this PhD, 83 bp, 1 kb and 11 kb qPCR assays were used (Figure 2.2). The StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA) and QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, USA) were used to amplify 83 bp and 1 kb PCR products. The 11 kb PCR product was amplified using the StepOnePlus™ Real-Time PCR System. Optimisation was performed to ensure that both machines functioned in the same way and generated the correct PCR product for the 83 bp and 1 kb qPCR assays (Figure 2.3). Each study was completed using the same PCR system. SYBR® Green chemistry was used for all qPCR reactions (Figure 2.4).

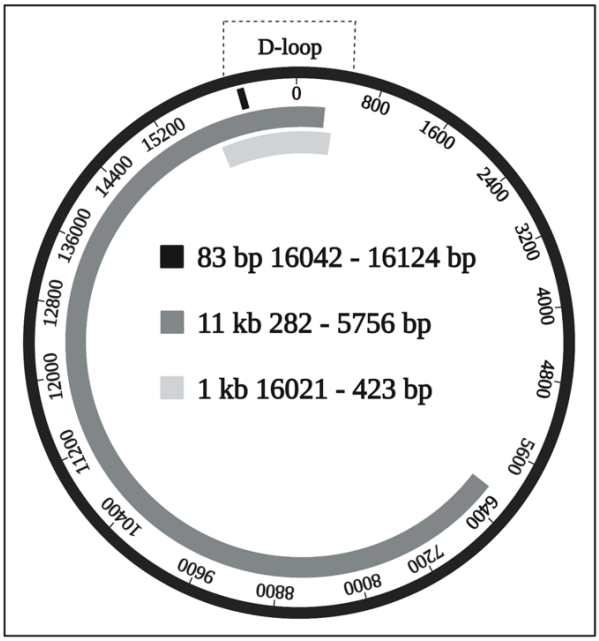


Figure 2.2 Amplification regions of the 83 bp, 1 kb and 11 kb qPCR assays within the mitochondrial genome.
83 bp primers bind to and amplify 16042-16234 bp, a region within the D-loop. 1 kb primers bind to and amplify 16021-423 bp, amplifying the majority of the D-loop (16024-576 bp). 11 kb primers bind to and amplify 282 – 5756 bp. Image created using Biorender.com.

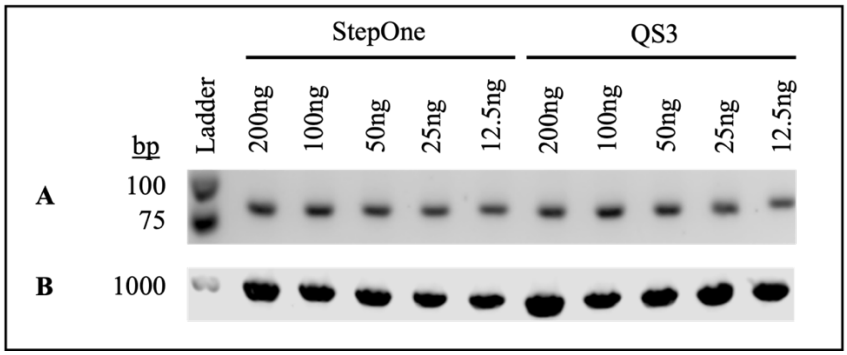


Figure 2.3 Gel confirmation of 83 bp and 1 kb PCR products amplified using the StepOnePlus Real-Time PCR system and QuantStudio 3 Real-Time PCR system.
Cellular DNA was diluted using a serial dilution. Standards were amplified with the 83 bp and 1 kb assay on both PCR systems to generate a standard curve. (A) Bands at 83 bp confirm that the correct PCR product has been generated on both the StepOnePlus Real-Time PCR system and QuantStudio 3 Real-Time PCR system. (B) Bands at 972 bp confirm that the correct PCR product has been generated on both the StepOnePlus Real-Time PCR system and QuantStudio 3 Real-Time PCR systems.

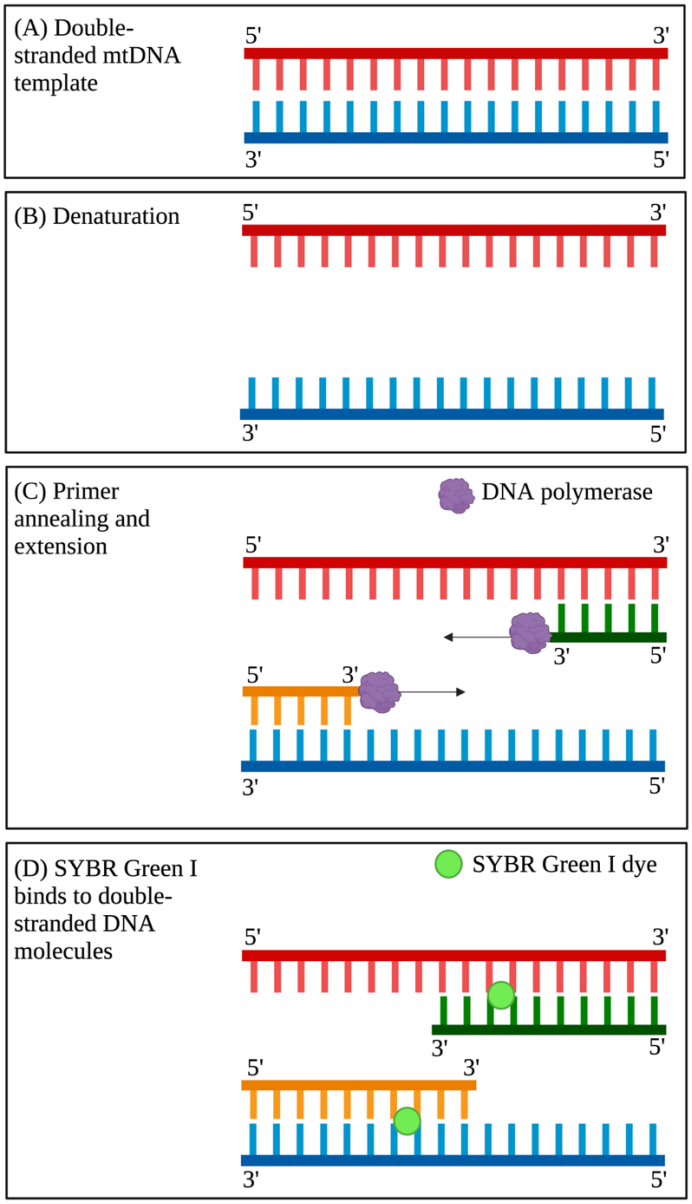


Figure 2.4 DNA amplification and detection through qPCR using SYBR® Green I.
(A) Double-stranded target DNA (dsDNA) is added to the reaction mix along with DNA polymerase, nucleotides and primers that are complementary to the target DNA sequence. (B) The solution is heated and dsDNA is denatured, separating the two strands. (C) The solution is cooled and primers anneal to the target sequences within the two separated DNA strands. DNA polymerase forms a new strand by extending the primers with nucleotides, creating a complementary copy of the target DNA sequence. When repeated, this cycle of denaturing, annealing and extending increases the number of target DNA sequences exponentially. (D) SYBR Green I binds to the minor groove of dsDNA. As SYBR Green binds to dsDNA, the intensity of the fluorescence increases. Image created using Biorender.com.

2.3.1 83 bp amplicon

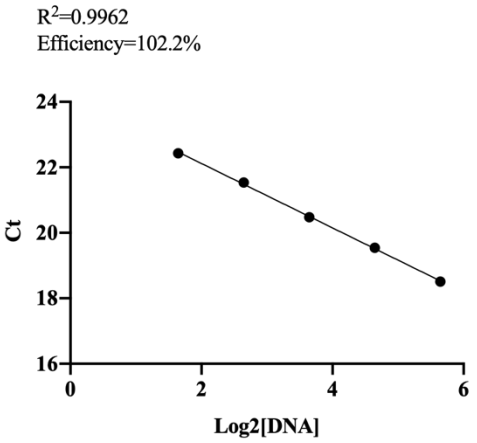
An 83 bp region known as the "housekeeping region" of the mitochondrial genome was amplified to quantify total mtDNA within each sample (Figure 2.2). Primers bind and amplify

16042-16234 bp, a region within the mitochondrial D-loop. Quantification using a spectrophotometer is not accurate as it measures total DNA concentration. The relative mtDNA copy number was determined by the number of cycles required for the level of amplified product to reach the threshold, known as the cycle threshold (Ct) value. Fewer number of cycles indicates a higher concentration of mtDNA within the sample as there is more DNA available to be amplified.

83 bp regions of the mitochondrial genome were amplified in a 25µl reaction. SYBR Green binds to double stranded DNA and fluoresces relative to a passive ROX reference dye. Each sample was assayed in triplicate, with a standard deviation threshold of ≤ 0.3 Ct. The threshold was set to automatic threshold. Melt curve analysis was used to determine product size, with a melt temperature of 79-90°C confirming amplification of the correct PCR product (Figure 2.5B), as well as a band at 83 bp on an agarose gel (Figure 2.3A). Master mix composition, amplification settings and primer sequences are reported by Hanna *et al.* (2019) (69). When performing the 83 bp assay using the QuantStudio 3 Real-Time PCR system, the ROX concentration was reduced to 0.1X due to increased sensitivity of the system. Reaction efficiency was calculated to be 102.2% with cellular DNA (Figure 2.5A).

A

DNA concentration/well (ng)	Log2[DNA]	Ct
50	5.643856	18.50876
25	4.643856	19.54292
12.5	3.643856	20.47615
6.25	2.643856	21.53835
3.125	1.643856	22.43038



B

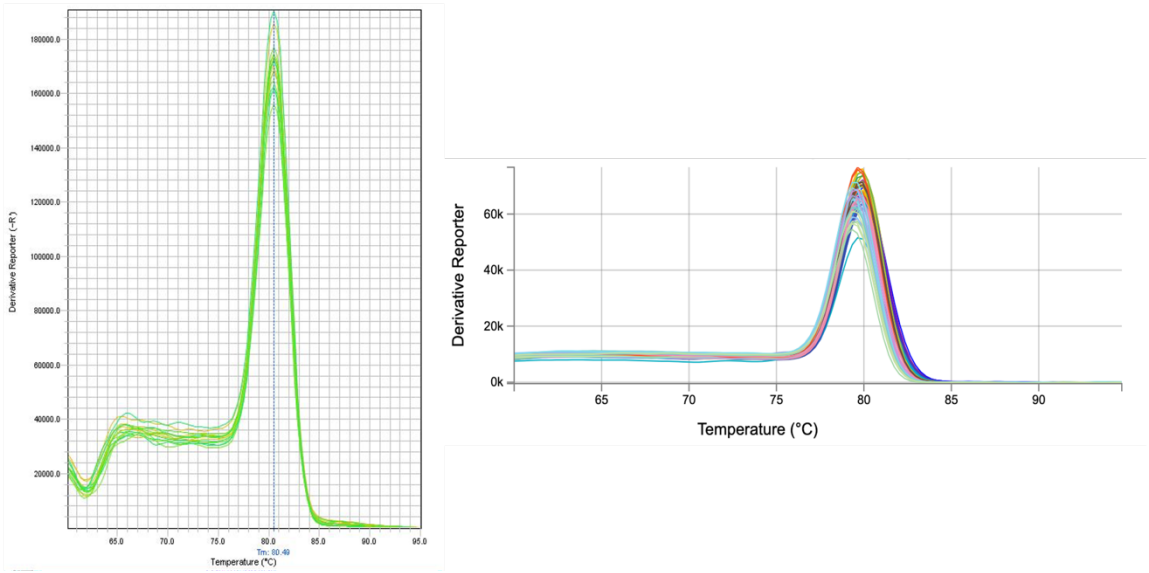


Figure 2.5 83 bp standard curve and melt curve plots.
(A) Standard curve generated with cellular DNA to determine reaction efficiency, with R^2 and reaction efficiency presented. Standards were generated using a serial dilution and show a difference of 1 Ct. (B) Melt curve analysis confirms the presence of the 83 bp PCR product on both the StepOnePlus Real-Time PCR system and QuantStudio3 Real-Time PCR system, with no evidence of non-specific binding or primer dimer.

2.3.2 1 kb amplicon

A 972 bp region of the mitochondrial genome was amplified to quantify damage within that region in the form of strand breaks (Figure 2.2). Primers bind and amplify 16021-423 bp which is the majority of the mitochondrial D-loop. A high Ct value corresponds to a large amount of mtDNA damage within a sample as there is less intact mtDNA that can be amplified and therefore requires more cycles to reach the cycle threshold.

1 kb sections of the mitochondrial genome were amplified in a 20µl reaction. SYBR Green binds to double stranded DNA and fluoresces relative to a passive ROX reference dye. Each sample was assayed in triplicate with a standard deviation threshold of ≤ 0.3 Ct. The threshold was set to automatic threshold. Melt curve analysis was used to determine product size, with a melt temperature of 83-84°C confirming that the correct product had amplified (Figure 2.6B) as well as a band at 1000 bp on an agarose gel (Figure 2.3B). Master mix composition and amplification settings were adapted from those reported by Rothfuss *et al*. (2010) and primer sequences were reported by Rothfuss *et al*. (2010) (70). SensiMix Hi-ROX (Scientific Laboratory Supplies, UK) was used with the StepOnePlus Real-Time PCR System and SensiMix Low-ROX (Scientific Laboratory Supplies, UK) was used with the QuantStudio3 Real-Time PCR system. Reaction efficiency was calculated to be 93.1% with cellular DNA (Figure 2.6A).

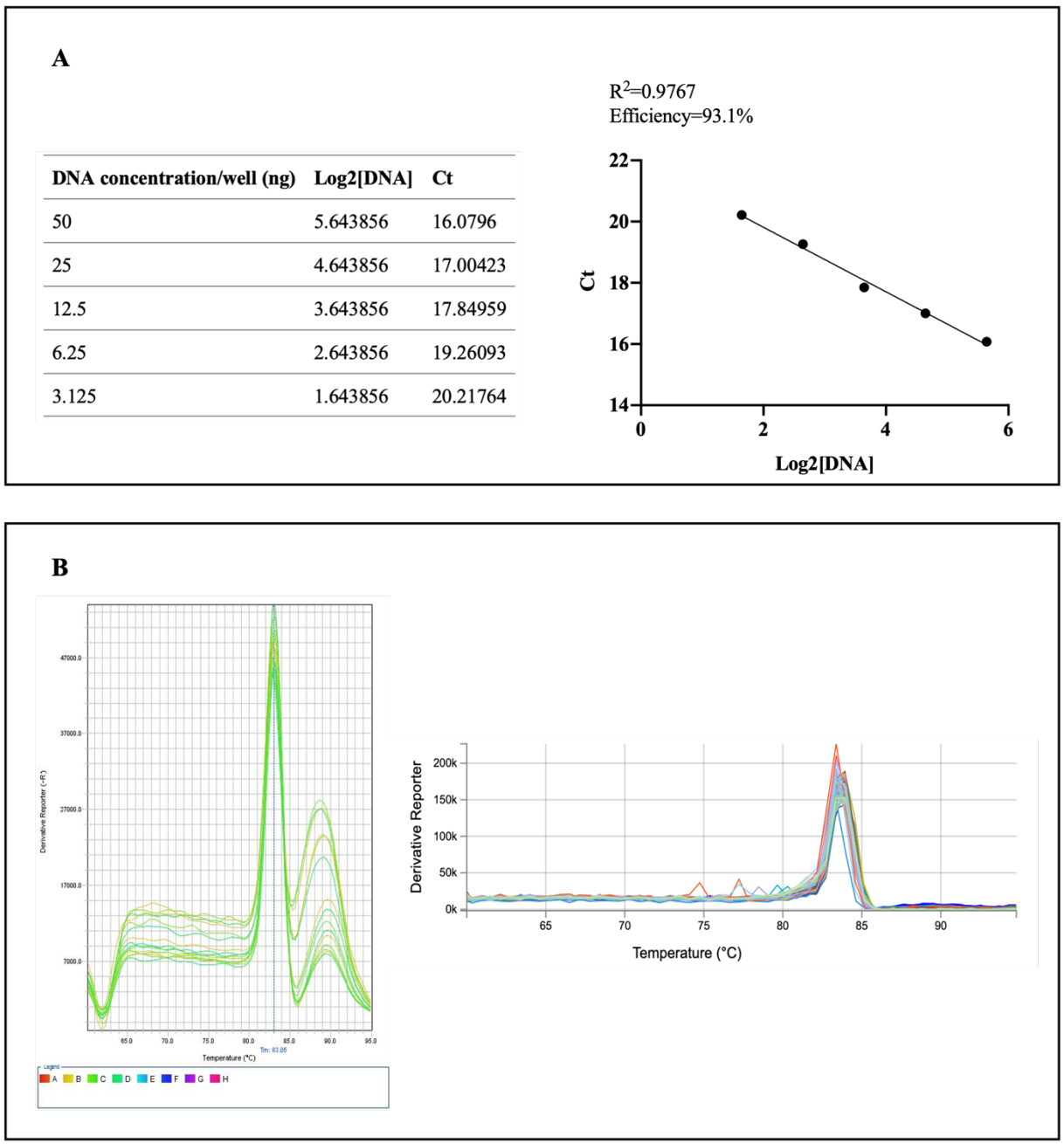


Figure 2.6 1 kb standard curve and melt curve plots.
(A) Standard curve generated with cellular DNA to determine reaction efficiency, with R^2 and reaction efficiency presented. Standards were generated using a serial dilution and show a difference of 1 Ct. (B) Melt curve analysis confirms the presence of the 83 bp PCR product on both the StepOnePlus Real-Time PCR system and QuantStudio3 Real-Time PCR system, with no evidence of non-specific binding or primer dimer.

2.3.3 11 kb amplicon

An 11,096 bp region of the mitochondrial genome, extracted from cells, was amplified to quantify damage within that region in the form of strand breaks (Figure 2.2). Primers bind and amplify 282-5756 bp which contains 11 of 13 mitochondrial genes which encode polypeptide components of the mitochondrial electron transport chain (69). A high Ct value corresponds to

a large amount of mtDNA damage within a sample as there is less intact mtDNA available to be amplified, therefore more cycles are required to reach the threshold.

11 kb sections of the mitochondrial genome were amplified in a 20µl reaction. SYBR Green binds to double stranded DNA and fluoresces relative to a passive ROX reference dye. Each sample was assayed in triplicate with a standard deviation threshold of ≤ 0.3 Ct. The threshold was set to automatic threshold. Melt curve analysis was used to determine product size, with a melt temperature of 84°C confirming that the correct product had amplified (Figure 2.7A), as well as a band at 11,000 bp on an agarose gel (Figure 2.7B). Master mix composition, amplification settings and primer sequences are reported by Hanna *et al.* (2019) (69).

Following the 83 bp mtDNA quantification assay, samples were diluted further if their Ct values weren't within 1 Ct to ensure that the same amount of mtDNA was added to the subsequent 11 kb assay. A difference of 1 Ct between samples represents a two-fold difference in the amount of mtDNA; therefore, the samples were diluted by a factor of $2^{\text{Difference in Ct}}$.

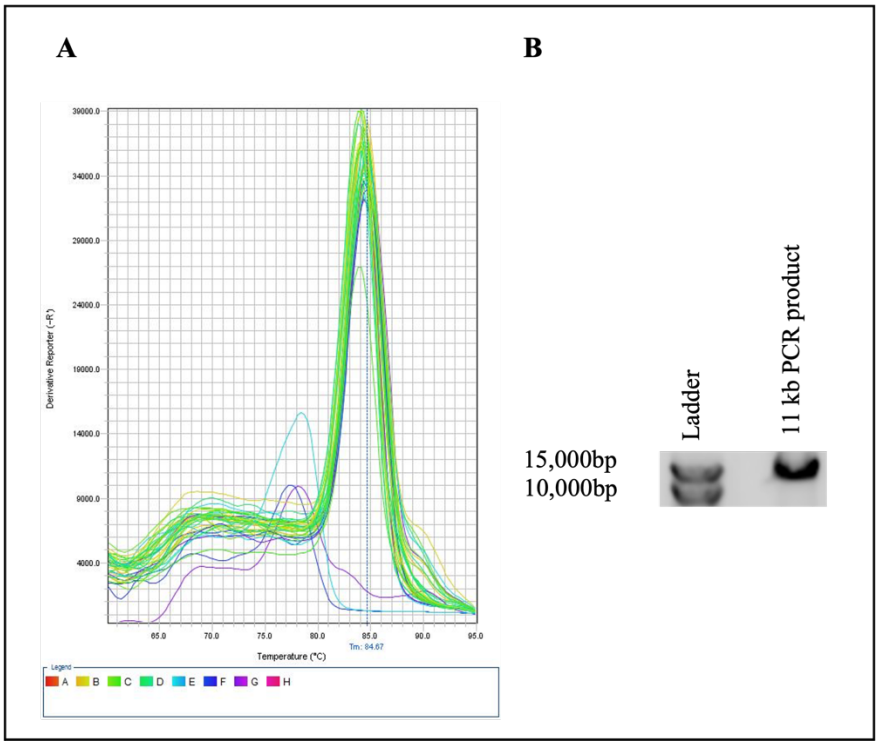


Figure 2.7 11 kb melt curve plot and agarose gel confirmation.
(A) Melt curve analysis confirms the presence of the 11 kb PCR product on the StepOnePlus Real-Time PCR system, with no evidence of non-specific binding or primer dimer. 3 individual peaks show negative control wells. (B) Agarose gel confirmation of correct PCR product.

2.4 Gel electrophoresis

2.4.1 83 bp products

A 4% w/v agarose (Merck, USA) gel in 1X TBE stained with 1X GelRed nucleic acid stain (Biotium, USA) was prepared. Amplified 83 bp products were loaded and run alongside a Low Molecular Weight DNA ladder (New England Biolabs, USA) or Ultra Low DNA ladder (Thermo Fisher Scientific, USA), according to manufacturer's guidelines. Electrophoresis was performed in 1X TBE at 80V for approximately 1.5 hours. The gel was imaged using a LI-COR Odyssey Fc (LI-COR, USA).

2.4.2 1 kb and 11 kb products

A 0.8% w/v agarose (Merck, USA) gel in 1X TAE stained with 1X GelRed Nucleic acid stain (Biotium, USA) was prepared. Amplified 1 kb and 11 kb products were loaded and run alongside a GelPilot High Range Molecular DNA Ladder (QIAGEN, Europe) or Hyperladder 1 (Bioline, Europe) according to manufacturer's guidelines. Electrophoresis was performed in 1X TAE at 100V for approximately 1.5 hours. The gel was imaged using a LI-COR Odyssey Fc (LI-COR, USA).

Chapter 3. Optimisation of skin swab methodology

3.1 Introduction

This chapter aimed to optimise methodology used to normalise mtDNA content within samples, as well as skin swab collection techniques. During the studies performed throughout this PhD, problems were encountered with normalisation methodology. The fold change method ($2^{-\Delta\Delta Ct}$) was initially used to normalise for mtDNA content; however, an internal control was required. Issues were discovered with the use of the inner arm as an internal control in initial studies (Section 4.3.1), such as extremely high levels of mtDNA damage as well as low mtDNA content, and samples frequently failing to amplify with qPCR. As well as this, fold change is a measure of relative qPCR and assesses the abundance of target DNA in relation to a control without showing their actual abundances, and the comparison can only be done for samples run within the same qPCR reaction. In comparison, absolute qPCR allows the precise quantification of target DNA based on a standard curve constructed in the same quantification assay as the samples. The standard curve is generated by amplifying a dilution series of standard DNA; such as plasmid DNA, carrying the target DNA. Plasmid DNA, particularly uncut and circular, is the most common choice due to its stability and reproducibility (71). We set out to develop a plasmid which contained both the 83 bp and 1 kb regions of interest, which could subsequently be used to determine the percentage of DNA damage within samples. This would also eliminate the need for an internal control collected from a sun-protected site.

Optimisation was initially performed to determine differences in mtDNA content between different sample sites using the skin swab technique, as well as to determine the importance of normalisation between samples. It is widely accepted that there are approximately 100-1000 mitochondria within the cytoplasm of a eukaryotic cell, varying depending on metabolic demand (32); therefore, it was hypothesised that mtDNA content would differ between sample sites, as well as with swab pressure. A buffer was also tested with the aim of increasing the number of cells obtained using the swab and therefore mtDNA content within samples, particularly the arm, due to the lack of amplification of some samples as a result of low mtDNA concentration in initial studies.

As well as the use of qPCR to determine mtDNA damage as a result of extrinsic influences such as UVR exposure, skin analysis instruments can also be used to measure alterations in pigmentation through the use of UV light (72). Although studies have shown sun damage detected by skin analysis instruments to correlate with phenotypic melanoma risk factors, it is unknown how reliable and reproducible data obtained from such instruments is. We aimed to

determine the consistency of readings generated by the VISIA® Skin Analysis system by collecting consecutive images.

Specific aims are as follows:

1. Investigate mtDNA content in different sample sites using a skin swab technique, including preliminary testing in different individuals
2. Determine whether the use of a skin swab buffer increases mtDNA content within samples
3. Develop a plasmid which contains 83 bp and 1 kb mitochondrial regions of interest to generate a standard curve and optimise normalisation methodology
4. Investigate consistency in results obtained from the VISIA® Skin Analysis system

3.2 Methods

3.2.1 Generation of 1 kb plasmid

This experiment was planned and designed by Roisin Stout. Laboratory work was performed by Lizzie Ruddy and Gewei Zhu, with the help of Roisin Stout. The aim was to generate a plasmid which contained the 83 bp and 1 kb mitochondrial regions of interest so that unknown sample concentrations could be interpolated from standard curves.

The 1 kb region; more specifically 972 bp, which also contains the 83 bp region, was cloned into a plasmid to overcome the limitations of previous methodology. The final plasmid generated is shown in Figure 3.1.

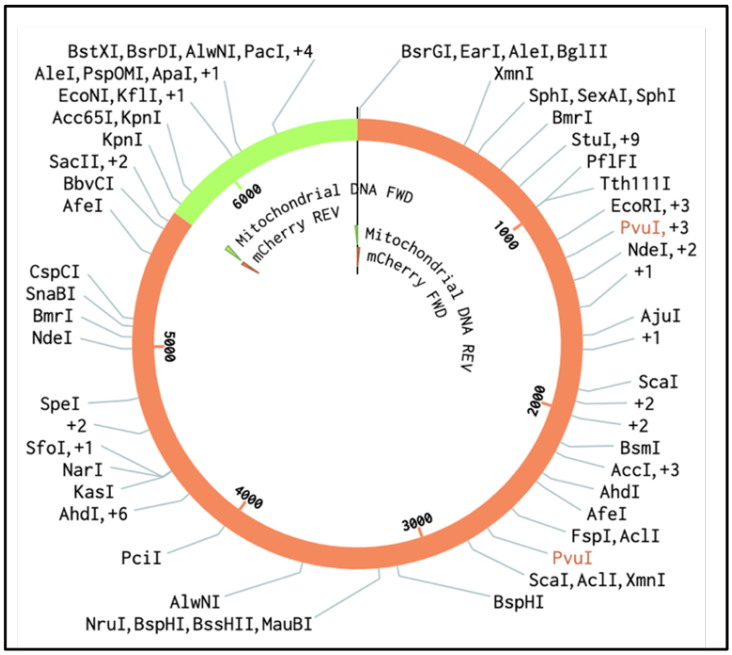


Figure 3.1 Final plasmid containing 83 bp and 1 kb regions of interest.

3.2.1.1 PCR amplification of 1 kb insert

The 1 kb region was amplified in a 50µl reaction to generate the insert with a product size of 972 bp. Master mix composition (Table 3.1) and amplification settings (Table 3.2) are shown below. Primer sequences were modified from Rothfuss *et al* (2010). The master mix was prepared quickly on ice and gently mixed. The PCR was run in a thermocycler (DNA Engine, Europe) and took 1 hour and 40 minutes.

Component	Volume (µl)	Final concentration
5X Q5 reaction buffer (New England Biolabs, USA)	10	1X
10mM dNTPs (Merck, USA)	1	200µM
10µM primer F (Eurofins, Europe)	2.5	0.5µM
10µM primer R (Eurofins, Europe)	2.5	0.5µM
Q5 polymerase (New England Biolabs, USA)	0.5	0.02U/µl
5X GC enhancer (New England Biolabs, USA)	10	1X
DNA	2	60ng
PCR grade H_2O (Thermo Fisher Scientific, USA)	21.5	
Reaction total	50	

Table 3.1 Master mix composition for amplification of the 1 kb insert.

Stage	Temperature (°C)	Time (minutes)	
Initial denaturation	98	3:00	
Denaturation	98	0:10	
Annealing	65	0:30	30 cycles
Extension	72	0:30	
Final extension	72	2:00	

Table 3.2 Amplification settings for the 1 kb insert.

The PCR product was confirmed using a 0.8% agarose gel, run alongside a GelPilot High Range Molecular DNA Ladder (QIAGEN, Europe) (Section 2.4.2). Remaining PCR reagents were removed using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA), following the manufacturer's protocol for extracting the DNA from gel.

3.2.1.2 PCR amplification of vector

The mCherry vector was amplified in a 50µl reaction to generate a product of 5635 bp. Amplification settings (Table 3.3) and primer sequences (Table 3.4) are shown below. The master mix composition used was the same as summarised in Table 3.1 and was assembled quickly on ice. The PCR was run in a thermocycler (DNA Engine, Europe) and took 4 hours. The mCherry vector was a kind gift from Dr Magomet Ausher (IGM, Newcastle University).

Stage	Temperature (°C)	Time (minutes)	
Initial denaturation	98	1:00	
Denaturation	98	0:10	
Annealing	66	0:30	25 cycles
Extension	72	4:37	
Final extension	72	2:00	
Hold	4		

Table 3.3 Amplification settings for the vector.

Name	Sequence
Vector (mCh) forward	5'CTTTTGGCGGTATGCACTTTGGGACTCCAGCCAAAGGCAG 3'
Vector (mCh) reverse	5'GCTTCCCCATGAAAGAACAGGGAGCCACCCTTCGAGTGG 3'

Table 3.4 Primer sequences for the vector.

A digest using PVUI (New England Biolabs, USA) was performed on the vector PCR product at 37°C for approximately 1 hour. Reaction components are present in Table 3.5. The digested products were confirmed on a 0.8% agarose gel (Section 2.4.2), run alongside a GelPilot High Range Molecular DNA Ladder (QIAGEN, Europe). The cut sites within the linear PCR product were at 1261 and 2669, generating 3 products that were 1260 bp, 1408 bp and 2967 bp (Table 3.6). The presence of 3 bands confirmed that the vector had amplified properly, due to the presence of 2 restriction enzyme sites within the linear molecule. Remaining PCR reagents were

removed using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA), following the manufacturer's protocol. Original plasmid was removed by performing a digest with DPNI (Thermo Fisher Scientific, USA) (Table 3.7), following the manufacturer's protocol.

Component	Volume (µl)
Vector PCR product	10
Nuclease-free water	33
NEB buffer (New England Biolabs, USA)	5
PVUI (New England Biolabs, USA)	2

Table 3.5 Reaction components for the digest of the vector PCR product using PVUI.

Start (bp)	End (bp)	Length (bp)
1	1260	1260
1261	2668	1408
2669	5635	2967

Table 3.6 PVUI cut sites of the vector PCR product.

Component	Volume (µl)
Vector PCR product	10
Nuclease-free water	6
Buffer Tango (Thermo Fisher Scientific, USA)	2
DPNI (Thermo Fisher Scientific, USA)	2

Table 3.7 Reaction components for the removal of leftover plasmid from the sample using DPNI.

3.2.1.3 Plasmid assembly

Assembly was performed using the NEBuilder Hifi DNA Assembly Cloning Kit (New England Biolabs, USA), following the manufacturer's protocol. Reaction components are shown below

(Table 3.8). Concentrations of the vector and insert were determined using the Nanodrop ND-100 (Nanodrop technologies, UK).

The number of base pairs for the insert and vector as previously mentioned are 972 bp and 5535 bp, respectively. Equation 3.1 was used to calculate weight in pmoles, which was used to calculate the volume of each that was required to make the required concentrations.

$$pmoles = \frac{(weight\ in\ ng) * 1000}{(base\ pairs * 650\ daltons)}$$

Equation 3.1 Equation used for plasmid assembly.

Components	2-3 fragment assembly
Recommended DNA molar ratio	Vector:insert = 1:2
Total amount of fragments	0.03-0.2pmols Xµl
NEBuilder HiFi DNA Assembly Master Mix	10µl
Deionised H_2O	10-Xµl
Total volume	20µl

Table 3.8 Reaction components for plasmid assembly.

3.2.1.4 Digest of recombinant plasmid and original mCherry plasmid

A digest of both the recombinant plasmid and original mCherry plasmid was performed using both PVUI and PVUII restriction enzymes (New England Biolabs, USA) to confirm that the final plasmid contains both the vector and the insert. The reaction mix is present in Table 3.9 and the digest was run at 37°C for approximately 2.5 hours. The PCR products were confirmed on a 0.8% agarose gel (Section 2.4.2), run alongside a GelPilot High Range Molecular DNA Ladder. Cut sites and product sizes for products generated by PVUI and PVUII digests are present in Table 3.10.

Component	Volume (ul)
Plasmid	5
Nuclease-free water	14.5
R buffer (Thermo Fisher Scientific, USA)	2.5
PVUI (New England Biolabs, USA)	1
PVUII (New England Biolabs, USA)	2

Table 3.9 Reaction components for the PVUI and PVUII digest of the recombinant plasmid and original mCherry.

mCherry				Recombinant plasmid			
Start (bp)	End (bp)	Length (bp)	Enzyme	Start (bp)	End (bp)	Length (bp)	Enzyme
2954	4345	1392	PVUI	1281	2688	1408	PVUI
4346	5753	1408	PVUI	2689	1280	5276	PVUI
5754	2953	3543	PVUII				

Table 3.10 PVUI and PVUII cut sites and product sizes of the recombinant plasmid and original mCherry.
PVUI cuts both mCherry and the recombinant plasmid twice, generating two PCR products; however, PVUII cuts only mCherry due to the lack of restriction enzyme site on the recombinant plasmid.

3.2.1.5 Agar plates and LB broth

Double concentrate agar was made by adding 7.5g agar to 250ml milliQ water, followed by autoclaving. Following autoclave, the agar was kept at 60°C to prevent it from setting. 250ml milliQ water was autoclaved separately, followed by the addition of 0.5ml 100mg/ml ampicillin (Thermo Fisher Scientific, USA), which was then mixed thoroughly with the agar. The plates were then poured next to a flame, left to set for approximately 1 hour, and then stored upside down in the fridge until required.

10g of LB was added to 500ml milliQ water and autoclaved. Once cooled, 0.5ml 100mg/ml ampicillin was added (Thermo Fisher Scientific, USA).

3.2.1.6 Transformation

5-alpha Competent E. coli cells (New England Biolabs, USA) were thawed on ice, followed by the addition of 5µl recombinant plasmid to tube 1, 1µl positive control plasmid to tube 2 (positive control) and no plasmid to tube 3 (negative control). Tubes were flicked 4-5 times to mix cells and plasmid DNA. The cells were placed on ice for 30 minutes, followed by a 42°C heat shock for 30 seconds, and then returned to ice for 5 minutes. 950µl room temperature SOC media (New England Biolabs, USA) was then added to the tubes, which were incubated at 37°C whilst being shaken vigorously (250rpm) for 60 minutes. Dilution tubes were prepared for the recombinant plasmid and positive control plasmid; 7 for each, by adding 900µl LB into each of the tubes. 100µl from cell tube 1 was added to tube 1 of 7 and then vortexed. 100µl from tube 1 was then added to tube 2 and so on, until tube 7 was reached. This same process was repeated for tubes 1-7 for the positive control plasmid. 100µl from tubes 1, 3 and 5 were added to separate petri dishes and the cells were spread carefully over the agar using a cell scraper. The same was done for the positive control plasmid as well as the negative control. The negative control was not diluted. Plates were incubated at 37°C overnight and placed upside down in the fridge the following morning, wrapped in parafilm.

5ml LB and 5µl ampicillin were added to 15ml falcon tubes, followed by a single bacterial colony, transferred using a pipette tip. Tubes were incubated to 37°C overnight and shaken at 250 rpm.

3.2.1.7 Plasmid purification

Plasmid was purified from E. coli using the plasmid mini kit (QIAGEN, Europe), following the manufacturer's protocol.

3.2.1.8 Interpolation of unknowns from plasmid standard curve

Unknown DNA concentrations within samples can be calculated from standard curves generated with plasmid standards that are run alongside samples. Interpolation values from standard curves are obtained as Log10DNA, which are antilogged to give DNA concentration per well.

Calculating DNA amplified with the 1 kb assay as a percentage of the 83 bp assay gives %DNA amplified (Equation 3.2). A higher %DNA amplifies corresponds to a lower level of mtDNA damage, as more intact DNA is available to amplify.

$$\%DNA\ amplified = \left(\frac{DNA\ amplified\ with\ 1\ kb\ (ng/well)}{DNA\ amplified\ with\ 83\ bp\ (ng/well)}\right) * 100$$

Equation 3.2 Calculation of %DNA amplified.

3.2.1.9 Previous methodology for normalisation

Fold change methodology ($2^{-\Delta\Delta Ct}$) (Equation 3.3) was used for initial studies involving the skin swab (Section 4.3.1 and Section 4.3.2). Although this is a robust method that is commonly used for the analysis of qPCR data, it is unclear how good a control the inner arm is due to multiple factors such as skin thickness.

$$Fold\ gene\ expression = 2^{\Delta Ct\ (sample) - \Delta Ct\ (control\ average)}$$

Equation 3.3 Calculation of fold gene expression.
Used for samples collected from the face, using the arm as the control.

3.2.2 93 bp amplicon

The 93 bp qPCR assay was used to amplify the ß2M gene within the nuclear genome in order to quantify nuclear DNA content with samples. Master mix components and cycle conditions are the same as those used for the 83 bp qPCR assay (Section 2.3.1). Primer sequences were designed and reported by Malik *et al*. (2011) (73) and are presented in Table 3.11.

Primer	Sequence (5'-3')	T_m (°C)
Forward hB2M	GCTGGGTAGCTCTAAACAATGTATTCA	79
Reverse hB2M	CCATGTACTAACAAATGTCTAAAATGGT	

Table 3.11 Primer sequences for the 93 bp qPCR assay.

3.2.3 3D full-thickness skin equivalents

3D full-thickness skin equivalents were purchased from Phenion (Germany, Europe) for use in irradiation experiments. Skin equivalents are composed of both primary fibroblasts and primary keratinocytes isolated from human neonatal foreskin specimens and utilise a dermal matrix of bovine cross-linked lyophilised collagen. Skin equivalents were cultured using the individual

equivalent setup with 5ml pre-warmed ALI-Medium each (Table 3.12). Equivalents were maintained at 37°C in 5% CO_2 for 24 hours prior to proceeding with irradiation experiments. Media was replaced every other day. Skin swab and sample collection was performed as described in Sections 2.1.1 and 4.2.4.3, respectively.

Phenion ALI-Medium Components	
DMEM with Glutamax	Hydrocortisone
Ham/F-12 Medium	Insulin
L-Ascorbic acid 2-phosphate	Bovine serum albumin (BSA)
Penicillin/Streptomycin	

Table 3.12 Components of ALI-Medium.

3.2.3.1 H&E staining of 3D full-thickness skin equivalents

Processing, embedding and hematoxylin and eosin (H&E) staining of skin equivalent tissue was conducted by NovoPath (Newcastle, UK). The hematoxylin stains cell nuclei purple/blue and eosin stains the extracellular cytoplasm pink, with other structures taking on different shades of combinations of these colours. Eosin also stains collagens which gives indication of any changes in collagen content.

Control, swab and tape strip skin equivalents were prepared in the same way. After cutting appropriately (Figure 3.2), skin equivalents were placed in 10% neutral buffered formalin for histological staining.

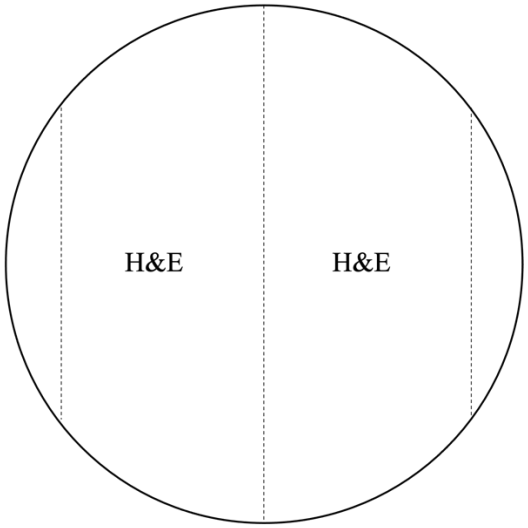


Figure 3.2 Cut sites of 3D full-thickness skin equivalents for H&E staining.
Skin equivalents were cut down the dotted lines. 2 samples for H&E staining were obtained from each skin equivalent.

3.2.4 Facial image collection

A 7th Generation VISIA® Skin Analysis system was used for image capture, with images stored under restricted access in line with the Data Protection Act/General Data Protection Regulation. Subjects placed their chin on the rest and closed their eyes and images were taken of the right, left and front of the face by rotating the VISIA around the subject.

4 consecutive images were taken from the front, left and right side of the face of 2 individuals. Results were saved as feature count, percentile, and score in order to determine relative standard deviation.

3.3 Results

3.3.1 Generation of 1 kb plasmid

Generation of the plasmid was required to provide a reliable method for normalisation of mtDNA content within samples.

3.3.1.1 PCR amplification of vector and insert

Three bands are visible at 1260 bp, 1408 bp and 2967 bp following digestion of the vector PCR product with PVUI; therefore, confirming amplification of the correct PCR product (Figure 3.3A). The 972 bp insert amplified, shown by bands at ~972 bp for both 40ng and 20ng positive control DNA (Figure 3.3B). 80ng and 60ng samples evaporated; hence, the absence of a band on the gel. 60ng was used for subsequent cloning experiments.

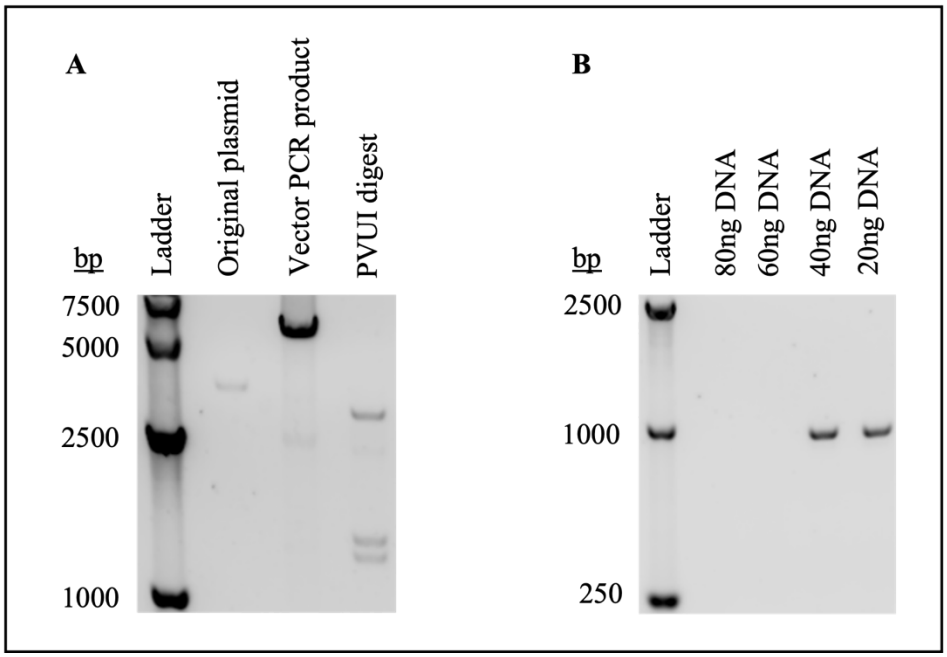


Figure 3.3 Gel confirmation of vector and insert PCR products.
(A) Gel confirmation of original plasmid, vector PCR product and vector PCR product following PVUI digest. 3 bands are visible following the vector digest as the product is linear and contains 2 restriction enzyme sites. Digest was run alongside original plasmid and vector PCR product for comparison. (B) Gel confirmation of insert PCR product. A band is present for reaction mixes containing 40ng and 20ng. Gels were run alongside a GelPilot High Range Molecular DNA ladder.

3.3.1.2 Confirmation of assembly

Two bands are present at 1408 bp and 5276 bp following digestion of the assembled plasmid with PVUI which confirmed that the assembly was successful (Figure 3.4). Bands are visible

at 3543 bp and approximately 1400 bp for the mCherry digest, with the lower band being a combination of 2 products of 1392 bp and 1408 bp. A third product is generated from the original mCherry plasmid due to the presence of the PVUII restriction site, which is not present in the amplified vector and therefore the transformed plasmid. This confirms that the plasmid has been transformed correctly.

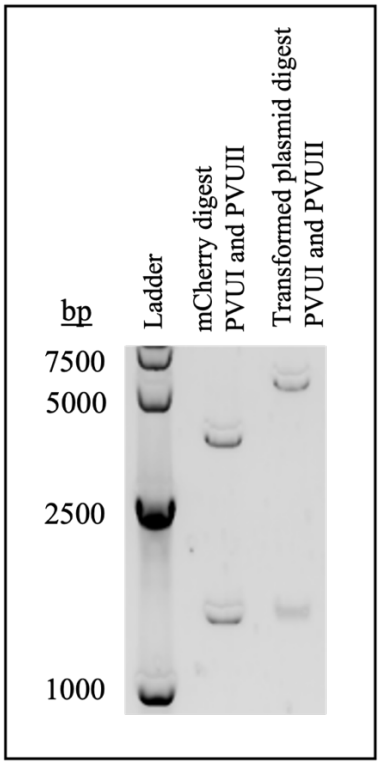


Figure 3.4 Gel confirmation of transformed plasmid.
Transformed plasmid and mCherry were digested with PVUI and PVUII. Gel confirmation of transformed plasmid due to difference in band weight between the final plasmid and original mCherry. An extra band is present due to the presence of the PVUII cut site in the original mCherry plasmid, with the smaller band being a combination of 2 bands.

3.3.1.3 Gel confirmation of 83 bp and 1 kb products generated using the 1 kb plasmid

Gel confirmation of 83 bp (Figure 3.5A) and 1 kb (Figure 3.5B) PCR products. Cell DNA was extracted from human dermal neonatal fibroblasts (HDFn) to use as positive control DNA.

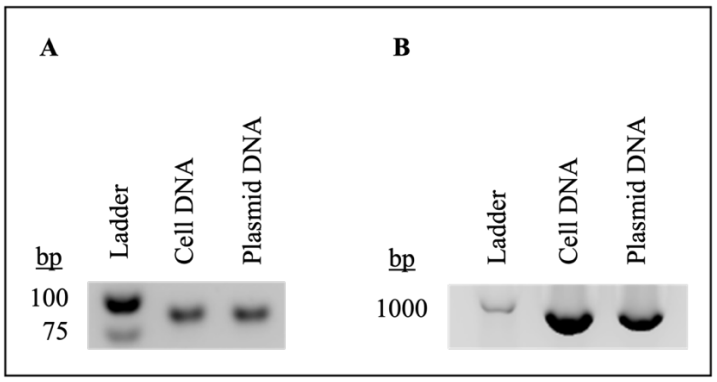


Figure 3.5 Gel confirmation of 83 bp and 1 kb PCR products.
Gel confirmation of (A) 83 bp and (B) 1 kb PCR products. Products were run on an agarose gel alongside a GelPilot High Range Molecular DNA ladder. Cell DNA was extracted from HDFn cells and used as a positive control.

3.3.1.4 83 bp and 1 kb standard curves

Standard curves were generated using plasmid DNA standards and plasmid concentration was interpolated from a standard curve of known cellular DNA concentration (Figure 3.6).

A

DNA/well	Log10[DNA]	83 bp Ct	1 kb Ct
528.060	2.723	14.225	12.714
52.806	1.723	17.909	16.630
5.281	0.723	21.320	20.643
0.528	-0.277	24.873	24.096
0.053	-1.277	27.942	28.098
0.005	-2.277	31.643	31.833

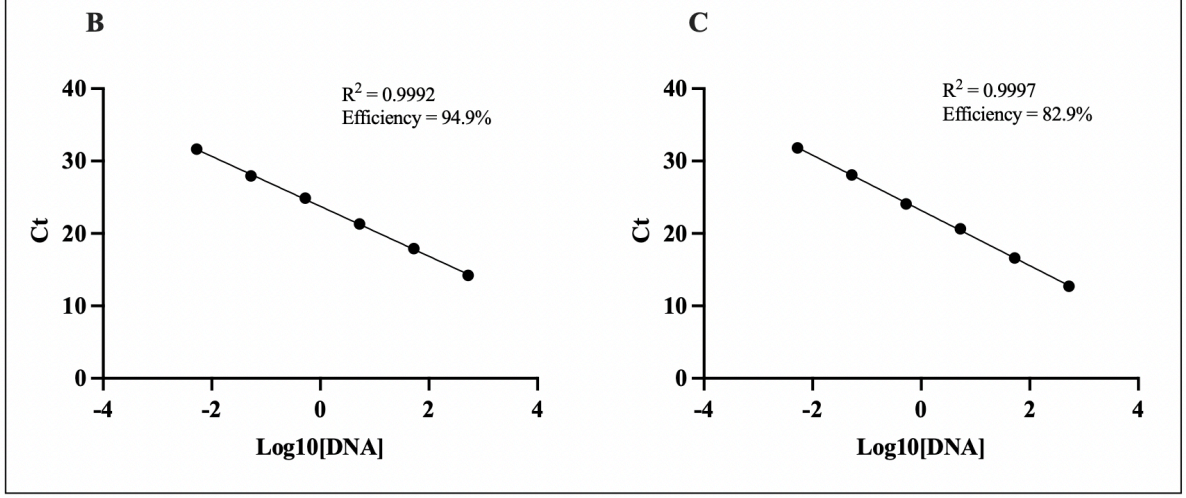


Figure 3.6 83 bp and 1 kb standard curves generated from plasmid DNA standards.
(A) Concentration of DNA (ng/well) and Ct values used to generate standard curves. (B) 83 bp standard curve. (C) 1 kb standard curve. R^2 values and efficiencies are presented on the graphs.

3.3.2 Investigating mitochondrial DNA content in different sample sites using a skin swab technique

Preliminary experiments were performed to investigate differences in mtDNA content between different sample sites, obtained using a skin swab technique. Figure 3.7A shows a significant difference in mtDNA content between samples obtained from the inner arm and nose with a skin swab ($p<0.01$), with Ct values of 25.50 and 22.19, respectively.

In order to control for variation in swab pressure which may increase the number of cells collected and therefore the yield of DNA, mtDNA content was normalised to nDNA to control for cell number (Figure 3.7B). A similar pattern was observed in participants 1-4, suggesting that cells within the nose have the highest mtDNA content, followed by the inner arm and then the heel. The difference between groups was not found to be statistically significant ($p=0.19$). Participants 5 and 6 showed a reverse pattern and reported having eczema.

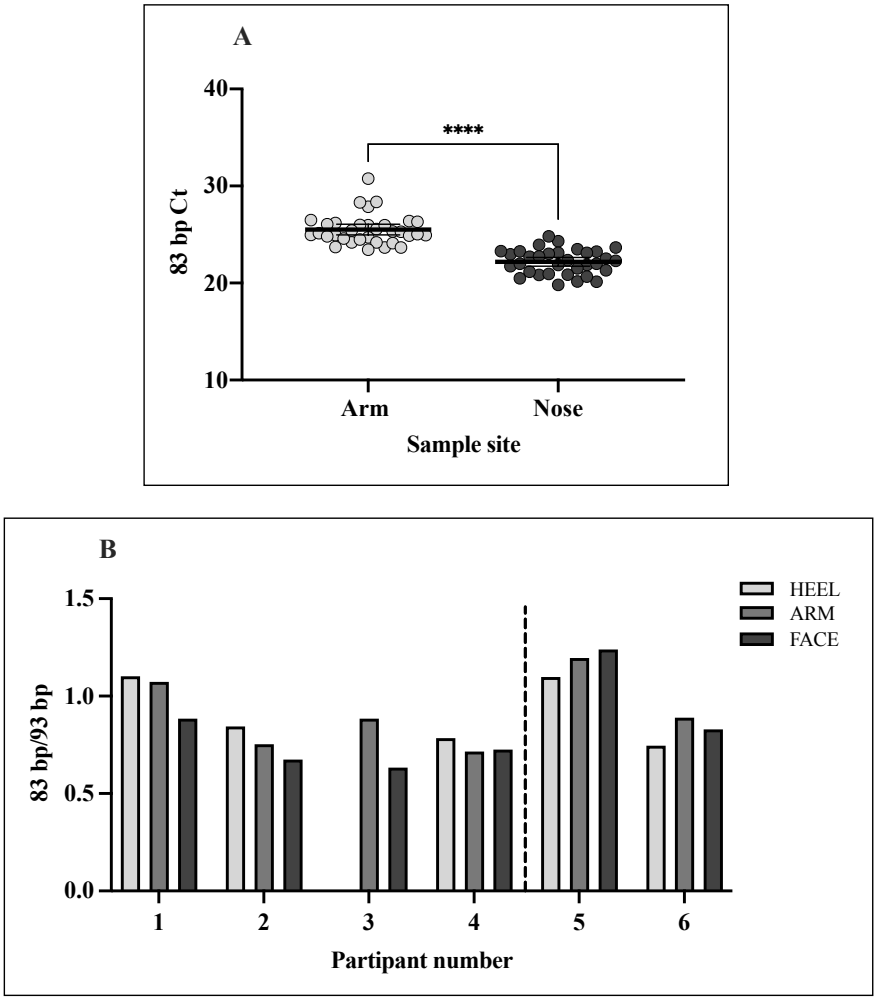


Figure 3.7 Mitochondrial DNA content varies between different sample sites.
(A) Mitochondrial DNA content in the arm and nose (n=33), determined using the 83 bp qPCR assay, expressed as raw Ct values. A higher Ct value corresponds to a lower starting concentration of mtDNA within the sample. Data are presented as mean+95%CI. Paired t-test was used to determine statistical difference. (B) Mitochondrial DNA content in the heel (n=5), inner arm (n=6) and face (n=6) was determined using the 83 bp qPCR assay and normalised to nDNA using the 93 bp qPCR assay. Data are expressed as an 83 bp/93 bp ratio, with a higher ratio corresponding to a lower mtDNA content within the sample. Friedman test was used to determine statistical difference. Participants 5 and 6 were removed prior to performing statistical analysis as they reported having eczema. All samples were collected using a skin swab collection technique.

3.3.3 Preliminary testing of the skin swab technique

A preliminary investigation into the level of mtDNA damage in individuals of different ages and skin types was conducted, using a skin swab technique. Figure 3.8 shows variation in %DNA amplified between different sample sites, as well as between individuals. In 4 of the 5 participants, %DNA amplified is lower in samples collected from the inner arm in comparison to those collected from the left and right cheek; however, differences between sample sites were not statistically significant (p=0.81). Although the sample size is small, age and skin type were not found to have an effect on %DNA amplified (data not shown).

The smallest %DNA amplified value observed was 1.86%, from a sample collected from the arm, and the greatest was 16.93%, collected from the left cheek. As expected, the %DNA amplified in samples from the left and right cheek were similar, with small differences of 1.16, 1.06, 2.11, 0.36 and 1.58 %DNA amplified.

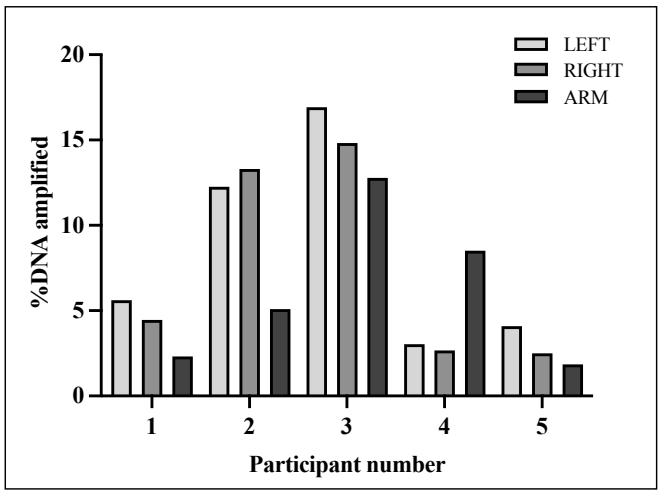


Figure 3.8 Mitochondrial DNA damage varies between individuals and different sample sites.
Variation in mtDNA damage in the left cheek, right cheek and arm of individuals (n=5). 83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified with a higher %DNA amplified corresponding to a lower level of mtDNA damage. Statistical difference between sample sites was determined using One-Way ANOVA.

Further studies were performed to investigate %DNA amplified in swabs collected consecutively from the same sample site, as well as on consecutive days and weeks in order to determine how mtDNA damage varies as corneocytes are removed from the stratum corneum. Skin swabs collected consecutively from the same individual from the left and right cheek show similar increases in %DNA amplified between swabs (Figure 3.9A). A similar finding was seen in a subsequent study; however, %DNA amplified continued to increase in swabs 1 and 2 that were collected from the same site on day 2, with the final swab showing a slight decrease in %DNA amplified (Figure 3.9B).

Differences in %DNA amplified between swabs collected consecutively from the same site as well as over a time course was also performed. As shown previously, samples collected from the arm had a lower %DNA amplified in comparison to those collected from the face (Figure 3.9C). The %DNA amplified increased between each arm swab on week 2 and 4; however, the same pattern was not observed on week 5. Skin swabs were not collected from the arm on week 1. Samples collected from the left and right cheek on week 1 showed a similar pattern in %DNA

amplified, increasing between swab 1 and 2 and slightly decreasing between swab 2 and 3. A pattern in samples collected during week 2 was not observed and there was increased variability between the left and right cheek. Samples collected during week 4 showed an increase in %DNA amplified between swabs 1 and 3 on the left cheek; as shown by previous optimisation studies; however, a decrease in %DNA amplified between swabs 1 and 3 was observed on the right cheek. Finally, %DNA amplified seemed to increase with consecutive swabs from the face during week 5; however, all samples collected from the right side of the face had a lower %DNA amplified, in comparison to those collected from the left.

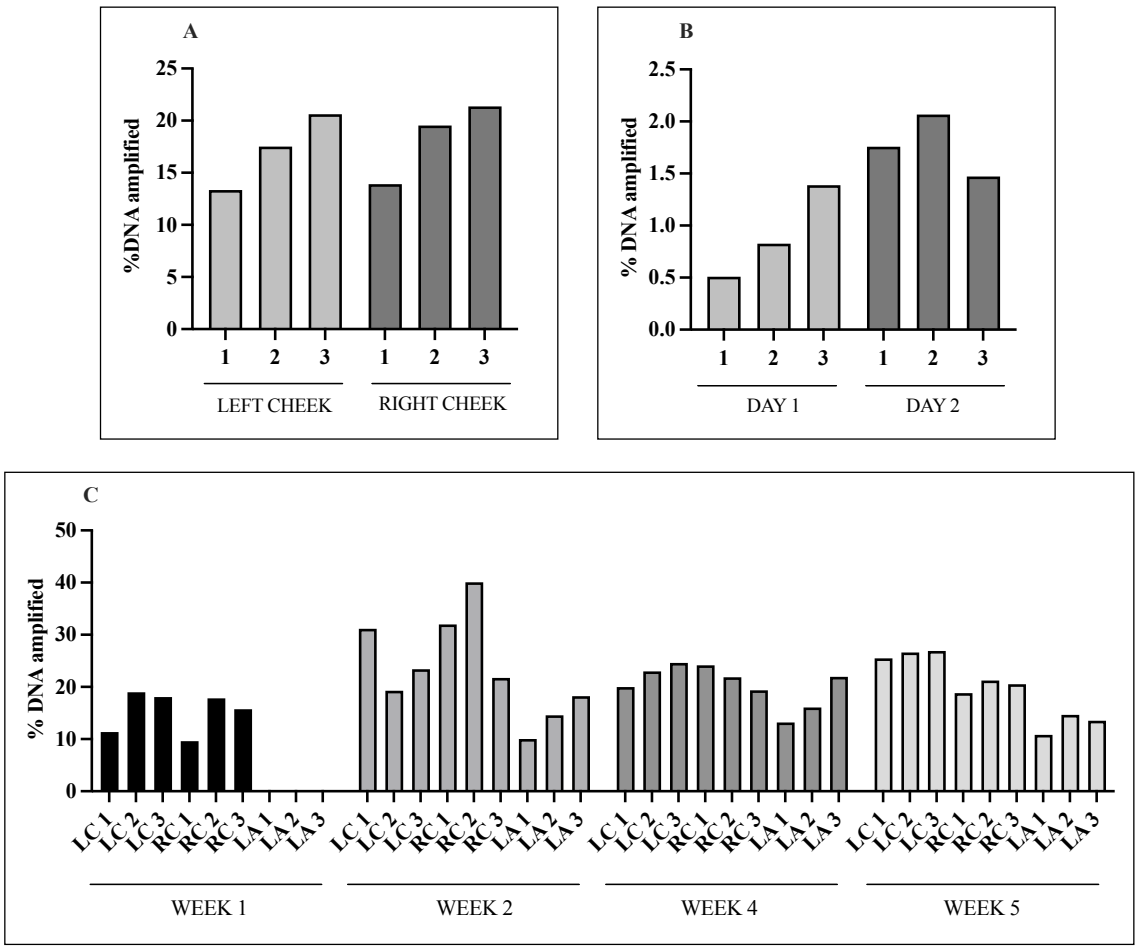


Figure 3.9 Mitochondrial DNA damage varies between different sample sites, consecutive swabs collected from the same sample site and swabs collected on consecutive weeks from the same sample site.

83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified with a higher %DNA amplified corresponding to a lower level of mtDNA damage. (A) Variation in mtDNA damage between 3 consecutive swabs, on the left and right cheek (n=1). (B) Variation in mtDNA damage between 3 consecutive swabs, on 2 consecutive days, on the right cheek (n=1). (C) Variation in mtDNA damage between swabs collected over a 5-week period, from the left cheek, right cheek and arm (n=1).

3.3.4 Using a skin swab buffer to increase mtDNA yield

Preliminary investigation of skin swab collection using buffer to increase mtDNA yield and subsequently qPCR amplification of samples was performed. A higher concentration of mtDNA was observed in all samples which were collected using skin swab buffer (Figure 3.10A); however, statistical significance was not observed between samples collected with and without buffer ($p>0.05$).

A greater %DNA amplified was observed in samples which were collected using buffer (Figure 3.10B); however, statistical significance was not observed between samples collected with and without buffer ($p>0.05$).

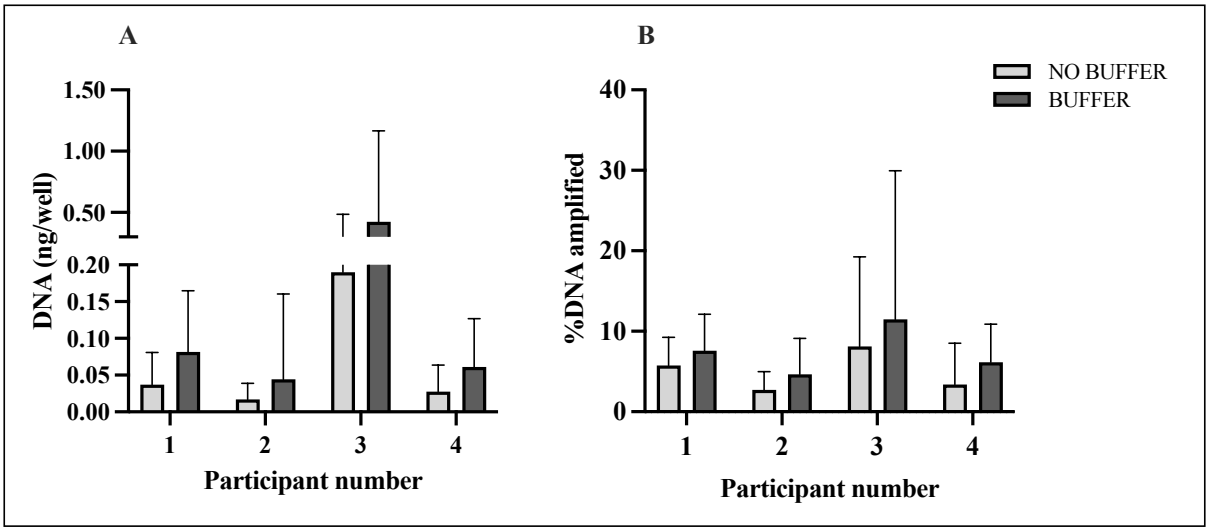


Figure 3.10 Skin swab buffer increases mtDNA yield and decreases mtDNA damage.
Samples obtained from the left cheek without buffer, and right cheek with buffer (n=4). 83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate DNA concentration and %DNA amplified. (A) Mitochondrial DNA concentration per well in the presence and absence of buffer. Statistical differences were determined using unpaired t-tests for participants 1-3 and Mann-Whitney test for participant 4. (B) %DNA amplified in the presence and absence of a skin swab buffer, with a higher %DNA amplified corresponding to a lower level of mtDNA damage. Statistical differences were determined using unpaired t tests for participants 1, 2 and 4 and Mann-Whitney test for participant 3. Data are presented as mean+95%CI.

3.3.5 Determining the effect of skin swab and tape strip collection on the stratum corneum of 3D full-thickness skin equivalents

Skin swabs and tape strips were collected from skin equivalents to investigate the effects of different sample collection techniques on the stratum corneum. Results show that the collection of swabs compacted the stratum corneum; shown in pink, (Figure 3.11A), in comparison to the

control (Figure 3.11B). The tape strip completely removed the epidermis from the skin equivalent (Figure 3.11C).

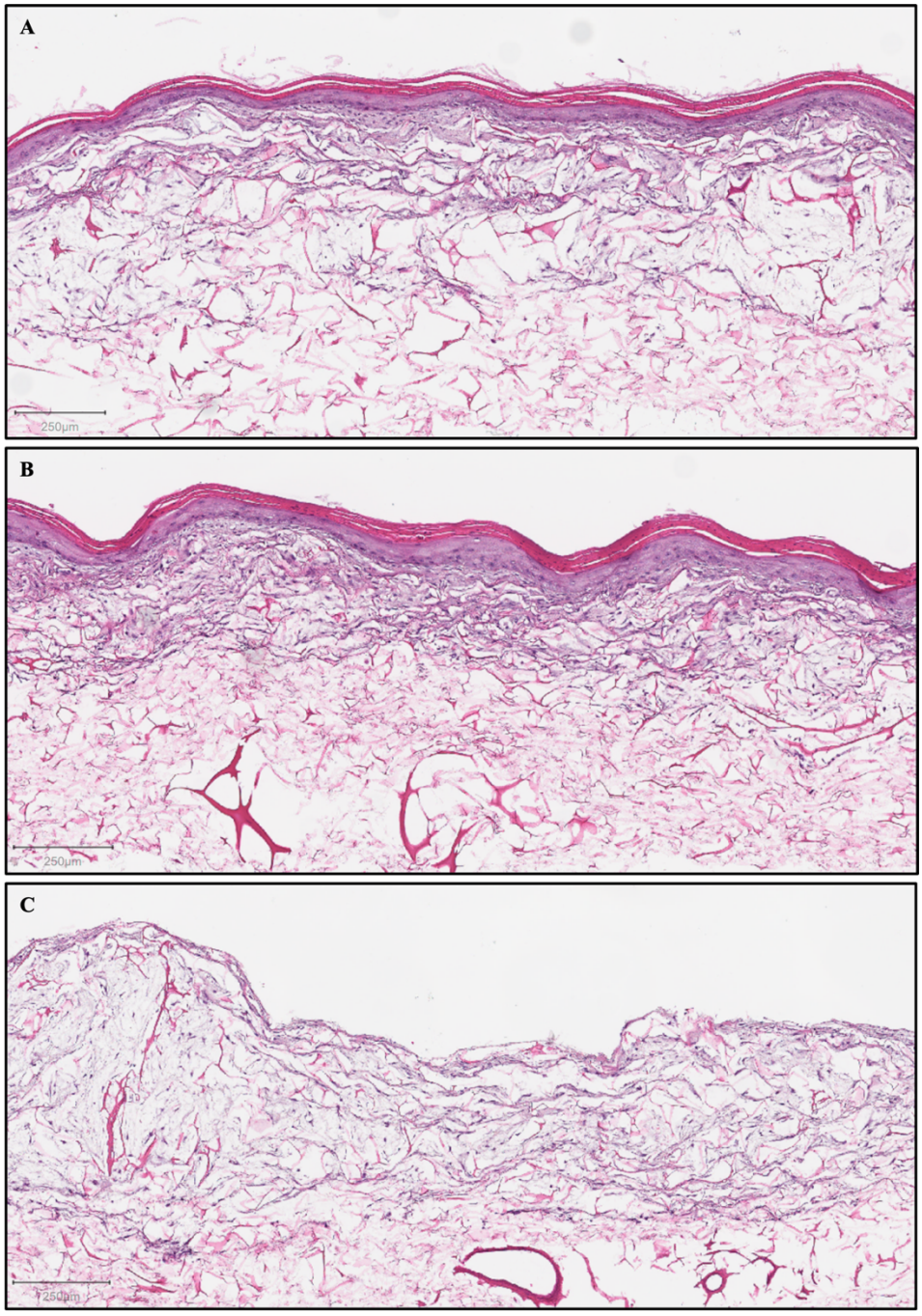


Figure 3.11 H&E staining shows differences in the stratum corneum of 3D full-thickness skin equivalents following skin swab sample collection.
Following sample collection using skin swabs and tape strips, skin equivalents were fixed and sent for H&E staining to investigate the effect of sample collection on the stratum corneum. (A) Control, (B) skin swab, (C) tape strip.

3.3.6 Determining the consistency of results obtained from facial images

Multiple facial images were taken consecutively in order to determine the relative standard deviations and therefore the reproducibility of results. Overall, red areas and wrinkles appear to have the greatest relative standard deviation (Figure 3.12). Feature count showed the highest relative standard deviation for both red areas and wrinkles, with values of 85.72 and 54.55, respectively. Rather than using data extracted from the VISIA software, images were exported into Image J to analyse for UV spots count and %Area (Section 4.2.4.5).

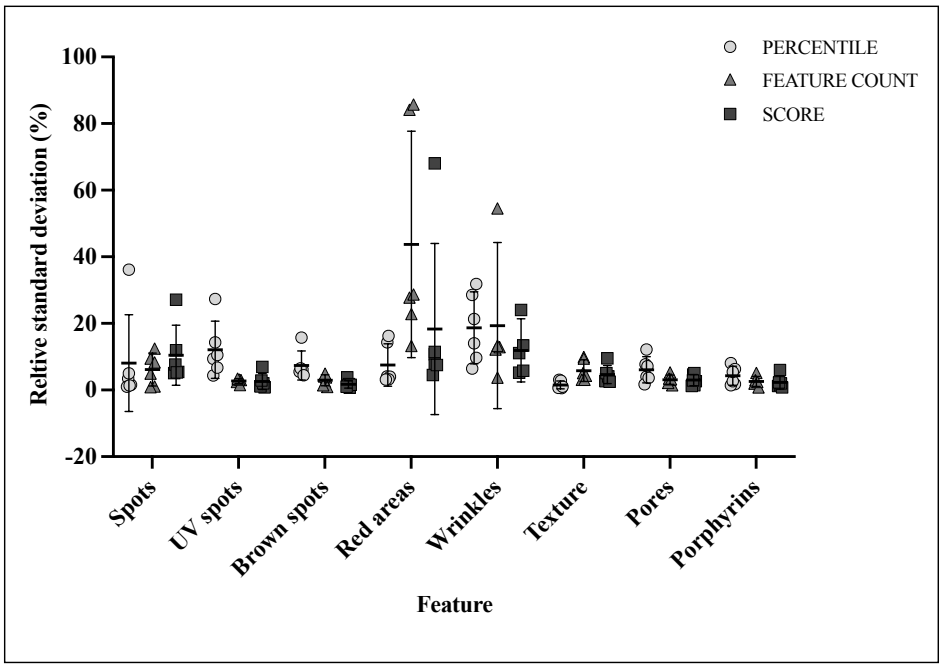


Figure 3.12 The VISIA® Skin Analysis system shows differences in relative standard deviation.
Facial images were collected to determine percentile, feature count and score values for each feature. 4 consecutive images were collected from the left, right and front of the face, from 2 individuals (n=2). Relative standard deviation was calculated from the 4 images collected from each side of the face.

3.4 Discussion

This chapter aimed to optimise methodology that is to be used throughout this PhD project such as the skin swab collection technique and methodology used to normalise mtDNA content and determine damage. It also aimed to determine the consistency of results obtained using the VISIA® Skin Analysis system.

3.4.1 Generation of 1 kb plasmid

The plasmid was generated to overcome the limitations of fold change methodology ($2^{-\Delta\Delta Ct}$) that was used for initial swab studies. As previously mentioned, an internal control is required to calculate fold change and it is unclear whether the inner arm can be used as a reliable control. Firstly, due to the low mtDNA content within samples obtained from the arm, not all samples amplify with qPCR; therefore, samples collected from the face cannot be used for analysis due to lack of normalisation. As well as this, fold change is relative qPCR and measures differences in abundances of target DNA between DNA samples without showing their actual abundances, and samples can only be compared if they are run within the same qPCR reaction (71). Preliminary studies also showed that a higher level of mtDNA damage was frequently seen in samples collected from the arm in comparison to the face (Figure 4.3), suggesting that the arm is not a reliable internal control. Finally, unlike with cellular DNA which tends to be of a high concentration, it is not possible to accurately quantify mtDNA within samples collected using a skin swab with the nanodrop due to the low concentration; therefore samples cannot be diluted down for normalisation purposes.

As previously mentioned, absolute qPCR allows precise quantification of target DNA through the generation of a standard curve, constructed in the same quantification assay as the samples. The standard curve is generated by amplifying a dilution series of plasmid DNA which carries the target sequence. This is a popular technique as plasmid DNA is stable, reproducible and easy to prepare (71, 74). Through interpolation, DNA concentration and subsequently %DNA amplified can be calculated (Equation 3.2). By using the same plasmid standards for generation of both 83 bp and 1 kb standard curves, it ensures that the same amount of DNA is added to both assays so that they are comparable. It also allows for comparison between samples run on different qPCR plates, unlike with fold change methodology. Prior to plasmid generation, cellular DNA was tested to generate standard curves; however, cellular DNA is less stable and will degrade more rapidly which will affect the reaction efficiency and therefore the overall results.

In most applications, including our own, circular plasmid DNA is used without linearisation by restriction digestion (71). Research has shown that Ct values are significantly higher when using circular plasmid standards; in comparison to linear standards, and that linear standards are more reliable for qPCR. This is due to uncut circular plasmid DNA being mostly supercoiled, resulting in the suppression of PCR due to decreased efficiency for primer binding and elongation. Restriction enzyme digest to generate linear DNA would relax the supercoiling and therefore increase the efficiency for primer binding and elongation (75, 76). Since plasmid molecules are circular and the mtDNA molecules within our samples are linear, future work could explore the effect of supercoiled vs. linear plasmid DNA on Ct values of the plasmid standards. Although the %DNA amplified calculated may be different if results were interpolated from standard curves generated with linear plasmid DNA, sample-sample comparison is relative; therefore, %DNA amplified between samples can be compared.

3.4.2 Investigating mitochondrial DNA content in different sample sites using a skin swab technique

Results from this chapter highlight differences in mtDNA content obtained from different sample sites, with samples collected from the nose having significantly higher mtDNA concentrations in comparison to samples collected from the inner arm (Figure 3.7A) ($p<0.01$). Although this finding could be due to differences in pressure applied during collection, this highlights the importance of normalising samples based on mtDNA content before investigating differences in mtDNA damage. It is widely accepted that mitochondrial content differs as a result of metabolic demand; therefore, an explanation for the increased content in samples collected from the face may be that cells within the face turn over more quickly. This may be a result of frequent exposure to damaging environmental influences; such as UVR, in comparison to the arm which is not often exposed due to clothing, particularly in those living within the UK (32).

To ensure that differences in mtDNA content obtained from different sample sites was not due to differences in pressure applied during swab collection, mtDNA content was normalised to nDNA to control for differences in cell number per swab. It was hypothesised that cell number would differ between sample sites as the face is more frequently exposed to UVR which has been shown to decrease intercellular cohesion; therefore, a greater number of corneocytes may be collected by the swab, which may subsequently increase mtDNA content within samples collected from the face (77). As well as this, studies have also shown epidermal thickness to increase following exposure to UVR, increasing the number of cells available to be collected

by the swab from sample sites more frequently exposed to UVR (20). A similar pattern was observed in all participants who did not report a skin condition, with mtDNA content being highest in the face, followed by the arm and the heel (Figure 3.7B); however, the differences weren't statistically significant. This pattern was similar to one reported by Harbottle *et al*. (2010) which found the highest level of 3895 bp deletion in a frequently sun-exposed site, in comparison to sites which are less sun exposed (50). The deletion was found to be highest in the face, followed by the arm and then heel. We propose that this finding is due to differences in mtDNA content rather than differences in the level of the deletion itself, due to an unreliable method of DNA quantification. Harbottle *et al*. (2010) used PicoGreen to quantify DNA within the samples; however, this method detects all single and double-stranded molecules and therefore doesn't differentiate between mtDNA and nDNA. This also supports our hypothesis that cell turnover is increased in sample sites that are more frequently exposed to UVR. Normalisation of mtDNA content within studies reported in this thesis involves primers specific to the mitochondrial genome; therefore, eliminating the need to separate mtDNA and nDNA (78).

Results from those with eczema were not in line with those who did not report a skin condition. The ratio was higher in those with eczema; therefore, less mtDNA damage was present in samples (Figure 3.7B). As well as this, a higher DNA yield was obtained from individuals with eczema which could be due to increased cell proliferation; a finding often reported in those with dry skin (79), which could result in the collection of more corneocytes with the skin swab. This may also explain the lower level of mtDNA damage in samples, as increased cell turnover could prevent damage accumulation. As well as this, studies have shown parakeratosis in individuals with inflammatory skin conditions including chronic eczema. It is characterised by incomplete keratinization of epithelial cells, with abnormal retention of keratinocyte nuclei in the stratum corneum (80). The increased proliferation rate, aberrant differentiation and retained nuclei within the stratum corneum may mean that the DNA degradation usually involved in cornification may not be complete; therefore, resulting in a greater yield obtained from swabs, as well as less mtDNA damage.

3.4.3 Preliminary testing of the skin swab technique

Testing of the swab technique was performed to investigate the level of mtDNA damage in individuals of different ages and skin type. Although variation is seen between individuals (Figure 3.8), it wasn't shown to correlate with age or skin type; however, the sample size was small. Previous studies have suggested that the skin swab detects recent UVR exposure, rather

than damage that has accumulated with age, and showed a higher level of a UVR-induced deletion in sites that are sun-exposed (50).

When swabs were collected consecutively from the same site, an increase in %DNA amplified was observed between samples (Figure 3.9A). It is widely accepted that the stratum corneum consists of 15-20 layers of corneocytes which undergo loss of nuclei and cytoplasmic organelles (1, 6); therefore, it was hypothesised that corneocytes were successively removed from the stratum corneum with each swab, leaving more in-tact mtDNA available to be amplified in cells deeper within the stratum corneum. The same finding was observed on day 2 (Figure 3.9B), with swabs 1 and 2 showing higher %DNA amplified in comparison to day 1. This suggests that cells removed from the stratum corneum on day 1 had not yet been replaced by day 2, which could be a result of the 28-day turnover time from cell division to shedding of the stratum corneum (1, 6).

Results also showed that %DNA amplified between consecutive swabs when collected from the left and right cheek (Figure 3.9A) as expected, providing evidence for accurate detection of mtDNA damage from the epidermis using a swab. As well as this, mtDNA damage fluctuated over the course of 5 weeks, which was expected due to lifestyle factors and the use of skincare products. Damage was also shown to be higher in the inner arm in comparison to the face, as seen previously (Figure 3.9C). As discussed in more detail in later chapters, this could be due to differences in stratum corneum thickness, cell turnover rates and exposure to UVB which has been shown to decrease cellular cohesion and therefore loosen corneocytes within the stratum corneum (77, 81, 82). It is important to note that preliminary studies were performed in February when UV levels are relatively low; therefore, it was expected that mtDNA damage would fluctuate slightly but not by great amounts.

Skin hydration and dry skin; also known as xerosis, may also affect results obtained using the skin swab technique. Research has shown that the development of dry skin is common as we age (79); however, it is also affected by multiple external factors such as cold and dry climates, as well as repeated washing. As well as this, evidence has also shown racial variability in the physiological properties of the skin, which directly impact water content of the stratum corneum (83).

Although differences between individuals due to skin health as well as differing lifestyle factors may mean that direct comparisons cannot be drawn, it may be possible to use individuals as their own control over time. Finally, as this is a relatively new technique, larger and more inclusive studies are required to determine what “normal” %DNA amplified values are.

3.4.4 Using a skin swab buffer to increase mtDNA yield

As previously mentioned, research has shown that exposure to UVB decreases intercellular cohesion between cells, which could explain why the yield is low in samples collected from the sun-protected inner arm (77). Results from a preliminary study (Section 4.3.1) highlighted amplification issues with samples collected from the inner arm due to low mtDNA content; therefore, a buffer was tested with the aim of increasing mtDNA content in samples collected using the swab.

Results show an increase in mtDNA content in samples collected using skin swab buffer (Figure 3.10A); however, differences were not statistically significant. Skin swab buffer contains Tris HCL, Tris base, EDTA and Triton X-100, with Tris HCL and Tris base maintaining a stable pH to maintain DNA stability. EDTA is also responsible for stabilising DNA through the chelation of metal atoms that are required for enzymes such as DNases to function; therefore, preventing DNA degradation. Finally, Triton X-100 lyses cells and is therefore thought to help remove the corneocytes from the stratum corneum to increase yield. As well as stabilising the DNA prior to DNA extraction, it was also hypothesised that the buffer reduces friction, allowing the swab to be pressed more firmly against the skin during sample collected, without it feeling uncomfortable.

Similarly, an increase in %DNA amplified was also observed in samples collected with skin swab buffer (Figure 3.10B); however, this wasn't statistically significant. It was hypothesised that the prevention of DNA degradation also results in more in-tact DNA available to be amplified through qPCR; therefore, resulting in less mtDNA damage detected within samples. It is important to note that samples collected with and without buffer, were collected from different cheeks. The study did not control for lifestyle factors which could influence differing UVR exposure between different sides of the face. Differences in %DNA amplified with and without buffer could be due to increased UVR exposure; for example, due to frequent driving. Due to time constraints this study was not repeated to control for lifestyle factors; however, the buffer was used for skin swab collection in subsequent studies.

3.4.5 Determining the effect of skin swab and tape strip collection techniques on the stratum corneum of 3D full-thickness skin equivalents

Due to differences in mtDNA damage with consecutive swabs, skin swabs and tape strips were collected from skin equivalents in order to investigate the effect on the stratum corneum and

which layers were removed by each method of collection. Results showed that the collection of swabs compacted the stratum corneum; therefore, it was not possible to draw any conclusions. As well as this, the tape strip completely removed the epidermal layer from the skin equivalent. Although the skin equivalents comprise both an epidermis and dermis embedded in a mechanically stable collagen sponge and synthesis major extracellular matrix proteins such as collagens, elastin and fibrillin-1, they lack some major skin components. Skin equivalents therefore may not be a good measure of collection techniques; however, methods such as optical coherence tomography (OCT) may be able to determine differences in stratum corneum thickness following sample collection (84).

3.4.6 Determining the consistency of results obtained from the VISIA® Skin Analysis system

Consecutive facial images were collected to determine the relative standard deviation and therefore the reproducibility of results obtained from the VISIA® Skin Analysis System. Red areas and wrinkles appeared to have the greatest relative standard deviations, suggesting that results generated from the VISIA are not reproducible and vary in consecutive images (Figure 3.12). This could be due to multiple factors such as positioning of the chin on the rest and lighting. With the aim of increasing accuracy, UV photographs were exported and analysed in Image J (described in Section 4.2.4.5) which accounted for any shadows around the edge of the face and controlled for area.

3.4.7 Key findings

- A plasmid was generated to eliminate the need for an internal control and allow direct comparison between a large number of samples
- Mitochondrial DNA content differed between sample sites
- Results from individuals with skin conditions differed to those collected from individuals who did not report a skin condition
- Mitochondrial DNA damage decreased in samples obtained from consecutive swabs
- The use of a skin swab buffer increased DNA yield and decreased mtDNA damage within samples
- Readings obtained from the VISIA® Skin Analysis software were not found to be consistent and reproducible

3.4.8 Conclusions

Overall, normalisation methodology was improved by introducing the use of a plasmid in order to normalise for mtDNA content and determine mtDNA damage within samples. As well as this, a skin swab buffer was introduced which was shown to increase mtDNA yield and therefore amplification through qPCR.

In order to further improve standard curve methodology, the plasmid should be linearized to assist primer binding. If the swab is to be rolled out for general public use, skin conditions should be investigated further, due to differences in results observed during this optimisation process. Although optimisation studies suggest that the swab can accurately detect mtDNA damage on swabs, large-scale studies are required to further our understanding. It was concluded that differences are less likely to be observed when drawing comparisons between different individuals; however, individuals could be used as their own control, comparing mtDNA damage values over time and potentially testing the protective effects of compounds against UVR.

Chapter 4. Aim I: Investigation of extrinsic influences on skin ageing

4.1 Introduction

Skin ageing results from a combination of intrinsic and extrinsic factors, also known as genetic and environmental factors. Research has shown that UVR is the main extrinsic insult to the skin, which has multiple damaging effects including DNA damage, oxidative stress, inflammation, pigmentation and suppression of the immune system (85). Over time, extrinsic factors accelerate skin ageing, which is characterised by deep wrinkles, rough texture, telangiectasia, lentigines and irregular pigmentation (51).

The use of mtDNA as a sensitive and reliable marker of cumulative exposure to UVR has been pioneered by multiple groups (59, 60, 64, 86). Research has also shown that mtDNA in primary dermal fibroblasts is more vulnerable to damage at wavelengths greater than 320nm, in comparison to nDNA (87). As well as this, mitochondria are short of NER pathways and therefore do not have the ability to repair UVR-induced photoproducts such as pyrimidine dimers. The mitochondrial genome also has a 10-fold higher mutation rate in comparison to nDNA due to its lack of protective histones and location within the mitochondrial matrix, close to the site of ROS production in the ETC. Finally, there are multiple copies of mtDNA per mitochondria and approximately 200-2000 mitochondria per cell which varies depending on the energy requirement of the cell; therefore, mtDNA has the ability to tolerate up to 90% of damaged DNA through complementation of the remaining wild-type (32, 34, 85).

Studies have shown an increase in specific deletions in photoaged skin in comparison to photo-protected skin, with the 4977 bp common deletion being the most frequently reported mtDNA mutation in human skin and shown to increase 10-fold in photoaged skin (58, 60). The 3895 bp deletion and T414G mutation are also found with increasing frequencies in UVR exposed skin (51, 59, 60). Multiple groups have reported methodology that detects strand breaks in mtDNA using qPCR; which have also been shown to result from UVR exposure, and have shown great potential (85). These assays work on the premise that single or double-stranded DNA breaks or significant mutation will prevent DNA polymerase progression; therefore, a more damaged sample will require a greater number of cycles to reach the set threshold (88). The length of the amplicon increases the region assessed for mtDNA damage; thus, increasing assay sensitivity (69).

Expanding upon the detection of the 3895 bp deletion as a result of exposure to UVR, a non-invasive skin swab technique has been developed by the Birch-Machin laboratory to assess

mtDNA damage within the epidermis as a result of recent UVR exposure (60). The 3895 bp deletion was detected with differing frequencies in three sample sites of differing UVR exposure. The data therefore suggests that the epidermis is a reliable site for assessing mtDNA damage caused by recent UVR exposure, despite the high turnover rate of the epidermis. The skin swab did not detect an increase in deletion frequency with increasing age; however, an increase was observed in biopsy samples. Previous research has shown the accumulation of mtDNA damage within the dermis due to its slow turnover; therefore, it was hypothesised that biopsy samples detect long-term chronological mtDNA damage due to collecting the dermis as well as the epidermis, whereas the skin swab detects recent UVR exposure only (50). As well as this, tape strips have also been used as a minimally invasive method of sampling the epidermis, removing the need for skin biopsies. They have been used to investigate keratinocyte number and morphology, skin barrier function, penetration of topically applied drugs and stratum corneum proteins; however, to our knowledge, the technique has not yet been used to investigate photodamage (89).

Although an increase in mtDNA damage as a result of UVR exposure is widely reported, the literature is yet to assess whether the skin swab has the ability to detect differences in lifestyle factors between individuals such as sun exposure and protection behaviours, as well as demographic factors. Previous studies have reported that polymorphisms of the MC1R gene correlate with skin fairness, sensitivity to UVR and enhanced cancer risk; however, a significant difference between skin type and mtDNA damage was not detected using the skin swab previously (20, 50).

Another method of analysing underlying skin damage is through the use of skin analysis instruments, which are able to record and measure surface and subsurface skin conditions. UV light; also known as Wood's light, has been used since 1903 to document alterations in pigmentation (72). The VISIA® Skin Analysis system is commonly used for UV photography, generating UV spot images due to the selective absorption of UV light by melanin within the epidermis in comparison to deeper melanin; therefore highlighting solar lentigines (90). The photographs reveal sun damage in the form of spotted pigmentation and it is currently accepted that increased spot frequency and size indicates greater damage (91).

Although this is a frequently used technique, the mechanism for UV spot formation is not well understood. It has previously been reported that solar lentigines may be induced by gene mutations of keratinocytes and/or melanocytes, which plays a pivotal role in pigment formation and melanin transfer (92). Previous research involving UV photography has shown sun damage to correlate with phenotypic melanoma risk factors, and that UV photography intervention

results in significantly stronger sun protection intentions and greater sun protection behaviours. This therefore suggests that UV photography is an effective intervention in altering poor sun protection behaviours and that highlighting current sun damage in an individual's face is particularly effective in comparison to less specific educational efforts (91, 93, 94). This highlights the importance of utilising UV photography to improve skin health (95).

Overall, this chapter aims to investigate variation in mtDNA damage *in vivo* and *in vitro*, as well as the effect of UVR, with a focus on demographics, sun exposure and sun protection behaviours.

Specific aims are as follows:

1. Investigate whether variation in mtDNA damage between individuals can be detected using a skin swab technique, and whether the variation correlates with sun exposure and sun protection behaviours
2. Investigate whether recent exposure to high intensity UVR can be detected using a skin swab technique
3. Investigate whether seasonal variation in UV levels can be detected using skin swab and facial imaging techniques, and whether the variation correlates with sun exposure, sun protection habits and demographics
4. Investigate variation in mtDNA damage obtained from skin equivalents following recent exposure to solar simulated light using a skin swab collection technique
5. Investigate whether variation in mtDNA damage can be detected as a result of age as well as recent UVR exposure in biopsy samples
6. Compare mtDNA damage in samples collected using skin swab and tape strip collection methods

4.2 Materials and Methods

4.2.1 Ethical approval

Ethical approval was granted by the Newcastle University Ethics Committee with reference numbers shown in Table 4.1.

Study number	Study name	Ethical approval reference
1	Pilot study evaluating mtDNA damage variation in the epidermis	12405/2018 and 14252/2018
2	Evaluating the effect of UVR exposure on mtDNA damage in the epidermis	14252/2018
3	Evaluating the effect of seasonal variation in UVR exposure on mtDNA damage in the epidermis	2075/9956 and 2075/9956
5	Evaluating the effect of UVR exposure on mtDNA damage in the epidermis and dermis	Samples collected under external ethics.
6	Evaluating differences in mtDNA damage between samples collected with skin swab and tape strip collection methods	11299/2020

Table 4.1 Ethical approval reference numbers for participant studies.
Study 4 did not involve human participants and therefore did not require ethical approval.

4.2.2 Study information

4.2.2.1 General information

General study information is presented in Table 4.2. Studies 2, 3, 5 and 6 involved repeated measurements. Sample collection dates for study 3 are presented in Table 4.3.

Study	Sample number	Collection dates	Collection sites	Inclusion criteria	PCR assays used	VISIA?	Questionnaire?
1	17	31/05/2019 - 7/06/2019	Inner arm Nose	Female Over the age of 30 years Fitzpatrick skin types 1 and 2	83 bp 1 kb		✓
2	9	26/07/2019 - 3/01/2020	Inner arm Nose	Female Over the age of 30 years Fitzpatrick skin types 1 and 2	83 bp 1 kb		
3	87	18/05/2021 - 23/11/2021	Left cheek Right cheek Inner arm	Over the age of 18 years	83 bp 1 kb	✓	✓
5	42	N/A	Upper outer arm	Over the age of 18 years	83 bp 11 kb		

Table 4.2 General overview of participant studies.

Sample collection number	Season	Start	End
1	Spring	18/05/2021	15/06/2021
2	Summer	7/09/2021	15/09/2021
3	Autumn/winter	17/11/2021	23/11/2021

Table 4.3 Sample collection dates for study 3.
Skin swabs and facial images were collected from each participant during spring, summer and autumn/winter. 3 samples in total were collected from each participant over the course of the study. Autumn and winter sample collection was combined due to time constraints as a result of COVID-19. Missing samples were due to COVID-19 restrictions as well as participants having other commitments.

4.2.2.2 Questionnaire variables

Questionnaire variables and scales for studies 1 and 3 are presented in Table 8.1 and Table 8.2, respectively (Appendix). The majority of questions were adapted from studies previously reported (96-99). The number of questionnaire responses obtained for studies 1 and 3 were 33 and 80, respectively. Full questionnaires for studies 1 and 3 are included in Section 8.1.1 and Section 8.2.1, respectively (Appendix).

Due to time constraints, selected variables were chosen for analysis based on which more closely aligned with the study aims.

4.2.3 Recruitment

Individuals were recruited through a range of advertisements such as University mailing lists, LinkedIn and VOICE.

4.2.4 Sample collection

4.2.4.1 Skin swabs

See general methods for skin swab collection technique (Section 2.1.1). Skin swab collection sites are presented in Table 4.2. Skin swab buffer was used for the collection of skin swabs in study 2, 3, 4 and 6. Samples collected in study 2 were collected as soon after exposure to UVR as possible.

4.2.4.2 Skin biopsies

Skin biopsy samples used in study 5 were collected by a commercial company and DNA was extracted prior to receiving samples.

4.2.4.3 Tape strips

Sample sites were cleaned using an ethanol wipe. A D-Squame® tape strip (CuDerm Corporation, USA) was applied to the site and the D-Squame® pressure instrument (CuDerm Corporation, USA) was used to apply equal pressure to the tape strip. The tape strip was removed and stored in a 1.5ml microcentrifuge tube until DNA extraction.

4.2.4.4 3D full-thickness skin equivalents

Details regarding the setup of skin equivalents are present in Section 3.2.3. Skin equivalents were irradiated with 2.16 SED complete solar light (Section 4.2.5) and used to investigate mtDNA damage using skin swabs and how it compares to damage extracted from epidermal and dermal layers of models. Day 1 skin swab samples were collected immediately after irradiation for both treatment and control groups and returned to the incubator until day 3. Day 3 skin swab samples were collected at the same time as those collected on day 1. Skin swab buffer was used for swab collection from skin equivalents.

Day 1 tissue samples were harvested immediately after irradiation for treatment and control groups and samples were harvested at the same time on day 3. Harvesting involved incubating skin equivalents at 4°C overnight in 10% dispase before separating epidermal and dermal layers with sterile forceps. The epidermis was placed into a sterile 1.5ml microcentrifuge tube ready for DNA extraction and the dermis was cut into quarters and ¼ was subsequently cut into small sections and placed into a sterile 1.5ml microcentrifuge tube. DNA extraction was then performed as detailed in Section 4.2.6.

4.2.4.5 Facial image collection, analysis and storage

A 7th Generation VISIA® Skin Analysis system was used for facial imaging, with images stored under restricted access in line with the Data Protection Act/General Data Protection Regulation. Subjects placed their chin on the rest and closed their eyes and images were taken of the right, left and front of the face.

UV images were exported (Figure 4.1A) and number of UV and pigmented spots were quantified using Image J (USA). Images were converted from 32-bit colour to 8-bit and a large Gaussian blur was added to the original image (sigma=70) to create a mask (Figure 4.1B). The original image was then divided by the blurred image to create the flat field image, and brightness and contrast were adjusted to view the dark image produced by this process (Figure 4.1C). Image threshold was adjusted to visualise dark spots (Figure 4.1D) and particle analysis was run, following the generation of a masked area (Figure 4.1E and F), with a range of 3-infinity (pixel^2) and circularity of 0.02-1. These parameters were optimised to ensure that all large spots were included and small spots corresponding with pores and other artifacts were not, and all fully linear objects corresponding with wrinkles were removed from the analysis. Dark spots within the masked area (Figure 4.1F) are UV spots. Under eye areas and creases around the mouth were not included in the masked area. The method of quantification was devised by Glyn Nelson (Bioimaging Unit, Newcastle University).

All data was manipulated in Microsoft Excel (Microsoft, USA) and statistical analysis was performed using GraphPad Prism 8 statistical software (GraphPad software, USA). UV spot analysis was performed using correlation analysis as well as comparison of means. Shapiro-Wilk, as well as kurtosis and skewness were used to check for normality. Analyses were controlled for covariates and collinearity was checked. Graphs were produced from raw values and statistical analysis was performed on unstandardized predicted values. Age was grouped into 19-39, 40-59 and 60+ for analysis purposes.

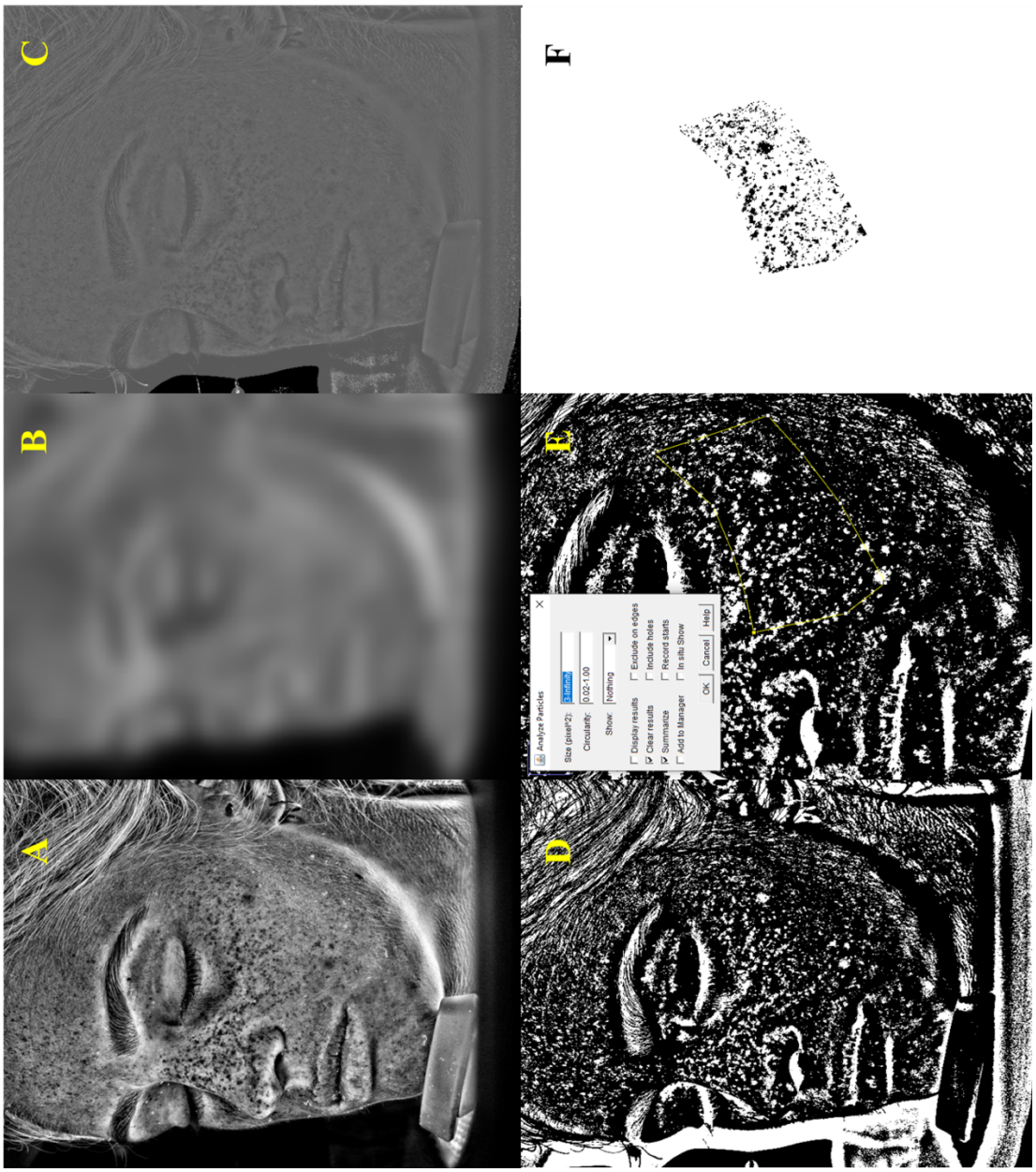


Figure 4.1 Example of particle analysis performed on UV spot images.
(A) Exported image from VISIA® Skin Analysis system. (B) Image converted from 32-bit colour to 8-bit and a Gaussian blur added (sigma=70). (C) Original image divided by the blurred image to create a flat field image and brightness and contrast were adjusted to view the dark image produced by this process. (D) Image threshold was adjusted to visualise dark spots. E and F Particle analysis was run following the generation of a masked area, with a range of 3-infinity (pixel^2) and circularity of 0.02-1.

4.2.5 Irradiation

A Newport solar simulator (MKS Instruments, Inc., USA) was used to illuminate treatment equivalents with controlled levels of weighted radiation across the UV, visible and IR solar spectrum. Treatment equivalents were irradiated for set times equivalent to 2.16 SED, to mimic approximately 20 minutes in the Mediterranean sun. An ILT-1400 (International Light Technologies, USA) handheld radiometer/photometer was used to measure radiant energy.

4.2.6 DNA extraction and quantitative real-time PCR analysis

Samples within studies 1, 2, 4 and tape strip samples within study 6 were extracted using the QIAamp DNA Mini Kit (QIAGEN, Europe) as per manufacturer's instructions.

Following separation of epidermal and dermal layers of skin equivalents in study 4, 180µl Buffer ATL and 20µl proteinase K were added to the tissue and incubated in a heat block for 3 hours at 56°C, vortexing every 30 minutes to ensure that sufficient lysis occurred. Regarding study 6, modifications made to the protocol are as follows:

1. Following removal of adhesive edges, tape strips were cut into 8 even segments and placed in a 1.5ml microcentrifuge tube.

2. 180µl buffer ATL and 20µl proteinase K were added and samples were incubated at 56°C for 3 hours, vortexing briefly every 30 minutes.

Samples within study 3 and skin swab samples from study 6 were extracted using the BuccalPrep Plus DNA Isolation Kit (Isohelix, UK), as per the manufacturer's instructions. In-house optimisation showed that the BuccalPrep Plus DNA Isolation Kit (Isohelix, UK) gave a greater yield when extracting mtDNA from skin swabs in comparison to the QIAampDNA Mini Kit (QIAGEN, Europe) (data not shown). DNA samples within study 5 (Section 4.4.5) were extracted externally following biopsy collection.

Samples within study 3, 4 and 6 were analysed using the Quant-Studio 3 Real-Time PCR System (Applied Biosystems, UK). All other samples within this chapter were analysed using the StepOnePlus Real-Time PCR System V2.1 (Applied Biosystems, UK). Upon the purchase of the newer, more sensitive Quant-Studio 3 model, optimisation experiments were conducted to ensure that the protocol was suitable for both PCR systems (Figure 2.3). For general methods including qPCR, refer to Section 2.3. For study 5, 10ng DNA was added to each reaction. DNA was not quantified prior to being added to PCR reactions. 5µl DNA was added to each reaction in study 1 and 2, which was subsequently reduced to 2µl for studies 3, 4 and 6.

83 bp master mix was adapted for studies 3 and 4 to enable both 83 bp and 1 kb qPCR assays to be run on the same plate using the same amplification settings and master mix composition, with the aim of eliminating variation between PCR plates. The reaction volume was reduced to 20µl and primer concentrations were the same as used in the original master mix. SensiMix Low-ROX (Scientific Laboratory Supplies, UK) was used as per manufacturer's protocol, rather than SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, USA) which was used in

the original master mix. Reaction efficiency of optimised 83 bp qPCR assay was 97.11% with plasmid DNA (Figure 4.2).

A

DNA concentration/well (ng)	Log10[DNA]	Ct
528.059669	2.722683	14.24469
52.805967	1.722683	17.62955
5.280597	0.722683	21.32298
0.528060	-0.277317	24.81561
0.052806	-1.277317	27.80747
0.005281	-2.277317	31.18973

B

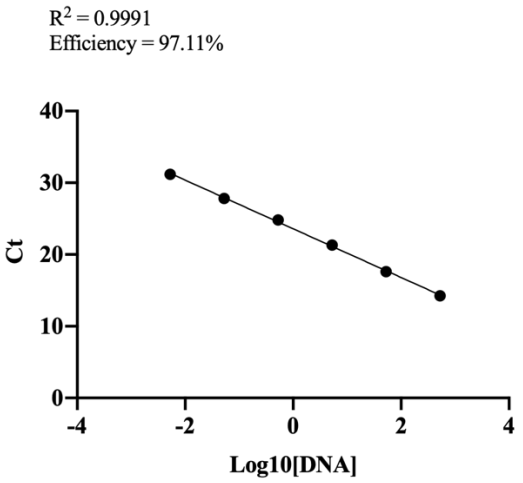


Figure 4.2 Standard curve generated with plasmid DNA for optimised 83 bp assay.
Standard curve generated to demonstrate efficiency of optimised 83 bp assay. (A) DNA concentrations of the plasmid DNA standards used to generate the standard curve. Standards were generated using a 1 in 10 dilution and show a difference of approximately 3.3 Ct. (B) Standard curve generated from values in (A) with R^2 and reaction efficiency presented.

4.2.6.1 Data manipulation and statistical analysis

Total cell DNA concentration for samples within study 5 were quantified using a Nanodrop ND-100 (Nanodrop technologies, UK) and diluted down to 5ng/µl before performing qPCR analysis. Dilutions were checked using the 83 bp assay to ensure that all samples were the same concentration; therefore, normalisation of Ct values following the 1 kb damage detection assay was not required. Statistical analysis was performed on raw Ct values. Samples within remaining studies were not diluted prior to running qPCR assays and the 83 bp assay was used to determine mtDNA content within samples. Samples within study 1 and 2 were analysed using fold change methodology ($2^{-\Delta\Delta Ct}$) and samples within study 3, 4 and 6 were analysed using interpolation from a standard curve (%DNA amplified) methodology, detailed in Chapter 3 (Section 3.2.1.8).

All data were manipulated in Microsoft Excel (Microsoft, USA) and statistical analysis was performed using GraphPad Prism 8 statistical software (GraphPad Software, USA). Fold change values were logged prior to performing statistical analysis. Qualitative data were analysed in SPSS Version 24 (IBM SPSS Statistics, USA).

Correlation analysis as well as comparison of means were performed. Linear regression was used to control for covariates. Shapiro-Wilk, kurtosis and skewness were used to check for normality. was used to test for normality. No significant covariates were observed when analysing mtDNA damage in study 3.

4.3 Results

4.3.1 Study 1: Pilot study evaluating mtDNA damage variation in the epidermis

4.3.1.1 Variation in mtDNA damage in sun-exposed skin

Skin swab samples were collected from the nose of 17 participants to investigate whether variation in mtDNA damage between individuals can be detected using a skin swab (Figure 4.3), and whether variation correlates with sun exposure and sun protection behaviours. Participants were female, had skin types 1 or 2 and were over the age of 30 years.

A higher level of mtDNA damage in samples collected from the nose in comparison to the inner arm was seen in 2 individuals, shown by a fold change greater than 1. The remaining 15 individuals had a higher level of mtDNA damage in samples collected from the inner arm.

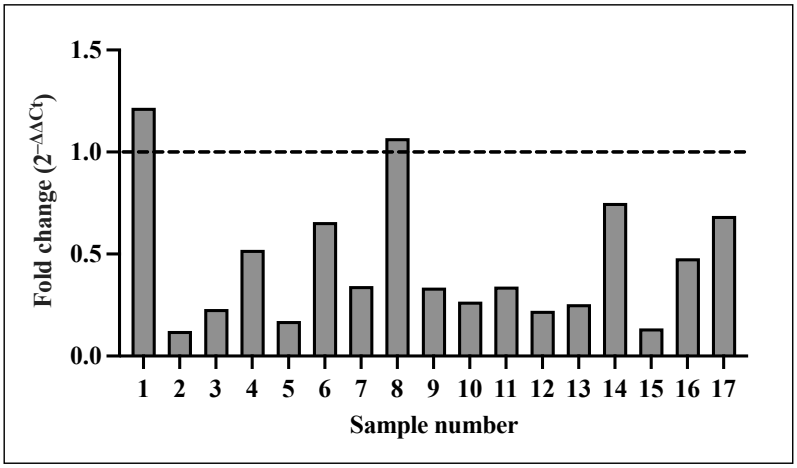


Figure 4.3 Individuals show variation in mtDNA damage within samples obtained from the nose using a skin swab technique.
Variation in mtDNA damage in nose swab samples when normalised to the inner arm (sun-protected internal control) (n=17). 83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively and data was analysed using the $2^{-\Delta\Delta Ct}$ method and presented as fold change. A fold change greater than 1 (shown by dotted line) corresponds to a higher level of mtDNA damage in samples obtained from the nose in comparison to the inner arm. 16 individuals were excluded due to incomplete data sets.

4.3.1.2 The effect of sun exposure and sun protection habits on mtDNA damage

Overall, questionnaires from 31 participants were collected and analysed. An overview of skin colour, skin reaction to midday sun and natural hair colour of participants is presented in Table 4.4. Overall, the largest proportion of individuals have pale skin (51.6%), burn easily and tan minimally with difficulty (n=66.7%) and have light brown hair (n=35.7%).

Table 4.5 provides an overview of the health behaviour frequencies of the sample population. 58.8% and 60.0% reported that they spent ≥4 hours outside between 10am and 4pm on

weekdays and weekends, respectively during a recent holiday abroad. Regarding sun protection behaviours when outside during a recent holiday on a warm sunny day, the largest proportion of individuals reported that they often or always wore sunscreen (100%), followed by the use of sunglasses (83.9%). Finally, 9.7% of individuals often or always spend time in the sun to obtain a tan, and 38.7% of individuals obtained at least 1 red or painful sunburn that lasted a day or more during their recent holidays.

Variable							Total
Skin colour	Very pale/reddish	Pale	Beige	Light brown (lightly tanned)	Moderately brown or tanned	Dark brown or black	
n (%)	9 (29%)	16 (51.6%)	5 (16.1%)	1 (3.2%)	0 (0%)	0 (0%)	n=31
Reaction to an initial sun exposure for 45-60 minutes (without sun protection), around midday in the early UK summer	Burn easily, never tan	Burn easily, tan minimally with difficulty	Burn moderately, tan moderately	Burn minimally, tan moderately and easily	Rarely burn, tan profusely	Never burn, tan profusely	
n (%)	7 (23.3%)	20 (66.7%)	3 (10.0%)	0 (0%)	0 (0%)	0 (0%)	n=30
Natural hair colour	Red	Blond	Light brown	Brown	Dark brown or black		
n (%)	7 (25.0%)	9 (32.1%)	10 (35.7%)	2 (7.1%)	0 (0%)		n=28

Table 4.4 Sample characteristics including skin type, skin sensitivity and hair colour for study 1. Summary data presented as n (%). Due to missing responses, total n varies between variables as presented in table

Health behaviours	Frequencies	Total
Sun exposure		
Sun exposure weekdays (% ≥ 4 hours)	17 (58.8%)	n=31
Sun exposure weekends (% ≥ 4 hours)	18 (60.0%)	n=30
Self-reported sun protection practices		
Sunscreen use (% often and always)	31 (100%)	n=31
T-shirt use (% often and always)	17 (54.8%)	n=31
Hat use (% often and always)	11 (35.5%)	n=31
Seek shade (% often and always)	12 (38.7%)	n=31
Sunglasses use (% often and always)	26 (83.9%)	n=31
Time spent in the sun for a tan (% often and always)	3 (9.7%)	n=31
Sunburns		
(% ≥ 1)	12 (38.7%)	n=31

Table 4.5 Health behaviour frequencies of the sample population in study 1.
Summary data presented as n (%). Due to missing responses, total n varies between questions as presented in table.

A significant correlation was not observed between mtDNA damage and sun protection behaviours (r=0.34, p=0.20), short term rationale attitudes (r=0.13, p=0.64), attitudes towards a tan (r=0.27, p=0.36), sun exposure (r=-0.33, p=0.21), short term affective attitudes (r=-0.02, p=0.94), long term attitudes (r=0.39, p=0.14) and sun protection intentions (r=0.18, p=0.51) (Figure 4.4).

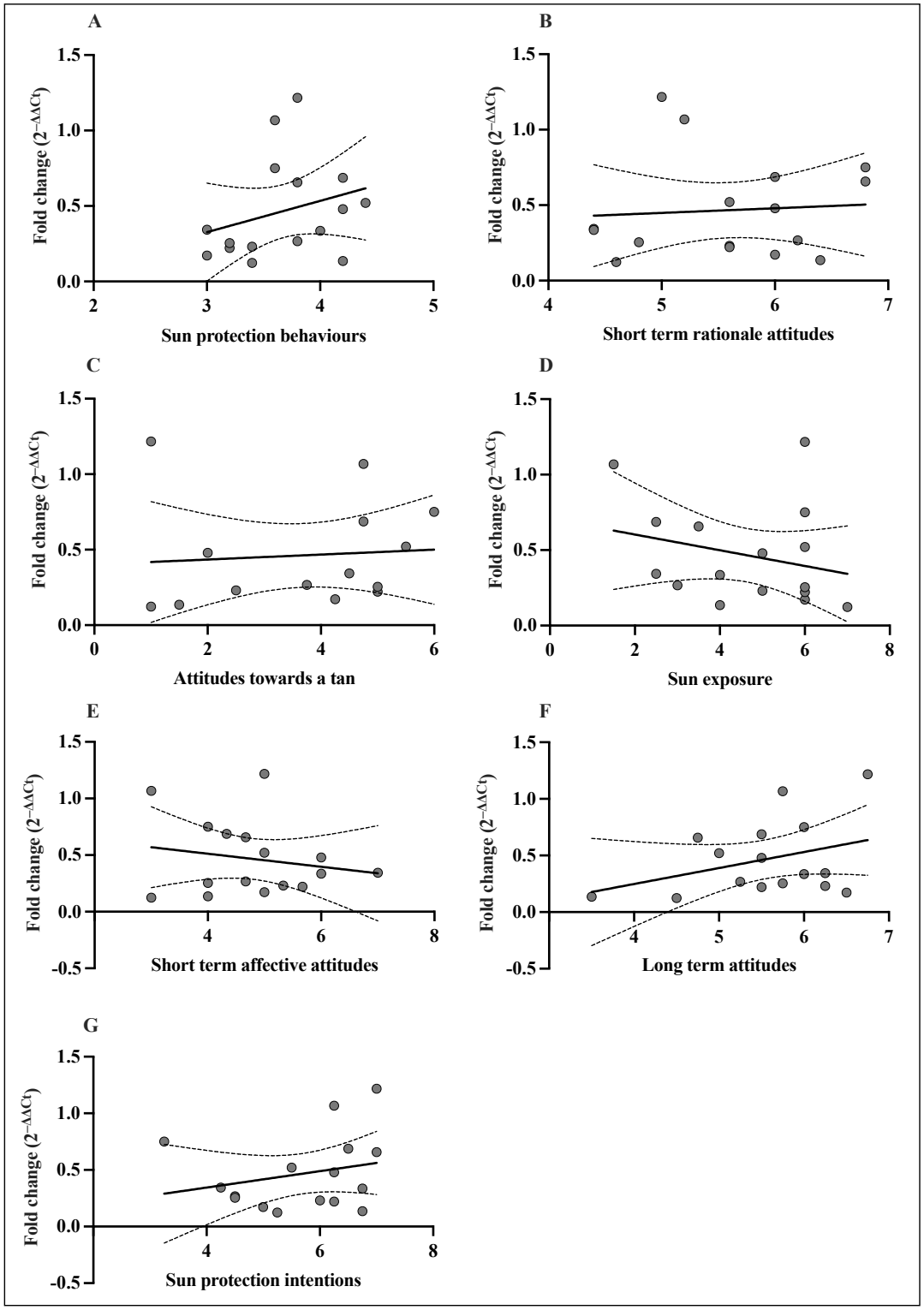


Figure 4.4 Significant correlations were not observed between sun exposure and protection variables and mtDNA damage.

83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Samples obtained from the nose were normalised to the inner arm using the $2^{-\Delta\Delta Ct}$ method, presented as fold change. A higher fold change value corresponds to a higher level of damage in the nose, in comparison to the inner arm. Data was logged prior to performing statistical analysis. Pearson's correlation analysis was performed between $\log2(2^{-\Delta\Delta Ct})$ and (A) sun protection behaviours (n=16), (B) short term rationale attitudes (n=16), (C) attitudes towards a tan (n=14), (D) sun exposure (n=16), (E) short term affective attitudes (n=16), (F) long term attitudes (n=16) and (G) sun protection intentions (n=16).

4.3.2 Study 2: Evaluating the effect of UVR exposure on mtDNA damage in the epidermis

Skin swab samples were collected from the nose and inner arm of 9 participants to investigate whether mtDNA damage following recent exposure to high intensity UVR can be detected using a skin swab (Figure 4.5). Participants were female, had skin types 1 or 2 and were over the age of 30 years.

A higher level of mtDNA damage in samples collected from the nose following UVR exposure was observed in 4 individuals, shown by a fold change greater than 1. Similarly, a higher level of damage in samples collected from the inner arm was also observed in 4 individuals; however, the increase was not seen in the same 4 individuals, and a trend in results was not seen (Figure 4.5A). A significant difference between mean fold change in samples collected from the inner arm and nose was not observed ($p=0.60$) (Figure 4.5B).

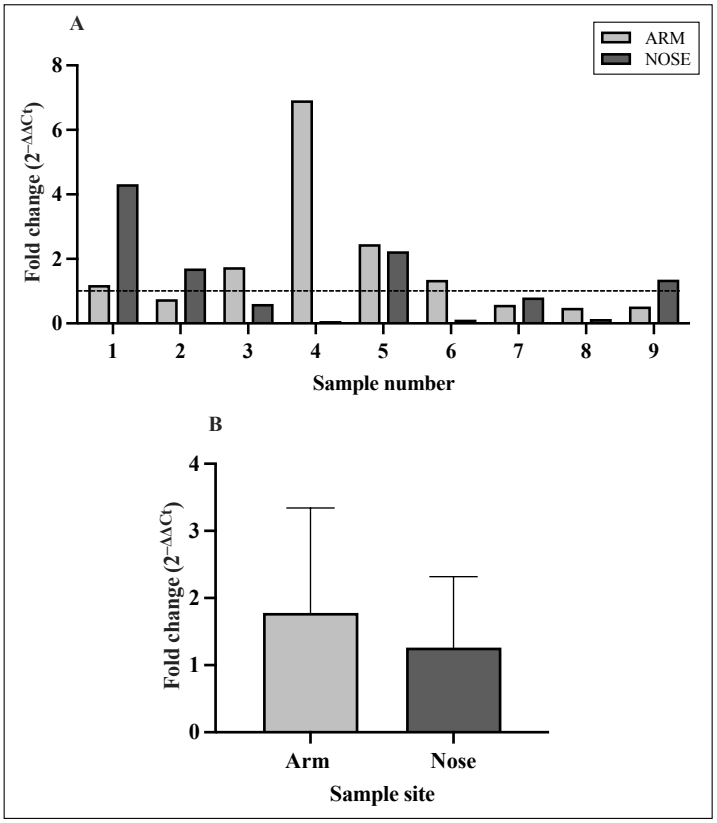


Figure 4.5 Individuals show variation in mtDNA damage following exposure to UVR.
Variation in mtDNA damage in the inner arm (n=9) and nose (n=9) following exposure to UVR. Samples normalised to an internal control (samples collected prior to UVR exposure) using $2^{-\Delta\Delta Ct}$ method, and presented as fold change. 83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. A fold change greater than 1 (shown by dotted line) corresponds to a higher level of mtDNA damage in samples obtained following exposure to UVR. (A) Fold change values for arm and nose samples following exposure to UVR, presented as individual values. (B) Mean fold change for arm and nose samples following exposure to UVR, presented as mean+95%CI. Statistical analysis was performed on logged values and statistical difference was determined using Mann-Whitney test. Samples from 20 individuals were excluded due to incomplete data sets.

4.3.3 Study 3: Evaluating the effect of seasonal variation in UVR exposure on mtDNA damage in the epidermis

Skin swab samples and facial images were collected during spring, summer and autumn months to determine whether seasonal variation in UV levels can be detected using skin swab and UV photography techniques, and whether variation correlates with sun exposure, sun protection habits and demographics.

4.3.3.1 Facial image analysis

UV photography was used to investigate fluctuations in UV spots across different seasons. A significant increase in UV spot count between summer and autumn was observed on the right cheek (p=0.04), increasing from 0.003505 to 0.003544 (Figure 4.6A). Overall, the same trend

was observed in both cheeks, with UV spot count decreasing in summer facial images, followed by an increase in autumn facial images.

A significant increase in %Area was observed between spring and summer on the left cheek, increasing from 27.69 to 27.75% ($p<0.01$) (Figure 4.6B). A significant decrease in %Area was also observed between spring and autumn on the left cheek, decreasing from 27.69 to 26.60% ($p<0.01$). The same pattern was observed on the right cheek, with a significant increase from 26.93 to 27.45% between spring and summer ($p<0.01$), and a significant decrease in %Area from 26.93 to 26.60% between spring and autumn ($p<0.01$). Overall, results showed that UV spot count decreases and %Area increases in summer, followed by an increase in count and decrease in size in autumn.

A significant negative correlation was observed between %Area and UV spot count ($r=-0.84$, $p<0.01$) (Figure 4.6C).

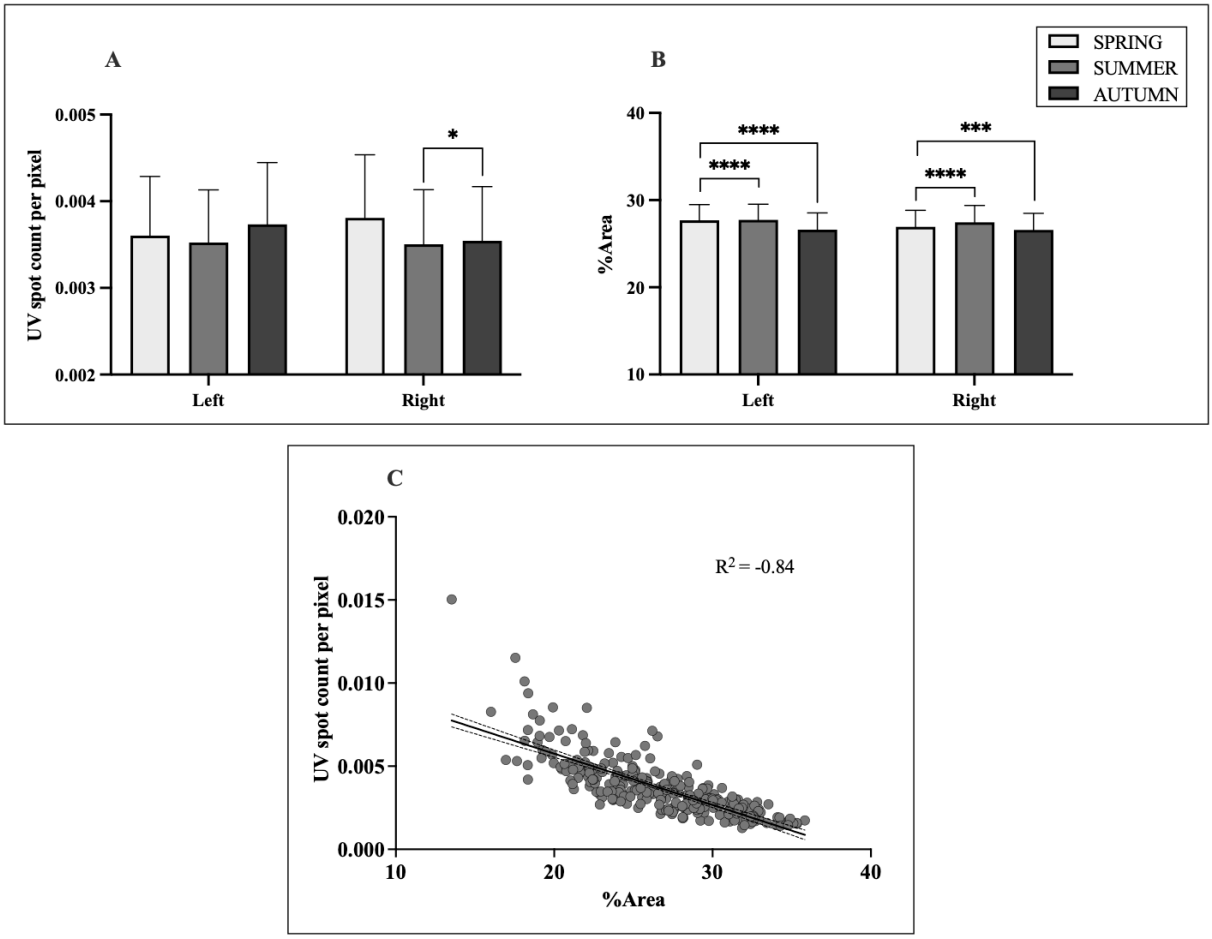


Figure 4.6 UV spots differed significantly between seasons and a significant correlation was observed between UV spot count and %Area.
Facial images were obtained using a VISIA® Skin Analysis system and exported images were analysed in Image J. Spot count was divided by total area to normalise for mask area. (A) UV spot count and (B) UV spot %Area for each season on the left and right cheek (n=27) are presented as mean+95%CI using paired data. Statistical difference was determined by Friedman with Dunn's multiple comparison's test. (C) Spearman's correlation analysis of UV spot count and %Area for facial images obtained in spring, summer and autumn combined (n=288), presented with 95%CI. *p<0.05, ***p<0.001, ****<0.0001. Analyses in (A) and (B) were performed on unstandardized predicted values when controlling for age, sex, skin type, sun exposure and sun protection habits.

A similar trend was observed between age groups from images collected each season from both cheeks, with UV spot count decreasing with age. A significant decrease in UV spot count from 0.00549 to 0.00320 was observed between groups 19-39 and 60+ years from images taken of the left cheek in summer (p=0.02) (Figure 4.7A). Similarly, a decrease in UV spot count from 0.00556 to 0.00325 was observed between groups 19-39 and 60+ years from images taken of the right cheek in summer (p=0.034) (Figure 4.7B).

Regarding UV spot %Area, a similar trend was observed between age groups from images collected each season from both cheeks, with %Area increasing with age. Figure 4.7C shows a significant increase in %Area between groups 19-39 and 60+ years from images taken in spring (p<0.01) and summer (p<0.01) from the left cheek, with increases of 4.73% and 5.28%,

respectively. Figure 4.7D also shows an increase in %Area between groups 19-39 and 60+ from images taken in spring (p=0.01) and summer (p=0.04) from the right cheek, with increases of 4.60% and 5.39%, respectively. Results are supported by Figure 4.8, which show UV spots converging with age; therefore, as %Area increases, UV spot count decreases.

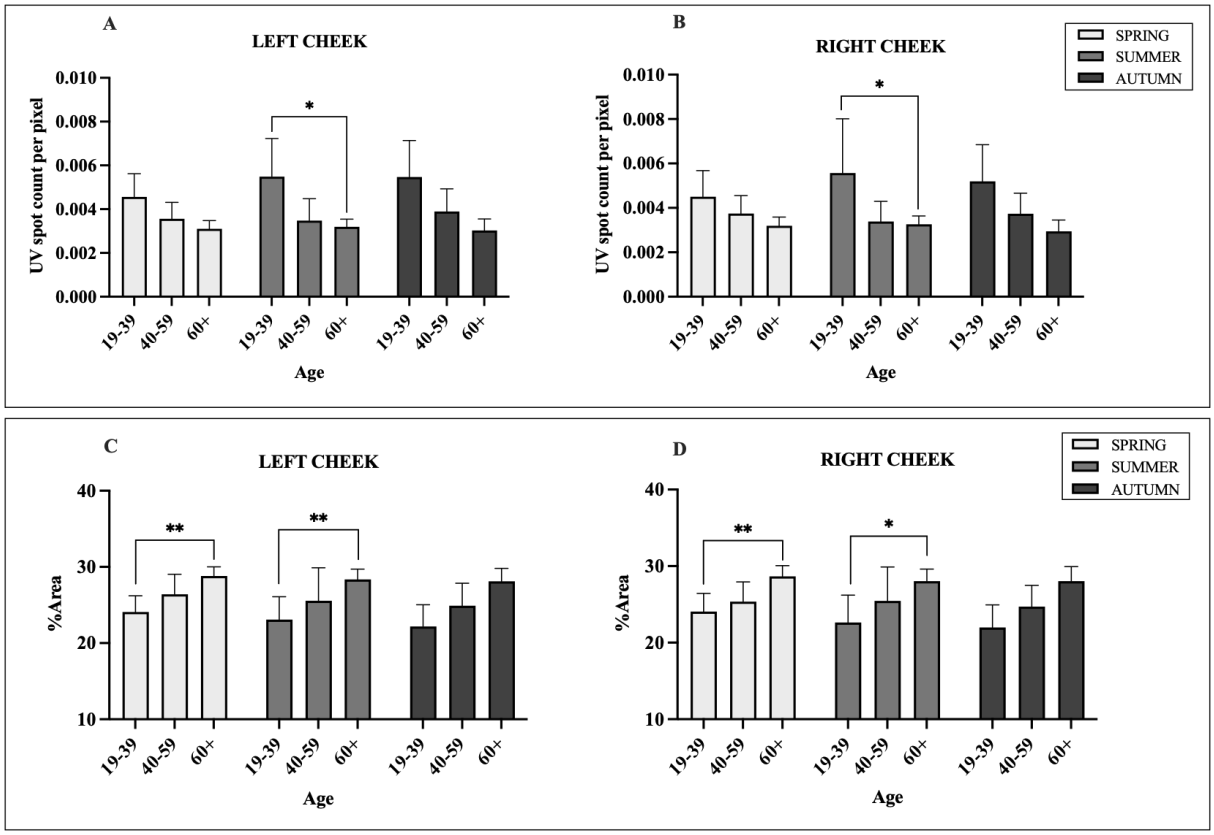


Figure 4.7 UV spot count significantly decreased and %Area significantly increased with age.
Facial images were obtained using a VISIA® Skin Analysis system and exported images were analysed using Image J. Spot count was divided by total area to normalise for mask area. Age was grouped into 19-39, 40-59 and 60+ to determine differences in UV spot count and %Area in spring (n=14, n=10, n=33), summer (n=11, n=7, n=26) and autumn (n=11, n=8, n=22). (A) Variation in UV spot count between age groups on the left and (B) right cheek. (C) Variation in UV spot %Area between age groups on the left and (D) right cheek. Data are presented as mean+95% CI. Statistical difference determined by Kruskal-Wallis test with multiple comparisons. Statistical analysis was performed on unstandardized predicted values when controlling for sex, skin type, sun exposure and sun protection habits. *p<0.05, **p<0.01.

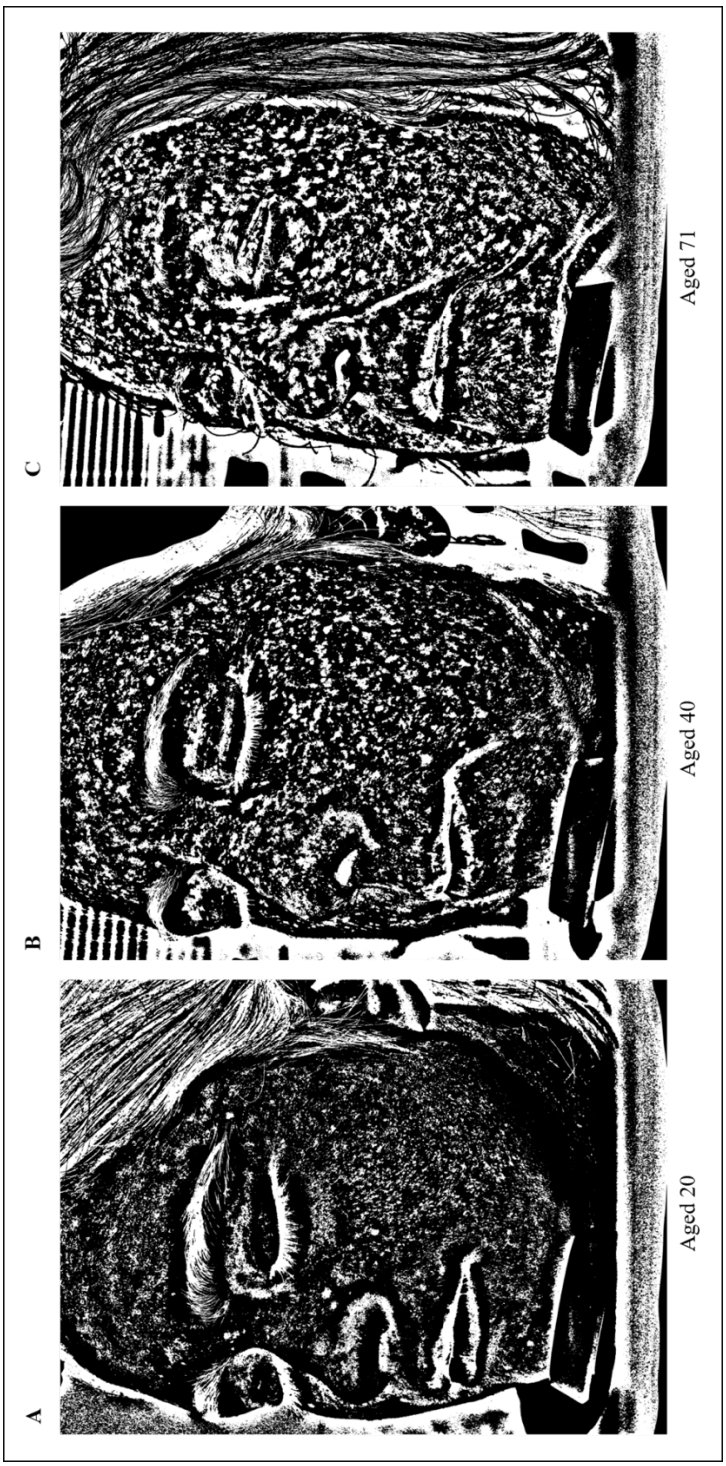


Figure 4.8 UV spots converge with increasing age resulting in greater %Area and lower spot count. Individuals of the same sex and skin type were selected at random from different age groups. UV spots are shown in white and converge with increasing age.

A similar trend in UV spot count was observed between skin type groups from facial images collected across all seasons, from both cheeks. UV spot count increased between skin type groups 1 and 3; however, the difference was not found to be significant (Figure 4.9A and B). A significant difference in %Area was not observed between skin type groups from facial images collected across all seasons, from both cheeks (Figure 4.9C and D).

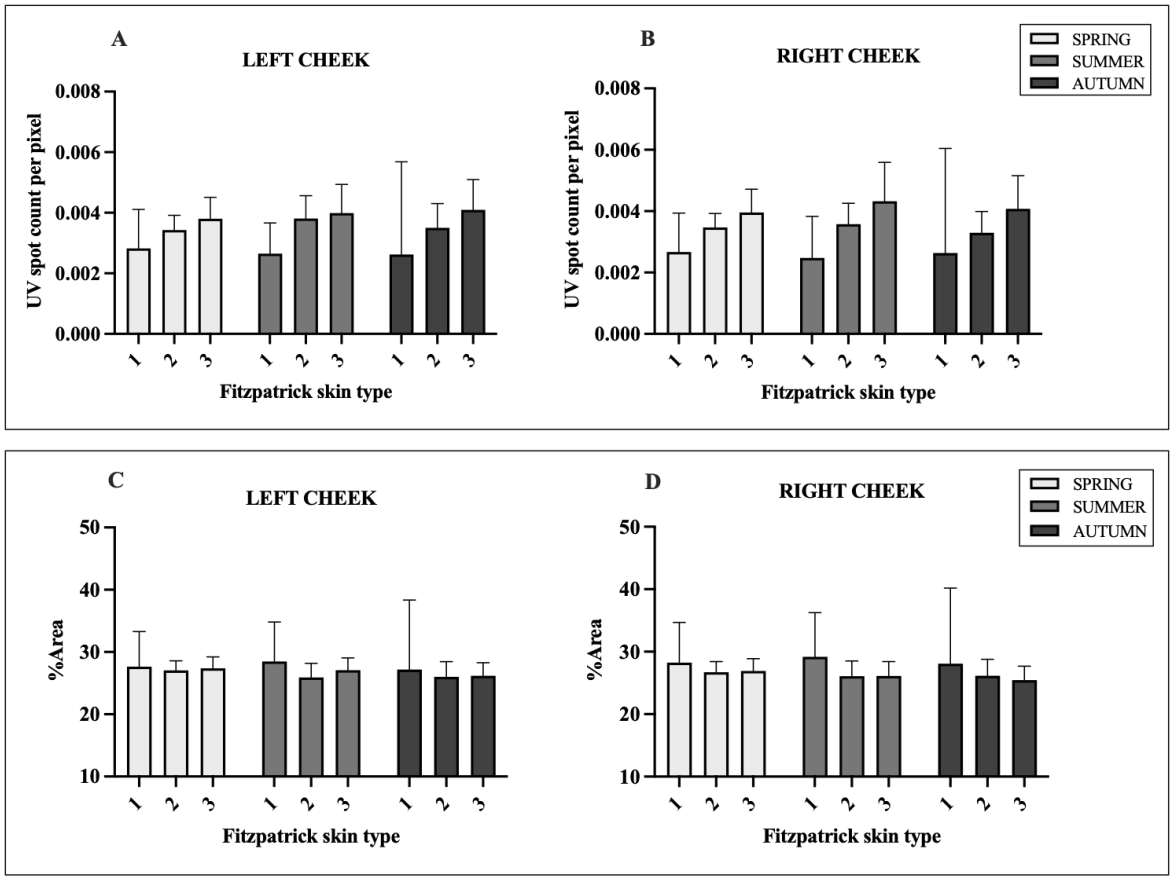


Figure 4.9 Skin type did not affect UV spot count and %Area.
Facial images were obtained using a VISIA® Skin Analysis system and exported images were analysed using Image J. Spot count was divided by total area to normalise for mask area. Data was grouped into Fitzpatrick skin types 1, 2 and 3 to investigate differences in UV spot count and %Area in spring (n=5, n=27, n=23), summer (n=4, n=18, n=21) and autumn (n=3, n=19, n=17). (A) Variation in UV spot count between skin type groups on the left and (B) right cheek. (C) Variation in UV spot %Area between skin type groups on the left and (D) right cheek. Data are presented as mean+95%CI. Statistical difference was determined by Kruskal-Wallis test. Statistical analysis was performed on unstandardized predicted values when controlling for age, sex, sun exposure and sun protection habits.

A significant negative correlation was observed between sun protection behaviours and UV spot count obtained from facial images collected in spring ($r=-0.46$, $p<0.01$), summer ($r=-0.44$, $p<0.01$) and autumn ($r=-0.42$, $p<0.01$) (Figure 4.10A). A significant positive correlation was observed between sun protection behaviours and UV spot %Area obtained from facial images collected in spring ($r=0.37$, $p<0.01$), summer ($r=0.41$, $p<0.01$) and autumn ($r=0.41$, $p<0.01$) (Figure 4.10B). Sun protection behaviours decreased in those with increased sun exposure in order to tan; however, this difference was not found to be significant when controlling for covariates ($p=0.31$) (Figure 4.10C). A significant difference in UV spots was not observed in individuals with different sun exposure behaviours (data not shown).

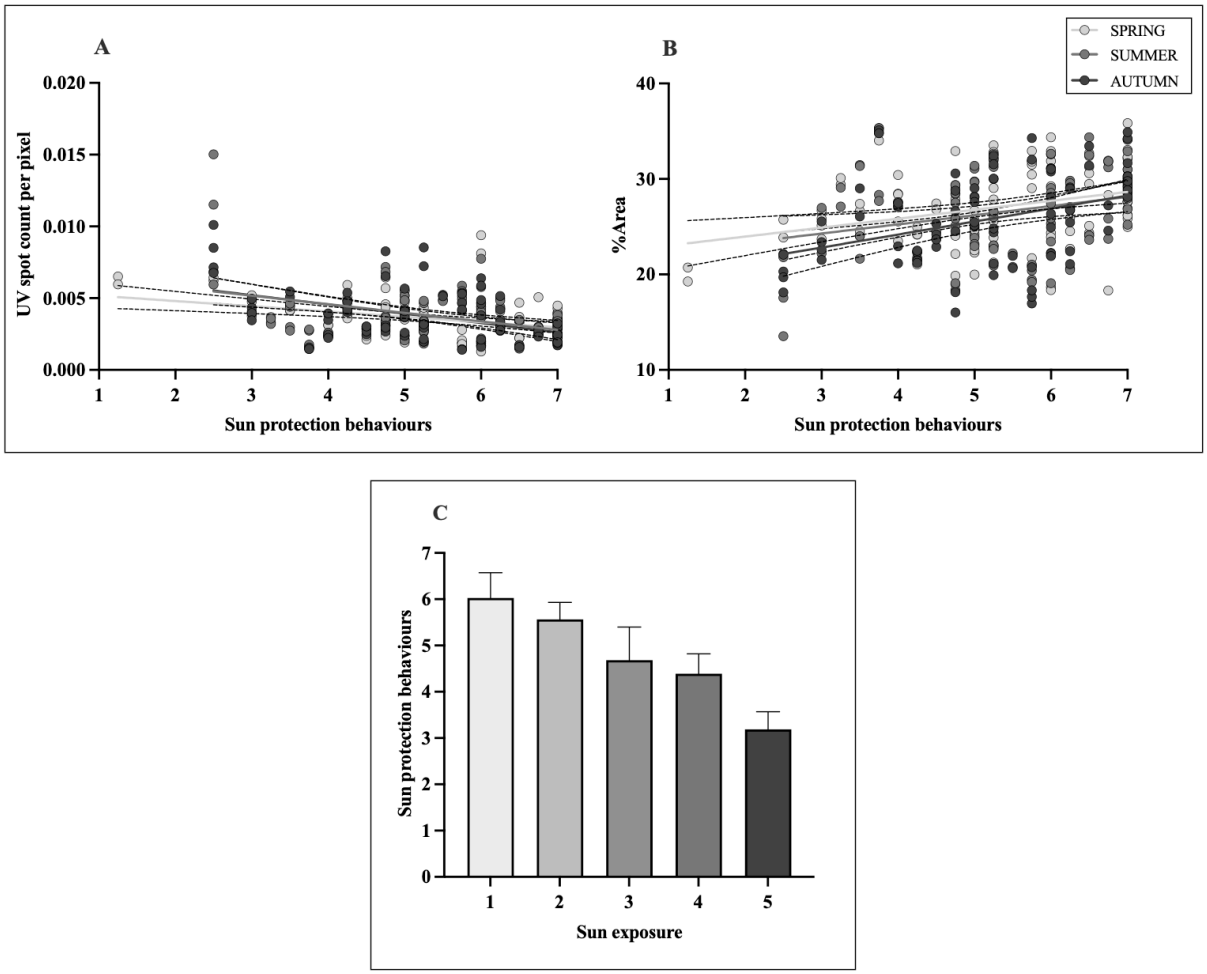


Figure 4.10 UV spot count decreased and %Area increased significantly as sun protection behaviours increased.

Facial images were obtained using a VISIA® Skin Analysis system. Images were exported and analysed using Image J. Spot count was divided by total area to normalise for mask area. (A) Spearman's correlation analysis between UV spot count and sun protection behaviours in spring (n=144), summer (n=88) and autumn (n=82), presented with 95%CI. Statistical analysis was performed on unstandardized predicted values, when controlled for age, sex, skin type and sun exposure. (B) Spearman's correlation analysis between UV spot %Area and sun protection behaviours in spring (n=144), summer (n=88) and autumn (n=82), presented with 95%CI. Statistical analysis was performed on unstandardized predicted values, when controlled for age, sex, skin type and sun exposure. (C) Variation in sun protection habits in individuals in sun exposure groups 1-5 (n=20, n=24, n=23, n=9 and n=4), presented as mean+95%CI. Statistical difference was determined by Kruskal-Wallis test. Analyses were controlled for age, sex and skin type.

4.3.3.2 Mitochondrial DNA damage

Skin swabs were collected to investigate mtDNA damage fluctuations across different seasons. From samples collected in summer, a significant decrease of 6.56 %DNA amplified was observed between the left cheek and the arm ($p<0.01$) (Figure 4.11A). A significant decrease of 7.15 %DNA amplified was also observed between the right cheek and arm ($p<0.01$). Significance was not observed between sample sites in samples collected in spring and autumn.

The same pattern was observed for each sample site between seasons, with the lowest %DNA amplified observed in spring and highest %DNA amplified in summer (Figure 4.11B). A significant increase of 13.91 %DNA amplified was observed between spring and summer ($p<0.0001$), an increase of 7.16 %DNA amplified between spring and autumn ($p<0.01$) and a decrease of 6.75 %DNA amplified between summer and autumn ($p<0.01$) in samples collected from the left cheek. Regarding the right cheek, a significant increase of 14.14 and 7.71 %DNA amplified was observed between spring and summer ($p<0.01$) and spring and autumn ($p=0.03$), respectively. Finally, samples collected from the arm showed a significant increase of 3.36 and 1.15 %DNA amplified between spring and summer ($p=0.02$) and spring and autumn ($p=0.05$), respectively.

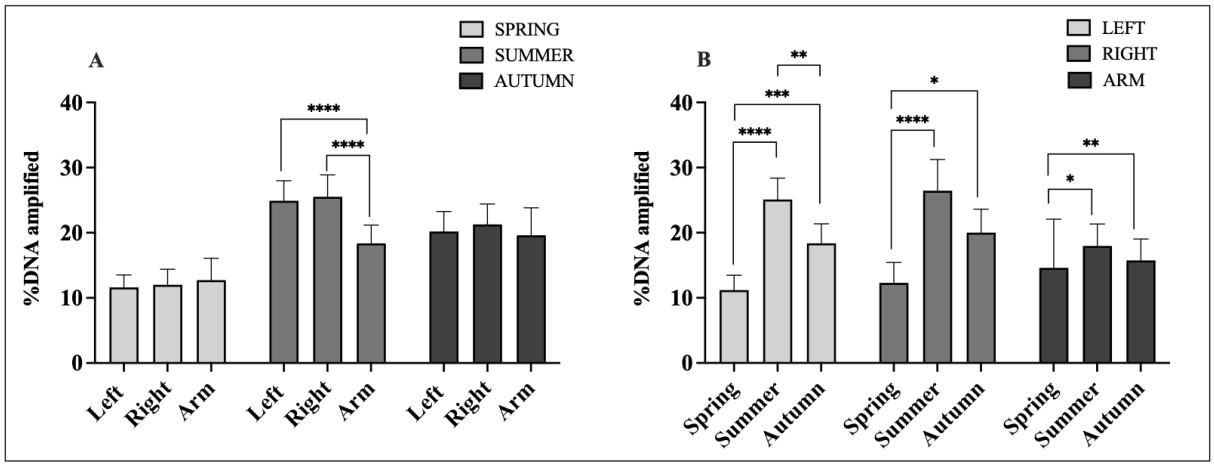


Figure 4.11 Mitochondrial DNA damage was significantly higher in samples collected in spring.
83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified. A higher %DNA amplified corresponds to a lower level of mtDNA damage. (A) Variation in mtDNA damage between different sample sites in spring (n=51), summer (n=49) and autumn (n=43). Statistical difference was determined by Friedman with Dunn's multiple comparisons test. Data are not paired between seasons and are presented as mean+95%CI. No significant covariates were found. (B) Seasonal variation in mtDNA damage in left cheek (n=38), right cheek (n=33) and arm (n=22). Statistical difference was determined by One-Way ANOVA with Tukey's multiple comparisons test and Friedman with Dunn's multiple comparisons test. Data are not paired between sample sites and are presented as mean+95%CI. No significant covariates were found. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

A significant difference in %DNA amplified was not observed between age groups for each sample site in spring (Figure 4.12A), summer (Figure 4.12B) or autumn (Figure 4.12C) ($p>0.05$). A consistent pattern was not observed.

A significant difference in %DNA amplified was not observed between Fitzpatrick skin type groups for each sample site in spring (Figure 4.12D), summer (Figure 4.12E) or autumn (Figure 4.12F) ($p>0.05$). A consistent pattern was not observed.

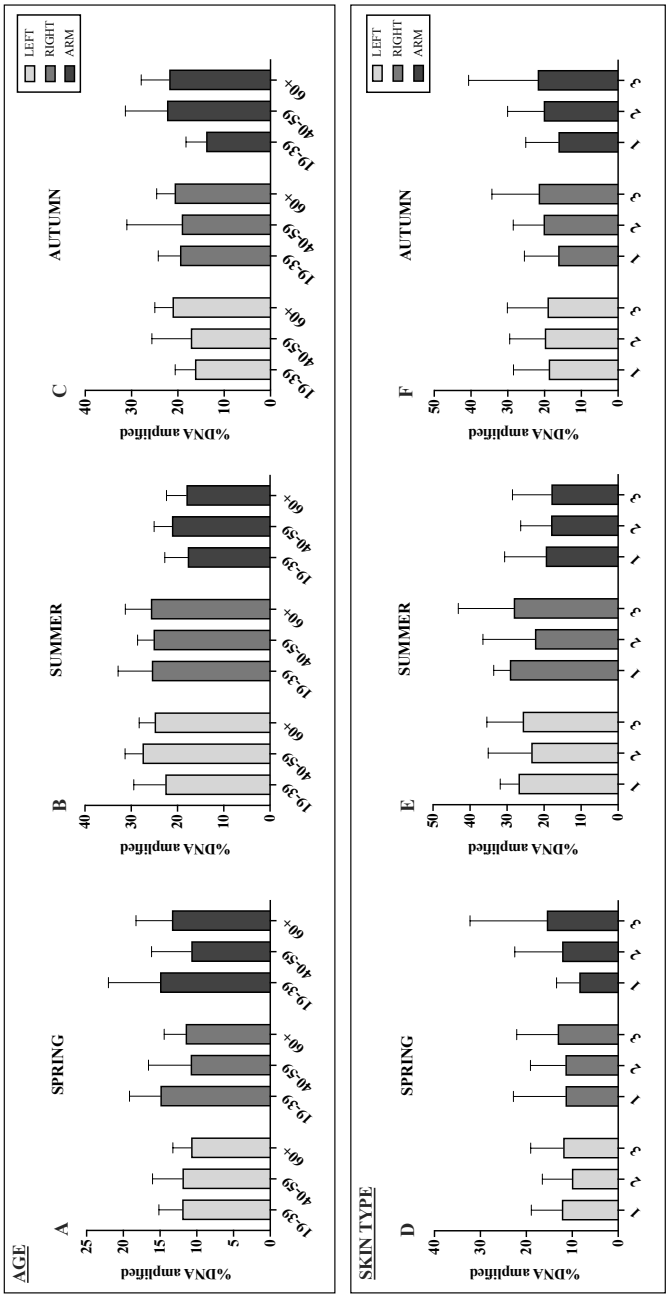


Figure 4.12 Age and skin type did not affect mtDNA damage.
83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified. A higher %DNA amplified corresponds to a lower level of mtDNA damage. (A) Variation in %DNA amplified between age groups in samples collected in spring from the left cheek (n=20, n=12, n=34), right cheek (n=19, n=11, n=35) and arm (n=10, n=11, n=31). (B) Variation in %DNA amplified between age groups in samples collected in summer from the left cheek (n=15, n=8, n=36), right cheek (n=16, n=8, n=33) and arm (n=16, n=9, n=27). (C) Variation in %DNA amplified between age groups in samples collected in autumn from the left cheek (n=13, n=9, n=31), right cheek (n=13, n=8, n=30) and arm (n=10, n=6, n=29). (D) Variation in %DNA amplified between skin type groups in samples collected in spring from the left cheek (n=6, n=29, n=27), right cheek (n=7, n=26, n=29) and arm (n=7, n=27, n=24). (E) Variation in %DNA amplified between skin type groups in samples collected in summer from the left cheek (n=6, n=26, n=25), right cheek (n=6, n=23, n=25) and arm (n=5, n=21, n=23). (F) Variation in %DNA amplified between skin type groups in samples collected in summer from the left cheek (n=5, n=24, n=22), right cheek (n=5, n=23, n=21) and arm (n=2, n=24, n=17). Data are not paired between sample sites and are presented as mean+95%CI. Statistical difference was determined by One-Way ANOVA and Kruskal-Wallis test. No significant covariates were found.

Correlations between %DNA amplified and UV spots were not statistically significant (p>0.05) (Figure 4.13); however, a trending positive correlation was observed between %DNA amplified and UV spot count in spring from samples collected and images taken from the right side of the face (r=0.24, p=0.09) (Figure 4.13A).

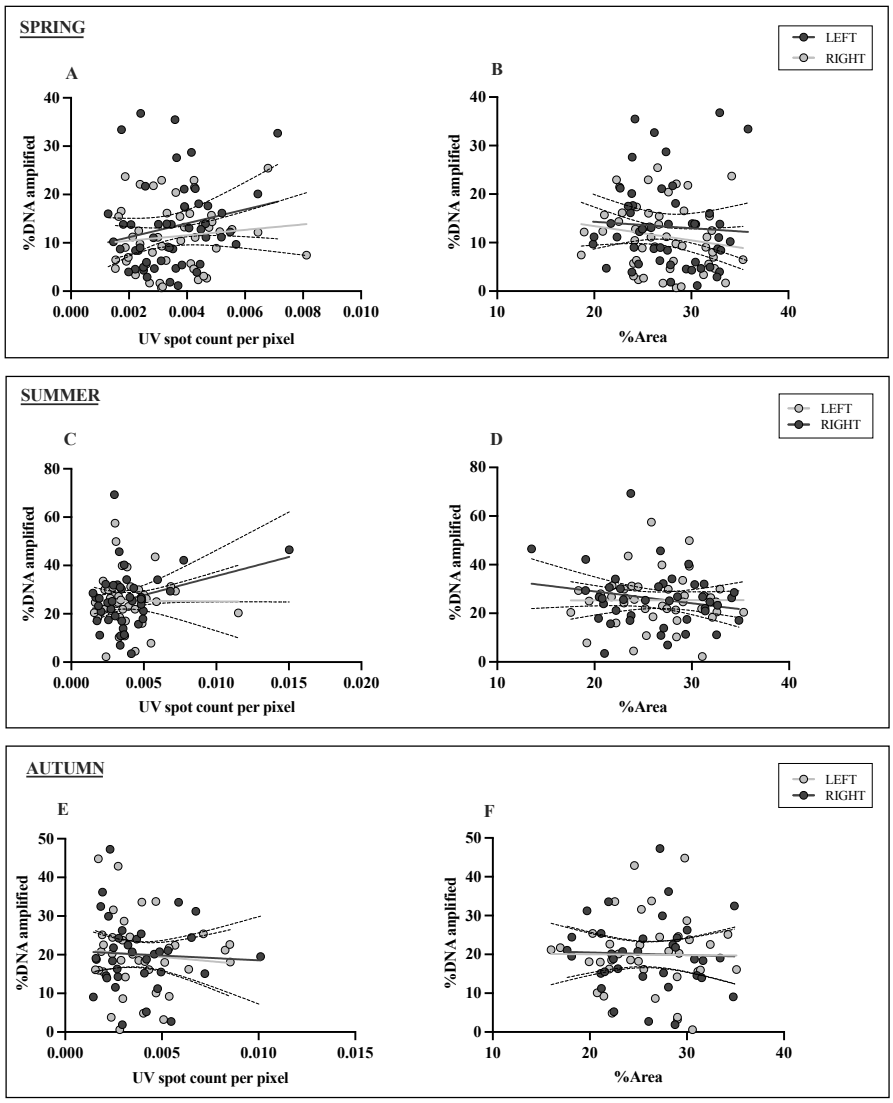


Figure 4.13 Significant correlations were not observed between mtDNA damage and UV spot count and %Area.
83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified, with a higher value corresponding to a lower level of damage. Facial images were obtained using a VISIA® Skin Analysis system and exported images were analysed using Image J. Spot count was divided by total area to normalise for mask area. (A) Spearman's correlation between %DNA amplified and count from spring samples on left (n=50) and right (n=49) cheeks. (B) Pearson's correlation between %DNA amplified and %Area from spring samples on left cheek (n=50) and Spearman's correlation on right cheek (n=49). (C) Spearman's correlation between %DNA amplified and count from summer samples on left (n=42) and right (n=41) cheeks. (D) Pearson's correlation between %DNA amplified and %Area from summer samples on left cheek (n=42) and Spearman's correlation on right cheek (n=41). (E) Spearman's correlation analysis between %DNA amplified and count from autumn samples on left (n=37) and right (n=35) cheeks. (F) Pearson's correlation between %DNA amplified and %Area from autumn samples on left (n=37) and right (n=35) cheeks. No significant covariates were found.

Correlations between %DNA amplified and sun protection behaviours in samples collected from the left and right cheek in spring (Figure 4.14A) and autumn (Figure 4.14C) were not found to be statistically significant ($p>0.05$). A significant positive correlation was observed between %DNA amplified and sun protection behaviours in samples collected from the left cheek in summer ($r=0.30$, $p=0.02$); however, a significant correlation was not observed in samples collected from the right cheek ($p>0.05$) (Figure 4.14B).

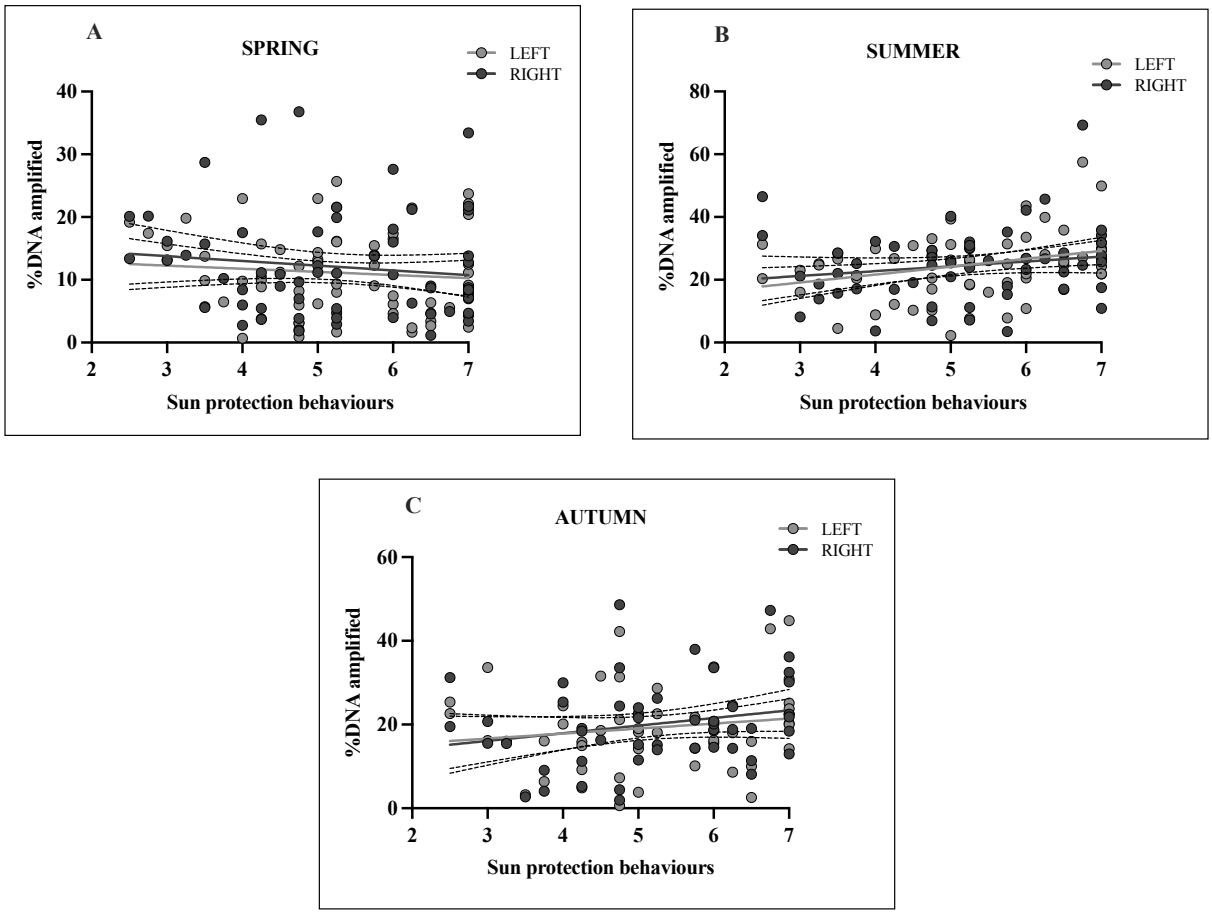


Figure 4.14 A significant correlation was observed between sun protection behaviours and mtDNA damage from samples collected in summer from the left cheek.
83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified, with a higher %DNA amplified corresponding to a lower level of damage. (A) Correlation between %DNA amplified and sun protection behaviours from samples collected in spring on the left (n=64) and right (n=63) cheek. (B) Correlation between %DNA amplified and sun protection behaviours from samples collected in summer on the left (n=60) and right (n=56) cheek. (C) %DNA amplified and sun protection behaviours from samples collected in autumn on the left (n=52) and right (n=50) cheek. Spearman's correlation analysis was performed. No significant covariates were found.

4.3.4 Study 4: Evaluating the effect of UVR on mtDNA damage in the epidermis and dermis *in vitro*

4.3.4.1 Investigation of damage in 3D full-thickness skin equivalents using a skin swab technique

Skin swabs were collected from skin equivalents following exposure to complete solar light to investigate levels of mtDNA damage detected immediately and on day 3.

Although a lower %DNA amplified was observed in the treatment group in comparison to the control group in day 1 skin equivalents, a significant difference between groups was not observed (Figure 4.15A). Similarly, a significant difference between day 3 groups was not observed.

Overall, the epidermis had significantly lower %DNA amplified for both control and treatment groups on day 1 and 3 in comparison to the dermis (significance not shown on graph) (Figure 4.15B). Statistical differences were not observed between epidermal groups. Although a decrease in %DNA amplified was observed in the treatment group in comparison to the control group in the dermis, the difference is not statistically significant. Both the control and treatment group on day 3 show similar levels of damage and no significance was observed between groups. A significant increase of 23.02 %DNA amplified was observed between treatment groups on day 1 and 3 in the dermis ($p=0.02$).

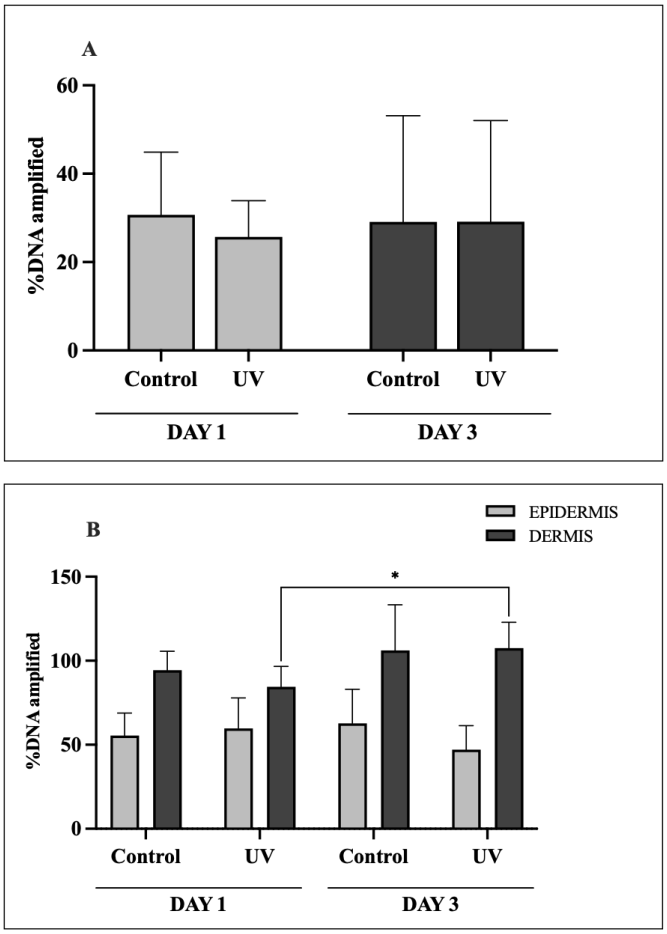


Figure 4.15 A decrease in mtDNA damage following exposure to complete solar simulated light was observed on day 3 in the dermis of 3D full-thickness skin equivalents.
Treatment skin equivalents were exposed to 2.16 SED complete solar simulated light on day 1. Control skin equivalents were not exposed to solar simulated light. 83 bp and 1kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified with a higher %DNA amplified corresponding to a lower level of mtDNA damage. (A) Skin swabs collected from control and treatment skin equivalents on day 1 (n=6) and day 3 (n=3). Statistical difference between control and treatment groups on day 1 and 3 was determined by unpaired t-test and Mann-Whitney test, respectively. Data are presented as mean+95%CI. (B) Variation in mtDNA damage in epidermal and dermal layers on day 1 and 3 (n=3). Statistical difference between groups was determined by One-Way ANOVA with Tukey's multiple comparisons test. Data are presented as mean+95%CI. *p<0.05.

4.3.5 Study 5: Evaluating the effect of UVR exposure on mtDNA damage in the epidermis and dermis

Biopsy samples were collected before and after exposure to UVR over summer months to investigate whether differences in mtDNA damage as a result of UVR exposure as well as age can be detected within skin biopsies.

A significant difference in mtDNA damage was not observed between samples collected prior to and post-UVR exposure in groups 20-40 years (p=0.13) and 41-60 years (p=0.45) (Figure 4.16A). Although significance was not observed, mtDNA damage decreased in both age groups

following UVR exposure. A significant increase in mtDNA damage was not observed in the absence of UVR between age groups (p=0.12); however, a significant 3.48-fold increase in mtDNA damage was observed between age groups following UVR exposure (p<0.01), with individuals within the 41-60 years group having greater damage.

A significant positive correlation was observed between age and mtDNA damage in samples collected following UVR exposure (r=0.43, p<0.01); however, a significant correlation was not observed in samples collected prior to UVR exposure (r=0.26, p=0.13) (Figure 4.16B).

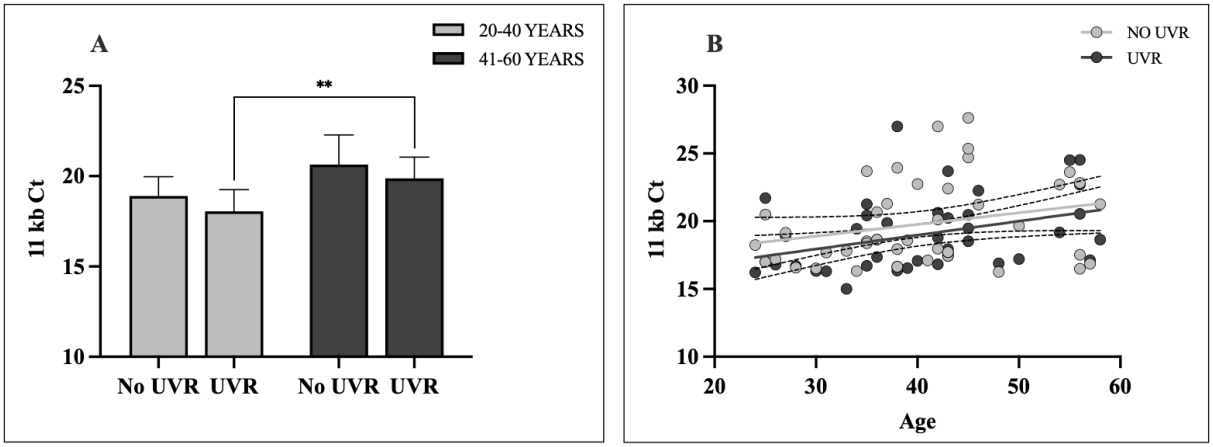


Figure 4.16 Mitochondrial DNA damage is higher in older individuals when using a skin biopsy technique.
Mitochondrial DNA content and damage were determined using the 83 bp and 11 kb qPCR assays, respectively. (A) Variation in mtDNA damage before and after UVR exposure between individuals within age groups 20-40 (n=21) and 41-60 years (n=20). Statistical difference between samples collected prior to UVR exposure and post-UVR exposure was determined by paired t-test and Wilcoxon test in groups 20-40 and 41-60 years, respectively. Statistical difference between different age groups in samples collected prior to UVR exposure and post-UVR exposure was determined by Mann-Whitney test. Data are presented as mean+95%CI. (B) Spearman's correlation analysis between age and mtDNA damage in the presence (n=41) and absence (n=42) of UVR. **p<0.01.

4.3.6 Study 6: Evaluating differences in mtDNA damage between samples collected with skin swab and tape strip collection methods

A preliminary investigation of the difference in %DNA amplified when samples were collected using skin swab and tape strip techniques was performed.

Overall, %DNA amplified seemed to increase between skin swabs 1 to 10 when collected from the same site consecutively; however, %DNA amplified remained consistent between tape strips (Figure 4.17A). Samples collected using a skin swab showed a higher %DNA amplified in the sample collected from the cheek in comparison to the arm; however, samples collected using a tape strip showed a much higher %DNA amplified in the sample collected from the arm

(Figure 4.17B). Comparing the collection methods, a lower %DNA amplified was observed in the sample collected from the cheek with the tape strip, in comparison to the swab. On the other hand, a lower %DNA amplified was observed in the sample collected from the arm with the swab, in comparison to the tape strip.

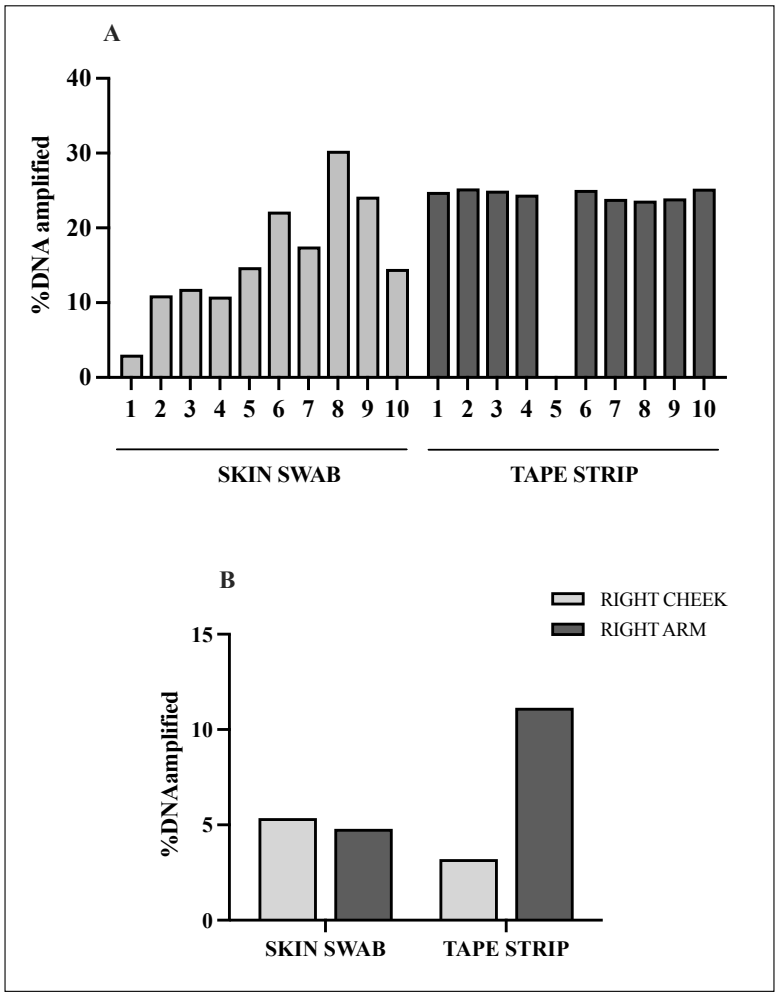


Figure 4.17 %DNA amplified varies between samples when collected using skin swab and tape strip techniques.
83 bp and 1 kb qPCR assays were used to determine mtDNA content and damage, respectively. Ct values were interpolated from a standard curve of known plasmid concentration and used to calculate %DNA amplified, with a higher %DNA amplified corresponding to a lower level of mtDNA damage. (A) 10 skin swabs were collected consecutively from the same site on the left arm and 10 tape strips were collected consecutively from the same site on the right arm in the same individual (n=1). (B) Skin swab and tape strip taken from the same sample site on the right arm and cheek in the same individual (n=1). Data are presented as individual values. Missing sample due to lack of qPCR amplification.

4.4 Discussion

The overall aim of this chapter was to investigate variation in mtDNA damage between individuals, as well as the effect of UVR on mtDNA damage, with a focus on sun exposure. Sun protection behaviours and demographics.

4.4.1 Study 1: Pilot study evaluating mtDNA damage variation in the epidermis

Previous research has highlighted the ability of a skin swab to detect increases in mtDNA damage; more specifically the 3895 bp deletion, as a result of UVR exposure within the epidermis of human skin (50). As the use of a skin swan is an underreported field of study, a pilot study was initially performed. The aim was to investigate whether variation in mtDNA damage; in the form of strand breaks, between individuals can be detected using a skin swab, and whether variation correlates with sun exposure and protection habits.

Of the 17 individuals, 2 showed a higher level of mtDNA damage in samples collected from the frequently sun-exposed nose; whereas, the remaining 15 showed more damage in samples collected from the sun-protected inner arm. Although the pilot study used a rapid and quantitative method to evaluate the relative levels of damage in mtDNA by real-time PCR by amplifying a 1 kb fragment of the mitochondrial genome (70); in comparison to quantification of the 3895 bp deletion used by Harbottle *et al*. (2010) (50), the findings were contradictory. All participants were over the age of 30 years; however, research suggests that skin thickness varies according to age and body site (81). Differences in skin thickness may therefore influence sample collection, which led us to question whether the inner arm is an effective internal control. In contrast, research has also shown that there is not a significant difference in morphological parameters between forearm skin in comparison to other body sites (100, 101). In addition to this, studies have also shown epidermal thickness to increase following exposure to UVR; also known as hyperkeratosis, which may result in the collection of more late stage differentiated keratinocytes or corneocytes with the swab; therefore, influencing results. UVR induces damage response pathways in keratinocytes as a result of cell injury. These damage signals such as p53 activation result in the alteration of keratinocyte physiology; therefore, mediating cell cycle arrest, activating DNA repair and inducing apoptosis if the damage is sufficient. A few hours after exposure to UVR, damage response signals decrease, and epidermal keratinocytes proliferate rapidly. Increased keratinocyte division following exposure to UVR results in the accumulation of epidermal keratinocytes which therefore increases epidermal thickness overall, protecting the skin against further UVR penetration (20, 102-105).

Exposure to UVB has also been shown to decrease intercellular cohesion which may affect sample collection between different sites and individuals due to differences in exposure, subsequently affecting the number of cells collected (77). Variation in daily exposure to UVR due to factors such as lifestyle, cosmetic products and occupation may therefore influence sample collection from the nose; however, significant correlations were not observed between mtDNA damage and sun protection and exposure variables. In comparison, other studies have linked sun exposure to visible and biological signs of skin ageing (104-106). As study results did not show any trends in results, further studies were planned to investigate whether the swab is able to detect mtDNA damage as a result of recent UVR exposure, as well as differences in demographics such as age and skin type.

The lack of qPCR amplification within mtDNA samples was assumed to be due to low concentrations within samples. Methodology was optimised to adjust the DNA volume added to each qPCR reaction as well as the DNA extraction kit used, which subsequently increased the yield of DNA obtained from skin swabs. Following the pilot study, a buffer was introduced which increased the number of cells obtained during sample collection and maintained DNA integrity before DNA extraction was performed (Section 3.3.4).

4.4.2 Study 2: Evaluating the effect of UVR exposure on mtDNA damage in the epidermis

The following study aimed to investigate whether mtDNA damage as a result of high intensity UVR such as during a summer holiday or as a result of using an artificial tanning bed, can be detected using a skin swab.

Although mtDNA damage has been assessed to confirm exposure to UVR in previous studies (107), consistency was not observed within our results obtained using a skin swab, with a higher level of mtDNA damage observed in samples collected from the nose following exposure to UVR in 4 individuals. Samples from the remaining 5 individuals showed a lower level of mtDNA damage in samples collected from the nose following exposure to UVR. Regarding the inner arm, a higher level of mtDNA damage following exposure to UVR was observed in 4 individuals and the remaining 5 individuals showed a lower level of mtDNA damage following exposure; however, the same individuals did not show a higher level of mtDNA damage in both the nose and arm. Samples were collected from individuals as shortly after exposure to UVR as possible; however, this wasn't controlled between individuals, which could potentially explain the differing results. Due to UVA having the ability to penetrate both the epidermis and dermis,

as well as the dermis having a slower turnover rate, studies have shown damage to accumulate within the dermis over time (45, 50). As a result, it was hypothesised that damage caused by UVR exposure may not be detectable with the swab immediately after exposure due to accessing only the epidermal layer. This is also supported by the fact that, in humans, the epidermis is it is estimated to turn over every 40-56 days (2); therefore, it may take time for mtDNA damage to reach the surface of the skin to be detected by a skin swab. Studies have also shown differences in skin turnover rates as a result of chronological ageing; with older cells turning over more slowly (82, 108). This may also influence results obtained using a skin swab as age was not controlled for during the study, although all individuals were over the age of 30 years.

The formation of apoptotic keratinocytes as a result of UVB; also known as sunburn cells, may also affect results obtained using a skin swab due to loss of the stratum corneum. Sunburn cells are keratinocytes which are undergoing apoptosis due to being exposed to a UVB dose that has irreversibly and severely damaged their DNA. Genomic DNA is the major chromophore for UVB; therefore, resulting in damage and subsequently activating the apoptotic pathway which is a protective mechanism against the carcinogenic effects of UVB (109). Studies have demonstrated the release of tumour necrosis factor-α (TNF-α), which has been shown to result in apoptosis in cells. In addition to this, research has also shown that UVB induces the release of TNF-α by keratinocytes and that keratinocytes express the 55-kD receptor for the ligand TNF-α (110). Differences in sun exposure and sunburn incidence between individuals may therefore affect results due to peeling of the stratum corneum following a large dose of UVB. As well as this, this is only expected to happen to sun-exposed sample sites such as the nose, rather than sun-protected sites such as the inner arm. This provides an argument for the development of a new method of normalising results, without the need for an 'internal control' sample site.

Finally, as previously mentioned, the epidermis is shown to increase in thickness following exposure to UVR in order to provide increased protection against UVR and may therefore impact the results both between individuals with different sun exposure habits, as well as between the sun-exposed and sun-protected sample sites (20, 102-105). The data obtained from the two studies described differ to those presented by Harbottle *et al*. (2010) (50), which showed the highest level of mtDNA damage in the face, followed by the arm and heel. Optimisation work (Section 3.3.2) showed the same pattern when assessing mtDNA content within the face, arm and heel. This suggests that the data previously published show differences in mtDNA content as opposed to differences in the amount of 3895 bp deletion present in each of the sites.

The method of optimisation used in the paper is PicoGreen, which works by quantifying all double-stranded DNA molecules and does not have the ability to distinguish between nDNA and mtDNA; therefore, variation in mtDNA content between sample sites has the potential to affect results.

Moving forward, the %DNA amplified method was developed (Section 3.2.1). This method quantifies mtDNA damage within samples and allows mtDNA damage between samples run on different plates to be compared more accurately. As well as this, it removes the ambiguity around the use of the inner arm as an internal control which is required for fold change methodology.

4.4.3 Study 3: Evaluating the effect of seasonal variation in UVR exposure on mtDNA damage in the epidermis

Due to the interindividual variation observed using the skin swab technique, a study involving the same individuals was performed to determine whether differences in mtDNA damage and UV spots can be detected as a result of seasonal variation in UVR levels, using skin swab and facial imaging techniques, respectively. The study also aimed to investigate whether correlations could be observed between damage detected using a skin swab and facial imaging, as well as between sun damage and sun exposure and protection habits. Work expanded on studies previously performed within this thesis as it included individuals between the age of 19 and 82.

UV imaging is a well-established technique for identifying underlying sun damage and has been previously shown to correlate with lifestyle as well as demographics (72, 91). Facial images were collected from individuals to investigate how UV spot count and %Area differs between seasons. Although it is currently accepted that more and larger UV spots indicate greater damage (91), we found a significant negative correlation between UV spot count and %Area (Figure 4.6C). This therefore highlights convergence of UV spots with increased damage, resulting in fewer UV spots overall (Figure 4.8). Regarding UV spot count, the expected results were observed on the left side cheek between seasons; however, statistical significance between groups was not observed (Figure 4.6A). Count decreased between spring and summer, followed by an increase between summer and autumn, suggesting that UV damage increased between spring and summer and decreased between summer and autumn. As previously mentioned, a higher UV spot count suggests less damage due to lack of convergence; therefore, results were in line with what would be expected. A slightly different pattern was observed on the right

cheek as UV spot count didn't increase as much between summer and autumn (Figure 4.6B); however, UV spot count did decrease between spring and summer and increase between summer and autumn. A significant increase between summer and autumn was observed, suggesting that underlying skin damage in the form of UV spots does vary between seasons as a result of variation in UV levels and damage can be detected using the VISIA® Skin Analysis system. The results also suggest that UV spots may repair to some degree in the absence of UVR exposure. An explanation for the small difference between the left and right cheeks could be that some individuals drive regularly and therefore get more frequent UVR exposure on the right side of the face, a finding previously reported (111). Regarding UV spot %Area, a slight increase is observed between spring and summer on both cheeks, with a significant decrease between summer and autumn on both cheeks (Figure 4.6C and Figure 4.6D). This result supports previous findings that UV spots vary between seasons and may repair to some degree in seasons with lower UVR levels. Previous research has also shown the skin to vary between seasons as a result of factors such as temperature and humidity (112-114).

In terms of age, the same pattern was observed in each season on both cheeks, with UV spot count decreasing and %Area increasing with age (Figure 4.7), also shown by previous studies through the use of UV technology (105). This therefore suggests that the VISIA® Skin Analysis system is able to detect the accumulation of underlying skin damage as a result of chronological ageing. A significant difference between age groups was not observed in autumn for count or %Area on both cheeks; however, the number of facial images obtained in autumn was lower than other seasons, which could be a potential cause of the lack of significance. As well as this, older skin may have less capacity to deal with UVR exposure and prevent damage as much as younger skin; therefore, differences may be more apparent in months with higher UVR levels.

Regarding skin type, although significance was not observed, UV spot count increased between Fitzpatrick skin types 1 and 3, suggesting a decrease in overall damage (Figure 4.9), a finding supported by other research (105). With a higher participant number, studies may observe significant changes in UV spot count with Fitzpatrick skin types. This is especially true in the current study as a small number of participants with skin types 5 and 6 were recruited and therefore could not be involved in statistical analysis. In addition to this, individuals graded their own skin type which could be subjective and may lead to incorrect Fitzpatrick scale placement, and therefore may skew any potential significance.

Correlation analysis showed that individuals with better sun protection behaviours had increased UVR damage, with significantly fewer UV spot count and significantly greater %Area. We hypothesised that individuals may use sun protection as a means of increasing their

sun exposure in order to obtain a tan; however, individuals with the highest sun exposure appeared to have the worst sun protection behaviours (Figure 4.10), although statistical significance was not seen. A limitation of the self-administered questionnaire is that participants may under-report harmful behaviours such as excessive sun exposure. As well as this, it is important to note that behavioural and exposure questions were scored using a Likert-type answers which are popular psychometric item scoring schemes. Although Likert-type responses are easily understood and quantifiable, the literature highlights multiple problems with Likert-type answers, such as middle terms within the scale could be very similar and space between each choice cannot be equidistance apart; therefore, they fail to measure the true attitudes of respondents (115). As well as this, behaviours may change daily depending on what the individual is doing while spending time outdoors in the sun.

In line with data from our previous studies, many individuals had a higher level of mtDNA damage in samples obtained from the arm; in comparison to the left and right cheek, in summer and autumn (Figure 4.11A). As previously mentioned, this was hypothesised to be due to differences in skin thickness due to anatomical site and differing exposure to UVR (20, 105). In each season, the left and right cheek appeared to have similar levels of damage which was expected, suggesting that the swab can accurately detect mtDNA damage. Although the inner arm was not required as an internal control for normalisation; unlike with fold change methodology ($2^{-\Delta\Delta Ct}$), samples were collected to assess any seasonal variation in a non-exposed sample site and to further our knowledge. A significant increase in %DNA amplified was observed between spring and summer samples for each sample site, suggesting that less damage was detected in summer (Figure 4.11B). Once again, this was assumed to be due to skin thickening due to UVR exposure (20, 102-105). %DNA amplified decreased between summer and autumn; however, %DNA amplified was still significantly higher in autumn in comparison to spring, suggesting that skin damage detected by a skin swab is highest in spring. A significant positive correlation was observed between %DNA amplified and sun protection behaviours in samples collected from the left cheek in summer, suggesting that damage detected using the swab may correlate with behaviours; however, significance between other groups was not seen. An explanation for this finding being present only in summer may be that individuals have greater exposure to UVR; therefore, the difference in those who use frequent sun protection and those who don't may be more apparent.

When comparing %DNA amplified and UV spots, correlations were not statistically significant. Although results show that UV spots vary based on age and seasonal variation, results suggest that the skin swab is not an accurate measure under our experimental conditions.

4.4.4 Study 4: Evaluating the effect of UVR on mtDNA damage in the epidermis and dermis *in vitro*

A study using 3D full-thickness skin equivalents was then performed to investigate variation in mtDNA damage following recent exposure to solar simulated light using skin swabs, in order to compare the findings to those *in vivo* using more standardised conditions. It also aimed to investigate mtDNA damage within the epidermal and dermal layers of skin equivalents following exposure in order to determine how the level of damage differs.

Although the difference wasn't statistically significant, a greater level of mtDNA damage was observed in the treatment group on day 1 (Figure 4.15A). On day 3, damage within the control and treatment groups were similar. This suggests that immediate UVR-damage can be detected from the epidermis of skin equivalents on day 1 using a skin swab; however, damage was no longer present or detectable using a swab on day 3, which may be a result of epidermal thickening (103). This study was only performed once and would need to be repeated to draw any firm conclusions. Although skin equivalents comprise both a dermis and epidermis both comprised from primary human cells, and the dermal fibroblasts; embedded in a mechanically stable collagen sponge, synthesise major extracellular matrix proteins like collagens, elastin and fibrillin-1, they lack some major skin components such as melanocytes.

Following extraction of mtDNA from epidermal and dermal layers of the skin equivalents after irradiating with complete solar light, mtDNA damage was significantly higher in the epidermis in comparison to the dermis (significance not shown on graph) (Figure 4.15B). This was expected as the epidermis is composed of 'dead' cornified cells and is the outermost layer of skin, frequently exposed to damaging environmental stressors (116). Levels of %DNA amplified remained relatively constant within the epidermis suggesting that epidermal mtDNA damage does not change much as a result of recent UVR exposure. In contrast, a significant increase in %DNA amplified was observed between treatment groups on day 1 and 3 within the dermis; therefore, suggesting that immediate damage is repaired by day 3 or no longer detectable.

4.4.5 Study 5: Evaluating the effect of UVR exposure on mtDNA damage in the epidermis and dermis

A collaborative study was performed to determine whether variation in mtDNA damage can be detected following exposure to UVR using a biopsy technique, as well as in individuals of different ages.

Following UVR exposure, mtDNA damage in both age groups decreased; however, statistical significance was not observed. Due to the nature of the study, biopsy samples were collected later in the year than planned which may explain the decrease in mtDNA damage in samples collected following UVR exposure. Previous research has shown that mtDNA damage accumulates within the dermis over time due to the slow turnover rate of the dermis in comparison to the epidermis (63, 117); however, it is unclear what occurs in the epidermis following exposure to UVR.

A greater level of mtDNA damage was observed in older individuals in comparison to younger individuals both before and after exposure to UVR, with significance observed following UVR exposure. This finding is in line with those published by Harbottle *et al*. (2010) which found an age-dependent increase in mtDNA damage in biopsy samples. It was concluded that this was due to the fact that a biopsy collects viable cells from both epidermis and dermis and therefore in-tact mtDNA is obtained. On the other hand, the swab collects corneocytes from the stratum corneum which is the dead layer of the epidermis; thus, the mtDNA is less likely to be in-tact (50).

4.4.6 Study 6: Investigating differences in mtDNA damage between samples collected with skin swab and tape strip collection methods

Samples were collected using both skin swabs and tape strips in order to compare mtDNA damage within samples obtained using both collection methods, as well as the consistency of results.

Results found that %DNA amplified increased overall between the 10 samples collected consecutively from the same sample site with the swab, with %DNA amplified values ranging from 3.08 to 30.30% (Figure 4.17A). This also supports the data shown within the optimisation section (Figure 3.9A) which showed an increase in %DNA amplified with 3 consecutive swabs collected from the same sample site. On the other hand, %DNA amplified was much more consistent in samples obtained using a tape strip, ranging from 23.65 to 25.27%. An explanation for the difference in results could be that swabs remove fewer cells from the sample site and

therefore each swab removes progressively removes corneocytes from the epidermis, hence the increase in %DNA amplified due to the removal of the 'dead' cells which may not contain much in-tact DNA. Due to the nature of the tape strips, more corneocytes are likely to be removed with each strip, accessing deeper layers. It is also important to note that this study was carried out on the inner arm as the collection of 10 tape strips may be slightly uncomfortable; however, for this technique to be used in future, optimisation must be performed using the face due to differences between sample sites. Although 10 tape strips were collected, research suggests that approximately 20 tape strips are required to completely strip the stratum corneum and access the stratum granulosum. This may be important for ensuring that all corneocytes are removed; however, is something that would be difficult to control for between individuals (89). The use of tape trips was not furthered during this project due to time constraints.

4.4.7 Key findings

- Skin swab does not consistently detect an increase in mtDNA damage following exposure to UVR under our experimental conditions
- Skin swab does not detect differences in mtDNA damage as a result of chronological ageing
- Mitochondrial DNA damage detected by the skin swab does not correlate with sun exposure and sun protection behaviours
- Mitochondrial DNA damage detected by the skin swab does not correlate with underlying skin damage obtained using UV technology
- UV technology can detect fluctuations in UV damage between seasons
- UV spots increase in size as a result of chronological ageing
- Skin swabs collected from 3D full-thickness skin equivalents show a greater level of mtDNA damage immediately after exposure to complete solar simulated light
- Mitochondrial DNA damage was found to be significantly higher within the epidermis in comparison to the dermis of 3D full-thickness skin equivalents
- Damage significantly decreased in the dermal layer of 3D full-thickness skin equivalents between day 1 and 3
- Biopsy samples detect differences in mtDNA damage as a result of chronological ageing in samples collected following UVR exposure
- Biopsy samples did not show an increase in mtDNA damage following UVR exposure
- Tape strips showed more consistency in %DNA amplified in comparison to skin swabs

- Tape strips showed opposite results to swabs, with less mtDNA damage in arm in comparison to the face

4.4.8 Limitations

The skin swab technique has multiple limitations that must be addressed through further optimisation and future work. Firstly, the swabs collect corneocytes from the dead stratum corneum layer; therefore, mtDNA obtained from this layer may not be representative of what is occurring in viable skin layers below. Optimisation also showed that results differed in those with skin conditions such as eczema, with mtDNA yield higher in those with skin conditions and mtDNA damage lower. This was thought to be due to parakeratosis, which has been shown to be present in individuals with inflammatory skin conditions including chronic eczema. As previously mentioned, it is characterized by incomplete keratinization of epithelial cells, with abnormal retention of keratinocyte nuclei in the stratum corneum. The retained nuclei may mean that DNA degradation that occurs during cornification may not be complete, resulting in less mtDNA damage but a greater yield, due to more intact DNA available to be amplified. As well as this, multiple factors may affect the results obtained using the swab. As damage has been shown to accumulate within the dermis, it may be important to control for how many days following UVR exposure the swabs are collected to allow the damage to reach the surface of the skin and be detected using the swab. Peeling of the skin following UVR exposure should also be taken into consideration, as removal of the top stratum corneum layers may reveal cells with more intact DNA; therefore, damage may seem lower, giving a false result. Finally, studies have shown epidermal thickness to increase following exposure to UVR, which may also affect the results due to the number of cells obtained from the stratum corneum using the swab.

4.4.9 Conclusions

Overall, we conclude that the skin swab is not able to detect recent UVR exposure, which could be due to multiple factors such as changes in lifestyle habits which are difficult to control for. As well as this, the skin swab is also not able to detect differences in age, similarly reported by a previous study (50). The use of facial imaging using UV technology in order to detect fluctuations in underlying sun damage in the form of UV spots with seasonal changes and age is effective when assessing %Area. Moving forward, it could be used to accurately measure damage as a result of UVR and test compounds for their protective effects.

Chapter 5. Aim II: A cross-sectional study investigating the impact of COVID-19 lockdown on sun exposure and behaviours within the UK

5.1 Introduction

The first lockdown within the UK due to SARS-CoV-2, more commonly known as COVID-19), was announced on 23rd March 2020, with restrictions being gradually lifted on 10th May 2020 and ending on 4th July 2020 (118, 119). The restrictions included closure of non-essential shops, a ban on social gatherings and severe restriction to movement, which allowed one form of exercise per day and banned international and UK travel (120). Individuals living within the UK were instructed to work from home where possible, with 46.6% of people in employment completing some work from home in April 2020. Of these individuals, 86.0% did so as a result of the COVID-19 pandemic (121, 122). As well as the closure of workplaces, schools and universities were also closed and furlough pay was also introduced (120). From mid-May new guidance was released which allowed individuals to spend more time outdoors, with the government announcing that individuals may go to parks and beaches to sunbathe, and that outdoor sports courts may reopen (123). Despite the easing of restrictions, the prevalence of homeworking among office workers remained high throughout summer in 2020 (121, 124).

Exposure to UVR is responsible for the induction of 80% of human skin cancers, including malignant melanoma. Malignant melanoma is the fifth most common cancer in the UK and its incidence has risen 360% since the 1970s. This rise is a result of changes in sun-related behaviours among the public, such as increased sun exposure with inadequate protection and greater accessibility to travel abroad and use artificial tanning beds (125-127). The risk of development of cutaneous melanoma in adulthood has been shown to double following childhood sunburn, making it an important risk factor (128). Sun exposure is a prevalent behaviour among adolescents, with research suggesting that a large proportion don't apply sunscreen whilst in the UK during the summer months, as well as the preference of younger individuals to have a tanned appearance and use artificial tanning beds (125, 126, 129, 130). The strongest predictor of recent sunburn is thought to be age, with individuals under the age of 35 being over twice as likely to report recent sunburn (131). Due to the prevalence of home working during lockdown, sun exposure may have been affected by the exceptionally sunny weather experienced by the UK, with April and May scoring the highest sunshine hours on record across the country (121).

Overall, this chapter aimed to assess the impact of the UK lockdown on sun protection behaviours and exposure.

Specific research questions are as follows:

1. Investigating the influence of sun exposure levels on sun protection behaviours during lockdown
2. Investigating the correlation between psychosocial variables, sun exposure and the occurrence of sunburns with sun protection behaviours, whether variables are predictors of sun protection behaviours and the effect of sun protection behaviours and exposure on sunburns in lockdown
3. Investigating the effect of sociodemographic factors on sun protection behaviours and sun exposure

5.2 Materials and Methods

5.2.1 Study design

We performed a cross-sectional study to determine the influence of lockdown within the UK as a result of the COVID-19 pandemic on sun exposure and protection behaviours. Ethical approval was granted by the Newcastle University Ethics Committee (Ref: 3901/2020). This study and related findings are reported in line with the STROBE checklist for cross-sectional studies from the EQUATOR Network website (132).

5.2.2 Participants

A total of 522 complete questionnaire responses were analysed, with responses from 99 males (19%), 422 females (80.8%), and 1 preferred not to say (.2%). Uncompleted responses were not saved. The eligibility criteria for the study was individuals over the age of 18 years. Convenience sampling was used and participants were recruited through social media sites such as Facebook, Twitter, Instagram and LinkedIn, as well as through Newcastle University mailing lists.

5.2.3 Questionnaire

The self-reported questionnaire consisted of 39 questions and was tested in advance (n=6) for readability, comprehension and length. The results of the test showed that it took approximately 13 minutes to complete and all questions were understood. Data was collected through an online survey (Qualtrics, USA) between 17th June 2020 and 16th July 2020. Questions were aimed mainly at sun exposure and sun protection habits. Full questionnaire is available in Section 8.3.1 (appendix). Most questions were adapted from previous studies (96-99); however, some questions were created for the purpose of the study. Variables and scales are presented in detail in Table 8.3 (appendix).

5.2.4 Statistical analysis

Data were analysed using SPSS version 24 (IBM SPSS Statistics, USA). Age was grouped into 18-30, 31-50 and >50 years for analysis purposes. Shapiro-Wilk was used to test for normality and non-parametric tests were used throughout as the data was not normally distributed. A two-tailed p-value <0.05 was considered statistically significant.

5.2.4.1 Research question 1: Investigating the influence of sun exposure levels on sun protection behaviours during lockdown

Mann-Whitney U test was used to determine whether those who spent more time outdoors during lockdown in comparison to pre-lockdown, had significantly better sun protection behaviours. Kruskal-Wallis test was used to determine differences in sun protection behaviours in those who reported that their sun protection behaviours are the same in the UK as they are abroad, less strict and the same. Spearman's correlation analysis was conducted between sun exposure and sun protection behaviours.

5.2.4.2 Research question 2: Investigating the correlation between psychosocial variables, sun exposure and the occurrence of sunburns with sun protection behaviours, whether variables are predictors of sun protection behaviours and the effect of sun exposure and sun protection behaviours on sunburns in lockdown

Spearman's correlation analysis was conducted between sun protection behaviours and intentions, short term affective attitudes, short term rationale attitudes, long term attitudes, attitudes towards a tan, self-efficacy, knowledge, time spent in the sun to tan and the occurrence of sunburns during lockdown. To test the prediction variables of sun protection behaviours, we used multivariate linear regression analysis. Primary analysis was conducted in order to ensure no violation of the assumptions of normality, linearity, multicollinearity and homoscedasticity. Individuals who answered with "I don't know" for sunburns obtained this year were removed from the analysis, leaving 519 responses remaining. Mann-Whitney U test was used to determine the difference in sun protection behaviours between those who did not obtain sunburn during lockdown, in comparison to those who obtained at least one sunburn.

5.2.4.3 Research question 3: The effect of sociodemographic factors on sun protection behaviours and sun exposure

Kruskal-Wallis test was used to determine whether age, marital status and region within the UK affected time spent outdoors during lockdown, time spent in the sun for a tan and sun protection behaviours. Kruskal-Wallis test was used to determine whether employment status affected time spent outdoors during lockdown. Mann-Whitney U was used to determine whether sex

affected time spent outdoors during lockdown, time spent in the sun for a tan and sun protection behaviours.

5.3 Results

5.3.1 Demographics and descriptive statistics

Table 5.1 provides an overview of the sample characteristics (n=522). 68.2% of individuals were aged 18-30 years and 80.8% were female. Regarding marital status, 52.3% were single. 39.5% of individuals reported that their skin type was medium. 59.2% of individuals were living in the North-East of England at the time they completed the questionnaire and 29.5% were working in their usual environment.

Table 5.1 Demographics of the sample population (n=522).

Variable									
Age	18-30	31-50	>50						
N (%)	*356 (68.2)*	*87 (16.7)*	*79 (15.1)*						
Gender	Male	Female	Prefer not to say						
N (%)	*99 (19)*	*422 (80.8)*	1 (0.2)						
Marital status	Married/civil partnership	Cohabiting	Single	Separated	Divorce/civil partnership dissolved	Prefer not to say			
	116 (22.2)	*109 (20.9)*	*273 (52.3)*	*7 (1.3)*	*13 (2.5)*	*4 (0.8)*			
Skin type	Very fair	Fair	Medium	Olive	Brown	Black			
N (%)	50 (9.6)	199 (38.1)	206 (39.5)	59 (11.3)	6 (2)	2 (0.4)			
Region within UK	North-East (England)	North-West (England)	Yorkshire and the Humber (England)	West Midlands (England)	East Midlands (England)	East (England)	South-East (England)	South-West (England)	Greater London (England)
N (%)	*309 (59.2)*	*56 (10.7)*	*28 (5.4)*	*8 (1.5)*	*17 (3.3)*	*10 (1.9)*	*14 (2.7)*	*12 (2.3)*	*20 (3.8)*
	Scotland	Wales	Northern Island	Outside of the UK					
N (%)	*11 (2.1)*	*20 (3.8)*	*1 (0.2)*	*16 (3.1)*					
Employment status	Working in usual environment	Working from home	Furloughed	Student	Unemployed	Prefer not to say			
N (%)	*154 (29.5)*	*132 (25.3)*	*69 (13.2)*	*138 (26.4)*	22 (4.2)	7 (1.3)			

Health behaviour descriptive statistics for the sample population are presented in Table 5.2. Almost half of the sample (44.1%) spent ≥4 hours outside on weekends, in comparison to 16.3% on weekdays, with 34.8% often or always spending time in the sun to tan. The largest proportion of individuals often or always wore sunglasses when they were outside during lockdown on a warm sunny day (56.5%). Most individuals (77.3%) stated that they have stricter sun protection habits when abroad in comparison to being in the UK. Approximately half of the sample population reported at least one sunburn during 2019 (56.9%) and lockdown up to the time of survey closure on 16th July 2020 (45.8%).

Health behaviours	**Frequencies**
Self-reported sun protection practices	
Sun exposure weekdays (% ≥ 4 hours)	16.3% (n=85)
Sun exposure weekends (% ≥ 4 hours)	44.1% (n=230)
Sunscreen use (% often and always)	45.8% (n=239)
T-Shirt use (% often and always)	41.8% (n=218)
Hat use (% often and always)	10.3% (n=54)
Seek shade (% often and always)	14.2% (n=74)
Sunglasses use (% often and always)	56.5% (n=295)
Number of sunburns	
This year (% ≥ 1)	45.8% (n=239)
Last year (% ≥ 1)	56.9% (n=297)
Sun exposure	
Spend time in the sun to tan (% often and always)	34.8% (n=182)
Stricter sun protection habits abroad in comparison to UK	77.3% (n=385)

Table 5.2 Health behaviour frequencies of the sample population.

5.3.2 Research question 1: Investigating the influence of sun exposure levels on sun protection behaviours during lockdown

No significant difference in overall sun protection behaviours between those who spent more time outdoors during lockdown and those who didn't was observed (p=0.18).

Table 5.2 shows that a large proportion (77.3%) of participants report having stricter sun protection habits when abroad in comparison to in the UK. Those who reported that their sun protection habits are the same in the UK as they are abroad showed significantly higher sun protection behaviours on a warm sunny day in lockdown in comparison to those who reported that their sun protection habits are less strict within the UK ($p<0.01$).

Spearman's correlation analysis showed a weak negative correlation between sun exposure and sun protection behaviours whilst outside on a warm sunny day in lockdown ($r=-0.12$, $p<0.01$) (Table 5.3). A weak negative correlation between sun exposure and how often individuals wore a shirt with sleeves that covered their shoulders ($r=-0.25$, $p<0.001$) and how often individuals stayed in the shade or under an umbrella ($r=-0.16$, $p<0.01$) was also seen.

		1	2	3	4	5	6	7
1	Sun exposure		-.117**	0.055	-.249**	-0.032	-.156**	0.040
2	Sun protection behaviours			.603**	.528**	.536**	.600**	.537**
3	Sunscreen				0.017	.116**	.223**	.297**
4	Shirt with sleeves that cover shoulders					.229**	.311**	0.004
5	Hat						.230**	0.063
6	Shade or under an umbrella							.108*
7	Sunglasses							

Table 5.3 Spearman's rank correlation analysis between sun exposure and sun protection behaviours. *p<0.05, **p<0.01 (2-tailed).

5.3.3 Research question 2: Investigating the correlation between psychosocial variables, sun exposure and the occurrence of sunburns with sun protection behaviours, whether variables are predictors of sun protection behaviours and the effect of sun protection behaviours and exposure on sunburns during lockdown

A moderate positive correlation between sun protection behaviours and intentions ($r=0.41$, $p<0.01$) (Table 5.4) was observed. A weak positive correlation was observed between sun protection behaviours and short-term rationale attitudes ($r=0.10$, $p=0.03$), long-term attitudes ($r=0.24$, $p<0.01$), self-efficacy ($r=0.14$, $p=<0.01$) and knowledge ($r=0.10$, $p<0.01$). A weak negative correlation was observed between sun protection behaviours and attitudes towards a

tan ($r=-0.20$, $p<0.01$), sunburns ($r=-0.18$, $p<0.01$) and time spent in the sun for a tan ($r=-0.33$, $p<0.01$) (Table 5.4).

Regression analysis was conducted to determine how several variables impact sun protection behaviour. Results show that 25.6% of the variance in sun protection behaviours can be accounted for by five predictors, collectively $F(5,513)=36.558$, $p<0.01$ (Table 5.5). Results shows that intentions ($\beta=.313$, $t=7.120$, $p<0.01$), long term attitudes ($\beta=.148$, $t=3.788$, $p<0.01$) and self-efficacy ($\beta=.089$, $t=2.339$, $p=0.02$) positively predict sun protection behaviours, whereas sunburns ($\beta=-.128$, $t=-3.283$, $p<0.01$) and time spent in the sun for a tan ($\beta=-.155$, $t=-3.599$, $p<0.01$) negatively predict sun protection behaviours.

Sun protection behaviours were found to be significantly higher in those who did not obtain sunburn during lockdown, in comparison to those who obtained at least one sunburn ($p<0.01$).

		1	2	3	4	5	6	7	8	9	10
1	Intentions		.107*	.159**	.222**	-.309**	0.076	.142**	.413**	-.441**	-.145**
2	Short-term affective attitudes			.372**	.272**	.093*	0.082	.122**	0.072	0.068	-0.015
3	Short-term rationale attitudes				.417**	.119**	.134**	.174**	.095*	0.051	-0.08
4	Long-term attitudes					.133**	0.072	.211**	.243**	-0.062	-.093*
5	Attitudes towards a tan						-.145**	-0.02	-.197**	.398**	.170**
6	Self-efficacy							.138**	.137**	0.015	-.102*
7	Knowledge								.100*	-0.007	-.096*
8	Sun protection behaviours									-.329**	-.177**
9	Average time spent in the sun for a tan										.190**
10	Occurrence of red painful sunburn during spring/summer this year										

Table 5.4 Spearman's rank correlation analysis between behavioural measures and psychosocial variables.
*p<0.05, **p<0.01 (2-tailed).

Variables	Std. βs	P-value
Intentions	.313	<0.001
Sunburns	-.128	<0.001
Long term attitudes	.148	0.001
Time spent in the sun for a tan	-.155	<0.001
Self-efficacy	.089	0.020
R^2	26.3%	

Table 5.5 Model summary.
Multivariate regression analysis testing associations between different predictor variables of sun protection behaviour with standard beta-values (Std. βs) and p-values.

5.3.4 Research question 3: The effect of sociodemographic factors on sun protection behaviours and sun exposure

5.3.4.1 Age

A significant difference in time spent outdoors during lockdown was observed between age groups (p=0.03), with those between the age of 31 and 50 years spending significantly longer outdoors in comparison to those between the age of 18 and 30 years (p=0.02). A significant difference in time spent in the sun for a tan between age groups was also seen (p<0.01). Individuals between the age of 18 and 30 years within the sample population spend longer in the sun for a tan on average, in comparison to those over the age of 50 years (p=0.01). A significant difference in sun protection behaviours was not observed between age groups (p=0.43).

5.3.4.2 Sex

The difference in time spent outdoors during lockdown between male and female participants was not statistically significant (p=0.35); however, results show that females within the sample population spend longer on average in the sun for a tan in comparison to males (p<0.01). A significant difference in sun protection behaviours was not observed between different sex groups (p=0.09).

5.3.4.3 Marital status

The difference in time spent outdoors during lockdown between marital status groups was not statistically significant ($p=0.19$); however, a significant difference in time spent in the sun for a tan was observed ($p<0.01$). Individuals that are single within the sample population spend longer in the sun for a tan on average in comparison to those who are married or in a civil partnership ($p<0.01$). A difference in sun protection behaviours was not observed between marital status groups ($p=0.66$).

5.3.4.4 Region of the UK

The difference in time spent outdoors during lockdown between individuals living within different regions of the UK was statistically significant ($p<0.01$), with individuals living in the North-West of England and Wales spending longer outdoors in comparison to those living in Greater London ($p<0.01$ and $p=0.02$, respectively). The difference in time spent in the sun for a tan on average was not statistically significant ($p=0.11$) and a difference in sun protection behaviours was not seen ($p=0.09$) between individuals living within different regions of the UK.

5.3.4.5 Employment group

A significant difference in time spent outdoors during lockdown between employment groups was seen ($p=0.05$), with those who were furloughed spending the longest outdoors.

5.4 Discussion

Overall, this chapter aimed to assess the impact of the UK lockdown on sun protection behaviours and exposure.

Despite multiple studies reporting that sunscreen is the most preferred and frequently used method of sun protection (133), there was no observed increase in sun protection behaviours in individuals who spent longer outdoors during lockdown in this study. This finding could be explained by 77.3% of individuals reporting that their sun protection habits are less strict in the UK in comparison to being abroad, a finding previously reported (126) and 54.2% of individuals choosing not wearing sunscreen often or always on a warm, sunny day in lockdown. This highlights the importance of education around sun exposure and UV levels, particularly due to the hot weather the UK experienced during lockdown and promotion of outdoor activities; such as sunbathing, in government guidelines to reduce COVID-19 transmission (123). We found that individuals engaged in fewer sun protection behaviours on a warm sunny day during lockdown as sun exposure increased. This may be due to the desire of individuals to spend time tanning and it should be acknowledged that this study was conducted within a population where current trends and culture emphasise a tanned appearance as desirable (134, 135).

We also found that sun protection behaviours during lockdown increased as intentions of using protective measures, positive attitudes towards using sun protection, self-efficacy and knowledge increased, and attitudes towards a tanned appearance, sun exposure and time spent in the sun in order to tan decreased. These are common findings which support correlations found in previous data (133, 135-138). Results, as well as supporting research, show self-efficacy, intentions and knowledge to be powerful motivators to engage in sun protection behaviours; therefore, incorporating such psychological factors into future skin cancer interventions may be effective. The strongest statistical correlation amongst this analysis is between sun protection behaviours and intentions, which is also common amongst previous data (139), supporting the important role of intention. On the other hand, studies report the 'intention-behaviour gap', which describes the failure to translate intentions into action, with data suggesting that intention predicts only 30-40% of the variation in health behaviour (140). Individuals who obtained at least 1 sunburn engaged in fewer sun protection behaviours, supporting previous data that shows an association between good sun protection behaviours and lower sunburn frequency (141). Results also showed that 45.8% of individuals had obtained at least 1 sunburn during lockdown at the time of questionnaire completion. Although this highlights the importance of education around the risks of sunburn, a significant difference in

knowledge score was not seen between those who had obtained at least 1 sunburn in lockdown and those who hadn't ($p=0.07$) (data not shown). This supports numerous studies which reveal that knowledge is not a good predictor of behaviour; therefore, knowledge may be necessary but insufficient for change (142).

Younger individuals spent significantly longer time outdoors and, in the sun, to obtain a tan during lockdown, suggesting that positive attitudes towards a tan are negatively related to age, a finding frequently reported (125, 129, 130, 143-145). Research has also shown that of the individuals who worked from home, 34.4% reported that they worked fewer hours than usual; therefore, may have spent longer outdoors (122). Although previous data suggests that females use more sun protection in comparison to men (146, 147), our results do not show a significant difference. This could be due to other factors during lockdown such as work commitments, with research showing that women were slightly more likely to do more work at home than men (47.5% and 45.7%, respectively) (122). On the other hand, females within the sample population spend significantly longer in the sun for a tan on average in comparison to males, following patterns previously identified (146, 148-150). Finally, we found that individuals living within the North-West of England and Wales spent longer time outdoors during lockdown in comparison to those living in Greater London. It may be that individuals living outside of London are more likely to own private outdoor space and were able to spend longer outdoors when exercising in public outdoor spaces was limited to once per day. Overall, 69.3% reported that they spent more time outdoors during lockdown; a similar result shown by a recent study (121), with furloughed individuals spending the most time outdoors.

5.4.1 Limitations

Regarding the investigation of the impact of COVID-19 lockdown on sun exposure and behaviours, findings may be limited due to the age, sex, skin type and location of participants. Although the sample size is large, the sample population is young and female orientated, with over half of the sample population living within the North-East of England which has fewer days of sun in comparison to other regions within the UK, shown by higher vitamin D levels within the south of England (151). This population bias may have affected protection behaviours during lockdown, with participants in the North engaging in fewer sun protection behaviours due to their perception of the weather, rather than due to associations with demographics and psychological factors. Limitations also include the recruitment method which could result in skewed data as social media connections are likely to share similar behaviours and opinions. A limitation of the self-administered questionnaire is that participants

may under-report harmful behaviours such as sun exposure. Finally, the analysis was performed using non-parametric tests which hold a lower statistical power, have a higher standard error and wider confidence intervals in comparison to parametric tests.

5.4.2 Conclusions

This research could help aid post-COVID-19 public health campaigns, targeting younger individuals and promoting the importance of sunscreen, particularly in the UK during summer months.

Chapter 6. Aim III: Exploring the protective effects of caffeine and forskolin in the presence of simulated solar light

6.1 Introduction

Bioactive molecules from plants have been widely used as cosmeceutical ingredients due to their ability to slow down the ageing process (152). Caffeine (1,3,7-trimethylxanthine) is a bioactive natural compound found in coffee, tea and multiple other food products (153). Studies have revealed that it may reduce the risk factors responsible for hypertension, cardiovascular conditions and type 2 diabetes, as well as act as a neuron protective agent in neurodegenerative diseases (154). As well as this, research has suggested that daily consumption of caffeine helps to reduce skin cancers, as well as wrinkle formation, through its protective effects against UVR (152, 155). Caffeine is being increasingly used in cosmetic products due to its high biological activity and ability to penetrate the skin barrier, with commercially available topical formulations usually containing approximately 3% caffeine (156). Creams containing caffeine have been shown to slow down photoageing due to its function as a sunscreen, in addition to its potent antioxidant properties (156, 157). Studies indicate that caffeine inhibits UVB-induced formation of thymine dimers and sunburn lesions in the epidermis of mice. As well as this, caffeine administration has a biological effect that enhances UVB-induced apoptosis, and therefore enhances the elimination of damaged precancerous cells. Treatment of primary human keratinocytes with caffeine has been shown to result in the inhibition of UVB-induced increases in ATR-mediated formation of p-Chk1 (Ser345); which is phosphorylated following DNA damage, and abolish the UVB-induced decrease in cyclin B1, resulting in premature mitosis, chromatin condensation and p53 independent cell death (157, 158).

Forskolin is a complex natural product that has become a standard research tool in biology (159). It is produced by the Indian Coleus plant and has been used for centuries to treat various diseases such as hypothyroidism, heart disease, respiratory disorder, and more recently is used for weight loss (160). Forskolin exerts its protective effects against UVR through the MC1R pigmentation pathway. The MC1R is a transmembrane G protein-coupled receptor that triggers the activation of adenylate cyclase and production of cAMP when bound to its ligand α-MSH, following exposure to UVR. Raising cytoplasmic levels of cAMP in melanocytes; either through α-MSH signalling or pharmacologically by direct activation of adenylate cyclase, initiates a pathway that involves activation/induction of the CREB and MITF transcription factors. This results in the generation of the UVR-protective pigment eumelanin. Forskolin exerts its protective effects by increasing intracellular levels of cAMP through activation of

adenylate cyclase, which catalyses the conversion of ATP to cAMP and pyrophosphate, subsequently generating eumelanin which is transported to keratinocytes to provide protection against UVB-induced apoptosis (161, 162). UVR-induced tanning is defective in numerous fair-skinned individuals who contain functional disruption of the MC1R (163); therefore, forskolin promotes sunless tanning in these individuals. Forskolin also enhances the removal of the two major types of UVB-induced DNA damage; CPDs and 6,4-photoproducts, by facilitating DNA repair, as well as epidermal thickening and enhancing melanocyte numbers in the skin (161, 162).

Overall, this chapter aims to investigate the protective effects of combination doses of caffeine and forskolin, against complete solar light exposure in cultured HDFn cells.

6.2 Materials and Methods

6.2.1 General cell culture

6.2.1.1 HDFn cells

The neonatal human dermal fibroblast (HDFn) cell line (Invitrogen, UK); obtained from human neonatal foreskin, was cultured in Dulbecco's modified Eagles medium (DMEM); 4.5g/l-glucose containing L-glutamine, sodium pyruvate and sodium bicarbonate (Sigma-Aldrich UK). DMEM was supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin. At 80-90% confluency, cells were washed with PBS and detached using trypsin-EDTA solution (Sigma-Aldrich, UK). Trypsin-EDTA solution was neutralised with medium. Cell numbers were established using a hemocytometer.

For experiments, a cell suspension of 5000 cells/ml was made up, and 100µl cell suspension was added to each well of a 96-well plate, including positive and negative control wells. For blank wells, 100µl media without cells was added. Cells were incubated overnight to adhere.

6.2.2 Irradiation

A Newport solar simulator (MKS Instruments, Inc., USA) was used to irradiate cells to the relevant standard erythemal dose (SED). Following optimisation experiments, cells were irradiated with 4.32 SED in the presence of compounds to mimic approximately 40 minutes in the Mediterranean sun. Media was removed and cells were washed twice with 100µl PBS, before replacing with 100µl PBS for irradiation. Cells were in PBS for no longer than 10 minutes to avoid cell stress. An ILT-1400 (International Light Technologies, USA) handheld radiometer/photometer was used to measure radiant energy. Calculations were performed previously in house to determine relevant dose timings. Following irradiation, PBS was replaced with 100µl warmed phenol red-free DMEM and the cells were returned to the incubator for 24 hours.

6.2.3 Compound preparation

6.2.3.1 Caffeine

A stock solution of caffeine was made up in dH_2O to a final concentration of 49.6mM and stored at room temperature. Sterile filtration was performed using 0.22µM Millipore-GP Syringe filter units (Thermo Fisher Scientific, USA). The required concentrations were made from the stock solutions in complete DMEM, warmed to 37°C on the day of the experiment, and discarded within 24 hours.

6.2.3.2 Forskolin

Forskolin was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 4.87mM and stored at -20°C in 500µl aliquots. Sterile filtration was performed using 0.22µM Millipore-GP Syringe filter units (Thermo Fisher Scientific, USA). The required concentrations were made from the stock solutions in complete DMEM, warmed to 37°C on the day of the experiment, and discarded within 24 hours.

6.2.4 Cell treatment

HDFn cells were incubated overnight to adhere. Cells were washed with 100µl PBS and 100µl compound dilutions were added to the wells. Cells were incubated for 24 hours. Warmed media was added to the positive and negative control wells and PBS was added to blank wells. Medium containing 0.35% DMSO was used as a vehicle control.

6.2.5 MTS viability assay

Cell viability was measured by the reduction of MTS tetrazolium compound by cells to produce a soluble purple/brown formazan product. Triton-X100 (1%) was prepared in culture media and applied to cells for 15 minutes as a positive control for cell death. Following adherence and treatment with compounds and/or irradiation, media was aspirated from wells, washed twice with 100µl PBS and replaced with 100µl phenol red-free DMEM. To all wells 20µl MTS reagent was added and the plates were incubated for 4 hours (37°C/5% CO_2), protected from light. MTS was added to 8 technical replicates. Formation of formazan was quantified by spectrophotometry (SpectraMax 250, USA) using an absorbance of 490nm. Raw data was manipulated in Microsoft Excel (Microsoft, USA) and analysed via One-Way ANOVA and Kruskal-Wallis test using GraphPad Prism 8 statistical software (GraphPad Software, USA).

6.3 Results

6.3.1 24-hour dose toxicity of caffeine, forskolin and combination

Cell viability following treatment with caffeine, forskolin and combination were assessed by Dr Matt Jackson to determine which doses to test in combination with complete solar irradiation. Concentration ranges were chosen based on those specified in literature.

A statistically significant decrease in cell viability was observed with caffeine doses of 2, 5, 10 and 20mM, with cell viability decreasing to 81.00% ($p=0.02$), 68.11% ($p<0.01$), 63.28% ($p<0.01$) and 63.33% ($p<0.01$), respectively (Figure 6.1A). A significant decrease in cell viability was observed with 500µM Forskolin, decreasing to 73.07% ($p=0.01$) (Figure 6.1B).

Dose combinations were selected using individual viability data. A significant decrease in cell viability was observed with Forskolin 250µM + Caffeine 10mM, decreasing to 59.19% ($p<0.01$) (Figure 6.1C).

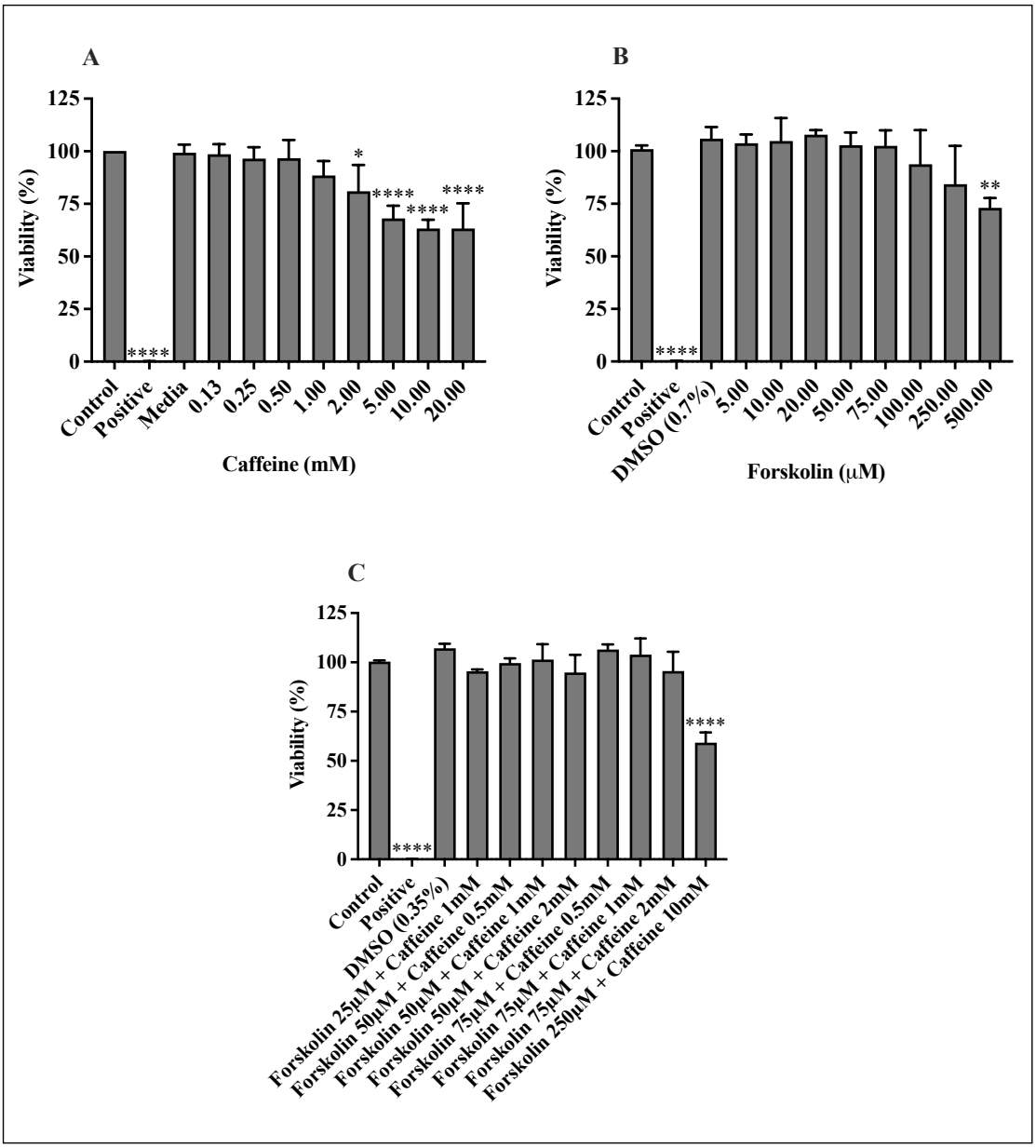


Figure 6.1 Caffeine, forskolin and combination 24-hour dose toxicity.
HDFn cells were treated with (A) caffeine, (B) forskolin and (C) combination for 24 hours. Viability was assessed using the MTS colorimetric assay. Data are presented as a percentage of the control (assuming that the cell viability of the non-irradiated control is 100%), and presented as mean+SD (n=3). Statistical difference was determined using One-Way ANOVA with Dunnett's multiple comparisons test and all groups were compared to the control group. Performed by Dr Matt Jackson. *p<0.05, **p<0.01, ****p<0.0001.

6.3.2 Determination of appropriate standard erythemal dose

Following irradiation with a range of different SEDs, cell viability was either immediately assessed, or assessed after a 24 hour incubation period, in order to determine which dose and incubation time to use for subsequent studies.

Cell viability decreased with increasing SED when measured 24 hours post-irradiation (Figure 6.2A). A significant difference in cell viability was observed with 4.32 SED and 8.64 SED,

decreasing to 42.99% (p=0.04) and 19.71% (p<0.01), respectively. A significant decrease in cell viability was not observed when assessed at 0 hours (Figure 6.2B). Cell viability was assessed 24 hours following irradiation with 4.32 SED for subsequent irradiation experiments.

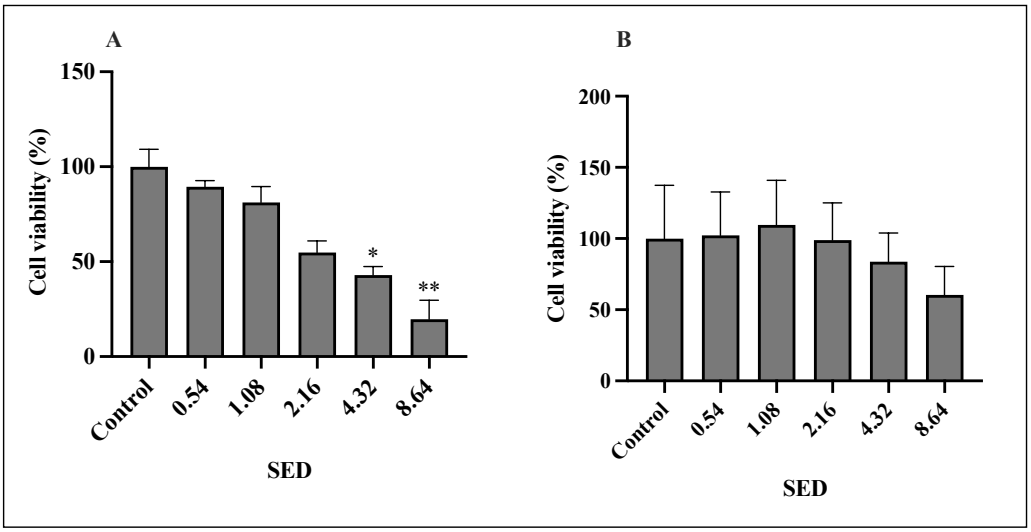


Figure 6.2 Cell viability decreased significantly with increasing SED when assessed at 24 hours. HDFn cells were irradiated with different SEDs, and viability was assessed using the MTS colorimetric assay at (A) 24 hours and (B) 0 hours following irradiation. Viability was assessed using the MTS colorimetric assay. Data are presented as a percentage of the control (assuming that the cell viability of the non-irradiated control is 100%), and presented as mean+SD (n=3). Statistical difference was determined using Kruskal-Wallis test and One-Way ANOVA for graphs A and B, respectively. All groups were compared to the control group. *p<0.05, **p<0.01.

6.3.3 24-hour dose toxicity of caffeine, forskolin and combination in the presence of 4.32 SED

After pre-treatment with caffeine, forskolin or a combination of the two, cells were irradiated with 4.32 SED complete solar light and the cell viability was assessed investigate protective effects of compounds.

Cell viability was maintained between 0.13 and 0.5mM caffeine (Figure 6.3A). A significant decrease in viability was observed with 5, 10 and 20mM caffeine, with viability decreasing to 10.04% (p=0.02), 1.39% (p=0.02) and -3.79% (p=0.04), respectively. Significance was not observed with forskolin; however, cell viability increased between 5 and 100µM (Figure 6.3B).

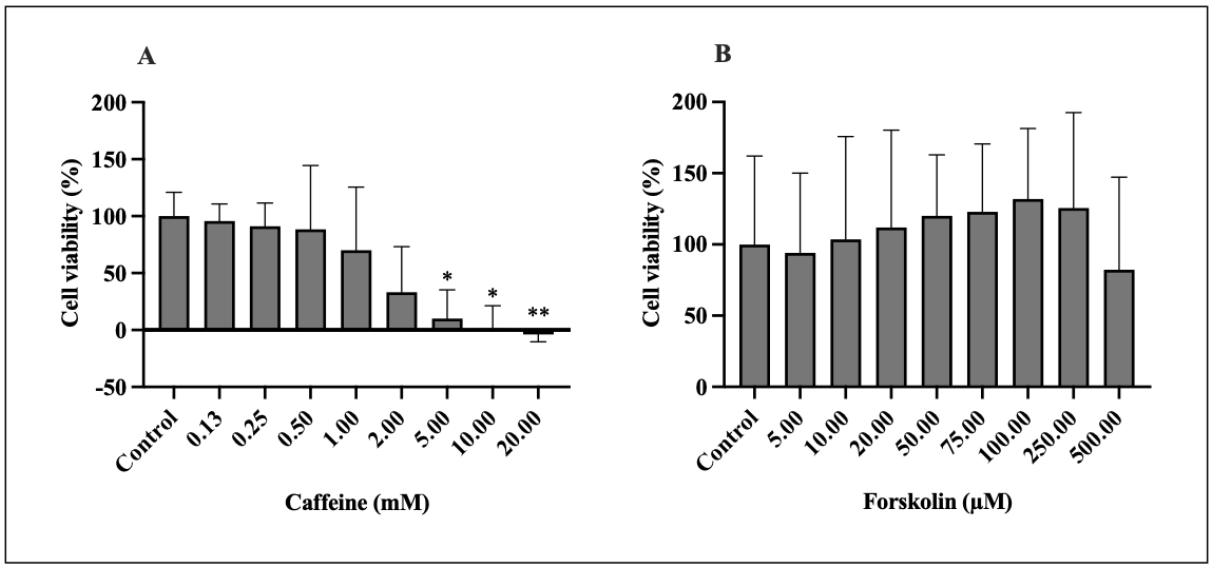


Figure 6.3 Caffeine and forskolin 24-hour incubation followed by 4.32 SED irradiation.
HDFn cells were treated with (A) caffeine and (B) forskolin for 24 hours prior to irradiation with 4.32 SED. Viability was assessed using the MTS colorimetric assay. Data are presented as a percentage of the control (assuming that the cell viability of the non-irradiated control is 100%), and presented as mean+SD (n=3). Statistical difference was determined using One-Way ANOVA with Dunnett's multiple comparisons test and all groups were compared to the control group. *$p<0.05$, **$p<0.01$.

A significant decrease in viability was not observed with combination doses (Figure 6.4A) and higher combination doses (Figure 6.4B).

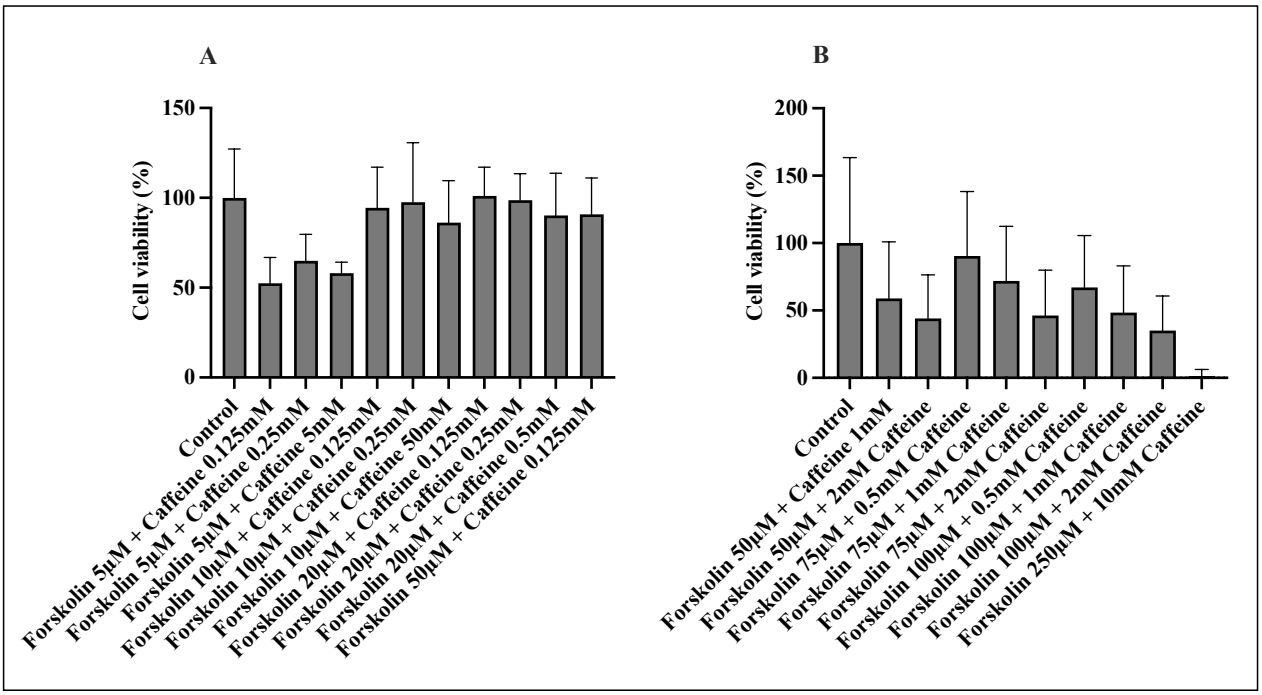


Figure 6.4 Caffeine and forskolin combination dose 24-hour incubation followed by 4.32 SED irradiation.

HDFn cells were treated with (A) caffeine and forskolin combination doses and subsequently (B) higher doses for 24 hours prior to irradiation with 4.32 SED. Viability was assessed using the MTS colorimetric assay. Data are presented as a percentage of the control (assuming that the cell viability of the non-irradiated control is 100%), and presented as mean+SD (n=3). Statistical difference was determined using One-Way ANOVA and all groups were compared to the control group.

6.4 Discussion

This chapter aimed to investigate the protective effects of combination doses of caffeine and forskolin against complete solar light exposure in cultured HDFn cells.

Although some studies have investigated the effect of caffeine on murine epidermis, little research into the effects of direct exposure of higher concentrations of caffeine in dermal fibroblasts exists (157). A statistically significant decrease in cell viability was observed with caffeine doses of 2, 5, 10 and 20mM; however, viability was maintained between 0.13 and 0.5mM (Figure 6.1A). This suggests that 0.13 and 0.5mM are safe doses to apply to cells and test in the presence of complete solar light moving forward. Our study used much higher doses of caffeine than currently reported in the literature; however, previous studies reported adding caffeine to murine epidermis, rather than cells. As well as this, multiple studies added caffeine following exposure to UVB, rather than prophylactically (157). Regarding forskolin, cell viability was maintained between 5 and 75µM, and a significant decrease in viability was observed with 500µM (Figure 6.1B). This suggests that concentrations between 5 and 75µM are safe doses to apply to cells, which is in line with other studies within the literature, which use 20µM forskolin (162). When in combination, cell viability is maintained between 94.82 and 106.5% for all combination doses, until the highest dose of 250µM forskolin + 10mM caffeine, which results in a decrease in viability to 59.19% (Figure 6.1C). This therefore suggests that doses aren't as toxic when compounds are combined.

The same doses of caffeine and forskolin were applied to cells, prior to irradiation. As observed in the absence of complete solar light, cell viability was maintained between 0.13 and 0.5mM caffeine; therefore, suggesting protective effects at lower doses of caffeine (Figure 6.3A). Regarding forskolin, cell viability increased between 5 and 100µM; therefore, suggesting that forskolin is providing a dose-dependent increase in protection up until 100µM (Figure 6.3B). Cell viability was above 100% which could be due to an increase in cell proliferation or mitochondrial activity, which can't be controlled for with the MTS assay. This is a similar finding to that observed in the absence of complete solar light. To our knowledge, the combination of caffeine and forskolin have not yet been tested together in order to determine their combined protective effects against solar simulated light; however, the two actives have been combined in order to develop a topical formulation able to reduce the visible appearance of cellulite (164). Research has also shown both caffeine and forskolin to stimulate glycerol release, which plays an important role in skin hydration, cutaneous elasticity and epidermal barrier repair (165). When in combination, cell viability was maintained with the following combination doses; 10µM forskolin + 0.13mM caffeine, 10µM forskolin + 0.25mM caffeine,

20µM forskolin + 0.13mM caffeine and 20uM forskolin + 0.25mM caffeine (Figure 6.4A). This suggests that 20µM forskolin + 0.25mM caffeine is the safest dose, which does not damage cells but also protects against complete solar light. When combination doses were increased (Figure 6.4B), cell viability was not maintained, suggesting that it is too high an antioxidant dose and is neutralising ROS to the point that cellular homeostasis is impaired; although, statistical significance was not observed. In contrast to these findings, studies have shown that caffeine inhibits forskolin-stimulated cAMP accumulation in rat brain (166); therefore, more work is required to further our understanding of the interaction between caffeine and forskolin.

6.4.1 Limitations

All experiments within this chapter were performed using the MTS assay which relies on tetrazolium salt reduction, a widely used and cost-effective indirect measure of cell viability. Originally, these assays involved the uptake of tetrazolium salts such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from culture medium and the analysis of the reduced product, following solubilisation of cells. This conversion is thought to be carried out by NAD(P)H-dependent dehydrogenase enzymes within the mitochondria. More recently, newer tetrazolium salts have been introduced which are converted by cells with active metabolism to a purple formazan product; able to pass back out of the cell following reduction, with an absorbance peak of 570nm. This eliminates the liquid solubilisation phase of MTT; therefore, increasing the convenience, as well as the accuracy, by removing potential errors such as cell loss (167, 168). The major advantage of the MTS assay is the ability to measure cell number without having to disturb the cells by the removal of the experimental culture medium; however, it is not possible to distinguish between changes in cell number and mitochondrial activity. As well as this, changes in proliferation rate and mitochondrial activity induced by a treatment can also affect results obtained using the assay (167). AlamarBlue is an alternative cell viability reagent to MTS which quantitatively measures cell proliferation, has greater sensitivity and can be used for time course experiments. Another limitation is that experiments were performed using HDFn cells which are present within the dermal layer. It must be noted that *in vivo*, most of the UV light would be absorbed by the epidermal layer and therefore may not reach the dermis. As well as this, crosstalk between fibroblasts and keratinocytes isn't present in monolayer cultures; therefore, it is unknown how keratinocyte interaction would affect the protective effect observed.

6.4.2 Conclusions

Overall, results show that doses of caffeine and forskolin provide protection against complete solar light in HDFns. As well as this, results also suggest that the compounds function together to provide protection and could therefore be used to generate a skincare formulation.

Chapter 7. General Discussion

7.1 Overview

Skin ageing is driven by both intrinsic and extrinsic influences, resulting in damage to mtDNA in the form of deletions, mutations and strand breaks. Previous studies have used a skin swab technique to detect differences in mtDNA damage between individuals; however, it has not been widely reported. The principle aim of this thesis was to investigate mtDNA variation between individuals and the influence of both intrinsic and extrinsic factors, and whether these variations can be accurately measured using a skin swab technique. This involved the analysis of demographics as well as sun exposure and sun protection behaviours.

7.2 Key findings

In this study, we built upon previous research involving the skin swab technique by performing large scale studies to investigate differences in mtDNA damage between individuals, as well as using qualitative data to determine whether differences in lifestyle factors and demographics can be detected using a skin swab. This technique provides a non-invasive, cheaper alternative that has no patient morbidity in comparison to biopsy collection, allowing at home sample collection. It has previously been suggested that a skin swab can be used to detect recent mtDNA damage as a result of UVR exposure, rather than chronological accumulation of damage with increasing age. Results from our studies did not consistently detect an increase in mtDNA damage following exposure to UVR or differences in damage as a result of chronological ageing. When comparing mtDNA damage to qualitative data, results obtained using a skin swab did not correlate with sun exposure, sun protection behaviours or demographics such as skin type.

Although UV spot count and %Area was shown to correlate with chronological ageing, as well as fluctuate across seasons, it did not correlate with mtDNA damage obtained using a skin swab. Importantly, we found that underlying UVR damage should be investigated using UV spot %Area, as opposed to UV spot count. This is due to an increase in %Area following UVR exposure as a result of UV spot convergence. We can therefore conclude that a skin swab cannot yet be used to detect recent UVR exposure, which could be due to general lifestyle factors, as well as biological factors such as the turnover rate of keratinocytes and level of cornification within the stratum corneum that are difficult to control for.

As it is widely accepted that individual behaviours and attitudes will affect biological principles, we investigated the impact of COVID-19 lockdown on behaviours and attitudes due to the prevalence of home working. We also investigated the effect this had on sunburn frequency, particularly due to the heatwave experienced by the UK in March 2020. Studies have frequently reported the major effects of COVID-19 in healthcare; however, the majority of dermatological studies showed a reduction in skin cancer diagnoses, which was thought to be due to patient reluctance to seek medical attention during the pandemic. This further highlighted a need for investigation, as well as to expand qualitative research which focused on sun exposure changes in lockdown alone. Importantly, we found that those who spent longer outdoors during lockdown did not adjust their sun protection behaviours accordingly, with 77.3% of individuals reporting that their sun protection habits are less strict in the UK in comparison to when abroad. We also found that under half of the sample population wore sunscreen often or always on warm sunny days during lockdown, highlighting the importance of education around sun exposure, particularly when in the UK during summer months. Individuals with greater sun exposure also engaged in fewer sun protection behaviours which could be due to the desire to obtain a suntan, and those who obtained at least 1 sunburn engaged in fewer sun protection behaviours. Overall, 69.3% of individuals reported that they spent more time outdoors during lockdown, with furloughed individuals spending longer outdoors in comparison to other occupation groups. Due to the damaging effect of UVR on the skin and the increased prevalence of sunbathing, there is a critical need for protection measures as well as increased education.

We explored the protective effects of caffeine, forskolin, and in combination against complete solar simulated light. Both compounds are frequently used in the cosmetic industry; however, have not been combined to investigate their protective effects against complete solar light. When caffeine and forskolin were combined, cell viability was maintained in concentration ranges of 10-20μM forskolin + and 0.13-0.25mM caffeine, with 20μM forskolin + 0.25mM caffeine found to be the safest dose. These findings demonstrate that forskolin and caffeine could be combined in skincare products to protect against UVR exposure as well as skin ageing, following further investigation.

7.3 Future work

Despite significant attempted optimisation of the skin swab technique, future work should include further optimisation with a focus on whether corneocytes from the stratum corneum are representative of the cells in viable layers below. It should also investigate how varying cornification rates; such as those observed in inflammatory skin conditions, may affect results

obtained using a skin swab. The timescale following UVR exposure for swab collection should also be considered, as well as the biological effects UVR may induce, including but not limited to epidermal thickening and skin peeling. Regarding epidermal thickening, future work could involve the irradiation of skin equivalents and the measurement of epidermal thickening over time. Future work could also include investigating how mtDNA strand breaks translate to actual expression/activity of ETC components, in order to determine how such breaks affect mitochondrial function. As well as this, further investigation could be performed to investigate how mtDNA damage varies over a time course, following irradiation. Future studies would also employ an objective measure of sunscreen use as well as questionnaire analysis, which would enable a clearer conclusion.

Investigation of the impact of COVID-19 lockdown on sun exposure and behaviour was limited by the possible recruitment bias. It is important to note that with self-reported questionnaire data, participants may under-report harmful behaviours such as sun exposure. Future work could use this research to help aid post-COVID-19 public health campaigns, targeting younger individuals and promoting the importance of sunscreen use within the UK. As well as this, future work could observe changes in sun protection behaviours and exposure at present in the absence of lockdown measures, to assess whether the COVID-19 pandemic had an overall effect on daily sun exposure. As well as this, findings may reflect an individual's daily sun exposure whilst on holiday, as well as at home in the UK during summer months. The findings presented may therefore have a broader impact, once again highlighting the importance of the promotion of sunscreen within the UK.

Cell viability work with caffeine and forskolin was performed using the MTS assay which does not distinguish between changes in cell number and mitochondrial activity, which may be induced by a treatment and affect results. As well as this, experiments were performed on HDFns which are present within the dermal layer; however, most of the UVR would be absorbed by the epidermal later *in vivo*, and the keratinocyte-fibroblast interaction cannot be investigated in monolayer cultures. Future work could include screening caffeine and forskolin in keratinocytes, or to mimic a true *in-vivo* response by screening the compounds within topical creams using skin equivalents. As well as this, stability testing of the two compounds when they are formulated should be performed to note any adverse reactions.

Overall, this thesis investigated the effect of intrinsic and extrinsic influences on skin ageing within associated demographics. Despite significant optimisation of the skin swab technique, further optimisation is required to further our understanding of the biological processes that may affect the results obtained. Our results suggest that forskolin and caffeine can be used in

combination to protect against complete solar light; however, further investigation is required. Finally, we have highlighted the importance of further education around exposure to UVR, as well as the use of sun protection methods including sunscreen.

Chapter 8. Appendix

8.1 Study 1: Pilot study evaluating mtDNA damage variation in the epidermis

8.1.1 Questionnaire

Sun protection habits and perceptions about engaging in sun-protection: study questionnaire

Section 1 – Sun Habits (Adapted from Glanz et al. 2008)

Think about your most recent holidays abroad. For each question listed, please select the one answer that is the best response to the question. There is no right or wrong answer.

1. On average, how many hours/day were you outside between 10 am and 4pm on WEEKDAYS (Monday-Friday)? (Please tick your answer).

30 minutes or less .....○.

31 minutes to 1 hour.....○

2 hours .....○

3 hours .....○.

4 hours .....○.

5 hours .....○

6 hours .....○

2. On average, how many hours/day were you outside between 10am and 4pm on WEEKEND DAYS (Saturday & Sunday)? (Please tick your answer).

30 minutes or less .....○.

31 minutes to 1 hour.....○

2 hours .....○

3 hours .....○.

4 hours .....○.

5 hours .....○.

6 hours .....○.

3. In your recent holidays, how many times did you have a red OR painful sunburn that lasted a day or more? (Please tick your answer).

0	1	2	3	4	5 OR MORE
○	○	○	○	○	○

4. For the following questions, think about what you did when you were outside <u>during your recent holidays on a warm sunny day</u>. (Please tick your answers).

	NEVER	RARELY	SOMETIMES	OFTEN	ALWAYS
a) How often did you wear **SUNSCREEN**?	○	○	○	○	○
b) How often did you wear a **SHIRT WITH SLEEVES that cover your shoulders**?	○	○	○	○	○
c) How often did you wear a **HAT**?.....	○	○	○	○	○
d) How often did you stay in the **SHADE or UNDER AN UMBRELLA**?	○	○	○	○	○

e) How often did you wear **SUNGLASSES**?	○	○	○	○	○

5. On average, how often do you spend time <u>in the sun</u> in order to get a tan?

Never ..... ○

Rarely ..... ○

Sometimes..... ○

Often ..... ○

Always ..... ○

<u>Skin sensitivity Assessment</u>

For each question listed, please select the one answer that is the best response to the question. There is no right or wrong answer. Please tick your answer.

1. How would you best describe the colour of your skin?

I. Very pale/Reddish
II. Pale
III. Beige
IV. Light brown (lightly tanned)
V. Moderate brown or tanned
VI. Dark brown or black

2. Which of the following best describes your reaction to an initial sun exposure of 45-60 minutes (without sun protection) around midday in the early UK summer?

I. Burn easily, never tan
II. Burn easily, tan minimally with difficulty
III. Burn moderately, tan moderately
IV. Burn minimally, tan moderately and easily
V. Rarely burn, tan profusely
VI. Never burn, tan profusely

3. What is the natural colour of your hair?

I. Red
II. Blond
III. Light Brown
IV. Brown
V. Dark brown or black

Section 2 – Perceptions about sun-protection

We are very interested in your views on sun experiences during a holiday. The following questions will help us to find out more about your experiences and preferences about sun protection.

1. Let's start with some general questions about how much you know about sun protection.

During which of the following time periods is sun protection most needed?

a) 11am -3pm
b) 12 noon - 1pm
c) 1pm - 4pm
d) 11am - 1pm

When buying a sunscreen what do you need to consider

a) Expiry date
b) Sun Protection Factor (SPF)
c) Provided protection against UVA and UVB
d) All of the above

What is the best way to protect your skin from sun damage?

a) Avoiding sun exposure
b) Finding shade, wearing a hat, clothing, sunglasses and sunscreen SPF 15+
c) Using sunscreen SPF 15+
d) Having a tan before going on holidays

What is the UV index?

a) A tool to measure waves length
b) A measurement of the intensity of the sun's ultraviolet (UV) radiation
c) A weather tool used to report hours of daylight
d) Don't know

Please, answer to the following questions below by selecting the option that best represents your views and experiences.

1. Your intentions

In this section, we are interested in your plans for sun protection and sun exposure during your holiday. For each statement, please circle the number in each line that best describes your opinion.

	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
I intend to seek shade when I go out in the midday sun	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
I intend to cover-up with protective clothing when I go out in the midday sun	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
I intend to use sunscreen with SPF 15 or higher when I go out in the midday sun	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
I intend to sunbathe to get a suntan	Strongly disagree	1	2	3	4	5	6	7	Strongly agree

2. People have different views about sun exposure. In the following questions, we would like to ask you to respond to a few statements about sun protection and sunbathing during your holiday.

For me, **using sun-protection in the midday sun** would be..

Uncomfortable	1	2	3	4	5	6	7	Comfortable
Unenjoyable	1	2	3	4	5	6	7	Enjoyable
Unpleasant	1	2	3	4	5	6	7	Pleasant

For me, **using sun-protection in the midday sun** would..

	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
Decrease my risk of sunburn	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
Make me tan less	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
Be costly/expensive	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
Decrease my risk of skin cancer	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
Protect my skin from aging	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely

In the long run, using sun protection in the midday sun will make me feel**..**

	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
More attractive	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
More comfortable about my skin	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
Better about myself	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely
Safer	Extremely unlikely	1	2	3	4	5	6	7	Extremely likely

For me, to **get a tan** would make me..

	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
Feel more confident about my appearance	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
Feel more attractive	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
Feel healthier	Strongly disagree	1	2	3	4	5	6	7	Strongly agree
Receive compliments about my appearance	Strongly disagree	1	2	3	4	5	6	7	Strongly agree

The questionnaire is complete. Thank you for your time.

8.1.2 Variables and scales

Category	Variables	Scoring
Skin sensitivity assessment		
Skin sensitivity	3 items (e.g. 'How would you best describe the colour of your skin?)	Scored on Likert scale with less sensitive skin scoring more highly.
Behavioural measures		
Sun exposure	2 items (e.g. 'Think about your most recent holidays. On average, how many hours/day were you outside between 10am and 4pm on WEEKDAYS?') (96)	Scored from '30 minutes or less' to '6 hours' on a Likert scale. A mean of both items was generated, giving a score between 1 and 7.
Sunburns	1 item (e.g. 'In your recent holidays, how many times did you have red OR painful sunburn that lasted a day or more?') (96)	Scored from '0' to '5+' on a Likert scale.
Sun protection behaviours	5 items (e.g. 'Think about what you did when you were outside during your recent holidays on a warm sunny day. How often did you wear sunscreen?') (96)	Scored from 'never' to 'always' on a Likert scale. A mean of the 5 items was generated, giving a score between 1 and 5. Individual behaviour scores were also used for analysis.
Time spent in the sun to tan	1 item (e.g. On average, how often do you spend time in the sun to get a tan?') (96)	Scored from 'never' to 'always' on a Likert scale.
Psychosocial variables		
Knowledge	4 items (e.g. 'What is UV index?') (97, 98)	Scored from '1' to '4' in no particular order. The correct answer was given a score of 1. Scores for all 4 of the questions were added together to give an overall knowledge score. Individuals were grouped depending on their overall knowledge score.

Intentions	3 items on sun protection (e.g. 'I intend to seek shade when I go out in the midday sun') and 1 item on tanning (e.g. 'I intend to sunbathe to get a tan') (99)	Sun protection items scored from 'strongly disagree' to 'strongly agree' on a Likert. Tanning item was reverse coded so that it was scored in the same direction. A mean of all 4 items was generated, giving an average score between 1 and 7.
Attitudes towards sun protection	3 items on affective short-term attitudes (e.g. 'For me, using sun protection in the midday sun would be… Uncomfortable/comfortable') (97, 98), 5 items on short term rationale attitudes (e.g. 'For me, using sun protection in the midday sun would decrease my risk of sunburn') (99) and 4 items on long-term attitudes (e.g. 'In the long run, using sun protection would make me feel more attractive') (97, 98)	Short-term affective attitudes scored from 'strongly agree' to 'strongly disagree' on a Likert scale, short term rationale attitudes scored from 'extremely likely' to 'extremely unlikely' on a Likert scale and long-term attitudes scored from 'extremely unlikely' to 'extremely likely' on a Likert scale. The 3 items for short-term affective attitudes, 5 items for short term rationale attitudes and 4 items for long-term attitudes were used to generate a mean for each of the 3 variables between 1 and 7.
Attitudes towards a tanned appearance	4 items (e.g. 'For me, to get a tan would make me feel more confident about my appearance') (99)	Scored from 'strongly agree' to 'strongly disagree' on a Likert scale. A mean of all 4 items was generated, giving a score between 1 and 7.

Table 8.1 Study 1 questionnaire variables and scales.

8.2 Study 3: Evaluating the effect of seasonal variation in UVR exposure on mtDNA damage in the epidermis

8.2.1 Questionnaire

Sun protection habits and perceptions questionnaire 2021

Page 1: Consent page

Thank you for your interest in our study. Please read the following information sheet provided separately carefully so that you understand what the study will entail. If after reading you still have some queries, please use the contact email below to ask any questions.

What is the purpose of this study?

The purpose of this study is to gain a more in depth understanding of individual variation in sun exposure and sun protection behaviours, outdoor physical activity levels and daily sugar intake.

What does the study entail?

From this page you will be able to access the questionnaire. It will take approximately 5-10 minutes to complete and all responses are anonymous.

Can I take part in this study?

You are warmly invited to participate in this research; however, participants must be over the age of 18.

If you have any questions or issues about the study then please email the researcher:

L.Ruddy1@newcastle.ac.uk

1. Do you consent and wish to take part in the study? ✱ *Required*

- ○ Yes

2. Please enter your unique study code in the box below ✱ *Required*

Page 2: Demographics

3. How old are you? ✱ *Required*

Please enter a whole number (integer).
Please make sure the number is between 18 and 100.

4. What is your skin type?

✱

Required

- Fitzpatrick skin type 1
- Fitzpatrick skin type 2
- Fitzpatrick skin type 3
- Fitzpatrick skin type 4
- Fitzpatrick skin type 5
- Fitzpatrick skin type 6

Page 3: Sun habits

Think about your most recent holidays abroad or time spent outside on a warm sunny day within the UK. For each question listed, please select one answer.

5. On average, how many hours/day were you outside between 10am and 4pm on WEEKDAYS (Monday-Friday)? ✱ *Required*

- ○ 30 minutes or less
- ○ 31 minutes to 1 hour
- ○ 2 hours
- ○ 3 hours
- ○ 4 hours
- ○ 5 hours
- ○ 6 hours

6. On average, how many hours/day were you outside between 10am and 4pm on WEEKEND DAYS (Saturday and Sunday)? ✱ *Required*

- ○ 30 minutes or less
- ○ 31 minutes to 1 hour
- ○ 2 hours
- ○ 3 hours
- ○ 4 hours
- ○ 5 hours
- ○ 6 hours

7. During a holiday abroad or time spent outside on a warm sunny day within the UK, would you be likely to obtain at least 1 sunburn? ✱ *Required*

- Yes
- No

8. For the following questions, think about what you do when you are outside during a holiday or within the UK during summer on a warm sunny day. ✱ *Required*

Please don't select more than 1 answer(s) per row.

Please select exactly 5 answer(s).

Please don't select more than 5 answer(s) in any single column.

	Never	Rarely	Sometimes	Often	Always
How often did you wear sunscreen?	☐	☐	☐	☐	☐
How often did you wear shirt with sleeves that cover your shoulders?	☐	☐	☐	☐	☐
How often did you wear a hat?	☐	☐	☐	☐	☐
How often did you stay in the shade or under an umbrella	☐	☐	☐	☐	☐
How often did you wear sunglasses?	☐	☐	☐	☐	☐

9. On average, how often do you spend time in the sun in order to get a tan? ✱ *Required*

- Never
- Rarely
- Sometimes
- Often
- Always

Page 4: Perceptions about sun protection

10. During which of the following time periods is sun protection most needed? ✱ *Required*

- 11am-3pm
- 12 noon-1pm
- 1pm-4pm
- 11am-1pm

11. When buying sunscreen what do you need to consider? ✱ *Required*

- Expiry date
- Sun Protection Factor (SPF)
- Provided protection against UVA and UVB
- All of the above

12. What is the best way to protect your skin from sun damage? ✱ *Required*

- Avoiding sun exposure
- Finding shade, wearing a hat, clothing, sunglasses and sunscreen SPF 15+
- Using sunscreen SPF 15+
- Having a tan before going on holiday

13. What is the UV index? ✱ *Required*

- A tool to measure wavelength
- A measurement of the intensity of the suns ultraviolet (UV) radiation
- A weather tool used to report hours of daylight
- Don't know

14. We are interested in your plans for sun protection and sun exposure during a holiday or within the UK on a warm sunny day. Please select the option that best describes your opinion.
✱ *Required*

Please don't select more than 1 answer(s) per row.

Please select exactly 4 answer(s).

Please don't select more than 4 answer(s) in any single column.

	1 - Strongly disagree	2	3	4	5	6	7 - Strongly agree
I intend to seek shade when I go out in the midday sun	☐	☐	☐	☐	☐	☐	☐
I intend to cover up with protective clothing when I go out in the midday sun	☐	☐	☐	☐	☐	☐	☐
I intend to use sunscreen with SPF 15 or higher when I go out in the midday sun	☐	☐	☐	☐	☐	☐	☐
I intend to sunbathe to get a tan	☐	☐	☐	☐	☐	☐	☐

15. For me, using sun protection in the midday sun would be... ✱ *Required*

Please don't select more than 1 answer(s) per row.

Please select exactly 3 answer(s).

Please don't select more than 3 answer(s) in any single column.

	1 - Strongly disagree	2	3	4	5	6	7 - Strongly agree
Comfortable	☐	☐	☐	☐	☐	☐	☐
Enjoyable	☐	☐	☐	☐	☐	☐	☐
Pleasant	☐	☐	☐	☐	☐	☐	☐

16. For me, using sun protection in the midday sun would... ✱ *Required*

Please don't select more than 1 answer(s) per row.

Please select exactly 5 answer(s).

Please don't select more than 5 answer(s) in any single column.

	1 - Extremely unlikely	2	3	4	5	6	7 - Extremely likely
Decrease my risk of sunburn	☐	☐	☐	☐	☐	☐	☐
Make me tan less	☐	☐	☐	☐	☐	☐	☐
Be costly/expensive	☐	☐	☐	☐	☐	☐	☐
Decrease my risk of skin cancer	☐	☐	☐	☐	☐	☐	☐
Protect my skin from ageing	☐	☐	☐	☐	☐	☐	☐

17. In the long run, using sun protection in the midday sun will make me feel... ✱ *Required*

Please don't select more than 1 answer(s) per row.

Please select exactly 4 answer(s).

Please don't select more than 4 answer(s) in any single column.

	1 - Extremely unlikely	2	3	4	5	6	7 - Extremely likely
More attractive	☐	☐	☐	☐	☐	☐	☐
More comfortable about my skin	☐	☐	☐	☐	☐	☐	☐
Better about myself	☐	☐	☐	☐	☐	☐	☐
Safer	☐	☐	☐	☐	☐	☐	☐

18. For me, to get a tan would make me... ✱ *Required*

Please don't select more than 1 answer(s) per row.

Please select exactly 4 answer(s).

Please don't select more than 4 answer(s) in any single column.

	1- Strongly disagree	2	3	4	5	6	7 - Strongly agree
Feel more confident about my appearance	☐	☐	☐	☐	☐	☐	☐
Feel more attractive	☐	☐	☐	☐	☐	☐	☐
Feel healthier	☐	☐	☐	☐	☐	☐	☐

Receive compliments about my appearance	☐	☐	☐	☐	☐	☐	☐

Page 5: Lifestyle

19. How often do you use a product with sun protection (SPF)? ✱ *Required*

- Always
- Sometimes
- Never
- When sunbathing
- When sunny outside

20. How often do you sunbathe? ✱ *Required*

- Never
- Rarely
- Sometimes
- When on holiday
- When sunny outside

21. Are you currently living in an urban environment (polluted city)? ✱ *Required*

- Yes
- No
- Don't know

22. How frequently do you do outdoor sports and physical activities such as walking? ✱ *Required*

- Often
- Somtimes

- Never

23. How much sugar do you consume each day? (e.g. fizzy drinks, chocolate, cakes/biscuits) ✱ *Required*

- A lot
- Some
- None

Page 6: Skin condition

24. Do you have any of the below skin conditions? ✱ *Required*

- [ ] Eczema
- [ ] Psoriasis
- [ ] Rosecea
- [ ] None

Page 7: Final page

The questionnaire is complete. Thank you for your time.

8.2.2 Variables and scales

Category	Variables	Scoring
Skin sensitivity assessment		
Skin sensitivity	1 item (e.g. 'What is your skin type?')	Scored on the Fitzpatrick skin type scale from '1' to '6'.
Behavioural measures		
Sun exposure	2 items (e.g. 'Think about your most recent holidays, or time spent outside on a warm sunny day in the UK. On average, how many hours/day were you outside between 10am and 4pm on WEEKDAYS?') (96)	Scored from '30 minutes or less' to '6 hours' on a Likert scale. A mean of both items was generated, giving a score between 1 and 7.
Sunburns	1 item (e.g. 'During a holiday abroad or time spent outside on a warm sunny day within the UK, would you be likely to obtain at least 1 sunburn?')	Scored as 'Yes' and 'No'. Individuals were grouped accordingly.
Sun protection behaviours	5 items (e.g. 'Think about what you did when you were outside during a holiday or within the UK during summer on a warm sunny day. How often did you wear sunscreen?') (96)	Scored from 'never' to 'always' on a Likert scale. A mean of the 5 items was generated, giving a score between 1 and 5.
Time spent in the sun to tan	1 item (e.g. On average, how often do you spend time in the sun to get a tan?') (96)	Scored from 'never' to 'always' on a Likert scale.
Psychosocial variables		
Knowledge	4 items (e.g. 'What is UV index?') (97, 98)	Scored from '1' to '4' in no particular order. The correct answer was given a score of 1. Scores for all 4 of the questions were added together to give an overall knowledge score. Individuals were grouped

		depending on their overall knowledge score.
Intentions	3 items on sun protection (e.g. 'I intend to seek shade when I go out in the midday sun') and 1 item on tanning (e.g. 'I intend to sunbathe to get a tan') (99)	Sun protection items scored from 'strongly disagree' to 'strongly agree' on a Likert scale. Tanning item was reverse coded so that it was scored in the same direction. A mean of all 4 items was generated, giving an average score between 1 and 7.
Attitudes towards sun protection	3 items on affective short-term attitudes (e.g. 'For me, using sun protection in the midday sun would be… Uncomfortable/comfortable') (97, 98), 5 items on short term rationale attitudes (e.g. 'For me, using sun protection in the midday sun would decrease my risk of sunburn') (99) and 4 items on long-term attitudes (e.g. 'In the long run, using sun protection would make me feel more attractive') (97, 98)	Short-term affective attitudes scored from 'strongly agree' to 'strongly disagree' on a Likert scale, short term rationale attitudes scored from 'extremely likely' to 'extremely unlikely' on a Likert scale and long-term attitudes scored from 'extremely unlikely' to 'extremely likely' on a Likert scale. The 3 items for short-term affective attitudes, 5 items for short term rationale attitudes and 4 items for long-term attitudes were used to generate a mean for each of the 3 variables between 1 and 7.
Attitudes towards a tanned appearance	4 items (e.g. 'For me, to get a tan would make me feel more confident about my appearance') (99)	Scored from 'strongly agree' to 'strongly disagree' on a Likert scale. A mean of all 4 items was generated, giving a score between 1 and 7.
Other		
Lifestyle	5 items (e.g. 'Are you currently living in an urban environment (polluted city)?)	N/A
Skin condition	1 item (e.g. 'Do you have any of the below skin conditions?')	N/A

Table 8.2 Study 3 variables and scales.

8.3 A cross-sectional study investigating the impact of COVID-19 lockdown on sun exposure and behaviours within the UK

8.3.1 Questionnaire

Sun protection habits and perceptions - Final

Start of Block: Consent

Section 1 **Consent**

Thank you for your interest in our study. Please read the following information carefully so that you understand what the study will entail. If after reading you still have some queries, please use the contact email below to ask any questions. What is the purpose of this study? The purpose of this study is to gain a more in depth understanding of individual variation in sun exposure behaviours, physical activity and alcohol consumption. The study will also look at the effect of the UK lockdown; implemented by the government on 23rd March 2020 as a result of Coronavirus (COVID-19), on these behaviours. What does the study entail? From this page you will be able to access the questionnaire. It will take roughly 15 minutes to complete and all responses are anonymous. Can I take part in this study? You are warmly invited to participate in this research; however, participants must be over the age of 18. Should I take part? Your participation in this study is voluntary. You do not have to take part in this study and you may leave at any point without giving reason. Once you have agreed to take part you are still free to change your mind and withdraw at any time. If you withdraw from the study any information already gathered from you will either be kept securely and confidentially or destroyed if you wish. Will my participation be kept confidential? Yes, all personal information collected will be kept confidential and anonymised in use. There will be no way of tracing your responses back to you. If you have any questions or issues about this study then please email the researchers: Jessica Moor (j.moor@newcastle.ac.uk) Lizzie Ruddy (L.Ruddy1@newcastle.ac.uk) **Do you consent and wish to take part in this study?** By clicking the consent button below you are acknowledging that your participation in the study is voluntary, you are aged 18 or over, and you are aware that you can withdraw yourself from the study at any point.

- ○ Yes, I consent (1)
- ○ No (2)

Skip To: End of Survey If Q1D3 != Yes, I consent

End of Block: Consent

Start of Block: Demographics

Page 1 of 24

Section 2 **Demographics**

Q1 How old are you?

_____

Q2 What is your sex?

- Male (1)
- Female (2)
- Prefer not to say (3)

Q3 What is your marital status?

- Married/civil partnership (1)
- Cohabiting (2)
- Single (3)
- Widowed (4)
- Separated (5)
- Divorce/civil partnership dissolved (6)
- Prefer not to say (7)

Q4 What is your ethnic group?

- ○ English/Welsh/Scottish/Northern Irish/British (1)
- ○ Irish (2)
- ○ Gypsy or Irish Traveller (3)
- ○ White and Black Caribbean (4)
- ○ White and Black African (5)
- ○ White and Asian (6)
- ○ Indian (7)
- ○ Pakistani (8)
- ○ Bangladeshi (9)
- ○ Chinese (10)
- ○ African (11)
- ○ Caribbean (12)
- ○ Arab (13)
- ○ Prefer not to say (14)
- ○ Other, please specify (16) _____

Q5 Where do you currently live?

- ○ North East (England) (1)
- ○ North West (England) (2)
- ○ Yorkshire and the Humber (England) (3)
- ○ West Midlands (England) (4)
- ○ East Midlands (England) (5)
- ○ East of England (England) (6)
- ○ South East (England) (7)
- ○ South West (England) (8)
- ○ Greater London (England) (9)
- ○ Scotland (10)
- ○ Wales (11)
- ○ Northern Ireland (12)
- ○ Outside the UK, please specify (13) _____

End of Block: Demographics

Start of Block: Current occupation

Section 3 **Current occupation**

Q6 Please select your current employment status

- Working in usual environment (1)
- Working from home (2)
- Furloughed (3)
- Student (4)
- Unemployed (5)
- Prefer not to say (6)

Display This Question:
If Q6 != Working in usual environment

Q6a If you are not working in your usual environment, are you spending more time outdoors?

- Yes (1)
- No (2)
- Not sure (3)

Display This Question:
If Q6 = Working in usual environment

Q6b If you are working in your usual environment, does this involve any work outdoors?

- Never (1)
- Rarely (2)
- Sometimes (3)
- Frequently (4)
- Always (5)

End of Block: Current occupation

Start of Block: Sun habits

Section 4 **Sun habits**

Q7
For each question listed, please select the one answer that is the best response to the question.

During spring/summer this year, on average, how many hours/day were you outside between 10am and 4pm on WEEKDAYS (Monday-Friday)?

- 30 minutes or less (1)
- 31 minutes to 1 hour (2)
- 2 hours (3)
- 3 hours (4)
- 4 hours (5)
- 5 hours (6)
- 6 hours (7)

Q8 During spring/summer this year, on average, how many hours/day were you outside between 10am and 4pm on the WEEKEND (Saturday & Sunday)?

- 30 minutes or less (1)
- 31 minutes to 1 hour (2)
- 2 hours (3)
- 3 hours (4)
- 4 hours (5)
- 5 hours (6)
- 6 hours (7)

Q9 During spring/summer **this year**, how many times did you have red OR painful sunburn that lasted a day or more?

- 0 (1)
- 1 (2)
- 2 (3)
- 3 (4)
- 4 (5)
- 5+ (6)
- Don't know (7)

Q10 During spring/summer **last year**, how many times did you have red OR painful sunburn that lasted a day or more?

- ○ 0 (1)
- ○ 1 (2)
- ○ 2 (3)
- ○ 3 (4)
- ○ 4 (5)
- ○ 5+ (6)
- ○ Don't know (7)

Q11 For the following questions, think about what you did when you were outside during spring/summer this year on a warm sunny day.

	Never (1)	Rarely (2)	Sometimes (3)	Often (4)	Always (5)
How often did you wear sunscreen? (1)	○	○	○	○	○
How often did you wear a shirt with sleeves that cover your shoulders? (2)	○	○	○	○	○
How often did you wear a hat? (3)	○	○	○	○	○
How often did you stay in the shade or under an umbrella (4)	○	○	○	○	○
How often did you wear sunglasses? (5)	○	○	○	○	○

Q12 On average, how often do you spend time in the sun in order to get a tan?

- ○ Never (1)
- ○ Rarely (2)
- ○ Sometimes (3)
- ○ Often (4)
- ○ Always (5)

Q13
Now we want to ask a couple of questions about your sun habits within the UK, especially during the UK lockdown period which was imposed on the 23rd March 2020 by the government as a result of the Coronavirus (COVID-19) pandemic. If this doesn't apply to you as you do not currently live within the UK, please answer with N/A.
Please select the statement which best applies to your sun habits within the UK.

- My sun protection habits are the same in the UK as they are abroad (1)
- My sun protection habits are not as strict in the UK as they are abroad (2)
- My sun protection habits are more strict in the UK as they are abroad (3)
- N/A (4)

Q14 Since the UK went into lockdown on the 23rd March 2020 as a result of COVID-19, would you say that you are spending/spent more time outdoors/in the garden?

- Yes (1)
- No (2)
- It's hard to say (3)
- N/A (4)

Display This Question:
If Q14 = Yes

Q14a If you selected "'yes" to the following question, what is/was the main outdoor activity that you have been/were doing? (e.g. sunbathing, walking, gardening)

_____

Q15 Would you say that the news reports that suggested a link between low levels of vitamin D and COVID-19 made you spend more time outdoors during the UK lockdown?

- ○ Yes (1)
- ○ No (2)
- ○ I don't know (3)
- ○ I didn't see the news reports (4)
- ○ N/A (5)

Q16 Are you aware of the importance of sunlight in the production of vitamin D?

- ○ Yes (1)
- ○ No (2)
- ○ It's hard to say (3)

End of Block: Sun habits

Start of Block: Skin sensitivity assessment

Section 5 **Skin sensitivity assessment**

Q17
Select the skin type which best represents your skin

- ○ 1 - Very Fair (1)
- ○ 2 - Fair (2)
- ○ 3 - Medium (3)
- ○ 4 - Olive (4)
- ○ 5 - Brown (5)
- ○ 6 - Black (6)

End of Block: Skin sensitivity assessment

Start of Block: Perceptions about sun protection

Section 6 **Perceptions about sun protection**

Q18 During which of the following time periods is sun protection most needed?

- ○ 11am-3pm (1)
- ○ 12pm-1pm (2)
- ○ 1pm-4pm (3)
- ○ 11am-1pm (4)

Q19 When buying a sunscreen what do you need to consider?

- ○ Expiry date (1)
- ○ Sun Protection Factor (SPF) (2)
- ○ Provided protection against UVA and UVB (3)
- ○ All of the above (4)

Q20 What is the best way to protect your skin from sun damage?

- ○ Avoiding sun exposure (1)
- ○ Finding shade, wearing a hat, clothing, sunglasses and sunscreen SPF 15+ (2)
- ○ Using sunscreen SPF 15+ (3)
- ○ Having a tan before going on holiday (4)

Q21 What is the UV index?

- ○ A tool to measure wave length (1)
- ○ A measurement of the intensity of the suns ultraviolet (UV) radiation (2)
- ○ A weather tool used to report hours of daylight (3)
- ○ Don't know (4)

Q22
In this question we are interested in your plans for sun protection and sun exposure during a holiday abroad, or within the UK during spring/summer.

For each statement please select the number in each line that best describes your opinion.

(1 - strongly agree, 7 - strongly disagree)

	1 (1)	2 (2)	3 (3)	4 (4)	5 (5)	6 (6)	7 (7)
I intent to seek shade when I go out in the midday sun (1)	○	○	○	○	○	○	○
I intend to cover up with protective clothing when I go out in the midday sun (2)	○	○	○	○	○	○	○
I intend to use suncream with SPF 15 or higher when I go out in the midday sun (3)	○	○	○	○	○	○	○
I intend to sunbathe to get a suntan (4)	○	○	○	○	○	○	○

Q23

In the following questions we would like to ask you to respond to a few statements about sun protection and sunbathing during a holiday abroad, or within the UK during spring/summer.

For me, using sun protection would be

(1 - strongly agree, 7 - strongly disagree)

	1 (1)	2 (2)	3 (3)	4 (4)	5 (5)	6 (6)	7 (7)
Uncomfortable (1)	○	○	○	○	○	○	○
Unenjoyable (2)	○	○	○	○	○	○	○
Unpleasant (3)	○	○	○	○	○	○	○

Q24 For me, using sun protection would

(1 - extremely likely, 7 - extremely unlikely)

	1 (1)	2 (2)	3 (3)	4 (4)	5 (5)	6 (6)	7 (7)
Decrease my risk of sunburn (1)	○	○	○	○	○	○	○
Make me tan less (2)	○	○	○	○	○	○	○
Be costly/expensive (3)	○	○	○	○	○	○	○
Decrease my risk of skin cancer (4)	○	○	○	○	○	○	○
Protect my skin from ageing (5)	○	○	○	○	○	○	○
Be negative to the environment (6)	○	○	○	○	○	○	○

Q25 In the long run, using sun protection will make me feel

(1 - extremely likely, 7 - extremely unlikely)

	1 (1)	2 (2)	3 (3)	4 (4)	5 (5)	6 (6)	7 (7)
More attractive (1)	○	○	○	○	○	○	○
More comfortable about my skin (2)	○	○	○	○	○	○	○
Better about myself (3)	○	○	○	○	○	○	○
Safer (4)	○	○	○	○	○	○	○

Q26 For me, to get a tan would make me

(1 - strongly agree, 7 - strongly disagree)

	1 (1)	2 (2)	3 (3)	4 (4)	5 (5)	6 (6)	7 (7)
Feel more confident about my appearance (1)	○	○	○	○	○	○	○
Feel more attractive (2)	○	○	○	○	○	○	○
Feel healthier (3)	○	○	○	○	○	○	○
Receive compliments about my appearance (4)	○	○	○	○	○	○	○

End of Block: Perceptions about sun protection

Start of Block: Self efficacy

Section 7 **Self efficacy**

Q27
Would you please indicate below how confident you are that you can do the following steps during a holiday abroad, or within the UK during spring/summer.
I am confident that I can

(1 - not confident at all, 7 - extremely confident)

	1 (1)	2 (2)	3 (3)	4 (4)	5 (5)	6 (6)	7 (7)
Pick a good sunscreen (i.e. SPF 15+, both UVA and UVB protection, expiry date) (1)	○	○	○	○	○	○	○
Apply sunscreen properly (i.e. how and where to apply it, the quantity, how long to wait before going out in the sun) (2)	○	○	○	○	○	○	○
Re-apply sunscreen properly (i.e. how often, after which activities) (3)	○	○	○	○	○	○	○
Use the right level of protection for my individual skin type and sun intensity (i.e. UV levels) (4)	○	○	○	○	○	○	○

Seek shade when I go out in the midday sun (5)	○	○	○	○	○	○	○
Cover up with protective clothing when I go out in the midday sun (6)	○	○	○	○	○	○	○
Get a suntan without burning (7)	○	○	○	○	○	○	○

End of Block: Self efficacy

Start of Block: Alcohol consumption

Section 8 **Alcohol consumption**

Q28 How often do you have a drink containing alcohol?

- ○ Never (1)
- ○ Monthly or less (2)
- ○ 2-4 times a month (3)
- ○ 2-3 times a week (4)
- ○ 4 or more times a week (5)

Q29 How many drinks containing alcohol do you have on a typical day when you are drinking?

- 1 or 2 (1)
- 3 or 4 (2)
- 5 or 6 (3)
- 7 to 9 (4)
- 10 or more (5)
- 0 (6)

Q30 How often do you have six or more drinks on one occasion?

- Never (1)
- Less than monthly (2)
- Monthly (3)
- Weekly (4)
- Daily or almost daily (5)

Q31 How often during the last year have you found that you were NOT able to stop drinking once you had started?

- Never (1)
- Less than monthly (2)
- Monthly (3)
- Weekly (4)
- Daily or almost daily (5)

Q32 During the UK lockdown period as a result of COVID-19, did you alcohol consumption increase?

If this doesn't apply to you as you do not currently live within the UK, please answer with N/A.

- Yes (1)
- No (2)
- It's hard to say (3)
- N/A (4)

End of Block: Alcohol consumption

Start of Block: Physical activity

Section 9 **Physical activity**

Q33 During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics or fast cycling?

_____

Q34 How many hours did you usually spend doing vigorous physical activities on one of those days?

- 0 (1)
- Less than 1 hour (2)
- 1-2 (3)
- 3+ (4)

*

Q35 During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace or doubles tennis? Do not include walking.

_____

Q36 How many hours did you usually spend doing moderate physical activities on one of those days?

- 0 (1)
- Less than 1 hour (2)
- 1-2 (3)
- 3+ (4)

*

Q37 During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

_____

Q38 How many hours did you usually spend walking on one of those days?

- 0 (1)
- Less than 1 hour (2)
- 1-2 (3)
- 3+ (4)

Q39 During the last 7 days, how many hours did you spend sitting on a weekday?

- 0 (1)
- 1-2 (2)
- 3-4 (3)
- 5-6 (4)
- 7+ (5)

Q40 Would you say that the majority of your physical exercise is done indoors or outdoors?

- Indoors (1)
- Outdoors (2)
- Mixture of both (3)
- N/A (4)

Q41 Since the government announced lockdown within the UK, has your level of physical activity increased?

- Yes (1)
- No (2)
- It's hard to say (3)
- N/A (4)

End of Block: Physical activity

8.3.2 Variables and scales

Category	Variables	Scoring
Demographics and occupation		
Demographics	5 items (e.g. 'How old are you?')	
Occupation	3 items (e.g. 'Please select your current employment status)	
Behavioural measures		
Sun exposure	2 items (e.g. 'During spring/summer this year, on average, how many hours/day were you outside between 10am and 4pm on WEEKDAYS?') (96)	Scored from '30 minutes or less' to '6 hours' on a Likert scale. A mean of both items was generated, giving a score between 1 and 7.
Sunburns	2 items (e.g. 'During spring/summer this year, how many times did you have red OR painful sunburn that lasted a day or more?') (96)	Scored from '0' to '5+' on a Likert scale.
Sun protection behaviours	5 items (e.g. 'When you were outside during spring/summer this year on a warm sunny day, how often did you wear sunscreen?') (96)	Scored from 'never' to 'always' on a Likert scale. A mean of the 5 items was generated, giving a score between 1 and 5.
Time spent in the sun to tan	1 item (e.g. 'On average, how often do you spend time in the sun in order to get a tan?') (96)	Scored from 'never' to 'always' on a Likert scale.
Sun protection habits within the UK	1 item (e.g. 'Please select the statement which best applies to your sun protection habits within the UK')	
Psychosocial variables		

Knowledge	4 items (e.g. 'What is UV index?') (97, 98)	Scored from '1' to '4' in no particular order. The correct answer was given a score of 1. Scores for all 4 questions were added together to give an overall knowledge score. Individuals were grouped depending on their overall score.
Intentions	3 items on sun protection (e.g. 'I intend to seek shade when I go out in the midday sun') and 1 item on tanning ('I intend to sunbathe to get a tan') (99)	Sun protection items scored from 'strongly disagree' to 'strongly agree' on a Likert scale. Tanning item was reverse coded so that it was scored in the same direction. A mean of all 4 items was generated, giving an average score between 1 and 7.
Attitudes towards sun protection	3 items on affective short-term attitudes (e.g. 'For me, using sun protection would be… Uncomfortable/comfortable') (97, 98), 5 items on short term rationale attitudes (e.g. 'For me, using sun protection would decrease my risk of sunburn') (99) and 4 items on long-term attitudes (e.g. 'In the long run, using sun protection would make me feel more attractive') (97, 98)	Short-term affective attitudes scored from 'strongly agree' to 'strongly disagree' on a Likert scale, short term rationale attitudes scored from 'extremely likely' to 'extremely unlikely' on a Likert scale and long-term attitudes scored from 'strongly agree' to 'strongly disagree' on a Likert scale. The 3 items for short-term affective attitudes, 5 items for short-term rationale attitudes and 4 items for long-term attitudes were used to generate a mean for each of the 3 variables between 1 and 7.
Attitudes towards a tanned appearance	4 items (e.g. 'For me, to get a tan would make me feel more attractive') (99)	Scored from 'strongly agree' to 'strongly disagree' on a Likert scale. A mean of all 4 items was generated, giving a score between 1 and 7.
Self-efficacy	7 items (e.g. 'I am confident that I can pick a good sunscreen') (99)	Scored from 'not confident at all' to 'extremely confident' on a Likert scale. A mean of all 7 items was

	generated, giving a score between 1 and 7.

Table 8.3 Variables and scales.

Chapter 9. References

1. Venus M, Waterman J, McNab I. Basic physiology of the skin. Surgery (Oxford). 2011;29(10):471-4.

2. Koster MI. Making an Epidermis. Annals of the New York Academy of Sciences. 2009;1170(1):7-10.

3. Barcaui EdO, Carvalho ACP, Piñeiro-Maceira J, Barcaui CB, Moraes H. Study of the skin anatomy with high-frequency (22 MHz) ultrasonography and histological correlation. Radiologia brasileira. 2015;48(5):324-9.

4. Lai-Cheong JE, McGrath JA. Structure and function of skin, hair and nails. Medicine (Abingdon 1995, UK ed). 2017;45(6):347-51.

5. Thingnes J, Lavelle TJ, Hovig E, Omholt SW. Understanding the melanocyte distribution in human epidermis: An agent-based computational model approach. PloS one. 2012;7(7):e40377-e.

6. Rittié L. Cellular mechanisms of skin repair in humans and other mammals. Journal of cell communication and signaling. 2016;10(2):103-20.

7. Koehler MJ, Vogel T, Elsner P, König K, Bückle R, Kaatz M. In vivo measurement of the human epidermal thickness in different localizations by multiphoton laser tomography. Skin research and technology. 2010;16(3):259-64.

8. Stout R, Birch-Machin M. Mitochondria's Role in Skin Ageing. Biology (Basel, Switzerland). 2019;8(2):29.

9. Papakonstantinou E, Roth M, Karakiulakis G. Hyaluronic acid: A key molecule in skin aging. Dermato-endocrinology. 2012;4(3):253-8.

10. Mphande FA. The Structure and Function of the Skin. Singapore: Singapore: Springer Singapore; 2020. p. 13-22.

11. Boer M, Duchnik E, Maleszka R, Marchlewicz M. Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function. Postępy dermatologii i alergologii. 2016;33(1):1-5.

12. Kalia YN, Pirot F, Guy RH. Homogeneous transport in a heterogeneous membrane: water diffusion across human stratum corneum in vivo. Biophysical journal. 1996;71(5):2692-700.

13. Elias PM. Skin barrier function. Current allergy and asthma reports. 2008;8(4):299-305.

14. Elias PM. The skin barrier as an innate immune element. Seminars in immunopathology. 2007;29(1):3-14.

15. Farage MA, Miller KW, Elsner P, Maibach HI. Intrinsic and extrinsic factors in skin ageing: a review. International journal of cosmetic science. 2008;30(2):87-95.

16. Wlaschek M, Tantcheva-Poór I, Naderi L, Ma W, Schneider LA, Razi-Wolf Z, et al. Solar UV irradiation and dermal photoaging. Journal of photochemistry and photobiology B, Biology. 2001;63(1):41-51.

17. Hearing VJ. Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. Journal of dermatological science. 2005;37(1):3-14.

18. Ghoreishi M. Heat shock proteins in the pathogenesis of inflammatory skin diseases. Journal of medical and dental sciences. 2000;47(2):143-50.

19. He SYBS, McCulloch CEP, Boscardin WJP, Chren M-MMD, Linos EMDD, Arron STMDP. Self-reported pigmentary phenotypes and race are significant but incomplete predictors of Fitzpatrick skin phototype in an ethnically diverse population. Journal of the American Academy of Dermatology. 2014;71(4):731-7.

20. D'Orazio J, Jarrett S, Amaro-Ortiz A, Scott T. UV radiation and the skin. International journal of molecular sciences. 2013;14(6):12222-48.

21. Denat L, Kadekaro AL, Marrot L, Leachman SA, Abdel-Malek ZA. Melanocytes as Instigators and Victims of Oxidative Stress. Journal of investigative dermatology. 2014;134(6):1512-8.

22. Roberts DW, Newton RA, Beaumont KA, Helen Leonard J, Sturm RA. Quantitative analysis of MC1R gene expression in human skin cell cultures. Pigment cell research. 2006;19(1):76-89.

23. Kanetsky PA, Ge F, Najarian D, Swoyer J, Panossian S, Schuchter L, et al. Assessment of Polymorphic Variants in the Melanocortin-1 Receptor Gene with Cutaneous Pigmentation Using an Evolutionary Approach. Cancer epidemiology, biomarkers & prevention. 2004;13(5):808-19.

24. Valverde P, Healy E, Jackson I, Rees JL, Thody AJ. Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. Nature genetics. 1995;11(3):328-30.

25. Wolf Horrell EM, Boulanger MC, D'Orazio JA. Melanocortin 1 receptor: Structure, function, and regulation. Frontiers in genetics. 2016;7(MAY):95-.

26. Harding RM, Healy E, Ray AJ, Ellis NS, Flanagan N, Todd C, et al. Evidence for Variable Selective Pressures at MC1R. American journal of human genetics. 2000;66(4):1351-61.

27. Alaluf S, Heath A, Carter NIK, Atkins D, Mahalingam H, Barrett K, et al. Variation in Melanin Content and Composition in Type V and VI Photoexposed and Photoprotected Human Skin: The Dominant Role of DHI. Pigment cell research. 2001;14(5):337-47.

28. Vierkötter A, Krutmann J. Environmental influences on skin aging and ethnic-specific manifestations. Dermato-endocrinology. 2012;4(3):227-31.

29. Nouveau-Richard S, Yang Z, Mac-Mary S, Li L, Bastien P, Tardy I, et al. Skin ageing: a comparison between Chinese and European populations. A pilot study. Journal of dermatological science. 2005;40(3):187.

30. Rijken F, Bruijnzeel PLB, van Weelden H, Kiekens RCM. Responses of Black and White Skin to Solar-Simulating Radiation: Differences in DNA Photodamage, Infiltrating Neutrophils, Proteolytic Enzymes Induced, Keratinocyte Activation, and IL-10 Expression. Journal of investigative dermatology. 2004;122(6):1448-55.

31. Halder RM, Ara CJ. Skin cancer and photoaging in ethnic skin. Dermatol Clin. 2003;21(4):725-32, x.

32. Tulah AS, Birch-Machin MA. Stressed out mitochondria: the role of mitochondria in ageing and cancer focussing on strategies and opportunities in human skin. Mitochondrion. 2013;13(5):444-53.

33. Taanman J-W. The mitochondrial genome: structure, transcription, translation and replication. BBA - Bioenergetics. 1999;1410(2):103-23.

34. Boulton SJ, Bowman A, Koohgoli R, Birch-Machin MA. Skin manifestations of mitochondrial dysfunction: more important than previously thought. Experimental dermatology. 2015;24(1):12-3.

35. Birch-Machin MA, Swalwell H. How mitochondria record the effects of UV exposure and oxidative stress using human skin as a model tissue. Mutagenesis. 2010;25(2):101-7.

36. Wickersham M, Wachtel S, Wong Fok Lung T, Soong G, Jacquet R, Richardson A, et al. Metabolic Stress Drives Keratinocyte Defenses against Staphylococcus aureus Infection. Cell reports (Cambridge). 2017;18(11):2742-51.

37. Feichtinger RG, Sperl W, Bauer JW, Kofler B. Mitochondrial dysfunction: a neglected component of skin diseases. Experimental dermatology. 2014;23(9):607-14.

38. Akram M, Akram M. Citric Acid Cycle and Role of its Intermediates in Metabolism. Cell biochemistry and biophysics. 2014;68(3):475-8.

39. Bowtell JL, Marwood S, Bruce M, Constantin-Teodosiu D, Greenhaff PL. Tricarboxylic Acid Cycle Intermediate Pool Size: Functional Importance for Oxidative Metabolism in Exercising Human Skeletal Muscle. Sports Medicine. 2007;37(12):1071-88.

40. Li Xb, Gu Jd, Zhou Qh. Review of aerobic glycolysis and its key enzymes – new targets for lung cancer therapy. Thoracic cancer. 2015;6(1):17-24.

41. Moor J, Ruddy E, Idowu O, Birch-Machin M. Mitochondrial DNA biomarkers in skin disease featuring skin ageing (In Press). Biomarkers in Dermatology: Springer; 2022.

42. Cogliati S, Lorenzi I, Rigoni G, Caicci F, Soriano ME. Regulation of Mitochondrial Electron Transport Chain Assembly. Journal of molecular biology. 2018;430(24):4849-73.

43. Fernie AR, Carrari F, Sweetlove LJ. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport: Physiology and metabolism. Current opinion in plant biology. 2004;7(3):254-61.

44. Sena Laura A, Chandel Navdeep S. Physiological Roles of Mitochondrial Reactive Oxygen Species. Molecular cell. 2012;48(2):158-67.

45. Seim I, Ma S, Gladyshev VN. Gene expression signatures of human cell and tissue longevity. npj aging and mechanisms of disease. 2016;2(1):16014-.

46. Farage MA, Miller KW, Elsner P, Maibach HI. Characteristics of the Aging Skin. Adv Wound Care (New Rochelle). 2013;2(1):5-10.

47. Harman D. Aging: a theory based on free radical and radiation chemistry. Journal of gerontology (Kirkwood). 1956;11(3):298-300.

48. Harman D. Free radicals in aging. Molecular and cellular biochemistry. 1988;84(2):155-61.

49. Beckman KB, Ames BN. The Free Radical Theory of Aging Matures. Physiological Reviews. 1998;78(2):547-81.

50. Harbottle A, Maki J, Reguly B, Wittock R, Robinson K, Parr R, et al. Real-time polymerase chain reaction analysis of a 3895-bp mitochondrial DNA deletion in epithelial

swabs and its use as a quantitative marker for sunlight exposure in human skin. British journal of dermatology (1951). 2010;163(6):1291-5.

51. Naidoo K, Hanna R, Birch-Machin MA. What is the role of mitochondrial dysfunction in skin photoaging? Experimental dermatology. 2018;27(2):124-8.

52. Birch-machin MA, Tindall M, Turner R, Haldane F, Rees JL. Mitochondrial DNA Deletions in Human Skin Reflect Photo- Rather Than Chronologic Aging. Journal of investigative dermatology. 1998;110(2):149-52.

53. Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein Composition of Catalytically Active Human Telomerase from Immortal Cells. Science (American Association for the Advancement of Science). 2007;315(5820):1850-3.

54. Tümpel S, Rudolph KL. The role of telomere shortening in somatic stem cells and tissue aging: lessons from telomerase model systems. Annals of the New York Academy of Sciences. 2012;1266(1):28-39.

55. Zole E, Zadinane K, Pliss L, Ranka R. Linkage between mitochondrial genome alterations, telomere length and aging population. Mitochondrial DNA Part A DNA mapping, sequencing, and analysis. 2018;29(3):431-8.

56. Harris SE, Martin-Ruiz C, von Zglinicki T, Starr JM, Deary IJ. Telomere length and aging biomarkers in 70-year-olds: the Lothian Birth Cohort 1936. Neurobiology of aging. 2012;33(7):1486.e3-.e8.

57. Swalwell H, Latimer J, Haywood RM, Birch-Machin MA. Investigating the role of melanin in UVA/UVB- and hydrogen peroxide-induced cellular and mitochondrial ROS production and mitochondrial DNA damage in human melanoma cells. Free radical biology & medicine. 2012;52(3):626-34.

58. Berneburg M, Plettenberg H, Krutmann J. Photoaging of human skin. Photodermatology, photoimmunology & photomedicine. 2000;16(6):239-44.

59. Birket MJ, Birch-Machin MA. Ultraviolet radiation exposure accelerates the accumulation of the aging-dependent T414G mitochondrial DNA mutation in human skin. Aging cell. 2007;6(4):557-64.

60. Krishnan KJ, Harbottle A, Birch-Machin MA. The Use of a 3895 bp Mitochondrial DNA Deletion as a Marker for Sunlight Exposure in Human Skin. Journal of investigative dermatology. 2004;123(6):1020-4.

61. Yang JH, Lee HC, Lin KJ, Wei YH. A specific 4977-bp deletion of mitochondrial DNA in human ageing skin. Archives of dermatological research. 1994;286(7):386-90.

62. Cortopassi GA, Shibata D, Soong NW, Arnheim N. A Pattern of Accumulation of a Somatic Deletion of Mitochondrial DNA in Aging Human Tissues. Proceedings of the National Academy of Sciences - PNAS. 1992;89(16):7370-4.

63. Lee H-C, Pang C-Y, Hsu H-S, Wei Y-H. Differential accumulations of 4,977 bp deletion in mitochondrial DNA of various tissues in human ageing. Biochimica et biophysica acta Molecular basis of disease. 1994;1226(1):37-43.

64. Pang CY, Lee HC, Yang JH, Wei YH. Human Skin Mitochondrial DNA Deletions Associated with Light Exposure. Archives of biochemistry and biophysics. 1994;312(2):534-8.

65. Durham SE, Krishnan KJ, Betts J, Birch-Machin MA. Mitochondrial DNA damage in non-melanoma skin cancer. British journal of cancer. 2003;88(1):90-5.

66. Harbottle A, Birch-Machin MA. Real-time PCR analysis of a 3895 bp mitochondrial DNA deletion in nonmelanoma skin cancer and its use as a quantitative marker for sunlight exposure in human skin. British journal of cancer. 2006;94(12):1887-93.

67. Shindo Y, Witt E, Han D, Epstein W, Packer L. Enzymic and Non-Enzymic Antioxidants in Epidermis and Dermis of Human Skin. Journal of investigative dermatology. 1994;102(1):122-4.

68. Birket MJ, Passos JF, von Zglinicki T, Birch-Machin MA. The Relationship between the Aging- and Photo-Dependent T414G Mitochondrial DNA Mutation with Cellular Senescence and Reactive Oxygen Species Production in Cultured Skin Fibroblasts. Journal of investigative dermatology. 2009;129(6):1361-6.

69. Hanna R, Crowther JM, Bulsara PA, Wang X, Moore DJ, Birch-Machin MA. Optimised detection of mitochondrial DNA strand breaks. Mitochondrion. 2019;46:172-8.

70. Rothfuss O, Gasser T, Patenge N. Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach. Nucleic acids research. 2010;38(4):e24-e.

71. Hou Y, Zhang H, Miranda L, Lin S. Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: microalgal pcna as the model gene. PloS one. 2010;5(3):e9545-e.

72. FULTON JEJ. Utilizing the Ultraviolet (UV Detect) Camera to Enhance the Appearance of Photodamage and Other Skin Conditions. Dermatologic Surgery. 1997;23(3):163-9.

73. Malik AN, Shahni R, Rodriguez-de-Ledesma A, Laftah A, Cunningham P. Mitochondrial DNA as a non-invasive biomarker: Accurate quantification using real time quantitative PCR without co-amplification of pseudogenes and dilution bias. Biochemical and biophysical research communications. 2011;412(1):1-7.

74. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. BioTechniques. 2005;39(1):75-85.

75. Hayes F. The function and organization of plasmids. Methods Mol Biol. 2003;235:1-17.

76. Chen J, Kadlubar FF, Chen JZ. DNA supercoiling suppresses real-time PCR: a new approach to the quantification of mitochondrial DNA damage and repair. Nucleic acids research. 2007;35(4):1377-88.

77. Biniek K, Levi K, Dauskardt RH. Solar UV radiation reduces the barrier function of human skin. Proceedings of the National Academy of Sciences - PNAS. 2012;109(42):17111-6.

78. Koch H, Wittern K-P, Bergemann J. In Human Keratinocytes the Common Deletion Reflects Donor Variabilities Rather Than Chronologic Aging and can be Induced by Ultraviolet A Irradiation. Journal of investigative dermatology. 2001;117(4):892-7.

79. Engelke M, Jensen JM, Ekanayake-Mudiyanselage S, Proksch E. Effects of xerosis and ageing on epidermal proliferation and differentiation. British journal of dermatology (1951). 1997;137(2):219-25.

80. Ruchusatsawat K, Wongpiyabovorn J, Protjaroen P, Chaipipat M, Shuangshoti S, Thorner PS, et al. Parakeratosis in skin is associated with loss of inhibitor of differentiation 4 via promoter methylation. Hum Pathol. 2011;42(12):1878-87.

81. Wei JCJ, Edwards GA, Martin DJ, Huang H, Crichton ML, Kendall MAF. Allometric scaling of skin thickness, elasticity, viscoelasticity to mass for micro-medical device translation: from mice, rats, rabbits, pigs to humans. Scientific Reports. 2017;7(1):15885.

82. Karin O, Agrawal A, Porat Z, Krizhanovsky V, Alon U. Senescent cell turnover slows with age providing an explanation for the Gompertz law. Nature Communications. 2019;10(1):5495.

83. Wan DC, Wong VW, Longaker MT, Yang GP, Wei F-C. Moisturizing different racial skin types. J Clin Aesthet Dermatol. 2014;7(6):25-32.

84. Gambichler T, Boms S, Stücker M, Kreuter A, Moussa G, Sand M, et al. Epidermal thickness assessed by optical coherence tomography and routine histology: preliminary results of method comparison. J Eur Acad Dermatol Venereol. 2006;20(7):791-5.

85. Birch-Machin MA, Russell EV, Latimer JA. Mitochondrial DNA damage as a biomarker for ultraviolet radiation exposure and oxidative stress. British journal of dermatology (1951). 2013;169(s2):9-14.

86. Berneburg M, Gattermann N, Stege H, Grewe M, Vogelsang K, Ruzicka T, et al. Chronically Ultraviolet-exposed Human Skin Shows a Higher Mutation Frequency of Mitochondrial DNA as Compared to Unexposed Skin and the Hematopoietic System. Photochemistry and photobiology. 1997;66(2):271-5.

87. Latimer JA, Lloyd JJ, Diffey BL, Matts PJ, Birch-Machin MA. Determination of the Action Spectrum of UVR-Induced Mitochondrial DNA Damage in Human Skin Cells. Journal of investigative dermatology. 2015;135(10):2512-8.

88. Ray AJ, Turner R, Nikaido O, Rees JL, Birch-Machin MA. The Spectrum of Mitochondrial DNA Deletions is a Ubiquitous Marker of Ultraviolet Radiation Exposure in Human Skin. Journal of investigative dermatology. 2000;115(4):674-9.

89. Hughes AJ, Tawfik SS, Baruah KP, O'Toole EA, O'Shaughnessy RFL. Tape strips in dermatology research*. British Journal of Dermatology. 2021;185(1):26-35.

90. Goldsberry A, Hanke CW, Hanke KE. VISIA system: a possible tool in the cosmetic practice. J Drugs Dermatol. 2014;13(11):1312-4.

91. Gamble RGMD, Asdigian NLP, Aalborg JMPH, Gonzalez VBA, Box NFP, Huff LSMD, et al. Sun damage in ultraviolet photographs correlates with phenotypic melanoma risk factors in 12-year-old children. Journal of the American Academy of Dermatology. 2011;67(4):587-97.

92. Takahashi Y, Fukushima Y, Kondo K, Ichihashi M. Facial skin photo-aging and development of hyperpigmented spots from children to middle-aged Japanese woman. Skin research and technology. 2017;23(4):613-8.

93. Mahler HIM, Kulik JA, Harrell J, Correa A, Gibbons FX, Gerrard M. Effects of UV photographs, photoaging information, and use of sunless tanning lotion on sun protection behaviors. Archives of dermatology (1960). 2005;141(3):373-80.

94. Lynn D, Jones T, Whitsitt J, Trikha R, Schlichte M, Karimkhani C, et al. The impact of ultraviolet photography on the sun safety awareness and behavior of skiers and snowboarders. Journal of Epidemiological Research. 2015;2.

95. Karimkhani C, Huff LS, Dellavalle RP. Measuring Sun Damage at the Grocery Store: Mychelle Dermaceuticals and Whole Foods Market Bring UV Photography to Aisle #7. JAMA dermatology (Chicago, Ill). 2014;150(6):589-90.

96. Glanz K, Yaroch AL, Dancel M, Saraiya M, Crane LA, Buller DB, et al. Measures of Sun Exposure and Sun Protection Practices for Behavioral and Epidemiologic Research. Archives of dermatology (1960). 2008;144(2):217-22.

97. Rodrigues AM, Sniehotta FF, Birch-Machin MA, Olivier P, Araújo-Soares V. Systematic and Iterative Development of a Smartphone App to Promote Sun-Protection Among Holidaymakers: Design of a Prototype and Results of Usability and Acceptability Testing. JMIR Res Protoc. 2017;6(6):e112-e.

98. Rodrigues A, Sniehotta F, Birch-Machin M, Olivier P, Araújo-Soares V. Acceptability and Feasibility of a Trial Testing Allocation to Sunscreen and a Smartphone App for Sun Protection: Discontinued Randomized Controlled Trial (Preprint)2017.

99. Jackson KM, Aiken LS. A Psychosocial Model of Sun Protection and Sunbathing in Young Women: The Impact of Health Beliefs, Attitudes, Norms, and Self-Efficacy for Sun Protection. Health psychology. 2000;19(5):469-78.

100. Maiti R, Duan M, Danby SG, Lewis R, Matcher SJ, Carré MJ. Morphological parametric mapping of 21 skin sites throughout the body using optical coherence tomography. Journal of the mechanical behavior of biomedical materials. 2020;102:103501-.

101. Kakasheva-Mazhenkovska L, Milenkova L, Gjokik G, Janevska V. Variations of the histomorphological characteristics of human skin of different body regions in subjects of different age. Prilozi / Makedonska akademija na naukite i umetnostite, Oddelenie za biološki i medicinski nauki = Contributions / Macedonian Academy of Sciences and Arts, Section of Biological and Medical Sciences. 2011;32:119-28.

102. Coelho SG, Choi W, Brenner M, Miyamura Y, Yamaguchi Y, Wolber R, et al. Short- and Long-Term Effects of UV Radiation on the Pigmentation of Human Skin. The Journal of investigative dermatology symposium proceedings. 2009;14(1):32-5.

103. Scott TL, Christian PA, Kesler MV, Donohue KM, Shelton B, Wakamatsu K, et al. Pigment-independent cAMP-mediated epidermal thickening protects against cutaneous UV injury by keratinocyte proliferation. Experimental dermatology. 2012;21(10):771-7.

104. Lavker RM, Gerberick GF, Veres D, Irwin CJ, Kaidbey KH. Cumulative effects from repeated exposures to suberythemal doses of UVB and UVA in human skin. Journal of the American Academy of Dermatology. 1995;32(1):53-62.

105. El-Domyati M, Attia S, Saleh F, Brown D, Birk DE, Gasparro F, et al. Intrinsic aging vs. photoaging: a comparative histopathological, immunohistochemical, and ultrastructural study of skin. Experimental dermatology. 2002;11(5):398-405.

106. Flament F, Bazin R, Laquieze S, Rubert V, Simonpietri E, Piot B. Effect of the sun on visible clinical signs of aging in Caucasian skin. Clin Cosmet Investig Dermatol. 2013;6:221-32.

107. Langton AK, Ayer J, Griffiths TW, Rashdan E, Naidoo K, Caley MP, et al. Distinctive clinical and histological characteristics of atrophic and hypertrophic facial photoageing. Journal of the European Academy of Dermatology and Venereology. 2021;35(3):762-8.

108. Tomasetti C, Poling J, Roberts NJ, London JNR, Pittman ME, Haffner MC, et al. Cell division rates decrease with age, providing a potential explanation for the age-dependent deceleration in cancer incidence. Proceedings of the National Academy of Sciences - PNAS. 2019;116(41):20482-8.

109. Laethem AV, Claerhout S, Garmyn M, Agostinis P. The sunburn cell: Regulation of death and survival of the keratinocyte. The international journal of biochemistry & cell biology. 2005;37(8):1547-53.

110. Schwarz A, Bhardwaj R, Aragane Y, Mahnke K, Riemann H, Metze D, et al. Ultraviolet-B-Induced Apoptosis of Keratinocytes: Evidence for Partial Involvement of Tumor Necrosis Factor-α in the Formation of Sunburn Cells. Journal of investigative dermatology. 1995;104(6):922-7.

111. Gordon JRS, Brieva JC. Unilateral dermatoheliosis. The New England journal of medicine. 2012;366(16):e25-E.

112. Engebretsen KA, Kezic S, Riethmüller C, Franz J, Jakasa I, Hedengran A, et al. Changes in filaggrin degradation products and corneocyte surface texture by season. British journal of dermatology (1951). 2018;178(5):1143-50.

113. Ishikawa J, Shimotoyodome Y, Ito S, Miyauchi Y, Fujimura T, Kitahara T, et al. Variations in the ceramide profile in different seasons and regions of the body contribute to stratum corneum functions. Archives of Dermatological Research. 2012;305(2):151-62.

114. Egawa M, Tagami H. Comparison of the depth profiles of water and water-binding substances in the stratum corneum determined in vivo by Raman spectroscopy between the cheek and volar forearm skin: effects of age, seasonal changes and artificial forced hydration. British journal of dermatology (1951). 2008;158(2):251-60.

115. Bishop PA, Herron RL. Use and Misuse of the Likert Item Responses and Other Ordinal Measures. Int J Exerc Sci. 2015;8(3):297-302.

116. Eckhart L, Lippens S, Tschachler E, Declercq W. Cell death by cornification. Biochim Biophys Acta. 2013;1833(12):3471-80.

117. Ikebe S-i, Tanaka M, Ohno K, Sato W, Hattori K, Kondo T, et al. Increase of deleted mitochondrial DNA in the striatum in Parkinson's disease and senescence. Biochemical and biophysical research communications. 1990;170(3):1044-8.

118. Ingram J, Maciejewski G, Hand CJ. Changes in Diet, Sleep, and Physical Activity Are Associated With Differences in Negative Mood During COVID-19 Lockdown. Frontiers in psychology. 2020;11:588604-.

119. Sallie SN, Ritou V, Bowden-Jones H, Voon V. Assessing international alcohol consumption patterns during isolation from the COVID-19 pandemic using an online survey: highlighting negative emotionality mechanisms. BMJ open. 2020;10(11):e044276-e.

120. Ogden RS. The passage of time during the UK Covid-19 lockdown. PloS one. 2020;15(7):e0235871-e.

121. Baczynska KA, Rendell RJ, Khazova M. Impact of COVID-19 Lockdown on Sun Exposure of UK Office Workers. International journal of environmental research and public health. 2021;18(8):4362.

122. Coronavirus and homeworking in the UK: April 2020 2020 [updated 8th July 2020. Available from: https://www.ons.gov.uk/employmentandlabourmarket/peopleinwork/employmentandemployeetypes/bulletins/coronavirusandhomeworkingintheuk/april2020.

123. New guidance on spending time outdoors 2020 [updated 13th May 2020. Available from: https://www.gov.uk/government/news/new-guidance-on-spending-time-outdoors.

124. Staying at home and away from others (social distancing) Gov.co.uk2020 [updated 1st May 2020. Available from: https://www.gov.uk/government/publications/full-guidance-on-staying-at-home-and-away-from-others/full-guidance-on-staying-at-home-and-away-from-others.

125. Cokkinides V, Weinstock M, Glanz K, Albano J, Ward E, Thun M. Trends in Sunburns, Sun Protection Practices, and Attitudes Toward Sun Exposure Protection and Tanning Among US Adolescents, 1998-2004. Pediatrics (Evanston). 2006;118(3):853-64.

126. Kirk L, Greenfield S. Knowledge and attitudes of UK university students in relation to ultraviolet radiation (UVR) exposure and their sun-related behaviours: a qualitative study. BMJ open. 2017;7(3):e014388-e.

127. Loughlin SM, Cox P, Ullrich SE, Ananthaswamy HN, Kripke ML, Evans RL. Sunlight and skin cancer: Inhibition of p53 mutations in UV-irradiated mouse skin by sunscreens. Nature medicine. 1997;3(5):510-4.

128. Dennis LK, Vanbeek MJ, Beane Freeman LE, Smith BJ, Dawson DV, Coughlin JA. Sunburns and Risk of Cutaneous Melanoma: Does Age Matter? A Comprehensive Meta-Analysis. Annals of epidemiology. 2008;18(8):614-27.

129. Gould M, Farrar MD, Kift R, Berry JL, Mughal MZ, Bundy C, et al. Sunlight exposure and photoprotection behaviour of white Caucasian adolescents in the UK. Journal of the European Academy of Dermatology and Venereology. 2015;29(4):732-7.

130. Stanton WR, Janda M, Baade PD, Anderson P. Primary prevention of skin cancer: A review of sun protection in Australia and internationally. Health promotion international. 2004;19(3):369-78.

131. Diffey BL, Norridge Z. Reported sun exposure, attitudes to sun protection and perceptions of skin cancer risk: a survey of visitors to Cancer Research UK's SunSmart campaign website. British journal of dermatology (1951). 2009;160(6):1292-8.

132. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies 2021 [Available from: https://www.equator-network.org/reporting-guidelines/strobe/.

133. Ghiasvand R, Lund E, Edvardsen K, Weiderpass E, Veierød MB. Prevalence and trends of sunscreen use and sunburn among Norwegian women. British journal of dermatology (1951). 2015;172(2):475-83.

134. Chang C, Murzaku EC, Penn L, Abbasi NR, Davis PD, Berwick M, et al. More skin, more sun, more tan, more melanoma. American journal of public health (1971). 2014;104(11):e92-e9.

135. Cafri G, Thompson JK, Roehrig M, van den Berg P, Jacobsen PB, Stark S. An investigation of appearance motives for tanning: The development and evaluation of the Physical Appearance Reasons For Tanning Scale (PARTS) and its relation to sunbathing and indoor tanning intentions. Body image. 2006;3(3):199-209.

136. Kamimura A, Nourian MM, Ashby J, Trinh HN, Tabler J, Assasnik N, et al. Sun Protection Behaviors Associated with Self-Efficacy, Susceptibility, and Awareness among Uninsured Primary Care Patients Utilizing a Free Clinic. Dermatology research and practice. 2015;2015:753681-7.

137. Støle HS, Nilsen LTN, Joranger P. Beliefs, attitudes and perceptions to sun-tanning behaviour in the Norwegian population: a cross-sectional study using the health belief model. BMC public health. 2019;19(1):206-.

138. Pichon LC. Prevalence and correlates of sun protection behaviors among African Americans. eScholarship, University of California; 2008.

139. Starfelt Sutton LC, White KM. Predicting sun-protective intentions and behaviours using the theory of planned behaviour: a systematic review and meta-analysis. Psychology & health. 2016;31(11):1272-92.

140. Faries MD. Why We Don't "Just Do It": Understanding the Intention-Behavior Gap in Lifestyle Medicine. Am J Lifestyle Med. 2016;10(5):322-9.

141. Pettigrew S, Jongenelis M, Strickland M, Minto C, Slevin T, Jalleh G, et al. Predictors of sun protection behaviours and sunburn among Australian adolescents. BMC public health. 2016;16(1):565-.

142. Ajzen I, Joyce N, Sheikh S, Cote NG. Knowledge and the Prediction of Behavior: The Role of Information Accuracy in the Theory of Planned Behavior. Basic and Applied Social Psychology. 2011;33(2):101-17.

143. BrÄNstrÖM R, Kasparian NA, Bruno W, Chan M, Cuellar F, Debniak T, et al. Predictors of Sun Protection Behaviors and Severe Sunburn in an International Online Study. Cancer epidemiology, biomarkers & prevention. 2010;19(9):2199-210.

144. Volkov A, Dobbinson S, Wakefield M, Slevin T. Seven-year trends in sun protection and sunburn among Australian adolescents and adults. Australian and New Zealand journal of public health. 2013;37(1):63-9.

145. Autier P, Boniol M, Doré JF. Sunscreen use and increased duration of intentional sun exposure: Still a burning issue. International journal of cancer. 2007;121(1):1-5.

146. Falk M, Anderson CD. Influence of age, gender, educational level and self-estimation of skin type on sun exposure habits and readiness to increase sun protection. Cancer epidemiology. 2013;37(2):127-32.

147. Nahar VK, Ford MA, Hallam JS, Bass MA, Vice MA. Sociodemographic and Psychological Correlates of Sun Protection Behaviors among Outdoor Workers: A Review. Journal of skin cancer. 2013;2013:453174-10.

148. Hansen MR, Bentzen J. High-risk sun-tanning behaviour: a quantitative study in Denmark, 2008–2011. Public health (London). 2014;128(9):777-83.

149. Prior SM, Fenwick KD, Peterson JC. Adolescents' reasons for tanning and appearance motives: A preliminary study. Body image. 2014;11(1):93-6.

150. Holman DMMPH, Watson MMPH. Correlates of Intentional Tanning Among Adolescents in the United States: A Systematic Review of the Literature. Journal of adolescent health. 2013;52(5):S52-S9.

151. Lin L-Y, Smeeth L, Langan S, Warren-Gash C. Distribution of vitamin D status in the UK: a cross-sectional analysis of UK Biobank. BMJ open. 2021;11(1):e038503-e.

152. Eun Lee K, Bharadwaj S, Yadava U, Gu Kang S. Evaluation of caffeine as inhibitor against collagenase, elastase and tyrosinase using in silico and in vitro approach. Journal of Enzyme Inhibition and Medicinal Chemistry. 2019;34(1):927-36.

153. Li Y-F, Ouyang S-H, Tu L-F, Wang X, Yuan W-L, Wang G-E, et al. Caffeine protects skin from oxidative stress-induced senescence through the activation of autophagy. Theranostics. 2018;8(20):5713-30.

154. O'Keefe JHMD, Bhatti SKMD, Patil HRMD, DiNicolantonio JJP, Lucan SCMDMPHMS, Lavie CJMD. Effects of Habitual Coffee Consumption on Cardiometabolic Disease, Cardiovascular Health, and All-Cause Mortality. Journal of the American College of Cardiology. 2013;62(12):1043-51.

155. Koo SW, Hirakawa S, Fujii S, Kawasumi M, Nghiem P. Protection from photodamage by topical application of caffeine after ultraviolet irradiation. British journal of dermatology (1951). 2007;156(5):957-64.

156. Herman A, Herman AP. Caffeine's Mechanisms of Action and Its Cosmetic Use. Skin Pharmacology and Physiology. 2013;26(1):8-14.

157. Conney AH, Lu Y-P, Lou Y-R, Kawasumi M, Nghiem P. Mechanisms of Caffeine-Induced Inhibition of UVB Carcinogenesis. Frontiers in oncology. 2013;3:144.

158. Kawasumi M, Lemos B, Bradner JE, Thibodeau R, Kim Y-S, Schmidt M, et al. Protection from UV-induced skin carcinogenesis by genetic inhibition of the ataxia telangiectasia and Rad3-related (ATR) kinase. Proceedings of the National Academy of Sciences - PNAS. 2011;108(33):13716-21.

159. Hylse O, Maier L, Kučera R, Perečko T, Svobodová A, Kubala L, et al. A Concise Synthesis of Forskolin. Angewandte Chemie (International ed). 2017;56(41):12586-9.

160. Ding X, Staudinger JL. Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. J Pharmacol Exp Ther. 2005;312(2):849-56.

161. Spry ML, Vanover JC, Scott T, Abona-Ama O, Wakamatsu K, Ito S, et al. Prolonged treatment of fair-skinned mice with topical forskolin causes persistent tanning and UV protection. Pigment cell and melanoma research. 2009;22(2):219-29.

162. Passeron T, Namiki T, Passeron HJ, Le Pape E, Hearing VJ. Forskolin protects keratinocytes from UVB-induced apoptosis and increases DNA repair independent of its effects on melanogenesis. J Invest Dermatol. 2009;129(1):162-6.

163. Fisher DE, D'Orazio JA, Nobuhisa T, Cui R, Arya M, Spry M, et al. Topical drug rescue strategy and skin protection based on the role of Mc1r in UV-induced tanning. Nature (London). 2006;443(7109):340-4.

164. Twice daily application of a topical formulation containing caffeine, forskolin, and the TPM delivery system was able to significantly reduce the visible appearance of cellulite. Journal of the American Academy of Dermatology. 2014;70(5):AB21.

165. Roure R, Oddos T, Rossi A, Vial F, Bertin C. Evaluation of the efficacy of a topical cosmetic slimming product combining tetrahydroxypropyl ethylenediamine, caffeine, carnitine, forskolin and retinol, In vitro, ex vivo and in vivo studies. Int J Cosmet Sci. 2011;33(6):519-26.

166. Mante S, Minneman KP. Caffeine inhibits forskolin-stimulated cyclic AMP accumulation in rat brain. European Journal of Pharmacology. 1990;175(2):203-5.

167. Huang KT, Chen YH, Walker AM. Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids. BioTechniques. 2004;37(3):406-12.

168. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnology Annual Review. 11: Elsevier; 2005. p. 127-52.