Can green tea catechin supplement protect against photoageing?

A thesis submitted to the University of Manchester for

The degree of Doctor of Philosophy in the Faculty of

Biology, Medicine and Health

2016

Nisamanee Charoenchon

School of Biological Sciences University of Manchester

Table of contents

Table of contents 2
List of figures 5
List of tables 7
Abstract 8
Declaration 9
Copyright Statement 9
Acknowledgement 10
Abbreviations 11
Chapter 1 – Introduction 12
1.1 Function and structure of skin 12
1.1.1 Functions of skin 12
1.1.2 Structure of skin 12
1.1.2.1 Epidermis 13
1.1.2.2 Dermis 14
1.2 Sun reactive skin type by Fitzpatrick scale 15
1.3 Extracellular matrices 17
1.3.1 Collagenous matrices 17
1.3.2 Elastic fibres 23
1.4 The clinical feature of ageing skin 28
1.5 The histological features of ageing skin 29
1.5.1 Intrinsically aged skin 29
1.5.2 Extrinsically aged skin 29
1.6 Optical cutaneous irradiation 30
1.6.1 Ultraviolet radiation properties and its impacts in human skin 30
1.6.2 Advantages of UVR in human skin as an influence on vitamin D synthesis 32
1.6.3 Vitamin D sufficiency 33
1.6.4 Erythema (sun burn) 34
1.6.5 Natural cutaneous chromophores in human skin 35

1.7 Alteration of collagen matrix and elastic fibre system in photo aged skin 37
1.7.1 Changes to the collagen matrix in photoageing..... 37
1.7.2 Elastic matrix..... 37
1.7.3 Potential mechanism..... 38
1.8 Photoprotection 41
1.8.1 Sunscreen 41
1.8.2 Systemic photoprotection..... 42
1.9 Green tea catechins (GTC).....44
1.9.1 Green tea sources, chemical ingredient and processing..... 44
1.9.2 Medical advantages of green tea relating to health 49
1.9.3 Advantages of green tea specifically to skin health 49
1.9.4 Role of green tea extract in collagen and elastic fibre protection 51
1.9.5 Dose usage and toxicity of green tea..... 53
1.10 Summary and hypotheses 53
1.11 Hypotheses: 53
Chapter 2 - Materials and methods.....54
2.1 Research ethics 54
2.2 Ultraviolet radiation sources.....54
2.2.1 Broadband UVB 54
2.2.2 Solar-simulated radiation (SSR) 56
2.3 Minimal erythema dose (MED) testing.....56
2.3.1 Irradiation procedure 56
2.3.2 MED assessment..... 59
2.4 Clinical studies 60
2.4.1 Effect of UVB and SSR on the dermal matrix 60
2.4.2 Randomised controlled trial (RCT) of dietary GTC 61
2.4.3 Timeline of study and study intervention..... 62
2.5 Skin biopsy and urinary sampling 63
2.5.1 Skin biopsy sampling 63
2.5.2 Urinary sampling 64

2.6 Slide preparation and staining 64
2.6.1 Picrosirius Red staining (PSR) for fibrillar collagens 65
2.6.2 Weigert's Resorcin Fuchsin staining (WRF) for elastic fibres 65
2.6.3 Immunohistochemistry (IHC) 66
2.7 Image capture 68
2.7.1 Photomicrograph recording procedure for PSR 68
2.7.2 Image analysis - PSR 68
2.7.3 Photomicrograph recording procedure for WRF and IHC stains 70
2.7.4 Image analysis – WRF and immunohistology 71
2.7.5 Quantification of procollagen I (pCI) IHC staining using a semi-quantitative analysis method 72
2.8 Statistical analysis 73
2.8.1 Effect of UVB and SSR on the dermal matrix 73
2.8.2 Randomised controlled trial of dietary green tea catechins 73
Chapter 3 - Ultraviolet wavelength results in differential reorganisation of cutaneous elastic fibres: A comparison of solar simulated radiation and UVB 74
Chapter 4 - Fibulin-5 microfibrils, essential components of cutaneous elastic fibers, are protected from UVR-induced damage by dietary green tea catechin supplementation 88
Chapter 5 - An acute dose of solar simulated radiation effects on cutaneous collagenous matrices and procollagens and protective efficacy of green tea catechins supplementation ... 106
Chapter 6 - Discussion 124
Bibliography 131

Word count 28,204

List of figures

Figure 1.1: Five principle layers in epidermis of human integumentary system. 14

Figure 1.2: Diagrammatic representation of the dermal ECM. 15

Figure 1.3: The schematic of correlation between quantity of melanin in epidermis and skin phenotypes and pie chart data. 16

Figure 1.4: The categories of collagen families classified by the their supramolecular structure. 18

Figure 1.5: Assembly of fibrillar collagens. 20

Figure 1.6: Collagen synthesis. 21

Figure 1.7: The processes of generating mature collagen. 22

Figure 1.8: The organization of elastin. 23

Figure 1.9: Fibrillins and fibulins are composed of repeating domains. 24

Figure 1.10: The domain structure of the fibulin family of proteins. 25

Figure 1.11: Clinical features of aged and photoaged human skin. 29

Figure 1.12: The electromagnetic spectrum specific to UV length and nearby radiation type. .. 30

Figure 1.13: Differentiation terrestrial of UVA and UVB in summer of UK on a clear sky day. .. 31

Figure 1.14: Vitamin D3 (D3) synthesis diagram. 33

Figure 1.15: Cutaneous erythema response of human skin to UV exposure. 34

Figure 1.16 : Absorption of energy causes damage to biological tissues. 35

Figure 1.17: The fibrillin microfibrillar network of healthy skin and in photoageing. 38

Figure 1.18: Potential ways UVR can disrupt protein. 39

Figure 1.19: The pathway of pCI regulation at the cellular level involving transforming growth factor-β (TGF- β). 40

Figure 1.20: Classification flow chart of subdivided groups within polyphenolic family 43

Figure 1.21: Illustrations of tea. 44

Figure 1.22: Tea production of various areas of cultivation in 1996 45

Figure 1.23: Tea production, 2009-2013. 46

Figure 1.24: Classes of polyphenols. 47

Figure 1.25: The chemical structure of six components in catechin group. 48
Figure 1.26: Potential model of GTC inhibition of direct and indirect photoageing mechanisms. 52
Figure 1.27: The oxidation of catechins. 52
Figure 2.1: UVB irradiation equipment. 55
Figure 2.2: The solar simulator. 57
Figure 2.3: MED template of SSR and UVB. 58
Figure 2.4: Measurement of erythema. 60
Figure 2.5: Dietary supplements. 62
Figure 2.6: Experimental timeline and study intervention. 63
Figure 2.7: Procedure of biopsy sampling. 64
Figure 2.8: Image capture of PSR-stained skin sections. 68
Figure 2.9: Image analysis of PSR-stained tissue sections. 70
Figure 2.10: Image capture of WG and IHC-stained skin sections. 71
Figure 2.11: Elastic fibre staining in human skin. 72
Figure 2.12: Evaluation of pCI staining using a semi-quantitative scale. 73
Figure 6.1: Indirect mechanism of UV-induced skin damage via over production of cellular oxygen species (ROS). 126
Figure 6.2: Bar graph displayed the percentage of different sub-unit of polyphenol components or flavonoids in green tea and black tea. 128

List of tables

Table 1.1: Classification of skin tone based on Fitzpatrick scale. 15

Table 1.2: Collagen assemblies with functions in dermal ECM. 19

Table 1.3: Proteins of the fibulin family 24

Table 1.4: The comparison of aged patterns between intrinsic aged skin and extrinsic aged skin 28

Table 1.5: Wavelength of three spectrum regions of different UV types based on timeline reports. 30

Table 1.6: Relation of estimated depth in fair Caucasian skin and the wavelength of solar ray penetration 32

Table 1.7: Tea consumption. 45

Table 1.8: Chemical elements of fresh green tea leaves measured in % dry weight 47

Table 2.1: List of biochemical compounds in green tea supplement provided to subjects 62

Table 2.2: Antibody details. 67

Table 6.1: Antioxidant activities of flavonoids and vitamin C and E *in vitro* 127

Abstract

Abstract for a PhD thesis submitted in September 2016 at the University of Manchester by Nisamanee Charoenchon titled "Can green tea catechin supplement protect against photoageing?"

Photoaged skin caused by chronic ultraviolet radiation (UVR) is characterised clinically by hyperpigmentation, coarse skin texture and deep wrinkles; the worst outcome is skin cancer. Histological investigation of the alteration within major extracellular matrices (ECM; elastic fibres, fibrillar collagens) is an essential study to understand the cellular effect on skin structure from UVR. This thesis used an acute dose of radiation to examine in humans *in vivo* the effect of UVR on ECM components before assessing whether a dietary intervention could protect skin from UVR damage. Green tea catechins (GTCs) have anti-oxidant properties and may be an interesting option as a systemic photoprotection agent. Hence this thesis assesses: 1) the effect of acute irradiation of skin on dermal ECM damage to see whether it mimics the changes observed in photoageing and; 2) whether dietary supplementation with GTC will provide dermal ECM protection.

UV-induced change in elastic fibre network. Initially, the effect of two different UV light sources on elastic fibre protein (elastic fibres, fibrillin-rich microfibrils and fibulin-2 and -5 microfibrils) remodelling was performed. The effect of UVB vs full-spectrum solar simulated radiation (SSR) was investigated in a small sample of healthy Caucasian volunteers (n = 6 per group). At 24 hour after 3x MED irradiation, Weigert's resorcin–fuchsin (WRF) stained elastic fibres showed a significant reduction regardless of irradiation protocol (UVB, $P<0.01$; SSR $P<0.05$). Specific components were identified by immunohistochemistry; a significant reduction in fibrillin-rich microfibrils (FRM) was observed in UVB irradiated skin ($P<0.05$), whilst fibulin-5-positive microfibrils were only affected by SSR ($P<0.05$). The data revealed, therefore, differential effects on UV wavelength on ECM remodelling. SSR, the more physiologically relevant light source was used in subsequent studies.

Supplement effect in SSR-induced damage in elastic fibre. Fifty healthy volunteers were recruited to this randomised control trial to investigate whether GTC can protect skin from photodamage. Volunteers were randomized to GTC (1080 mg plus 100 mg vitamin C; n=25) or placebo (maltodextrin; n = 25) daily for 12-weeks with compliance assessed biochemically in urine samples. Of the n = 50 recruited, 44 volunteers completed the study. At baseline, UVR challenge resulted in a significant remodelling of the cutaneous elastic fibre system ($P<0.001$), particularly fibulin-2 and fibulin-5-positive microfibrils at 24-hr after 3x MED irradiation. After supplementation, fibulin-5 positive microfibrils were protected from UVR remodeling (% staining, mean ± SE; no UV, 18.1±0.89; UVR, 17.1±0.61; $P=0.30$) whilst no protection was seen in the placebo group (no UVR, 19.41±0.79; UVR, 17.69±0.61; $P<0.05$).

Supplement effect in SSR-induced damage in collagenous matrix. In the identical experiment, collagenous matrices including synthesis of procollagen I (pCI) was also examined as fibrillar collagens are the major ECM components providing strength within dermis. The fibrillar collagen and newly synthesised pCI were stained by Picrosirius red (PSR) and immunohistochemistry (IHC) respectively. At baseline, acute irradiation significantly reduced papillary dermal fibrillar collagens ($P<0.001$) and induced deposition of newly synthesised pCI ($P=0.02$). In post-supplementation, GTC enhanced the deposition of thin collagen fibres in the dermis. Whilst placebo showed no effect on the altered organisation of fibrillar collagens or deposition of pCI following the irradiation challenge, GTC protected the organisation of fibrillar collagens in the papillary dermis ($P=0.97$).

This novel *in vivo* human study may be used to recapitulate elastic fibre and collagen changes associated with photoageing and may be useful for dissecting out the mechanisms underlying extracellular matrix damage in response to chronic sunlight exposure. Furthermore, in a randomized control trial, dietary GTC protected fibulin-5 microfibrils and collagen fibres in the papillary dermis from UV-mediated degradation. The mechanism by which this protection occurs requires further study.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

Copyright Statement

Acknowledgement

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Rachel Watson for her continuous kind support of my PhD study and all related activities in my 4 years in Manchester, for her patience, motivation, and immense knowledge. Her guidance, empathy and enthusiasm helped me in all the time of research writing of this thesis as well as conduct of life abroad in the University. I could not have imagined having a better supervisor and mentor for my PhD study.

Secondly my co-supervisors, Professor Lesley Rhodes and Dr. Mark Farrar provided me an opportunity to join their team as co-supervisee. The doors to their office were always warmly opened whenever I ran into a spot of trouble or had a question about my research or writing. They kindly steered me in the right the direction whenever they thought I needed it.

My sincere thanks thirdly also goes to all staff in my supervisory team, particularly Dr. Sarah Mason for encouraging me in the tough times of my first year of study abroad, Lindsay Cotterell and Patrick Costello for looking after me very well and for general help in the laboratory, additionally Dr. Helen Graham, Dr. Sarah Hibbert, Dr. James McConnell, Dr. Suzy Pilkington, Dr. Victoria Newton and Dr. Abigail Langton for all their kind support in teaching me specific lab methods, suggestions for my research including providing skin tissue samples to practice with. Without their precious support it would not be possible to conduct this research.

Likewise I greatly appreciate Professor Anna Nicolaou from the University of Manchester and Professor Gary Williamson from the University of Leeds as dedicated collaborators for their pioneering works in mass spectrometry and measuring catechins and their metabolites respectively.

Last but not the least, I would like to not only thank my father Veeraphol, mother Chadakan and younger brother Patchaphol Charoenchon with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis, but also gave the special thanks to Development and Promotion of Science and Technology Talents Project (DPST) which is the Royal Government of Thailand scholarship that provided me this inestimable opportunity to study at this outstanding University with full funding throughout 4 years of study. This accomplishment would not have been possible without them.

Abbreviations

ANOVA	Analysis of variance
cb-EGF	Calcium binding epidermal growth factor
DEJ	Dermal-epidermal junction
ECM	Extracellular matrix
EGCG	Epigallo catechin gallate
EI	Erythema index
EP	Epidermis
FB	Fibroblast
FRM	Fibrillin-rich microfibril
GAGs	Glycosaminoglycans
GTC	Green tea catechin
IHC	Immunohistochemistry
IR	Infrared radiation
KC	Keratinocyte
MED	Minimal erythemal dose
MHD	Minimum heat dose
MMPs	Matrix metalloproteinases
NF-κβ	Nuclear factor-kappa-β
PBS	Phosphate-buffered saline
pC	Procollagen
PFA	Paraformaldehyde
PSR	Picrosirius red
RCT	Randomized controlled trial
SC	Stratum corneum
SED	Standard erythema dose
SLE	Systemic lupus erythematosus
TGF-β	Transforming growth factor-β
TBS	Tris–buffered saline
UV	Ultraviolet
UVR	Ultraviolet radiation

Chapter 1 – Introduction

1.1 Function and structure of skin

1.1.1 Functions of skin

Skin has many functions. The first is thermoregulation; in response to changing environmental temperatures, it is essential for humans to maintain thermal homeostasis [1]. The skin is an excretory organ; small amount of water, salts and several organic compounds (component of perspiration) are excreted by sweat glands [2]. Sweat glands in skin can help decrease temperature by releasing sweat to the skin's surface, allowing evaporation when the body temperature becomes too high. In contrast, skin can preserve heat when body temperature falls by reducing blood circulation from the skin and extremities [3]. Secondly, the skin provides a physical, chemical and biological barrier. Skin physically separates the body from the environment, secretes the acidic pH 4-6 anti-bacterial proteins, i.e., defensins and cathelicidins, and provides a habitat for normal bacterial flora that prevent outgrowth of pathogenic bacteria [4, 5]. The skin also contains abundant sensory nerve ending and sensory receptors that detect stimuli related to temperature, touch, pressure and pain [6]. It monitors immunity, primarily via the Langerhans' cell of the epidermis, an important component of the immune system, which identifies potential foreign antigens [7]. Finally, the skin synthesises vitamin D; the exposure of skin to ultraviolet radiation (UVR) initiates synthesis of the active form of vitamin D, a substance that aids in the absorption of calcium and phosphorus from the gastrointestinal tract into the blood [8].

1.1.2 Structure of skin

Structurally, the skin comprise of 2 main compartments supported by the hypodermis or adipose fat layer: the outer epidermis and inner dermis. The epidermis is composed of an epithelium and is attached to the deeper, thicker, connective tissue dermis [9].

1.1.2.1 Epidermis

The epidermis is a keratinized stratified squamous epithelium containing 4 types of cells arranged in 4 or 5 layers [10], (*figure 1.1*). In most regions of the body the epidermis has four layers and is called thin skin. Thick skin occurs where exposure to friction is greatest, such as on the palms and the soles; five layers appear in thick skin. The five layers of strata from the deepest to the most superficial are: *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum* and *stratum corneum*. The *stratum basale* is a single layer of cuboidal or columnar keratinocyte: the cells undergo differentiation, during which cells formed in the basal layer are pushed to the surface. As the cells move toward the surface they make keratin,
a protein that helps protect the skin and underlying tissues. These basal cells are capable of continued cell division [10]. The epidermis also contains melanocytes, which have long slender projections extending between the keratinocytes and transfer melanin granules to them. Melanin is a brown-black pigment that contributes to skin colour and absorbs damaging ultraviolet radiation (UVR) [11]. In addition, Langerhans' cells and Merkel cells are located in this layer. Langerhans' cells arise from red bone marrow and migrate to the epidermis, where they constitute a small proportion of epidermal cells. They participate in immune responses. Merkel cells are the least numerous of all cell types in the epidermis, they are located in the deepest layer of epidermis, where they contact the flattened process of a sensory neurone (nerve cells) and function in sensation [6, 7]. The *stratum spinosum* consists of 8 to 10 layers of many-sided keratinocytes with spine-like projections. Langerhans' cells and projections of melanocytes also appear in this layer. The *stratum granulosum* contains about 5 layers of flattened keratinocytes with granules that release lipids that functions as a water-repelling sealant [12]. The secretion retards both loss of body fluid from the epidermis and entry of foreign materials into the epidermis [13]. The *stratum lucidum* consists of about five layers of clear, flat, dead cells and is present in only the thick skin of the palms and soles. The final layer, the *stratum corneum (SC)*, consists of 30 layers of flat, dead keratinocytes [9].

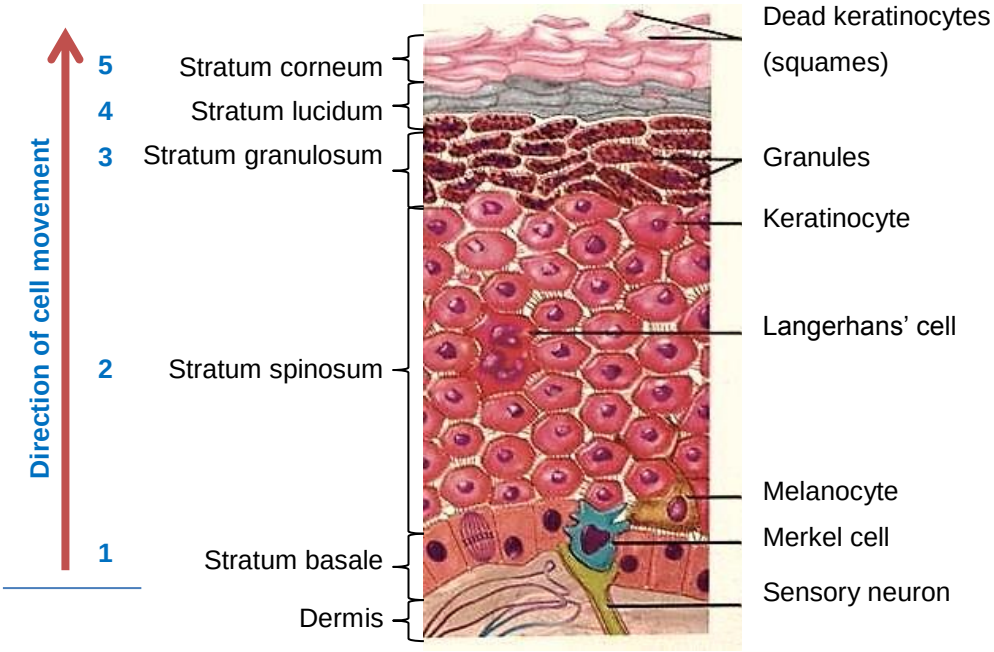


Figure 1.1: Five principle layers in epidermis of human integumentary system.
The stratum basale contains stem cells which divide by mitosis to produce daughter cells which transit to superficial layers. Merkel cells disperse also in this layer. Next, the stratum spinosum is mostly occupied by keratinocytes (KC) which produce keratin fibres. The Langerhans' cells and melanocytes are inserted among these KCs, though are much less frequent. Stratum granulosum, the third layer, is where keratohyalin granules are synthesised and contained in envelope form. In thick skin, the stratum lucidum, an additional layer comprised of many dead cells packed with keratohyalins, is found. At the superficial surface, the dead keratinocytes of the stratum corneum are shed (from Tortora, 2001 [14]).

1.1.2.2 Dermis

The dermis is the second principle component of skin and composed of loose connective tissue or extracellular matrix (ECM) containing collagens and elastic fibres. The combination of these fibres gives the skin its strength, extensibility and elasticity. There are various cells types in the dermis, the main cell being fibroblasts (FBs), but it also contains nerve cells and circulating cells of the immune system (e.g. T-cells, dermal dendritic cells, macrophages and mast cells) (see review at [15]). The dermis is sub-divided into 2 regions: the superficial papillary dermis and the deeper reticular dermis. The papillary dermis is the dermal area around the dermal papillae; these papillae help to maintain skin integrity by increasing surface area and cause ridges in the epidermis which produce fingerprints on the fingers which help us to grip objects. Veins and arteries are scattered throughout the tissue [16], as are blood capillaries (which are organised into superficial and deep plexus), corpuscles of touch (Meissner's corpuscles) and nerve endings [9].

The inner reticular dermis consists of dense irregular connective tissue, adipose tissue, hair follicles, nerves, oil glands and sweat glands. It is attached to the underlying bone and muscle by the subcutaneous layer [14], (*figure 1.2*). The arrangement of collagen in the dermal ECM provides strength for the skin with elastic fibres giving the skin extensibility [14].

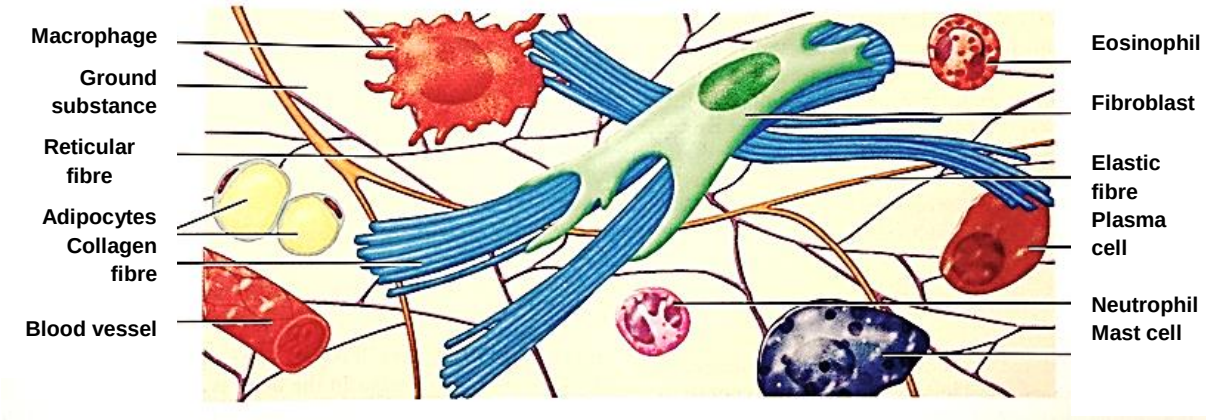


Figure 1. 2: Diagrammatic representation of the dermal ECM.
The cartoon shows the various cells and fibres contained in connective tissue (from Tortora, 2001 [14]).

As well as collagen and elastin, the dermal ECM also contains glycosaminoglycans (GAGs), proteoglycans and glycoproteins and functions to support the tissue. These molecules all play important roles in the function of dermal tissue.

1.2 Sun reactive skin type by Fitzpatrick scale

Fitzpatrick skin type is the most widely used method to characterise skin responses to UVR. It is based on three features: non-irradiated skin colour or pigmentary phenotype; skin response to UVR (likelihood to burn) and history of tanning [17]. This provides a six-point scale by which a dermatologist can categorise someone's skin (*table 1.1* and *figure 1.3*).

Table 1.1: Classification of skin tone based on Fitzpatrick scale.

Skin type	Skin tone	Skin response to UV	History of Tanning
I	Pale white	Always burn	Never
II	White	Always burns easily	A little
III	Light brown	Burns moderately	Gradually
IV	Moderate brown	Burns minimally	Well
V	Dark brown	Rarely burns	Profusely
VI	Deeply pigmented dark brown to black	Never burns	N/A

Table adapted from Gogia et al 2013 and Fasugba et al 2014 [18, 19].

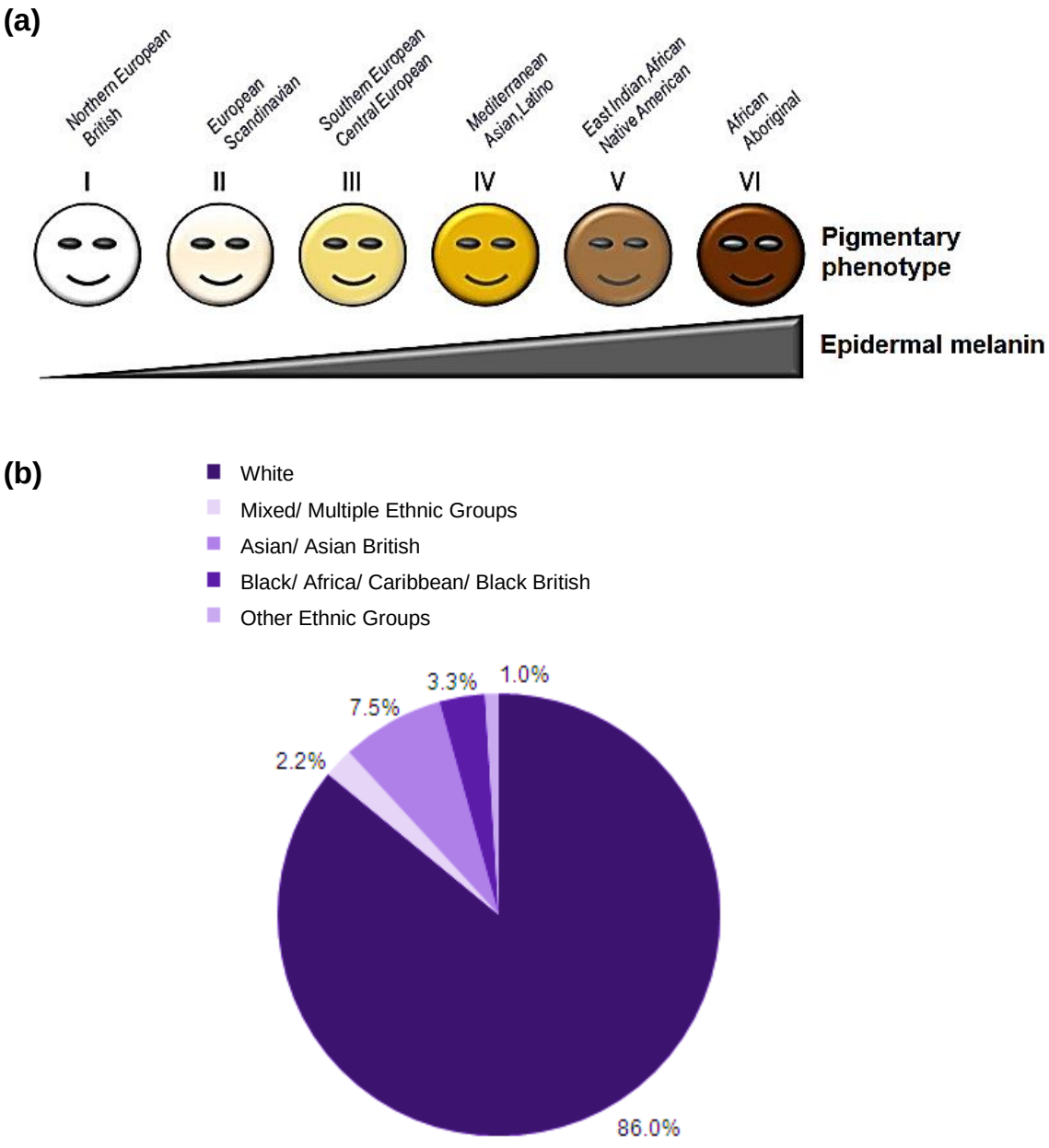


Figure 1.3: The schematic of correlation between quantity of melanin in epidermis and skin phenotypes and pie chart data.

(a) Schematic describes the increasing amounts of epidermal melanin in different ethnic skin types (Fitzpatrick scale I-VI). (b) Pie chart of census data identifies the proportion of skin types of the resident population in England and Wales. Image adapted from D'orazio et al 2013 [20] and White, 2011 [21] respectively.

1.3 Extracellular matrices

1.3.1 Collagenous matrices

Collagens are the major insoluble fibrous protein components in connective tissues throughout the human body and are mainly synthesized by FBs [22, 23]. Collagens are categorized into 28 different types depending on their supramolecular structure, for example: fibril forming, anchoring fibrils and network-forming [24]. Various collagens are found in diverse tissues and organs, and the types of collagens expressed gives the tissues their various functions [25]. Fibril-forming collagens types I, II, III, V and XI are the most abundant fibrillar collagen assemblies [26-30]. Collagen VI is a microfibril-forming collagen that is thought to maintain muscle and skin integrity and functions as the intermediary for connection between cell-cell and cell-matrix [31-33]. It is thought that type VI collagen is involved in the control of tissue growth and remodelling [31]. Other forms of collagen are relatively minor components of tissues; however every collagen type plays a specific role in the matrix. For example, collagen VII is found in anchoring fibrils, found specifically close to the basement membrane zone and attaching epithelial tissue layers to their underlying stroma [34]. Specially collagen VII not only is produced by epithelial cells but also by epidermoid carcinoma cell line, transformed amniotic cell line and fibroblasts [35], *(figure 1.4b).* Collagen IX is a FACIT collagens (Fibril-Associated Collagens with Interrupted Triple-helices); these are thought to act as molecular bridges and help in the organization and stability of extracellular matrices [36]. This class is linked to thrombospondin (another extracellular matrix protein; see review at [37]), to form networks (*figure 1.4a*).

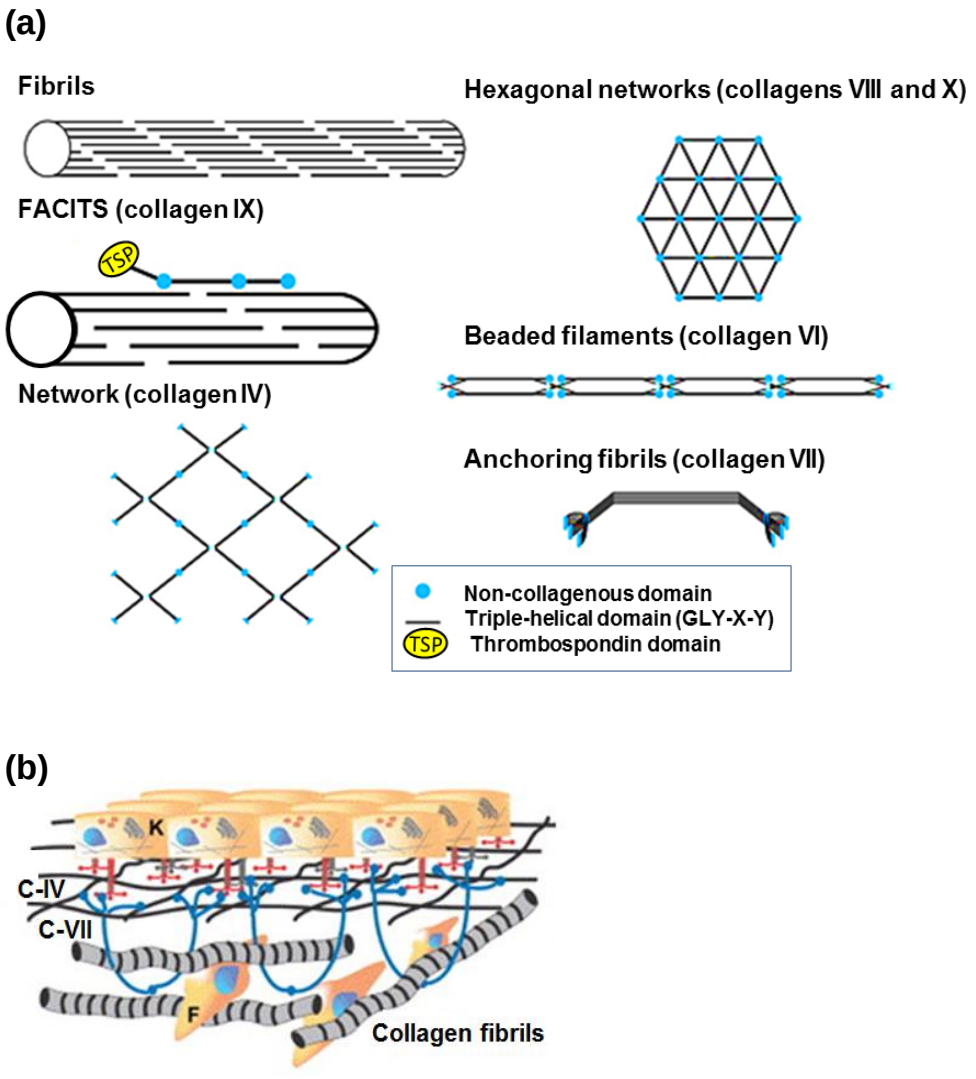


Figure 1.4: The categories of collagen families classified by the their supramolecular structure. *(a) Distinct collagens form different type of assembly. (b) Systematic relationship among various forms of collagen at DEJ. Image adapted from Ricard-Blum 2011 and Chung and Uitto 2010 [24, 38].*

Collagen types which are thought to be specifically located and play vital roles in skin are described in *table 1.2*.

Table 1.2: Collagen assemblies with functions in dermal ECM.

Type	Class identification	Function	Area specification	References
I	Fibrils (diameter ~40-100 nm)	Tensile Strength	Dermis (main feature in reticular dermis)	[26, 27]
III	Fibrils (diameter ~20-40 nm)	Tensile Strength	Boundary zones of the dermis that lack thick fibre bundles, such as around blood vessel or area adjacent to DEJ	[27]
IV	Network forming	A component of basement membrane (BM) and induce cytoskeletal rearrangements	Lamina densa of BM	[39]
V	Fibrils (inconsequential component to COL type I and III)	Determination of fibril structure	Basal and periderm cells of the epidermis and dermal cell surfaces	[28, 30]
VI	Beading filament forming	• Cell-cell and cell-matrix communication • Regulates ECM assembly and fibroblast motility	Papillary dermis immediately below DEJ	[33, 40, 41]
VII	Anchoring fibrils	Attach cells to DEJ	DEJ	[42, 43]
XVII	Transmembrane	Formation of hemidesmosomes	BM	[44]
XIX	fibril-associated collagens with interrupted triple helices	Formation of specialized BM	BM	[45-47]

Collagen synthesis and assembly

Collagen molecules are made up of three separate α amino acid chains to form a triple helical collagen molecule. These α chains are encoded by more than 45 genes and the resultant proteins are packed firstly into fibrils and then into thicker fibres [25, 29], (*figure 1.5*).

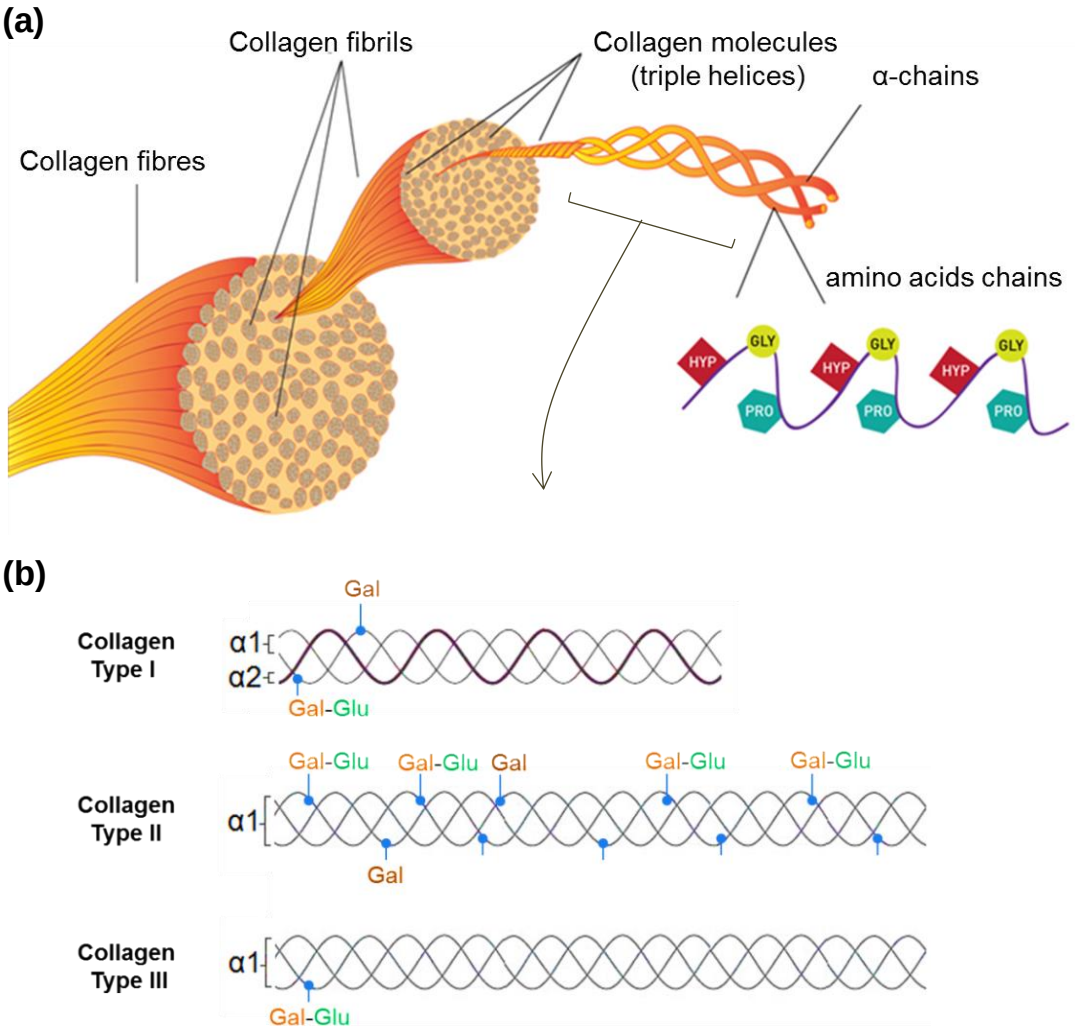


Figure 1.5: Assembly of fibrillar collagens.
(a) The collagen fibres are formed from small subunits. Firstly the amino acids which are the smallest unit, are composed into an α chain and introduced into a triple helix formation (composed of three α-chains per helix). Then the collagen molecules/helices are be arranged into collagen fibrils. Lastly, these fibrils aggregate into collagen fibres. Image adapted from Cradto 2016 [48]. (b) Three types of collagen are compared to show the type of the α-chains in the triple helices and associated glycosylation patterns; collagen type I is comprised of 2 α1(I) alpha chains and 1 α2(I) chain to form a heterotrimer whilst collagen types II and III are homotrimeric, that is all three chains are made up of the same molecule.

Fibrillar collagens are dominant proteins in the bodies of vertebrates [49]. The primary amino acid sequence conforms to a regular repeating pattern of 'glycine–X–Y' to produce a right hand helix; the glycine position (hydrophobic residue) is usually fixed whereas X and Y can be any amino acids, though they are frequently proline and hydroxyproline [50]. The synthesis process starts with the production and secretion of procollagen which is subsequently processed to the mature collagen fibre [51], (*figure 1.6*).

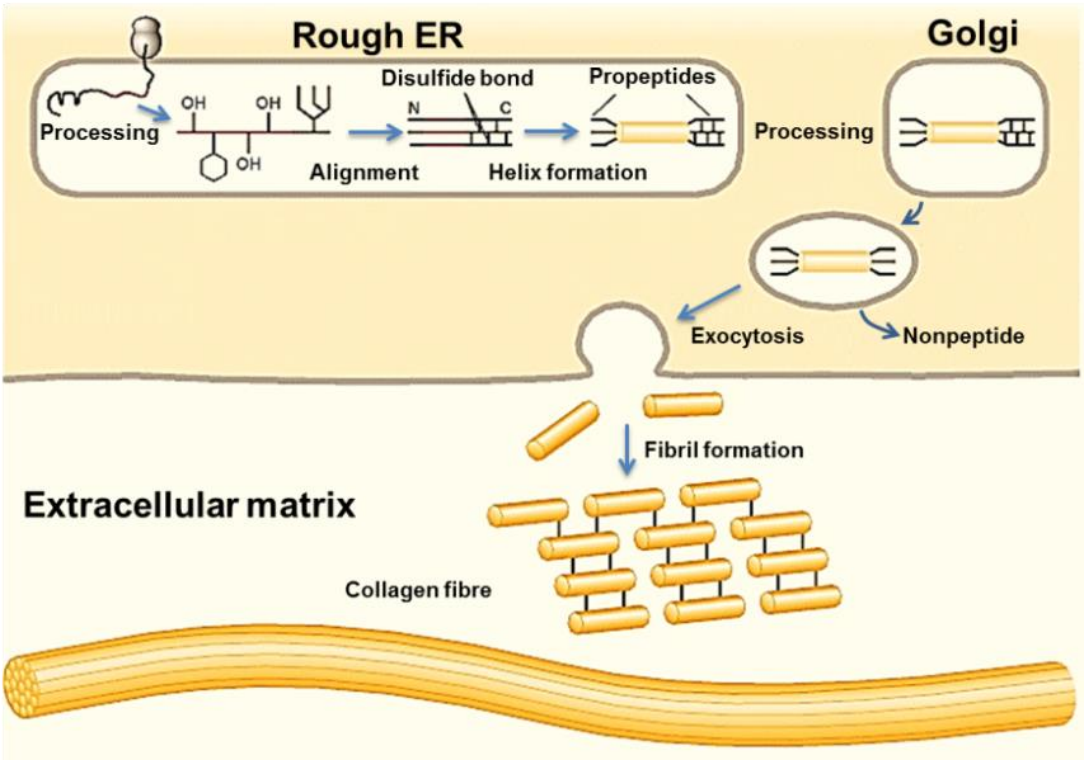


Figure 1.6: Collagen synthesis.
Procollagen is synthesized by fibroblasts in the endoplasmic reticulum. Triple chain of amino acids binds together with disulphide bonds to form helical shape as amino propeptides. Then it would be delivered and contained in the Golgi body and continuously released into ECMs as collagen fibre subunits, which aggregate into completed collagen fibre in the ECM. Image adapted from Lodish et al 2000 [23].

Procollagen molecules have 2 termini: carboxyl terminal (C-terminal) and amino terminal (N-terminal) propeptides. The C-terminal peptide has a non-collagenous (NC) domain whilst the N-terminal peptide has both NC and a shortened collagenous (COL) domain. The number of COL and NC domains are specific to each type of collagen [25]. C- and N- terminal domains induce the process of collagen fibril clustering, resulting in the formation of tropocollagen. Unique enzymes are needed for synthesis of each collagen fibre type [51], (*figure 1.7*).

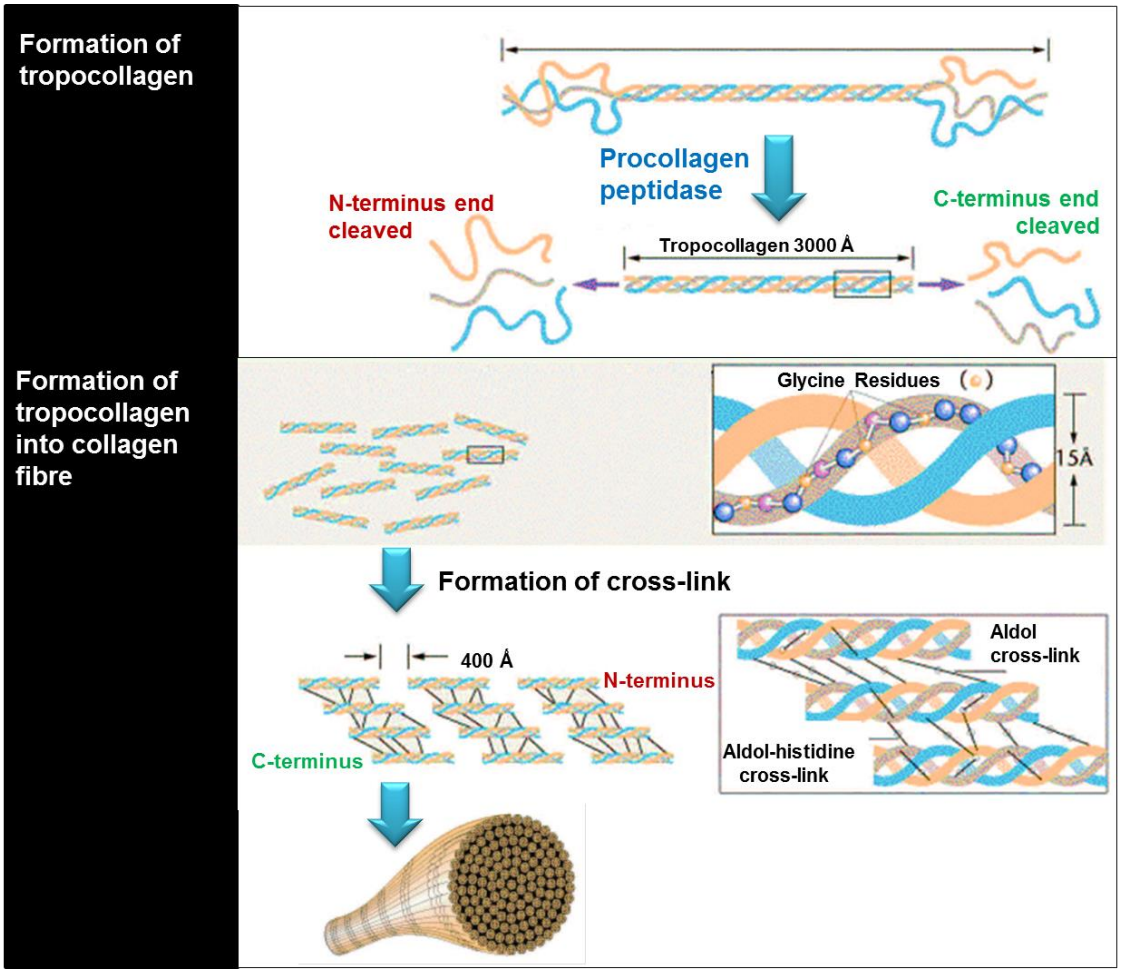


Figure 1.7: The processes of generating mature collagen.
Procollagen C- and N- termini were cleaved by procollagen peptidase.. These tropocollagens are cross-linked to form into mature collagen fibres (aldo and aldo-histidine cross-links). Image adapted from Bhattacharjee and Bansal 2005 [52].

Factors, particularly tissue biomechanics, can influence the structure of the molecule, for example, the diameter of collagen fibre and the ground substance (glycosaminoglycans; GAGs) in which it sits. The amount and variety of collagen within a tissue is not static and alters in response to increasing age - soluble collagens have been shown to decline progressively whilst insoluble collagen may increase [53]. Importantly collagen synthesis and deposition can alter in response to internal or external (environmental) stimuli.

1.3.2 Elastic fibres

The second important fibre present in the dermis is the elastic fibre. The basic molecules in elastic fibres are elastin protein and the microfibrillar glycoprotein, fibrillin [54]. Fibrillin-rich microfibrils (FRM) enclose elastin to stabilize the elastic fibre [55]. The special characteristic of elastic fibre is, as its name suggests, elasticity or recoil, allowing this fibre type to flexibly stretch and contract [56]. This property usefully helps various tissues to perform their function, e.g. walls of blood vessels and lung that need to enlarge in size [14].

The characteristics of elastic fibre system‘s architecture was commonly observed in 3 types which vary in different depths of dermis and proportion of two major proteins: FRM and elastin. Candelabra-like structural fibres, oxytalan fibres represent more plentiful FRM than elastic in papillary dermis area where is the closest area to DEJ. Elaunin fibres which contain approximately half and half between two main proteins appear in standing upright and link the oxytalan fibres to the elastic fibres with much higher elastic fibre contain than FRM in the reticular dermis [57].

Elastin is highly hydrophobic; the precursor of elastin, tropoelastin, is a single polypeptide chain. It has different isoforms dependant on alternative splicing allowing the protein to play specific roles in in different tissues (tissue-specific functions; [58], (*figure 1.8*).

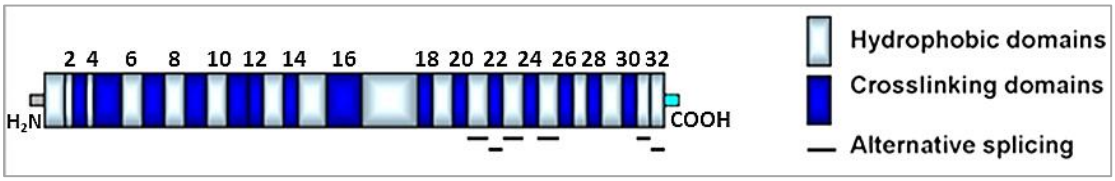


Figure 1.8: The organization of elastin.
The protein has both hydrophobic and crosslinking domains from Kielty et al 2002 [59].

Fibrillin-rich microfibrils are composed of the large multiple domain glycoprotein fibrillin (350 KDa) [60]) Three isoforms of fibrillin have been identified: fibrillin-1, -2 [61] and -3 [62], (*figure 1.9*). Fibrillin-1 is the most abundant isoform and is the main constituent in FRM, with a proline-rich hinge region [63]. Fibrillin-2 is glycine-rich [61] has been found in initial stage of development; fibrillin-3 is enriched in proline and glycine residues [64] as is found in brain tissue. In cross section, the microfibril is thought to be made up of eight fibrillin-1 monomers [54, 65] in developed tissue.

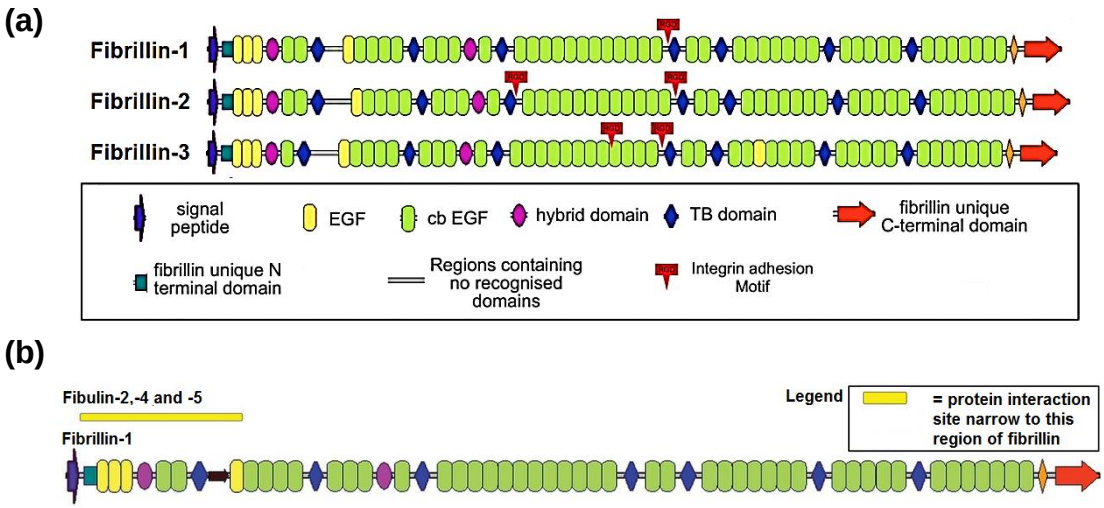


Figure 1.9: Fibrillins and fibulins are composed of repeating domains.
(a) The comparative chart of multiple protein domain organization within in fibrillin-1,-2 and -3. (b) Similarity of protein alignment of fibrillin to fibulins (-2,-4 and -5). Image adapted from Robertson et al 2011 [66].

Proteins in the fibulin family have been found to share some similarity or overlapping domains with both the fibrillins and tropoelastin (see review in [67]); fibrillin-1 has been shown to interact with fibulins through an N-terminal binding site [68]. Five different fibulins have been discovered to date (*table 1.3*). Fibulin-1 is thought to have multiple different isoforms [69], (*figure 1.10*).

Table 1.3: Proteins of the fibulin family

Isoform*	Species	No. of amino-acid residues‡	Accession number§
Fibulin-1A	Human	537 (29)	NM_006487
Fibulin-1B	Human	572 (29)	NM_006485
Fibulin-1C	Human Mouse	654 (29) 656 (29)	NM_001996 X 70853
Fibulin-1D	Human Mouse	675 (29) 675 (29)	NM_006486 NM_010180
Fibulin-2	Human Mouse	1157(27) 1204 (27) 1196 (26)	NM_001998 AH011811 NM_007992
Fibulin-3 (S1-5, EFEMP1)	Human Mouse	466 (27) 466 (27)	NM_004105/ NM_018894 NM_146015
Fibulin-4 (EFEMP2, PH1, MBP1)	Human Mouse	416 (27) 416 (27)	NM_016938 NM_021474
Fibulin-5 (DANCE, EVEC)	Human Mouse	367 (23) 367 (23)	NM_006329 NM_011812

*Alternative names are given in parentheses.‡The number of amino acids in the predicted mature protein and in the signal peptide (in parentheses) is shown. §The accession number refers to information in the GenBank database (see Online links). DANCE, developmental arteries and neural crest epidermal growth factor (EGF)-like; EFEMP, EGF-containing fibulin-like extracellular matrix protein; EVEC, embryonic vascular EGF-like repeat-containing protein; MBP1, mutant p53-binding protein 1.

Each fibulin isoform has specific protein size that was discovered in mammalians such as human or mice. The smallest isoform with only 327 amino acids is fibulin-5 while the largest one is fibulin-2 with 1,157 or 1,204 amino acids. From Timpl et al 2003 [67].

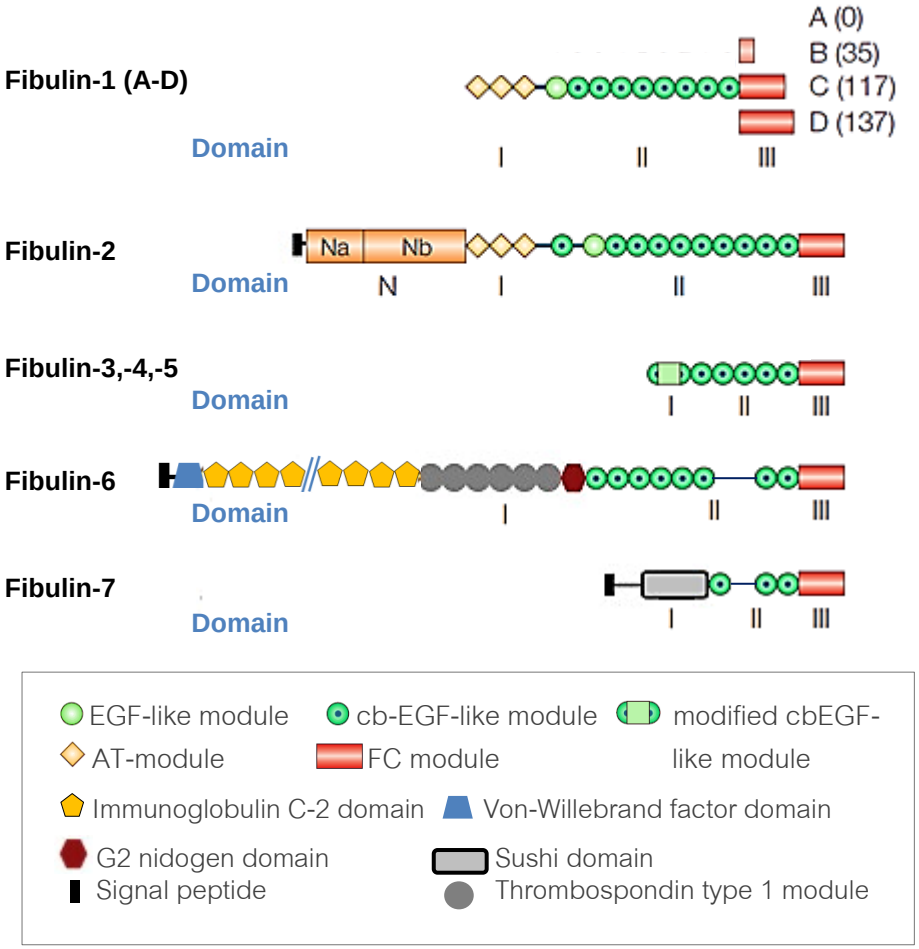


Figure 1.10: The domain structure of the fibulin family of proteins.
Every fibulin is basically composes of 3 domains (I, II and III). Long microfibril fibulins are type 1, 2 and 6 whereas the short microfibril fibulins are types 3, 4, 5 and 7. In fibulin-1, the various subgroups display 4 different COOH-terminal modules (FC module; isotypes A-D). Fibulin-2 has a characteristicN-terminal domain: cysteine (Cys)-rich sector (Na) and a Cys-free sector (Nb). Fibulins-3, -4 and -5 share structural similarities with modified calcium-binding (cb) epidermal growth factor (EGF)-like module at amino terminus (domain I; as seen in fibrillin molecules). Domain II or modified cbEGF-like modules or a central rod-like elements is inserted between C- and N- terminals. Fibulin-6 and fibulin-7 revealed distinct domain I. Fibulin-6 has complex domain I with sequences, hence, it contained 5,635 amino acid residues [70]. The novel fibulin-7 contained II and III domains homologous to those in the fibulin family, however, it additionally displays sushi domain at the N terminus. AT=anaphylatoxin-like. Image adapted from Timpl et al 2003 [67].

Fibulin-1 was identified histochemically in 1995 as an intercellular component of connective tissue, abundantly found in tissues rich in elastic fibres, i.e., dermis, cervix and blood vessels. This protein shared immunoreactive characteristics with elastin and fibrillins when stained by either fibulin-1 antibodies or Verhoeff's elastin stain [71]. Fibulin-1 specifically locates to the amorphous core of elastic fibres, however, it does not co-localise with FRM in mature elastic fibres (which surround the elastic fibre) [71].

Fibulin-2 also shares homology in domain structure with fibulin-1, both binding calcium and extracellular ligands [72]. This isoform is the largest of the family with a size of 195-kDa [73]. Fibulin-2 and -5 have been shown to interact with fibrillin-1 and tropoelastin based on the evidence of formation process of ternary structural protein of recombinant fibrillin-1, fibulin and tropoelastic molecules [68]. Fibulin-2 is expressed in various adult body sites, for example, the heart, the neural system and skin [74-76]. Expression in the skin is high and can be seen in the epidermis, at the basement membrane, and as discrete structures in the dermis and subcutaneous layer [77]. In 2001, Hunzelmann and colleagues concluded that fibulin-2 and elastin are closely related; additionally the team confirmed that this protein is influential to elastic fibre and microfibril formation [73].

Fibulins-3, -4 and -5 share similarity of structure (central segment of 5 cbEGF-like modules) and size [67, 78]. These fibulins were subsumed in the short protein fragment which related to interaction between two proteins of the protein composition in basement membrane and ECMs [79]. In 1999, Giltay and the team reported that fibulins-3 and -4 showed closely similarity with fibulin-1 type C and have short amino acid residues 487 and 443 amino acids respectively in human. Yet these two fibulins did not have cross-reactivity [78].The same study also revealed the localization of fibulins-3 and -4 in adult mice as being in vessel walls, in basement membrane zones and some other extracellular regions. Specifically fibulin-4 shows high expression in heart valves (though less than fibulin-1), veins, arteries (even in small capillaries) but fibulin-3 was only found exclusively in small capillaries.

Fibulin-3 could also be detected in close proximity to cardiomyocytes, where fibulin-1 expression is rare.

Fibulin-5 is a small isoform at ~66KDa and is expressed with elastic fibres in elastogenic organs (aorta, lung and skin) [80]. Fibulin-5 is connected to elastic fibre development (see review at [79] but did not directly relate to elastic fibre recovery following wound healing [81]. However, fibulin-5 was suggested to be a protein reporter for early UV-induced skin damage as well as being associated with intrinsic skin ageing [82]. In 2009, the role of fibulin-5 were reported based on a gene knockout (*Fbln5−/−*) study in mice. The results showed that fibulin-5 controlled the aggregation and elongation of tropoelastin monomers and in microfibril bundle formation when associated with elastic protein [83].

In a similar way like other proteins in the fibulin family, fibulin-6 (or hemicentin-1) functions to link ECM to cellular components at basement membranes of epithelia and blood vessels [70]. Fibulin-6 was found in studies to impact on migration of cardiac fibroblast [84] and was expressed in human salivary gland epithelial cells [85].

The most recently discovered family member is fibulin-7 or TM14; this localies to teeth, cartilage, hair follicles and extraembryonic tissues of the placenta. This proteins impacts on odontoblast differentiation including dentin development. Specifically fibulin-7 is thought to link dental mesenchymal cells to odontoblasts as a dental ECM adhesion molecule [86].

1.4 The clinical feature of ageing skin

Skin ageing can be separated into two clinical phenotypes depending on the underlying cause of the ageing process. The first is intrinsic ageing, the changes that occur because of increasing time. The other is extrinsic ageing; this is concerned with impact of various external environmental factors which induce changes to human skin.

Intrinsic ageing causes a slow decline in structure and function of skin. The changes relate to all aspects of skin (biological, chemical and physical). Two important extrinsic factors that can be harmful to skin are UVR from any light sources and smoking; these factors are influential in skin ageing, causing characteristic alterations which are different from those seen in intrinsically aged skin. The clinical features of both intrinsic and extrinsic ageing are compared and shown in *table 1.4* [87].

Table 1.4: The comparison of aged patterns between intrinsic aged skin and extrinsic aged skin *(from Langton et al 2010 [87]).*

Intrinsically aged skin	Extrinsically aged skin
Fine wrinkling	Coarse wrinkling
Smooth texture	Roughened texture
Clear complexion	Sallow complexion
Uniform pigmentation	Mottled pigmentation
Gradual loss of elasticity	Marked loss of elasticity

In intrinsically aged skin, the skin appears pale and has delicate wrinkles. Even though the general skin texture is smooth and unblemished, the wrinkles are incidentally found with magnified expression lines. Intrinsically aged skin is drier and has decreased elasticity than young skin. In extrinsically aged skin, we observe deep and course wrinkles with changes in pigmentation (hyper- and hypo-pigmentation). Extrinsically aged skin is less elastic and less capable to reshape. The outcome of extrinsic skin ageing by UV exposure is termed photoageing [88], *(figure 1.11)***.**

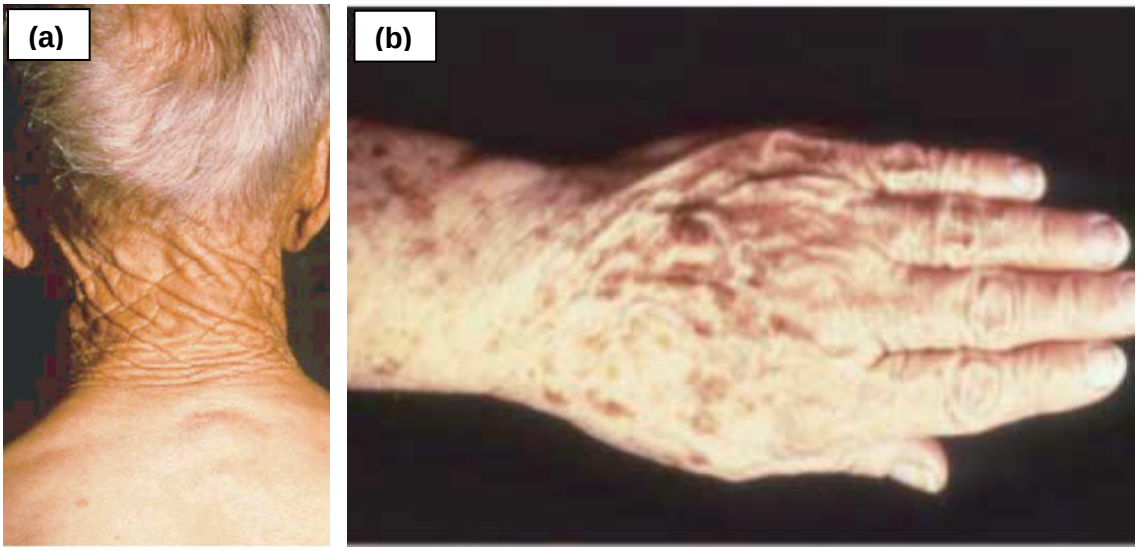


Figure 1.11: Clinical features of aged and photoaged human skin.
Intrinsic aged skin reveals fine wrinkles on smooth texture skin surface without any pigment spots like the skin area under the neck base line. On the contrary, the neck skin which had severe sunlight exposure demonstrates the skin patterns of photoageing (a). The characters of extrinsic aged skin show coarse wrinkles, toughened texture with many pigment spots (b) (from Watson and Griffiths, 2005 [88]).

1.5 The histological features of ageing skin

1.5.1 Intrinsically aged skin

Epidermal skin thickness declines gradually as it ages [89]. Moreover, the numbers of cells, e.g. fibroblasts, are reduced [90]. The structure of the dermal-epidermal junction (DEJ) becomes more flattened [91].

1.5.2 Extrinsically aged skin

Some skin sites are always exposed to environmental factors such as sunlight. The prolonged UVR exposure results in photoageing. After acute sunlight exposure, skin can be burned (inflammation or erythema) and acute UV exposure can suppress the immune system. Long term (chronic) UVR exposure can damage dermal connective tissues and may result in skin cancer [87, 88].

1.6 Optical cutaneous irradiation

1.6.1 Ultraviolet radiation properties and its impacts in human skin

Ultraviolet radiation (UVR) contains UVA, UVB and UVC light categorised by way of wavelength (*table 1.5*). UVR travels from the Sun to the Earth's surface and can affect all life; however, only UVA and UVB penetrate to the Earth's biosphere [92, 93] while UVC is filtered out by the ozone layer [94, 95]. Hence the remaining electromagnetic radiation of UVA and UVB affects biological structures [96] and impacts on skin function [94, 97]; detail of UVR are summarised in *figure 1.12 - 1.13*.

Table 1.5: Wavelength of three spectrum regions of different UV types based on timeline reports. *Image adapted from Diffey, 2002 [98].*

UV types	First report in Copenhagen meeting of the Second International Congress on Light held during August 1932	Normal definition and use by environmental and dermatological photobiologists
UVA	400–315nm	400–320nm
UVB	315–280nm	320–290nm
UVC	280–100nm	290–200nm

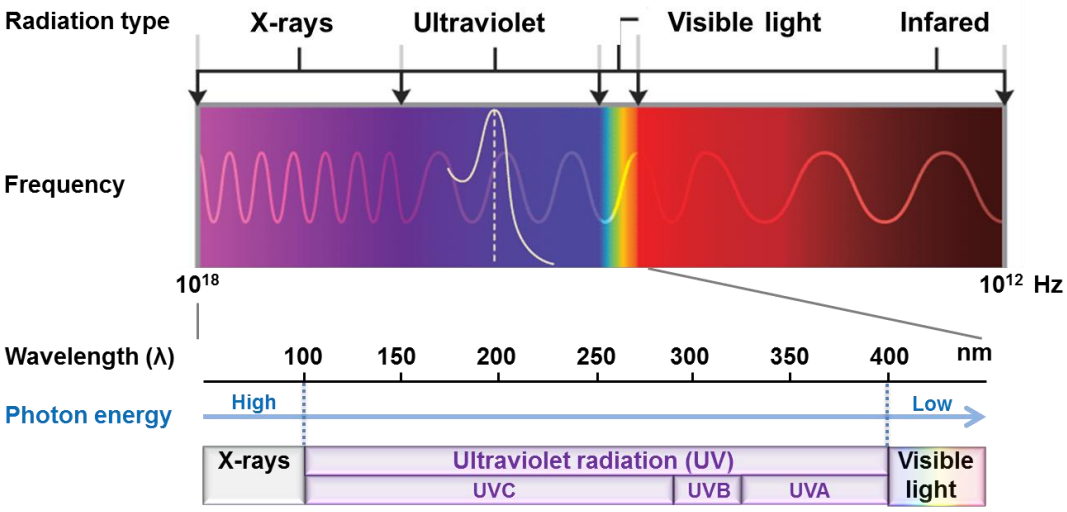


Figure 1.12: The electromagnetic spectrum specific to UV length and nearby radiation type. *Three types of UV (UVA, UVB and UVC) are covered in wavelength 100-400 nm. However, only UVA and UVB are present on the earth surface because UVC is filtered out by the ozone layer in the earth atmosphere. Image adapted from Matsumura and Ananthaswamy 2005 [99].*

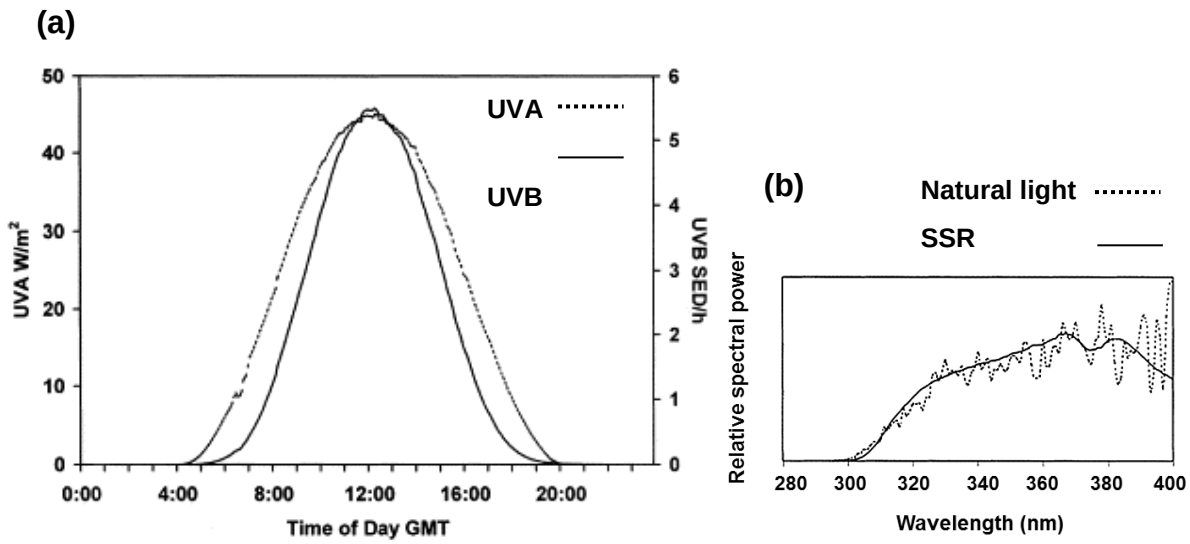


Figure 1.13: Differential terrestrial UVA and UVB in summer of UK on a clear sky day.
(a) and intensity of terrestrial UVR in clear sky at noon in summer season at latitude of 38˚S compared to those from solar simulator (b). The SSR source was from xeno arc filtered with a WG320 (2 mm thick) and UG5 (1 mm thick) filter (from Diffey, 2002 [98]).

The efficacy of each UV wavelength to penetrate skin depends on it is wavelength and energetic properties *(table 1.6)*. The solar spectrum contains around 6% UVB (290–320nm), a much smaller proportion than UVA (320-400nm; approximately 94%; [98]. Biologically UVB has more diverse and potentially severe effects on both plants [100] and animals [101] than UVA. The *stratum corneum* absorbs about 70% of all UVB that radiates to the skin's surface whilst approximately 30% penetrates to the deeper layers [102]. This is due to its photon energy matching to biological chromophores, i.e., proteins, melanin and DNA [103, 104]. Even though UVA has less energy, its higher amount in sunlight may influence different changes in skin after UVB has been filtered out by the epidermis [92], such as by influencing the production of reactive oxygen species (ROS;[105] which can affect biological molecules in an indirect manner).

Table 1.6: Relation of estimated depth in fair Caucasian skin and the wavelength of solar ray penetration

Wavelength (nm)	Depth (μm)
250	2
280	1.5
300	6
350	60
400	90
450	150
500	230
600	550
700	750
800	1,200
1,000	1,600
1,200	2,200

Table from Anderson and Parrish, 1981 [94] .

1.6.2 Advantages of UVR in human skin as an influence on vitamin D synthesis

UVR, specifically UVB, plays a key role in the synthesis of cholecalciferol or vitamin D_3 in human skin. Vitamin D_3 is involved directly with bone heath [106, 107] as cholesterols in skin are converted to the hormone calcitriol which controls calcium, phosphorus and bone metabolism [108], (*figure1.14*). The major factor in vitamin D_3 deficiency is dependent on the lack of UVR in daily life [109]. In 2016, Farrar et al reported insufficient vitamin D_3 production in an adolescent white Caucasian group (n=131; 12-15 years old) because of low sun exposure behaviour in the UK even though testing was in the peak of summer and this negatively affected bone mineral density (BMD) [110]. As well as reduced vitamin D_3, reduced exposure to UVR has been linked to other health problems, such as ovarian cancer [111, 112], autoimmune diseases (e.g. rheumatoid arthritis [113], psoriasis [114] and Lupus Erythematosus or SLE [115], hypertension and infectious diseases, all of which may affect quality of life [109].

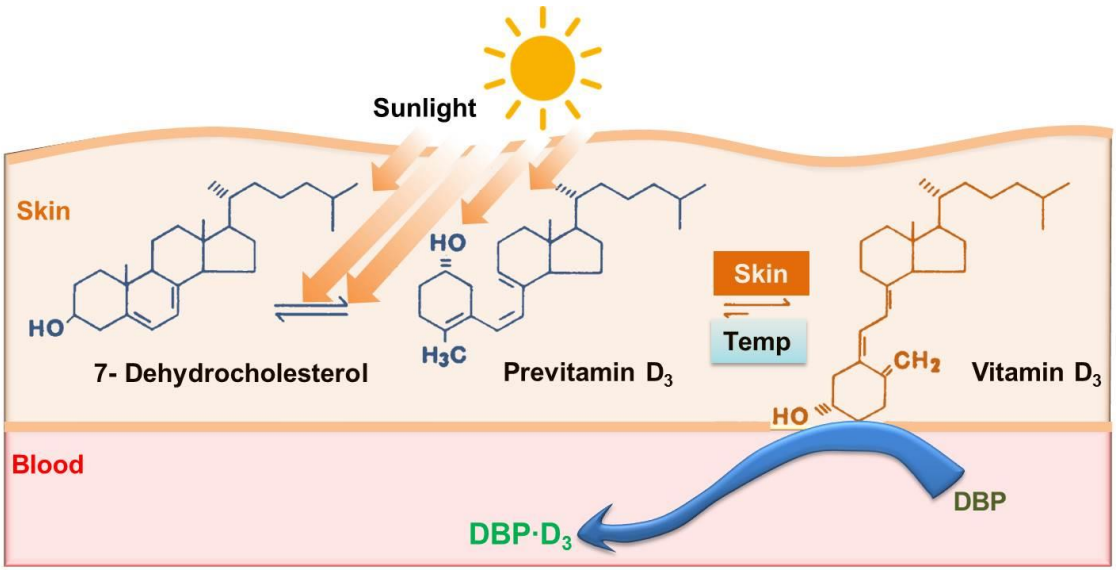


Figure 1.14: Vitamin D3 (D3) synthesis diagram.
Previtamin D3 is derived by the action of sunlight (UVR) on 7-dehydrocholesterol. Thr previtamin is converted to the vitamin D3 in the skin, where it binds to its cognate binding protein, DPB, allowing trafficking to the circulation. Image adapted from Holick et al. 1980 [116].

1.6.3 Vitamin D sufficiency

Appropriate doses of natural light provide benefit; essential levels of vitamin D to maintain health is only 1 minimal erythemal dose (MED) of sunlight equivalent to 600–1000 IU of vitamin D ingestion. Hence vitamin D synthesised from irradiated hands, arms, and face by natural sunlight (~5 minutes for skin-type-2 adult in Boston at noon in July) in the spring, summer, and autumn equals to 1/3 or 1/2 of a person's MED, two to three exposure times per week are thought to be suitable to make enough vitamin D [117].

Reichrath (2006) suggests exposing no more than 18 % of total body surface area for ~5 minutes (adult skin type II; Boston, MA, USA; at noon in July; [118]. However, excessive exposure and chronic repetitive exposure both cause skin problems.

1.6.4 Erythema (sun burn)

Skin damage from over dose of strong sunlight irradiated on skin brings about mild clinical signs such as cutaneous redness (*figure 1.15*) that is sore, warm, tender and itchy. These basic symptoms can last at least a week. However, in serious cases skin can be burnt with blistering or swelling [119].

UV irradiated skin shows a biological response via dermal vasodilatation and immunological mechanisms [120, 121] that can be observed as skin burn or erythema [122]. Erythema is noticeable redness of UV-exposed skin and is observed at approximately 24 hour after UV radiation [121, 123] and able to be measured level of skin sensibility to UV by MED that referred blood content flowing in the superficial dermis and standard erythema dose (SED) which is similar measurement but dose of UVR will be fixed at 100J/m^2 representing 1 SED [122].

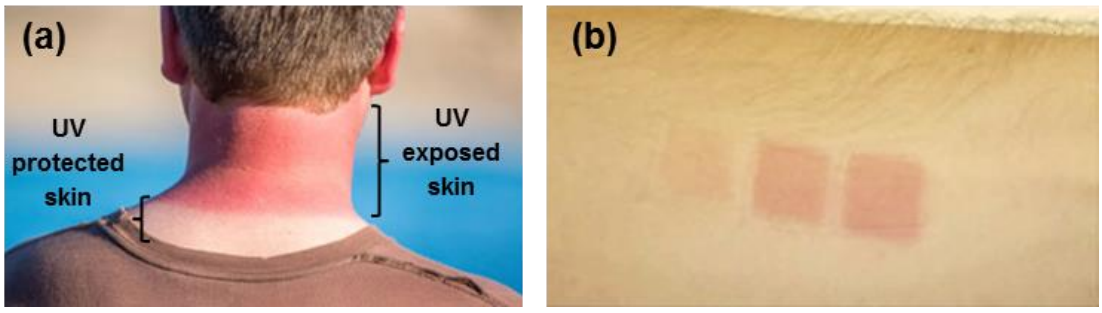


Figure 1.15: Cutaneous erythema response of human skin to UV exposure.
(a) Burnt neck skin (upper neck) and cloth protected skin (lower neck) in the individual, image adapted from National Health Service, 2015 [119]. (b) Forearm skin appears noticeably red after 24hr radiation from 1x MED (left) and darker red in higher doses at 2x (middle) and 3x MED (right). Image adapted from D'Mello et al (2016) [124].

1.6.5 Natural cutaneous chromophores in human skin

UV is known to interact with a number of biological molecules in the skin including DNA, urocanic acid (UCA), some amino acids, melanin and their building block and metabolites. These chromophores have the capability to transfer photon energy within the molecule to produce excited electrons (for review see [104, 125], (*figure 1.16*)). This phenomenon is capable of causing damage to the molecules themselves and surrounding proteins, including protein assemblies in the ECM.

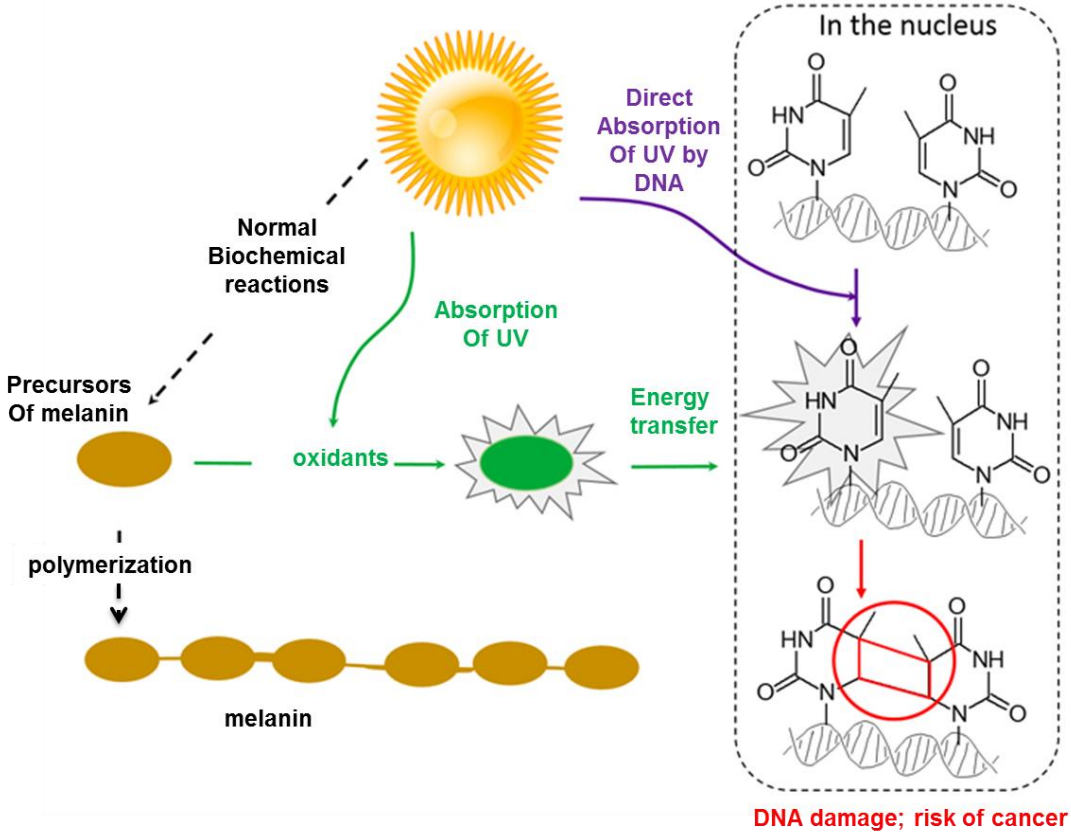


Figure 1.16 : Absorption of energy causes damage to biological tissues.
Photon from UV radiation impels the photon receptors of chromophore, such as melanin, DNA and singlet oxygen species through energy transferation and brings about diverse mechanism. Image from [126].

1.6.5.1 DNA

Although the significant mutagenic waveband is in UVC (range 200-290 nm), UVA and UVB still clearly cause DNA damage [104]. UV induced-DNA base mutation produces pyrimidine-pyrimidone (6–4) photoproducts (6–4PPs) [127] and cyclobutane pyrimidine dimer (CPDs) formation [128]. If DNA photoproducts are not repaired, they can result in cell death [129], mutation induction and onset of carcinogenic events [130].

1.6.5.2 Melanin

Melanin absorbs energy from UVR and so acts as a natural 'sunscreen', stopping at least some of the UVR energy penetrating deeper into the skin [131, 132]. Melanin absorbs in the UVA and UVB (280-400 nm). UV exposure also stimulates melanogenesis.

1.6.5.3 UCA

UCA is a key biological photoreceptor found in cutaneous epidermis. *Trans*-UCA is synthesized from histidine by deamination and is capable of photoisomerization by UVR to *cis*-UCA (see review at [133]). *Cis*-UCA is thought to be a mediator for immunosuppression as it binds to serotonin [5-hydroxytryptamine (5-HT)] receptors [134].

1.6.5.4 Amino acids

There are twenty amino acids which are precursors for protein synthesis. However, only five of them (tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), histidine (His) and cysteine (Cys)) can absorb energy from UVR and so function as chromophores in ECMs [135]. Such UV chromophores are major components of the proteins found in the elastic fibre system; the primary amino acid sequence of fibrillin-1 contains about 20% UV chromophores and this is thought to play a role in their early loss in photoageing (see review at [136, 137]).

1.7 Alteration of collagen matrix and elastic fibre system in photo aged skin

Photoageing in skin is thought to occur from repeated doses of UVR over a long period of time and affects both the epidermis and dermis.

1.7.1 Changes to the collagen matrix in photoageing

Chronic ultraviolet radiation has the capacity to induce molecular alteration in skin fibroblasts which results in a deterioration of their function. In intrinsically aged skin, fibroblasts have less ability to produce collagen fibres [138], resulting in short length collagen fragments [139]. In addition, the cell's ability to bind collagen is reduced and the fibroblasts may become detached from the fibre [140]. A number of biological processes occur when skin becomes photo exposed, including augmentation of AP-1 and subsequent MMP expression, oxidization of biomolecules and diminution of collagen synthesis [141]. The basic components supporting cell structure in dermis is type I and type III collagen [142]. However, in 2004, Quan and colleagues [143] reported that UVR exposure is capable of initiating pCI production and also induces prominent changes in collagens such as abnormal fibre organization, fibre bundle separation and fragmentation. Therefore, UVR exposure has the ability to damages collagen in two ways: repression of the procollagen producing pathway and collagen degeneration. However, topical all-*trans* retinoic acid (tretinoin) treatment has been shown to partially restore the collagenous matrix from UVR-induced damage by inhibiting matrix metalloproteinases [144, 145].

1.7.2 Elastic matrix

One of the distinguishing features in photoaged skin related to elastic tissue is the occurrence of solar elastosis. Solar elastosis is a disordered assemblage of great amounts of elastic material in the reticular dermis [146-148]. An early event in the photoageing process is loss of the microfibrillar apparatus of elastic fibres at the DEJ [57, 149]. These microfibrils are made up mainly by the glycoprotein fibrillin-1 but also contain other proteins, for example, fibulin-5 [82]. The two major repeating domains in fibrillin-1 are calcium-binding epidermal growth factor- like (cbEGF) domains [65] and a single TGFβ1-binding protein-like (TB) domain [150]. This structure is maintained by Cys-Cys disulphide bonds which are instantly disintegrated by UVB radiation

[54, 151]. Moreover at the boundary of the DEJ, oxytalan fibres (fibrillin-rich microfibrils) are degraded early in the photoageing process. In spite of detectable fibrillin-1 fibres, by using immunohistochemical method, the structure and quantity of this fibres have been altered that microfibrils are less in number than in normal healthy UV-protected skin *[87], (figure 1.17).*

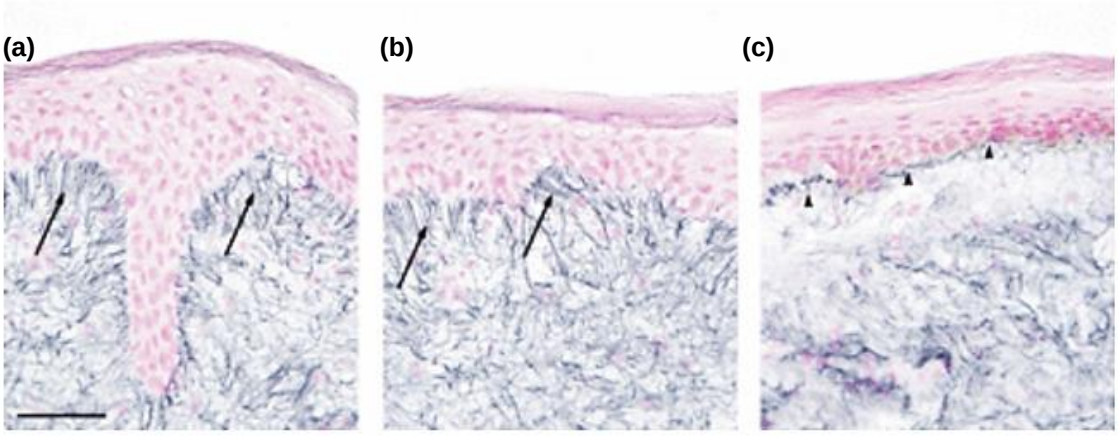


Figure 1.17: The fibrillin microfibrillar network of healthy skin and in photoageing.
Unexposed skin area of buttock in young adult (a) shows an intricate system in bundles of fibrillin-1-positive microfibrillar (arrow) that start from DEJ then coagulate to elastic fibres in reticular dermis. In intrinsically aged photoprotected buttock skin (b) we see an identical organization to those skins in young adult of bundles of fibrillin-1-positive microfibrils (arrow). In addition epidermis is thinner and has lost the curves of papillae at the DEJ. (c) Photoaged skin old adult demonstrates thin epidermal layer similar to intrinsically aged buttock skin and a marked loss of fibrillin-1-positive microfibril bundles (arrow head). Measurement scale bar is 50 µm (from Langton et al. 2010 [87]).

1.7.3 Potential mechanism

Studies have demonstrated wrinkle formation in hairless mice following regular UVB irradiation. UVB is able to increase the expression of the enzyme 'fibroblast elastase' or membrane-bound type metalloprotease [152] by inciting cytokine secretion in keratinocytes [153].

The ECM may be continually damaged owing to the UVR-induced secretion of enzymes from neutrophils, for example MMPs-1, -8 (neutrophil collagenase), -9 and neutrophil elastase.

Hence the neutrophil is one of the most important cell types involved with photoageing process [154]. Ultraviolet radiation appears to play a key role as the starter in cellular mechanism, via the induction of MMPs. In 2010, Sherratt [135] and team suggested that at low dose (20-100 mJ/cm^2) UVB is able to change molecular structures in isolated fribrillin-1 microfibrils *in vitro* but not collagen type I. At this dose of UVB-irradiation impacts on both mature and maturing

microfibrils. It is also possible that an acellular process is also initiated by UVR, so dividing ECM degradation into indirect and direct mechanisms [155], *(figure 1.18)*.

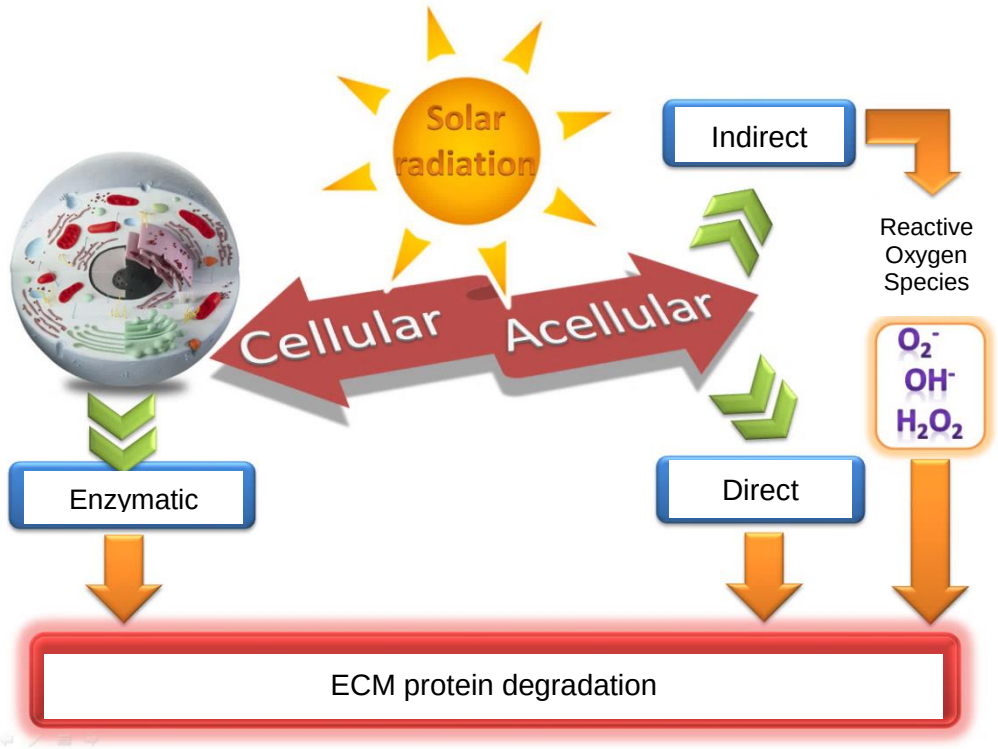


Figure 1.18: Potential ways UVR can disrupt protein.
The effect from UVR exposure can induce ECM degradation in two possible ways: cellular and acellular pathways. Firstly, UVR influences enzyme activation (especially MMPs) which are involved with demolishing the ECM. Secondly, UVR interacts to create reactive oxygen species, then these molecules can degrade ECM called 'indirect' way whist UVR exposes to skin and is adsorbed my amino acid in ECM called 'direct'. Image adapted from Thurstan et al. 2012 [136].

The mechanism of pCI suppression involves the ubiquitous multifunctional cytokine, transforming growth factor-β (TGF- β) as pC production controller [156, 157]. The cellular pathway operates by TGF-β binding to a complex of TGF-β receptors type I (TβRI) or type II (TβRII) on the cell membrane. Receptor/ligand binding activates serine/threonine kinase activity which has the potential to phosphorylate Smad-2 and -3 and form a complex with Smad-4. The phosphorylated Smad complex may then be transferred to nucleus to initiate specific gene transcription. These genes, for example *COL1A1*, are regulated by TGF-β via their promoters *(figure 1.19)*. Hence TGF-β is a key molecule in the control of ECM synthesis. Experiments in mice show that if TGF- β1 is over expressed, fibrosis results [143].

Quan et al. (2004) reported that UVR has the potential to suppress the expression of pCl.

In addition, it decreases expression of TβRII significantly [143]. However, irradiation does not affect the expression of mRNA of TβRII or protein levels. However, when experiments were repeated in human skin *in vivo*, the results revealed that UVR reduces TβRII mRNA expression.

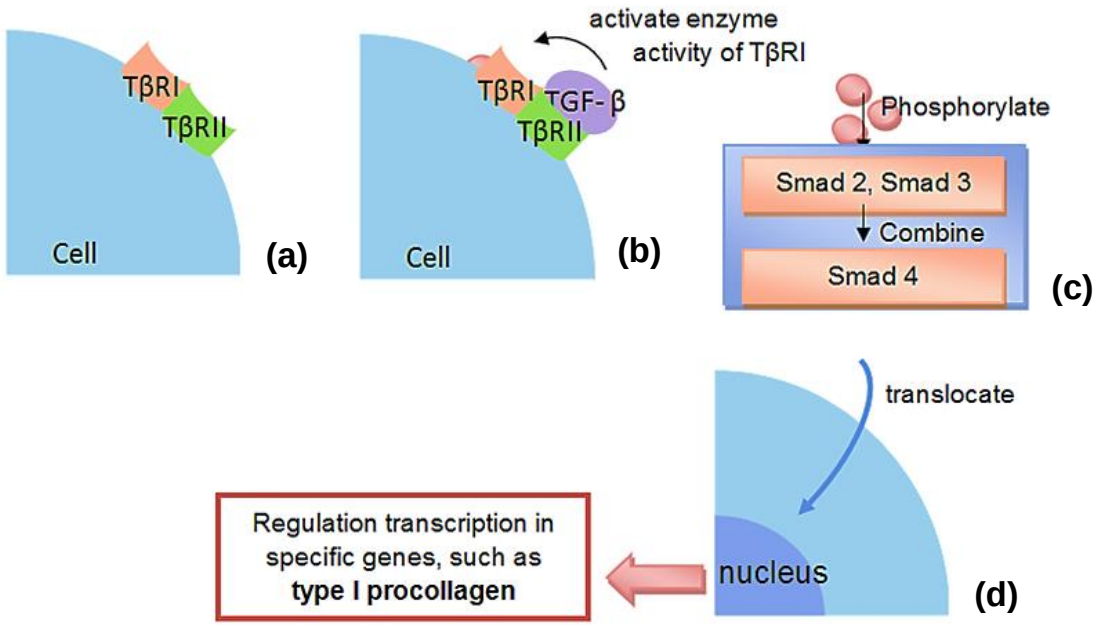


Figure 1.19: The pathway of type I procollagen regulation at the cellular level involving transforming growth factor-β (TGF- β).
Complex of TGF- β type I (TβRI) and TGF- β type II (TβRII) on the cell membrane (a) is the receptors for TGF- β. When TGF- β binds with TβRII, it causes enzyme "serine /threonine kinase" activity of TβRI to activate (b). The function of activated enzyme is to phosphorylate Smad 2 and Smad 3 to combine with Smad 4 (c). Then compound molecules have been moved to nucleus eventually (d) as regulator of type I procollagen genes.

Skin may also be subject to direct UVR damage. The energy from UVR may induce chemical alteration in chromophores or the energy may be passed from chromophores to other adjacent molecules instead. It may also result in the production of reactive oxygen species (ROS) which damage essential biological components in tissue such as proteins, lipids and DNA. The free radical theory of ageing [158] suggests that ROS, by products from oxidative metabolism, in cells has great efficiency to damage key biomolecules. The oxidized DNA contributes to mutagenesis generation [159]. The oxidized proteins cause alterations in structure and function [160]. The oxidized lipid which is a main component of cell membranes results in decreasing chemical transportation and signal transduction.

1.8 Photoprotection

The consequences from photoageing cause a range of skin symptoms from mild (signs of premature aged skin) to severe (melanoma and non-melanoma skin cancers). Hence avoidance and protection of skin from UV is the best way to reduce a person's risk of skin problems caused by UV. Though wearing appropriate clothes efficient photoprotection can be provided [161], particularly in summer, and in areas near the equinoctial line. However, clothing is only one way of protecting the skin; photoprotective agents and supplementation play a role in preventing UVR effects in daily life.

1.8.1 Sunscreen

The topical application of sunscreen as a photoprotective agent is a well-known option to improve and protect skin from UV. The chemical compounds popularly used in sunscreens are divided based on their photoprotective mechanism into inorganic and organic sunscreens (see review at [162]). Inorganic sunscreens are inert compounds, for example, zinc oxide and titanium dioxide, used to reflect UV to reduce UV photons penetrating into skin [163]; organic sunscreens are aromatic compounds bonded with carbonyl groups which will absorb photon energy [164].

A study in a Japanese population showed that use of a chemical sunscreen with UVB sun protection factor (SPF) of around 30.4 and a UVA protection factor of 8, comprised of six chemical UV absorbers for 18 months, maintained skin health (number of spots and uniformity of skin colour tone) compared to those who did not use sunscreen [165]. However, sunscreen improvements in the cosmetic market by adding natural extracted ingredients, such as aloe vera, evening primrose and green tea which have antioxidant property, may enhance sunscreen capability (see review at [166, 167]) and may be further developed to replace chemical sunscreen that might cause skin problems from long term use [168]. Nevertheless topical sunscreens may be inappropriate in some skin conditions such as skin with an underlying clinical complaint or sensitive or oily skin [169]. Topically applied sunscreens may also cause contact dermatitis, phototoxic and delayed hypersensitivity reactions, cross-sensitivity reactions including discoloration and staining of clothes (see review at [170]).

1.8.2 Systemic photoprotection

The oral consumption of supplements for photoprotection have been studied and discovering in both human and animal models to be an alternative choice to prevent photoageing [171-174]. Many studies have investigated natural or organic products, such as plant and herb extracts [175], vitamins [176, 177] and food or drink [171] for convenient consumption, potentially providing photoprotection with less toxicity.

1.8.2.1 Vitamin C

Vitamin C or ascorbic acid is a six-carbon compound which humans cannot synthesise because of a lack of the key enzyme, L-gulonolactone oxidase. Deficiency of vitamin C results in 'scurvy' characterised by fatigue, bleeding gums, red-blue skin spots and poor wound healing responses [178, 179]. The recommended dietary allowance (RDA) of vitamin C from any sources (food, drink or supplement) thought to maintain normal health is 60 mg/day [180], whereas the suggested optimal dose is around 200 mg/day [181]. Despites some researchers reporting that vitamin C had antioxidant properties, some experiments also show that high dose vitamin C (500-1000 mg/day for 1-2 months; combined with other vitamins A or E) did not exhibit a benefit on oxidative marker suppression [182, 183]. Particularly consumption of only vitamin C at 500 mg/day for 8 weeks had no effect on the mild oxidative stress seen in human skin following UVR exposure [184]. Vitamin C is also required for the synthesis of collagen [185].

1.8.2.2 Polyphenols

Polyphenols can be defined as 'compounds containing more than one phenolic hydroxyl group' (Oxford English Dictionary, online edition, accessed 15th September 2016), and are generally found in plant food stuffs i.e. green beans, various legumes, apples, oranges, chocolate, red wine, coffee and tea [186-188] which have antioxidant activity correlating with the amount of phenolic compounds they contain [189-191], (*figure 1.20*). They are thought to be beneficial to health, enhancing the body's antioxidant capacity and are often taken as a dietary supplement [192-199]. More recently, they have been suggested to have anti-ageing effects (see review at [200]), with potential, in a cosmetic application, to support skin maintenance and improvement of appearance (see review at [201, 202]).

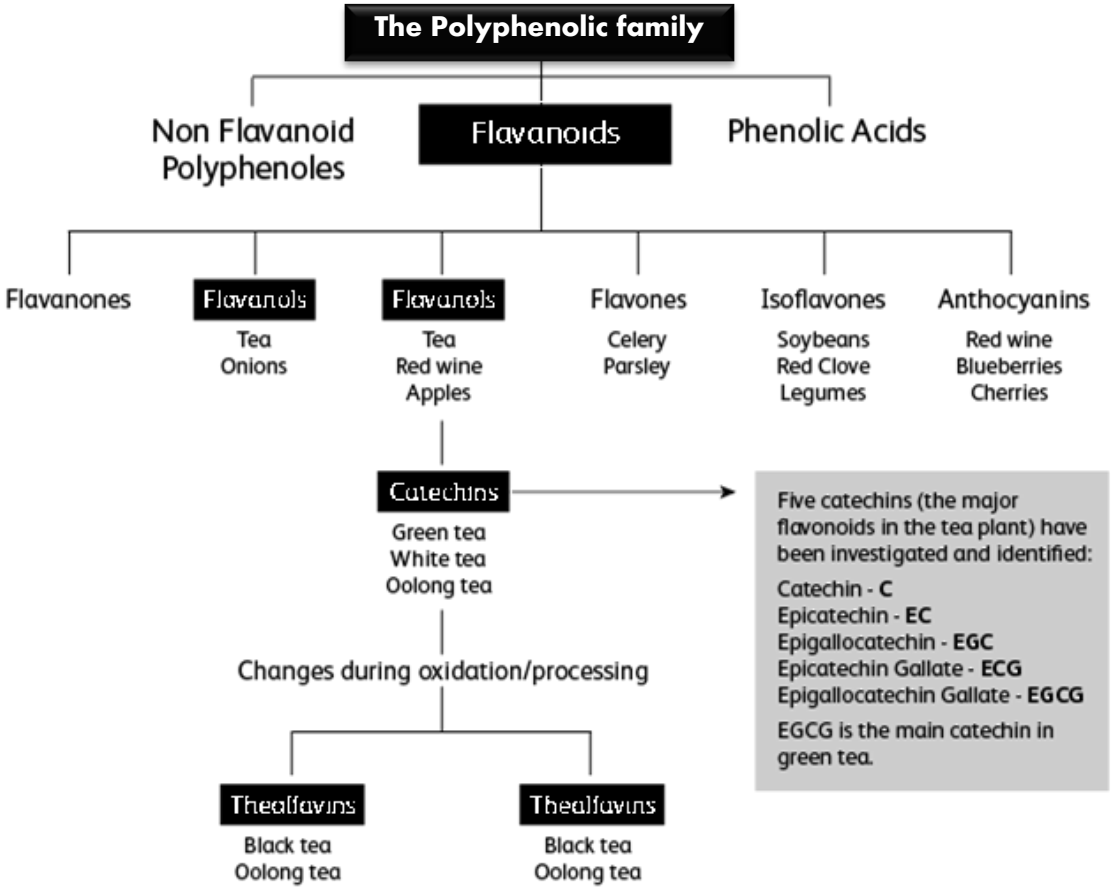


Figure 1.20: Classification flow chart of subdivided groups within polyphenolic family *from Petros, 2014 [203].*

1.9 Green tea catechins (GTC)

1.9.1 Green tea sources, chemical ingredient and processing

Tea (*Camellia sinensis L.*) has many varieties which are extensively grown in south-east Asia and in India. It is a well-known beverage for more than 3,000 years in China. Wild type tea possibly originated from southern part of China or Indonesia and Assum. In the 18th century, teas began to be produced in a much larger large scale in India and subsequently have been introduced into other geographical areas such as Africa [204], (*figure 1.21*).

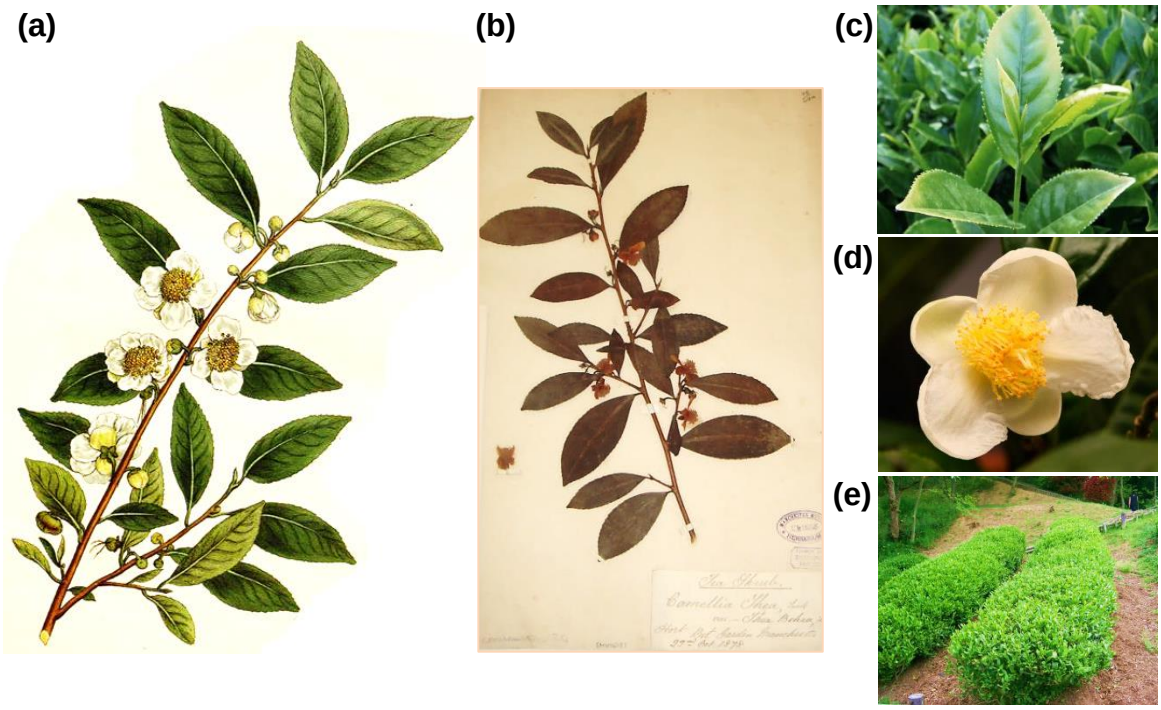


Figure 1.21: Illustrations of tea (a-e).
Tea is a woody evergreen shrub categorised in the family Theacea. This plant can maximally grow to 15-metre heights in wide type. Generally tea height in cultivation for traditional purposes is kept about 0.6-1 metre for convenient harvesting of the tea tip and young leaves [205]. (a) Completed mature branch of tea read for pollination displayed in 17th century books; (b) Dry tea specimen collected in herbarium sheet format from Stephen Welsh (Curator of Living Cultures at Manchester Museum); (c) tea tip with young leaves; (d) tea flower and (e) short tea shrubs. (a) Painting adapted from Lettsom, 1799 [206] and (b-e) images adapted from Jemma, 2015 [207].

The parts of the tea plant used for beverage manufacturing are the leaf buds and young leaves [208]. The popularity of tea has consecutively increased due to taste and aroma [205], so that tea markets have been expanded (*table 1.7*).

Table 1.7: Tea consumption.
The expansion of tea consumption measured in million pound spreading in non-producing countries around mid-19th century over a seven year period.

Countries	1995	1962
UK	464	510
Ireland	26	24
Netherlands	16	19
The Union of Soviet Socialist Republics	--	27
Other Europe	31	59
Total	537	639

Image adapted from Eden, 1965 [209].

Although tea consumption has oscillated in the UK, tea demand is increasing about 10% per year [209]. About 30 year later, the demand for tea beverage worldwide continually increases and this results in multiplying tea production in many countries [210], *(figure 1.22).*

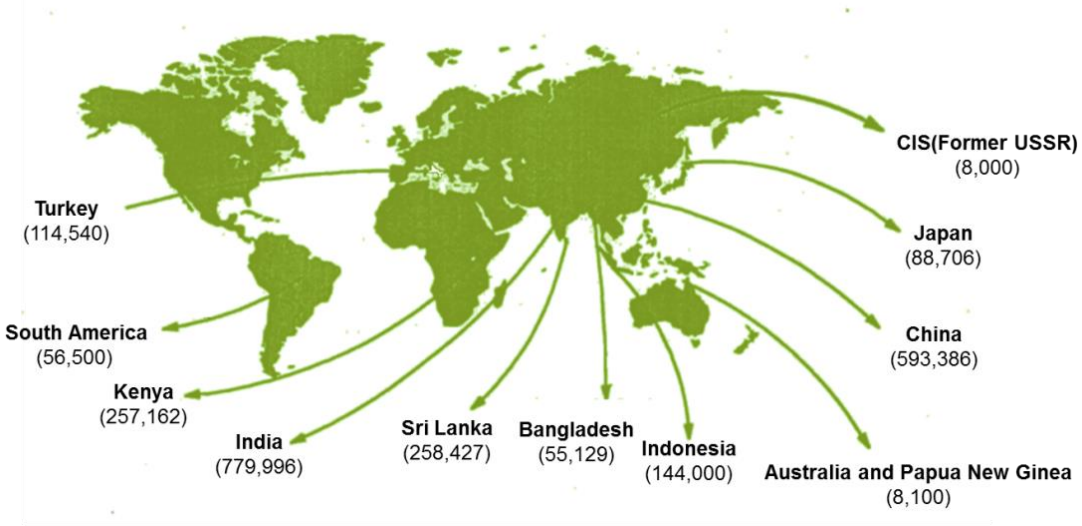


Figure 1.22: Tea production of various areas of cultivation in 1996 was roughly displayed as 2,610,569 metric tons. *Image adapted from Balentine et al 1997 [210]).*

Until 2009, more than 3 billion cups of tea were consumed around the world [211]. And since then tea industrial production has increased every year *(figure 1.23).*

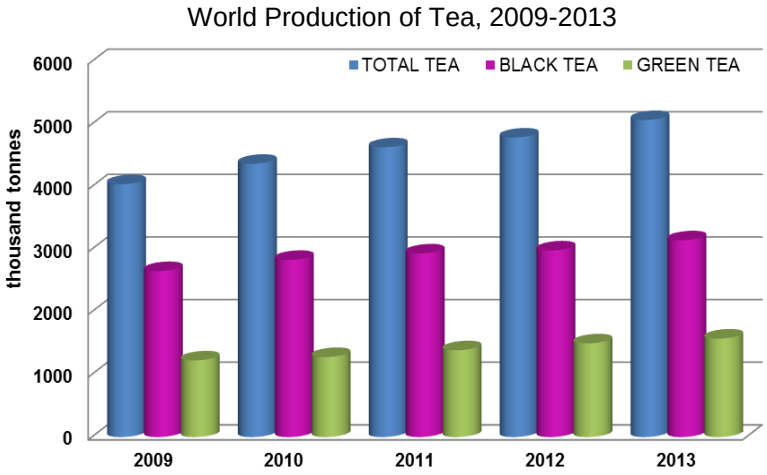


Figure 1.23: Tea production, 2009-2013.
Different processing (mainly fermentation) produces two kinds of tea: unfermented green tea and fermented black tea [208]. Bar graph displays that demand for black tea and green tea is increased among worldwide customers. Hence the sources of tea products can be conveniently purchased. Image adapted from Chang, 2015 [212].

Tea is abundant in polyphenols, which are estimated to be approximately one-third of young leaves' dry weight [208]. Fermentation uses digestion of the tea with, among other enzymes, pholyphenoloxidase, which digests catechins; hence, green tea is catechin-rich whilst black tea is catechin-poor. Green tea is processed by passing through stream just for 15-20 seconds; the temperature is then quickly reduced, hence the green appearance of young leaves is preserved [204, 209].

Polyphenols are secondary metabolites found in diverse plants. Their function is thought to protect the plant against UV and pathogens. There are four subgroups of polyphenol: phenolic acids, flavonoids, stilbenes and lignans [213], (*figure 1.24*).

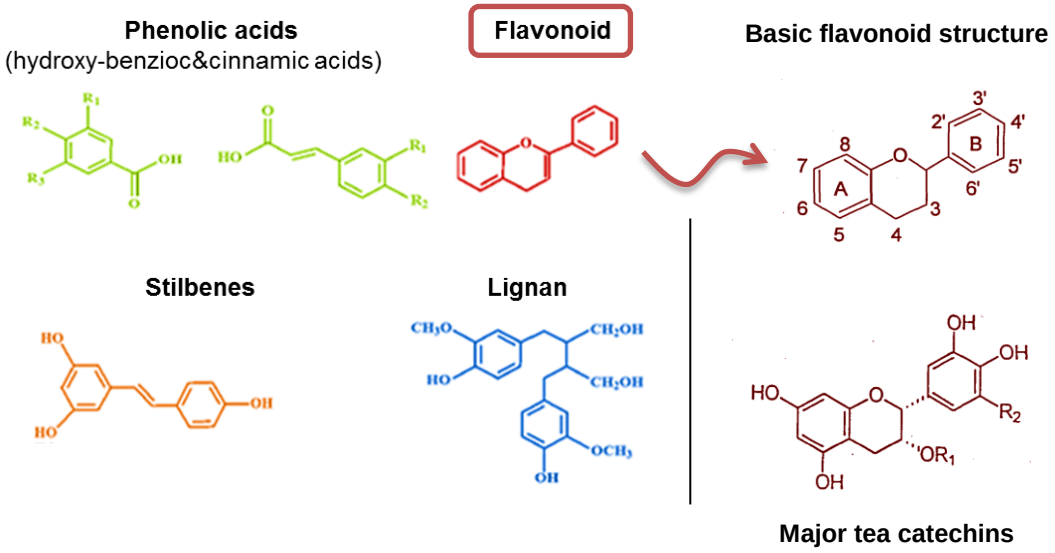


Figure 1.24: Classes of polyphenols.
These are divided in to 4 classes (left side) which are phenolic acids, flavonoids, stilbenes and lignans. The dominant biochemical ingredient in green tea is flavonoids (right side) which has sub-group in polyphenol 'catechins'. Image adapted from Balentine et al 1997 [210] and Pandey and Rizvi 2009 [213].

Flavanols are extensive in green tea, the major sub-group being flavanoid. In fresh green tea leaves these comprise four major catechins: (+)-Catechin, (-)-Epicatechin, (-)-Epicatechin gallate, (+)-Gallocatechin, (-)-Epigallocatechin, and (-)-Epigallocatechin gallate [208, 210], (*table 1.8 and figure 1.25*). Many foods, especially fruits, are rich in molecules which are thought to provide health benefits, such as various vitamins (A, E and C) which have the capacity to protect skin from free radicals caused by UVR exposure [176, 177, 214].

Table 1.8: Chemical elements of fresh green tea leaves measured in % dry weight

(+)-Catechin	1-2	(+)-Gallocatechin	1-3
(-)-Epicatechin	1-3	(-)-Epigallocatechin	3-6
(-)-Epicatechin gallate	3-6	(-)-Epigallocatechin gallate	7-13
Total	**16-30**		

Table adapted from Harler, 1964 [208]

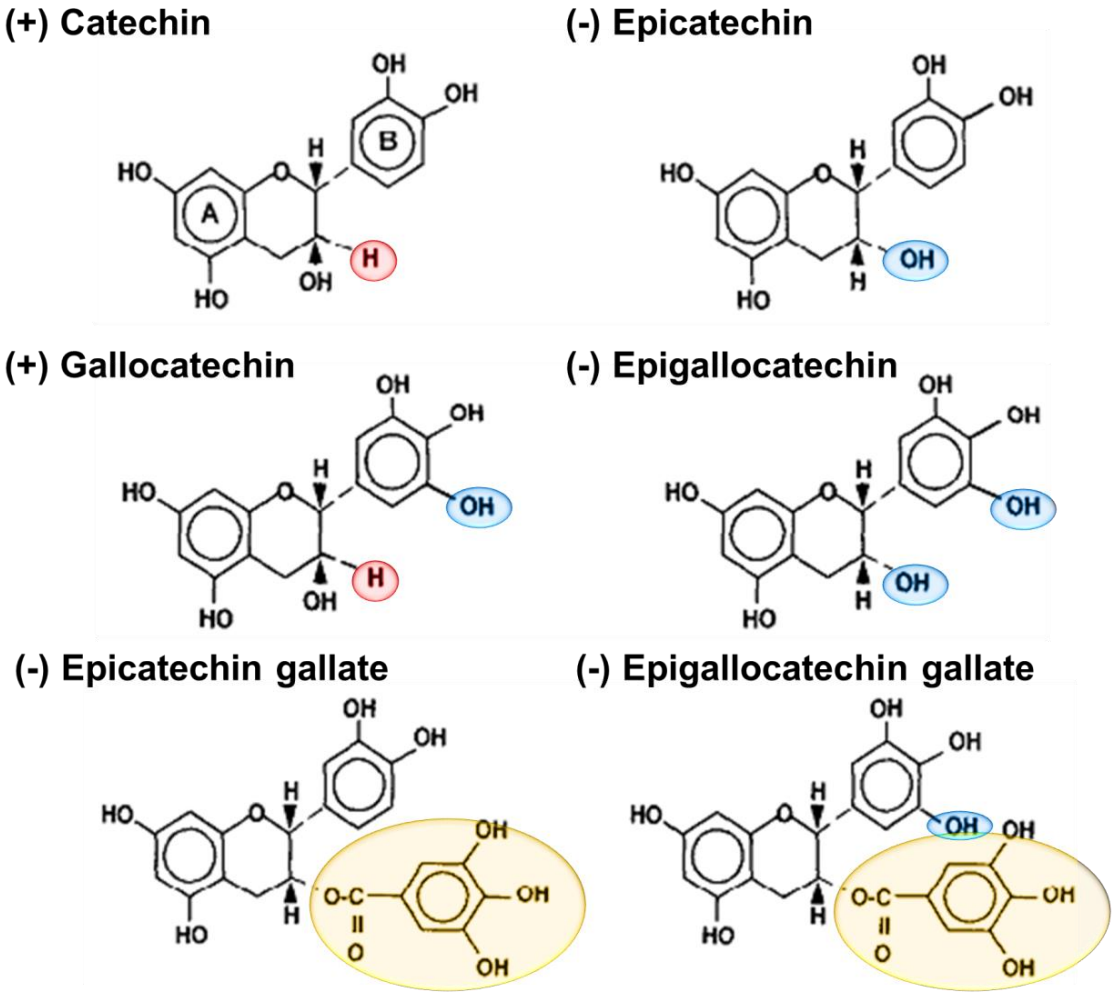


Figure 1.25: The chemical structure of six components in catechin group.
(+)-Catechin and (-)-Epicatechin have the basic structures of the group. The molecular weight of Epicatechin is higher than another because of the hydroxyl group which replaces the single hydrogen molecule (arrow). (-)-Epicatechin gallate and (+)–Gallocatechin have an additional hydroxyl group in the B ring. The –gallate group are appended by a gallic acid group in pyran ring by esterification from Graham, 1992 [215].

1.9.2 Medical advantages of green tea relating to health

Green tea consumption may have abundant medical benefits. Firstly, it contains high concentrations of flavonoids and catechins which act as antioxidants to prevent damages in tissue [216] and cells [217]. Secondly, *in vitro* experiment of green tea catechins indicated that catechins stimulate apotosis in cancer cells to prevent against their expansion, via the suppression of nuclear factor-kappa-β (NF-κβ) expression, a member of the family of transcription factors involved mainly in stress responses, immunity and inflammatory [218]. Furthermore, these compounds oppose tumour development [219]. Some *in vitro* and *in vivo* experiments have shown that green tea polyphenols have potential anti-cancer properties. For example, the effect of GTC treatment have been shown to restrain the growth of lung carcinoma cells in mice [220]. High rate of green tea consumption reduced risk breast cancer and preventive effect in [221].

The main catechin, (-)-Epigallocatechin gallate (EGCG), reduces the ability of cancer cells to attach to each other; EGCG can inhibit carcinoma cells sticking to endothelial basement membrane which is composed of fibronectin and laminin [222-226].

GTCs also appear to suppress the growth of bacteria, and so impacts upon diseases such as diarrhea and influenza [227] . Lastly, it diminishes cholesterol levels in blood. LDL cholesterol oxidation are reduced in *in vitro* studies [228, 229]. The dominant pharmacological property of catechins is the reduction of cholesterol which has been seen experimentally in both rats and human [230-233].

1.9.3 Advantages of green tea specifically to skin health

Presently green tea is not only wide-spread in cuisine and beverage for its aroma and colour, but it also is thought to provide benefits to skin (taken topically or orally). There have been many such experiments reported to provide beneficial results in skin protection in both human and animal models.

In 2001, Kim and team studied a hairless mouse model to see whether EGCG affected collagen and collagenase expression. They topically applied green tea in vivo after UVA radiation and showed that EGCG could protect skin against collagen degradation following multiple dose of UVA exposure 3 times/week in total period of 10 weeks. The mRNA encoding MMP-1 (human collagenase) declined in expression with ECGC treatment; hence collagen in the dermis was preserved. Fibroblasts *in vitro* showed the increasing of fibrillar collagen synthesis after UV radiation; however, when EGCG solution was added in fibroblast culture, the synthesis was reduced [234].

In 2004, Vayalil and colleagues studied the histopathology of SKH-1 hairless mouse skin following chronic UVB exposure. Initially mice were dosed with GTC 10-20 mg per day via drinking water before observation. Normally, mouse epidermis is very thin; following chronic UVB exposure the epidermis becomes hyperplastic (two times as thick as normal skin). Additionally, the size of sebaceous glands increase and the skin contained vacuoles. The skin of mice irradiated but treated with GTC histologically appeared more like that of untreated mice. Hence, GTC appeared to protect skin from chronic UVB exposure. Immunohistochemistry was used to establish whether protein oxidation occurred in skin following UVB irradiation. The result showed that GTC was capable of reducing protein oxidation in skin in those mice which had been treated with GTC and irradiated chronically with UVB.

Oral GTC consumption also reduced the expression of MMPs-2, -3, -7 and -9 the main MMPs which can remodel collagen I and III in the dermis and collagen IV in the basement membrane of the DEJ [235, 236] .

In the same study, Vayalil and co-workers studied the effect of GTP *in vitro* on human skin fibroblast HS68 cells. The cell line was irradiated with UVB at 30 mJ/cm^2. The results showed that GTP acts as an antioxidant to prevent UVB and H_2O_2-mediated damage to proteins. The results showed that higher concentrations of green tea were the more protective than lower doses.

The topical application of green tea (at a concentration of 2.5 mg/cm^2 skin) was studied using an acute irradiation system to assess its effect on oxidative DNA damage in man. Skin was

pretreated topically with green tea 15 minutes before and immediately after UVR exposure (SSR). The results showed that in green tea-treated skin, epidermal Langerhans' cells were preserved and there was reduced nuclear staining for 8-hydroxy-2' -deoxyguanosine (8-OHdG) which is a biomarker of oxidative stress [237, 238]. This study also reported that this effect was not via the green tea cream acting as a sunscreen (measured as sun protection factor 1 only).

In addition, human adult skin fibroblasts (84BR) incubated with green tea at concentration of 250 μM for 1 hr before UV irradiation could be protected from UV-induced DNA damage [175]. In the same study, 10 healthy participants were recruited to provide peripheral blood samples; volunteers who were assigned to drink 3 cups of green tea (2.5 g of pure green tea) continuously after irradiation demonstrated lower levels of DNA damage comparing to those who drank green tea prior to irradiation.

In the same year, a double-blinded study used a combination of topical product (10% w/w) and oral green tea (300 mg green tea supplement) in women (n=40) twice a day for 6 weeks. This study showed a benefit in skin elastic fibre content and clinical signs of photoageing [239]

1.9.4 Role of green tea extract in collagen and elastic fibre protection

In vitro it has been shown that an excess of GTCs (100 times) can protect the structure of extracted collagen I when exposed to an external UVA light source (330 nm wavelength; [240]) compared to untreated collagen. However, collagen contains few UV chormophores [135] and so very high doses of UVR must be given to produce any effect on the structure of this protein; hence, the potential benefits of GTC on protecting collagen remains unclear. In the same year, Madhan et al (2007) reported inhibitory ability of GTCs on the activity of *Clostridium histolyticum* collagenase IA (ChC) by interfering with the enzymes secondary structure at high concentrations. Hence, collagen showed less degradation in the presence of enzyme when incubated with both catechins and EGCG [241]. A year later, the *in vitro* experiments by Bae and colleagues studied the activity of human MMPs derived from human fibroblasts in culture and concluded that GTC impacted on MMP activity by blocking ROS and the JNK, p38 and ERK1/2 signalling cascades, all of which have been implicated in ECM turnover and remodelling in photoageing [242], (*figure 1.26*).

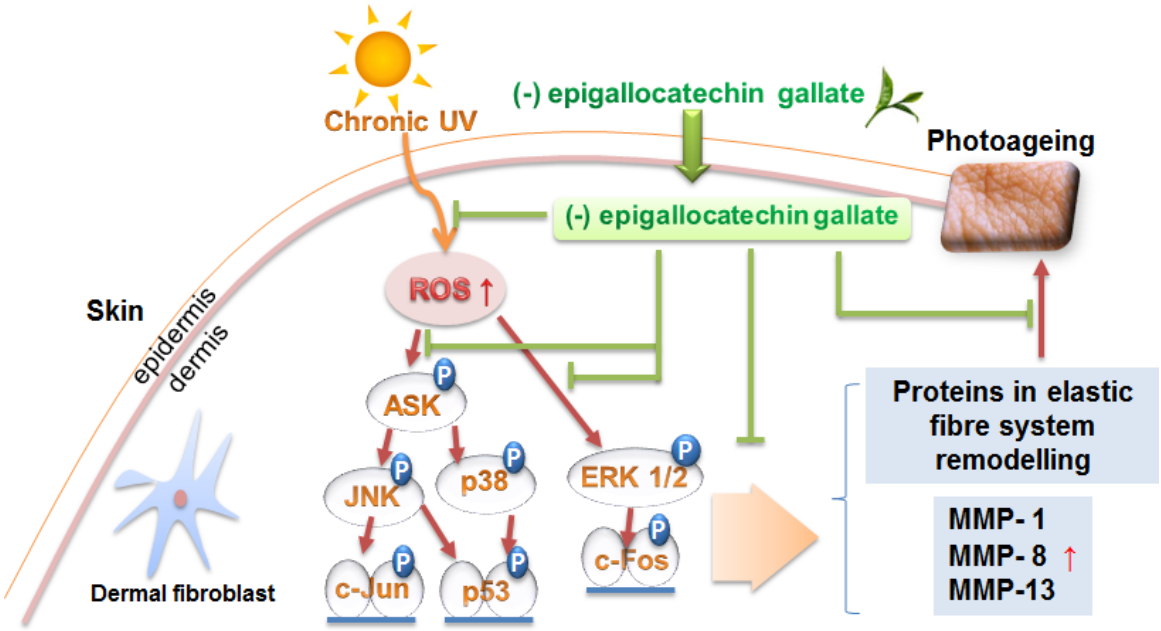


Figure 1.26: Potential model of GTC inhibition of direct and indirect photoageing mechanisms.
Diagram of photoprotective effect from EGCG on UVB-induced ECM damage in dermis. The cascades leading to produce MMPs which have ability to breakdown ECMs are bog down from EGCGs as blocker in between the reaction. ASK-1, apoptosis signal-regulating kinase-1; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinase. Image adapted from Bae et al 2008 [242].

Another mechanism by which GTCs may protect ECM from damage is the reduction of ROS via electron transfer [243], (*figure 1.27*); hydroxyl groups on phenolic rings A and B can perform electrochemical oxidisation. The study in 2002 by Cren-Olivé et al stated that –OH groups in benzene ring B are better electron donors than those in ring A [244], so quenching ROS activity.

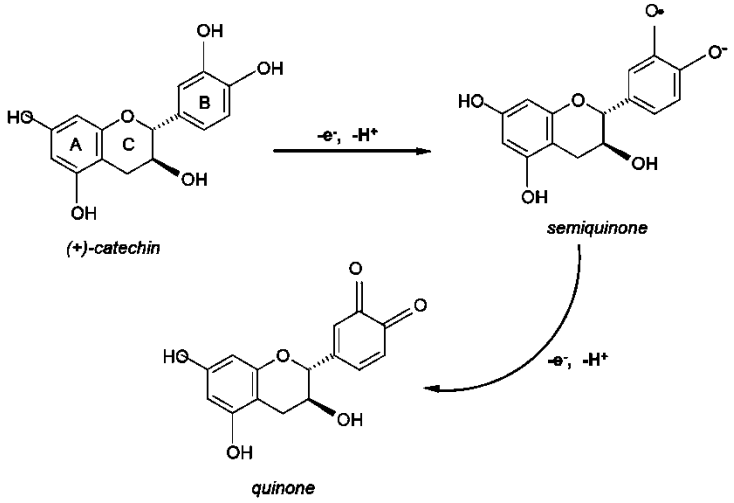


Figure 1.27: The oxidation of catechins.
Two site of pharmacophores: catechol group in benzene ring B and resorcinol group in benzene ring A provide their –OH groups from each ring are independent and unconjugated. Additionally the other hydroxyl group is in ring C specifically in position 3. Image adapted from Janeiro and Oliveira 2004 [243]

1.9.5 Dose usage and toxicity of green tea

Taking large doses of green tea orally may be toxic [245]. Hence an appropriate dose for consumption should be considered before starting any human intervention study. In 2005, Chow et al. stated in their study that 800 mg EGCG oral consumption (comparable to 8–16 cups of green tea) for 4 weeks in healthy volunteers was a safe, well-tolerated dose. The systemic availability of free EGCG was found to be >60% at the end of 4th week [239]. However, in the same year, green tea extract mixed in vehicle cream applied topically on skin was reported to induce some level of irritation [246], with some individuals reporting sun sensitivity relating to green tea topical usage. Recently, Of the 25 participants in the GTC arm of the trial described feelings of nausea when taking this high dose supplement; nausea was also reported in one individual from the placebo arm [247].

1.10 Summary and hypotheses

In these studies we wished to assess whether consumption of green tea could provide some systemic benefit to skin when challenged with a robust dose of UVR. Initial studies compared the ability of solar simulated radiation with a UVB light source to produce changes to the elastic fibre network, as seen in chronically photoaged skin (chapter 3). The findings of this study were used to perform a gold-standard randomised control study to establish whether a dietary intervention rich in green tea catechins could protect skin from damage caused by acute irradiation; the elastic matrix (chapter 4) and collagen matrix (chapter 5) were considered. Result chapters are presented as stand-alone scientific papers for publication. Hence the hypotheses which directed this work were:

1.11 Hypotheses:

1) Acute irradiation of skin with UVR will damage the dermal ECM and mimic the changes observed in photoageing (addressed in Chapter 3);
2) Dietary supplementation with GTC will provide protection against UVR-induced ECM damage (addressed in Chapters 4 and 5).

Chapter 2 - Materials and methods

2.1 Research ethics

All studies described in this thesis were conducted according to the principles of the Declaration of Helsinki (as revised 2013) and were approved by NHS research ethics committees. These were as follows:

(A) 'Identifying the network of lipid mediators responsible for maintenance and resolution of ultraviolet radiation–induced skin inflammation', Greater Manchester North Research Ethics Committee, ref: 11/NW/0567;

(B) 'The effect of dietary bioactive compound on skin health *in vivo*" North Manchester Research Ethics Committee, ref: 08/H1006/79.

2.2 Ultraviolet radiation sources

2.2.1 Broadband UVB

Waldmann UV6 bulbs were used to irradiate with broadband UVB (emission 280–360nm, *figure 2.1a,b*; Herbert Waldmann, GmbH & Co., Villingen-Schwenningen, Germany) in a partial body UV therapy tool (Waldmann UV236B, Herbert Waldmann, GmbH & Co.). The output (irradiance) of the lamp was measured continuously during irradiation procedures using an integrated radiometer, and the lamp switched off when the required dose was reached.

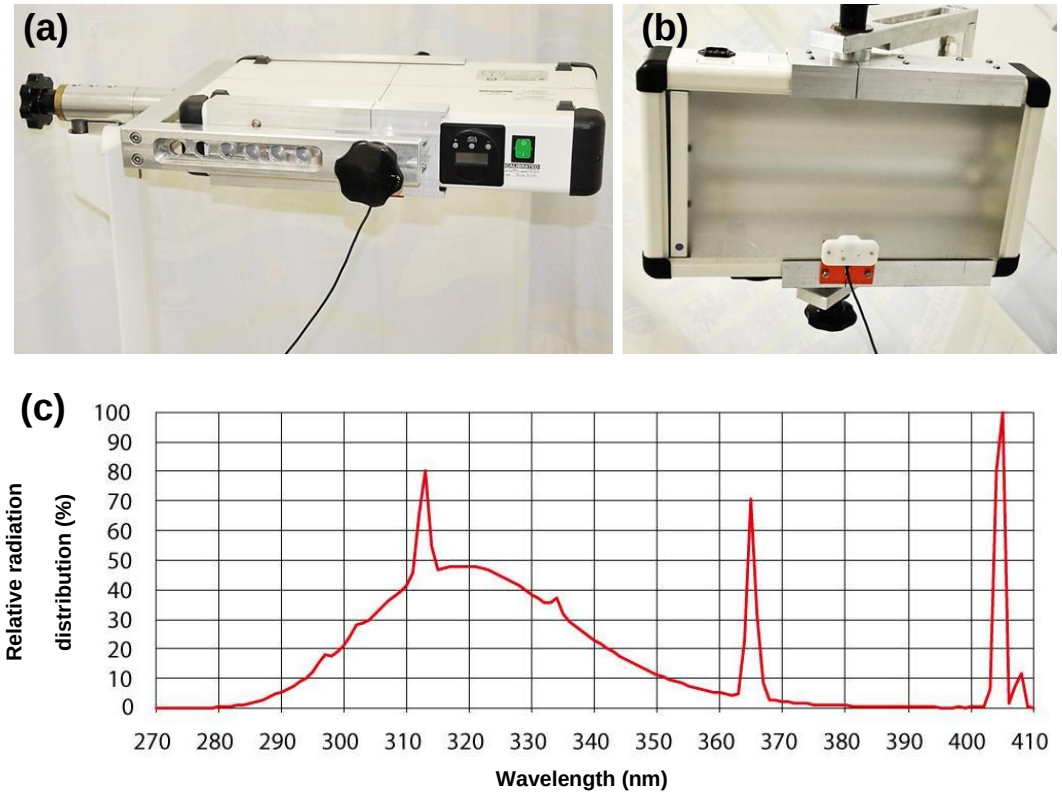


Figure 2.1: UVB irradiation equipment.
(a), (b) Waldmann UV6 bulbs were used as the UVB broadband source, installed under acrylic glass. (c) A typical UVB (UV6) lamp emission spectrum. Image adapted from http://www.waldmann.com/.

2.2.2 Solar-simulated radiation (SSR)

A solar simulator (*figure 2.2a*; Newport Spectra-Physics Ltd; Didcot, UK) was used to provoke skin erythema using a UVR spectrum approximate to that of natural sunlight (3.35% UVB combined with 96.68% UVA; wavelength range 290-400 nm). This machine was composed of light shutter power supply, a light shutter, collimating optics, an arc lamp arouser and an arc lamp (1kW xenon). A dichroic mirror eliminated unnecessary light signals (infrared and the majority of visible; *figure 2.2c*). Prior to experimentation, the SSR output was measured using a radiometer [247] (*figure 2.2b*; Waldmann UVR meter; Herbert Waldmann, GmbH & Co.). The SS was switched on and allowed to acclimatise for 15 minutes. The output was measured by radiometer at the middle of solar beam and approximately 9.5cm away from the light source; the stabilised reading was then noted. This value of SSR irradiance was used to calculate the exposure time needed to deliver the required dose.

2.3 Minimal erythema dose (MED) testing

2.3.1 Irradiation procedure

Lamp irradiance was measured in milliWatts (mW) per unit area (cm^2). The exposure time needed to deliver a defined dose (measured in mJ/cm^2) was calculated using the following formula:

$$\textbf{Exposure time} \text{ (seconds)} = \frac{\textbf{UVR dose } (mJ/cm^2)}{\textbf{Irradiance } (mW/cm^2)}$$

According to the equation, if irradiance was fixed at a stable point, the highest UVR dose required the longest time for irradiation, whist the lowest MED needed less. Hence this rule was applicable with the irradiation process to produce skin erythema.

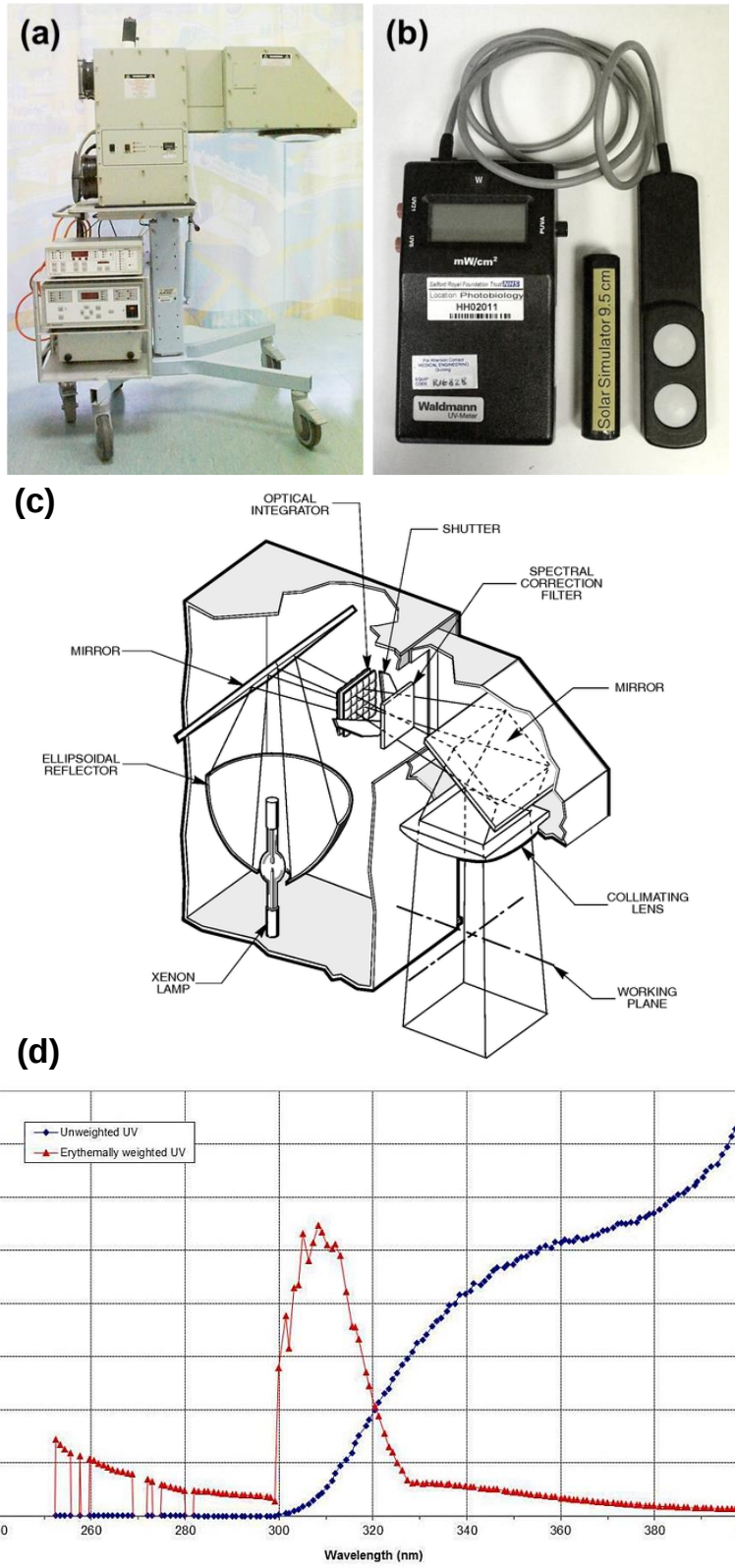


Figure 2.2: The solar simulator.
The Newport SS was used to imitate natural sunlight (a). The UVR output was measured using a radiometer (b). The basic elements of solar simulator shown in (c). Image (c) taken from http://www.newport.com/. (d) Sample SSR emission spectrum. The graph plotted from the actual measured output of SS shows the total irradiance in blue and the erythemally-weighted irradiance in red, which is the output just for those wavelengths that cause erythema.(provided by Dr Donald Allan, Medical Physics Department, Salford Royal Hospital, Salford, UK).

In these experiments, each volunteer received 3x their individual MED. Calibrated UVR sources can cause skin inflammation within the calculated period. For SSR, an individual's MED was determined by applying a geometric series of erythemally-weighted UVR doses specifically to SSR as shown in the box below [247]:

7, 9, 12, 16, 20, 28, 36, 48, 64, 80 mJ/cm^2

A thin flat rubber material (Medical Physics department, Salford Royal NHS Foundation Trust, Manchester, UK) which has 10x10mm diameter punctured holes was used as a template for the dose series (*figure 2.3a*). This material would be set down on buttock skin and fixed with a latex-free, microporous surgical tape (3M Micropore^{TM}; Healthcare Ltd; Bracknell, UK). At the beginning, all holes were opened for UV irradiation. When the smallest dose (7mJ/cm^2) was completed, that particular hole would be closed by small square piece of rubber fitted to cover the hole (*figure 2.3b*). The next holes for higher doses would be closed until the last dose was applied.

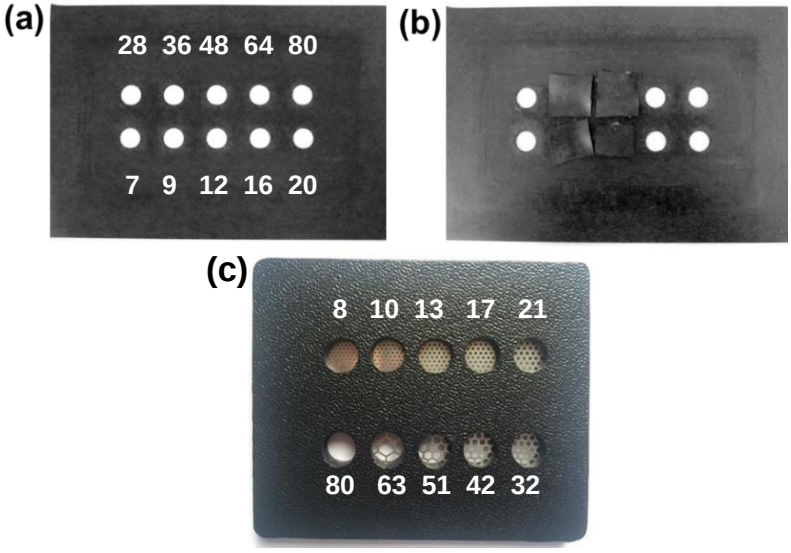


Figure 2.3: MED template of SSR and UVB.
(a-b) The rubber template was used to deliver the 10 doses of UVR to defined areas for MED testing. (c) The hard black plastic phototesting template was applicable for UV6 bulbs that generate MED from UVB. This tool presents the grids which delivers light intensity for 10 doses as labelled.

To produce MED by broadband UVB, an MED template is used that has grids to allow delivery of different doses. The series for this is replaced with data shown in this beneath box:

8, 10, 13, 17, 21, 32, 42, 51, 63, 80 mJ/cm^2

In addition, these dose series have the identical sorting on rubber template as shown in *figure 2.3c.*

2.3.2 MED assessment

UVR-induced skin erythema is caused by local vasodilatation and increased blood flow in the skin, peaking at around 24 hr post-exposure [248]. The MED is defined as the UVR dose (mJ/cm^2) that produces just perceptible skin reddening [249], detected 24 hr after irradiation [123]. Skin erythema can be measured visually [249, 250] or by using specific tools, such as a laser Doppler flowmeter, a spectrophotometer, a two-channel erythema meter or a Minolta chromameter [251].

In this study, MED evaluation task was completed by means of visual observation (subjective measurement), which was the easiest method and can be done without specific or expensive equipment, and by using an objective measure, the Dia-Stron erythema meter (Dia-Stron Ltd, Andover, UK) which is permanent equipment in the laboratory as shown in *figure 2.4*.

Twenty-four hours after irradiation, all subjects would return to the clinic and their personal MEDs determined. Dia-Stron erythema meter measurement was performed to read the degree of erythema, reported as an erythema index (EI). The mechanism of this meter uses the reflectance principle [252]. Using a tungsten halogen lamp emitting through fibre optic bundle both UVR irradiated and adjacent non-irradiated controlled skin was measured. By using narrow pass interference filters, the meter measures green (wavelength, λ 546nm) and red (λ 632nm). The red channel is set as a constant, whereas the green channel is flexible and alters depending on the haemoglobin content of the skin.

The EI was calculated according to ratio of red and green light absorption as formula below:

$$\textbf{Erythema Index (EI)} = \log_{10}\left(\frac{\text{reflected red light}}{\text{reflected green light}}\right)$$

All participants were asked to lie down for 15 minutes on the hospital bed to allow their body to adjust to the room temperature (approximately 25˚C). Then EI was measured at each UVR dose site along with two non-irradiated adjacent sides as controls. The EI at irradiated skin and control were read, recorded and repeated 3 times to find the average. After that the mean from control area would be deducted from the mean from each exposure site to obtain the change in erythema value (ΔE).

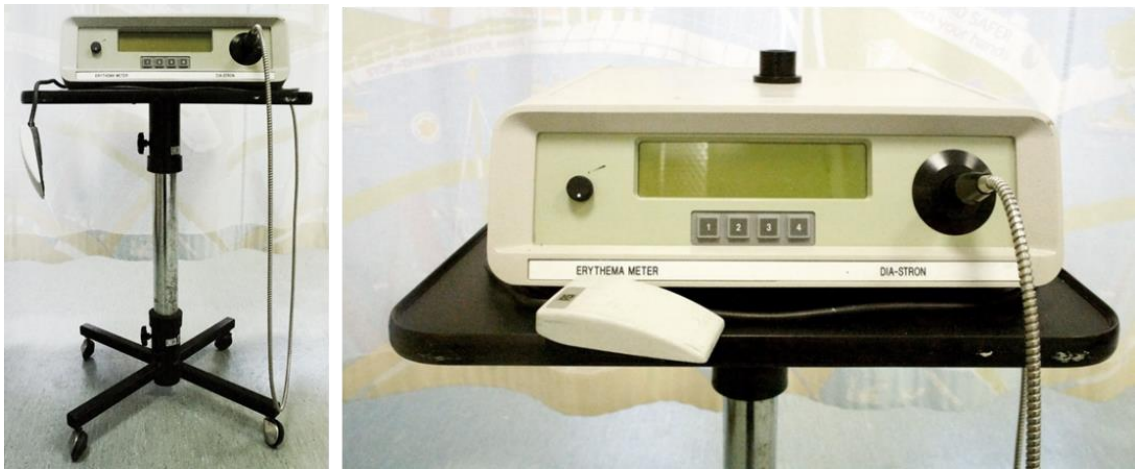


Figure 2.4: Measurement of erythema.
the Dia-stron erythema meter is based on the reflectance principle [252] and was used to objectively estimate skin redness as an erythema index (EI), 24 hr after UVR exposure. This meter measures haemoglobin (Hb) which is one type of chromophores in dermal skin and their quantity can be altered due to skin conditions, such as inflammation, oedema, and burn (from Dolotov et al. 2004 [253]).

2.4 Clinical studies

2.4.1 Effect of UVB and SSR on the dermal matrix

Samples of unexposed, UVB-exposed and SSR-exposed (both 3x MED) skin were obtained from baseline biopsies taken as part of the studies described in Section 2.1. Study A was an investigation into the role of bioactive lipids in UVR-induced skin inflammation. Volunteers were healthy white Caucasian adults recruited by open advertisement. Exclusion criteria for this study were the participants who were treated with phototherapy including using sunbed or sunbathing for 3 months prior to the study start, had skin cancer in health history, were pregnant or were taking medication that was photoactive or anti-inflammatory.

Study B is described in section 2.4.2. Samples from 6 volunteers from each study (age, sex and skin type matched) were selected. Dermal elastic fibres and three related proteins: fibrillin-1, fibulins -2 and -5 in buttock skins would be histologically analysed in order to compare the impact between 3x MED of UVB and SSR on those matrices.

2.4.2 Randomised controlled trial (RCT) of dietary GTC

We performed a randomised, controlled trial to assess the potential protective effects of GTC on the dermal ECM and its remodelling in human skin, following an acute inflammatory dose of SSR. Healthy white Caucasian volunteers (n=50; age range 18-65 years old) were recruited into the study from public advertisement. All were Fitzpatrick sun-reactive skin types I-II [17]; Exclusion criteria were: history of skin cancer, skin photosensitivity disorders; regular black or green tea drinkers (more than 2 cups of tea/day); UVR exposure from either natural sunlight (sunbathing) or artificial sunbeds in the previous 3 months; pregnant or breastfeeding women. Subjects were randomised into active or control treatment groups (n=25 each) by block randomization method (StatsDirect v2.7.8, StatsDirect Ltd.). In the active group, volunteers were directed to consume a high dose of GTC (1080 mg per day; equal to 5 cups of green tea) together with a low dose of ascorbic acid (vitamin C,100 mg per day; to help GTC stabilisation in the intestine [254] but has been shown to have no effect on either skin erythemal responses [184] or the level of oxidative damage [255]), whilst those in the control group consumed control capsules contained maltodextrin powder. The chemical components in each capsule of green tea extract provided to the active group are described in *table 2.1*. All subjects were advised to take three capsules twice daily with morning and evening meals. Both active and placebo treatments were supplied in identical capsules by Nestlé Limited (Vevey, Switzerland) and manufactured in similar packages by Laboratoire LPH (*figure 2.5)*.

Table 2.1: List of biochemical compounds in green tea supplement provided to subjects in active group

Green tea extract ingredients		Amount (mg in 450 mg capsule) mean + SD
1	Gallic acid	0.4 + 0.0
2	Catechin	2.1 + 0.0
3	Epicatechin	12.5 + 0.2
4	Gallocatechin	12.4 + 0.6
5	Epigallocatechin	49.3 + 3.9
6	Catechin gallate	0.3 + 0.0
7	Epicatechin gallate	26.0 + 0.2
8	Gallocatechin gallate	4.5 + 0.4
9	Epigallocatechin gallate	72.6 + 3.1
total		180.0 + 8.3

Table adapted from previous publications Rhodes et al 2013, Darby et al, 2014 and Farrar et al 2015 [247, 256, 257].

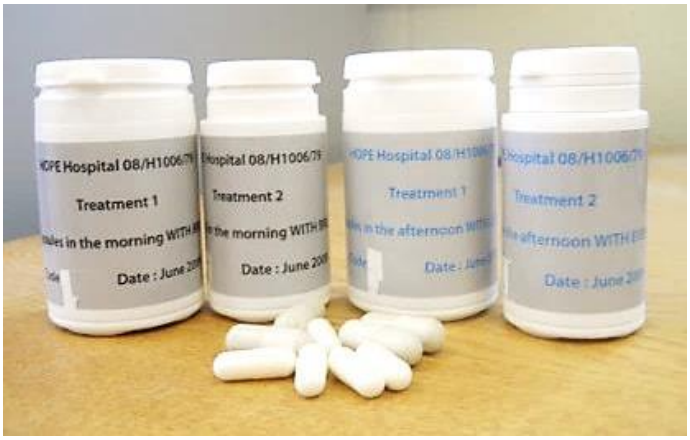


Figure 2.5: Dietary supplements.
Green tea extract (active compound), ascorbic acid (catechin stabilizer in gut lumen) and maltodextrin powder (placebo) were enclosed in identical white capsules.

2.4.3 Timeline of study and study intervention

Volunteers took the dietary intervention for 12 weeks. Prior to beginning the supplementation, a baseline urine sample was taken and the MED for each volunteer was established. Skin biopsies (from unirradiated and irradiated skin sites) were taken at the beginning and the end of the study period, whilst compliance was assessed by assay of green tea metabolites in urine at the beginning, middle and end of the supplementation period. The study timeline is displayed in *figure 2.6*.

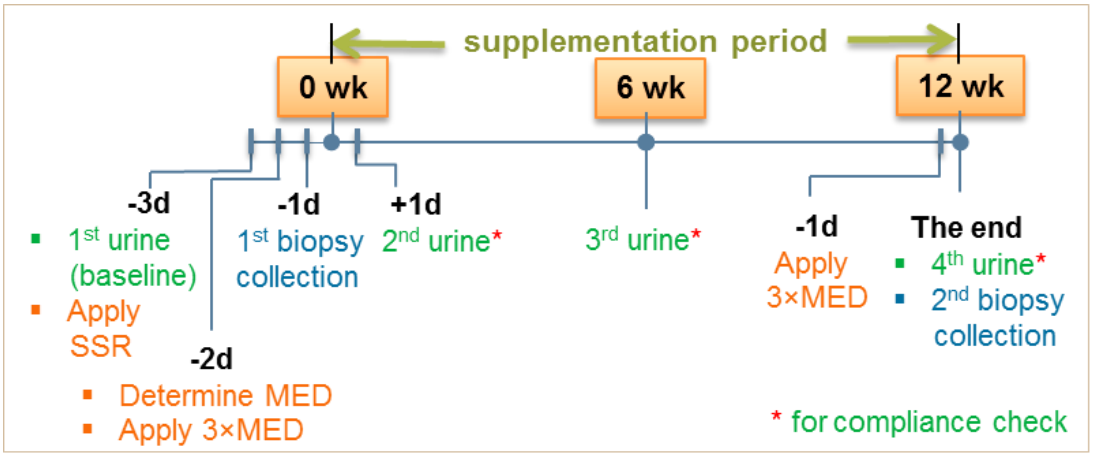


Figure 2.6: Experimental timeline and study intervention.
At 3 days prior to the supplementation period, the minimal erythemal dose (MED) of subjects were individually determined by applying a geometric series of SSR doses to photoprotected buttock skin. After 24 hr, the MED was read, subsequently allowing each volunteer to be further irradiated with 3x MED SSR. Twenty-four hr after this experimental irradiation, each volunteer provided 5mm diameter punch biopsies from both non-SSR exposed and 3x MED exposed buttock skin. Irradiation and tissue sampling was performed again at the end of supplementation period. To ensure compliance with the intervention, urinary analysis for GTC and their metabolites were performed at baseline and 24 hr after commencement of the intervention. This was repeated at 6 weeks and at the end of the study (12 weeks).

2.5 Skin biopsy and urinary sampling

2.5.1 Skin biopsy sampling

A day prior to supplementation started, skin biopsies were collected. Two sides of buttock were zoned for tissue sampling into UV and non-UV sides (*figure 2.7a*). An acute dose of 3x individual's MED of SSR was applied on an area of upper buttock skin, defined by a thin rubber template (*Figure 2.7b*), to stimulate an erythemal response in all subjects for 24 hr prior to skin sampling. On the following day, 5mm diameter punch biopsies were taken: firstly the biopsy area was cleaned by wiping with a 70% ethanol wipe and allowed to air dry. Local anaesthetic infiltration was carried out by subcutaneous injection of lignocaine, and when numb, a punch biopsy cutter was used to sample through to the subcutaneous fat, (*figure 2.7c*) which was removed (*figure 2.7d*).

Finally the tissue was embedded in optimal cutting temperature compound (OCT; Fisher Chemicals; Loughborough, United Kingdom), snap-frozen in liquid nitrogen and transferred to -80°C freezer prior to preparation of tissue sections [121]. This protocol was utilized in either pre-supplement or post-supplement side of buttocks and would be repeated at a day before ending of supplementation intake following the study timeline as shown in *figure 2.6.*

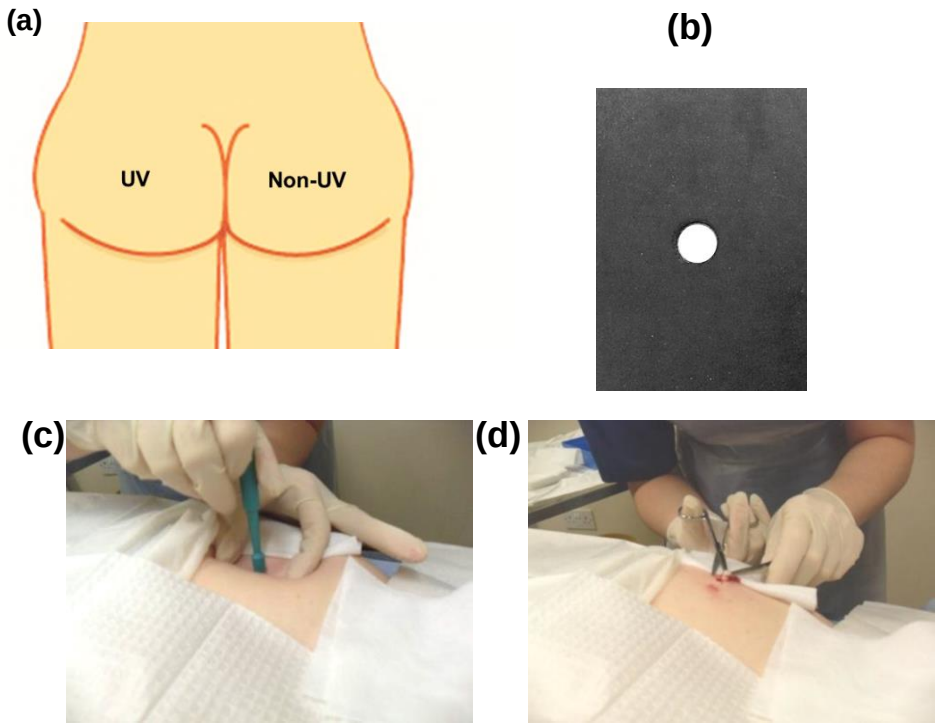


Figure 2.7: Procedure of biopsy sampling.
Begining with allocating two sides of buttocks to be one side for UV protection and other side for irradiation (a). Both sides were assigned to be collected 5 mm-diameter of circle biopsies with 5 mm-depth. The rectangular rubber template was applied to limit the UVR exposure area (b). The sterile punch (c) and sterile surgical scissors (d) were key equipment for skin penetration and biopsy trimming (Image c – d taken by Darby, 2014 [257]).

2.5.2 Urinary sampling

To ensure compliance with the intervention, urinary analysis for GTC and urinary analysis was performed at baseline and 24 hr after commencement of the intervention. This was repeated at 6 weeks and at the end of the study (12 weeks). Totally all subjects were assigned to provide urinary sample for 4 times within the experimental period. Each urinal container (Urisafe Polypropylene 24 hr Urine Collection Containers size 3 litre; VWR International Ltd; Lutterworth, UK) contained 3g acidified ascorbate (Sigma; Poole, Dorset, UK) to maintain the excreted GTC metabolites and was processed by collaborators at the University of Leeds.

2.6 Slide preparation and staining

The tissues were cryosectioned at a nominal thickness of 7µm (OTF cryostat; Bright Instruments Ltd; Cambridge, UK) and arranged as 3 tissue sections per slide.

2.6.1 Picrosirius Red staining (PSR) for fibrillar collagens

Sections were air-dried at room temperature 5 minutes prior to staining and fixed in 90% industrial methylated spirit (IMS; 4x 2 minutes). To stain the collagen, slides were briefly immersed in 0.1% (w/v) of Sirius red F3BA in saturated aqueous picric acid (Direct red 80) dissolved in saturated aqueous picric acid (10 seconds). Excess staining solution was removed off the slides before a brief rinse in 0.1% acetic acid (slides dipped in and out of solution twice). The aqueous solution helped to remove excess stain from the tissue sections. Slides were then dehydrated through serial alcohols (immersion in 70% (v/v) IMS, subsequently 90% (v/v) IMS, 100% (v/v) IMS) and cleared in xylene (5 minutes each step). Finally all slides were permanently mounted using DePex (Fisher Chemicals) as mounting media.

2.6.2 Weigert's Resorcin Fuchsin staining (WRF) for elastic fibres

Frozen tissue sections were air-dried at room temperature for 5 minutes, prior to fixation in 4% (w/v) paraformaldehyde (PFA)/phosphate-buffered saline (PBS) solution for 10 minutes. Slides were then immersed for 5 minutes in Tris-buffered saline (TBS; 20mM Tris and 150mM NaCl) to rehydrate tissue before placing into Weigert's haematoxylin working solution (Millipore; Darmstadt, Germany) to stain nuclei. Following brief washes in IMS, slides are submerged in Weigert's Resorcin Fuchsin solution (ClinTech Limited; Guildford, UK) for 90 minutes. Slides were rinsed in IMS, then dH_2O before counterstaining slide with Van Gieson's solution (Clin-Tech Limited). Following a brief rinse in dH_2O, tissue sections were dehydrated and mounted following *2.6.1*.

2.6.3 Immunohistochemistry (IHC)

Immunohistochemistry was used to identify specific components of the collagenous and elastic ECMs. The specific details for each primary antibody used in these experiments are shown in *table 2.2*. All of them were processed using the same basic protocol. In brief, frozen tissue sections were air-dried at room temperature for 5-10 minutes. Sections were fixed by (please refer to *table 2.2* for specifics) for 10 minutes to retain cellular and subcellular structure [258] before hydrating in TBS. Sections were then exposed for 10 minutes to 0.5%(v/v) Triton X-100, a detergent which has capability to dissolve lipid in cell, nuclear and organellar membranes, thus allowing antibodies to access antigens in the cytoplasm and nucleus. Following washing, endogenous peroxidase activity was abolished by incubating sections with H_2O_2 (in methanol). A broad blocking solution was then applied to limit non-specific antibody binding (2%(v/v) normal serum, 1%(w/v) bovine serum albumin (BSA; stabilizer), 0.1%(v/v) cold fish skin gelatine (blocking), 0.1%(v/v) Triton X-100 (penetration enhancer), 0.05%(v/v) Tween 20 (detergent and surface tension reducer) and 0.05%(w/v) sodium azide ; Vector Labs; Peterborough, UK) (*table 2.2*). Primary antibody diluted in blocking solution was applied overnight at 4°C. Negative controls were run alongside every experiments, with primary antibody replaced with blocking solution The next day, slides were washed in TBS (2x3 minutes) and appropriate secondary biotinylated antibody was applied (Vector *Elite* ABC; Vector Labs) for 30 minutes at the room temperature. Following washing in TBS, sections were incubated for 30 minutes with VECTASTAIN® *Elite*® ABC reagent (Vector Labs). Then chromogen (peroxidase substrate solution; Vector SG®; Vector Labs) was applied to sections for about 90 second or until staining appears explicitly. The chromogenic reaction was stopped by immersing slides in TBS (2x 3 minutes). Slides were counterstained with nuclear fast red (Vector Labs) for 5 minutes and washed in dH_2O until clear. Finally, slides were dehydrated and permanently mounted in DePeX (Fisher Chemicals) as described in *2.6.1*.

Table 2.2: Antibody details.
Summary detail of specific primary antibodies to ECM proteins, fixative methods, chemical concentration, epitope specification, clonality, suitable dilution and providers in the study

Immunogen	Clone	Fixative	Peroxidase (v/v)	Epitope	Dil^{n}	Source
Rat monoclonal anti-human procollagen I	MAB 1912	cold acetone (-20°C)	0.6%	amino pro-peptide α-1(I) chain	1: 200	Millipore Darmstadt, Germany
Mouse monoclonal anti-human fibrillin-rich microfibril	11C1.3	4% PFA	0.6%	aa 451 - 909	1:1000	NeoMarkers CA, USA
Rabbit polyclonal anti-human fibulin-2	HPA001934	4% PFA	3%	aa 739 - 861	1:1000	Sigma Prestige MO, USA
Rabbit polyclonal anti-human fibulin-5	HPA000848	4% PFA	3%	aa 268 - 414	1:180	Sigma Prestige MO, USA

2.7 Image capture

2.7.1 Photomicrograph recording procedure for PSR

The positively stained fibrillar collagen in tissue sections from all experimental treatments (baseline and following irradiation, both pre- and post- supplementation) were observed and photographed using bright field and polarised field microscopy. This allows the quantification of collagen fibres by image analysis [259, 260], (*figure 2.8)*.

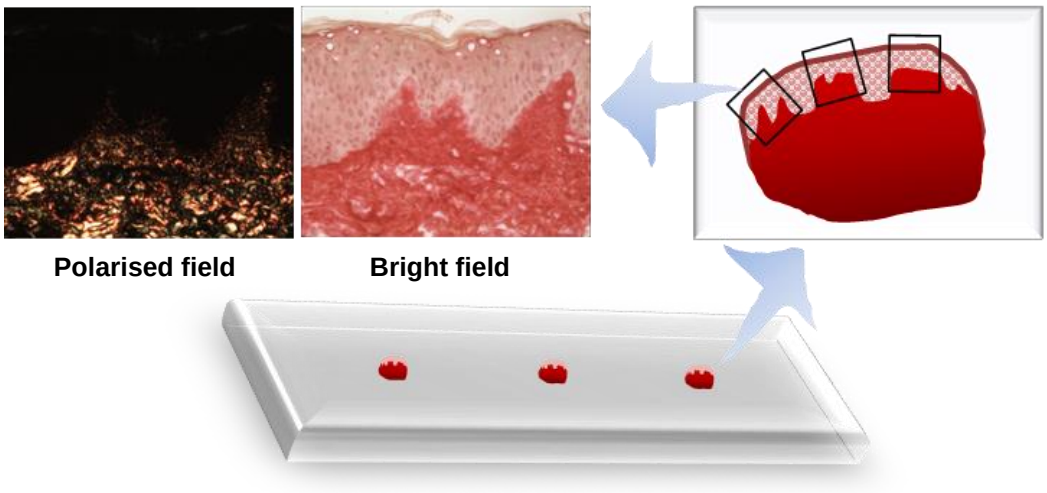


Figure 2.8: Image capture of PSR-stained skin sections.
Three non-overlapping fields of view were captured using both polarised and bright field microscopy at a magnification of x20. Resultant micrographs were subsequently subjected to image analysis using ImageJ (NIH, Bethesda, MA, USA) and the data used to calculate the fibrillary collagen percentage area per treatments.

2.7.2 Image analysis - PSR

Image analysis was used to quantify the percentage of fibrillar collagen (analysed from the polarised field image) in the stained tissue sections (analysed from bright field image; ImageJ; National Institutes of Health, MA, USA). Three independent areas were measured per slide, so allowing the calculation of the average amount of fibrillar collagen in each treatment.

Primarily the target area in bright field microphotographs was scoped by creating the boundary of upper papillary dermis with 100µm depth along DEJ margin. Next, non-assessment area outside the border was eliminated and replaced with a white background (*figure 2.9, a-b*). Similarly, polarised field micrographs which were taken from the same tissue section position as the bright field image were processed in the same manner (black used to screen out areas not for quantification; *figure 2.9, c-d*).

Polarised field images allow identification of collagenous structures, providing images of red, orange, yellow and green. Red represents mature or thick collagenous fibrils [259, 260], whereas thin collagen fibres and fibrin (a non-collagenous protein; [261] may also be seen as green under polarised light. To reduce noise, the images were separated into 3 separate colour channels (red, green and blue); the red channel was chosen to analyse data, the program automatically subtracting green and blue signals. In a similar way, the bright field image was also separated into 3 channels but for this imaging, the blue channel was chosen as it represents the area that fibrillar collagens were located in. Images were thresholded to maximise accuracy with the program evaluating both the area covered by fibrillar collagen as a function of the pixel areas. These two values would be calculated in following formula:

$$\textbf{fibrillar collagen percentage area } (\%) = \frac{\text{fibrillar collagen area in pixel}}{\text{collagen habitat area in pixel}}\ x100$$

The depth to which the images could be analysed may also be varied using this method (i.e. 100 µm versus 50 µm).

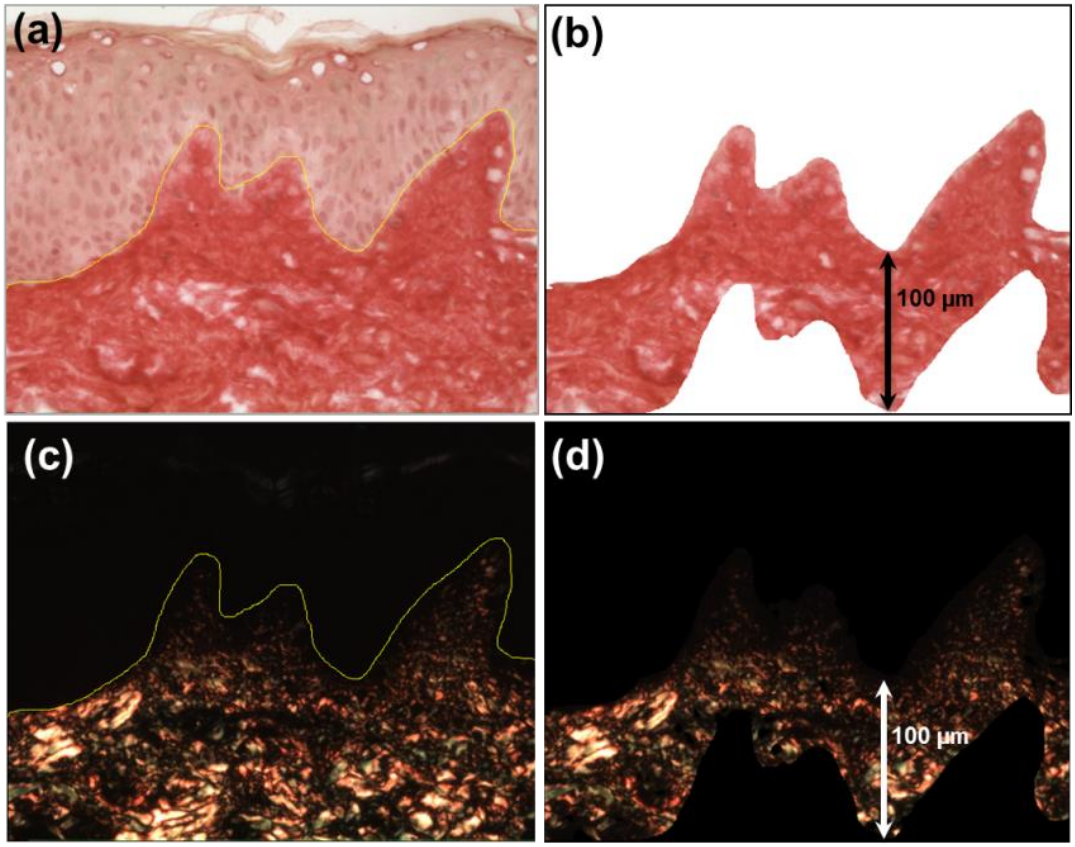


Figure 2.9: Image analysis of PSR-stained tissue sections.
The original bright field micrograph with drawing line (yellow line) along the DEJ (a). The lower dark red zone represents the collagenous area whereas the upper pale red presents non-collagenous or non-dermal area that must be excluded before threshold adjustment. Ultimately the target area in papillary dermis covering 100µm depth from DEJ was prepared for analysis (b). The polarised field micrograph were scoped the area with the same boundary as in bright field (c) and then the non-related area was deleted (d).

2.7.3 Photomicrograph recording procedure for WRF and IHC stains

The positively stained elastic fibres and specific proteins, namely, fibrillin rich microfibril, fibulin-2 and -5 in tissue sections from all experimental treatments (baseline and following irradiation, both pre- and post-supplementation) were observed and photographed using only bright field (*figure 2.10)*. This allows the quantification of these components by image analysis following the identical procedures in *2.6.4* .

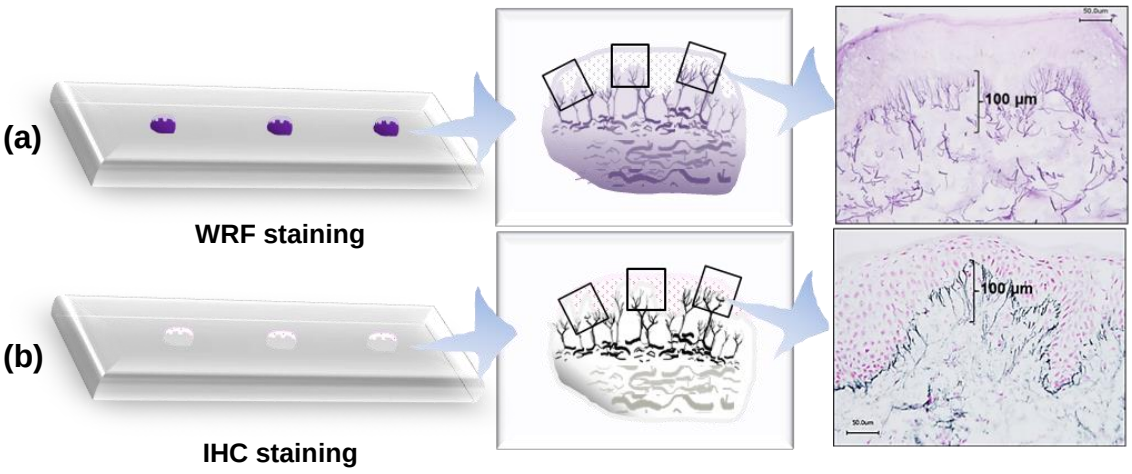


Figure 2.10: Image capture of WG (a) and IHC (b)-stained skin sections.
Three non-overlapping fields of view were captured using bright field microscopy at a magnification of x20 with scale bar 50µm. Resultant micrographs were subsequently subjected to image analysis using ImageJ (NIH) and the data used to calculate the fibrillar in each protein percentage area per treatments.

2.7.4 Image analysis – WRF and immunohistology

Image analysis was also used to quantify the occurrence of microfibrillar elements in the papillary dermis (ImageJ, National Institutes of Health). Fibres were quantified in pixels and calculated as a percentage of the papillary dermal area of each figure, in the region directly below the DEJ boundary with the epidermis (as show in the *figure 2.11*). The original bright field image (a) was separated into 3 colour channels (red, green and blue) which were subsequently displayed in black and white. The red and green channels were chosen to analyse data as the positively-stained fibre area and all dermal area respectively. In the lower zone of DEJ represents the elastic fibre area whereas the upper one presents non-elastic fibre or non-tissue area that must be excluded before threshold adjustment.

Finally, the ratio between elastic fibre area (c) and total area (b) was expressed as a percentage of tissue cover. All tissue sections were photographed 3 times (non-overlapping fields of view) to produce a mean. All analytical procedures were also applicable for the assessment of immunohistochemical staining of fibrillin-rich microfibril (*figure 2.11, d-f*) and fibulins-2 and -5 as above was used to quantify staining in the first 50 um of the papillary dermis.

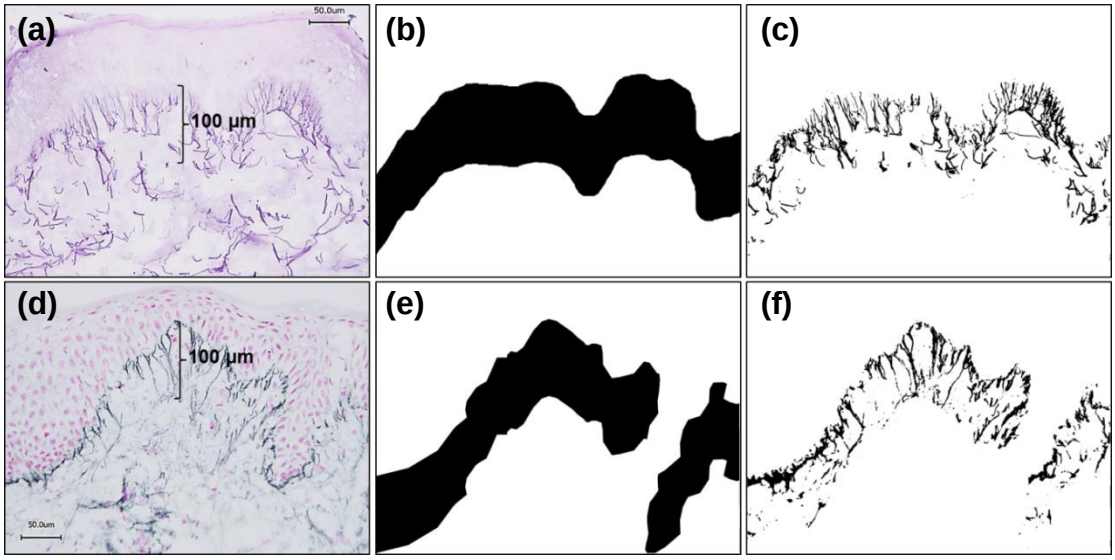


Figure 2.11: Elastic fibre staining in human skin.
Weigert's resorcin fuchsin (WRF) stained buttock tissues; (a) Bright field image; (b) Image split and masked for region of interest, and; (c) Image split and masked for fibre area. NB: at 100 µm depth. The ratio between elastic fibre area (c) and total area (b) was expressed as a percentage of tissue cover. These identical methods were applied for all IHC staining, i.e., fibrillin-rich microfibril; (d) Bright field; (e) Image of region of interest and (f) Image shown fibre area.

2.7.5 Quantification of procollagen I (pCI) IHC staining using a semi-quantitative analysis method

The semi-quantitative scale was set to 5 points (from 0 to 4) as previously described [262] based upon the abundance of staining as shown in the representative photomicrographs in *figure 2.12.* This measurement scale was applied to all pCI-stained immunohistochemistry slides. Three non-overlapping areas for each section (3 sections per treatment total) were evaluated to determine a mean staining.

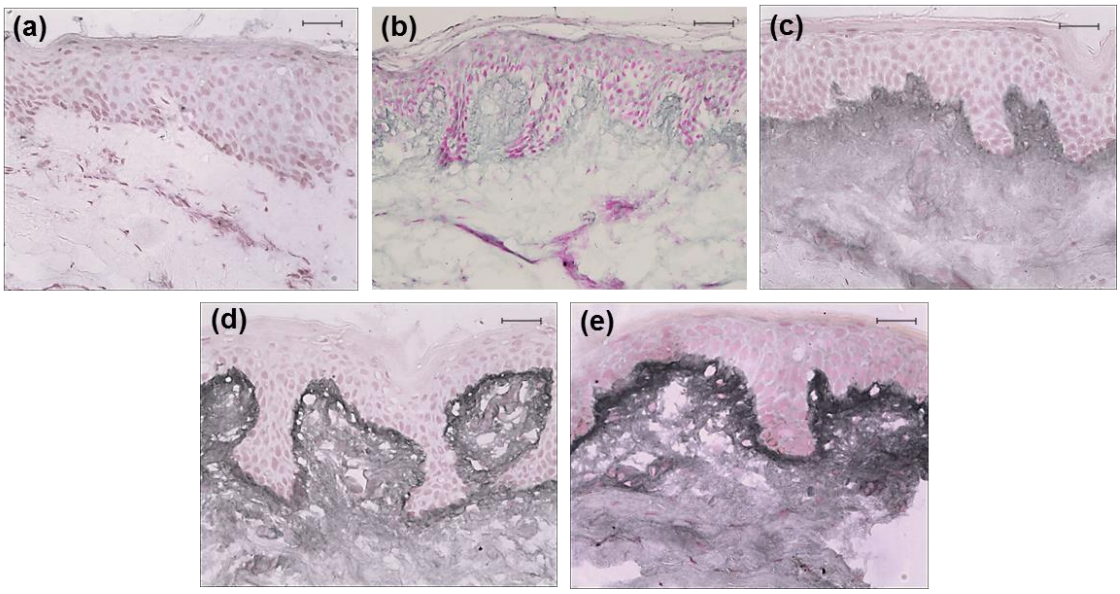


Figure 2.12: Evaluation of pCI staining using a semi-quantitative scale.
pCI was identified by immunohistochemistry procedures before evaluation with a semi-quantitative method. The scale was: 0 = no stain (a); 1 = diffuse with light grey stain at dermal-epidermal junction (DEJ) (b); 2 = dark grey at DEJ (c); 3 = very dark grey at DEJ (d) and; 4 = maximal staining with very dark grey and black appearance at DEJ (e). Original magnification x20 and scale bar equals to 50 µm.

2.8 Statistical analysis

2.8.1 Effect of UVB and SSR on the dermal matrix

In UVB vs SSR effect on ECMs study, Student's t-test was applied to compare between no UVR and UVR-exposed skin in each group independently.

2.8.2 Randomised controlled trial of dietary green tea catechins

The sample size for this study was calculated based on the primary outcome measure of UVR-induced erythema and indicated n=22 volunteers were required per group. A total of n=50 were recruited to allow for drop-out [247]. Paired Student's t-tests were used to compare no UVR and UVR-exposed skin for all subjects pre-supplementation, and by group post-supplementation. Differences in UVR-induced effects between placebo control and active group post-supplementation were analysed by analysis of covariance (ANCOVA) with baseline data as the covariate. Statistical tests were performed using the statistical software program, SPSS (v 22; IBM Corp.; Armonk, NY, USA).

Chapter 3 - Ultraviolet wavelength results in differential reorganisation of cutaneous elastic fibres: A comparison of solar simulated radiation and UVB

Nisamanee Charoenchon, Lesley E Rhodes, Mark D Farrar, Rachel E B Watson*

Centre for Dermatology Research, The University of Manchester M13 9PT and Salford Royal NHS Foundation Trust M6 8HD, Manchester Academic Health Science Centre, UK;

*Corresponding author: Dr Rachel E B Watson

Centre for Dermatology Research

2.100 Stopford Building

The University of Manchester

Manchester M13 9PT

UK

Email: rachel.watson@manchester.ac.uk

Tel: +44 161 275 5505

Abstract

Long-term exposure of ultraviolet radiation (UVR) on human skin impacts its appearance and function. Prolonged solar irradiation results in skin which is characteristically leathery and rough in appearance, with deep wrinkles. These clinical features of photodamage are thought to result from a drastic remodelling of the dermal extracellular matrix, particularly the elastic fibre system. Different wavelengths of solar UVR have specific effects on biological molecules; UVB is absorbed by DNA, melanin and some amino acids whilst UVA, being less energetic rarely interacts with chromophores in the epidermis and so may penetrate to deeper skin depths. In this study, we aimed to monitor the differential effect of UVB and full-spectrum solar simulated radiation (SSR) on components of the dermal elastic fibre system (elastic fibres, fibrillin-rich microfibrils and fibulin-2 and -5 microfibrils) following a robust challenge with either UVB or SSR. Healthy Caucasian volunteers (n = 6 per group; skin type II-III) were recruited and were assigned to receive either 3x MED of UVB or SSR on photoprotected buttock skin; biopsies were procured prior to and 24-hr after irradiation. Histological assessment of elastic fibres revealed a significant reduction in prevalence following both UVB and SSR irradiations ($P<0.01$ and $P<0.05$ respectively). However, a significant reduction in fibrillin-rich microfibrils was only observed in UVB irradiated skin ($P<0.05$), whilst fibulin-5-positive microfibrils were only affected by SSR ($P<0.05$). Hence, choice of irradiation system should be considered when designing and implanting studies on the pathomechanisms of UVR-induced skin damage.

Introduction

The skin is the outermost organ and, as well as providing a number of essential physiological roles such as thermoregulation and vitamin D synthesis, separates the body from its external environment. The structure of human skin is complex, consisting of a superficial, continuously regenerating cell-rich epidermis and a deeper, relatively cell-poor dermis rich in extracellular matrix [1]. The predominant cell type in the epidermis is the keratinocyte; as cells divide and differentiate they form the outermost stratum corneum (SC), a lipid-rich barrier to infection by microbes and chemicals that would pass through to the deeper down layer if it were not there [2]. The SC also preserves water balance and prevents excessive water loss [3]. Melanocytes scattered amongst the epidermal keratinocytes produce melanin, a natural 'sunscreen', which is gifted to the surrounding keratinocytes packaged in melanosomes and provides some protection to nuclear DNA from solar irradiation [4, 5].

Solar UVR is categorised based on its wavelength and is a combination of UVA (315-400 nm), UVB (280-315 nm) and UVC (200-280 nm). UVA and UVB are only two types that can pass through the Earth's atmosphere and penetrate to the Earth's surface (UVC is depleted by the ozone layer [6]). UVB has more diverse and potentially more severe effects on plants [7] and animal [5] than UVA, possibly due to it having higher photon energy at wavelengths that can be absorbed by biological materials such as DNA and protein. Even though UVA has less energy, the proportion of UVA in the full solar spectrum (~95%) is greater than UVB (only ~5%) and this may influence the potential for longer term effects when UVB is filtered out by, for example, interacting with DNA and proteins in the superficial epidermis [6, 8].

Long term exposure of skin to UVR causes photodamage and premature skin ageing [9, 10]. Clinically, this presents as skin with a leathery and rough appearance, with deep wrinkles and areas of hypo- and hyperpigmentation [10]. These changes to the appearance of photodamaged skin are thought to be driven by the effect of chronic UVR on the dermal extracellular matrix (ECM); the majority of ECM is produced by fibroblasts within the dermal compartment of skin and includes collagens, which provide strength, and elastic fibres which provide flexibility and recoil (see review [11]). Components of the elastic fibre system,

particularly fibrillin-rich microfibrils, are especially susceptible to photodamage. Work by Watson et al (1999) showed that these structures were lost from the papillary dermis of skin with only minimal clinical signs of photodamage. More recently, Sherratt and colleagues [12, 13] have shown that fibrillin-rich microfibrils are enriched in amino acids which can directly absorb energy from UVR, termed UV-chromophores; fibrillin-rich microfibrils (FRM), a major component in elastic fibre system, have one of the highest reported chromophore content up to 21.1% of their primary amino acid sequence and so this may account for their specific and early loss from photodamaged skin. Fibrillin-1 is the major family member of a group of modular proteins which also contains fibulins-2 and -5; these too localise to the dermal elastic fibre (see review [14]) and have been shown to be affected by photodamage [15].

There have been numerous studies which have sought to understand the mechanisms by which UVR affects skin structure [15, 16] and function [17, 18]. Such *in vivo* human studies commonly use artificial UVB to elicit a response, but one can argue that SSR is physiologically more relevant as it has an analogous spectrum and intensity to solar UVR, combining visible, infrared and both UVA and UVB wavelengths [19]. Choice of irradiation light source has been shown to elicit differential skin responses; for example, SSR has been shown to produce significant pigmentation when compared to UVB alone [20, 21], which induces much more skin reddening or erythema [22]. Therefore in this study we aimed to assess whether SSR or UVB could produce equivalent levels of ECM remodelling – specifically remodelling of components of the elastic fibre system - in response to a single robust UVR challenge.

Methods

Study design

Caucasian healthy volunteers (n=12; Fitzpatrick skin type II-III, 21-53 years old) were recruited to the study, ensuring that none had any previous skin or photoreactive condition and were not pregnant or breast feeding. A single irradiation of 3x individual's minimal erythemal dose (MED) was used to elicit a skin response; either UVB (n=6; ethics reference 11/NW/0567) or SSR (n=6; ethics reference 08/H1006/79). Studies were conducted according to the Declaration of Helsinki 2009, with participants providing written informed consent.

MED assessment and skin sampling

At study initiation, the MED of all participants was assessed using an ascending geometric dose of UVR (7-80 mJ/cm^2, SSR; 8-80 mJ/cm^2 UVB) to photoprotected buttock skin. The SSR was generated using a solar simulator with output spectra 290–400 nm (3.32% UVB, 96.68% UVA; Newport Spectra-Physics Ltd: Didcot, UK); UVB was generated using Waldmann UV6 bulbs with output spectra 280-360 nm (Herbert Waldmann GmbH, Villingen-Schwenningen, Germany). Twenty-four hours after irradiation, participants returned to clinic and their MED were assessed visually prior to irradiation of a further photoprotected buttock site with 3x their individual MED. Samples of unirradiated (UVR-naïve) and irradiated skin were sampled 24 hr after irradiation under local anaesthesia. Punch biopsies (5 mm diameter) were embedded in OCT compound (Tissue-Tek® OCT; Sakura Finetek USA INC., Torrance, CA, USA), snap frozen in liquid nitrogen and stored at -80˚C prior to histological investigation.

Histological staining

Frozen tissue were sectioned at 7µm (OTF cryostat; Bright Instrument Ltd, Cambridge, UK) to identify the entirety of the elastic fibre system (Weigert's resorcin fuchsin stain [23, 24]) or specific protein components of this system (FRM, fibulins-2 and -5) by immunohistochemistry. All staining was performed in triplicate. In brief, to perform Weigert's resorcin fuchsin staining, tissue sections were fixed in 4% (w/v) paraformaldehyde, nuclei were stained with Weigert's haematoxylin (Millipore; Damstatdt, Germany) with excess removed by industrial methylated spirits (IMS) prior to immersion in Weigert's resorcin fuchsin for 90 minutes (Clin-Tech Limited;

Guilford, UK). Stained slides were again washed briefly in 70% IMS prior to dehydration through serial alcohols, cleared in xylene and permanently mounted (DePex; Fisher Chemicals; Loughborough, UK).

For immunohistochemistry, sections were in 4% (w/v) paraformaldehyde and solubilised with 0.5% Triton X-100 (Fisher Chemicals). Endogenous peroxidase activity was abolished by incubation with 0.6% hydrogen peroxide in methanol, with non-specific binding blocked by incubation with 3% bovine serum albumin/1% normal sera at room temperature for 1 hr. Sections were incubated overnight in primary antibody. These were: fibrillin-rich microfibrils (clone 11C1.3, dilution 1:1000; NeoMarkers; Fremont, CA USA); fibulin-2, (clone HPA001934, dilution 1:1000; Atlas Antibodies AB; Stockholm, Sweden) and; fibulin-5 (clone HPA000848, dilution 1:180; Atlas Antibodies AB; Stockholm, Sweden). Sections were stringently washed and visualised using a commercially available kit utilising a secondary biotinylated antibody (Vector *Elite* ABC; Vector Labs., Burlingame, CA, USA). Vector SG® was used as chromogen with nuclear fast red used as contrast (Vector Labs). Finally, sections were serially dehydrated, cleared and permanently mounted as previously described.

Photomicrography, image analysis and statistics

All sections were randomised and blinded prior to image capture (Biozero-800 All-in-One microscope; Keyence; Osaka, Japan); three section per slide, three slides per treatment. Positive staining was quantified using program ImageJ (National Institutes of Health, Maryland, USA) [25] and expressed as a percentage of the imaged section, with paired Student's t-test used to assess differences in staining between irradiated and unirradiated sites (SPSS v22, IBM Corp., New York, NY, USA).

Results

Elastic fibres in skin have a characteristic architecture depending on their location; close to the dermal-epidermal junction (DEJ), candelabras of oxytalan fibres can be seen. These are fibrillin-rich microfibrils, largely devoid of elastin and they converge with perpendicular elaunin fibres in the papillary dermis. Elaunin fibres are a composite of fibrillin-rich microfibrils plus elastin and they coalesce with elastic fibres proper in the reticular and deeper dermis [26]; here elastin is

the major component of the fibre, with microfibrils found on the periphery of the structure (see review at [27]). Weigert's resorcin fuchsin staining revealed this ordered pattern in unirradiated skin (Fig. 1a). Irradiation significantly impacted on the pattern of elastic fibres in papillary dermis; image analysis of the papillary dermis from the dermal-epidermal junction to a depth of 100 μm was performed to enumerate the percentage of dermis occupied by positive stain. In participants irradiated with 3x MED UVB, the amount of staining was significantly reduced (mean ± SE; unirradiated, 13.64% ± 0.74; UVB, 8.57% ± 1.39; P=0.004; Fig. 1b); this reduction was less marked in those who received 3x MED of full spectrum SSR (unirradiated, 15.23% ± 0.74; SSR, 12.34 ± 1.05 P=0.043; Fig. 1c); data is presented graphically in Fig. 1d.

To establish which microfibrillar protein components of the elastic fibre network were affected by acute irradiation immunohistochemistry was performed to identify fibrillin-rich and fibulin-positive microfibrils. The differential effects of UVB and SSR in the organisation and abundance of fibrillin-rich microfibrils is shown in Fig. 2 (a-d). Prior to irradiation, this population of microfibrils (oxytalan fibres) are easily identified in the superficial papillary dermis, occupying the area immediately below the DEJ (Fig. 2a). On irradiation with UVB, there is significant rearrangement and loss of these elastic fibre elements (unirradiated, 25.21 ± 1.38; UVB, 18.96 ± 1.45; P=0.023, Fig. 2b). Surprisingly, little alteration in the distribution of fibrillin-rich microfibrils was seen in SSR irradiated skin (unirradiated, 25.13 ± 1.58; SSR, 21.85 ± 1.38; P>0.05, Fig. 2c). Data is displayed graphically in Fig. 2d. Fibulin-2-positive microfibrils also appeared to emanate from the DEJ, but were generally of shorter length than fibrillin-rich microfibrils and so occupied less area of the papillary dermis. When quantified, neither UVB nor SSR elicited a significant remodelling (Fig. 2e-h). Fibulin-5 microfibrils had a similar distribution to fibrillin-rich microfibrils with longer fibres coalescing with elaunin fibres in the papillary dermis (Fig. 2i-k). However, when quantified, these microfibrillar elements responded specifically to SSR irradiation (unirradiated, 19.40 ± 1.94; SSR, 15.92 ± 1.44; P=0.04, Fig. 2k), as opposed to UVB (unirradiated, 13.54 ± 1.19; UVB, 10.90 ± 0.61; P>0.05, Fig. 2j). Data is displayed graphically in Fig 2l.

Discussion

Sunlight (solar UVR) is required for life on Earth [28, 29]; UVC is filtered out by the ozone layer, meaning that only UVA and UVB can influence biological activity of plants and animals on the planet's surface [28]. In man, exposure of skin to UVR benefits the individual by stimulating vitamin D synthesis [30], but the skin further protects the body from deleterious effects of UVR. One consequence of this long term exposure to UVR is photoageing [31]. To understand the mechanisms by which UVR alters skin and skin cells' behaviour, previous mechanistic studies have used UVB to elicit biological responses, mainly because of its higher energy than UVA, meaning irradiation times have been shorted and generally more acceptable to volunteers, in addition to its capability to interact with biological molecules, producing reproducible effects [32].

In 2002, Walker et al. used a UVB light source to assess the effect of irradiation on the occurrence of FRM in non-radiated and 3x MED UVB exposed skin and discovered that these structures significantly declined 24-hours after irradiation [16]. Similarly in 2005, Kadoya et al. found that fibulin-2 and -5-containing microfibrils, in addition to fine elastin fibres (as identified histologically using Weigert's resorcin fuchsin stain) were also reduced 24-hr after a single dose of 2x MED UVB compared to UV-protected skin [15]. However, UVB only makes up approximately 6% of the solar spectrum reaching Earth [33]; SSR therefore is more physiologically relevant and can be argued to be a more suitable system to replicate 'real life' sun exposure. Chronic photodamage is the product of years of sunlight exposure, which incrementally alters in the elastic fibre system of the skin (elastosis, loss of FRM, altered fibulin expression) [34-36]. Exposure is not necessarily constant; independent variables such as longitude, season and outdoor activity all play a role in accelerating UVR-induced skin damage. Hence to truly recapitulate photodamage in a laboratory setting, it may be necessary to subject individuals to multiple sub-erythemal doses of SSR. This will provide a better understanding of the pathways which underpin the pathology and may provide a model system for examining interventions.

In our study, we directly compared the effect of UVB and SSR irradiation in healthy volunteers (n=6 per group). We confirmed the results first reported by Kadoya et al. (2005) that, within the first 50 μm of the papillary dermis, elastic fibres and microfibrils positive for fibulins-2 and -5 were significantly remodelled following such an acute dose of UVB (P=0.005, P=0.028 and P=0.048 respectively) [15]. However, at deeper depths (up to 100 μm into the papillary dermis), this remodelling was no longer observed for both fibulin-2 (P=0.10) and fibulin-5 (P=0.11); hence significant remodelling of these microfibrillar elements by UVB appears to happen close to the DEJ. Examination of the FRM network, in agreement with previous work [16], identified loss of microfibrils deeper in the papillary dermis (to 100 μm; P=0.02).

Whilst irradiation with SSR induced remodelling of the elastic fibre network, we identified differences in the components affected and the degree of the induced damage; elastic fibres were significantly reduced, but the observed remodelling was less than when skin was irradiated with UVB. Likewise, irradiation with SSR had a smaller effect on the organisation and amount of FRMs in the papillary dermis. Fibulin-5 appeared particularly sensitive to SSR irradiation (P=0.04) whereas remodelling to fibulin-2-positive microfibrils, whilst occurring, did not reach significance (P=0.06). Hence, we show here that UVB and SSR irradiation protocols produce distinct patterns of damage with the dermal elastic fibre network.
It is likely that this is due to the differences in wavelength components between the two spectra.

The mechanisms by which remodelling occurs requires further investigation; two possible mechanisms of protein damage have been discussed in the literature. The first is a direct mechanism by which the primary amino acid sequence of the protein plays a large role in its susceptibility to damage by energy transfer from UV photons (UVR chromophore content; Cys, His, Phe, Trp and Tyr) [11]. This theory suggests that even low doses of UVB (up to 50 mJ/cm^2 [13]) are capable of interacting with molecules with high chromophore content (e.g. FRM, up to ~21%; fibrillar collagens, only ~2%); experimentally, molecules with higher chromophore content showed more damage from the same UVR dose challenge. The second possible mechanism suggests cell- or reactive oxygen species (ROS)-mediated damage. It is known that ROS can cause damage to biological molecules, such as DNA [37], lipids [38] and proteins [39]; as UVR generates ROS it is possible that these are the mediators of damage to the elastic

fibres within skin. Irradiation also elicits a cellular response where enzymes, such as matrix metalloproteinases (MMPs) are released or activated [40]; irradiation also induces the accumulation of immune cells into the skin which are capable of expressing other classes of enzymes e.g. neutrophils and neutrophil elastase which can impact on the resident elastic fibres. It is possible that both mechanisms play a role in UVR-induced ECM remodelling. Regardless of mechanism, this data shows that it is important to consider the means of irradiation, as different light sources can produce differential remodelling effects in vivo. It is therefore important that a researcher consider how they produce skin damage and whether the challenge is appropriate to the research question under study.

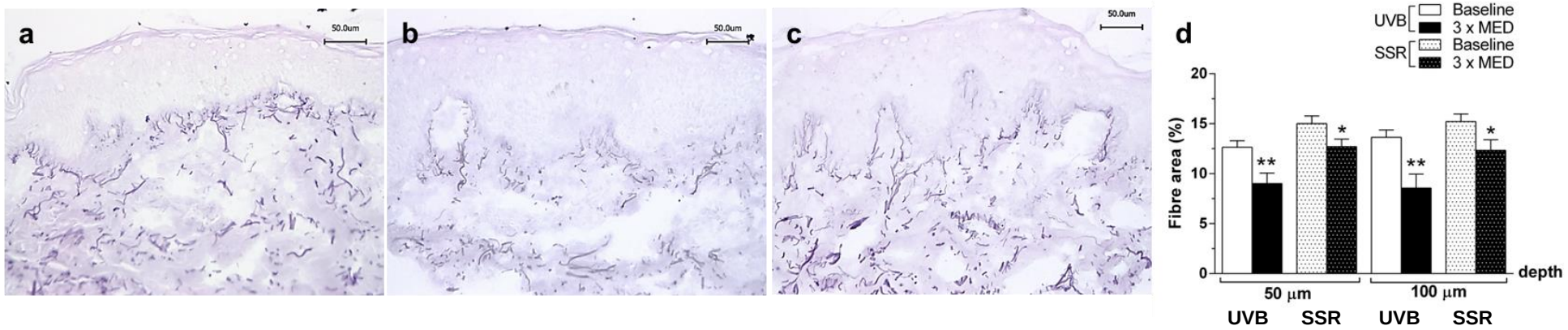


Fig1. The distribution of papillary dermal elastic fibres is affected by both SSR and UVB irradiation. *The distribution of elastic fibres was identified histologically using Weigert's resorcin fuchsin; (a) unirradiated photoprotected skin shows the classic arrangement of cutaneous elastic fibres. (b) Irradiation with 3x MED of UVB results in significant breakdown and/or reorganisation of elastic fibres 24 hr after the irradiation event whilst (c) 3x MED irradiation with full-spectrum SSR appeared to have less of an impact. Scale bar, 50 μm. (d) Bar graphs representing the area of the papillary dermis occupied by elastic fibres in percentages, *** P ≤ 0.001, ** P ≤ 0.010 , *P ≤ 0.050.*

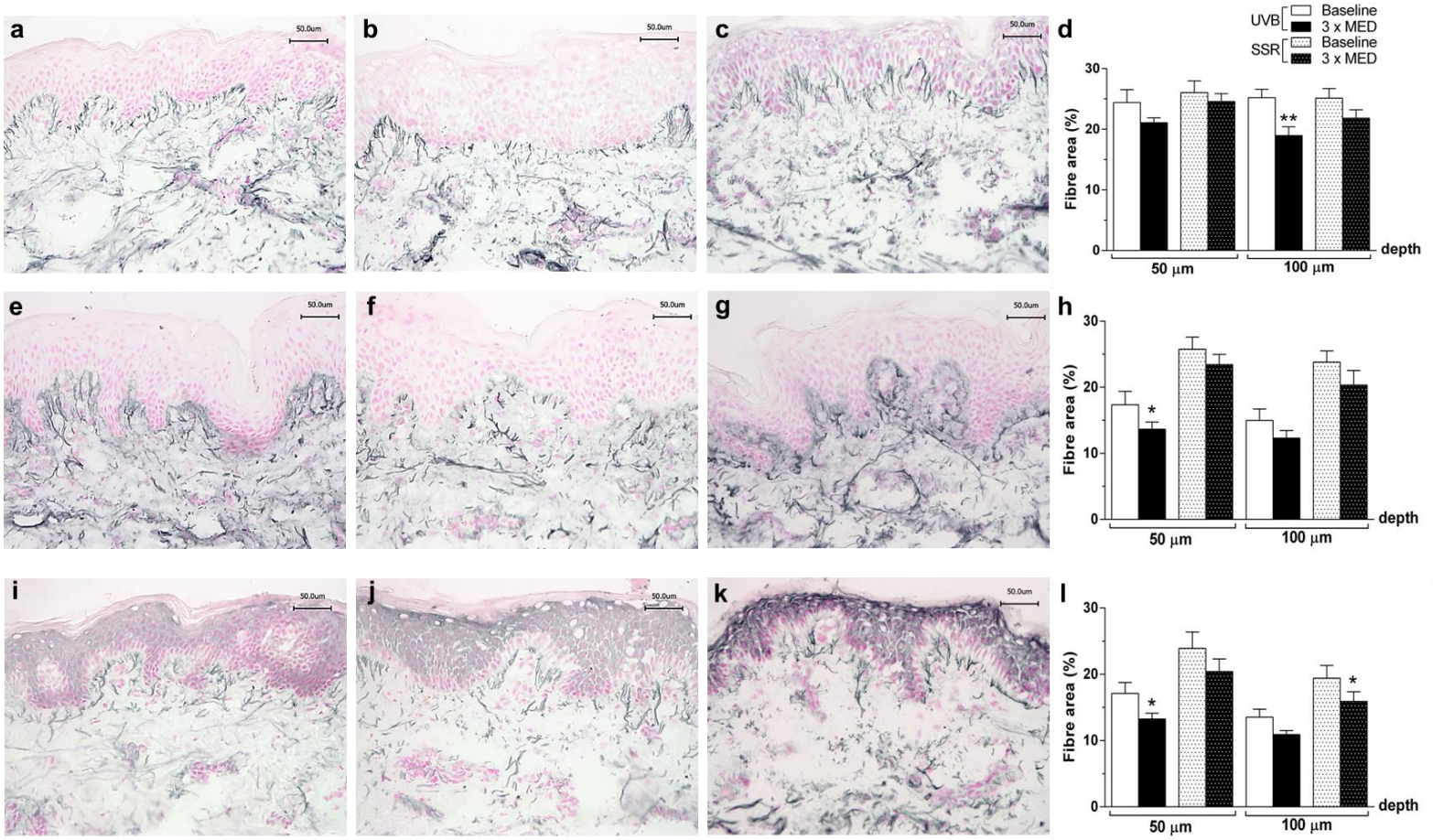


Fig 2. Three protein types stained by IHC staining*: FRM (a-c); fibullin-2(e-g) and fibulin-5(i-k) showed decline trend affected by 3xMED UVB and SSR radiation with overall bar graphs individually on the right of each protein type. However, only some proteins were significantly decreased. *** P ≤ 0.001, ** P ≤ 0.010 , *P ≤ 0.050*

References

1. Kielbassa, C., L. Roza, and B. Epe, *Wavelength dependence of oxidative DNA damage induced by UV and visible light*. Carcinogenesis, 1997. **18**(4): p. 811-816.
2. Bouwstra, J.A. and P.L. Honeywell-Nguyen, *Skin structure and mode of action of vesicles. Advanced Drug Delivery Reviews*, 2002. **54**, Supplement(0): p. S41-S55.
3. Rawlings, A.V. and C.R. Harding, *Moisturization and skin barrier function*. Dermatologic Therapy, 2004. **17**: p. 43-48.
4. Kvam, E. and R.M. Tyrrell*, Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation*. Carcinogenesis, 1997. **18**(12): p. 2379-2384.
5. Ravanat, J.-L., T. Douki, and J. Cadet, *Direct and indirect effects of UV radiation on DNA and its components*. Journal of Photochemistry and Photobiology B: Biology, 2001. 63(1–3): p. 88-102.
6. Paul, N.D. and D. Gwynn-Jones, *Ecological roles of solar UV radiation: towards an integrated approach.* Trends in Ecology & Evolution, 2003. **18**(1): p. 48-55.
7. Seité, S., et al., *A broad-spectrum sunscreen prevents cumulative damage from repeated exposure to sub-erythemal solar ultraviolet radiation representative of temperate latitudes*. Journal of the European Academy of Dermatology and Venereology, 2010. **24**(2): p. 219-222.
8. Meinhardt, M., et al., *Wavelength-dependent penetration depths of ultraviolet radiation in human skin.* Journal of Biomedical Optics, 2008. **13**(4): p. 044030-044030.
9. Marshall, D.R., *The Clinical and Pathological Effects of Prolonged Solar Exposure Part I.-the association with ageing of the skin*. Australian and New Zealand Journal of Surgery, 1965. **34**(3): p. 161-172.
10. Tsoureli-Nikita, E., R.E.B. Watson, and C.E.M. Griffiths, *Photoageing: the darker side of the sun. Photochemical & Photobiological Sciences*, 2006. **5**(2): p. 160-164.
11. Watson, R.E.B., et al., *Damage to Skin Extracellular Matrix Induced by UV Exposure. Antioxidants & Redox Signaling*, 2014. **21**(7): p. 1063-+.
12. Thurstan, S., et al., *Chemical consequences of cutaneous photoageing.* Chemistry Central Journal, 2012. **6**(1): p. 1-7.
13. Sherratt, M.J., et al., *Low-dose ultraviolet radiation selectively degrades chromophore-rich extracellular matrix components.* Journal of Pathology, 2010. **222**(1): p. 32-40.
14. Timpl, R., et al., *Fibulins: a versatile family of extracellular matrix proteins*. Nat Rev Mol Cell Biol, 2003. **4**(6): p. 479-489.
15. Kadoya, K., et al., *Fibulin-5 deposition in human skin: decrease with ageing and ultraviolet B exposure and increase in solar elastosis*. British Journal of Dermatology, 2005. **153**(3): p. 607-612.
16. Walker, S., Rhodes, L.E., Chatelus, Y., Griffiths, C.M., Watson, R.B. *Fibrillin-rich microfibrils are reduced following acute UVB irradiation*. in British Society for Investigative Dermatology Annual Meeting. 2002. University of East Anglia, Norwich, U.K.: Br J Dermatol.
17. Andesen, P.H., et al., *Time-correlation study of ultraviolet B-induced erythema measured by reflectance spectroscopy and laser Doppler flowmetry*. Photodermatology Photoimmunology & Photomedicine, 1991. **8**(3): p. 123-128.
18. Huang, M.-W., P.-Y. Lo, and K.-S. Cheng, *Objective Assessment of Sunburn and Minimal Erythema Doses: Comparison of Noninvasive In Vivo Measuring Techniques after UVB Irradiation.* Eurasip Journal on Advances in Signal Processing, 2010.
19. Brenner, M. and V.J. Hearing, *The Protective Role of Melanin Against UV Damage in Human Skin†*. Photochemistry and Photobiology, 2008. **84**(3): p. 539-549.
20. Wolber, R., et al., *Pigmentation effects of solar-simulated radiation as compared with UVA and UVB radiation*. Pigment Cell & Melanoma Research, 2008. **21**(4): p. 487-491.
21. Choi, W., et al., *Regulation of human skin pigmentation in situ by repetitive UV exposure: molecular characterization of responses to UVA and/or UVB.* J Invest Dermatol, 2010. **130**(6): p. 1685-96.
22. Mehta, R.V., et al., *Minimal erythema response (MED) to solar simulated irradiation in normal Indian skin.* Indian J Dermatol Venereol Leprol, 2004. **70**(5): p. 277-9.
23. Crescenzi, A., et al., *Physicochemical analysis of resorcinol-fuchsin reagents and its relevance to staining quality of elastic fibres.* European Journal of Basic and Applied Histochemistry, 1991. **35**(1): p. 37-44.

24. Proctor, G.B. and R.W. Horobin, *Chemical structures and staining mechanisms of Weigrt resorcin-fuchsin and related elastic fiber stains*. Stain Technology, 1988. **63**(2): p. 101-111.
25. Abramoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with ImageJ*. Biophotonics international, 2004. **11**(7): p. 36-42.
26. Frances, C., et al., *Elastic fibers in normal human skin. variations with age: a morphometric analysis*. Archives of Gerontology and Geriatrics, 1990. **10**(1): p. 57-67.
27. Kielty, C.M., M.J. Sherratt, and C.A. Shuttleworth, *Elastic fibres*. Journal of Cell Science, 2002. **115**(14): p. 2817-2828.
28. Cockell, C.S., *Biological effects of high ultraviolet radiation on early Earth - a theoretical evaluation*. Journal of Theoretical Biology, 1998. **193**(4): p. 717-729.
29. Rozema, J., et al., *UV-B as an environmental factor in plant life: Stress and regulation*. Trends in Ecology & Evolution, 1997. **12**(1): p. 22-28.
30. Hakim, O.A., et al., *Vitamin D production in UK Caucasian and South Asian women following UVR exposure.* The Journal of Steroid Biochemistry and Molecular Biology.
31. Watson, R.E.B. and C.E.M. Griffiths, *Pathogenic aspects of cutaneous photoaging. Journal of cosmetic dermatology,* 2005. **4**(4): p. 230-6.
32. de Gruijl, F.R., Photocarcinogenesis: *UVA vs UVB, in Methods in Enzymology*. 2000, Academic Press. p. 359-366.
33. Diffey, B.L., *Sources and measurement of ultraviolet radiation*. Methods, 2002. **28**(1): p. 4-13.
34. Suwabe, H., et al., *Degenerative processes of elastic fibers in sun-protected and sun-exposed skin: Immunoelectron microscopic observation of elastin, fibrillin-1, amyloid P component, lysozyme and α1-antitrypsin*. Pathology International, 1999. **49**(5): p. 391-402.
35. Watson, R.E.B., et al., *Fibrillin-rich microfibrils are reduced in photoaged skin. Distribution at the dermal-epidermal junction.* Journal of Investigative Dermatology, 1999. **112**(5): p. 782-787.
36. Watson, R.E. and C.E. Griffiths, *Fibulin-2 and its association with fibrillin-rich microfibrils in the papillary dermis of photoaged skin*. Br J Dermatol, 2002. **146**(4): p. 712.
37. Jena, N.R., DNA damage by reactive species: *Mechanisms, mutation and repair.* J Biosci, 2012. **37**(3): p. 503-17.
38. Barrera, G., *Oxidative Stress and Lipid Peroxidation Products in Cancer Progression and Therapy*. ISRN Oncology, 2012. 2012: p. 137289.
39. Berlett, B.S. and E.R. Stadtman, *Protein oxidation in aging, disease, and oxidative stress.* J Biol Chem, 1997. **272**(33): p. 20313-6.
40. Quan, T.H., et al., *Matrix-Degrading Metalloproteinases in Photoaging.* Journal of Investigative Dermatology Symposium Proceedings, 2009. **14**(1): p. 20-24.

Chapter 4 - Fibulin-5 microfibrils, essential components of cutaneous elastic fibers, are protected from UVR-induced damage by dietary green tea catechin supplementation

Nisamanee Charoenchon[1], Lesley E Rhodes[1], Anna Nicolaou[2], Gary Williamson[3], Mark D Farrar[1], Rachel E B Watson[1]*

[1] Centre for Dermatology Research, The University of Manchester M13 9PT and Salford Royal NHS Foundation Trust M6 8HD, Manchester Academic Health Science Centre, UK;

[2] Manchester Pharmacy School, The University of Manchester M13 9PT, Manchester Academic Health Science Centre, UK;

[3] School of Food Science & Nutrition, University of Leeds, Leeds LS2 9JT, UK

*Corresponding author:

Rachel E B Watson BSc (hons), PhD

Centre for Dermatology Research

2.100 Stopford Building

The University of Manchester

Manchester M13 9PT

UK

Email: rachel.watson@manchester.ac.uk

Tel: +44 161 275 5505

Abstract

Background

The cutaneous elastic fiber system undergoes extensive remodeling in chronically photodamaged skin due to long-term exposure to solar ultraviolet radiation (UVR). *Camellia sinensis* contains high concentrations of green tea catechins (GTC) which have been reported to provide skin photoprotection *in vitro* and *in vivo*. In this study, we performed a double-blind randomized controlled trial to evaluate whether oral GTC supplementation protected cutaneous elastic fibers from UVR challenge.

Methods

Healthy volunteers (N=50) were randomized to either active (1080mg GTC plus 100mg vitaminC) or placebo (maltodextrin) groups for 12-weeks (n=25 per group); compliance was assessed by urinary analysis. At pre- and post-supplementation time points, participants provided biopsies of unirradiated skin and skin challenged 24-hours previously with 3x MED of solar simulated UVR which was subsequently analyzed for elastic fiber components.

Results

Of the recruited participants, 44 completed the supplementation protocol and provided all biopsies. Pre-supplementation, UVR challenge resulted in a significant remodeling of the cutaneous elastic fiber system ($P<0.001$), particularly fibulin-2- and fibulin-5-positive microfibrils. Post-supplementation with GTC, fibulin-5 positive microfibrils were protected from UVR remodeling (% staining, mean ± SE; no UV, 18.1±0.89; UVR, 17.1±0.61; $P=0.30$) whilst no protection was seen in the placebo group (no UVR, 19.41±0.79; UVR, 17.69±0.61; $P<0.05$).

Conclusions

Dietary supplementation with oral GTC preserved components of the elastic fiber system known to be remodeled by UVR. This effect was differential, with GTC protecting fibulin-5-positive microfibrillar bundles against UVR damage. Hence, dietary supplementation with GTC may be a useful adjunct to conventional skin protection regimens, protecting the skin from features of ageing.

Introduction

Skin is a dynamic organ which provides the body with many essential functions including synthesis of vitamins [1] and hormones [2] plus protection from infection and the external environment [3]. Whilst the epidermis is a continuously renewing cell layer, the underlying dermis, composed of a cell-sparse but complex extracellular matrix (ECM) is comparatively static; the components of this dermal ECM, e.g. elastic fiber proteins, fibrillar collagens and glycosamino- and proteoglycans, are long-lived and so may accrue damage over the life course [4].

Skin is continually exposed to solar ultraviolet radiation (UVR) with some body sites such as the face and hands becoming chronically photoexposed over time (termed photodamage). One of the first pathological changes observed in photodamaged skin is loss of oxytalan fibers in the papillary dermis (comprised of fibulin-positive and fibrillin-rich microfibrils; [5, 6]); with continued UVR exposure, dystrophic elastic fibers are deposited within the reticular dermis [7] which contributes to the sallow complexion often observed in individuals with chronic photodamage [8, 9]. Ultraviolet radiation can also induce acute responses in skin such as sunburn (inflammation), producing DNA damage and initiating photocarcinogenesis. Hence, appropriate sun protection behaviour such as the use of topical sunscreens, which can absorb and scatter UVR wavelengths, should protect skin from both photodamage and skin cancer [10]. However, such topical photoprotective agents are rarely used as directed [11], making methods of systemically protecting the skin an interesting potential therapy.

The leaves of *Camellia sinensis* (tea bush) are rich in flavan-3-ol polyphenols (green tea catechins; GTCs). These polyphenolic compounds have been shown in both *in vitro* and *in vivo* studies to impact on cancer incidence [12], cardiovascular inflammation [13] and metabolism [14]. Chui et al (2005) performed a short-term intervention of topical GTC in combination with oral GTC supplementation to assess whether treatment slowed the clinical progression of photodamage; it did not. However, some benefit was observed in the organization of the already dystrophic cutaneous elastic fiber network [15]. In a longer term study, oral supplementation with GTC improved the clinical appearance of photodamaged skin [16]. Finally, Heinrich et al (2011) reported that oral GTC, taken for 12-weeks, improve skin elasticity and improved barrier function and increased the dose of UVR required to elicit erythema [17].

Our own studies have suggested that, when given orally, GTCs can protect against cutaneous inflammation at high UVR doses [18]. Hence, we have performed a double-blind randomized controlled trial (RCT) of adequate power to examine the potential of oral GTC against remodeling of the elastic fiber system in response to UVR.

Methods

Study design and implementation

Fifty healthy white Caucasians (18-65 years old; male and female) with Fitzpatrick sun-reactive skin types I-II were recruited to the study by open advert. Potential participants were screened to ensure no history of: skin cancer or any photosensitivity disorder; photoactive medications or dietary supplements; sunbed use in the 3 months prior to commencement of the study; or currently pregnant or breastfeeding. In addition, a food history for the 3 months prior to enrolment was explored to ensure consumption of tea <2 cups per day. The study was approved by NHS Research Ethics Committee (reference 08/H1006/79) and was performed according to the principles of the Declaration of Helsinki, with participants providing written informed consent. The study was performed between November 2010 and August 2011 at the Photobiology Unit, The Dermatology Centre, Salford Royal Hospital, Manchester UK. Participants were assigned randomly to receive either active GTC plus vitamin C supplement or placebo maltodextrin (1:1; block randomization with random block sizes of 4-8; StatsDirect v2.7.8, StatsDirect Ltd). Supplements were identically packaged in gelatin capsules and sequentially numbered, with both participants and investigators blinded to their contents (supplements provided by Nestec Ltd. and packaged by Laboratoire LPH). Coding was only broken following completion of the study.

Supplements were given with breakfast and evening meal; the active green tea group taking 3 capsules (450 mg green tea extract containing 180 mg GTC/capsule) and 2 capsules of vitaminC (25 mg/capsule) twice daily, providing a total daily dose of 1080 mg GTC (equivalent to ~ 5 cups of green tea) and 100 mg vitamin C (the vitamin C stabilizing the GTC in the gut, so supporting absorption)[50]. The placebo group took supplements containing maltodextrin alone. The intervention lasted 12 weeks with compliance assessed by examining any remaining

capsules when participants returned the dispensing containers plus measurement of urinary epigallocatechin glucuronide (a breakdown product of GTC; at the beginning, middle and end of the study. The outcome measure was change in percentage coverage of elastic fibres in the papillary dermis following UVR challenge at 12 weeks [19].)

UVR challenge

UVR challenge was performed using a solar simulator with an emission spectrum that mimicked sunlight (290-400 nm; 95% UVA, 5% UVB; Newport Spectra-Physics Ltd.). Lamp output was measured by radiometer at a distance of 10 cm from the source prior to each irradiation (IL 730A; International Light). The minimal erythemal dose (MED; sunburn threshold) for each individual participant was assessed at study initiation; this was performed following irradiation with an ascending geometric dose series of solar simulated UVR (erythemally weighted, 7-80 mJ/cm^2) to photoprotected buttock skin. Irradiation sites were observed 24-hours following irradiation and the MED defined as the lowest dose producing just discernable erythema.

At study initiation and following supplementation, a UVR dose 3 times that of the participants' MED was given to photoprotected buttock to initiate elastic fiber remodeling. Skin punch biopsies (5 mm diameter) were taken under 1% lidocaine anesthesia from irradiated and contralateral unirradiated buttock sites 24-hours following challenge; hence, each participant provided 4 biopsy samples. Samples were embedded in Tissue-Tek® OCT (Sakura Finetek USA INC., Torrance, CA, USA), snap frozen in liquid nitrogen and stored at -80ºC prior to histological evaluation.

Identification of cutaneous elastic fibers

Frozen 7 µm sections were produced (OTF cryostat; Bright Instruments Ltd, Cambridge, UK) for histological investigation of the cutaneous elastic fiber system, particularly oxytalan fibers. Weigert's resorcin fuchsin stain was used to identify the network in its entirety [20, 21]. In brief, sections were fixed in 4% (w/v) paraformaldehyde (in phosphate-buffed saline; PFA/PBS) prior to immersion in Weigert's hematoxylin working solution (Millipore; Darmstadt, Germany). Sections were washed with industrial methylated spirits (IMS), rinsed in distilled water and stained with Weigert's resorcin fuchsin solution (Clin-Tech Ltd.; Guilford, UK). Following

staining, tissue sections were dehydrated through serial alcohols, cleared in xylene and permanently mounted using DePex (Fisher Chemicals; Loughborough, UK).

Immunohistochemistry was performed to identify specific components of oxytalan fibers (fibrillin-rich microfibrils, fibulin-2 and fibulin-5 [5, 6, 22]). Sections were fixed in 4% PFA/PBS prior to solubilization of lipid membranes with 0.5% Triton-X-100. Sections were blocked to reduce non-specific binding of antibodies (normal sera plus bovine serum albumin) prior to overnight incubation with primary antibody (fibrillin-rich microfibrils, clone 11C1.3 dilution 1:1000 [NeoMarkers; Fremont, CA USA]; fibulin-2, clone HPA001934 dilution 1:1000 [Atlas Antibodies AB; Stockholm, Sweden], and; fibulin-5, clone HPA000848 dilution1:180 [Atlas Antibodies AB; Stockholm, Sweden]. Binding was identified via a secondary biotinylated antibody using a commercially available kit (Vector *Elite* ABC; Vector Labs., Burlingame, CA USA), using Vector SG® as chromogen (Vector Labs.). Nuclei were counterstained with Nuclear Fast Red (Vector Labs.), serially dehydrated, cleared and permanently mounted.

Image capture and analysis

Stained slides (3 sections per participant per biopsy) were captured on a Biozero-800 all-in-one microscope (Keyence; Osaka, Japan). To assess whether there was any difference in the distribution of elastic fibers following UVR irradiation prior to and after dietary supplementation, images were subjected to image analysis (ImageJ; NIH, MA USA) [23]. The microfibrillar elements from the dermal-epidermal junction (DEJ) to 100 µm into the papillary dermis were quantified as percentage coverage.

Statistical analysis

To analyze the effect of supplementation on collagen deposition in UVR-exposed skin, analysis of covariance (ANCOVA) was used with baseline data as the covariate. Prior to supplementation, the effect of UVR challenge was assessed using paired Student's t-test across all participants. Post-supplementation, paired Student's t-test were used for intragroup comparisons of irradiated and unirradiated skin. Significance was taken at the 95% confidence level and employed SPSS (v22; IBM Corp., Armonk, NY USA).

Results

Subjects and compliance

Fifty healthy participants were recruited to the RCT, with random allocation to each of the treatment groups (active, n = 25; placebo, n = 25). Of the 25 individuals in the active group, 4 were non-compliant as identified by urinary analysis and a further participant withdrew from the study for unrelated reasons. One participant in the placebo group had high EGCG in the urine at study initiation and was therefore excluded from analysis [24], (*figure 1*).

Solar simulated UVR causes the remodeling of fibulin-positive microfibrils but not fibrillin-rich microfibrils in the papillary dermis

Weigert's resorcin fuchsin was used to identify the entirety of the cutaneous elastic fiber network. Prior to supplementation, UVR challenge with 3xMED of solar simulated UVR resulted in a significant decrease in the amount of histologically identifiable elastic fibers in the papillary dermis quantified as percentage coverage (mean ± SE of 15.24 ± 0.58 % in unirradiated skin and 12.43 ± 0.65 % in UVR-exposed skin, $P < 0.001$; *figure 2a, b*). When we examined specific components of oxytalan fibers resident in the papillary dermis, we observed markedly different patterns of staining following UVR challenge. As oxytalan fibers are multi-component ECM assemblies, we investigated each of their major components in turn: fibrillin-rich microfibrils as well as fibulin-2- and fibulin-5-positive microfibrils [25, 26]. When fibulin-2 and fibulin-5 were examined, we observed significant loss of fibres following UVR challenge for each fiber component, comparable to that observed with Weigert's resorcin fuchsin (fibulin-2: 22.31 ± 0.68 % coverage in unirradiated skin and 19.25 ± 0.63 % in UVR-exposed skin, $P < 0.001$; fibulin-5 19.87 ± 0.69 % in unirradiated skin and 16.59 ± 0.51 % in UVR-exposed skin, $P < 0.001$; *figure 2c-f*). UVR challenge with solar simulated UVR also impacted on the organization or deposition of fibrillin-rich microfibrils, elastic fiber elements known to be lost early in the pathological progression of photodamage with, 26.82 ± 0.56 % coverage in unirradiated skin and 25.11 ± 0.62 % in UVR-exposed skin, $P = 0.02$; *figure 2g, h*).

Green tea catechin supplementation specifically protects fibulin-5 microfibrils from UVR-induced remodeling

Compared with placebo, supplementation with GTC had no effect on percentage coverage of papillary dermal elastic fibers following UVR challenge, as identified by Weigert's resorcin fuchsin stain (placebo 12.48 ± 0.61 %; active 11.15 ± 0.78 %; ANCOVA P =0.56; *figure 1a-d*) or on fibrillin–rich microfibrils following UVR challenge (placebo 26.37 ± 0.87 %; active, 25.48 ± 1.09 %; ANCOVA P = 0.28; *figure 3e-h*). Examination of fibulin-2-positive fibers following the intervention showed no significant difference between groups in percent coverage following UVR challenge with 20.62 ± 0.84 % in the placebo and 20.37 ± 0.84 % in the active (ANCOVA P = 0.82; *figure 3i-l*). Similarly, there was no significant difference between groups post-supplementation in percentage coverage of fibulin-5 following UVR challenge (placebo 17.69 ± 0.82 %; active 17.07 ± 0.61 %; ANCOVA P = 0.65; *figure 3m-p*). Notably, the significant UVR-induced reduction in fibulin-5 observed at baseline was preserved post-supplementation in the placebo group (P = 0.012) but not in the active group (P = 0.30). A similar trend was seen for fibulin-2 post-supplementation with UVR-induced loss approaching significance in the placebo group (P = 0.06) but not in the active group (P = 0.90).

Discussion

Skin is a physical and biological barrier which protects the body from external environmental stressors and insults, enabling the maintenance of organismal homeostasis [27]. Sited at the interface with the environment, skin is in direct and often continuous contact with UVR from sunlight [28]; prolonged UVR exposure disrupts tissue architecture, remodeling components of the dermal ECM (photodamage) [29, 30] and in some cases, eliciting photocarcinogenesis [31, 32]. Hence robust methods of photoprotection are necessary to maintain long term skin health. Such photoprotection can be provided by topical sunscreens [33]; however, these are rarely used by the general public as advised by either manufacturers or health care providers [10]. Hence, systemic methods by which the harmful effects of solar UVR can be mitigated are attractive.

Dietary intervention to boost skin defense is an interesting concept. Evidence from numerous research studies has suggested that foods rich in, for example, omega-3 polyunsaturated fatty acids or polyphenols, may have a number of health benefits, including benefits to skin [18, 34, 35]. *Camellia sinensis* (tea bush) is especially rich in polyphenolic catechins (for a review, see [36]). However, processing of leaves reduces catechin content and so focus has fallen onto the least processed form of tea, green tea, which contains the highest concentration of potential actives [37, 38]. There is significant evidence from animal models and more recently in human intervention studies that GTCs can provide health benefits (for a recent review, see [39]). Specifically in skin, studies have shown that GTC, given orally or topically, increases erythema threshold [40, 41] and systemic antioxidant capacity [42]; the increase in erythema threshold has been further observed in man [16, 24, 43].

Elastic fibers are key elements of tissues which require the ability to deform and recoil to function [44]. In skin, they have a hierarchical but continuous distribution: mature elastic fibers, a composite of elastin encased and reinforced by microfibrils, reside in the deep dermis. These give way to finer elaunin fibers in the reticular dermis (containing less elastin) which finally arborize with oxytalan fibers (fibrillin-rich and fibulin-rich microfibrils) that terminate at the DEJ.

It is these oxytalan fibers which are destroyed in the early stages of photodamage [5]. The mechanism by which these elastic fiber elements are degraded is controversial; certainly they are amenable to remodeling by matrix metalloproteinases (MMPs; [45] but the broad specificity of MMPs make it difficult to reconcile why oxytalan fibers are specifically lost as an early event in the pathogenesis of photodamage, whilst other ECM components such as the fibrillar collagens only reduce at much later stages of the pathology [46, 47]. We have postulated that one factor which may account for the specific loss of oxytalan fibers is that their component proteins are enriched in amino acids which are able to directly absorb UVR (Trp, Met, Cys, His, Tyr) [4, 48, 49]. These amino acids are also oxidation sensitive and so reactive oxygen species, released indirectly from the tissue during irradiation, may further cause addition damage to these specific structures.

To assess whether GTCs could protect these especially vulnerable oxytalan fibers in skin from UVR damage we performed a gold standard, randomized controlled dietary intervention study. Our robust UVR challenge system had a significant effect on the organization of cutaneous elastic fibers as identified by the histological stain, Weigert's resorcin fuchsin ($P<0.001$). Microfibrils containing fibulins-2 and -5 were also significantly remodeled following irradiation ($P<0.001$; both molecules). Surprisingly, only a small reduction in the abundance of fibrillin-rich microfibrils was observed after UVR challenge. Previous work examining the remodeling of fibrillin-rich microfibrils by UVR used extracted microfibrils from *in vitro* and tissue samples (to mitigate the effect of MMPs), irradiated with a broadband UVB light source [48]; it is possible that UVB, with its higher energetic potential, causes more damage to these structures than that which would naturally occur in sunlight. The solar simulated UVR used in the current study reflects that of sunlight (95% UVA, 5% UVB) and so is more likely to reflect the *in vivo* situation.

The intervention described herein used a high dose of GTC plus low dose vitamin C to stabilize the supplement in the gut and aid absorption [50, 51]. Low dose vitamin C has been shown not to effect levels of systemic oxidation *in vivo* [52, 53], nor to be an effective treatment for photodamaged skin [54]. Our previous work shows that dietary GTCs are bioavailable in the skin and so have potential for delivering benefit [19]. Overall, GTC did not protect the entire oxytalan fiber network in skin, as identified by histological staining. However, using the more

sensitive approach of immunohistochemistry allowed us to dissect the effect of supplementation on specific protein components of this network. As our UVR challenge did not impact the abundance of fibrillin-rich microfibrils, the intervention could provide little or no benefit. However, immunohistochemistry identified a specific protection of fibulin-5 microfibrils following irradiation in those supplemented with GTC as opposed to placebo (GTC, no UVR *versus* UVR, P = 0.297; placebo, no UVR *versus* UVR, P = 0.012). Examination of fibulin-2-positive microfibrils identified that GTC provided protection from UVR challenge, but so did the placebo treatment (GTC, no UVR *versus* UVR, P = 0.904; placebo, no UVR *versus* UVR, P = 0.061).

Hence we have shown in a double-blind randomized controlled trial with biochemical confirmation of participant compliance, that daily consumption of 1080 mg GTC (equivalent to 5 cups of green tea; stabilized with 100 mg of vitamin C) protects fibulin-5 microfibrils of the cutaneous elastic fiber network from UVR-induced degradation. The exact mechanism of this protective effect requires further study, but it is plausible that GTCs may act as antioxidants in the skin, buffering the direct and indirect effects of reactive oxygen species elicited when skin is irradiated.

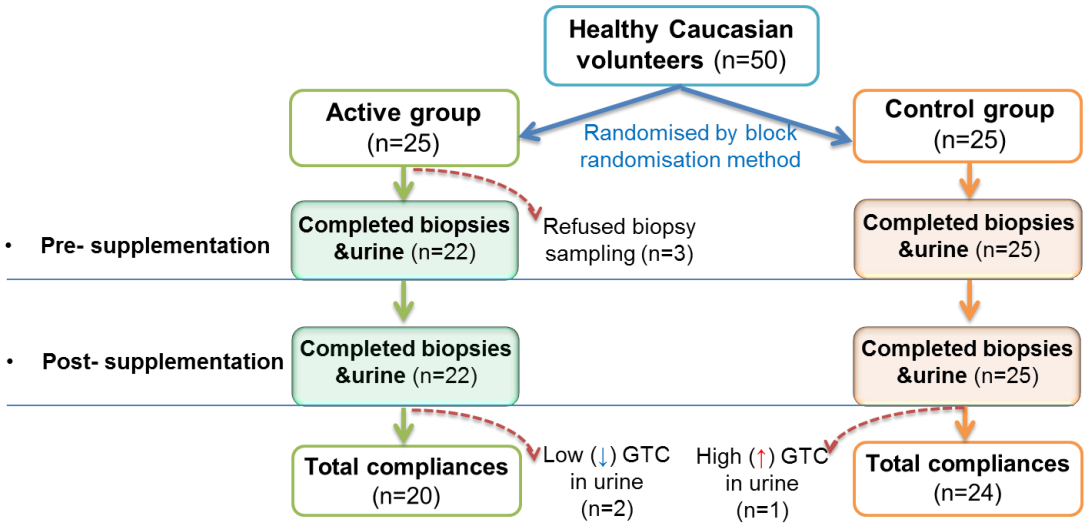


Figure 1: Consort diagram of the randomized placebo-controlled trial to assess the potential of GTC to protect cutaneous elastic fibers from UVR-induced damage. *Some subjects were excluded from the study due to either not following the study process or missing the criteria as green tea catechin chemical traces in urine were quantifiable (under the assay's limit of detection). In total, 5 individuals from the active group were excluded. In control group, only 1 was selected out as found the high level of green tea substance in urine sampling from day 1. For all detail of urinary analysis results were previously informed [18, 24]. Time frame of research was covered from November 2010 to May 2011.*

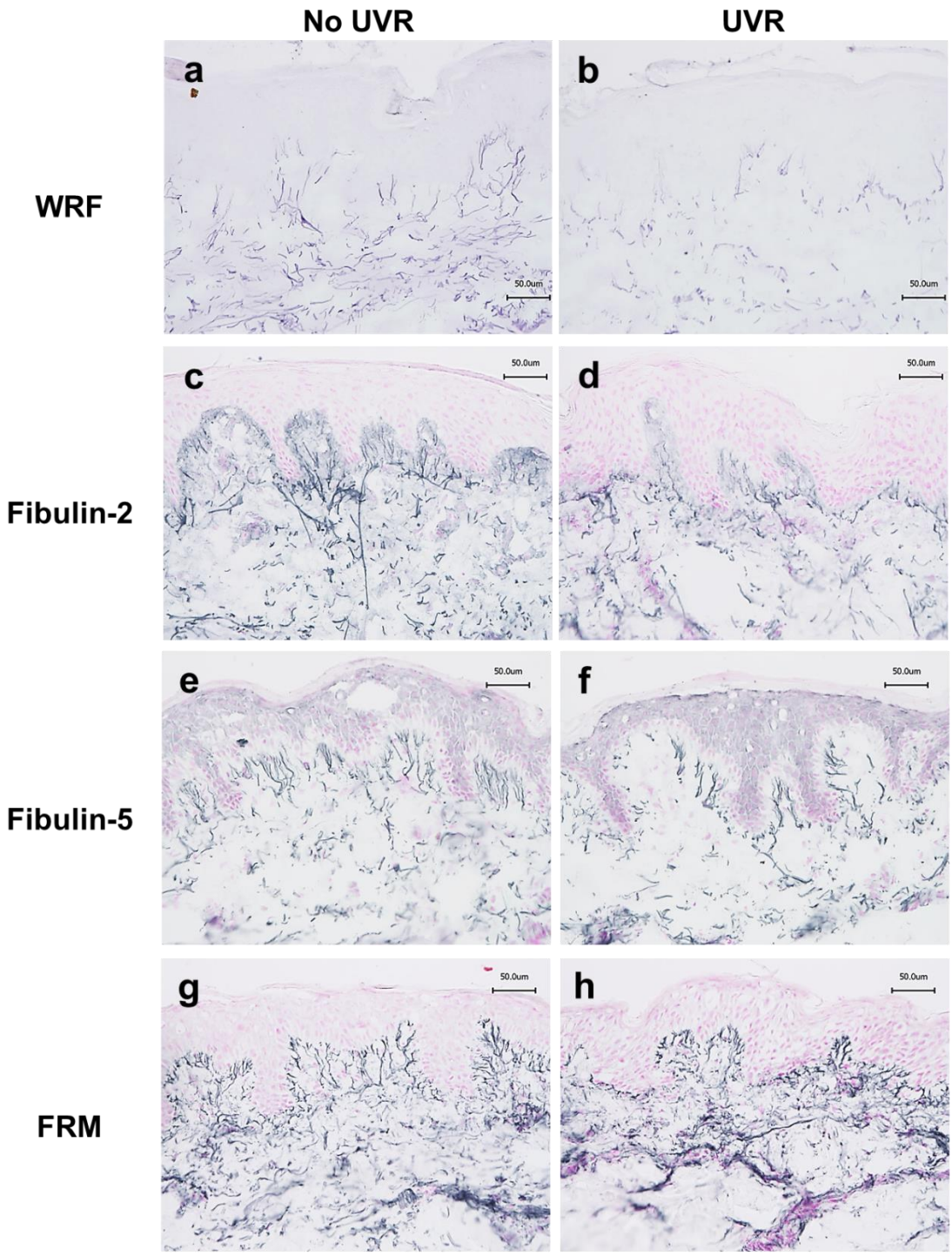


Figure 2: UVR challenge induced remodeling of the elastic fiber network, specifically fibulin-positive microfibrils, in skin. *Histological staining (WRF) reveals elastic fibers in cutaneous tissue (a) unirradiated and (b) 24-hours following UVR challenge with 3x MED of solar simulated radiation. Fine oxytalan fibers can be seen in the papillary dermis perpendicular to DEJ and forming a candelabra-like architecture in unirradiated skin but are lost following UVR. Immunohistochemistry was used to identify specific components of these oxytalan fibers: (c, d) fibulin-2; (e, f) fibulin-5, and; (g, h) fibrillin-rich microfibrils (FRMs). Following irradiation, fibulin-positive microfibrils exhibit remodeling, whilst the quantity of FRMs are unaffected. Scale bar, 50 μm.*

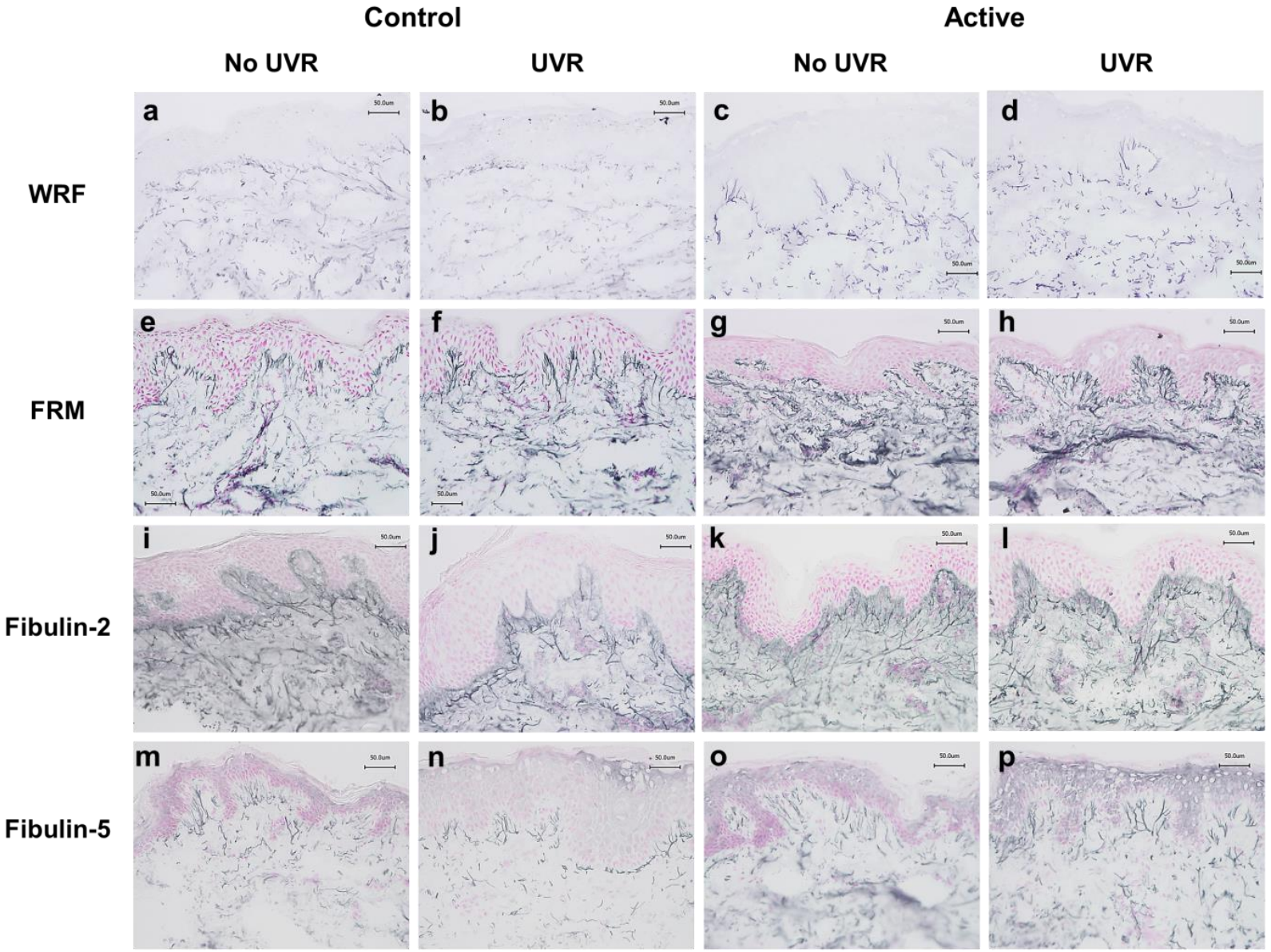

Control
Active
No UVR
UVR
No UVR
UVR
WRF
FRM
Fibulin-2
Fibulin-5
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

Figure 3: Dietary GTC specifically protects fibulin-5 microfibrils from UVR-induced damage. *The effect of GTC on elastic fibers components following UVR challenge: (a-d) Weigert's resorcin fuchsin (WRF); (e-h) fibrillin-rich microfibrils; (i-l) fibulin-2-positive microfibrils, and (m-p) fibulin-5-positive microfibrils. Post-supplementation placebo and GTC failed to protect oxytalan fibers identified by WRF (b, d) or FRM immunohistochemistry (f, h). Both placebo and GTC supplementation had a mitigating effect on loss of fibulin-2 microfibrils following UVR challenge (j, l). However, there was evidence of specific protection with GTC supplementation on the fibulin-5-positive microfibrils (n, p).*

References

1. Wacker, M. and M.F. Holick, *Sunlight and Vitamin D: A global perspective for health.* Dermato-endocrinology, 2013. **5**(1): p. 51-108.
2. Zouboulis, C.C., *The skin as an endocrine organ.* Dermato-endocrinology, 2009. **1**(5): p. 250-252.
3. Brodell, L.A.R., K. S. , *Skin Structure and Function: The Body's Primary Defense Against Infection.* Infectious Diseases in Clinical Practice, 2008. **16**(2): p. 113-117.
4. Watson, R.E.B., et al., *Damage to Skin Extracellular Matrix Induced by UV Exposure.* Antioxidants & Redox Signaling, 2014. **21**(7): p. 1063-+.
5. Watson, R.E.B., et al., *Fibrillin-rich microfibrils are reduced in photoaged skin. Distribution at the dermal-epidermal junction.* Journal of Investigative Dermatology, 1999. **112**(5): p. 782-787.
6. Kadoya, K., et al., *Fibulin-5 deposition in human skin: decrease with ageing and ultraviolet B exposure and increase in solar elastosis.* British Journal of Dermatology, 2005. **153**(3): p. 607-612.
7. Tsuji, T., *Scanning electron microscope studies of solar elastosis.* British Journal of Dermatology, 1980. **103**(3): p. 307-312.
8. Marshall, D.R., *The Clinical and Pathological Effects of Prolonged Solar Exposure Part I.-the association with ageing of the skin.* Australian and New Zealand Journal of Surgery, 1965. **34**(3): p. 161-172.
9. Griffiths, C.M., et al., *A photonumeric scale for the assessment of cutaneous photodamage.* Archives of Dermatology, 1992. **128**(3): p. 347-351.
10. Jansen, R., et al., *Photoprotection Part I. Photoprotection by naturally occurring, physical, and systemic agents.* Journal of the American Academy of Dermatology, 2013. **69**(6).
11. Jou, P.C. and K.J. Tomecki, *Sunscreens in the United States: current status and future outlook.* Advances in experimental medicine and biology, 2014. **810**: p. 464-84.
12. Lambert, J.D., *Does tea prevent cancer? Evidence from laboratory and human intervention studies.* The American Journal of Clinical Nutrition, 2013.
13. Zheng, X.-X., et al., *Green tea intake lowers fasting serum total and LDL cholesterol in adults: a meta-analysis of 14 randomized controlled trials.* American Journal of Clinical Nutrition, 2011. **94**(2): p. 601-610.
14. Liu, K., et al., *Effect of green tea on glucose control and insulin sensitivity: a meta-analysis of 17 randomized controlled trials.* American Journal of Clinical Nutrition, 2013. **98**(2): p. 340-348.
15. Chiu, A.E., et al., *Double-Blinded, Placebo-Controlled Trial of Green Tea Extracts in the Clinical and Histologic Appearance of Photoaging Skin.* Dermatologic Surgery, 2005. **31**: p. 855-860.
16. Janjua, R., et al., *A Two-Year, Double-Blind, Randomized Placebo-Controlled Trial of Oral Green Tea Polyphenols on the Long-Term Clinical and Histologic Appearance of Photoaging Skin.* Dermatologic Surgery, 2009. **35**(7): p. 1057-1065.
17. Heinrich, U., et al., *Green Tea Polyphenols Provide Photoprotection, Increase Microcirculation, and Modulate Skin Properties of Women.* Journal of Nutrition, 2011. **141**(6): p. 1202-1208.
18. Rhodes, L.E., et al., *Oral green tea catechin metabolites are incorporated into human skin and protect against UV radiation-induced cutaneous inflammation in association with reduced production of pro-inflammatory eicosanoid 12-hydroxyeicosatetraenoic acid.* British Journal of Nutrition, 2013. **110**(5): p. 891-900.
19. Clarke, K.A., et al., *Green tea catechins and their metabolites in human skin before and after exposure to ultraviolet radiation.* Journal of Nutritional Biochemistry, 2016. **27**: p. 203-210.
20. Crescenzi, A., et al., *Physicochemical analysis of resorcinol-fuchsin reagents and its relevance to staining quality of elastic fibres.* European Journal of Basic and Applied Histochemistry, 1991. **35**(1): p. 37-44.
21. Proctor, G.B. and R.W. Horobin, *Chemical structures and staining mechanisms of Weigrt resorcin-fuchsin and related elastic fiber stains.* Stain Technology, 1988. **63**(2): p. 101-111.

22. Watson, R.E.B., et al., *A Short-Term Screening Protocol, Using Fibrillin-1 as a Reporter Molecule, for Photoaging Repair Agents.* J Investig Dermatol, 2001. **116**(5): p. 672-678.
23. Abramoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with ImageJ.* Biophotonics international, 2004. **11**(7): p. 36-42.
24. Farrar, M.D., et al., *A randomized controlled trial of green tea catechins in protection against ultraviolet radiation-induced cutaneous inflammation.* American Journal of Clinical Nutrition, 2015. **102**(3): p. 608-615.
25. Watson, R.E. and C.E. Griffiths, *Fibulin-2 and its association with fibrillin-rich microfibrils in the papillary dermis of photoaged skin.* Br J Dermatol, 2002. **146**(4): p. 712.
26. Freeman, L.J., et al., *Fibulin-5 interacts with fibrillin-1 molecules and microfibrils.* Biochemical Journal, 2005. **388**: p. 1-5.
27. Rawlings, A.V. and C.R. Harding, *Moisturization and skin barrier function.* Dermatologic Therapy, 2004. **17**: p. 43-48.
28. Akiba, S., et al., *Influence of chronic UV exposure and lifestyle on facial skin photo-aging--results from a pilot study.* Journal of epidemiology / Japan Epidemiological Association, 1999. **9**(6 Suppl): p. S136-42.
29. Smith, J.G., et al., *Alterations in Human Dermal Connective Tissue with Age and Chronic Sun Damage.* Journal of Investigative Dermatology, 1962. **39**(4): p. 347-350.
30. Schwartz, E., *Connective Tissue Alterations in the Skin of Ultraviolet Irradiated Hairless Mice.* Journal of Investigative Dermatology, 1988. **91**(2): p. 158-161.
31. Weill, F.S., et al., *Skin Exposure to Chronic But Not Acute UV Radiation Affects Peripheral T-Cell Function.* Journal of Toxicology and Environmental Health-Part a-Current Issues, 2011. **74**(13): p. 838-847.
32. Kozma, B. and M.J. Eide, *Photocarcinogenesis: An Epidemiologic Perspective on Ultraviolet Light and Skin Cancer.* Dermatologic Clinics, 2014. **32**(3): p. 301-313.
33. Seité, S. and A.M.A. Fourtanier, *The benefit of daily photoprotection.* Journal of the American Academy of Dermatology, 2008. **58**(5, Supplement 2): p. S160-S166.
34. Paul, N.D. and D. Gwynn-Jones, *Ecological roles of solar UV radiation: towards an integrated approach.* Trends in Ecology & Evolution, 2003. **18**(1): p. 48-55.
35. Rhodes, L.E., et al., *Dietary fish oil reduces basal and ultraviolet B-generated PGE2 levels in skin and increases the threshold to provocation of polymorphic light eruption.* Journal of Investigative Dermatology, 1995. **105**(4): p. 532-535.
36. Zillich, O.V., et al., *Polyphenols as active ingredients for cosmetic products.* International Journal of Cosmetic Science, 2015. **37**(5): p. 455-464.
37. Botten, D., et al., *Structural Properties of Green Tea Catechins.* Journal of Physical Chemistry B, 2015. **119**(40): p. 12860-12867.
38. Harler, C.R., *The culture and marketing of tea*. 1964, London: London : Oxford University Press.
39. Afzal, M., A.M. Safer, and M. Menon, *Green tea polyphenols and their potential role in health and disease.* Inflammopharmacology, 2015. **23**(4): p. 151-161.
40. Jeon, H.Y., et al., *Effects of Oral Epigallocatechin Gallate Supplementation on the Minimal Erythema Dose and UV-Induced Skin Damage.* Skin Pharmacology and Physiology, 2009. **22**(3): p. 137-141.
41. Kim, J., et al., *Protective effects of (-)-epigallocatechin-3-gallate on UVA- and UVB-induced skin damage.* Skin Pharmacology and Applied Skin Physiology, 2001. **14**(1): p. 11-19.
42. Jeon, H.Y., et al., *Beneficial Effect of Dietary Epigallocatechin-3-Gallate on Skin via Enhancement of Antioxidant Capacity in Both Blood and Skin.* Skin Pharmacology and Physiology, 2010. **23**(6): p. 283-289.
43. de Gruijl, F.R., *Photocarcinogenesis: UVA vs UVB*, in *Methods in Enzymology*. 2000, Academic Press. p. 359-366.
44. Sandberg, L.B., N.T. Soskel, and J.G. Leslie, *Elastin Structure, Biosynthesis, and Relation to Disease states.* New England Journal of Medicine, 1981. **304**(10): p. 566-579.
45. Ashworth, J.L., et al., *Fibrillin degradation by matrix metalloproteinases: implications for connective tissue remodelling.* Biochemical Journal, 1999. **340**: p. 171-181.
46. Fisher, G.J., et al., *Pathophysiology of premature skin aging induced by ultraviolet light.* New England Journal of Medicine, 1997. **337**(20): p. 1419-1428.
47. Rittie, L. and G.J. Fisher, *Natural and Sun-Induced Aging of Human Skin.* Cold Spring Harbor Perspectives in Medicine, 2015. **5**(1).

48. Sherratt, M.J., et al., *Low-dose ultraviolet radiation selectively degrades chromophore-rich extracellular matrix components.* Journal of Pathology, 2010. **222**(1): p. 32-40.
49. Hibbert, S.A., et al., *A potential role for endogenous proteins as sacrificial sunscreens and antioxidants in human tissues.* Redox Biology, 2015. **5**: p. 101-113.
50. Chung, J.-H., et al., *Green Tea Formulations with Vitamin C and Xylitol on Enhanced Intestinal Transport of Green Tea Catechins.* Journal of Food Science, 2013. **78**(5): p. C685-C690.
51. Chen, Z.-Y., et al., *Stabilizing Effect of Ascorbic Acid on Green Tea Catechins.* Journal of Agricultural and Food Chemistry, 1998. **46**(7): p. 2512-2516.
52. McArdle, F., et al., *UVR-induced oxidative stress in human skin in vivo: effects of oral vitamin C supplementation.* Free Radical Biology and Medicine, 2002. **33**(10): p. 1355-1362.
53. Herbert, K.E., et al., *Dietary supplementation with different vitamin C doses: no effect on oxidative DNA damage in healthy people.* European Journal of Nutrition, 2006. **45**(2): p. 97-104.
54. Brenner, M. and V.J. Hearing, *The Protective Role of Melanin Against UV Damage in Human Skin†.* Photochemistry and Photobiology, 2008. **84**(3): p. 539-549.

Chapter 5 - An acute dose of solar simulated radiation effects on cutaneous collagenous matrices and procollagens and protective efficacy of green tea catechins supplementation

Running head: Dietary GTC and protection of dermal collagen I from SSR-induced damage

N. Charoenchon[1], L.E. Rhodes[1,2], A. Nicolaou[3], G. Williamson[4], M.D. Farrar[1,2], R.E.B. Watson[1]*

[1]Centre for Dermatology Research, The University of Manchester, Manchester Academic Health Science Centre, Manchester M13 9PT, UK;

[2]Photobiology Unit, Salford Royal NHS Foundation Trust, Manchester Academic Health Science Centre, Salford M6 8HD, UK;

[3]Manchester Pharmacy School, The University of Manchester, Manchester Academic Health Science Centre, Manchester M13 9PT, UK;

[4]School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK.

*Corresponding author: Dr Rachel Watson
2.100 Stopford Building
The University of Manchester
Oxford Road
Manchester M13 9PT
UK
Telephone: +44 161 275 5505
Email: rachel.watson@manchester.ac.uk

Word count (3500): **Tables:** **Figures:** 2

ORCiD references: N. Charoenchon (0000-0001-7043-1218)
L.E. Rhodes
A. Nicolaou
G. Williamson
M.D. Farrar
R.E.B. Watson (0000-0002-5162-7503)

Funding: Supported by grants BB/G005575/1 (to LER), BB/G005540/1 (to AN) and BB/G005559/1 (to GW) from the Biotechnology and Biological Sciences Research Council, Diet and Health Research Industry Club.

Conflict of interest: None

What is already known about this topic?

- Fibrillar collagens and procollagens type I (pCI) and III (pCIII) are decreased in chronically photoaged skin compared to photoprotected skin.
- Long-term of green tea catechin (GTC) supplementation significantly enhanced overall of clinical signs of chronic photodamaging in facial skin in human *in vivo*.

What does this study add?

- Acute dose of solor simulated radiation (SSR) significantly reduced mature collagens whist the same dose influence newly synthesized of pCI.
- High doses of GTC supplementation efficiently supported mature collagens from acute photodamage in human *in vivo*.

Abbreviations:

GTC, green tea catechins; IHC, immunohistochemistry; IMS, industrial methylated spirit; MMP, matrix metalloproteinase; pCI, procollagen I; PSR, picrosirius red; SSR, solar simulated radiation; TBS, Tris–buffered saline

Summary

Background: Dermal fibrillar collagens provide tissue strength and resilience; with long-term photoexposure, this collagen matrix is remodelled with dermal fibroblasts exhibiting reduced capacity to synthesise new collagen. Green tea catechins (GTC) are dietary polyphenols thought to act as antioxidants with the ability to reduce and/or prevent oxidative tissue damage. Hence, GTC consumption may provide protection to dermal collagens following photoexposure.

Objectives: To assess whether GTC protect the dermal collagen network following an acute challenge of solar simulated radiation (SSR).

Methods: Fifty healthy volunteers were recruited and randomized to GTC (1080 mg plus 100 mg vitamin C; n=25) or placebo (maltodextrin; n=25) daily for 12-weeks with compliance assessed biochemically in urine samples. Prior to and following the intervention, skin biopsies were obtained from unirradiated skin and 24-hrs following irradiation challenge (3x minimal erythemal dose). Biopsies were processed to identify the organisation of fibrillar collagens (collagens I and III by picrosirius red staining) and deposition of newly synthesised procollagen (pCI).

Results: Of the n=50 recruited, 44 volunteers completed the study. Prior to supplementation, acute irradiation significantly reduced papillary dermal fibrillar collagens ($P<0.001$) and induced deposition of newly synthesised pCI ($P=0.02$). Following supplementation, GTC enhanced the deposition of thin collagen fibres in the dermis. Whilst placebo supplementation had no additional effect on the altered the organisation of fibrillar collagens or deposition of pCI following the irradiation challenge, GTC protected the organisation of fibrillar collagens in the papillary dermis ($P=0.97$).

Conclusions: Dietary GTC protects the arrangement of fibrillar collagens in the papillary dermis, but does not appear to impact collagen synthesis.

Introduction

Solar ultraviolet radiation (UVR) has significant biological effects, causing an acceleration of the ageing process and initiating cutaneous skin cancer. Whilst UVC (wavelengths 100-280 nm) is filtered via the ozone layer before reaching the Earth's surface [1, 2], UVA (320-400 nm) and UVB (280-320 nm) penetrates to the surface and has the ability to induce changes to skin structure [3] and function [4]. These wavelengths of UVR are thought to be a significant factor in accelerated skin ageing, termed photoageing [for a review, see [5]]

The clinical appearance of photoaged skin is distinct from that seen in natural skin ageing, with the appearance of profound wrinkles, skin laxity, a sallow complexion and the occurrence of hyper- and hypopigmented lesions [6]. These changes to the appearance of skin are thought to be underpinned by specific pathologies including the remodelling of extracellular matrix (ECM) components, particularly loss of collagens [7] and deposition of amorphous and non-functioning elastin (known as solar elastosis; [8] in the severe chronic photoageing case, rhomboidal architecture would be replaced the smooth skin [9]. Fibrillar collagens, the major isoforms being collagens type I and III provide skin with firmness and strength [10]; with photoageing, these components are lost [11]. Mechanistically, it is unlikely that fibrillar collagens can directly absorb energy from UVR, having only a very small number of UVR-absorbing amino acids [12, 13]. More likely is that UVR initiates the production and activation of matrix metalloproteinases (MMPs) via induction of the AP-1 pathway in both keratinocytes and fibroblasts [14, 15].

The action of MMPs on fibrillar collagen results in the production of gelatin and small peptide fragments [16]; such matrikines may further initiate MMP activation, so making a vicious cycle which results in further ECM degradation [for a review, see [17]].

Procollagens, soluble precursor of insoluble fibrillar collagen produced and secreted from dermal fibroblasts, have been shown to significantly reduce their synthesis of collagen mRNA species by up to 50% following UVR exposure *in vitro* [7]. Such a response has also been observed *in vivo*, where an acute dose of 1x MED UVR was shown to reduce both procollagen I (pCI) and procollagen III (pCIII) within the dermal ECM [18].

Plant extracts are purported to contain molecules beneficial to human health, such as antioxidant vitamins (A, C and E) and polyphenols. In skin, numerous groups have suggested that such compounds, either applied topically or taken orally have the ability to protect skin from UVR-induced damage [19-22]. Catechins are a class of polyphenols which have been shown *in vitro* and in animal models to partially protect skin from the unwanted adverse effects of UVR (e.g. inflammation, immunosuppression and DNA damage) [23, 24]. Green tea is particularly enriched in these catechins and is drunk in large quantities globally [25]. Hence, green tea catechins (GTC) may make a potentially interesting dietary supplement which may have the ability to systemically enhance skin photo-protection.

To clarify whether dietary supplementation with GTC can protect skin from UV-induced damage to the collagen matrix, we have performed a gold standard randomised placebo controlled trial. A robust UVR challenge was performed prior to, and following, dietary intervention, with subsequent quantification of collagen (deposition of both mature fibrillar collagen and the amino pro-peptide of pCI).

Materials and methods

Randomized control trial

The study was conducted according to the principles of the Declaration of Helsinki and was reviewed and approved by North Manchester Research Ethics Committee (study ref: 08/H1006/79). The trial was registered at clinicaltrials.gov (identifier: NCT01032031). Healthy Caucasian volunteers (n=50; male and female, 18-65 years) with Fitzpatrick skin type I-II enrolled in the study in response to a public advert. Pre-screening ensured that potential volunteers did not have existing skin problems (previous skin cancer, dermatologist-verified skin disease or condition), were not regular sunbed users or consumed >2 cups of tea daily in the 3 month period immediately prior to study initiation. Currently pregnant or breastfeed women were also excluded from the study.

Volunteers were allocated to either active or placebo control groups in a ratio of 1:1 by block randomization method (Statsdirect v2.7.8, StatsDirect Ltd.). All supplements were produced by Nestec Ltd. (Vevey, Switzerland) and packed by Laboratoire LPH in identically appearing gelatin capsules. Active group volunteers were given 1080 mg GTC supplement (1.8 mg gallic acid; 12.6 mg catechins; 75.0 mg epicatechin; 74.4 mg gallocatechin;295.8 mg epigallocatechin; 1.8 mg catechin gallate; 156.0 mg epicatechin gallate; 28 mg gallocatechin gallate; 435.6 mg epigallocatechin gallate) and 100 mg vitamin C which acted to stabilise the GTC in the gut lumen to so increase GTC absorption [26]; those in placebo control group took maltodextrin in identical gelatin capsules. The dietary intervention was taken daily for 12-weeks. Compliance was performed by analysis of urinary epigallocatechin glucouronide in all subjects at study initiation, at study mid-point (6-weeks) and at study completion (12-weeks) [27].

Irradiation protocol and tissue sampling

Sunlight is composed of UVA and UVB wavelengths (95% UVA, 5% UVB; 290-400 nm); hence we chose a solar simulator to provide a robust UVR challenge (SSR; Newport Spectra-Physics Ltd.; Didcot, UK). Prior to irradiation, the output of the lamp was measures to ensure consistency between volunteers (IL 730A radiometer; International Light Technologies; Peabody, MA, USA). The minimal erythemal dose (MED) for each volunteer was assessed prior to and following intervention; each person providing an unirradiated sample and a sample 24-hrs after a 3x MED SSR challenge at each time point. Skin punch biopsies (5 mm diameter) were obtained following 1% lidocaine anaesthesia. All biopsies were embedded in Tissue-Tek® OCT (Sakura Finetek USA INC., Torrance, CA, USA) and snap frozen in liquid nitrogen. Frozen tissue blocks were stored at -80ºC prior to histological processing.

Picrosirius red histology

Frozen sections (7 µm thickness; OTF cryostat; Bright Instruments Ltd, Cambridge, UK) were prepared and stained with picrosirius red (PSR) to identify the mature fibrillar collagen network of the skin [28]. Sections were fixed in 90% industrial methylated spirits (IMS) before brief emersion into 0.1% (w/v) of sirius red F3BA in saturated aqueous picric acid. Excessive staining was removed by rinsing in 0.1% acetic acid and deionised water. Finally, sections were dehydrated through serial alcohols (70%-100% IMS), cleared in xylene and permanently mounted using DePex (Fisher Scientific; Loughborough, UK).

Immunohistochemistry

Immunohistochemistry (IHC) was used to identify new synthesised pCI [29]. Sections were fixed in 4% (v/w) paraformaldehyde in phosphate-buffered saline, with 0.5% Triton-X-100 used to solubilise lipids. Endogenous peroxidase activity was abolished by incubating tissue with hydrogen peroxide in methanol. Following blocking with normal rabbit serum plus bovine serum albumin, sections were probed with a rat anti-human antibody directed to the amino pro-peptide of collagen I (pCI; MAB1912, dilution 1:200 [Millipore, Darmstadt, Germany]). Following incubation, binding was visualised using a commercially-available horseradish peroxidase-based kit (Vector *Elite* ABC; Vector Labs.; Burlingame, CA USA) and Vector SG® chromogen

solution (Vector Labs.). Sections were counterstained with Nuclear Fast Red (Vector Labs.), serially dehydrated, cleared and permanently mounted as above.

Image analysis and quantification

Sections subjected to PSR staining were captured under both polarised and bright field microscopy (Leitz DMRB; Leica Microsystems Inc.; Buffalo Grove, IL, USA) [30, 31]. Three photomicrographs were analysed per volunteer per treatment using ImageJ (NIH; Bethesda, MA, USA; [32]); these identified the percentage coverage of fibrillar collagens in each section.

Tissue positively stained to identify pCI were captured using bright field microscopy (Biozero-800 'all-in-one' microscope; Keyence; Osaka, Japan) and were analysed using a 5-point semi-quantitative method as previously described [33] where 0 = absence of stain to 4 = maximal staining in the papillary dermis proximal to the dermal epidermal junction (DEJ).

For both methods of analysis, a numerical mean was calculated for each treatment (with and without irradiation) for pre- and post-intervention biopsies. This data was subsequently used for statistical analysis.

Statistical analysis

Differences in SSR-induced alterations in collagen coverage between active GTC supplementation and placebo control groups following 12-weeks of intervention were analysed by ANCOVA, with baseline data taken as the covariate. Paired Student's t-tests were applied to compare irradiated and unirradiated skin both prior to and following the intervention.

The statistical package SPSS was used for all analyses (v22; IBM Corp.; Armonk, NY, USA).

Results

Volunteer demographics and compliance

Fifty healthy volunteers were recruited to this study (13M, 37F) and randomly allocated to active GTC supplementation or placebo (n = 25 per group); of the fifty recruited, 44 completed the study according to the protocol (4 failed compliance tests and 2 withdrew consent to biopsy) (*figure 1*). Hence, the analyses included *n* = 20 volunteers in the active GTC group and *n* = 24 volunteers in the placebo group.

Acute SSR challenge induces a significant reduction in fibrillar collagen, as identified by PSR staining

At baseline, we found a significant reduction in the mean ± SE percentage area coverage by collagen fibres, as determined by PSR staining, following irradiation with 3x MED of SSR (unirradiated, 51.8 ± 1.2 %; SSR, 46.3 ± 1.4 %, P = 0.001; n = 44; *figure 2*). Post-supplementation, ANCOVA analysis revealed no significant difference in percentage coverage of collagen in SSR-exposed skin between the active and placebo groups (P = 0.12; *figure 2*).

In contrast to baseline findings, intragroup analyses showed no significant change in fibrillar collagen coverage following SSR challenge in the active group (52.0 ± 1.6 % and 52.1 ± 2.0 % in unirradiated and SSR-exposed skin respectively; P = 0.97) or the placebo group (51.8 ± 1.8 % and 48.4 ± 1.8 % respectively; P = 0.15; *figure 2*).

Acute SSR challenge induces the deposition of procollagen I

At baseline, irradiation with SSR induced a significant accumulation of pCI in the papillary dermis (mean ± SE staining score in unirradiated skin 2.7 ± 0.1; SSR, 2.9 ±0.10; n = 44;

P = 0.02; *figure 3*). Post-supplementation, ANCOVA analysis of newly synthesised pCI deposition in SSR-exposed skin revealed no significant difference in the staining score between active and placebo groups (P = 0.81). In line with baseline findings, intragroup analyses showed a significant increase in pCI following SSR challenge in both the active group (unirradiated

2.8 ± 0.1, SSR 3.1 ± 0.1; P = 0.005) and placebo group (unirradiated, 2.6 ± 0.2, SSR, 2.9 ± 0.2; *P* < 0.001).

Discussion

Fibrillar collagens (type I and type III) are the most abundant collagens in skin, with collagen I comprising approximately 85-90 % of the total [34]. Anatomically, some areas of human skin, such as the face and hands, are subject to long-term, chronic irradiation from sunlight; in these regions, skin becomes clinically photodamaged, exhibiting fine and coarse wrinkles and altered pigmentation. One of the major pathological features associated with photodamage is the significant loss of dermal fibrillar collagens [7]. In human skin, it has long been recognised that long-term chronic photoexposure results in a loss of mature papillary dermal fibrillar collagen [35] and altered deposition of newly synthesised material [36, 37];, estimated as a reduction in chronically photodamaged skin by scoring for the intensity of collagen immunostaining >20% for pCI and >30% for pCIII.

Here we show, using a placebo controlled, randomised trial design that oral supplementation of GTC protects mature collagen fibres within the dermal ECM from UVR-induced damage (placebo $P=0.17$; active $P=0.79$) but has no effect on pCI synthesis (placebo $P<0.001$; active $P=0.005$).

Experimentally, repeated irradiation with UVR impacts on the expression and deposition of collagens, mediated potentially by UV-induced oxidative damage and matrix metalloproteinase expression and activity. Multiple doses of SSR (16 weeks; 3x per week) have been shown in Skh:HR1 mice to induce the deposition of abnormal collagen deposits in the papillary dermis [3]. Repetitive acute UVR irradiations (for a period of 10 days) has further been shown to up-regulate protein oxidation in human skin *in vivo* [38]. However, the effect of acute SSR irradiation on the organisation of the mature collagenous matrix *in situ* has not been studied in depth. In this novel *in vivo* human study, we show that a single robust dose of SSR (3x MED) caused a significant reduction in the amount of mature collagen fibres within the superficial papillary dermis ($P<0.001$) 24 h after UVR challenge. To assess whether this observation was simply a reorganisation of the existing fibres or reflects breakdown of collagen matrix warrants further investigation; acute UVR irradiation is also known to induce the expression of a number of matrix metalloproteinases with the ability to turnover ECM components [14]. Irradiation

induces the expression and enhanced activity of MMPs-1, -2, -3, -7, -8, -9, -10 and -12; with MMPs-1, -3 and -9 known to have particular efficacy in causing damage to collagens [39-41].

Irradiation may also impact the collagenous matrix by influencing the synthesis of fibrillar collagens by fibroblasts resident in the dermis. Collagen is synthesised as a soluble pro-peptide or pC which is composed a triple helix of molecule-specific α-chains; the amino and carboxy termini of immature collagen is cleaved to allow processing to mature collagen [42]. Hence, one method of assessing collagen synthesis is to examine the deposition of, for example, the amino-pro-peptide. This method has been used extensively to assess the efficacy of topical treatments for photodamaged skin [29]. Here we show that deposition of newly synthesised pCI is significantly increased 24 h after irradiation (P=0.015); this is in agreement with a report by Chung et al. (2001) which found a significant increase in gene expression of COL1(α1) mRNA in fibroblasts by *in situ* hybridization was significantly higher in photodamaged as compared to photoprotected skin [43]. It is possible that this increase in COL1(α1) expression is an attempt by the fibroblast to counteract ECM degradation caused by UVR. Matrikines – short degradation products of proteolytic ECM cleavage - can further influence suppression or activation of ECM synthesis. Collagen degradation could be indirectly promoted from collagenous fragments that arise from oxidative stress in human fibroblasts [44]. However, some small peptide fragments, e.g. KTTKS pentapeptide (Lys-Thr-Thr-Lys-Ser) has been used topically in over-the-counter anti-ageing formulations to induce collagen I expression [45].

An upregulation of collagen gene expression has also been observed after acute infrared irradiation (IR; emission spectra 600-1,120 nm; [46]. Experiments in human volunteers showed that acute IR induced pCI gene expression examined through IHC staining, semi-quantitative RT-PCR and Western blot analysis. Hence, some of the actions seen here in our *in vivo* SSR study might be due to the production of heat stress within the tissue.

Supplementation of cell culture media with GTC has identified a stimulation of collagen synthesis [47]. Using a murine model of photodamage, Vayalil et al (2004) discovered that GTC inhibited protein oxidation and the induction of MMP expression following UVB irradiation [48]. Subsequently, GTC supplementation with additional GTC-containing topical therapy has been studied in humans [49]; this relatively short intervention study (8 weeks) found no benefit to the active treatment when histological features such as epidermal thickness, melanin content,

dermis including dermal collagen were assessed. A later study of long term oral supplementation with 250 mg GTPs twice daily (24 months) confirmed that GTPs did not elevate dermal collagen compared to placebo [50]. Although these studies have assessed some aspects of change to the collagen matrix *in vivo*, the studies did not challenge the skin at all.

In our study, we delivered a controlled dose of solar simulated radiation; hence, it is possible that the beneficial effects of GTC are only observed when the skin is subjected to stress. To note is that our dietary intervention further contained low dose vitamin C in the active group; vitamin C is known to stabilise GTC in the gut lumen, allowing increased absorption [26, 51]. Vitamin C is an essential vitamin for the synthesis of collagens [52]; it is possible that taken in combination, these dietary supplements influence collagen production. However, there is evidence that oral supplementation with vitamin C alone has little or no effect on the deposition of collagen in the skin [53].

Major constituents of green tea polyphenol [(+)-gallocatechin; EGC, (−)-epigallocatechin; C, (+)-catechin; EC, (−)-epicatechin; EGCg, (−)-epigallocatechin gallate; GCg, (+)-gallocatechin gallate; ECg, (−)-epicatechin gallate] potentially have antioxidant activity, particularly EGCG [54]. Phenolic groups in GTCs are easily oxidized and turned to be quinone (see review [55]). GTCs directly affect free radicals via hydrogen atom transfer (HAT) or single electron transfer reactions (SET) that have relativity with hydroxyl groups on B- (in EC and EGC) and both B- and D- rings (in ECG and EGCG) of GTC structure [56].

Hence, it appears from these studies that GTC supplementation has a positive effect on the remodelling of the collagenous matrix caused by acute UVR irradiation. The exact mechanisms behind this benefit requires further study but is likely to include an increase in the antioxidant capacity of the tissue, making it better equipped to withstand exogenous stressors, such as UV irradiation.

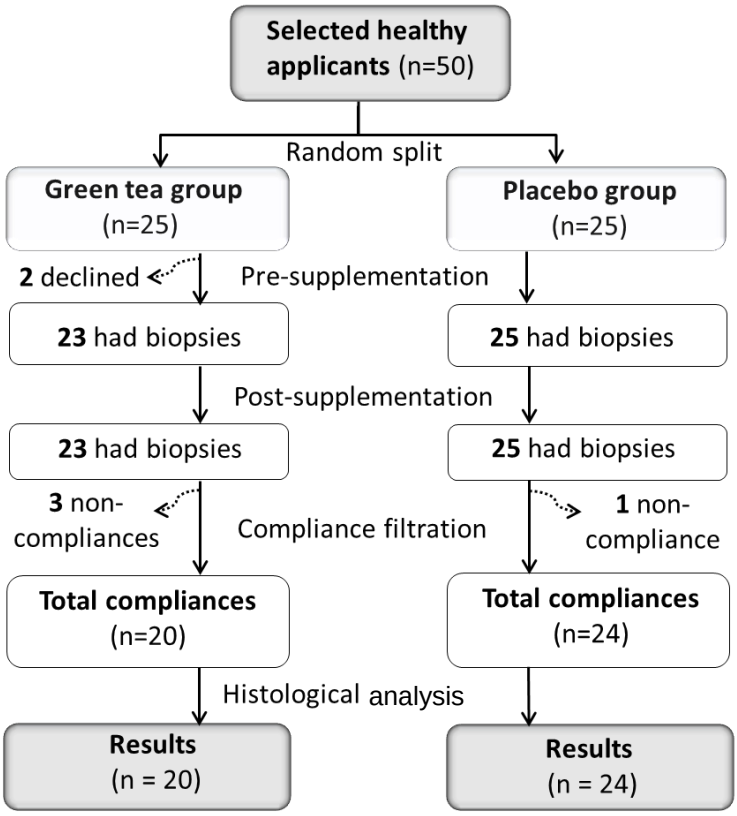


Figure 1. Diagram describes the recruitment, randomisation and sampling within this study.
Healthy participants were recruited (n = 50) and randomized to placebo maltodextrin or GTC supplement respectively.

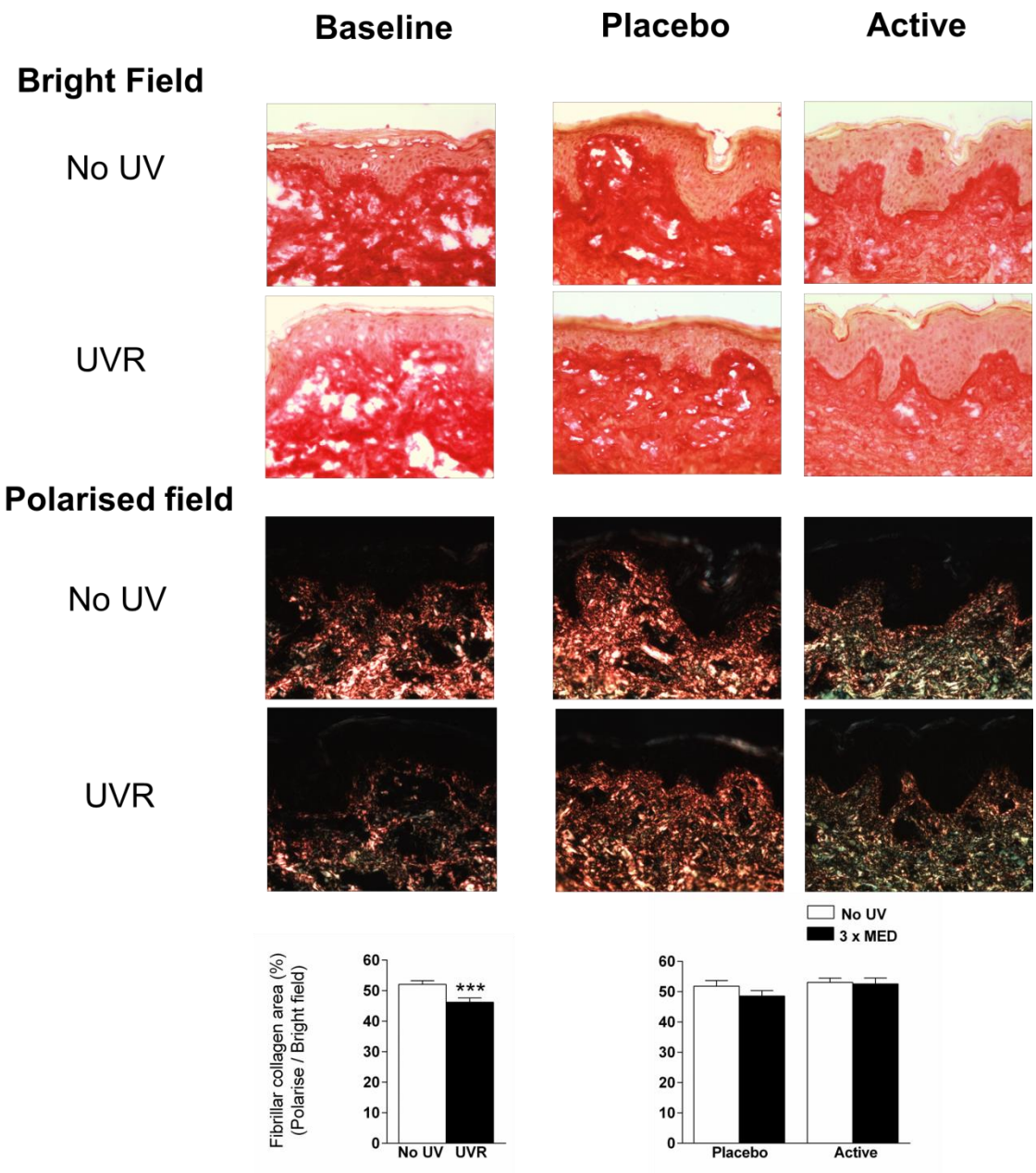


Figure 2: Picrosirius red histology reveals the organisation of the fibrillar collagenous matrix in human skin*. All samples were stained with PSR to identify areas of collagenous ECM, prior to and following a robust UVR challenge with 3x MED of SSR. Biopsy samples were taken prior to, and following 12-weeks intervention with either a placebo (maltodextrin) or active (GTC plus vitamin C) supplementation. In bright field view, staining indicated the area of ECM. Under polarised light, discrete collagen fibres can be observed: red fibres, thick or mature collagen and; green fibres, thin or immature collagen (final magnification, x200). Polarised field view is performed for the percentage occupied by fibrillar collagens calculation. Quantification of collagen percentage occupancy is displayed in the bottom panel. Black bar, SSR; white bar, no UV.*

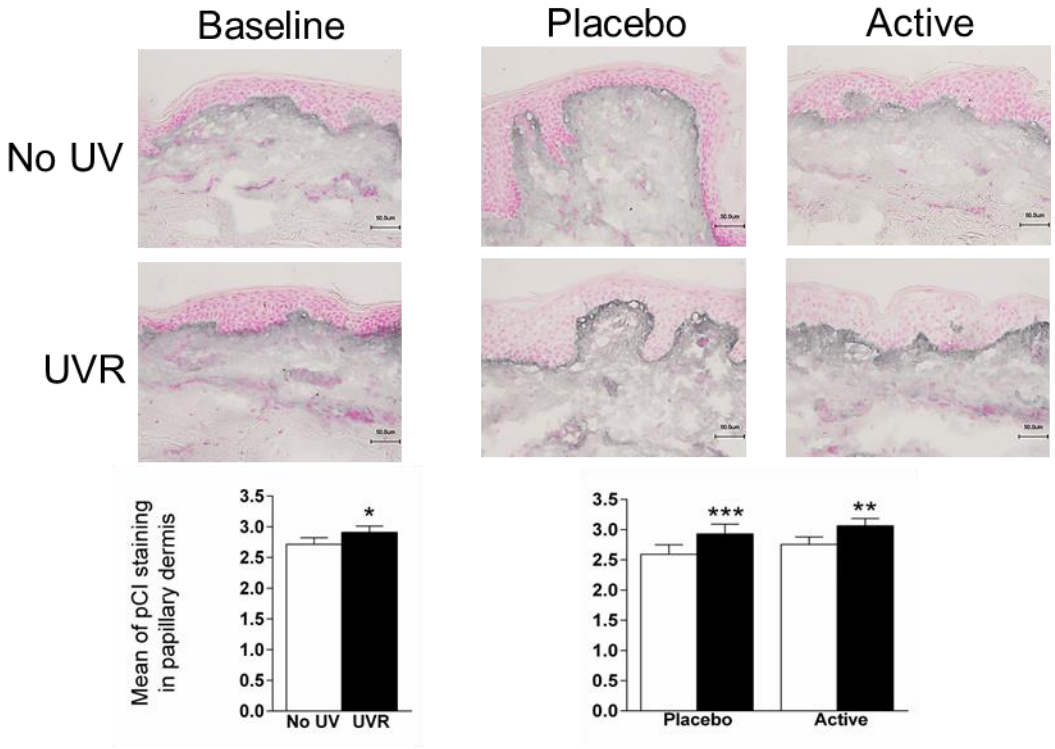


Figure 3. Acute SSR induces the deposition of procollagen I in the papillary dermis of skin. *In positive staining, dermis would be developed to be grey representing pCI component whereas pale pink represents the counter staining nucleus in epidermal cells. Scale bar =50 µm. pCI was increased in both active and control group at 24 hour after UV-protected buttock skin irradiated with 3x MED of SSR.*
** P< 0.05, ** P< 0.01 and *** P< 0.001, compared to irradiated skin (Student T-test).; pCI, procollagen I; UVR, ultraviolet radiation; MED, minimal erythema dose; post-supp, after supplementation. Black bar, SSR; white bar, no UV.*

References

1. Anderson, R.R. and J.A. Parrish, *The Optics of Human Skin.* Journal of Investigative Dermatology, 1981. **77**(1): p. 13-19.
2. Popov, A.P., et al., *Biophysical mechanisms of modification of skin optical properties in the UV wavelength range with nanoparticles.* Journal of Applied Physics, 2009. **105**(10).
3. Bissett, D., D. Hannonand, and T. Orr, *An animal model of solar-aged skin: Histologic, physical and visible changes in UV-irradiated mouse skin.* Photochemistry and Photobiology, 1987. **46**(3): p. 367-378.
4. Biniek, K., K. Levi, and R.H. Dauskardt, *Solar UV radiation reduces the barrier function of human skin.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(42): p. 17111-17116.
5. Naylor, E.C., R.E.B. Watson, and M.J. Sherratt, *Molecular aspects of skin ageing.* Maturitas, 2011. **69**(3): p. 249-256.
6. Flament, F., et al., *Effect of the sun on visible clinical signs of aging in Caucasian skin.* Clinical, Cosmetic and Investigational Dermatology, 2013. **6**: p. 221-232.
7. Quan, T.H., et al., *Solar ultraviolet irradiation reduces collagen in photoaged human skin by blocking transforming growth factor-beta type II receptor/Smad signaling.* American Journal of Pathology, 2004. **165**(3): p. 741-751.
8. Seite, S., et al., *Elastin changes during chronological and photo-ageing: the important role of lysozyme.* Journal of the European Academy of Dermatology and Venereology, 2006. **20**(8): p. 980-987.
9. Ma, W., et al., *Chronological ageing and photoageing of the fibroblasts and the dermal connective tissue.* Clinical and Experimental Dermatology, 2001. **26**(7): p. 592-599.
10. Fleischmajer, R., et al., *Ultrastructural identification of extension aminopropeptides of type I and III collagens in human skin.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1981. **78**(12): p. 7360-7364.
11. Schwartz, E., et al., *Collagen alterations in chronically sun-damaged human skin.* Photochemistry and Photobiology, 1993. **58**(6): p. 841-844.
12. Sherratt, M.J., et al., *Low-dose ultraviolet radiation selectively degrades chromophore-rich extracellular matrix components.* Journal of Pathology, 2010. **222**(1): p. 32-40.
13. Watson, R.E.B., et al., *Damage to Skin Extracellular Matrix Induced by UV Exposure.* Antioxidants & Redox Signaling, 2014. **21**(7): p. 1063-+.
14. Fisher, G.J., et al., *Molecular basis of sun-induced premature skin ageing and retinoid antagonism.* Nature, 1996. **379**(6563): p. 335-339.
15. Wang, X.Y. and Z.G. Bi, *UVB-irradiated human keratinocytes and interleukin-1alpha indirectly increase MAP kinase/AP-1 activation and MMP-1 production in UVA-irradiated dermal fibroblasts.* Chin Med J (Engl), 2006. **119**(10): p. 827-31.
16. Fisher, G.J., et al., *Pathophysiology of premature skin aging induced by ultraviolet light.* New England Journal of Medicine, 1997. **337**(20): p. 1419-1428.
17. Maquart, F.X., et al., *Matrikines in the regulation of extracellular matrix degradation.* Biochimie, 2005. **87**(3–4): p. 353-360.
18. Seité, S., et al., *Changes in matrix gene and protein expressions after single or repeated exposure to one minimal erythemal dose of solar-simulated radiation in human skin in vivo.* Photochemistry and Photobiology, 2004. **79**(3): p. 265-271.
19. Eberlein-Konig, B., M. Placzek, and B. Przybilla, *Protective effect against sunburn of combined systemic ascorbic acid (vitamin C) and d-alpha-tocopherol (vitamin E).* Journal of the American Academy of Dermatology, 1998. **38**(1): p. 45-48.
20. Stahl, W., et al., *Carotenoids and carotenoids plus vitamin E protect against ultraviolet light-induced erythema in humans.* American Journal of Clinical Nutrition, 2000. **71**(3): p. 795-798.
21. Mittal, A., C.A. Elmets, and S.K. Katiyar, *Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation.* Carcinogenesis, 2003. **24**(8): p. 1379-1388.
22. Thring, T., P. Hili, and D. Naughton, *Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants.* Bmc Complementary and Alternative Medicine, 2009. **9**(1): p. 27.

23. Afaq, F. and S.K. Katiyar, *Polyphenols: Skin Photoprotection and Inhibition of Photocarcinogenesis.* Mini-Reviews in Medicinal Chemistry, 2011. **11**(14): p. 1200-1215.
24. Katiyar, S.K., *Green tea prevents non-melanoma skin cancer by enhancing DNA repair.* Archives of Biochemistry and Biophysics, 2011. **508**(2): p. 152-158.
25. Harler, C.R., *The culture and marketing of tea*. 1964, London: London : Oxford University Press.
26. Chung, J.-H., et al., *Green Tea Formulations with Vitamin C and Xylitol on Enhanced Intestinal Transport of Green Tea Catechins.* Journal of Food Science, 2013. **78**(5): p. C685-C690.
27. Clarke, K.A., et al., *Green tea catechins and their metabolites in human skin before and after exposure to ultraviolet radiation.* Journal of Nutritional Biochemistry, 2016. **27**: p. 203-210.
28. Lattouf, R., et al., *Picrosirius Red Staining: A Useful Tool to Appraise Collagen Networks in Normal and Pathological Tissues.* Journal of Histochemistry & Cytochemistry, 2014. **62**(10): p. 751-758.
29. Talwar, H.S., et al., *Reduced type I and type III procollagens in photodamaged adult human skin.* Journal of Investigative Dermatology, 1995. **105**(2): p. 285-290.
30. Graham, H.K. and A.W. Trafford, *Spatial disruption and enhanced degradation of collagen with the transition from compensated ventricular hypertrophy to symptomatic congestive heart failure.* American Journal of Physiology - Heart and Circulatory Physiology, 2007. **292**(3): p. H1364-H1372.
31. Graham, H.K., et al., *Localised micro-mechanical stiffening in the ageing aorta.* Mechanisms of Ageing and Development, 2011. **132**(10): p. 459-467.
32. Abramoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with ImageJ.* Biophotonics international, 2004. **11**(7): p. 36-42.
33. Watson, R.E.B., et al., *A cosmetic 'anti-ageing' product improves photoaged skin: a double-blind, randomized controlled trial.* British Journal of Dermatology, 2009. **161**(2): p. 419-426.
34. Smith, L.T., K.A. Holbrook, and J.A. Madri, *Collagen types I, III, and V in human embryonic and fetal skin.* Am J Anat, 1986. **175**(4): p. 507-21.
35. Kligman, A.M., *Early destructive effect of sunlight on human skin.* JAMA, 1969. **210**(13): p. 2377-2380.
36. Griffiths, C.E.M., et al., *Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid).* New England Journal of Medicine, 1993. **329**(8): p. 530-535.
37. Bernstein, E.F., et al., *Long-term sun exposure alters the collagen of the papillary dermis - comparison of sun-protected and photoaged skin by Northern analysis, immunohistochemical staining, and confocal laser scanning microscopy.* Journal of the American Academy of Dermatology, 1996. **34**(2): p. 209-218.
38. Sander, C.S., et al., *Photoaging is Associated with Protein Oxidation in Human Skin In Vivo.* 2002. **118**(4): p. 618-625.
39. Quan, T.H., et al., *Matrix-Degrading Metalloproteinases in Photoaging.* Journal of Investigative Dermatology Symposium Proceedings, 2009. **14**(1): p. 20-24.
40. Tewari, A., et al., *Upregulation of MMP12 and Its Activity by UVA1 in Human Skin: Potential Implications for Photoaging.* Journal of Investigative Dermatology, 2014. **134**(10): p. 2598-2609.
41. Pittayapruek, P., et al., *Role of Matrix Metalloproteinases in Photoaging and Photocarcinogenesis.* International Journal of Molecular Sciences, 2016. **17**(6): p. 868.
42. Kadler, K.E., et al., *Collagen fibril formation.* Biochemical Journal, 1996. **316**(Pt 1): p. 1-11.
43. Chung, J.H., et al., *Modulation of Skin Collagen Metabolism in Aged and Photoaged Human Skin In Vivo.* 2001. **117**(5): p. 1218-1224.
44. Fisher, G.J., et al., *Collagen Fragmentation Promotes Oxidative Stress and Elevates Matrix Metalloproteinase-1 in Fibroblasts in Aged Human Skin.* American Journal of Pathology, 2009. **174**(1): p. 101-114.
45. Tsai, W.C., et al., *The pentapeptide KTTKS promoting the expressions of type I collagen and transforming growth factor-beta of tendon cells.* J Orthop Res, 2007. **25**(12): p. 1629-34.
46. Kim, M.-S., et al., *Regulation of type I procollagen and MMP-1 expression after single or repeated exposure to infrared radiation in human skin.* Mechanisms of Ageing and Development, 2006. **127**(12): p. 875-882.

47. Kim, S. and J.H. Chung, *Berberine prevents UV-induced MMP-1 and reduction of type I procollagen expression in human dermal fibroblasts.* Phytomedicine, 2008. **15**(9): p. 749-753.
48. Vayalil, P.K., et al., *Green tea polyphenols prevent ultraviolet light-induced oxidative damage and matrix metalloproteinases expression in mouse skin.* Journal of Investigative Dermatology, 2004. **122**(6): p. 1480-1487.
49. Chiu, A.E., et al., *Double-Blinded, Placebo-Controlled Trial of Green Tea Extracts in the Clinical and Histologic Appearance of Photoaging Skin.* Dermatologic Surgery, 2005. **31**: p. 855-860.
50. Janjua, R., et al., *A Two-Year, Double-Blind, Randomized Placebo-Controlled Trial of Oral Green Tea Polyphenols on the Long-Term Clinical and Histologic Appearance of Photoaging Skin.* Dermatologic Surgery, 2009. **35**(7): p. 1057-1065.
51. Chen, Z.-Y., et al., *Stabilizing Effect of Ascorbic Acid on Green Tea Catechins.* Journal of Agricultural and Food Chemistry, 1998. **46**(7): p. 2512-2516.
52. Chung, J.H., et al., *Regulations of collagen synthesis by ascorbic acid, transforming growth factor-β and interferon-γ in human dermal fibroblasts cultured in three-dimensional collagen gel are photoaging- and aging-independent.* Journal of Dermatological Science, 1997. **15**(3): p. 188-200.
53. McArdle, F., et al., *UVR-induced oxidative stress in human skin in vivo: effects of oral vitamin C supplementation.* Free Radical Biology and Medicine, 2002. **33**(10): p. 1355-1362.
54. Lee, L.-S., et al., *Quantitative Analysis of Major Constituents in Green Tea with Different Plucking Periods and Their Antioxidant Activity.* Molecules, 2014. **19**(7): p. 9173-9186.
55. Singh, B.N., S. Shankar, and R.K. Srivastava, *Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications.* Biochemical Pharmacology, 2011. **82**(12): p. 1807-1821.
56. Lambert, J.D. and R.J. Elias, *The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention.* Archives of Biochemistry and Biophysics, 2010. **501**(1): p. 65-72.

Chapter 6 - Discussion

Sunlight is essential for life on Earth and is required for photosynthesis in plants [263], vitamin D synthesis in mammals [264] and maintaining biological clocks in living organisms [265]. However, in man, there are some negative effects of long term exposure to solar UVR including melanoma and non-melanoma skin cancers, photosensitivity disorders and photoageing. These problems occur in skin as the skin is the major organ which interacts with the environment [263] and such problems can be affected by a person's choices about activities related to regular natural sunlight or mimicked by UVR from manmade source (e.g. sun-beds [266]).

In these studies we have focussed on damage which happens following long-term exposure to UVR termed photodamage or photoageing; clinical characteristics are coarse wrinkles, altered pigmentation (loss and gain of pigment in focussed areas) and uneven, rough skin texture [88]. Photoageing is seen particularly in Caucasian populations who appear to be more sensitive to UVR-induced damage (Fitzpatrick skin types I-III; [267]). Experimentally, UVB has been shown to be the key modulator of biomolecular damage to the skin, its short wavelength (high photon energy) being easily absorbable by biomolecules [268]. Hence this wavelength of light is often used in photobiological studies in skin. For example the effect of UVB has been used to investigate the effect of irradiation on elastic fibres and related proteins(Walker et al 2002 [269]; Kadoya et al 2005 [82]); the results from these two projects pointed out that UVB affected the abundance and organisation of FRM, and fibulin-2 and -5-containing microfibrils. In addition, Kadoya *et al* showed a UVB-induced reduction in elastic fibres 24 hr after irradiation using the histological stain, Weigert's resorcin fuchsin.

However, it can be argued that using only UVB to study skin-sunlight interactions is flawed; the amount of UVB in natural sunlight is much lower than UVA (UVB~6%: UVA~94%). This ratio of UVA:UVB can be produced artificially in the laboratory by using solar simulated radiation (SSR), so producing data which may be more similar to natural sunlight spectrum [98]. Previously many sources had reported negative photoageing effects in skin (elastosis, loss of FRM, altered fibulin expression) owing to regular continuous UV irradiation in long range or UV chronic exposure [57, 270, 271]. One evident benefit of using SSR experimentally as opposed to natural

UVR is that there is no need to take into account other variable environment factors, i.e. longitude and season (that can vary the intensity and consistency of UVR; [98]).

Under the controlled environment in the lab, we can control the wavelength of UVR and its dose, enabling the researcher to compare the effects of irradiation on skin. By using SSR, in contrast to UVA alone or UVB alone, we can model as closely as possible the effect of UVR (a better estimation of the real atmosphere).

In the studies described in this thesis, the potential differential effect of UVB versus SSR irradiation was assessed in healthy Caucasian (Fitzpatrick skin type I-III) volunteers. These results were in agreement with that reported by Kadoya *et al*. (2005); following UVB irradiation, elastic fibres and microfibrils positive for fibulins-2 and -5 were significantly remodelled within the first 50 µm of the papillary dermis (P=0.005, P=0.028 and P=0.048 respectively). At deeper depths (100 µm) of papillary dermis, this total amount of both fibulin-2 (P=0.10) and fibulin-5 (*P*=0.11) were not significantly reduced. Hence, UVB-mediated remodelling occurs superficially in human skin and may be because some of the UVB can penetrate to this level, and can be absorbed by proteins enriched in the UVR chromophores, such as microfibrils of the fibulin microfibrils [135]. Additionally, it was possible to observe a significant decline in the FRM network following UVB irradiation (to 100 µm; P=0.02); this data is in agreement with previous work [269] examining the network in the papillary dermis.

The first results from this thesis was the comparison between different wavelengths of UV on the distribution of elastic fibres. This was studied in a small sample of volunteers with Fitzpatrick skin type I-III (n = 6 per irradiation protocol); this studywas essential to establish the protocol before extension of sample size to the larger cohort (n = 50). These findings indicated that both UVB and SSR had serious harmful effects on skin health involving the significant decreassome of elastic fibre components in the papillary dermis.

However, SSR (imitated UVR specturm representing natural sunlight) was used for the subsequent randomised controlled trial because it better represents everyday life exposure. Thus the latter results of this thesis focus on acute SSR-induced elastic fibre damage.

From previous papers on the pathology of photodamaged skin, it is established that protein components of the elastic fibre system (elastic fibres, FRM, fibulin-2 and -5) are affected.

The possible mechanisms which may help us to understand the SSR effects on these biologically photosensitive-rich components as direct mechanism [135, 136] along with indirect mechanism through ROS accelerating [272], are shown in (*figure 6.1*).

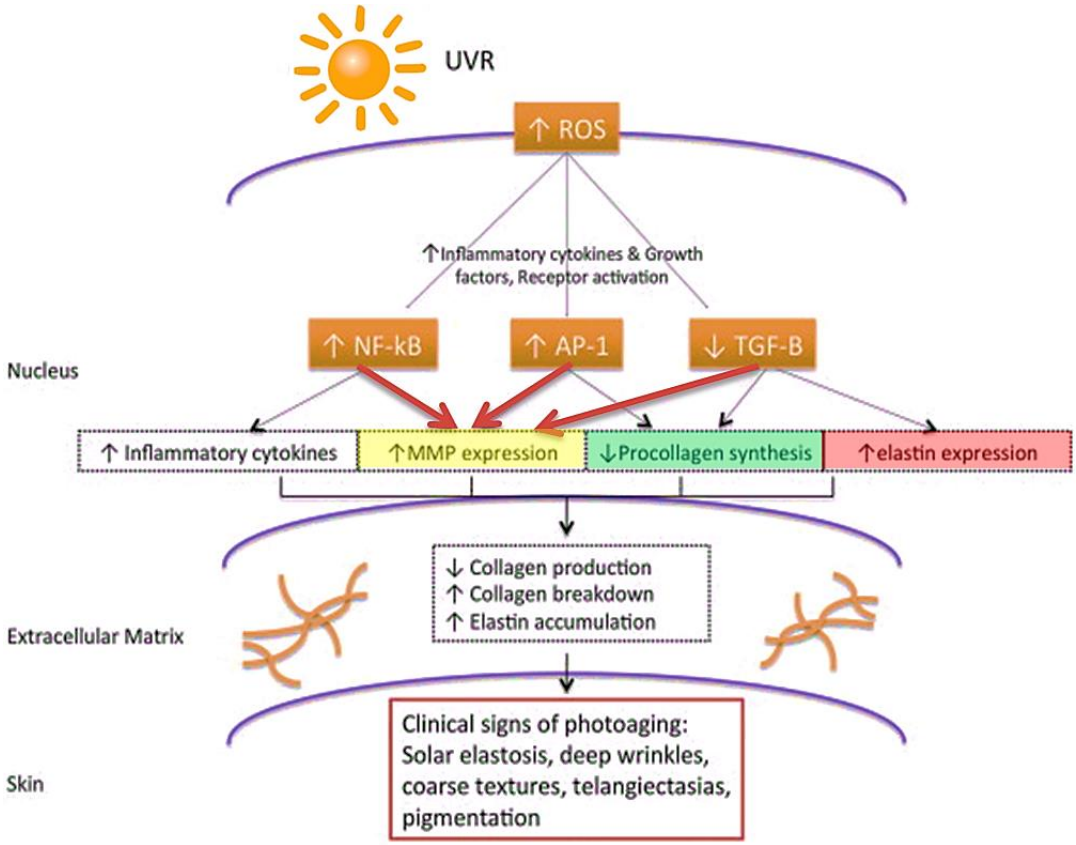


Figure 6.1: Indirect mechanism of UV-induced skin damage via over production of cellular oxygen species (ROS).
The trigger of ECMs breakdown is UV radiation that causes excessive ROS emerging in cells to influence signal transduction cascade of AP-1, NF-kB up-regulation and transforming growth factor (TGF)-β down-regulation. These higher AP-1, NF-kB and lower TGF-B synthesis lead MMP expression that have ability to suppress in collageneous and elastic fibre matrices accordingly abnormal elastotic material can be possibly appeared. Image adapted from Chen et al 2012 [272].

So to avoid the consequences of harmful UVR exposure on skin, routine application of photoprotective agents is important, as is wearing proper clothes [273] to maintain and protect skin from the negative effects of UVR. Although sunscreen is widely used presently, this product has been spread and contacted directly on skin and may affect other skin problems (blemish, oily skin even irritating) [169]. Clothes have high UV protection [161] but may not suitable or convenient for every place or season. Hence oral photoprotective supplementation, for example vitamin C plus Zinc and marine complex [274], vitamin C and E [176] and polyphenols [194] is an interesting options to prevent skin from UV damage [275] without the need of re-applying product to increase protective efficacy like sunscreen.

Even though vitamin C or vitamin E are options as antioxidants, these provide little or no benefit if were separately consumed [176]. Furthermore the antioxidant activity (mM) by Trolox equivalent antioxidant activity (TEAC) at the same concentration when compared to other antioxidant types like catechin in polyphenol group are much lower [276], *table 6.1*.

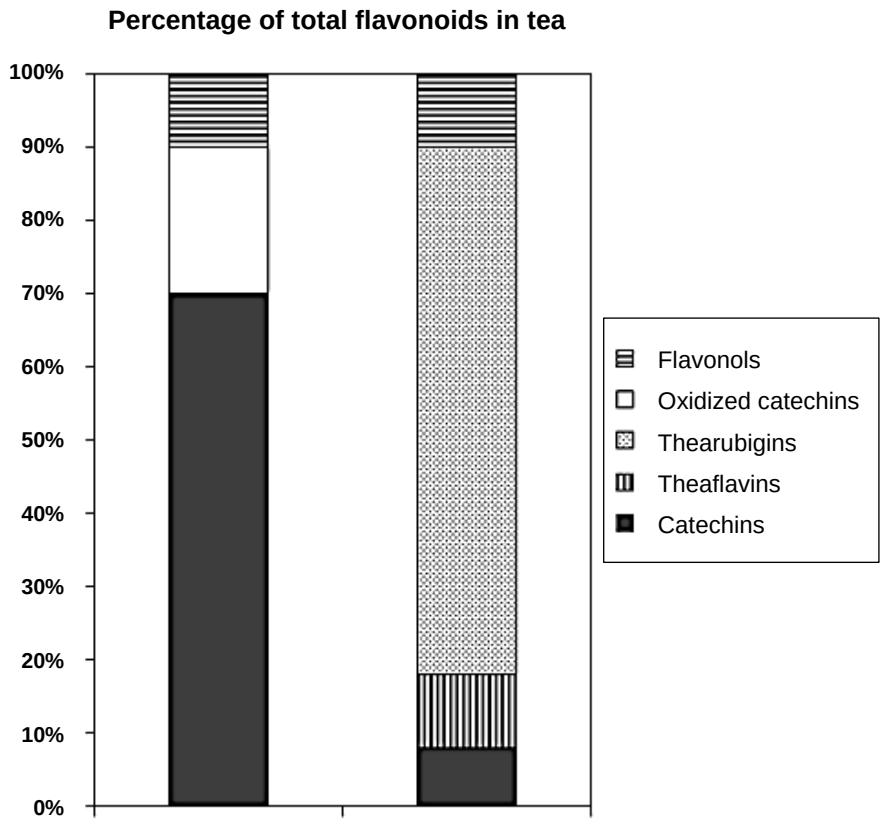
Table 6.1: Antioxidant activities of flavonoids and vitamin C and E in vitro

Antioxidant	Antioxidant activity (mM)
(-)-Epicatechin	2.4 ± 0.02
(-)-Epigallocatechin	3.8 ± 0.06
(-)-Epicatechin gallate	4.9 ± 0.02
(-)-Epigallocatechin gallate	4.8 ± 0.06
Theaflavin	2.9 ± 0.08
Theaflavin digallate	6.2 ± 0.43
Green tea (1000 ppm)	3.8 ± 0.03 ←
Black tea (1000 ppm)	3.5 ± 0.03
Vitamin E	1.0 ± 0.03 ←
Vitamin C	1.0 ± 0.02 ←

Table adapted from Higdon and Frei 2003 [276].

Polyphenols are commonly found in vegetables and fruits. This secondary dietary metabolite may have various manifest advantages in skin; people are accustomed to popular polyphenol related-products, i.e. red wine, green beans and green tea [187]. Particularly, green tea is a well-known brew with high annual consumption [212] with a safe, well-tolerated dose [239]. Green tea contains catechins which play an important role as an antioxidant [276, 277], giving advantages to many systems throughout the body, such as helping decrease cholesterol [278], decreasing risk of cancers [279, 280] and being used as supplement for cosmetic supplement to

anti-ageing [281-283]. Particularly this tea type is passed the process that preserved catechins than black tea; it therefore is contains high catechins content [204, 209], *figure 6.2*.



	Green tea	Black tea
Flavonols	7-9%	7-9%
Oxidized catechins	20-30%	
Thearubigins		63-74%
Theaflavins		5-12%
Catechins	60-80%	6-24%

Figure 6.2: Bar graph displayed the percentage of different sub-unit of polyphenol components or flavonoids in green tea and black tea.
Catechin compounds in green tea is evidently higher than those in black tea, in the contrast Thearubigins which are polymeric polyphenols formed during the enzymatic oxidation is much higher than those in green tea owing to passing the fermented process. Image from Higdon and Frei 2003 [276].

Hence, in a clinical research study we tested the photoprotective efficacy of green tea supplement on skin following an acute dose of SSR damage. Supplements were consumed daily for 12 weeks as part of a randomised controlled trial (RCT; gold standard for knowing whether a tratement, in this case consumption of dietary green tea, will work). The RCT is a well-known and robust experimental design for clinical studies. This method is suggested to apply in 1-2 interventions in the test including some essential criteria: 1) the previous founding facts support the hypothesis in this study; 2) some established evidences were clearly

presented the systemic mechanism; 3) the result are conveniently estimated or measured and; 4) outcome from the trial may yield a huge impact [284]. The double blind style was set to reduce bias of volunteers and analysis in the lab.

In this novel *in vivo* human study, the testing impact of SSR on an enlarged sample size (n= 50) confirmed that an acute dose of SSR irradiation affected proteins of the elastic fibre network ($P<0.001$), particularly the significant reduction of fibulin family proteins (fibulin-2 and -5; $P<0.001$ both molecules). Whereas decreasing of fibulin-2 did not reach significant level in smaller group (n=6). However, small reduction of FRM is present in this study butwas different from Sherratt et al 2010 [135] report that FRM was declined significantly after one strong dose of UVB radiation; however, this paper looked at microfibrils extracted from human skin and then irradiated. It may be concluded that UVB with high intensity from a laboratory UVB source influences the structure of extracted FRM but UVB within the SSR spectrum, in a much lower ratio (95% UVA, 5% UVB) did not damage elastic fibre significantly.

Overall, GTC supplement did not protect the entire oxytalan fibre network of elastic fibre matrix within the papillary dermis, as identified by histological staining. However it prevented specific protein remodeling (fibulin-5 microfibrils) from UV damage (GTC, no UVR *versus* UVR, $P = 0.297$; placebo, no UVR *versus* UVR, $P = 0.012$). While the other protein in the same family, fibulin-2 was positively protected, this did not reach statistical significance (GTC and placebo; GTC, no UVR *versus* UVR, $P = 0.904$; placebo, no UVR *versus* UVR, $P = 0.061$).

The last part of this study involved the same experiment model but examined the fibrillar collageneous matrix, also known to be damaged by UVR, including the deposition of newly synthesized pCI (schemetic in *figure 6.1*). If green tea intervention for 12 weeks protected the reduction of fibulin-5 from SSR damage, by mechanisms currently undetermined but which might indirectly involve ECMs proteinase, such as collagenase, this supplement may also protects against other ECM damage in the same situation.

The data indicated that amount of mature collagen fibres was lower within the superficial papillary dermis within 100 µm depth ($P<0.001$) 24 h after radiated with SSR. The newly synthesised pCI revealed the an increase in newly synthesised collagen entering the papillary dermal ECM ($P=0.015$); this data is in agreement with work published by Chung and team

(2001) [38]. In the collagenous study area, the oral supplementation of GTC protected mature collagen fibres within the papillary dermis (placebo P=0.17; active P=0.79) whist has no impact on pCI synthesis following UV (placebo P<0.001; active P=0.005). Further investigation in this area is essential to establish the mechanisms by which these alterations take place.

Further work

The data presented in this thesis only relates to protein changes observed histologically *in vivo* as a basis of the structure of dermal tissue. However, it would be important to assess mechanistically how these changes occur. Gene expression levels for the proteins shown to be affected by UVR should also be examined. This could be done by qPCR, or via transcriptomic methods.

In addition to induction of gene expression, it would be also important to assess levels of breakdown. This can be done by looking at the effect of GTC on the expression and activity of remodelling enzymes, such as the MMPs. The expression of these enzymes are controlled by the activity of the AP-1 transcription factor. Hence it would interesting to assess the expression of c-fos and c-jun in systemically treated tissue. According to direct mechanism of photoageing, ECM components could be broken down from catalytic enzymes, triggered by higher ROS levels within the tissue. Hence enzymatic and ROS measurement experiment could be performed.

If the study were repeated, the sample size could be increased to provide even more confidence with less error in these results. Vitamin C could be used alone as a third arm and compared to GTC plus vitamin C; vitamin C was reported to influence collagen synthesis *in vitro.* If repeated, it might also be interesting to look at biopsy tissue taken at different time points for RNA and protein expression work.

Bibliography

1. Charkoudian, N., *Skin blood flow in adult human thermoregulation: how it works, when it does not, and why.* Mayo Clin Proc, 2003. **78**(5): p. 603-12.
2. Mykytowycz, R. and B.S. Goodrich, *Skin glands as organs of communication in mammals.* J Investig Dermatol, 1974. **62**(3): p. 124-131.
3. Charkoudian, N., *Skin Blood Flow in Adult Human Thermoregulation: How It Works, When It Does Not, and Why.* Mayo Clinic Proceedings, 2003. **78**(5): p. 603-612.
4. Wollenweber, U., Korevaar, K., Rawlings, A.V. and Schick, U., *Application of a skin-identical lipid concentrate for enhanced skin moisturization and protection.* SÖFW-Journal, 2004. **130**(9): p. 6.
5. Marples, M.J., *The normal fllora of the human skin.* British Journal of Dermatology, 1969. **81**: p. 2-13.
6. Munger, B.L. and C. Ide, *The structure and function of cutaneous sensory receptors.* Archives of histology and cytology, 1988. **51**(1): p. 1-34.
7. Bos, J.D. and M.L. Kapsenberg, *The skin immune system: progress in cutaneous biology.* Immunology Today, 1993. **14**(2): p. 75-78.
8. Chen, T.C., et al., *Factors that influence the cutaneous synthesis and dietary sources of vitamin D.* Archives of Biochemistry and Biophysics, 2007. **460**(2): p. 213-217.
9. Bouwstra, J.A. and P.L. Honeywell-Nguyen, *Skin structure and mode of action of vesicles.* Advanced Drug Delivery Reviews, 2002. **54, Supplement**(0): p. S41-S55.
10. Nelson, W.G. and T.T. Sun, *The 50- and 58-kdalton keratin classes as molecular markers for stratified squamous epithelia: cell culture studies.* The Journal of Cell Biology, 1983. **97**(1): p. 244-251.
11. Costin, G.-E. and V.J. Hearing, *Human skin pigmentation: melanocytes modulate skin color in response to stress.* The FASEB Journal, 2007. **21**(4): p. 976-994.
12. Elias, P.M., *Lipids and the epidermal permiablelity barrier.* Archives of Dermatological Research, 1981. **270**(1): p. 95-117.
13. Madison, K.C., et al., *Lamellar granule extrusion and stratum corneum intercellular lamellae in murine keratinocyte cultures.* Journal of Investigative Dermatology, 1988. **90**(2): p. 110-116.
14. Tortora, G.J. and S.R. Grabowski, *Introduction to the human body : The essentials of anatomy and physiology*. 5th ed, ed. S. Reynolds. 2001, New York: Chichester: Wiley.
15. Di Meglio, P., Gayathri K. Perera, and Frank O. Nestle, *The Multitasking Organ: Recent Insights into Skin Immune Function.* Immunity, 2011. **35**(6): p. 857-869.
16. Yannas, I.V., et al., *Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin.* Proceedings of the National Academy of Sciences, 1989. **86**(3): p. 933-937.
17. Fitzpatrick, T.B., *The validity and practicality of sun-reactive skin types I through VI.* Archives of Dermatology, 1988. **124**(6): p. 869-871.
18. Fasugba, O., A. Gardner, and W. Smyth, *The Fitzpatrick skin type scale: a reliability and validity study in women undergoing radiation therapy for breast cancer.* J Wound Care, 2014. **23**(7): p. 358, 360-2, 364 passim.
19. Gogia, R., et al., *Fitzpatrick skin phototype is an independent predictor of squamous cell carcinoma risk after solid organ transplantation.* Journal of the American Academy of Dermatology, 2013. **68**(4): p. 585-591.
20. D'Orazio, J., et al., *UV Radiation and the Skin.* International Journal of Molecular Sciences, 2013. **14**(6): p. 12222-12248.
21. White, E. *Ethnicity and National Identity in England and Wales: 2011*. 2011 11th December 2012 [cited 2016 12th September]; Available from: http://www.ons.gov.uk/peoplepopulationandcommunity/culturalidentity/ethnicity/articles/ethnicityandnationalidentityinenglandandwales/2012-12-11.
22. Narayanan, A.S., R.C. Page, and J. Swanson, *Collagen synthesis by human fibroblasts. Regulation by transforming growth factor-β in the presence of other inflammatory mediators.* Biochemical Journal, 1989. **260**(2): p. 463-469.
23. Lodish H, B.A., Zipursky SL, et al., *Molecular Cell Biology*. 4th ed. 2000, New York, US: W. H. Freeman and Company.
24. Ricard-Blum, S., *The collagen family.* Cold Spring Harb Perspect Biol, 2011. **3**(1): p. a004978.

25. Mecham, R.P., *The Extracellular Matrix: an Overview*. 2011, Berlin, Heidelberg.
26. Meigel, W.N., S. Gay, and L. Weber, *Dermal architecture and collagen type distribution.* Archives of Dermatological Research, 1977. **259**(1): p. 1-10.
27. Fleischmajer, R., et al., *Ultrastructural identification of extension aminopropeptides of type I and III collagens in human skin.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1981. **78**(12): p. 7360-7364.
28. Smith, L.T., K.A. Holbrook, and J.A. Madri, *Collagen types I, III, and V in human embryonic and fetal skin.* Am J Anat, 1986. **175**(4): p. 507-21.
29. Mayne, R., *Structure and function of collagen types / edited by Richard Mayne, Robert E. Burgeson*, ed. R. Mayne and R.E. Burgeson. 1987, Orlando ; London: Orlando ; London : Academic Press.
30. Chanut-Delalande, H., et al., *Development of a functional skin matrix requires deposition of collagen V heterotrimers.* Mol Cell Biol, 2004. **24**(13): p. 6049-57.
31. Kielty, C.M., et al., *Type VI collagen microfibrils: evidence for a structural association with hyaluronan.* J Cell Biol, 1992. **118**(4): p. 979-90.
32. Urciuolo, A., et al., *Collagen VI regulates satellite cell self-renewal and muscle regeneration.* Nat Commun, 2013. **4**: p. 1964.
33. Watson, R.E.B., et al., *Distribution and expression of type VI collagen in photoaged skin.* British Journal of Dermatology, 2001. **144**(4): p. 751-759.
34. Keene, D.R., et al., *Type VII collagen forms an extended network of anchoring fibrils.* J Cell Biol, 1987. **104**(3): p. 611-21.
35. Sakai, L.Y., et al., *Type VII collagen is a major structural component of anchoring fibrils.* J Cell Biol, 1986. **103**(4): p. 1577-86.
36. Shaw, L.M. and B.R. Olsen, *FACIT collagens: diverse molecular bridges in extracellular matrices.* Trends Biochem Sci, 1991. **16**(5): p. 191-4.
37. Adams, J.C. and R.P. Tucker, *The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development.* Dev Dyn, 2000. **218**(2): p. 280-99.
38. Chung, H.J. and J. Uitto, *Type VII Collagen: The Anchoring Fibril Protein at Fault in Dystrophic Epidermolysis Bullosa.* Dermatologic Clinics, 2010. **28**(1): p. 93-105.
39. Feru, J., et al., *Aging decreases collagen IV expression in vivo in the dermo-epidermal junction and in vitro in dermal fibroblasts: possible involvement of TGF-beta1.* Eur J Dermatol, 2016. **26**(4): p. 350-60.
40. Keene, D.R., E. Engvall, and R.W. Glanville, *Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network.* J Cell Biol, 1988. **107**(5): p. 1995-2006.
41. Theocharidis, G., et al., *Type VI Collagen Regulates Dermal Matrix Assembly and Fibroblast Motility.* Journal of Investigative Dermatology, 2016. **136**(1): p. 74-83.
42. O'Toole, E.A., *Type VII collagen and squamous cell carcinoma.* British Journal of Dermatology, 2014. **170**(6): p. 1215-1215.
43. Richer, B.C. and K. Seeger, *The hinge region of type VII collagen is intrinsically disordered.* Matrix Biol, 2014. **36**: p. 77-83.
44. Pulkkinen, L. and J. Uitto, *Hemidesmosomal variants of epidermolysis bullosa. Mutations in the alpha6beta4 integrin and the 180-kD bullous pemphigoid antigen/type XVII collagen genes.* Exp Dermatol, 1998. **7**(2-3): p. 46-64.
45. Khaleduzzaman, M., et al., *Structure of the human type XIX collagen (COL19A1) gene, which suggests it has arisen from an ancestor gene of the FACIT family.* Genomics, 1997. **45**(2): p. 304-12.
46. Myers, J.C., et al., *Biochemical and immunohistochemical characterization of human type XIX defines a novel class of basement membrane zone collagens.* Am J Pathol, 1997. **151**(6): p. 1729-40.
47. Sumiyoshi, H., et al., *Embryonic expression of type XIX collagen is transient and confined to muscle cells.* Dev Dyn, 2001. **220**(2): p. 155-62.
48. Cradto, A.M. *Printing with Collagen*. 2016 [cited 2016 31 August]; Available from: http://worlddrugtracker.blogspot.co.uk/2016/05/printing-with-collagen.html.
49. Exposito, J.-Y., et al., *Tracing the evolution of vertebrate fibrillar collagens from an ancestral α chain.* Matrix Biology, 2000. **19**(3): p. 275-279.
50. Woessner, J.F., Jr., *Matrix metalloproteinases and their inhibitors in connective tissue remodeling.* Faseb j, 1991. **5**(8): p. 2145-54.

51. Nimni, M.E., *Collagen: Biochemistry volume 1*. 1988, United States: CRC Press. 384.
52. Bhattacharjee, A. and M. Bansal, *Collagen structure: the Madras triple helix and the current scenario.* IUBMB Life, 2005. **57**(3): p. 161-72.
53. Bailey, A.J., R.G. Paul, and L. Knott, *Mechanisms of maturation and ageing of collagen.* Mechanisms of Ageing and Development, 1998. **106**(1–2): p. 1-56.
54. Kielty, C.M., et al., *Fibrillin microfibrils.* Fibrous Proteins: Coiled-Coils, Collagen and Elastomers, 2005. **70**: p. 405-+.
55. Faury, G., *Function–structure relationship of elastic arteries in evolution: from microfibrils to elastin and elastic fibres.* Pathologie Biologie, 2001. **49**(4): p. 310-325.
56. Fleming, W.W., C.E. Sullivan, and D.A. Torchia, *CHARACTERIZATION OF MOLECULAR MOTIONS IN C-13-LABELED AORTIC ELASTIN BY C-13-H-1 MAGNETIC DOUBLE-RESONANCE.* Biopolymers, 1980. **19**(3): p. 597-617.
57. Watson, R.E.B., et al., *Fibrillin-rich microfibrils are reduced in photoaged skin. Distribution at the dermal-epidermal junction.* Journal of Investigative Dermatology, 1999. **112**(5): p. 782-787.
58. Hay, E.D., *Cell biology of extracellular matrix / edited by Elizabeth D. Hay*, ed. E.D. Hay. 1991, New York ; London: New York ; London : Plenum.
59. Kielty, C.M., M.J. Sherratt, and C.A. Shuttleworth, *Elastic fibres.* Journal of Cell Science, 2002. **115**(14): p. 2817-2828.
60. Sakai, L.Y., D.R. Keene, and E. Engvall, *Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils.* Journal of Cell Biology, 1986. **103**(6): p. 2499-2509.
61. Zhang, H., et al., *Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices.* Journal of Cell Biology, 1994. **124**(5): p. 855-863.
62. Nagase, T., et al., *Prediction of the coding sequences of unidentified human genes. XX. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro.* DNA Research, 2001. **8**(2): p. 85-95.
63. Ritty, T.M., et al., *Processing of the fibrillin-1 carboxyl-terminal domain.* Journal of Biological Chemistry, 1999. **274**(13): p. 8933-8940.
64. Corson, G.M., et al., *Differential expression of fibrillin-3 adds to microfibril variety in human and avian, but not rodent, connective tissues.* Genomics, 2004. **83**(3): p. 461-472.
65. Downing, A.K., et al., *Solution structure of a pair of calcium-binding epidermal growth factor-like domains: Implications for the Marfan syndrome and other genetic disorders.* Cell, 1996. **85**(4): p. 597-605.
66. Robertson, I., S. Jensen, and P. Handford, *TB domain proteins: evolutionary insights into the multifaceted roles of fibrillins and LTBPs.* Biochemical Journal, 2011. **433**(2): p. 263-276.
67. Timpl, R., et al., *Fibulins: a versatile family of extracellular matrix proteins.* Nat Rev Mol Cell Biol, 2003. **4**(6): p. 479-489.
68. El-Hallous, E., et al., *Fibrillin-1 interactions with fibulins depend on the first hybrid domain and provide an adaptor function to tropoelastin.* J Biol Chem, 2007. **282**(12): p. 8935-46.
69. Zhang, Y. and L.Y. Marmorstein, *Focus on molecules: Fibulin-3 (EFEMP1).* Experimental Eye Research, 2010. **90**(3): p. 374-375.
70. Schultz, D.W., et al., *HEMICENTIN-1 (FIBULIN-6) and the 1q31 AMD locus in the context of complex disease: review and perspective.* Ophthalmic Genet, 2005. **26**(2): p. 101-5.
71. Roark, E.F., et al., *The association of human fibulin-1 with elastic fibers: an immunohistological, ultrastructural, and RNA study.* J Histochem Cytochem, 1995. **43**(4): p. 401-11.
72. Miosge, N., et al., *The extracellular matrix proteins fibulin-1 and fibulin-2 in the early human embryo.* Histochem J, 1996. **28**(2): p. 109-16.
73. Hunzelmann, N., et al., *Increased deposition of fibulin-2 in solar elastosis and its colocalization with elastic fibres.* Br J Dermatol, 2001. **145**(2): p. 217-22.
74. Missan, D.S., S.V. Chittur, and C.M. DiPersio, *Regulation of fibulin-2 gene expression by integrin alpha3beta1 contributes to the invasive phenotype of transformed keratinocytes.* J Invest Dermatol, 2014. **134**(9): p. 2418-27.
75. Radice, P.D., et al., *Fibulin-2 is a key mediator of the pro-neurogenic effect of TGF-beta1 on adult neural stem cells.* Mol Cell Neurosci, 2015. **67**: p. 75-83.

76. Serra, N., et al., *Simvastatin Increases Fibulin-2 Expression in Human Coronary Artery Smooth Muscle Cells via RhoA/Rho-Kinase Signaling Pathway Inhibition.* PLoS ONE, 2015. **10**(7): p. e0133875.
77. Pan, T.C., et al., *Structure and expression of fibulin-2, a novel extracellular matrix protein with multiple EGF-like repeats and consensus motifs for calcium binding.* Journal of Cell Biology, 1993. **123**(5): p. 1269-1277.
78. Giltay, R., R. Timpl, and G. Kostka, *Sequence, recombinant expression and tissue localization of two novel extracellular matrix proteins, fibulin-3 and fibulin-4.* Matrix Biol, 1999. **18**(5): p. 469-80.
79. Papke, C.L. and H. Yanagisawa, *Fibulin-4 and fibulin-5 in elastogenesis and beyond: Insights from mouse and human studies.* Matrix Biol, 2014. **37**: p. 142-9.
80. Kobayashi, N., et al., *A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization.* J Biol Chem, 2007. **282**(16): p. 11805-16.
81. Zheng, Q., et al., *Normal wound healing in mice deficient for fibulin-5, an elastin binding protein essential for dermal elastic fiber assembly.* J Invest Dermatol, 2006. **126**(12): p. 2707-14.
82. Kadoya, K., et al., *Fibulin-5 deposition in human skin: decrease with ageing and ultraviolet B exposure and increase in solar elastosis.* British Journal of Dermatology, 2005. **153**(3): p. 607-612.
83. Choi, J., et al., *Analysis of dermal elastic fibers in the absence of fibulin-5 reveals potential roles for fibulin-5 in elastic fiber assembly.* Matrix Biol, 2009. **28**(4): p. 211-20.
84. Chowdhury, A., et al., *Expression of fibulin-6 in failing hearts and its role for cardiac fibroblast migration.* Cardiovasc Res, 2014. **103**(4): p. 509-20.
85. Sisto, M., et al., *Fibulin-6 expression and anoikis in human salivary gland epithelial cells: implications in Sjogren's syndrome.* Int Immunol, 2009. **21**(3): p. 303-11.
86. de Vega, S., et al., *TM14 is a new member of the fibulin family (fibulin-7) that interacts with extracellular matrix molecules and is active for cell binding.* J Biol Chem, 2007. **282**(42): p. 30878-88.
87. Langton, A.K., et al., *Review Article: A new wrinkle on old skin: the role of elastic fibres in skin ageing.* International Journal of Cosmetic Science, 2010. **32**(5): p. 330-339.
88. Watson, R.E.B. and C.E.M. Griffiths, *Pathogenic aspects of cutaneous photoaging.* Journal of cosmetic dermatology, 2005. **4**(4): p. 230-6.
89. Neerken, S., et al., *Characterization of age-related effects in human skin: A comparative study that applies confocal laser scanning microscopy and optical coherence tomography.* Journal of Biomedical Optics, 2004. **9**(2): p. 274-281.
90. Fenske, N.A. and C.W. Lober, *Structural and functional changes of normal aging skin.* Journal of the American Academy of Dermatology, 1986. **15**(4): p. 571-585.
91. Langton, A.K., et al., *The impact of intrinsic ageing on the protein composition of the dermal-epidermal junction.* Mechanisms of Ageing and Development, 2016. **156**: p. 14-16.
92. Paul, N.D. and D. Gwynn-Jones, *Ecological roles of solar UV radiation: towards an integrated approach.* Trends in Ecology & Evolution, 2003. **18**(1): p. 48-55.
93. Wolber, R., et al., *Pigmentation effects of solar-simulated radiation as compared with UVA and UVB radiation.* Pigment Cell & Melanoma Research, 2008. **21**(4): p. 487-491.
94. Anderson, R.R. and J.A. Parrish, *The Optics of Human Skin.* Journal of Investigative Dermatology, 1981. **77**(1): p. 13-19.
95. Popov, A.P., et al., *Biophysical mechanisms of modification of skin optical properties in the UV wavelength range with nanoparticles.* Journal of Applied Physics, 2009. **105**(10).
96. Bissett, D., D. Hannonand, and T. Orr, *An animal model of solar-aged skin: Histologic, physical and visible changes in UV-irradiated mouse skin.* Photochemistry and Photobiology, 1987. **46**(3): p. 367-378.
97. Biniek, K., K. Levi, and R.H. Dauskardt, *Solar UV radiation reduces the barrier function of human skin.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(42): p. 17111-17116.
98. Diffey, B.L., *Sources and measurement of ultraviolet radiation.* Methods, 2002. **28**(1): p. 4-13.
99. Matsumura, Y. and H.N. Ananthaswamy, *Toxic effects of ultraviolet radiation on the skin.* Toxicology and Applied Pharmacology, 2004. **195**(3): p. 298-308.

100. Seité, S., et al., *A broad-spectrum sunscreen prevents cumulative damage from repeated exposure to sub-erythemal solar ultraviolet radiation representative of temperate latitudes.* Journal of the European Academy of Dermatology and Venereology, 2010. **24**(2): p. 219-222.
101. Ravanat, J.-L., T. Douki, and J. Cadet, *Direct and indirect effects of UV radiation on DNA and its components.* Journal of Photochemistry and Photobiology B: Biology, 2001. **63**(1–3): p. 88-102.
102. Anderson, R.R. and J.A. Parrish, *The Optics of Human Skin.* J Investig Dermatol, 1981. **77**(1): p. 13-19.
103. Kielbassa, C., L. Roza, and B. Epe, *Wavelength dependence of oxidative DNA damage induced by UV and visible light.* Carcinogenesis, 1997. **18**(4): p. 811-816.
104. Antony, R.Y., *Chromophores in human skin.* Physics in Medicine and Biology, 1997. **42**(5): p. 789.
105. Kvam, E. and R.M. Tyrrell, *Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation.* Carcinogenesis, 1997. **18**(12): p. 2379-2384.
106. Mager, D.R., et al., *Vitamin D3 supplementation, bone health and quality of life in adults with diabetes and chronic kidney disease: Results of an open label randomized clinical trial.* Clinical Nutrition.
107. Zhang, L., et al., *The effects of different intensities of exercise and active vitamin D on mouse bone mass and bone strength.* Journal of Bone and Mineral Metabolism, 2016: p. 1-13.
108. Kovacs, C.S., *Calcium, phosphorus, and bone metabolism in the fetus and newborn.* Early Human Development, 2015. **91**(11): p. 623-628.
109. Holick, M.F. and T.C. Chen, *Vitamin D deficiency: a worldwide problem with health consequences.* The American Journal of Clinical Nutrition, 2008. **87**(4): p. 1080S-1086S.
110. Farrar, M.D., et al., *Sun exposure behavior, seasonal vitamin D deficiency and relationship to bone health in adolescents.* The Journal of Clinical Endocrinology & Metabolism, 2016: p. jc.2016-1559.
111. Lefkowitz, E.S. and C.F. Garland, *Sunlight, vitamin D, and ovarian cancer mortality rates in US women.* Int J Epidemiol, 1994. **23**(6): p. 1133-6.
112. Garland, C.F., et al., *Role of ultraviolet B irradiance and vitamin D in prevention of ovarian cancer.* Am J Prev Med, 2006. **31**(6): p. 512-4.
113. Cutolo, M., et al., *Vitamin D in rheumatoid arthritis.* Autoimmun Rev, 2007. **7**(1): p. 59-64.
114. Mattozzi, C., et al., *Peripheral blood regulatory T cell measurements correlate with serum vitamin D level in patients with psoriasis.* Eur Rev Med Pharmacol Sci, 2016. **20**(9): p. 1675-9.
115. Yap, K.S., et al., *Association of low vitamin D with high disease activity in an Australian systemic lupus erythematosus cohort.* Lupus Science & Medicine, 2015. **2**(1): p. e000064.
116. Holick, M., et al., *Photosynthesis of previtamin D3 in human skin and the physiologic consequences.* Science, 1980. **210**(4466): p. 203-205.
117. Holick, M.F., *Sunlight "D"ilemma: risk of skin cancer or bone disease and muscle weakness.* Lancet, 2001. **357**(9249): p. 4-6.
118. Reichrath, J., *The challenge resulting from positive and negative effects of sunlight: How much solar UV exposure is appropriate to balance between risks of vitamin D deficiency and skin cancer?* Progress in Biophysics and Molecular Biology, 2006. **92**(1): p. 9-16.
119. NHS. *Sunburn*. 2015 [cited 2016 2nd, August]; Available from: http://www.nhs.uk/Conditions/Sunburn/Pages/Introduction.aspx.
120. Weill, F.S., et al., *Skin Exposure to Chronic But Not Acute UV Radiation Affects Peripheral T-Cell Function.* Journal of Toxicology and Environmental Health-Part a-Current Issues, 2011. **74**(13): p. 838-847.
121. Rhodes, L.E., et al., *The sunburn response in human skin is characterized by sequential eicosanoid profiles that may mediate its early and late phases.* Faseb Journal, 2009. **23**(11): p. 3947-3956.
122. Harrison, G.I. and A.R. Young, *Ultraviolet radiation-induced erythema in human skin.* Methods, 2002. **28**(1): p. 14-19.

123. Huang, M.-W., P.-Y. Lo, and K.-S. Cheng, *Objective Assessment of Sunburn and Minimal Erythema Doses: Comparison of Noninvasive In Vivo Measuring Techniques after UVB Irradiation.* Eurasip Journal on Advances in Signal Processing, 2010.
124. Lopes, D.M. and S.B. McMahon, *Ultraviolet Radiation on the Skin: A Painful Experience?* CNS Neurosci Ther, 2016. **22**(2): p. 118-26.
125. Pattison, D.I., A.S. Rahmanto, and M.J. Davies, *Photo-oxidation of proteins.* Photochem Photobiol Sci, 2012. **11**(1): p. 38-53.
126. Douki, T. *Melanin synthesis: a paradoxical effect against UV*. 2015 2015 [cited 2016 3rd September]; 24th February,]. Available from: http://inac.cea.fr/en/Phocea/Vie_des_labos/Ast/ast.php?t=fait_marquant&id_ast=1097.
127. Osakabe, A., et al., *Structural basis of pyrimidine-pyrimidone (6-4) photoproduct recognition by UV-DDB in the nucleosome.* Sci Rep, 2015. **5**: p. 16330.
128. Besaratinia, A., et al., *Wavelength dependence of ultraviolet radiation-induced DNA damage as determined by laser irradiation suggests that cyclobutane pyrimidine dimers are the principal DNA lesions produced by terrestrial sunlight.* Faseb j, 2011. **25**(9): p. 3079-91.
129. de Lima-Bessa, K.M., et al., *CPDs and 6-4PPs play different roles in UV-induced cell death in normal and NER-deficient human cells.* DNA Repair (Amst), 2008. **7**(2): p. 303-12.
130. Drouin, R. and J.P. Therrien, *UVB-induced cyclobutane pyrimidine dimer frequency correlates with skin cancer mutational hotspots in p53.* Photochem Photobiol, 1997. **66**(5): p. 719-26.
131. Kaidbey, K.H., et al., *Photoprotection by melanin--a comparison of black and Caucasian skin.* J Am Acad Dermatol, 1979. **1**(3): p. 249-60.
132. Montagna, W. and K. Carlisle, *The architecture of black and white facial skin.* Journal of the American Academy of Dermatology, 1991. **24**(6): p. 929-937.
133. Norval, M., N.K. Gibbs, and J. Gilmour, *The role of urocanic acid in UV-induced immunosuppression: recent advances (1992-1994).* Photochem Photobiol, 1995. **62**(2): p. 209-17.
134. Walterscheid, J.P., et al., *Cis-urocanic acid, a sunlight-induced immunosuppressive factor, activates immune suppression via the 5-HT2A receptor.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17420-5.
135. Sherratt, M.J., et al., *Low-dose ultraviolet radiation selectively degrades chromophore-rich extracellular matrix components.* Journal of Pathology, 2010. **222**(1): p. 32-40.
136. Thurstan, S., et al., *Chemical consequences of cutaneous photoageing.* Chemistry Central Journal, 2012. **6**(1): p. 1-7.
137. Watson, R.E.B., et al., *Damage to Skin Extracellular Matrix Induced by UV Exposure.* Antioxidants & Redox Signaling, 2014. **21**(7): p. 1063-+.
138. Harman, D., *Aging: a theory based on free radical and radiation chemistry.* Journals of Gerontology, 1956. **11**(3): p. 298-300.
139. El-Domyati, M., et al., *Intrinsic aging vs. photoaging: a comparative histopathological, immunohistochemical, and ultrastructural study of skin.* Experimental Dermatology, 2002. **11**(5): p. 398-405.
140. Puschel, H.U., et al., *Attachment of intrinsically and extrinsically aged fibroblasts on collagen and fibronectin.* Journal of photochemistry and photobiology. B, Biology, 1995. **27**(1): p. 39-46.
141. Fisher, G.J., et al., *Collagen Fragmentation Promotes Oxidative Stress and Elevates Matrix Metalloproteinase-1 in Fibroblasts in Aged Human Skin.* American Journal of Pathology, 2009. **174**(1): p. 101-114.
142. Naylor, E.C., R.E.B. Watson, and M.J. Sherratt, *Molecular aspects of skin ageing.* Maturitas, 2011. **69**(3): p. 249-256.
143. Quan, T.H., et al., *Solar ultraviolet irradiation reduces collagen in photoaged human skin by blocking transforming growth factor-beta type II receptor/Smad signaling.* American Journal of Pathology, 2004. **165**(3): p. 741-751.
144. Griffiths, C.E.M., et al., *Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid).* New England Journal of Medicine, 1993. **329**(8): p. 530-535.
145. Fisher, G.J., et al., *Pathophysiology of premature skin aging induced by ultraviolet light.* New England Journal of Medicine, 1997. **337**(20): p. 1419-1428.
146. Warren, R., et al., *Age, sunlight, and facial skin: A histologic and quantitative study.* Journal of the American Academy of Dermatology, 1991. **25**(5): p. 751-760.

147. Fisher, G.J., et al., *Molecular basis of sun-induced premature skin ageing and retinoid antagonism.* Nature, 1996. **379**(6563): p. 335-339.
148. Yaar, M. and B.A. Gilchrest, *Photoageing: mechanism, prevention and therapy.* British Journal of Dermatology, 2007. **157**(5): p. 874-887.
149. Carta, L., et al., *Fibrillins 1 and 2 perform partially overlapping functions during aortic development.* Journal of Biological Chemistry, 2006. **281**(12): p. 8016-8023.
150. Yuan, X.M., et al., *Solution structure of the transforming growth factor beta-binding protein-like module, a domain associated with matrix fibrils.* Embo Journal, 1997. **16**(22): p. 6659-6666.
151. Kerwin, B.A. and R.L. Remmele, *Protect from light: Photodegradation and protein biologics.* Journal of Pharmaceutical Sciences, 2007. **96**(6): p. 1468-1479.
152. Godeau, G. and W. Hornebeck, *Morphometric analysis of the degradation of human skin elastic fibres by human leukocyte elastase (EC 3-4-21-37) and human skin fibroblast elastase (EC 3-4-24).* Pathologie Biologie, 1988. **36**(9): p. 1133-1138.
153. Imokawa, G., *Recent advances in characterizing biological mechanisms underlying UV-induced wrinkles: a pivotal role of fibrobrast-derived elastase.* Archives of Dermatological Research, 2008. **300**(1): p. 7-20.
154. Rijken, F. and P.L.B. Bruijnzeel, *The Pathogenesis of Photoaging: The Role of Neutrophils and Neutrophil-Derived Enzymes.* Journal of Investigative Dermatology Symposium Proceedings, 2009. **14**(1): p. 67-72.
155. Thurstan, S.A., et al., *Chemical consequences of cutaneous photoageing.* Chemistry Central Journal, 2012. **6**.
156. Massague, J. and Y.G. Chen, *Controlling TGF-beta signaling.* Genes & Development, 2000. **14**(6): p. 627-644.
157. Massague, J. and D. Wotton, *Transcriptional control by the TGF-beta/Smad signaling system.* Embo Journal, 2000. **19**(8): p. 1745-1754.
158. Harman, D., *Free radical theory of aging.* Mutation Research/DNAging, 1992. **275**(3–6): p. 257-266.
159. Richter, C., J.W. Park, and B.N. Ames, *Normal oxidative damage to mitochondrial and nuclear DNA is extensive.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(17): p. 6465-6467.
160. Butterfield, D.A., et al., *Structural and Functional Changes in Proteins Induced by Free Radical-mediated Oxidative Stress and Protective Action of the Antioxidants N-tert-Butyl-α-phenylnitrone and Vitamin Ea.* Annals of the New York Academy of Sciences, 1998. **854**(1): p. 448-462.
161. Ghazi, S., et al., *Interest of external photoprotection by means of clothing and sunscreen products in young children.* J Eur Acad Dermatol Venereol, 2012. **26**(8): p. 1026-30.
162. Saewan, N. and A. Jimtaisong, *Natural products as photoprotection.* J Cosmet Dermatol, 2015. **14**(1): p. 47-63.
163. More, B.D., *Physical sunscreens: on the comeback trail.* Indian J Dermatol Venereol Leprol, 2007. **73**(2): p. 80-5.
164. Dean, J.C., et al., *Plant sunscreens in the UV-B: ultraviolet spectroscopy of jet-cooled sinapoyl malate, sinapic acid, and sinapate ester derivatives.* J Am Chem Soc, 2014. **136**(42): p. 14780-95.
165. Mizuno, M., et al., *The effects of continuous application of sunscreen on photoaged skin in Japanese elderly people - the relationship with the usage.* Clin Cosmet Investig Dermatol, 2016. **9**: p. 95-105.
166. Korać, R.R. and K.M. Khambholja, *Potential of herbs in skin protection from ultraviolet radiation.* Pharmacognosy Reviews, 2011. **5**(10): p. 164-173.
167. Serafini, M.R., et al., *Natural compounds for solar photoprotection: a patent review.* Expert Opin Ther Pat, 2015. **25**(4): p. 467-78.
168. Dromgoole, S.H. and H.I. Maibach, *Sunscreening agent intolerance: contact and photocontact sensitization and contact urticaria.* J Am Acad Dermatol, 1990. **22**(6 Pt 1): p. 1068-78.
169. Xu, S., et al., *Sunscreen Product Performance and Other Determinants of Consumer Preferences.* JAMA Dermatol, 2016. **152**(8): p. 920-7.
170. Pathak, M.A., *Sunscreens: topical and systemic approaches for protection of human skin against harmful effects of solar radiation.* J Am Acad Dermatol, 1982. **7**(3): p. 285-312.

171. Aust, O., et al., *Supplementation with tomato-based products increases lycopene, phytofluene, and phytoene levels in human serum and protects against UV-light-induced erythema.* Int J Vitam Nutr Res, 2005. **75**(1): p. 54-60.
172. Cho, S., et al., *Differential effects of low-dose and high-dose beta-carotene supplementation on the signs of photoaging and type I procollagen gene expression in human skin in vivo.* Dermatology, 2010. **221**(2): p. 160-71.
173. Sugimoto, S., et al., *Photoprotective effects of Bifidobacterium breve supplementation against skin damage induced by ultraviolet irradiation in hairless mice.* Photodermatol Photoimmunol Photomed, 2012. **28**(6): p. 312-9.
174. El-Haj, N. and N. Goldstein, *Sun protection in a pill: the photoprotective properties of Polypodium leucotomos extract.* Int J Dermatol, 2015. **54**(3): p. 362-6.
175. Morley, N., et al., *The green tea polyphenol (−)-epigallocatechin gallate and green tea can protect human cellular DNA from ultraviolet and visible radiation-induced damage.* Photodermatology, Photoimmunology & Photomedicine, 2005. **21**(1): p. 15-22.
176. Eberlein-Konig, B., M. Placzek, and B. Przybilla, *Protective effect against sunburn of combined systemic ascorbic acid (vitamin C) and d-alpha-tocopherol (vitamin E).* Journal of the American Academy of Dermatology, 1998. **38**(1): p. 45-48.
177. Stahl, W., et al., *Carotenoids and carotenoids plus vitamin E protect against ultraviolet light-induced erythema in humans.* American Journal of Clinical Nutrition, 2000. **71**(3): p. 795-798.
178. Burns, J.J., *Missing step in man, monkey and guinea pig required for the biosynthesis of L-ascorbic acid.* Nature, 1957. **180**(4585): p. 553.
179. Carpenter, K.J., *The discovery of vitamin C.* Ann Nutr Metab, 2012. **61**(3): p. 259-64.
180. Bendich, A. and L. Langseth, *The health effects of vitamin C supplementation: a review.* Journal of the American College of Nutrition, 1995. **14**(2): p. 124-136.
181. Frei, B., I. Birlouez-Aragon, and J. Lykkesfeldt, *Authors' Perspective: What is the Optimum Intake of Vitamin C in Humans?* Crit Rev Food Sci Nutr, 2012. **52**(9): p. 815-829.
182. Prieme, H., et al., *No effect of supplementation with vitamin E, ascorbic acid, or coenzyme Q10 on oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2'-deoxyguanosine excretion in smokers.* Am J Clin Nutr, 1997. **65**(2): p. 503-7.
183. Witt, E.H., et al., *Exercise, oxidative damage and effects of antioxidant manipulation.* J Nutr, 1992. **122**(3 Suppl): p. 766-73.
184. McArdle, F., et al., *UVR-induced oxidative stress in human skin in vivo: effects of oral vitamin C supplementation.* Free Radical Biology and Medicine, 2002. **33**(10): p. 1355-1362.
185. Boyera, N., I. Galey, and B.A. Bernard, *Effect of vitamin C and its derivatives on collagen synthesis and cross-linking by normal human fibroblasts.* Int J Cosmet Sci, 1998. **20**(3): p. 151-8.
186. Scalbert, A., et al., *Dietary polyphenols and the prevention of diseases.* Crit Rev Food Sci Nutr, 2005. **45**(4): p. 287-306.
187. Gonzalez, S., et al., *Dietary intake of polyphenols and major food sources in an institutionalised elderly population.* J Hum Nutr Diet, 2014. **27**(2): p. 176-83.
188. Cheynier, V., F.A. Tomas-Barberan, and K. Yoshida, *Polyphenols: From Plants to a Variety of Food and Nonfood Uses.* J Agric Food Chem, 2015. **63**(35): p. 7589-94.
189. Lee, K.W., et al., *Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine.* J Agric Food Chem, 2003. **51**(25): p. 7292-5.
190. Lamien-Meda, A., et al., *Polyphenol content and antioxidant activity of fourteen wild edible fruits from Burkina Faso.* Molecules, 2008. **13**(3): p. 581-94.
191. Sariburun, E., et al., *Phenolic content and antioxidant activity of raspberry and blackberry cultivars.* J Food Sci, 2010. **75**(4): p. C328-35.
192. Shrubsole, M.J., et al., *Drinking green tea modestly reduces breast cancer risk.* J Nutr, 2009. **139**(2): p. 310-6.
193. Fu, Y.C., et al., *Ultraviolet radiation and reactive oxygen generation as inducers of keratinocyte apoptosis: protective role of tea polyphenols.* J Toxicol Environ Health A, 2000. **61**(3): p. 177-88.
194. Afaq, F. and S.K. Katiyar, *Polyphenols: Skin Photoprotection and Inhibition of Photocarcinogenesis.* Mini-Reviews in Medicinal Chemistry, 2011. **11**(14): p. 1200-1215.
195. Singh, A., S. Holvoet, and A. Mercenier, *Dietary polyphenols in the prevention and treatment of allergic diseases.* Clin Exp Allergy, 2011. **41**(10): p. 1346-59.

196. Fuller, H.R., E.L. Humphrey, and G.E. Morris, *Naturally occurring plant polyphenols as potential therapies for inherited neuromuscular diseases.* Future Med Chem, 2013. **5**(17): p. 2091-101.
197. Chirumbolo, S., *Dietary assumption of plant polyphenols and prevention of allergy.* Curr Pharm Des, 2014. **20**(6): p. 811-39.
198. Lecci, R.M., A. Logrieco, and A. Leone, *Pro-oxidative action of polyphenols as action mechanism for their pro-apoptotic activity.* Anticancer Agents Med Chem, 2014. **14**(10): p. 1363-75.
199. Noad, R.L., et al., *Beneficial effect of a polyphenol-rich diet on cardiovascular risk: a randomised control trial.* Heart, 2016. **102**(17): p. 1371-9.
200. Uysal, U., et al., *Consumption of polyphenol plants may slow aging and associated diseases.* Curr Pharm Des, 2013. **19**(34): p. 6094-111.
201. Korkina, L.G., et al., *Metabolism of plant polyphenols in the skin: beneficial versus deleterious effects.* Curr Drug Metab, 2008. **9**(8): p. 710-29.
202. Korkina, L., C. De Luca, and S. Pastore, *Plant polyphenols and human skin: friends or foes.* Ann N Y Acad Sci, 2012. **1259**: p. 77-86.
203. Petros, S. *The Polyphenolic Family*. [Health & Nutrition Blog] 2014 10th February 2014 [cited 2016 14th September]; Available from: https://www.justvitamins.co.uk/blog/the-polyphenolic-family/.
204. Banerjee, B., *Tea : production and processing*. 1993, New Delhi: Oxford & IBH Publishing Company. 336.
205. Mondal, T.K., *Beverage Crops: Tea*, in *Transgenic Crops V*, E.-C. Pua and R.M. Davey, Editors. 2007, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 519-535.
206. Lettsom, J.C., et al., *The natural history of the tea-tree : with observations on the medical qualities of tea and on the effects of tea drinking*. 1799, London, UK: Missouri Botanical Garden.
207. Jemma. *Camellia sinensis*. 2015 [cited 2016 28 August]; Available from: https://herbologymanchester.wordpress.com/tag/camellia-sinensis/.
208. Harler, C.R., *The culture and marketing of tea*. 1964, London: London : Oxford University Press.
209. Eden, T., *Tea (Tropical Agriculture)* 1965, Logmans, Green & Co., London: Prentice Hall Press.
210. Balentine, D.A., S.A. Wiseman, and L.C.M. Bouwens, *The chemistry of tea flavonoids.* Crit Rev Food Sci Nutr, 1997. **37**(8): p. 693-704.
211. Hicks, A., *Current status and future development of global tea production and tea products.* AU Journal of Technology, 2009. **12(4)**: p. 251-264.
212. Chang, K. *World tea production and trade: current and future development*. [A Subsidiary Body of the FAO Committee on Commodity Problems (CCP)] 2015 [cited 2016 26 August]; Available from: http://www.fao.org/economic/est/est-commodities/tea/en/.
213. Pandey, K.B.a.R., S.I., *Plant polyphenols as dietary antioxidants in human health and disease.* Oxidative medicine and cellular longevity, 2009. **2**(5): p. 270-8.
214. Mittal, A., C.A. Elmets, and S.K. Katiyar, *Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation.* Carcinogenesis, 2003. **24**(8): p. 1379-1388.
215. Graham, H.N., *Green tea composition, consumption, and polyphenol chemistry.* Preventive Medicine, 1992. **21**(3): p. 334-350.
216. Skrzydlewska, E., et al., *Protective effect of green tea against lipid peroxidation in the rat liver, blood serum and the brain.* Phytomedicine, 2002. **9**(3): p. 232-238.
217. Wiseman, S., T. Mulder, and A. Rietveld, *Tea flavonoids: Bioavailability in vivo and effects on cell signaling pathways in vitro.* Antioxidants & Redox Signaling, 2001. **3**(6): p. 1009-1021.
218. Ahmad, N., Gupta, S. and Mukhtar, H., *Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells.* Archives of biochemistry and biophysics, 2000. **15; 376**(2): p. 338-46.
219. Sato, D., *Inhibition of urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine in rats by green tea.* International Journal of Urology, 1999. **6**(2): p. 93-99.
220. Sazuka, M., et al., *Inhibitory effects of green tea infusion on in vitro invasion and in vivo metastasis of mouse lung carcinoma cells.* Cancer Letters, 1995. **98**(1): p. 27-31.
221. Zhang, M., et al., *Green tea and the prevention of breast cancer: a case-control study in Southeast China.* Carcinogenesis, 2007. **28**(5): p. 1074-8.

222. Ogata, K., Mukae, N., Suzuki, Y., Satoh, K., Narumi, K., Nukiwa, T. and Isemura, M., *Effects of catechins on the mouse tumor cell adhesion to fibronectin.* Planta medica, 1995. **61**(5): p. 472-4.
223. Isemura, M., Suzuki, Y., Satoh, K., Narumi, K. and Motomiya, M., *Effects of catechins on the mouse lung carcinoma cell adhesion to the endothelial cells.* Cell biology international, 1993. **17**(6): p. 559-64.
224. Suzuki, Y.a.I., M., *Inhibitory effect of epigallocatechin gallate on adhesion of murine melanoma cells to laminin.* Cancer letters, 2001. **173**(1): p. 15-20.
225. Yamaguchi, Y., Isemura, M., Yosizawa, Z., Kurosawa, K., Yoshinaga, K., Sato, A. and Suzuki, M., *Changes in the distribution of fibronectin in the placenta during normal human pregnancy.* American journal of obstetrics and gynecology, 1985. **15; 152**(6 Pt 1): p. 715-8.
226. Kurosawa, K., Isemura, M., Yamaguchi, Y., Yosizawa, Z., Furuyama, T., Yoshinaga, K. and Ishii, T., *Changes in distribution of connective tissue components of human placentae with maturation.* The Tohoku journal of experimental medicine, 1985. **147**(3): p. 261-5.
227. Mason, P., *Dietary Supplements*. 3 ed. 2007: Pharmaceutical Press.
228. Van het Hof, K.H., de Boer, H.S., Wiseman, S.A., Lien, N., Westrate, J.A. and Tijburg, L.B., *Consumption of green or black tea does not increase resistance of low-density lipoprotein to oxidation in humans.* American Society for Clinical Nutrition, 1997. **66**(5): p. 1125-32.
229. Yokozawa, T. and E.B. Dong, *Influence of green tea and its three major components upon low-density lipoprotein oxidation.* Experimental and Toxicologic Pathology, 1997. **49**(5): p. 329-335.
230. Muramatsu, K., Fukuyo, M. and Hara, Y., *Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats.* Journal of nutritional science and vitaminology, 1986. **32**(6): p. 613-22.
231. Erba, D., Riso, P., Bordoni, A., Foti, P., Biagi, P.L. and Testolin, G., *Effectiveness of moderate green tea consumption on antioxidative status and plasma lipid profile in humans.* The Journal of nutritional biochemistry, 2005. **16**(3): p. 144-9.
232. Gomikawa, S., Ishikawa, Y., Hayase, W., Haratake, Y., Hirano, N., Matuura, H., Mizowaki, A., Murakami, A. and Yamamoto, M., *Effect of ground green tea drinking for 2 weeks on the susceptibility of plasma and LDL to the oxidation ex vivo in healthy volunteers.* The Kobe journal of medical sciences, 2008. **23; 54**(1): p. E62-72.
233. Lee, S.M., Kim, C.W., Kim, J.K., Shin, H.J. and Baik, J.H., *GCG-rich tea catechins are effective in lowering cholesterol and triglyceride concentrations in hyperlipidemic rats.* Lipids, 2008. **43**(5): p. 419-29.
234. Kim, J., et al., *Protective effects of (-)-epigallocatechin-3-gallate on UVA- and UVB-induced skin damage.* Skin Pharmacology and Applied Skin Physiology, 2001. **14**(1): p. 11-19.
235. Vayalil, P.K., et al., *Green tea polyphenols prevent ultraviolet light-induced oxidative damage and matrix metalloproteinases expression in mouse skin.* Journal of Investigative Dermatology, 2004. **122**(6): p. 1480-1487.
236. Mantena, S.K., et al., *Orally administered green tea polyphenols prevent ultraviolet radiation-induced skin cancer in mice through activation of cytotoxic T cells and inhibition of angiogenesis in tumors.* J Nutr, 2005. **135**(12): p. 2871-7.
237. Camouse, M.M., et al., *Topical application of green and white tea extracts provides protection from solar-simulated ultraviolet light in human skin.* Experimental Dermatology, 2009. **18**(6): p. 522-526.
238. Valavanidis, A., T. Vlachogianni, and C. Fiotakis, *8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis.* J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, 2009. **27**(2): p. 120-39.
239. Chow, H.H.S., et al., *Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals.* Clinical Cancer Research, 2005. **11**(12): p. 4627-4633.
240. Fathima, N.N., et al., *Role of green tea polyphenol crosslinking in alleviating ultraviolet-radiation effects on collagen.* Journal of Applied Polymer Science, 2007. **106**(5): p. 3382-3386.
241. Madhan, B., et al., *Role of green tea polyphenols in the inhibition of collagenolytic activity by collagenase.* Int J Biol Macromol, 2007. **41**(1): p. 16-22.

242. Bae, J.-Y., et al., *(−)Epigallocatechin gallate hampers collagen destruction and collagenase activation in ultraviolet-B-irradiated human dermal fibroblasts: Involvement of mitogen-activated protein kinase.* Food and Chemical Toxicology, 2008. **46**(4): p. 1298-1307.
243. Janeiro, P. and A.M. Oliveira Brett, *Catechin electrochemical oxidation mechanisms.* Analytica Chimica Acta, 2004. **518**(1–2): p. 109-115.
244. Cren-Olivé, C., et al., *Free Radical Chemistry of Flavan-3-ols: Determination of Thermodynamic Parameters and of Kinetic Reactivity from Short (ns) to Long (ms) Time Scale.* Journal of the American Chemical Society, 2002. **124**(47): p. 14027-14038.
245. Halliwell, B., *Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies?* Archives of Biochemistry and Biophysics, 2008. **476**(2): p. 107-112.
246. Chiu, A.E., et al., *Double-Blinded, Placebo-Controlled Trial of Green Tea Extracts in the Clinical and Histologic Appearance of Photoaging Skin.* Dermatologic Surgery, 2005. **31**: p. 855-860.
247. Farrar, M.D., et al., *A randomized controlled trial of green tea catechins in protection against ultraviolet radiation-induced cutaneous inflammation.* American Journal of Clinical Nutrition, 2015. **102**(3): p. 608-615.
248. Andesen, P.H., et al., *Time-correlation study of ultraviolet B-induced erythema measured by reflectance spectroscopy and laser Doppler flowmetry.* Photodermatology Photoimmunology & Photomedicine, 1991. **8**(3): p. 123-128.
249. Dornelles, S., J. Goldim, and T. Cestarl, *Determination of the Minimal Erythema Dose and Colorimetric Measurements as Indicators of Skin Sensitivity to UV-B Radiation.* Photochemistry and Photobiology, 2004. **79**(6): p. 540-544.
250. Rhodes, L.E. and P.S. Friedmann, *A comparison of the ultraviolet B-induced erythemal response of back and buttock skin.* Photodermatology Photoimmunology & Photomedicine, 1992. **9**(2): p. 48-51.
251. Lahti, A., et al., *Assessment of skin erythema by eye, laser Doppler flowmeter, spectroradiometer, two-channel erythema meter and Minolta chroma meter.* Archives of Dermatological Research, 1993. **285**(5): p. 278-282.
252. Diffey, B.L. and P.M. Farr, *Quantitative aspects of ultraviolet erythema.* Clinical Physics and Physiological Measurement, 1991. **12**(4): p. 311-325.
253. Dolotov, L.E., et al., *Design and evaluation of a novel portable erythema-melanin-meter.* Lasers in Surgery and Medicine, 2004. **34**(2): p. 127-135.
254. Chen, Z.Y., et al., *Stabilizing effect of ascorbic acid on green tea catechins.* Journal of Agricultural and Food Chemistry, 1998. **46**(7): p. 2512-2516.
255. Herbert, K.E., et al., *Dietary supplementation with different vitamin C doses: no effect on oxidative DNA damage in healthy people.* European Journal of Nutrition, 2006. **45**(2): p. 97-104.
256. Rhodes, L.E., et al., *Oral green tea catechin metabolites are incorporated into human skin and protect against UV radiation-induced cutaneous inflammation in association with reduced production of pro-inflammatory eicosanoid 12-hydroxyeicosatetraenoic acid.* British Journal of Nutrition, 2013. **110**(5): p. 891-900.
257. Darby, G., *Impact of oral green tea catechins on UVR-induced skin inflammation*, in *Faculty of Medical and Human Sciences*. 2014, The University of Manchester: Manchester. p. 107.
258. Novikoff, A.B., W.Y. Shin, and J. Drucker, *Cold acetone fization for enzyme localization in frozen sections.* Journal of Histochemistry & Cytochemistry, 1960. **8**(1): p. 37-40.
259. Graham, H.K. and A.W. Trafford, *Spatial disruption and enhanced degradation of collagen with the transition from compensated ventricular hypertrophy to symptomatic congestive heart failure.* American Journal of Physiology - Heart and Circulatory Physiology, 2007. **292**(3): p. H1364-H1372.
260. Graham, H.K., et al., *Localised micro-mechanical stiffening in the ageing aorta.* Mechanisms of Ageing and Development, 2011. **132**(10): p. 459-467.
261. Rich, L.a.W., P., *collagen and picrosirius red staining: a polarized light assessment of fibrillar hue and spatial distribution.* Journal of Morphological Science, 2005. **2**(22): p. 97-107.
262. Watson, R.E.B., et al., *A cosmetic 'anti-ageing' product improves photoaged skin: a double-blind, randomized controlled trial.* British Journal of Dermatology, 2009. **161**(2): p. 419-426.

263. Cockell, C.S., *Biological effects of high ultraviolet radiation on early Earth - a theoretical evaluation.* Journal of Theoretical Biology, 1998. **193**(4): p. 717-729.
264. Hakim, O.A., et al., *Vitamin D production in UK Caucasian and South Asian women following UVR exposure.* The Journal of Steroid Biochemistry and Molecular Biology.
265. Ramkisoensing, A. and J.H. Meijer, *Synchronization of Biological Clock Neurons by Light and Peripheral Feedback Systems Promotes Circadian Rhythms and Health.* Frontiers in Neurology, 2015. **6**: p. 128.
266. Cust, A.E., et al., *Sunbed use during adolescence and early adulthood is associated with increased risk of early-onset melanoma.* International journal of cancer. Journal international du cancer, 2011. **128**(10): p. 2425-2435.
267. Wilkes, M., et al., *Fitzpatrick skin type, individual typology angle, and melanin index in an african population: Steps toward universally applicable skin photosensitivity assessments.* JAMA Dermatology, 2015. **151**(8): p. 902-903.
268. de Gruijl, F.R., *Photocarcinogenesis: UVA vs UVB*, in *Methods in Enzymology*. 2000, Academic Press. p. 359-366.
269. Walker, S., Rhodes, L.E., Chatelus, Y., Griffiths, C.M., Watson, R.B. *Fibrillin-rich microfibrils are reduced following acute UVB irradiation*. in *British Society for Investigative Dermatology Annual Meeting*. 2002. University of East Anglia, Norwich, U.K.: Br J Dermatol.
270. Suwabe, H., et al., *Degenerative processes of elastic fibers in sun-protected and sun-exposed skin: Immunoelectron microscopic observation of elastin, fibrillin-1, amyloid P component, lysozyme and α1-antitrypsin.* Pathology International, 1999. **49**(5): p. 391-402.
271. Watson, R.E. and C.E. Griffiths, *Fibulin-2 and its association with fibrillin-rich microfibrils in the papillary dermis of photoaged skin.* Br J Dermatol, 2002. **146**(4): p. 712.
272. Chen, L., J.Y. Hu, and S.Q. Wang, *The role of antioxidants in photoprotection: A critical review.* Journal of the American Academy of Dermatology, 2012. **67**(5): p. 1013-1024.
273. Seité, S. and A.M.A. Fourtanier, *The benefit of daily photoprotection.* Journal of the American Academy of Dermatology, 2008. **58**(5, Supplement 2): p. S160-S166.
274. Stephens, T.J., et al., *A Randomized, Double-blind, Placebo-controlled Clinical Trial Evaluating an Oral Anti-aging Skin Care Supplement for Treating Photodamaged Skin.* J Clin Aesthet Dermatol, 2016. **9**(4): p. 25-32.
275. Tundis, R., et al., *Potential role of natural compounds against skin aging.* Curr Med Chem, 2015. **22**(12): p. 1515-38.
276. Higdon, J.V. and B. Frei, *Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions.* Crit Rev Food Sci Nutr, 2003. **43**(1): p. 89-143.
277. Lambert, J.D. and R.J. Elias, *The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention.* Archives of Biochemistry and Biophysics, 2010. **501**(1): p. 65-72.
278. Zheng, X.-X., et al., *Green tea intake lowers fasting serum total and LDL cholesterol in adults: a meta-analysis of 14 randomized controlled trials.* American Journal of Clinical Nutrition, 2011. **94**(2): p. 601-610.
279. Beltz, L.A., et al., *Mechanisms of cancer prevention by green and black tea polyphenols.* Anticancer Agents Med Chem, 2006. **6**(5): p. 389-406.
280. Baba, Y., et al., *Reduction of oxidative stress in liver cancer patients by oral green tea polyphenol tablets during hepatic arterial infusion chemotherapy.* Exp Ther Med, 2012. **4**(3): p. 452-458.
281. Janjua, R., et al., *A Two-Year, Double-Blind, Randomized Placebo-Controlled Trial of Oral Green Tea Polyphenols on the Long-Term Clinical and Histologic Appearance of Photoaging Skin.* Dermatologic Surgery, 2009. **35**(7): p. 1057-1065.
282. Jeon, H.Y., et al., *Effects of Oral Epigallocatechin Gallate Supplementation on the Minimal Erythema Dose and UV-Induced Skin Damage.* Skin Pharmacology and Physiology, 2009. **22**(3): p. 137-141.
283. Heinrich, U., et al., *Green Tea Polyphenols Provide Photoprotection, Increase Microcirculation, and Modulate Skin Properties of Women.* Journal of Nutrition, 2011. **141**(6): p. 1202-1208.
284. Sullivan, G.M., *Getting Off the "Gold Standard": Randomized Controlled Trials and Education Research.* Journal of Graduate Medical Education, 2011. **3**(3): p. 285-289.