Ultrastructural characterisation of fibrillin microfibrils from photoaged and all-\textit{trans} retinoic acid-treated skin.

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Abstract

The University of Manchester, Elizabeth C. Naylor, Doctor of Philosophy, Ultrastructural characterisation of fibrillin microfibrils from photoaged and all-trans retinoic acid-treated skin, 2013.

Photoageing of human skin by cumulative exposure to ultraviolet radiation (UVR) is characterised by extensive remodelling of the long-lived dermal elastic fibre network. Fibrillin microfibrils are key components of this network and are a useful biomarker of photoageing and its repair with topical all-trans retinoic acid (t-RA) treatment. Investigations have shown that fibrillin microfibrils are highly susceptible to UVR in vitro, yet it is unknown how the full contributing effects of photoageing in vivo will affect their complex ultrastructure. Furthermore, whilst t-RA-treatment of photoaged skin leads to an increase in immunohistochemical staining for fibrillin microfibrils, still unanswered is whether treatment leads to new synthesis or repair of structurally and functionally competent fibrillin microfibrils, comparable to those found in photoprotected young skin.

Healthy, but severely photoaged, volunteers (mean age 71.3 years) were recruited to the study (n=10). Samples (3mm biopsies) were obtained from photoprotected (intrinsically aged) upper inner arm and photoaged forearm untreated or treated with 0.025% t-RA for four-days. Biopsies were sectioned and stained for fibrillin microfibrils using immunohistochemistry (performed on nine volunteers) or used for extraction of fibrillin microfibrils by bacterial collagenase and size exclusion chromatography (performed on five volunteers). The following parameters were measured on extracted fibrillin microfibrils both by scanning transmission electron microscopy (STEM) and atomic force microscopy (AFM): (i) Abundance (n=10) (ii) length (n=50) (iii) mass per repeat (n=250, STEM only) (iv) flexion angle (n=250) and (v) periodicity (n=250).

In eight of the nine volunteers there was a reduction in fibrillin microfibril staining grade of photoaged skin. Fibrillin microfibrils extracted from photoaged skin had a lower mass per repeat than those extracted from photoprotected skin of the same volunteer; which was localised to the centre of the bead. All volunteers displayed a significant increase or decrease in periodicity, but no change in flexibility or abundance. Fibrillin microfibrils extracted from t-RA-treated photoaged skin showed alterations in periodicity in four of the five volunteers and an increase in mass in two of four volunteers, but no change in length, flexibility or abundance. Fibrillin microfibrils extracted from t-RA-treated photoprotected (intrinsically aged) skin, were apparently normalised in terms of length and periodicity, with decreases in mass in three of five volunteers, but no alterations in flexibility or abundance. There was wide individual variation across all fibrillin microfibril populations.

Results showed for the first time that fibrillin microfibrils are susceptible to photoageing-related remodelling and damage in vivo, which is likely mediated by a combination of direct UVR absorption by chromophores, ROS-mediated damage and enzyme-mediated remodelling. t-RA was found not to significantly alter the ultrastructure of fibrillin microfibrils, perhaps due to the short length of the patch-test study, the complex macromolecular nature of fibrillin microfibrils, or individual variation in retinoid response.
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Ultrastructural characterisation of fibrillin microfibrils from photoaged and all-trans retinoic acid-treated skin.
Chapter 1

Introduction
1 Introduction, aims, literature review and thesis hypotheses.

This chapter provides a brief review of the relevant literature and background for the investigation before providing an overview of the aims and structure of the thesis followed by the specific research hypotheses.

1.1 Human skin is a multilayer organ.

Skin is a multilayer organ which functions as a barrier to external challenges such as water, heat, pathogens, ultra-violet radiation (UVR) and mechanical stress. It can be divided into two distinct layers; the epidermis and the dermis which vary in both structure and function (figure 1.1).

1.1.1 The epidermis is highly cellular and acts as the primary barrier to external challenges.

The human epidermis is the outer layer of the skin which varies in thickness between body sites from 80 μm on the forearms, to 1 mm on palms and soles (Sandby-Moller et al., 2003). It is highly cellular with the main cell type being the keratinocyte. These cells migrate to form distinct layers as they mature and differentiate from the lowest level of the epidermis (stratum basale) to the skin’s surface (stratum corneum) in a process that takes approximately 40 to 56 days (Halprin, 1972; Mackenzie, 1975).

The epidermis is the primary barrier of the skin as keratinocytes link together by tight junctions to prevent water loss or gain, as well as form the stratum corneum where enucleated keratin-rich cells are shed in a process known as desquamation to prevent bacterial overpopulation (Goldschmidt and Kligman, 1967; O’Neill and Garrod, 2011; Tsuruta et al., 2002). The epidermis is also host to melanocytes which produce melanin, the chemical pigment of skin, which can absorb harmful UVR (Brenner and Hearing, 2008). Melanin is contained within melanosomes which are transported dendritically to neighbouring keratinocytes, thereby increasing the area it can protect (Brenner and Hearing, 2008; Friedmann and Gilchrest, 1987; Lavker and Kaidbey, 1982; Wolff, 1973). The epidermal barrier, as well as immune components of the skin such as Langerhans cells, provide protection from pathogenic insults (Romani et al., 2003) (Figure 1.1).
1.1.2 The dermis contains a complex extracellular matrix.

The dermis lies beneath the epidermis and can be subdivided into the upper papillary dermis and the lower reticular dermis. In contrast to the epidermis, the dermis has relatively few cells, the most common of which are fibroblasts which synthesise and organise components of the extracellular matrix (ECM), as well as playing roles in cellular communication via integrin binding (Sorrell and Caplan, 2004). The dermal ECM provides structural support to the skin and resistance/compliance to mechanical insults (Daly, 1982). However, the dermis also contains complex capillary, nervous and lymphatic networks, as well as a population of immune cells such as mast cells and Langerhan's cells (Benyon, 1989; Braverman, 1983; Romani et al., 2003; Ryan, 1989; Salmon et al., 1994). There are also hair follicles, sebaceous and sweat glands and striated muscle (Briggaman, 1982; Smith et al., 1982).

The dermis is secured to the epidermis via the dermal-epidermal junction (DEJ) which undulates to form rete-ridges and a complex basement membrane which consists of hemidesmosomes, collagen VII anchoring filaments and collagen IV supports (Briggaman and Wheeler, 1975; Ray and Gately, 1996; Stanley et al., 1982; Swanson and Helwig, 1968).

1.2 The dermal extracellular matrix is dynamic and complex.

The dermal ECM consists of proteins and glycosaminoglycans found in the intercellular spaces underlying epithelia and basement membranes. It provides structural support as well as elastic resilience and recoil, and has multiple functions in cellular anchorage and communication. The dermal ECM is a dynamic and complex mixture of collagens, elastic fibre proteins and glycosaminoglycans which are synthesised by the dermal fibroblasts and keratinocytes (Carrel and Ebeling, 1921; Haynes et al., 1997; Raghunath et al., 1996; Sorrell and Caplan, 2004). Unlike intracellular proteins, the proteins of the ECM are extremely long-lived and are often stable over a human lifetime (Naylor et al., 2011; Ritz-Timme et al., 2003; Sherratt, 2009; Verzijl et al., 2000).

1.2.1 Elastic fibres of the ECM provide recoil and resilience to skin.

The dermal ECM contains a complex elastic fibre network of elastin and fibrillin microfibrils which provide skin with resilience and recoil to mechanical insults. Elastin is a highly elastic protein with an amorphous, random coiled structure that allows it to be reversibly stretched to almost double its length (1.2.4) (Aaron and
Gosline, 1981). It is a vital component of the skin dermis and is secreted first as tropoelastin, its soluble precursor (Indik et al., 1987). Fibrillin microfibrils are macromolecular complexes comprised mainly of fibrillin-1. They have a ‘beads-on-a-string’ appearance and, like elastin, have the ability to elastically recoil, although not on the same magnitude (1.2.4) (Sherratt et al., 2003).

Development of elastic fibres is highly regulated and involves the deposition of tropoelastin onto a preformed fibrillin microfibril template (Lin et al., 2002; Low, 1962; Mecham, 1991; Robb et al., 1999; Trask et al., 1999, 2000b). Therefore mature elastic fibres have an inner core of cross-linked elastin surrounded by a fibrillin microfibril mantle (Mecham, 1991; Sherratt, 2009). However, in the papillary dermis, adjacent to the DEJ, elastic fibres are almost exclusively fibrillin microfibrils, which form a candelabra-like network termed ‘oxytalan fibres’. These oxytalan fibres join with elastin-rich ‘elainin fibres’ in the reticular dermis (Gilchrest, 1996; Juckett, 1987; Schwartz and Fleischmajer, 1986) (Figure 1.1). It is this continuous cascade of elastic fibres throughout the dermis which provides the skin with elasticity and recoil (Daly, 1982; El-Domyati et al., 2002; Schwartz and Fleischmajer, 1986; Sherratt et al., 2003).

1.2.2 Collagens of the ECM provide strength and resistance to skin.

Collagens are one of the most abundant proteins in the dermal ECM and there are many different types which vary in structure and function (Birk and Bruckner, 2005). Fibrillar collagens I and III are the major supporting collagens in the human dermis and provide high tensile strength and resistance as they are virtually inextensible compared to elastin and fibrillin microfibrils (1.2.4) (Akhtar et al., 2011; Daly, 1982; Fleischmajer et al., 1990; Heim et al., 2006). As well as fibrillar collagens, there are numerous other collagens which contribute to the complex architecture of skin. Collagen VII, which is the major component of anchoring fibrils at the DEJ, forms specialised fibrous structures which secure the epidermis to the dermis (Sakai et al., 1986a; Swanson and Helwig, 1968). Collagen VI microfibrils are also widely distributed throughout a variety of connective tissue including the dermis. They have a double-beaded structure and are thought to play a role in cell-to-cell and cell-to-matrix communication (Baldock et al., 2003).

1.2.3 Proteoglycans and oligosaccharides of the ECM provide hydration.

Throughout the whole of the dermis, hydrated oligosaccharide glycosaminoglycans (GAGs) and proteoglycans form a gel like ‘ground substance’ surrounding the elastic
fibres and collagenous matrix. GAGs are large structures of repeating negatively charged disaccharide units, often attached to a protein core. The most prevalent structures are hyaluronic acid and chondroitin sulphates (Silbert, 1982; Smith et al., 1982). The negative charge from the acidic and sulphate groups attract cations such as sodium (Na\(^+\)) which in turn attract water creating a high amount of turgor (Figure 1.1).

1.2.4 The dermal ECM is a complex mixture of both stiff and elastic proteins.

The dermal ECM provides structural support as well as elastic resilience and compliance to mechanical insults (Daly, 1982). A good measure of the stiffness of any elastic component is to calculate the Young’s modulus, which is the ratio of tensile stress over tensile strain along an axis if Hooke’s law holds and can be experimentally tested. Since strain is free of units, Young’s modulus is expressed as the unit of stress which is pressure measured in Pascals.

In the dermal ECM fibrillar collagens are the most stiff component, with a Young’s modulus of 1-2 GPa (Heim et al., 2006). The most compliant is elastin with a Young’s modulus of around 1.1 MPa (Aaron and Gosline, 1981). The Young’s modulus of fibrillin microfibrils appears to differ with species, but the average for human microfibrils is 78-96 MPa (Sherratt et al., 2003). Therefore the stiffness of the collagen network is complemented by the elasticity and recoil of the elastic fibre network.
Figure 1.1 The molecular architecture of the skin. Left to right: human skin consists of an epidermis and dermis. The dermis may be split into papillary (upper) dermis and reticular (lower) dermis. The epidermis is secured to the dermis via hemidesmosomes and collagen VII of the DEJ. The epidermis is cell-rich, mainly keratinocytes. In contrast, the dermis has relatively few cells, mainly fibroblasts. Elastic fibres form a cascade throughout the dermis, with fibrillin microfibrils adjacent to the DEJ in the papillary dermis and elastin-rich fibres in the reticular dermis. Fibrillar collagens I and III are the most abundant collagens in the dermal ECM and are organized roughly parallel to the skin surface. GAGs are abundant throughout the dermis (Naylor et al., 2011).
1.3 As well as intrinsic ageing, skin is subject to external influences.

The process of skin ageing is extremely complex and the age of an individual plays a large role in the appearance of skin. The passage of time (intrinsic ageing) leads to a slow deterioration of skin structure and function which progresses with age, but due to the position on the outside of our bodies, skin is also subject to external factors such as sunlight. The most damaging component of sunlight on our skin is ultraviolet radiation (UVR; which is split into UVA, 400 – 315 nm; UVB, 315 – 280 nm; UVC 280 – 100 nm), and chronic exposure of the skin to sunlight and UVR is termed ‘photoageing’. Photoageing can influence the skin over the course of a lifetime so effects accumulate with increasing age. The signs of skin ageing can be monitored over time, both clinically through the appearance of wrinkles and age spots (Gilchrest, 1996; Griffiths et al., 1992a) and histologically through examination of thin cross-sections of skin biopsies (El-Domyati et al., 2002; Talwar et al., 1995; Watson et al., 1999).

1.3.1 The change in clinical appearance of intrinsically aged skin is subtle.

Intrinsic ageing is ageing of cells and tissues due to the passage of time and a predetermined genetic background (Bakaysa et al., 2007; Borrás et al., 2003; Kimura et al., 2008; Martin et al., 2007). The clinical signs of intrinsic ageing are difficult to examine, since changes are subtle until around the 8th decade. However, the most notable clinical signs are the appearance of pale skin with fine wrinkling, dryness and a mild reduction in the skin’s ability to elastically recoil (Boss and Seegmiller, 1981; El-Domyati et al., 2002; Escoffier et al., 1989; Fenske and Conard, 1988; Fenske and Lober, 1986; Gilchrest, 1989, 1996; Smalls et al., 2006; Takema et al., 1994) (Figure 1.2).

1.3.2 The change in clinical appearance of photoaged skin is caused by cumulative UVR exposure.

Photoageing is a result of the added effects of intrinsic ageing and cumulative UVR exposure (El-Domyati et al., 2002; Gilchrest, 1989; Kligman, 1969). Photoaged skin exhibits many distinct properties, including deep wrinkles, actinic lentigines (also known as age spots), rough sallow skin and a marked loss of skin elasticity (Griffiths et al., 1992a; Kligman, 1969; Smalls et al., 2006; Takema et al., 1994; Tsoureli-Nikita et al., 2006) (Figure 1.2). Photoageing can also be asymmetrical and regular drivers are more likely to have severe photodamage on their right arm, or the right side of...
their face (in the UK) due to the ability of UVA to pass through untreated glass leading to cumulative exposure (Brenner and Hearing, 2008; Mac-Mary et al., 2010; Schaefer et al., 1998; Singer et al., 1994). This is why most glass in automobiles, aircraft and eye-wear have specially treated glass, such as laminated glass, reflective glass or an insulated glass unit (Tuchinda et al., 2006).

<table>
<thead>
<tr>
<th>Intrinsic Ageing</th>
<th>Photoageing</th>
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<tbody>
<tr>
<td>* Smooth skin,</td>
<td>* Leathery appearance,</td>
</tr>
<tr>
<td>* Unblemished,</td>
<td>* Deep wrinkles,</td>
</tr>
<tr>
<td>* Some loss of elasticity and recoil,</td>
<td>* Dark spots and areas of</td>
</tr>
<tr>
<td>* Fine wrinkles.</td>
<td>hyperpigmentation (actinic lentigines),</td>
</tr>
<tr>
<td></td>
<td>* Rough, sallow skin.</td>
</tr>
</tbody>
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Figure 1.2 Comparison of the effects of intrinsic ageing and photoageing. Table above (a) lists the clinical appearance of both intrinsically aged skin (below the neckline (b)) and photoaged skin (above the neckline (b)). The effects of photoageing in skin are much more pronounced than the subtle changes seen in intrinsic ageing. Photograph from (Tsoureli-Nikita et al., 2006).

1.4 The dermal ECM is influenced by intrinsic ageing and photoageing.

In intrinsic ageing the changes in the ECM are very subtle and are described as a general decrease in the abundance of ECM proteins. However, the changes seen in photoaged skin are much more pronounced (El-Domyati et al., 2002; Naylor et al., 2011).

1.4.1 The dermal ECM undergoes subtle changes with intrinsic ageing.

The changes in intrinsic ageing can be mostly attributed to a general atrophy of the proteins of the ECM (El-Domyati et al., 2002; Fenske and Lober, 1986). Whilst the majority of elastic fibre network mRNA is unchanged with age as examined by microarray (Langton et al., 2012b), there are decreases in amount of immunohistochemical staining of elastin, fibrillin, fibrillar collagens I and III (El-Domyati et al., 2002; Schwartz et al., 1993; Uitto, 2008; Uitto et al., 1989) and
collagen VII (Craven et al., 1997), suggesting that it is protein translation or the resident proteins which are affected. There is also a reduction in the abundance of glycosaminoglycans/proteoglycans, which is associated in a lack of hydration in aged skin (El-Domyati et al., 2002; Ghersetich et al., 1994; Li et al., 2013; Oh et al., 2011a, 2011b), as well as a noticeable increase in the immunohistochemical staining of LTBP-2 and LOXL, which have roles in maintaining elastic fibre deposition, assembly and structure (Langton et al., 2012b) (Figure 1.3).

1.4.2 The dermal ECM is greatly reorganised in photoaged skin.

1.4.2.1 Elastic fibres are severely damaged in photoaged skin.

In photoageing there is a selective deposition of dysfunctional elastotic material in the lower papillary and reticular dermis (Mitchell, 1967), which has since been identified as elastin, fibrillin-1, fibulin-5 and versican (Bernstein et al., 1994, 1995a, 1995b; Chen et al., 1986; Dahlback et al., 1990; Hasegawa et al., 2007; Kadoya et al., 2005; Schwartz, 1988; Schwartz et al., 1993). This build-up of dysfunctional proteins is termed solar elastosis and is often linked to the regions of the skin where dark spots appear on the surface (Chen et al., 1986; Schwartz, 1988). However, it is not known whether solar elastosis is a result of increased production of matrix material and abnormal deposition (Bernstein et al., 1994, 1995a; Uitto, 2008) or an increase in protease activity and matrix reorganisation since it is often coupled with lower abundance in the papillary dermis (Chen et al., 1986, 2004; Fisher et al., 1999; Muthusamy and Piva, 2010; Rijken and Bruijnzeel, 2009). For example, fibrillin microfibrils adjacent to the DEJ are significantly reduced in photoaged skin, but increased in the deeper dermis (Bernstein et al., 1994; Dahlback et al., 1990; Suwabe et al., 1999). The candelabra-like cascade of fibrillin microfibrils in the upper papillary dermis are lost even in minimally photoaged skin, suggesting that it is one of the early molecules to be affected by UVR and could therefore be used as a marker for identifying photoaged skin (Watson et al., 1999) (Figure 1.3).

1.4.2.2 Collagens are profoundly reduced in photoaged skin

Immunohistochemical staining of skin sections of volunteers with severe photoageing clearly show a marked reduction in the levels of collagen I and III and/or procollagen I and III, which often correlate with the severity of photoageing (El-Domyati et al., 2002; Schwartz, 1988; Schwartz et al., 1993; Talwar et al., 1995) and is thought to contribute to wrinkle formation (Chauhan and Shakya, 2009).
Collagen VII abundance is also severely depleted in photoageing (Craven et al., 1997) (Figure 1.3).

1.4.2.3 GAGs are increased but lose function with photoageing.

In a similar pattern to elastin, the abundance of GAGs in the dermis significantly increases in photoaged skin, but is disorganised with a lack of function. This increase in GAGs in the dermis is correlated with the increase in elastin deposition and immunohistochemical co-localisation indicates that the GAGs accumulate on the elastin depositions (Bernstein et al., 1995b, 1996; El-Domyati et al., 2002; Hasegawa et al., 2007; Tzellos et al., 2009; Uitto, 2008) (Figure 1.3).
Figure 1.3 Comparing young photoprotected, photoprotected intrinsically aged and photoaged skin. Immunohistochemical localisation of collagens (picro-Sirius red stain), elastic fibres (Miller’s elastin stain) and glycosaminoglycans (periodic acid Schiff stain) alongside a pictorial representation. In intrinsically aged skin there is a general atrophy, however in photoaged skin there is a dramatic difference in the architecture and abundance of the ECM (Naylor et al., 2011).
1.5 The mechanisms of intrinsic ageing and photoageing are complex.

1.5.1 The mechanism of intrinsic ageing is not fully understood.

The factors influencing intrinsic ageing are complex and there are a number of theories as to why we age, such as alterations in mitochondrial function, changes in cell cycle and decline of physiological hormones, as well as theories related to telomere shortening and increases in the number of senescent cells (Baird and Kipling, 2004; Bowles, 1998; Campisi, 2013; Carrel and Ebeling, 1921; Coppé et al., 2008; Juckett, 1987). Cell senescence occurs during a particular stage of the cell lifetime of dividing cells, usually after the cell has undergone a high number of replications. The cell becomes incapable of proliferation and mitosis, but is still viable, often utilising it’s energy resources in increased protein synthesis and secretion, thereby altering its microenvironment (Campisi, 2013; Coppé et al., 2008; Kipling et al., 2004; Rodier and Campisi, 2011). There is also a theory of reactive oxygen species (ROS) damage, although ROS can be generated by UVR exposure, they can also be produced as a byproduct of cell respiration via electron leakage in mitochondria and accumulate with age (Birch-Machin and Swalwell, 2010; Jastroch et al., 2010; Poljšak et al., 2012; Tulah and Birch-Machin, 2013). When coupled with the fact that both enzymatic and non-enzymatic antioxidants decline with age, oxidative damage is able to occur and accumulate over a lifetime (Hoppe et al., 1999; Yasui and Sakurai, 2003) (Figure 1.5).

1.5.2 There are two mechanisms for photoageing, cellular and acellular.

The epidermis has several innate defence mechanisms to help protect the skin from UVR damage, such as the stratum corneum and melanin pigment (Brenner and Hearing, 2008). However, following excessive UVR exposure these defences cannot absorb all the harmful radiation, allowing some to penetrate into the dermis where it can damage both cells and components of the ECM (Brenner and Hearing, 2008). UVR can act to damage components of the skin in two distinct ways, acellular via intra- and extracellular chromophore photosensitisation and cell-mediated via generation of ROS and increased protease activity (Rabe et al., 2006; Sherratt et al., 2010; Watson et al., 2013) (Figure 1.5).
1.5.2.1 Cellular mechanisms of UVR in photoageing are non-specific.

UVR is able to influence cell behaviour either via induction of cell signalling pathways to alter protein expression or by ROS which can either promote protein oxidation or further promote cell signalling.

UVR can promote the expression of c-jun, which, along with c-fos, forms an extremely transcriptionally active activator protein one (AP-1) complex (Fisher et al., 1999). AP-1 can enhance the expression of matrix metalloproteinases (MMPs), specifically MMP-1 (Chung et al., 1996), and reduce the activation of tissue inhibitors of MMPs (TIMPs) further increasing MMP activity (Denhardt et al., 1993). Furthermore, an important regulator of collagen homeostasis in skin, cysteine-rich protein 61 (CCN1) has also been found to be increased (both mRNA and protein) following UVR exposure, where it can down-regulate collagen production and increase MMP activity (Quan et al., 2006, 2011). Finally, UVR can also directly activate the transcription factor ‘nuclear factor kappa enhancer of B cells’ (NF-κB) which leads to the increased expression of inflammatory cytokines which attract neutrophils to the site of UVR influence (Muthusamy and Piva, 2010; Pattison et al., 2011; Rabe et al., 2006). Neutrophils produce neutrophil elastase, a serine protease which can also degrade proteins of the ECM (Figure 1.5; table 1.2).

ROS can directly damage both intracellular proteins, ECM proteins and DNA (Berlett and Stadtman, 1997; Birch-Machin et al., 1998; Chakravarti and Chakravarti, 2007; Kuluncsics et al., 1999; Pattison et al., 2011; Wondrak et al., 2003; Yasui and Sakurai, 2003), as well as activating cellular cytokines which can begin a signalling cascade leading to the up-regulation of ECM proteases which can degrade a wide range of ECM proteins (Brenner et al., 2005; Pattison et al., 2011; Wondrak et al., 2006). ROS activation of signalling cascades (such as mitogen-activating protein kinase (MAPK) cascade) can either reduce protein synthesis or increase protein degradation (Pattison et al., 2011; Rabe et al., 2006).

Cell-mediated consequences of UVR are non-selective, since ROS and activation of matrix proteases can lead to the degradation of almost all ECM proteins including collagens, fibronectin, elastin and fibrillin microfibrils (Ashworth et al., 1999; Woessner, 1991). However, in photoageing there is a selective loss of the fibrillin microfibrils (and associated fibulin-5) adjacent to the DEJ - even in minimally photoaged skin (Kadoya et al., 2005; Watson et al., 1999). It was therefore suggested that an alternative method of selective degradation due to amino acid
composition, susceptibility to glycation, location in the dermis and macromolecular structure, may occur following excessive UVR exposure (Sherratt et al., 2010; Watson et al., 2013).

1.5.2.2 Chromophore content of extracellular proteins leads to increased susceptibility to UVR damage.

UVR is absorbed by specific molecular structures known as chromophores (Young, 1997). Following exposure to UVR, chromophores are excited to a short-lived singlet higher energy state. The energy from the collapse of this state can disrupt peptide bonds or be converted to light or heat energy (Pattison et al., 2011; Ravanat et al., 2001; Young, 1997). Out of the essential twenty amino acids, four of them are known to be UV chromophores either due to their aromatic structure (His, Tyr and Trp) or the presence of a sulphur group (Cys) (Kerwin and Remmele, 2007; Pattison et al., 2011; Wondrak et al., 2003) (Figure 1.4). However, only Trp, Tyr and disulphide bonded Cys (Cystine) are likely to be photosensitisers and mediators of UV damage due to their absorption spectra (Creed, 1984; Pattison et al., 2011; Watson et al., 2013).

![Graph](image-url)

**Figure 1.4 (a)** The amino acids which are known to be UV absorbing chromophores (Cysteine, Tyrosine and Tryptophan). **(b)** The UV chromophore content of ECM components shown as a percentage of the amino acid sequence which is a chromophore. Fibrillin microfibril associated proteins have the highest percentage content, whilst elastin and fibrillar collagens have the lowest percentage content (Naylor et al., 2011).
The percentage of chromophore amino acid residues in the primary sequence of a variety of dermal ECM components was investigated (Sherratt et al., 2010). It was clear from this that elastic fibre associated proteins (with the exception of elastin), mainly the fibrillins, fibulins, LTBPs and LOX/LOXLs, had the highest percentage chromophore content of any of the ECM proteins examined, with the fibrillins being the highest at 21%, as well as having a high number of cysteine residues and hence, disulphide bonds (Sherratt et al., 2010). Conversely, elastin and fibrillar collagens had the lowest percentage chromophore content of dermal ECM proteins (Sherratt et al., 2010).

Whilst UVR can induce chromophore excitation resulting in direct molecular damage, this short-lived singlet state is also able to undergo intersystem crossing to form a longer-lived triplet state eventually resulting in the formation of ROS; which as described, can further promote ECM protein degradation via cell signalling pathways and oxidative damage (Chakravarti and Chakravarti, 2007; Pattison et al., 2011; Wondrak et al., 2003; Young, 1997).

![Figure 1.5 The mechanisms of intrinsic ageing and photoageing.](image)

*Intrinsic ageing occurs due to a general decline with age linked to telomere shortening, cell senescence and mitochondrial-generated ROS. Photoageing and UVR damage can occur through two arms; acellular (via amino acid chromophores and disulphide bonds) or cellular (via ROS and cell-mediated upregulation of ECM remodelling enzymes) (Naylor et al., 2011).*
1.5.3 Photoageing and inflammation.

The effects of chronic sunlight exposure can lead to inflammation, which can be observed clinically as sunburn; erythema (redness), oedema (swelling), pruritus (itching), pain, peeling and blisters. Since UVR can directly activate NF-κB, an inflammatory cascade is stimulated which not only leads to increased neutrophil, mast cell and macrophage infiltrate (Muthusamy and Piva, 2010; Pattison et al., 2011; Rabe et al., 2006), but also an increase in pro-inflammatory cytokines such as interleukins, tumour necrosis factor alpha (TNF-α) and transforming growth factor-beta (TGF-β) (Bennett et al., 2008; Bosset et al., 2003; Seo et al., 2001), as well as matrix degrading enzymes such as neutrophil elastase and MMPs (Bosset et al., 2003; Ohnishi et al., 2000; Rijken and Bruijnzeel, 2009). It is thought that inflammation following UVR exposure is to promote remodelling and repair of ECM proteins (such as solar elastosis) (Bosset et al., 2003; Ohnishi et al., 2000).

1.6 Summary I.

The dermal ECM is a dynamic and complex mixture of collagens, elastic fibre proteins and glycosaminoglycans. Whilst the consequences of intrinsic ageing and photoageing are non-specific, often affecting more than twenty ECM proteins including collagens, fibronectin and elastin, there appears to be a selective loss of the fibrillin microfibrils adjacent to the DEJ - even in minimally photoaged skin. Therefore, as well as non-selective damage via MMPs and ROS, fibrillin microfibrils were predicted to undergo direct damage by UVR via their high chromophore content and reliance on disulphide bonds. This suggests that they are one of the key molecules in the process of photoageing, perhaps contributing to the clinical appearance observed in photoaged skin, hence the increasing amount of research on fibrillin microfibrils in photoageing (Langton et al., 2010; Sherratt, 2009; Sherratt et al., 2010; Suwabe et al., 1999; Thurstan et al., 2011, 2012a, 2012b; Watson et al., 1999).

1.7 Fibrillin microfibrils are long-lived, macromolecular components.

Fibrillin microfibrils are long-lived macromolecular complexes and are key components of the dermal ECM, conferring elasticity and resilience to skin. They are thought to be metabolically stable over the human lifespan, but are highly susceptible to damage and degradation, accelerated by exposure of the skin to UVR.
1.7.1 Fibrillin microfibrils have an invariant ultrastructure in vertebrate tissues.

Atomic force microscopy (AFM), scanning transmission electron microscopy (STEM) and rotary shadowing (RS) identify fibrillin microfibrils as a ‘beads-on-a-string’ structure with a diameter of roughly 10-20 nm, a regular bead-to-bead distance (periodicity) of roughly 56 nm and an average flexion angle (angle between three beads) of roughly 165° (Baldock et al., 2001; Keene et al., 1991; Thurstan et al., 2011) (Figure 1.6). When mature, fibrillin microfibrils have an invariant mass of 2500-2700 kDa per repeat (Kielty et al., 2005; Sherratt et al., 2010).

![Figure 1.6 Fibrillin microfibrils imaged by scanning transmission electron microscopy (STEM) and atomic force microscopy (AFM). (STEM, left; AFM, right) Scale bars 200 nm.](image)

1.7.1.1 Fibrillin microfibrils have multiple biomechanical and biochemical functions.

The skin is a dynamic organ which requires the ability to stretch and recoil in response to its changing environment. Fibrillin microfibrils have been shown to possess extensible properties which allow them to contribute to long range deformability (Goldfischer et al., 1983; Kielty et al., 2002; Sherratt et al., 2003; Thurmond and Trotter, 1996). This property of fibrillin microfibrils can be tested by molecular combing and AFM imaging, which can be used to measure the Young's modulus (Hodson et al., 2009; Sherratt et al., 2003, 2006) (Figure 1.7).
Figure 1.7 Molecular combing exerts tensile strain onto partially adsorbed fibrillin microfibrils and can be used to measure their Young’s modulus and tensile strength. (A) Fibrillin microfibrils are allowed to partially adsorb to the glass surface of the specimen disc. (B) The majority of buffer is removed (by centrifugal force). (C and D) The thin film of buffer on the surface of the glass can exert strain on the fibrillin microfibril as the buffer dries and the receding meniscus moves across the surface. (E) The remaining fibrillin microfibril exhibits regions of native periodicities along-side elongated periodicities.

Fibrillin microfibrils also function as a template for elastin deposition in elastic fibre development (Lin et al., 2002; Low, 1962; Mecham, 1991; Robb et al., 1999; Trask et al., 1999, 2000b) where the inner core of elastin is surrounded by a fibrillin microfibril mantle (Fleischmajer et al., 1991a; Goldfischer et al., 1983; Mecham, 1991; Schwartz and Fleischmajer, 1986; Sherratt, 2009).

As well as their mechanical and structural functions, fibrillin microfibrils also have a function in modulating the bioavailability of molecules of the transforming growth factor-beta (TGF-β) superfamily, including TGF-β itself and bone morphogenic proteins (BMPs) (Chaudhry et al., 2007; Koenders et al., 2009; Massam-Wu et al., 2010; Neptune et al., 2003; Raghunath et al., 1998). TGF-βs are protein cytokines involved in cell proliferation, differentiation, ECM production, immune system regulation and apoptosis (Chaudhry et al., 2007; Kubiczkova et al., 2012; Massague,
Although it has been shown that fibrillins themselves cannot bind TGF-β directly, they can regulate its bioavailability via the interaction with LTBP proteins (Isogai et al., 2003; Ono et al., 2009; Raghunath et al., 1998) (table 1.1). BMPs are a group of cytokines, one of which, BMP-7, can directly interact and bind to the N-terminus of fibrillin-1. Binding of BMP to its cellular receptor (BMPR) can also cause a signalling cascade leading to cell proliferation and apoptosis. It also has roles in development, and as the name suggests a role in mesenchymal cell transformation into bone and cartilage (Gregory et al., 2005; Koenders et al., 2009; Sengle et al., 2008).

It is also thought that fibrillin microfibrils play a role in cell attachment and spreading of cells via their RGD (Arginine-Glycine-Aspartate) site which has the ability to bind α\textsubscript{v}β\textsubscript{3} and α\textsubscript{5}β\textsubscript{1} integrins (Bax et al., 2003; Maslen et al., 1991; Pereira et al., 1993; Pfaff et al., 1996).

### 1.7.2 Fibrillin microfibril assembly and organisation is highly regulated and intricate.

#### 1.7.2.1 Fibrillin-1 is the main component of fibrillin microfibrils.

Fibrillin-1 is part of a family of fibrillin proteins, it has a mass of 347 kDa and is made of a primary sequence of 2871 amino acids (Gibson et al., 1989; Pereira et al., 1993). The N- and C-termiini are both unique and the protein folds to create: 47 epidermal growth factor-like (EGF) domains, 43 of which are calcium binding (cb-EGF) (Corson et al., 1993; Maslen et al., 1991; Pereira et al., 1993); seven 8-cysteine (TB) modules (Pereira et al., 1993; Robertson et al., 2011); two hybrid motifs (Downing et al., 1996; Jensen et al., 2009; Lee et al., 2004; Smallridge et al., 2003) and a unique proline-rich hinge domain (Pereira et al., 1993). The proline-rich domain is a potential source of flexibility of fibrillin-1, as well as the N- and C-termiini and the third and seventh TB module (Baldock et al., 2001; Handford et al., 2000; Yuan et al., 1997). The fourth TB module in the protein contains the RGD site involved in cell signaling (Lee et al., 2004; Mariko et al., 2010; Maslen et al., 1991; Pereira et al., 1993; Pfaff et al., 1996).

Fibrillin-1 has a high concentration of cysteine residues, each of the 43 cb-EGF domains contain six cysteine residues, which can form three disulphide bonds in the tertiary structure (Corson et al., 1993; Jensen et al., 2009; Pereira et al., 1993). Furthermore, each of the seven TB modules contain eight cysteine residues, which
form four disulphide bonds in the tertiary structure (Handford et al., 2000; Jensen et al., 2009; Maslen et al., 1991; Pereira et al., 1993).

1.7.2.2 Other components of fibrillin microfibrils.

There are many other proteins which are thought to associate with fibrillin microfibrils and dermal elastic fibres both in vitro and in vivo (Table 1.1).

Microfibril associated glycoprotein-1 (MAGP-1) is known to bind fibrillin microfibrils in the bead region, roughly at the N-terminus of fibrillin-1 (Gibson et al., 1989; Henderson et al., 1996; Hubmacher et al., 2008; Jensen et al., 2001; Kielty and Shuttleworth, 1997; Massam-Wu et al., 2010). Mass spectrometry analysis of native microfibrils extracted from skin biopsies found that MAGP-1 was the only other microfibril-associated protein to be identified in the sample along with fibrillin-1 (Cain et al., 2006). The C-terminal domain of MAGP-1 has an overall positive charge and is rich in cysteine residues. This part of the protein is thought to bind to the microfibrils and facilitate interactions with fibrillin-1 and -2 (Henderson et al., 1996; Weinbaum et al., 2010).

Latent transforming growth factor-β-binding proteins (LTBPs) have been shown to bind to fibrillin-1 the C-terminal and also to fibulin-5 via the N-terminal. LTBP-1, -3 and -4 have the ability to bind TGF-β and are therefore the link between fibrillin microfibril sequestration and activation of TGF-β (Isogai et al., 2003; Ono et al., 2009; Raghunath et al., 1998). LTBP-2 is thought to be important for elastic fibre development and structure but has been found not to bind TGF-β (Hirai et al., 2007; Hirani et al., 2007; Moren et al., 1994)
Table 1.1 Summary of all the proteins and molecules which are thought to be associated with fibrillin microfibrils and elastic fibres in human skin.

<table>
<thead>
<tr>
<th>Molecule:</th>
<th>Found:</th>
<th>Function:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillin-1</td>
<td>Fibrillin microfibrils</td>
<td>Fibrillin-1 is a glycoprotein with a mass of 347kDa. Once processed, many fibrillin-1 molecules polymerise and interact with various other proteins and molecules to form fibrillin microfibrils. RGD sites of fibrillin-1 in the TB4 module can bind to ( \alpha \beta_3 ) integrins on dermal cell surfaces for cell signaling (Keene et al., 1991; Sakai et al., 1986b).</td>
</tr>
<tr>
<td>Fibrillin-2</td>
<td>Fibrillin microfibrils</td>
<td>Fibrillin-2 shares a high homology with fibrillin-1, with one of the only differences being its glycine-rich region in place of fibrillin-1’s proline-rich region. Fibrillin-2 can form heterotypic interactions with fibrillin-1 in microfibrils predominantly in early foetal development (Lin et al., 2002; Zhang et al., 1994).</td>
</tr>
<tr>
<td>MAGP-1</td>
<td>Fibrillin microfibrils</td>
<td>(Microfibril associated glycoprotein-1) Bound to the N-terminus of fibrillin-1 via disulphide bonds, MAGP-1 stabilises fibrillin/fibrillin interactions as well as forming cross links between fibrillin and tropoelastin. It can also link the elastic fibre to other associated molecules such as collagen VI (Cain et al., 2006; Gibson et al., 1986; Henderson et al., 1996; Jensen et al., 2001).</td>
</tr>
<tr>
<td>MAGP-2</td>
<td>Fibrillin microfibrils</td>
<td>(Microfibril associated glycoprotein-2) Binds to the N-terminus of fibrillin via disulphide bonds and contains an RGD site of the fibrillin microfibril which allows cell signalling via ( \alpha \beta_3 ) integrin. It is thought that this protein is mainly involved in development and differentiation (Gibson et al., 1998).</td>
</tr>
<tr>
<td>LTBP-2</td>
<td>Fibrillin microfibrils</td>
<td>(Latent TGF-( \beta ) binding protein-2) Very similar in structure to fibrillins. It is also thought to be important for elastic fibre development and structure but has been found not to bind TGF-( \beta ) (Hirai et al., 2007; Hirani et al., 2007; Moren et al., 1994).</td>
</tr>
<tr>
<td>LTBP-1, -4</td>
<td>Fibrillin microfibrils</td>
<td>(Latent TGF-( \beta ) binding proteins) Very similar in structure to fibrillins (numerous cb-EGF domains and TB modules). Bind TGF-( \beta ) and involved in LTBP-mediated sequestration and activation of TGF-( \beta ) (Isogai et al., 2003; Ono et al., 2009; Raghunath et al., 1998).</td>
</tr>
<tr>
<td>Decorin</td>
<td>Fibrillin microfibrils</td>
<td>A leucine-rich small chondroitin sulphated proteoglycan which interacts with both MAGP-1 and fibrillin-1 to form a ternary complex. It can also bind to tropoelastin (Reinboth et al., 2002; Sherratt et al., 1997; Trask et al., 2000a).</td>
</tr>
<tr>
<td>Versican</td>
<td>Fibrillin microfibrils</td>
<td>A large chondroitin sulphated proteoglycan. Its C-terminal lectin domain binds the N-terminus of fibrillin-1. It is thought to influence fibrillin microfibril integration in the ECM (Isogai et al., 2002).</td>
</tr>
<tr>
<td>Type VI collagen</td>
<td>Fibrillin microfibrils</td>
<td>Interacts with fibrillin-1 and MAGP-1. Thought to provide an anchoring function for the elastic fibres during stretching (Everts et al., 1998; Kiely et al., 1993).</td>
</tr>
<tr>
<td>Fibrillin-2</td>
<td>Elastic fibre interface</td>
<td>Fibulin-2 colocalises with fibrillin-1. It is thought to bind fibrillin-1 at the DEJ which could stabilise the interactions between microfibrils and the lamina densa. Their interaction could also be important for development (Reinhardt et al., 1996).</td>
</tr>
<tr>
<td>Fibrillin-4</td>
<td>Elastic fibre interface</td>
<td>Interact with elastin and fibrillin-1 and strongly binds LOX. It can form homodimers in the presence of calcium and is thought to regulate the formation of cross links in tropoelastin by regulating LOX activation (Choudhury et al., 2009).</td>
</tr>
<tr>
<td>Fibrillin-5</td>
<td>Elastic fibre interface</td>
<td>Interacts with elastin, LOX and fibrillin-1 and is essential in promoting elastic fibre formation, potentially as a chaperone by directing tropoelastin deposition onto fibrillin-1 (Freeman et al., 2005; Hirai et al., 2007; Katsuta et al., 2008; Yanagisawa et al., 2009).</td>
</tr>
<tr>
<td>LOX and LOXL(1-4)</td>
<td>Elastic fibres</td>
<td>(Lysyl Oxidase) Forms covalent lysyl-derived desmosine cross links and stabilises tropoelastin, allows maturation of tropoelastin to elastin and aids in elastic fibre formation.</td>
</tr>
</tbody>
</table>
1.7.2.3 Assembly of a mature untensioned fibrillin microfibril.

Fibrillin microfibril assembly is a cell-regulated process; fibroblasts first secrete profibrillin-1 of approximately 350 kDa which has globular N- and C-termini. After secretion fibrillin-1 is processed by furin to produce a mature form of approximately 320 kDa. Since average mass per repeat has been reported as 2500-2700 kDa, mature fibrillin microfibrils are thought to contain eight fibrillin-1 monomers (Baldock et al., 2001; Lonnqvist et al., 1998; Milewicz et al., 1992; Ritty et al., 1999; Trask et al., 1999; Wallis et al., 2003) as well as post-translational modifications of N-linked glycosylation units and binding of microfibril associated proteins (Baldock et al., 2001; Henderson et al., 1996; Jensen et al., 2001; Trask et al., 2000a). Assembly of the processed fibrillin-1 monomers is a cell mediated process which occurs via interactions of RGD sites with αVβ3 integrins on the cell surface (Bax et al., 2003; Pfaff et al., 1996).

C-terminal fragments of fibrillin-1 could be important for directing linear (N-C) (Marson et al., 2005; Trask et al., 1999) and lateral (N-N, C-C) interactions (Hubmacher et al., 2008; Trask et al., 1999) since they have been found associated with intact microfibrils (Cain et al., 2006). Fibrillin microfibrils are also rich in disulphide bonds and inter- and intra-molecular transglutaminase links (Hubmacher et al., 2006; Kojima et al., 1993; Qian and Glanville, 1997; Reinhardt et al., 2000a).

Organisation of fibrillin-1 within the fibrillin microfibril is controversial and there are three opposing structural models. The first proposed organisation was the $\frac{1}{3}$ staggered arrangement, which was suggested by the extrapolation of molecular dimensions and organisation of crystal structures of fibrillin-1 (Lee et al., 2004). There is also a $\frac{1}{2}$ staggered model for formation which has been suggested (Kuo et al., 2007). However, these theories have been opposed by the molecular folding model, which, in contrast to previous models, is based on detailed STEM mass mapping, AFM data, automated electron tomography and antibody mapping together (Baldock et al., 2001, 2006; Glab and Wess, 2008; Sherratt et al., 2003). This folding model reinforces the process of microfibril formation as described above by predicting a N-to-C alignment of fibrillin-1 monomers which then mature and pack, with the help of transglutaminase crosslinks, to form an ‘untensioned’ mature microfibril (Baldock et al., 2001, 2006; Glab and Wess, 2008; Sherratt et al., 2003). This folded arrangement can help to explain the extensibility of fibrillin
microfibrils, as well as accounting for several known fibrillin microfibril features such as mass profiles, axial distributions and interaction with other fibrillin microfibril proteins (Baldock et al., 2001; Glab and Wess, 2008) (Figure 1.8).

Fibrillin microfibrils are thought to be highly negatively charged and slightly hydrophilic, since the positively charged N- and C-termini are located within the bead interior and there are multiple N-linked oligosaccharides throughout fibrillin-1 (Baldock et al., 2001; Sherratt et al., 2004, 2007).

![Molecular folding model of fibrillin microfibril assembly](image)

*Figure 1.8 Molecular folding model of fibrillin microfibril assembly is based on detailed STEM mass mapping, AFM data, automated electron tomography and antibody mapping. This model would lead to a repeat (bead and interbead) of eight fibrillin monomers, a mass of 2560 kDa and an untensioned bead periodicity of 56 nm. Images based on (Baldock et al., 2001; Jensen et al., 2012)*
1.7.2.4 *Fibrillin microfibrils mature over time.*

Immunohistochemistry and STEM has revealed that fibrillin microfibrils may undergo a maturation process in developing tissues. In previous studies, STEM mass mapping was used to demonstrate that the mass of the repeating units of the fibrillin microfibrils increased with age during foetal development (Sherratt et al., 1997). Furthermore, fibrillin microfibrils extracted from foetal bovine skin showed increases in abundance and length during the stages of development suggesting that these parameters may be linked to developmental stage (Kielty et al., 1993).

In a more recent paper, it was concluded that the mass per repeat of microfibrils extracted from a variety of foetal tissues were significantly different to those extracted from adult tissues, but that the mass per repeat of those extracted from COS-1 (Green monkey kidney fibroblasts) cell cultures resembled foetal microfibrils (Sherratt et al., 2010). Furthermore, immunodetection of *in vitro* cultured fibrillin microfibrils with the monoclonal antibody 11C1.3 showed a time-dependent detection. The antibody-binding site appeared roughly two weeks post-confluency in human dermal fibroblast cell culture matrices (Baldock et al., 2001).

In an investigation into wound healing and the use of cultured epithelial autografts in burn victims, a model for fibrillin microfibril maturation at the DEJ was proposed. Fine candelabra-like microfibrils adjacent to the DEJ form first (as early as five-days after treatment) before cascading into the deeper dermis where they joined with elastin (which is deposited much later) after one to three months. The full maturation into fine elastic fibres took up to 17 months (Raghunath et al., 1996).

1.7.3 *Fibrillin microfibrils can accumulate damage due to their longevity.*

Elastic fibres are extremely long-lived proteins which are produced during midgestation before being completed during post-natal development (Ritz-Timme et al., 2003). Aspartic acid racemisation and $^{14}$C nuclear weapon-related carbon-dating of elastic fibre proteins from lung tissues suggested that the half-life far exceeds that of intracellular proteins and in most cases can persist for a lifetime, with the lifespan of fibrillin microfibrils highly correlated to the lifespan of elastin (Shapiro et al., 1991). Furthermore, skin-derived elastin is also highly stable over a human lifetime, with low-turnover (Ritz-Timme et al., 2003) (Figure 1.9). Proteins which have a long lifespan in human tissues will have increased risk to molecular ageing and an accumulation of damage over time (Dobberstein et al., 2010; Naylor et al., 2011;
Ritz-Timme et al., 2000, 2003; Shapiro et al., 1991; Sherratt et al., 2010; Varadi, 1972).

<table>
<thead>
<tr>
<th>Intracellular</th>
<th>Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine decarboxylase</td>
<td>0.2</td>
</tr>
<tr>
<td>Tryptophan oxygenase</td>
<td>2.5</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>12.0</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>24.0</td>
</tr>
<tr>
<td>Monamine oxidase</td>
<td>2.3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5.4</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>8.1</td>
</tr>
<tr>
<td>Histone</td>
<td>18.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extracellular matrix</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteoglycans</td>
<td>1-23</td>
</tr>
<tr>
<td>Fibrillar collagen</td>
<td>15-95</td>
</tr>
<tr>
<td>Elastin</td>
<td>&gt;lifespan</td>
</tr>
<tr>
<td>Fibrillin microfibrils</td>
<td>&gt;lifespan</td>
</tr>
</tbody>
</table>

*Figure 1.9 Extracellular matrix proteins have relatively long turnover rates when compared to intracellular proteins. Elastic fibre proteins are thought to last a lifetime. Figure from (Naylor et al., 2011).*

1.7.4 Fibrillin microfibrils can be imaged using multiple microscopic techniques.

Fibrillin microfibrils are complex macromolecular structures which may undergo ultrastructural changes undetectable via IHC or mRNA expression profiles (Sengle et al., 2012). Therefore, to characterise and locate changes within the ultrastructure, microscopic techniques can be employed, such as atomic force microscopy (AFM) and scanning transmission electron microscopy (STEM) (Figure 1.6). Intact beaded fibrillin microfibrils can be extracted from a variety of *in vitro* cell cultures and tissues before imaging for ultrastructural measurements (Table 1.2).

1.7.4.1 Imaging fibrillin microfibrils by STEM: mass and ultrastructural data.

Scanning transmission electron microscopy (STEM) is a type of transmission electron microscopy (TEM). The process of STEM involves the use of an electron gun which can omit a beam of electrons able to scan over a sample. When the electrons collide with the atoms of the sample, they are elastically scattered. The amount, and angle, of electron scattering is directly related to the atomic number. By using STEM equipped with a high-angle annular dark-field detector (HAADF), it is possible to form atomic resolution images, as well as extract quantitative mass
distribution data, without the need for staining. This absence of chemical staining or shadowing makes it able to detect small changes in molecular mass distribution in varying samples (Engel, 1982; Engel and Colliex, 1993; Engel et al., 1982; Holmes, 1995; Holmes et al., 2001; Rasband, 1997; Sherratt et al., 2009) (Figure 1.10).

![Figure 1.10 Schematic showing the process and theory of STEM. An electron beam is generated by a filament which first passes through a condenser aperture in a vacuum before colliding with the sample. The resulting electron scattering is detected with a HAADF.](image)

1.7.4.2 Imaging fibrillin microfibrils via AFM: tensile strength and ultrastructural data.

Atomic force microscopy (AFM) is a type of high-resolution scanning probe microscopy. The process involves a probe that is scanned over the sample surface to create an image. The AFM can be used to generate surface topology (height data) by the use of a nanoscale mechanical probe (or cantilever tip) which scans the sample surface via intermittent contact (tapping). The basic idea of tapping AFM is that the local attractive or repulsive force (atomic force) between the tip and the sample/surface causes measurable deflection of the cantilever (Cappella and Dietler, 1999; Hanssen et al., 1998; Hodson et al., 2009; Sherratt et al., 2003, 2006) (Figure 1.11).
Atomic force microscopy takes advantage of atomic attractive and repulsive forces between the cantilever probe and the sample surface. Deflections of the cantilever can be detected using a laser and photodiode detector.

1.7.4.3 Differences between ultrastructural data from STEM and AFM.

Whilst the STEM requires the fibrillin microfibrils to be adsorbed to a thin carbon film, the AFM can utilise a variety of surfaces such as negatively charged and hydrophilic mica, neutral and hydrophilic (poly-L-lysine) PLL-coated mica or positively charged hydrophobic glass. Previous studies have shown how the local substrate environment has a profound impact on the adsorption and morphology of fibrillin microfibrils (Hodson et al., 2009; Sherratt et al., 2004, 2005, 2007; Wess et al., 1998). For example, calcium concentration differentially affects how fibrillin microfibrils adsorb to either carbon-coated grids or mica-PLL stubs (Sherratt et al., 2007; Wess et al., 1998) (Table 1.2). Therefore, ultrastructural parameters measured by AFM may not always be comparable to those measured by STEM. Although since AFM and STEM have the ability to measure different parameters (AFM can measure tensile strength via molecular combing, STEM can measure mass per repeat) it is often useful to perform both techniques on the same sample (Reilly et al., 2007; Sherratt et al., 2006, 2010).

1.7.5 Ultrastructural remodelling of fibrillin microfibrils in vitro and in vivo.

Although fibrillin microfibrils are long-lived proteins of the ECM, they can be extensively remodeled both by the action of multiple enzymes also located in the ECM and by external influences such as ROS, cigarette smoke and direct UVR.
(Ashworth et al., 1999; Kielty et al., 1994; Merrick et al., 2009; Reilly et al., 2007; Sherratt et al., 2006, 2010; Thurstan et al., 2011, 2012a, 2012b, 2013).

1.7.5.1 Ultrastructural remodelling of fibrillin microfibrils following intrinsic ageing and in vitro UVR irradiation.

For fibrillin microfibrils, intrinsic ageing does not seem to affect the dermal organisation of fibres until roughly the 8th decade, and even then, there is little loss of the fibrillin microfibril candelabra network adjacent to the DEJ (El-Domyati et al., 2002; Langton et al., 2010; Sherratt et al., 2006). However, in photoaged skin, this candelabra-like cascade of fibrillin microfibrils is significantly reduced, as well as being greatly reorganised (Watson et al., 1999) (Figure 1.12). This occurs even in minimally photoaged skin, suggesting that it is one of the early markers to be affected by UVR in photoageing (Watson et al., 1999). Furthermore, there is a decrease in the number of fibroblasts positive for fibrillin microfibril mRNA (Watson et al., 1999). Conversely, there is also an accumulation of fibrillin-1 immunoreactive material in the lower reticular dermis as part of the solar elastosis. This indicates that in photoaged skin there may be both a reduction in fibrillin microfibrils, as well as reorganisation of the elastic fibre architecture (Bernstein et al., 1994; Suwabe et al., 1999; Watson et al., 1999).

![Intrinsically aged and Photoaged](image)

**Figure 1.12 Comparison between intrinsically aged and photoaged skin by IHC detection of fibrillin microfibrils.**

Black staining shows fibrillin microfibrils as detected by 11C1.3 monoclonal antibody. Photoprotected (but intrinsically aged) upper inner arm skin and photoaged forearm skin from a 78 year old male individual. The intrinsically aged site still exhibits long cascading candelabra-like microfibrils adjacent to the DEJ (indicated by arrows). However, in photoaged skin from the same individual the candelabras are significantly reduced (indicated by red arrows) with an accumulation in the deeper dermis (indicated by green arrows).
Whilst IHC and abundance analyses show no significant differences, ultrastructurally, fibrillin microfibrils undergo significant changes with intrinsic ageing. In fibrillin microfibrils extracted from photoprotected buttock skin of aged individuals (> 70 years) there is a marked reduction in mass per repeat compared to photoprotected buttock skin of young individuals (18-30 years), as well as a reduction in tensile strength as tested by molecular combing (Langton et al., 2011; Sherratt et al., 2006) (Table 1.2).

Whilst ultrastructural analyses have not yet been performed on fibrillin microfibrils extracted from photoaged skin, we can hypothesise the effects of UVR based on studies performed in vitro. The first of these studies investigated whether physiologically relevant doses of broadband UVB could damage fibrillin microfibrils extracted from both COS-1 (green monkey kidney fibroblasts) and young photoprotected human buttock skin microfibrils irradiated in vitro. Irradiation of COS-1 fibrillin microfibrils leads to increased fragmentation, flexibility and periodicity, as well as a reduction in mass. To show that these changes were not due to microfibril source, fibrillin microfibrils from photoprotected buttock skin of a young individual were also irradiated. There was also a reduction in mass per repeat of these fibrillin microfibrils, but a subpopulation was found to have accreted mass laterally so that there was a bimodal distribution of mass following UVB exposure (Sherratt et al., 2010) (Table 1.2).

Subsequent studies have shown how broadband UVB degradation in vitro is mediated (in part) by reactive oxygen species (Thurstan et al., 2011). With exposure to UVB alone, fibrillin microfibrils increased in flexibility. However, in the presence of deuterium oxide (D2O; which extends the half-life of singlet oxygen (Merkel et al., 1972)), UVB exposure increased the flexibility of fibrillin microfibrils much more than UVB alone. Furthermore, when oxygen was depleted in the buffer solution, UVB irradiation did not increase flexibility as much as UVB alone (Thurstan et al., 2011). So although UVB was shown to damage fibrillin microfibrils via ROS, there was still some damage that could be attributed to direct absorption of UVB by chromophores.

A further study into the effect of chromophore concentration of ECM components showed that whilst both UVA and solar simulated radiation (SSR) irradiation in vitro profoundly damaged fibrillin microfibrils (decrease in periodicity and increase in flexibility), direct irradiation of extracted fibrillar collagen I and tropoelastin (low
percentage of chromophores) showed no changes in structure. Additionally, fibronectin (intermediate percentage chromophore content compared to collagen and fibrillin microfibrils) showed some structural damage (aggregation) (Thurstan et al., 2012a, 2012b) (Table 1.2).

Previous studies have been performed on fibrillin microfibrils from a variety of species, tissues and cellular populations. However, focusing on human skin-derived microfibrils only, it could be hypothesised that photoaged fibrillin microfibrils from human skin would have both increased and decreased periodicity, a reduction in mass per repeat and a reduction in tensile strength (Table 1.2). However, skin-resident fibrillin microfibrils will not only be susceptible to direct UVR damage via chromophores and ROS, but increased activity of proteases such as neutrophil elastase and MMPs as well, which are known to be upregulated by UVR (Figure 1.5; Table 1.2).

1.7.5.2 *Enzyme-mediated ultrastructural remodelling of fibrillin microfibrils in vitro.*

Matrix metalloproteinases (MMPs) exist in both active and inactive forms within the dermis and are responsible for the continual remodeling of the proteins in the ECM. MMPs are zinc dependent endopeptidases which have highly conserved modular structures (Massova et al., 1998). There are twenty six known human MMPs which all cleave specific but multiple proteins within the ECM. MMPs are tightly regulated by tissue inhibitors of MMPs (TIMPs) (Denhardt et al., 1993). To date fibrillin microfibrils have been shown to be degraded by five different MMPs; MMP-2, -3, -9, -12 and -13 (Ashworth et al., 1999), although the activities and consequences amongst the enzymes vary. MMPs-2 and -9 fragment the fibrillin microfibrils whereas, MMPs-12 and -13 significantly increase their bead-to-bead periodicity, but do not alter their length (Table 1.2).

A recent study has suggested that MMPs could selectively degrade UVR damaged fibrillin microfibrils (Thurstan et al., 2011). In an investigation where UVB-irradiated fibrillin microfibrils were exposed to MMP-3, there was a loss of microfibrils which had increased flexibility, resulting in a population with flexibility similar to unirradiated microfibrils (Thurstan et al., 2011).

As well as MMPs, serine proteases also have the ability to degrade fibrillin microfibrils, for example, chymotrypsin, trypsin and neutrophil elastase, which is
secreted by neutrophil infiltrates (Kielty et al., 1994). These proteases have been found to fragment fibrillin microfibrils and increase periodicity, as well as in some cases, completely degrade the microfibrils (Table 1.2).

1.7.5.3 Other extrinsic factors affecting fibrillin microfibrils

It had previously been reported that cigarette smoking leads to increased levels of MMP mRNA, specifically MMP-1, in the skin (Lahmann et al., 2001), but more recently, a study was conducted to assess the effects of smoking on fibrillin microfibrils directly. Fibrillin microfibrils extracted from photoprotected sites from smoker’s skin had significantly reduced lengths (measured in beads), tensile strength (measured by molecular combing) and an increased periodicity of 66 nm compared to 56 nm for microfibrils from non-smoker skin (Merrick et al., 2009).

Diabetes mellitus is a metabolic disease associated with impaired production or response to insulin, leading to an increase in blood sugar abundance. One of the key mechanisms in the pathogenesis of diabetes is non-enzymatic glycation of proteins via the Maillard reaction to form structures referred to as advanced glycation end products (AGEs). The effect of non-enzymatic glycation of fibrillin-1 was investigated, suggesting that fibrillin-1 was able to form AGEs due to its long half-life and stable incorporation into long-lived elastic fibres. Glycated fibrillin-1 proteins could lead to protein cross-linking, polymerisation and decreased sensitivity to enzyme degradation (Atanasova et al., 2009). In the case of fibrillin microfibrils, diabetes induced AGEs lead to a reduction in periodicity compared to age-matched controls in mouse aorta (Akhtar et al., 2010).
Table 1.2 Previous investigations both in vitro and in vivo show extensive ultrastructural remodelling of fibrillin microfibrils.

<table>
<thead>
<tr>
<th>Paper:</th>
<th>Fibrillin microfibril source:</th>
<th>Imaged by:</th>
<th>Method of remodelling:</th>
<th>Length</th>
<th>Periodicity</th>
<th>Flexibility</th>
<th>Mass</th>
<th>Tensile strength</th>
<th>Degraded?</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Kiely et al., 1994)</td>
<td>Fetal bovine skin</td>
<td>RS</td>
<td>Trypsin, chymotrypsin, neutrophil elastase</td>
<td>↓</td>
<td>↑</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
</tr>
<tr>
<td>(Wess et al., 1998)</td>
<td>Fetal bovine skin, aorta, nuchal ligament &amp; human ciliary zonules</td>
<td>STEM &amp; RS</td>
<td>EDTA and EGTA</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CaCl₂</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td>(Ashworth et al., 1999)</td>
<td>Recombinant peptides</td>
<td>PAGE/WB</td>
<td>MMPs -2, -3, -9, -12, -13 and -14</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
</tr>
<tr>
<td>(Sherratt et al., 2006)</td>
<td>Human ciliary zonules</td>
<td>STEM &amp; RS</td>
<td>MMPs 2 and 9</td>
<td>↓</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MMPs 12 and 13</td>
<td>-</td>
<td>↑</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
</tr>
<tr>
<td>(Sherratt et al., 2007)</td>
<td>Fetal bovine aorta</td>
<td>AFM</td>
<td>CaCl₂</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td>(Reilly et al., 2007)</td>
<td>Photoprotected buttock</td>
<td>AFM &amp; STEM</td>
<td>Broadband UVB</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>↓</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td>(Merrick et al., 2009)</td>
<td>Photoprotected buttock</td>
<td>AFM</td>
<td>Cigarette smoking (in vivo)</td>
<td>↓</td>
<td>↑</td>
<td>-</td>
<td>n/a</td>
<td>↓</td>
<td>No</td>
</tr>
<tr>
<td>(Sherratt et al., 2010)</td>
<td>3-week COS-1s</td>
<td>AFM &amp; STEM</td>
<td>Broad-band UVB</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td>(Thurstan et al., 2011)</td>
<td>Photoprotected buttock</td>
<td>AFM</td>
<td>Broad-band UVB</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>↓ (T) bimodal</td>
<td>No</td>
</tr>
<tr>
<td>(Thurstan et al., 2012a)</td>
<td>6-week HDFs</td>
<td>AFM</td>
<td>UVB (O₂ depleted)</td>
<td>n/a</td>
<td>-</td>
<td>↑</td>
<td>n/a</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td>(Thurstan et al., 2012b)</td>
<td>6-week HDFs</td>
<td>AFM</td>
<td>D₂O</td>
<td>n/a</td>
<td>-</td>
<td>↑↑↑</td>
<td>n/a</td>
<td>n/a</td>
<td>No</td>
</tr>
<tr>
<td>(Thurstan et al., 2013)</td>
<td>Photoprotected buttock</td>
<td>AFM</td>
<td>UVB + MMP-3</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.8 Summary II.

Fibrillin microfibrils are a key component of the dermal ECM with functions in skin elasticity, homeostasis and cellular mobility and communication. In both intrinsic ageing and in vitro UVR exposure, their ultrastructure and tensile strength is compromised, potentially contributing to the clinical appearance of photoaged skin. Although IHC of photoaged skin shows marked differences in the abundance and architecture of the fibrillin microfibril network, studies have yet to be performed on the ultrastructure of extracted fibrillin microfibrils. Whilst previous investigations of in vitro UVR irradiation of human skin microfibrils suggest photoageing may induce increases and decreases in periodicity and a localised loss of mass, fibrillin microfibrils in skin are susceptible to both UVR and enzyme-mediated remodelling. This suggests the effects of photoageing may be unpredictable and highly variable.

However, whilst photoageing might profoundly affect the architecture and ultrastructure of fibrillin microfibrils, there are a class of vitamin A-derived drugs which can be topically applied to skin and have been proven to reduce the signs of photoageing, both clinically (reduction of wrinkles) and histologically (increased immunodetection of fibrillin microfibrils and other ECM components).

1.9 Repair of intrinsically aged and photoaged skin.

1.9.1 Retinoids are the gold standard for the treatment of photoageing.

Retinoids are a group of molecular compounds derived from vitamin A (retinol). They have essential roles in embryogenesis, reproductive ability, vision and cellular growth and differentiation (Chytil, 1986; Mukherjee et al., 2006; Reichrath et al., 2007; Roos et al., 1998). They cannot be manufactured by the body and instead are absorbed naturally through our food, for example vegetables high in beta-carotene such as carrots or sweet potatoes (Harrison, 2005; Mukherjee et al., 2006). Usually, the plasma concentration of retinol is between 0.035 and 0.075 µg/ml, with all-trans retinoic acid plasma levels between 0.55 and 1.2 ng/ml (Matsuoka et al., 1991). However, retinoids can be used as supplements either as an oral capsule or topical application in a gel or cream vehicle.

Topical application of retinoids were first used to treat acne before the accidental discovery of their clinical efficacy in improving the clinical signs of photoageing (Kligman et al., 1986; Pedace and Stoughto, 1971). Since then, investigations have
shown how both clinically and histologically, retinoids can reverse the signs of both intrinsic ageing (Chung and Eun, 2007; Kligman et al., 1993) and photoageing (Chen et al., 1997; Griffiths and Voorhees, 1993; Griffiths et al., 1993a; Watson et al., 2001a; Woodley et al., 1990b). The most commonly used retinoid in the treatment of photoageing is all-trans retinoic acid (t-RA; tretinoin) but other retinoids, including retinol and synthetic retinoids, are also used.

1.9.1.1 Cell signalling pathways of t-RA.

Topically applied t-RA is thought to cross the stratum corneum via traversing intracellular spaces (Elias, 1981) before moving through the lower layers of the epidermis quickly via endocytosis (Reichrath et al., 2007). The rate and amount of absorption is directly dependent on vehicle formulation and concentration (Darlenksi et al., 2010; Reichrath et al., 2007; Trotta et al., 2003; Vieira et al., 1995). By this method, t-RA can be transported throughout the entire epidermis and even into the dermis. Whilst some studies have shown that following topical application, almost ~80% of the active t-RA remains at the skin surface (Fresno Contreras et al., 2005; Reichrath et al., 2007; Roos et al., 1998), ex vivo experiments with radiolabelled t-RA have shown that it has the potential to almost fully penetrate human skin, with only ~1% remaining at the surface (~75% in epidermis, ~20% in dermis, 2-6% in plasma) (Bailly et al., 1998). Yet, in practice, following topical t-RA treatment, plasma levels are not significantly increased relative to daily fluctuations in normal levels (Buchan et al., 1994; Reichrath et al., 2007; Trotta et al., 2003).

t-RA is photochemically unstable and can be reversibly isomerised to 13- and 9-cis retinoic acid (13-c-RA; isotretinoin and 9-c-RA; alitretinoin) (Lehman and Malany, 1989; Randolph and Simon, 1997; Del Rosso et al., 2012) as well as being isomerised by the action of cytochrome P450 (CYP26) (Heise et al., 2006; Reichrath et al., 2007; Reynolds et al., 1993) and retinoic acid 4-hydroxylase into both 13- and 9-c-RA and inactive polar isomers such as 4-hydroxy retinoic acid and 4-oxo retinoic acid (Bailly et al., 1998; Kang et al., 1996) (Figure 1.13).
The metabolism of all-trans retinoic acid (t-RA) in skin to its polar metabolites 4-hydroxy retinoic acid and 4-oxo retinoic acid occurs via retinoic acid 4-hydroxylase. The reversible isomerisation of t-RA to 13- and 9-cis retinoic acid occurs via cytochrome P450 retinoic acid hydroxylase (CYP26) or can occur spontaneously or by photoisomerisation.

Once within cells, t-RA acts by binding to nuclear hormone receptors (Petkovich et al., 1987). There are two families of retinoid binding nuclear hormone receptors with three isoforms in each family; retinoic acid receptors (RARs α, β and γ) (Mangelsdorf et al., 1990) and the retinoid-X receptors (RXRs α, β and γ) (Heyman et al., 1992). The most abundant receptors in human skin cells are RARγ (which constitutes around 90% of the RARs) and RARα (around 10%); RARβ is expressed at extremely low levels in skin (Fisher et al., 1994; Watson et al., 2004). For RXR receptors, RXRα constitutes around 90%, with both RXRβ and RXRγ expressed at very low levels (Fisher et al., 1994; Mukherjee et al., 2006). These receptors are found in the cell cytoplasm of both keratinocytes and fibroblasts, but the abundance is significantly greater in epidermal keratinocytes. Interestingly, intrinsically aged
(photoprotected) epidermal keratinocytes has significantly increased abundance of RARα compared to young (photoprotected) skin, perhaps to compensate for a lack of function, or a reduction in concentration of endogenous retinoids (Watson et al., 2004).

Both RAR and RXR receptors form homo- or hetero-dimers when bound to t-RA and its isomers to promote a conformational change exposing a nuclear localisation signal and allowing entrance into the nucleus. Once in the nucleus, the receptor-ligand complex can bind to specific retinoic acid response elements (RAREs) or retinoid-X response elements (RXREs) in gene promoters, to alter the transcription of specific genes (Chambon, 1996; Heyman et al., 1992; Mangelsdorf et al., 1990; Petkovich et al., 1987; Schmuth et al., 2007; Watson et al., 2004). t-RA binds only to RARs whereas 9-c-RA can bind both RARs and RXRs (Figure 1.14).

**Figure 1.14** Cell signalling pathway of t-RA. t-RA enters the cell via endocytosis where it can be reversibly isomerised to various derivatives including 9-c-RA. t-RA can bind RARs (depicted as yellow) and 9-c-RA can bind both RARs and RXRs (depicted as green). Upon binding to receptors a conformational change occurs allowing entry to the nucleus where both hetero- (RAR/RXR) and homo- (RAR/RAR or RXR/RXR) dimers can bind to RAREs and RXREs and activate mRNA transcription.

### 1.9.1.2 Effects of retinoids on skin and dermal ECM components of skin.

Histological investigations of t-RA-treated photoaged skin have established that the predominant epidermal feature in both the short- and long-term are enhanced cell
turnover and increased thickness (Griffiths et al., 1992b, 1993a; Kligman et al., 1986; Reichrath et al., 2007; Watson et al., 2001a). t-RA is thought to increase epidermal thickness by shortening the mitotic phase of the keratinocyte cell cycle, as well as delaying the onset of terminal keratinisation which leads to desquamation (Aneskievich and Fuchs, 1992).

As well as enhancing proliferation and thickness, t-RA also decreases the cohesiveness of keratinocytes and the function of the permeability barrier leading to peeling and dryness associated with t-RA use (Darlenski et al., 2010; Lehman and Franz, 2012; Reichrath et al., 2007). However, retinoids do not always thicken the epidermis, and are often regarded clinically as a proliferation ‘normaliser’ since in hyperproliferative tissue, such as in psoriasis where the epidermis in the active plaque is very thick to start with, retinoids can reduce the thickness to a level comparable with disease-free skin (Jean et al., 2011; Reichrath et al., 2007).

One of the main tools for detecting changes following t-RA treatment is immunohistochemistry (IHC) of micron-thin skin sections. Immunohistochemical detection of several ECM proteins found significant changes in both long- and short-term investigations using topical t-RA treatment of photoaged skin (table 1.3) (Bhawan et al., 1996; El-Domyati et al., 2004; Griffiths et al., 1993b; Kligman et al., 1986, 1993; Rafal et al., 1992; Varani et al., 1994; Watson et al., 2001a; Weiss et al., 1988; Woodley et al., 1990b). Most studies were long-term investigations requiring volunteers to topically apply t-RA cream daily for up to four years.

Many studies have investigated the abundance of collagens following t-RA treatment. Whilst short-term studies (6 months or less) found only minor changes in the dermal ECM, with no improvement in the deposition of collagen I and III (Bhawan J et al., 1991; El-Domyati et al., 2004; Kligman et al., 1986; Weiss et al., 1988), longer studies (10 months or above) showed deposition of these proteins and their precursors (procollagens I and III) in the papillary dermis (Bhawan et al., 1996; El-Domyati et al., 2004; Griffiths et al., 1993b; Kligman et al., 1986) (table 1.3). Long-term studies also showed deposition of collagen VII anchoring fibrils (Woodley et al., 1990b), increases and decreases in glycosaminoglycans staining (El-Domyati et al., 2004; Weiss et al., 1988), decreases in the levels of dysfunctional elastotic material in the deeper dermis (El-Domyati et al., 2004; Watson et al., 2001a) and repair of the fibrillin microfibrillar network adjacent to the DEJ (Watson et al., 2001a) (Table 1.3).
Table 1.3 Investigations on the effects of t-RA-treatment of photoaged and intrinsically aged skin.

<table>
<thead>
<tr>
<th>Paper:</th>
<th>Formulation:</th>
<th>Concentration:</th>
<th>Length of application:</th>
<th>Subject:</th>
<th>Results:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kligman et al., 1986</td>
<td>Retin-A™</td>
<td>0.05%</td>
<td>12-weeks</td>
<td>Photoaged forearm</td>
<td>Reversed epidermal abnormalities, ↑ epidermal thickness, ↑ fibroblast activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>52-weeks</td>
<td></td>
<td>↑ collagen in papillary dermis, ↑ angiogenesis, ↑ blood flow, ↑ TEWL and skin permeability</td>
</tr>
<tr>
<td>Weiss et al., 1988</td>
<td>Retin-A™</td>
<td>0.1%</td>
<td>16-weeks</td>
<td>Photoaged forearm</td>
<td>↑ GAGs, clinical improvement</td>
</tr>
<tr>
<td>Woodley et al., 1990</td>
<td>Retin-A™</td>
<td>0.1%</td>
<td>16-weeks</td>
<td>Photoaged forearm</td>
<td>↑ density of anchoring fibrils (double)</td>
</tr>
<tr>
<td>Rafal et al., 1992</td>
<td>Retin-A™</td>
<td>0.1%</td>
<td>40-weeks</td>
<td>Photoaged regions</td>
<td>↓ clinical appearance of actinic lentigines, ↓ melanin pigment in basal keratinocytes and deep epidermis</td>
</tr>
<tr>
<td>Griffiths et al., 1993</td>
<td>Retin-A™</td>
<td>0.1%</td>
<td>40 to 52-weeks</td>
<td>Photoaged forearm</td>
<td>↑ Procollagen I in papillary dermis</td>
</tr>
<tr>
<td>Kligman et al., 1993</td>
<td>Retin-A™</td>
<td>0.025%</td>
<td>36-weeks</td>
<td>Photoprotected intrinsically aged inner thigh</td>
<td>↑ epidermal thickness, ↑ rete ridges, ↑ GAGs, ↑ elastic fibres, ↑ angiogenesis</td>
</tr>
<tr>
<td>Varani et al., 1994</td>
<td>In vitro t-RA</td>
<td>3µM</td>
<td>12-days</td>
<td>Organ culture (PP and PA)</td>
<td>↑ fibronectin, ↑ epidermal thickness</td>
</tr>
<tr>
<td>Bhawan et al., 1996</td>
<td>Retin-A™ and Retinova</td>
<td>0.001-0.05%</td>
<td>156 to 208-weeks</td>
<td>Photoaged facial skin</td>
<td>Initial ↑ epidermal thickness, then decrease, ↓ dystrophic elastin</td>
</tr>
<tr>
<td>Watson et al., 2001</td>
<td>Retin-A™</td>
<td>0.05%</td>
<td>196-weeks</td>
<td>Photoaged facial skin</td>
<td>↑ fibrillin microfibril staining adjacent to DEJ ↓ dystrophic elastin</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>4-days</td>
<td>Occluded photoaged forearm</td>
<td>↑ fibrillin microfibril staining adjacent to DEJ</td>
</tr>
<tr>
<td>El-Domyati et al., 2004</td>
<td>Not stated</td>
<td>0.05%</td>
<td>12-24-weeks</td>
<td>Photoaged facial skin</td>
<td>↓ Collagen I, no change collagen III, ↓ elastin, ↓ GAGs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>~40-weeks</td>
<td></td>
<td>↑ Collagen I, ↑ collagen III, no further ↓ in elastin, variability in GAGs, ↑ fibroblast number and activity, ↑ microfibrils adjacent to DEJ</td>
</tr>
</tbody>
</table>
1.9.1.3 Mechanism of action of t-RA in repairing photoaged skin.

In the dermal ECM of photoaged skin there are thought to be two pathways of t-RA action. The first is an increase in manufacture and reorganisation of ECM proteins and the second a decrease in the matrix-degrading enzymes with an upregulation of their inhibitors.

Whilst t-RA activation of RAREs or RXREs in human skin DNA promotes the transcription of specific genes such as those involved in the production of fibrillin-1 (Watson et al., 2001a), fibronectin (Varani et al., 1994) and CRABP II (Aström et al., 1991; Elder et al., 1993), the increase in IHC staining of ECM proteins can also be attributed to decreases in the activity of matrix-degrading enzymes (Figure 1.15).

Following short term (two weeks) topical 0.05% t-RA treatment, there was a reduction in the migration of inflammatory neutrophils throughout the skin, as detected by a reduction in neutrophil elastase (Wozel et al., 1991). Furthermore, 0.1% t-RA pre-treatment for just 48 hours was able to reduce the over-expression of c-jun in UVA irradiated skin, thereby reducing the highly active AP-1 complex (Fisher et al., 1999, 2000) and reducing MMP levels and activity (Fisher et al., 1997). Moreover, longer term 13-c-RA treatment for four months at 0.05% was found to directly decrease the levels of MMPs, specifically MMP-9 and -13, known to be potent fibrillin microfibril remodeling enzymes (Papakonstantinou et al., 2005).

![Figure 1.15 Downstream effects of topical application of t-RA can be divided into two arms. t-RA can firstly stimulate both keratinocytes and fibroblasts to increase proliferation and ECM manufacture. Secondly, it can decrease the levels and activity of matrix-degrading enzymes such as MMPs and neutrophil elastase.](image_url)
1.9.1.4 Side effects of retinoids are completely reversible.

There are side effects to the topical application of t-RA, such as skin dryness, irritation, erythema or bleaching, peeling and alopecia – all of which are completely reversible after stopping treatment (Griffiths et al., 1995; Kligman et al., 1986; Lehman and Franz, 2012; Reichrath et al., 2007; Roos et al., 1998; Tsoureli-Nikita et al., 2006). The irritant effect of t-RA had been linked to its mechanism of action. However, studies have shown that using a known irritant as a control (such as sodium lauryl sulphate (SLS)) did not have as much of a positive effect as t-RA treatment (Griffiths and Voorhees, 1993; Griffiths et al., 1993a; Watson et al., 2001a), indicating a specific effect other than irritation. Furthermore, lower concentrations of t-RA (0.025%) were shown to have the same efficacy as higher concentrations (0.05% or 0.1%) without the irritation (Griffiths et al., 1993a, 1995).

1.9.2 t-RA repairs of the fibrillin microfibrillar network in photoaged skin.

Almost all studies investigating the effects of t-RA in human skin have noted that whilst epidermal changes occur quickly (even as quickly as two-days) (Griffiths et al., 1993a), most dermal changes require a longer use (40-weeks for increased abundance of collagen) (Table 1.3). However, one of the exceptions to this rule are fibrillin microfibrils, and research has shown that their immunohistochemical detection occurs after just four-days following 0.025% t-RA under occlusion (Watson et al., 2001a). This could be due to the added effects of an up-regulation in the production of Fbn1 mRNA in keratinocytes which was observed, or the down-regulation in fibrillin microfibril-degrading enzymes, such as MMP-9, MMP-13 and neutrophil elastase (Figure 1.15). This suggests that fibrillin microfibrils are key components in the dermal ECM that need to be replaced in photoaged skin. They are also useful biomarkers for detecting retinoid response in a short-term patch test (Watson et al., 2001a).

The distribution of fibrillin microfibrils observed after treatment with t-RA are similar to those observed in a wound healing model utilising cultured epithelial autografts in burn victims (Raghunath et al., 1996). A model for fibrillin microfibril regeneration at the DEJ was proposed where fine candelabra-like microfibrils adjacent to the DEJ formed as early as five-days after treatment, before cascading into the deeper dermis where they joined with elastin after one to three months (Raghunath et al., 1996). However, there have been no controlled studies which have aimed to identify the mechanism of t-RA-induced differences in the fibrillin
microfibrillar network. t-RA could either repair existing fibrillin microfibrils structures that are damaged by photoageing – which could be quick and result in apparent intact microfibrils very quickly. Alternatively, t-RA could promote the manufacture and assembly of entirely new fibrillin microfibrils, which could lead to a population of microfibrils with characteristics similar to those seen in foetal development or wound healing. In addition, no previous research has investigated the ultrastructure of these complex microfibrillar structures following t-RA-treatment. Fibrillin microfibrils are manufactured and assembled during development and are thought to last a lifetime in human tissues. If the fibrillin microfibrils are newly formed, it is unknown whether their structure will resemble the original skin-resident microfibrils before they were affected by photoageing or intrinsic ageing.

1.10 Summary III.

Fibrillin microfibrils are a useful biomarker of photoageing and repair of photoageing with topical t-RA treatment. Previous investigations have shown how they are highly susceptible to UVR in vitro, yet it is unknown how the full contributing effects of photoageing in vivo will affect their complex ultrastructure. Furthermore, whilst t-RA-treatment of photoaged skin showed an increase in IHC staining for fibrillin microfibrils, still unanswered is whether treatment leads to repair of existing fibrillin microfibrils or newly synthesised fibrillin microfibrils and, more importantly, whether these are comparable to the long-lived microfibrils resident in photoprotected young skin. Therefore, it is important to understand the complexity and ultrastructure of these fibrillin microfibrils both before, and after, photoageing, as well as after treatment with t-RA. A detailed ultrastructural analysis of t-RA-treated fibrillin microfibrils may elucidate a mechanism of action of retinoid effect on skin and on the repair of the fibrillin microfibrillar network adjacent to the DEJ.

1.11 Aims, structure and investigation hypotheses.

Following this review of the relevant literature, a detailed account of all materials and methods will be listed along with the experiments performed which lead to the results of each chapter.

This thesis has four results chapters, the first of which (chapter three) aims to characterise the ultrastructure of fibrillin microfibrils extracted from both
photoprotected (intrinsically aged) skin and photoaged skin of ten healthy volunteers. The photoprotected (intrinsically aged) site of each volunteer will serve as an internal control for the comparison to the photoaged site. It is hypothesised that:

**H1.** Photoageing of the skin will lead to ultrastructural changes observed in extracted fibrillin microfibrils.

Chapter four will then address which of the volunteers included in the investigation can be categorised as retinoid responders based on increases in epidermal thickness and abundance of fibrillin microfibrils, an analysis which is performed retrospectively.

The investigation then aims to examine the ultrastructure of fibrillin microfibrils extracted from retinoid treated photoaged skin (chapter five) and retinoid treated photoprotected (intrinsically aged) skin (chapter six) of the same ten individuals. In both cases the untreated site will serve as an internal control, and volunteers will be stratified according to retinoid response in chapter four. It is hypothesised that:

**H2.** t-RA treatment of photoaged skin of retinoid responders will have a positive effect on fibrillin microfibril ultrastructure. In partial-responders there will be some positive effects, but no effects in non-responders.

**H3.** t-RA treatment of photoprotected, intrinsically aged, skin of retinoid responders will have a positive effect on fibrillin microfibril ultrastructure. There will be some positive effects in partial-responders, but no effects in non-responders.

Within each chapter a short summary of results is provided; however discussion points for each chapter are combined in a final discussion chapter (chapter seven).
Chapter 2

Materials and methods
2 Materials and methods.

The aim of this thesis was to characterise the distribution, organisation and ultrastructure of fibrillin microfibrils extracted from photoprotected skin, photoaged skin, retinoid-treated photoaged skin and retinoid-treated photoprotected skin. In order to achieve this aim ten volunteers were recruited and biopsies were taken from their photoprotected (upper inner arm) and photoaged (forearm) skin both with and without treatment with t-RA. For each population (photoprotected, photoaged and t-RA-treated photoprotected and photoaged), two biopsies were taken – one for fibrillin microfibril abundance and architecture analysis (via immunohistochemistry) and one for ultrastructural and functional analysis (via STEM and AFM) of extracted fibrillin microfibrils.

The first part of the investigation (chapter three) characterises the architecture and ultrastructure of fibrillin microfibrils extracted from both photoprotected (intrinsically aged) skin and photoaged skin of ten healthy volunteers. Here, the photoprotected (intrinsically aged) site of each volunteer served as an internal control for the comparison to the photoaged site. Volunteers included in the investigation were then categorised as retinoid responders based on increases in epidermal thickness and abundance of fibrillin microfibrils, an analysis which was performed retrospectively (chapter four). The investigation then examined the abundance and ultrastructure of fibrillin microfibrils extracted from retinoid treated photoaged skin (chapter five) and retinoid treated photoprotected (intrinsically aged) skin (chapter six) of the same ten individuals. In both cases the untreated site served as an internal control.

2.1 Recruitment of volunteers and biopsies for in vivo studies.

All methods described in the subsection were based on published protocols developed by the Dermatopharmacology Unit at the University of Manchester (Watson et al., 2001a).

Ten healthy volunteers, with no underlying skin conditions, were recruited to a local research ethics committee-approved study. After a thorough clinical assessment of the skin, performed by Sister Gill Aarons, t-RA cream was applied in occluded patches for four-days, before biopsies were taken for laboratory assessment.
2.1.1 Ethics applications and inclusion and exclusion criteria.

Healthy volunteers were recruited under Salford and Trafford research ethics committee study 10/H1014/02 by Sr Aarons, which allowed a maximum of ten recruits. Volunteers were asked to join the study if they matched the inclusion and exclusion criteria:

Inclusion criteria

- Aged over 40 years;
- Willing to submit to examination of photoaged forearm and photoprotected upper inner arm;
- Willing to wear test patches on forearm and upper inner arm skin for four-days;
- Willing to submit to 3mm diameter punch biopsies from each patch test site on the forearm and upper inner arm, plus negative (untreated) control sites;
- Signed informed consent from the subject.

Exclusion criteria

- Disease state that would impair evaluation of the test sites;
- Topical or systemic retinoids applied to the skin within the past six or twelve months respectively, prior to entry to the study;
- Topical steroids or other topical drugs applied within the past two weeks prior to entry to the study;
- History of use of experimental drug or experimental device in the thirty days prior to entry into the study;
- History of keloid scars.

Since part of this investigation was to assess the effects of photoageing, volunteers were only asked to partake if they had clinically moderate to severe photoaged facial skin, which was judged using a previously published clinical scale (Griffiths et al., 1992a), where 0 signifies no clinical signs of photoageing and 9 represents clinically severely photoaged skin (therefore all volunteers had facial skin of grade 5 or above). All volunteers gave informed consent. The study was conducted under the guidelines set out by the Declaration of Helsinki (2008; (Williams JR, 2008)).

2.1.2 Skin biopsies from recruited volunteers.

Eight 3mm diameter punch biopsies were taken from each volunteer; four from a photoprotected site (upper inner arm) and four from a photoaged site (forearm).
Retinoid patches were applied to volunteers’ skin on the day of clinical assessment and biopsies were acquired four-days later and the skin sutured. Volunteers were advised to return to the department for suture removal after seven to ten days.

**Flow diagram of study**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Clinical assessment of skin;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Written and informed consent;</td>
</tr>
<tr>
<td></td>
<td>Application of occluded patches.</td>
</tr>
<tr>
<td>Day 5</td>
<td>Clinical assessment of skin;</td>
</tr>
<tr>
<td></td>
<td>Biopsies and sutures.</td>
</tr>
<tr>
<td>Days 12 - 15</td>
<td>Removal of sutures.</td>
</tr>
</tbody>
</table>

### 2.1.2.1 Choosing sites for biopsies.

After a thorough clinical assessment of the skin, areas on the arm were selected for biopsy using the criteria below.

**Forearm**

The region of skin selected was required to be clinically moderate to severely photoaged as judged by an unpublished in-house scale (3 or above on a 0-4 scale, where 0 signifies no signs of photoageing and 4 represents severely photoaged skin). If the volunteer was a frequent driver, the right arm was selected for biopsy.

**Upper inner arm**

This sample was used as both a photoprotected control and an anatomical site control. The same arm that was used for the forearm sample was used for the upper inner arm. An area no more than 3 inches from the armpit was used. The region of skin selected was also required to be clinically mild to not-photoaged (1 or below on the 0-4 scale).

### 2.1.2.2 Applying retinoid patches.

To prepare a retinoid patch for application, a (Janssen-Cilag Ltd.) or 0.025% Retacnyl® cream (Galderma UK Ltd.) was applied. Four Finn-chambers® (SmartPractice®) (two containing retinoid cream, two occluded with just the filter paper as baseline controls) were attached firmly to the skin at each site and covered with a waterproof dressing (Tegaderm™; 3M™), the patches were applied in one
continuous strip roughly one centimetre apart (Figure 2.1). If the volunteer had particularly thick or dense hair, the area was depilated by shaving before patches were applied so as not to hinder the absorption of the cream.

No vehicle cream was applied to the skin in this study since previous investigations detailed the near-negligible effects of a vehicle as compared to t-RA formulations (Watson et al., 2001a). Furthermore, the addition of a vehicle in this investigation would have required a further four biopsies per person (twelve in total) which may have hindered recruitment and ethical application.

![Figure 2.1 A Finn-chamber® was covered with sterile filter paper attached using Vaseline® before either 20μl of t-RA cream was applied (a; retinoid treated) or the chamber was left blank (b; baseline). This was applied in duplicate.](image)

**2.1.2.3 Biopsy, wound suture and volunteer after-care.**

The volunteers were asked to return to the Dermatopharmacology Unit after four-days. The retinoid patches were removed from the skin and the regions where the patches had been applied were marked. Volunteers were then asked to wait 15 minutes whilst their skin acclimatised to the clinic room. Notes were taken about the volunteers’ skin reaction since it was known that application of 0.025% retinoid cream can sometimes cause erythema (skin reddening) or blanching (skin whitening). None of the volunteers exhibited severe erythema or irritation and there were no drop-outs or exclusions due to side-effects. The selected biopsy sites were sterilized using Sterets® Tisept™ (Medlock Medical Ltd.) and anaesthetised with 1% w/v lignocaine™ (Antigen Pharmaceuticals Ltd.). The eight biopsies were taken using a 3mm diameter punch biopsy cutter (Integra™ Miltex®) and the wounds were sutured with 4/o ethilon™ (Ethicon Inc.). Volunteers were asked to return to the Unit after 7-10 days for suture removal and were given the contact details of the site nurse.
2.1.2.4 Preserving the biopsies.

Biopsies were either snap frozen in liquid nitrogen for fibrillin microfibril extraction or placed in optimal cutting temperature (OCT) embedding matrix (CellPath®) before being frozen in liquid nitrogen for sectioning and histological analysis (Figure 2.2).

![Figure 2.2 Biopsy collection and preservation. Tissue samples from all four sites were processed for both histological analysis and microfibril extraction.](image)

2.2 Immunodetection and localisation of fibrillin microfibrils in skin.

All methods described in this subsection were based on published protocols (Watson et al., 1999, 2001a). Biopsies were sectioned using a cryostat and stained for fibrillin microfibrils using IHC before imaging and analysis.

2.2.1 Biopsy preparation and cryosectioning.

In this investigation the cryostat used was a Bright OTF 5000 (Bright Instruments Co., Ltd.) with a 190mm blade microtome blade (Bright Instruments Co., Ltd.). Biopsies were preserved in OCT embedding matrix and stored at -80°C until required. The OCT was allowed to reach room temperature briefly to allow mounting onto a sectioning chuck before being frozen inside the cryostat. Several 30µm sections were cut first to ensure the series of sections cut for histological analysis were not near the wounded edge of the biopsy. Section thickness was set
between 5µm and 8µm depending on the fragility of the biopsies. In all cases the section thickness was recorded and set to the same thickness within a volunteer (i.e. the most fragile biopsy – in all cases, the upper inner arm baseline biopsy - was sectioned first and subsequent biopsies were then sectioned at the same thickness).

Sections were collected on both gelatin-subbed slides and Superfrost® Ultra-Plus Adhesion slides (©Thermoscientific) which were kept at room temperature since some IHC techniques require gelatin-free slides. Three serial sections were collected on each slide and were stored at -80°C until required.

2.2.2 Immunohistochemical staining to detect fibrillin microfibrils in human skin sections.

The primary antibody used to detect fibrillin microfibrils was antibody clone 11C1.3, a monoclonal mouse antibody specific to fibrillin microfibrils (manufactured by NeoMarkers® for Lab Vision Corporation (a division of Thermoscientific®).

Slides which had been stored at -80°C were first allowed to warm to room temperature and a hydrophobic barrier pen; ImmEdge™ (Vector Laboratories, Inc.) was used to circle each of the three sections on the slide. Sections were fixed in 4% paraformaldehyde in PBS (1.3 M PFA in 1 x PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4 (Sigma-Aldrich® Co.)) for 10 minutes before washing for two minutes in tris-buffered saline (TBS, 150 mM sodium chloride, 10 mM Tris, pH 7.4) two times. Sections were solubilised in 0.5% Triton-X100 (Sigma-Aldrich® Co.) before washing for two minutes in TBS twice. To ensure that any endogenous peroxide activity did not induce false positive staining, sections were bathed in 3% hydrogen peroxide (Sigma-Aldrich® Co.) in methanol for five minutes, before washing for two minutes in TBS twice. To inhibit any non-specific binding of antibodies, sections were blocked in Vector® 2.5% normal horse serum (Vector Laboratories, Inc.) for one hour at room temperature. Following this, fibrillin microfibril primary antibody done 11C1.3 (Thermoscientific®) was applied to the sections overnight at 4°C, diluted 1 in 100 in Vector® 2.5% normal horse serum. All three sections of each slide were stained following this method. One extra slide was included as an antibody negative where no primary antibody was applied, instead Vector® 2.5% normal horse serum block was added. Following primary incubation, slides were washed for two minutes in TBS twice before the secondary antibody, VECTASTAIN® Elite ABC Anti-Mouse IgG Biotinylated Secondary Antibody
(Vector Laboratories, Inc.), was applied for thirty minutes at room temperature. Slides were washed for two minutes in TBS twice and the Avidin-biotinylated-HRP complex, VECTASTAIN® ABC Reagent (Vector Laboratories, Inc.), was applied for thirty minutes at room temperature. Slides were washed for two minutes in TBS a final two times before the peroxidase substrate, Vector® ImmPACT™ SG Peroxidase Substrate (Vector Laboratories, Inc.), was applied for five minutes at room temperature. Slides were rinsed in distilled water and counterstained with Vector® Nuclear Fast Red (Vector Laboratories, Inc.) for four minutes at room temperature. After rinsing in tap water, the slides were dehydrated by submersion in increasing concentrations of IMS (from 50% to 100%), followed by submersion in two changes of Xylene; the last of which had been warmed to 37°C. Slides were permanently mounted using DPX mounting media and a glass cover-slip (Figure 2.3).

![Diagram](image)

*Figure 2.3 Schematic showing the theory of immunodetection of fibrillin microfibrils via the ABC IHC method.*

### 2.2.3 Grading IHC sections for fibrillin microfibril staining.

Fibrillin microfibril architecture and abundance was quantified using a randomised and blinded semi-quantitative grading scale under light microscopy at 400x magnification. The sections were graded using a published zero to four scale where;

0  = No oxytalan fibres adjacent to the DEJ; a completely unstained grenz zone.

1  = Truncated oxytalan fibres (not connecting to lower papillary dermis) present in patches throughout the section.

2  = Truncated oxytalan fibres present throughout the whole section (not connecting to lower papillary dermis), or patches of longer fibres (not connecting to lower papillary dermis).
3 Long oxytalan fibres that are either present throughout the whole dermis (not connecting to lower papillary dermis), or present in patches (connecting to the lower papillary dermis).

4 Candelabra oxytalan fibres present throughout the whole section (connecting to the lower papillary dermis).

For each section on each slide, three non-overlapping fields of view were graded to give nine grades per sample. A mean (± SEM) for each sample was then calculated.

2.2.4 Imaging IHC sections for epidermal thickness measurements.

Sections were imaged using a Biozero BZ-800K optical microscope (© Keyence Corporation) at a magnification of 200x (for analysis) and 400x (for image display purposes). For each sample, three non-overlapping fields of view were imaged; one field of view from each section (since the sections are in series). Using ImageJ (Rasband, 1997-2012; Abramoff, M.D. et al., 2004), the distance between the DEJ and the top of the outermost cellular layer of the epidermis (stratum granulosum) was measured using the line drawing tool. In each image, ten measurements were made to give thirty measures in total. The line drawing tool was also used to measure the scale bar for the images to give a conversion factor so that measurement output was in μm.
2.3 Ultrastructural analysis of fibrillin microfibrils isolated from human skin.

All methods described in this subsection were based on published protocols (Kielty et al., 1991; Sherratt et al., 1997, 2003). Fibrillin microfibrils were extracted and purified from full thickness skin biopsies before being adsorbed to microscopic sample discs or stubs for imaging and analysis.

2.3.1 Extraction of fibrillin microfibrils from human skin biopsies

Biopsies were first dissected using a sterile scalpel blade (Swann-Morton®) before being incubated for six hours in rotation with 0.5 mg ml⁻¹ type IA bacterial collagenase (Sigma-Aldrich® Co.) in collagenase buffer (400 mM sodium chloride, 50 mM Tris, 10 mM calcium chloride, pH 7.4) and the presence of protease inhibitors: phenylmethanesulfonyl fluoride (PMSF) and N-ethylmaleimide (NEM) (2 mM PMSF, 5 mM NEM (Sigma-Aldrich® Co.) in 100% methanol). After incubation, the sample was centrifuged at 5,000 g for five minutes (to pellet cell nuclei, mitochondria and any intact cells). Type IA bacterial collagenase degrades all ECM components except fibrillin microfibrils and collagen VI microfibrils; these are suspended in the supernatant along with smaller molecules, debris and cytosol.

2.3.2 Purification of intact fibrillin and collagen VI microfibrils.

The gel filtration chromatography system utilised an ÄKTA PrimePlus™ (©GE Healthcare) and an XK 16/100 Column (©GE Healthcare) that was packed with 70ml Sepharose® CL-2B media (©GE Healthcare). Before the supernatant was injected onto the column, both the ÄKTA PrimePlus™ and the column were washed with 20 ml distilled water which had been vacuum filtered using a 0.22 μm Stericup® (©EMD Millipore) to remove air bubbles. The column was then equilibrated with 40 ml column buffer (400 mM sodium chloride, 10 mM Tris, pH 7.4) which had been vacuum filtered using a 0.22 μm Stericup® to remove any precipitate and air. The sample inlet tubing was then cleaned with three times the sample volume (6 ml) of column buffer. The sample supernatant (2 ml) was then loaded into the sample inlet tubing before being injected onto the column by the ÄKTA PrimePlus™. The column was set at a flow rate of 0.5 ml min⁻¹ with a maximum pressure limit of 0.45 MPa. The fraction size was 1 ml and the eluent’s absorbance at a wavelength of 280 nm was monitored in real-time using the in-built UV spectrophotometer, the results of which were displayed using PrimeView™ version 5.0 (©GE Healthcare) on a nearby
computer. The filtration was run for an elution volume of 80 ml (one hundred and sixty minutes) or until the $V_T$ had been completely eluted and the absorbance reading returned to baseline. The fractions corresponding to the $V_0$ eluent contained abundant fibrillin microfibrils and were pooled ready for sample preparation and analysis (Figure 2.4).

![Size-exclusion chromatography for microfibril purification. The column is packed with a porous medium which allows the entry of smaller molecules and proteins. Larger molecules, which cannot enter the pores, are able to flow through the column quickly and are eluted in the void volume ($V_0$). The smaller molecules are eluted together after one total column volume ($V_T$).](image)

**Figure 2.4**

2.4 Imaging fibrillin microfibrils by STEM.

All methods described in this subsection were based on published protocols (Engel, 1982; Engel and Collieux, 1993; Engel et al., 1982; Holmes, 1995; Holmes et al., 1991; Müller et al., 1992; Sherratt et al., 1997, 2000, 2003, 2009)

As well as measuring length, periodicity and flexion angle, the purpose of STEM in this investigation was to accurately measure the mass of each bead-repeat of fibrillin microfibrils extracted from various populations. The sample presentation in STEM requires a 3.05 mm diameter 400-mesh copper grids. In order to place the samples on the copper grid, a scaffold must be placed on top of the copper mesh. This scaffold must have a consistent mass over the whole grid and allow the electron beam to pass through with minimal scattering so that the fibrillin microfibrils are clearly observable. Therefore, a thin layering of carbon (graphite) was applied to the mesh copper grid (Figure 2.5).
2.4.1 Preparing carbon-coated mica.

Carbon was first evaporated onto a mica sheet which can then be used to coat the copper grids. The end of a 6 mm graphite rod (©Agar Scientific) was prepared using a bench lathe until it formed a thin pointed end. The rod was attached to an electrical circuit and spring-loaded against another 6 mm graphite rod. When a current was sent through the rod inside a vacuum, the tip became white-hot and readily evaporated carbon. The point of the graphite rod fell directly over a mask of a clean ricochet cylinder, aligned over a sheet of freshly cleaved mica (©Agar Scientific) (freshly cleaved mica generally has an atomically flat surface). By evaporating carbon in this way, it only coated the mica by an indirect (or ricochet) method, leading to smoother carbon film than direct evaporation. The process took place inside a vacuumed dome pressurised to 3-4 x 10^{-5} Mbar. The voltage sent through the graphite rod was usually between 6-7 volts. The thickness of the carbon was within a 1.5 – 3.5 nm range, which was clearly visible on a piece of white filter paper that was positioned next to the mica as a carbon-thickness visualisation guide.

2.4.2 Preparing carbon-coated copper grids.

A small number of 3.05 mm Athene old 400-mesh copper grids (©Agar Scientific) were positioned shiny-surface-down onto carbon-coated mica. The mica and grids were then submerged slowly into a beaker of filtered ultrapure water at an angle of 10-20° (Figure 2.6). The surface tension of the water at this angle of entry caused the carbon to detach from the mica and float (carrying the copper grids) on the water's surface. Grids were collected from the surface in small groups (3-4 grids at a time) using sections of newspaper only slightly larger than the area of the grids. The newspaper was placed over the grids and left until saturated. The newspaper was then lifted carefully from the water holding the carbon-coated grids and left to dry in a dust-free environment.
Figure 2.6 Surface tension enables carbon to be taken from the mica to the copper grid. 3.05 mm copper grids were placed shiny-side down onto the carbon-coated mica. Using forceps, the mica was submerged into filtered ultrapure water at an angle of ~15°. The surface tension of the water caused the carbon to detach from the mica and float on the surface carrying the copper grids.

For the production of thick carbon-coated grids suitable for calibration and standardising, carbon was evaporated straight on to 3.05mm titanium 400 mesh grids (©Agar Scientific) by placing them directly inside the carbon evaporation chamber on filter paper. The thickness of these carbon-coated grids was between 35-40 nm.

2.4.3 Applying fibrillin microfibrils to carbon-coated copper grids.

The fibrillin microfibril-containing V₀ (6 µl) was allowed to adsorb onto the carbon surface for one minute at room temperature. The liquid was removed by capillary action using Whatman™ cellulose filter paper. The grid was washed three times in one drop (~10 µl) of filtered distilled water and excess water was removed by capillary action using Whatman™ cellulose filter paper. The grids were then left to dry at room temperature for a minimum of five minutes.

2.4.4 Applying TMV mass calibration standard to carbon-coated copper grids.

STEM mass mapping techniques are equilibrated to a known mass standard. Tobacco mosaic virus is often used as a mass standard as it has an easy to measure linear structure, stability in air-dried preparations and under an electron beam and a generally invariant mass of 131 kDa/nm (standard deviation 4%) compared to other mass standards such as fd-phage (standard deviation 7%) (Engel and Colliex, 1993; Holmes, 1995; Müller et al., 1992). Clean carbon-coated copper grids were treated with 20 mA of positive glow discharge for twenty seconds. 6 µl of TMV (kindly provided by Dr. John Carr, Biochemistry Department, Cambridge
University), diluted 1 in 10 in filtered PBS, was immediately allowed to adsorb onto the charged carbon surface for one minute at room temperature. The liquid was removed by capillary action using Whatman™ cellulose filter paper. The grid was then left to dry at room temperature for a minimum of five minutes.

2.4.5 Technique for image capture of fibrillin microfibrils using STEM

In this investigation, STEM images were generated using the FEI™ Tecnai™ 12 microscope (©FEI Company), with a TWIN objective lens (©FEI Company). This was equipped with a JEOL 1200EX scanning unit (©JEOL Ltd) interfaced with an FEI™ computing system to provide optimal facilities for quantitative operation. The STEM also had a high-angle annular dark-field detector (©FEI Company) equipped. Digital images were acquired with condenser aperture C2 inserted (70 μm diameter), a high tension voltage of 120 kV, a gun filament emission of roughly 5 μA, using a spot size of 9 (approximately 3 nm) and a camera length of 250 mm. Images of TMV and fibrillin microfibrils were collected at a magnification of 34,000x, with a sampling frequency of 2.52 nm in both spatial dimensions (1024 x 1024 pixels) and an acquisition time of 23.84 seconds. Images for fibrillin microfibril abundance and length measurements were collected at 17,000x magnification with a pixel size of 5.2 nm pixels−1 (1024 x 1024 pixels). The instrument was calibrated using tobacco mosaic virus (TMV) mass standard and a thick solid carbon film standard. Images of the thick carbon film were captured at a magnification of 12,000x. For each image captured, the small screen exposure (SSexp), emulsion setting and time were recorded. TMV images were captured at the beginning and end of the microscopy session (and roughly every two hours in between) and thick carbon-film images were captured roughly every twenty minutes. All images were exported as 16-bit unsigned raw '.bin' files, with little-endian byte order and a 10 byte offset to the first image.

2.5 Imaging fibrillin microfibrils by AFM.

All methods described in this subsection were based on published protocols (Binnig et al., 1986; Engel et al., 1997; Fotiadis et al., 2002; Hodson et al., 2009; Sherratt et al., 2003).

As well as measuring length, periodicity and flexion angle, the AFM images can be used to assess the tensile strength of fibrillin microfibrils via molecular combing (1.7.1.1). Fibrillin microfibrils were allowed to partially adsorb to a glass surface of
a specimen disc and a force was applied to the un-adsorbed portions as the buffer dried and the receding meniscus moved across the surface (Figure 1.7). AFM requires the fibrillin microfibrils to be adsorbed onto 15 mm specimen discs which can be coated in a variety of substrates (mica, mica-PLL, glass etc). In this investigation glass-coated specimen discs washed in ethanol were used since fibrillin microfibrils exhibit the least morphological changes and can be successfully combed due to an only moderately hydrophilic local environment (Hodson et al., 2009; Sherratt et al., 2005).

2.5.1 Preparing glass-coated specimen discs

15 mm SPM specimen discs (© Agar Scientific) were prepared in advance; 15 mm glass coverslips (Fisher Scientific UK Ltd.) were washed to remove contaminants overnight at room temperature in 99.9% ethanol (Fisher Scientific UK Ltd.), before being attached to the specimen disc using clear nail varnish (and allowed to dry in a clean environment.

2.5.2 Applying fibrillin microfibrils to specimen discs for molecular combing.

The glass covered specimen disc was attached to a table which could be rotated using double-sided sticky tape. 100 µl of the fibrillin microfibril-containing V₀ was allowed to adsorb onto the glass surface for one minute at room temperature. The rotator was then switched to 10 Volts (equivalent to roughly 1500 rpm) for two minutes. Ten seconds into the rotation (after most of the water had been removed) the edge of the rotating specimen disc was touched with a Whatman™ filter paper to remove the excess buffer held by surface tension. Once the rotation was complete, the glass surface was allowed to dry in the sample buffer for a minimum of thirty minutes. Once dry, the disc was attached again to the rotating table using double-sided sticky tape. The glass surface was washed three times in 300 µl of distilled water filtered through a 0.22 µm syringe driven filter unit (Millex® GP). Each time, the water was removed by rotating the disc at 10 Volts for ten seconds. On the last wash the disc was left rotating for two minutes, but ten seconds into the rotation the edge of the rotating disc was touched with a Whatman™ cellulose filter paper. The specimen disc was allowed to dry for a minimum of two hours before sample imaging.
2.5.3 Applying fibrillin microfibrils to specimen discs for non-combed samples.

The fibrillin microfibril-containing V₀ (100 µl) was allowed to adsorb onto the glass surface for one minute at room temperature. The liquid was removed by capillary action using Whatman™ cellulose filter paper and the glass surface was allowed to dry in the sample buffer for a minimum of thirty minutes. Once dry, the glass surface was washed three times in 300 µl of filtered distilled water. Excess water was removed by capillary action using Whatman™ cellulose filter paper and allowed to dry for a minimum of two hours before sample imaging.

2.5.4 Technique for image capture of fibrillin microfibrils using AFM

In this investigation both a NanoScope™ Multimode™ (©Bruker Corporation) AFM and a NanoWizard® 3 (©JPK Instruments AG) were used.

2.5.4.1 Image capture using the ©Bruker NanoScope™ Multimode™

Samples were imaged by TappingMode™ (intermittent contact) in air using a Multimode™ AFM with a NanoScope™ IIIa controller fitted with an E scanner (©Bruker Corporation). The probes used were OTESPA® (©Bruker Corporation) fitted into a Fluid Cell cantilever holder (©Bruker Corporation). A NanoScope™ optical viewing system (©Bruker Corporation) was used to aid laser alignment on the cantilever and to locate the cantilever over the sample surface. The drive frequency and amplitude were determined by the NanoScope™ version 5.0 software (©Bruker Corporation) and phase shift was determined using the AutoTune™ feature. Images were captured at a scan rate of 1.97 Hz, a scan size of 2x2 µm with pixel size of 3.925 nm pixel⁻¹ and a setpoint which was adjusted for each image scan. All measurements were conducted on height data which had been flattened using the NanoScope™ version 5.0 software and exported as 8-bit ASCII.

2.5.4.2 Image capture using the JPK® NanoWizard® 3

Samples were imaged by AC Mode™ (intermittent contact) in air using a NanoWizard® 3 BioAFM mounted on a manual precision stage (©JPK Instruments AG). As with the Multimode™, the probes used were OTESPA® (©Bruker Corporation) fitted into a supercut cantilever holder with removable spring (©JPK Instruments AG). A Zeiss Axio Observer D1 optical viewing system (©Carl Zeiss) was used to aid laser alignment on the cantilever. The drive frequency and amplitude
was determined the NanoWizard® Control version 4.0 software (©JKP Instruments AG) and phase was determined using the manual tuning system in the software. Images were captured at a scan rate of 1.5 Hz, a scan size of 10x10 μm and a setpoint which was adjusted for each image scan. As with the Multimode™ the pixel size was 3.925 nm pixel⁻¹. All measurements were conducted on height data which was exported as a ‘.tiff’ image file.

### 2.6 Analysis of fibrillin microfibril images generated by STEM and AFM.

To visualise and measure specific parameters of the fibrillin microfibrils extracted from various populations, two microscopic techniques; AFM and STEM, were employed. Images generated by these microscopes were used for measuring microfibril abundance, length, flexibility, periodicity, mass per repeat and mechanical strength as previously described (Cardy and Handford, 1998; Kielty et al., 1991; Sherratt et al., 1997, 2003, 2005, 2007; Thurstan et al., 2011).

#### 2.6.1 Measuring abundance of fibrillin microfibrils using STEM.

Ten STEM grid-squares were selected in TEM at 500x magnification depending on their quality of carbon coverage. No microfibrils can be seen at this magnification therefore selection of a grid-square was unbiased, but made sure that the image could be taken on an area of intact, un-contaminated carbon film. STEM images were captured at a magnification of 17000x at a random area within the selected grid square. Image capture at this magnification generates a resolution of 5.2 nm pixel⁻¹ in a capture time of 23.84 seconds. Images were imported into ImageJ (Rasband, 1997) as raw data files (.bin) and a 5x5 grid was overlayed on top of the image (where each square was 205 pixels² or 516.6 nm²). Since bacterial collagenase leaves both fibrillin and collagen VI microfibrils intact, the number of squares which contained microfibrils were counted, as well as the number of squares containing collagen VI microfibrils. Immunohistochemical investigations have shown that type VI collagen microfibrils remain unchanged in photoaged skin (Watson et al., 2001b) and are thought to be unchanged by topical t-RA application (Figure 2.7).
2.6.2 Measuring length of fibrillin microfibrils in both AFM and STEM.

Since the structure of a fibrillin microfibril is a beads-on-a-string appearance, length was measured simply by counting the number of beads in each fibrillin microfibril. This method only required the use of the raw image files, since microfibril beads are counted by eye.

2.6.3 Measuring mass per repeat of fibrillin microfibrils imaged by STEM.

2.6.3.1 Calculating the drift of each session using images of thick carbon film.

Detector efficiency was calibrated throughout the STEM sessions against a thick carbon film (Equation 2.1; Equation 2.2).
Average intensity was measured using the 'Measure' feature in ImageJ (Rasband, 1997) from the 'mean pixel intensity' measure.

2.6.3.2 Analysis of TMV images and calculating the mass calibration factor.

For the extraction of data from the TMV filaments, a custom macro was used in ImageJ (Rasband, 1997). TMV images generated by STEM were imported into ImageJ (Rasband, 1997) with the option to 'scale when converting' turned off. The image was converted to a 32-bit image and the look-up table was changed to 'fire' to improve the visibility of the TMV without affecting the raw data. The TMV was then straightened with a height of 81 pixels (one partial filament at a time (excluding the very ends of the filament)) using the 'straighten curved objects' java plugin for ImageJ (Kocsis et al., 1991; Rasband, 1997). The macro was run to extract the integrated density (total pixel intensity minus background) of a 75x81 pixel area around the TMV (Figure 2.8).

The integrated density was then used to calculate the mass calibration factor for STEM on that day of analysis (Equation 2.3). This process was repeated for TMV filaments imaged during each day of microscopy.
Equation 2.3

\[
mcf = \frac{131 \times n_L \times \frac{100}{SS_{exp}}}{\Sigma_{int} \times p}
\]

Where:
\(\Sigma_{int} = \text{(total pixel intensity - mean background intensity)} \times nL;\)
\(nL = \text{box width (75 pixels)};\)
\(SS_{exp} = \text{small screen exposure time of that image};\)
\(131 = \text{mass of TMV (131kDa/nm).}\)

2.6.3.3 Preparing straightened fibrillin microfibrils for input into LFA4.

Images generated by STEM were imported into ImageJ (Rasband, 1997) with the option to ‘scale when converting’ turned off. The image was converted to a 32-bit image and the look-up table was changed to ‘fire’ to improve the visibility of the fibrillin microfibrils without affecting the raw data.

Most STEM images contained data interference from an unknown source which was displayed as extremely high intensity pixels. To ensure that this interference did not influence the analysis the option to ‘remove outliers’ was used, where if a single pixel in the image was above a set threshold it was replaced by a pixel of the average intensity of the eight surrounding pixels. In every image measured, this threshold was set to 1500 AU.

The mean background intensity of the area around the microfibril was measured by producing a histogram in ImageJ. The data from the histogram was copied into OriginPro® 8 (OriginLabs®) and an amplitude unimodal Gaussian was fitted to the data to find the mean pixel intensity. The fibrillin microfibril was then straightened using the ‘straighten curved objects’ java plugin for ImageJ (Kocsis et al., 1991; Rasband, 1997). The image of the straightened fibrillin microfibril was then saved as a text image and later used to determine mass distribution.

2.6.3.4 Extraction of fibrillin microfibril mass pixel intensity data using LFA4.

A Windows® Visual Basic 6 program (LFA4) was written for the purposes of measuring mass per repeat of straightened fibrillin microfibrils (M. J. Sherratt). The calculated mean background intensity was entered into the program before the straightened fibrillin microfibril text file was imported. The value for mean background was automatically subtracted from the entire straightened section. The centre of each bead was then manually selected by looking at both the microfibril
image and the intensity plot (Figure 2.9). Once the periodicity was generated the program extracted total pixel intensity of each bead repeat, the axial pixel intensity profile, the centre-bead pixel intensity profile, the centre-inter-bead pixel intensity profile and the intensity of each pixel (Figure 2.9).

Figure 2.9 The process of extracting mass data from STEM. Visual basic program ‘LFA4’ generates an intensity profile through the centre point of the straightened section (width = 41 pixels). It can also extract total pixel intensity of the bead and the individual pixel intensities.
2.6.3.5 Converting fibrillin microfibril mass pixel intensity data into mass data.

The LFA4 extracted pixel intensity data was imported into Microsoft® Excel® (Windows®, Microsoft® 2010) under comma and tab delimited import. Pixel intensity data was converted to mass data using the mass calibration factor calculated using TMV imaged during the same session as the fibrillin microfibrils (Equation 2.4).

Equation 2.4

$$\text{mass} = \text{mcf} \times p^2 \times \frac{\Sigma \text{int}}{\left(\frac{100}{SS_{\text{exp}}}\right)} \times \frac{E_{TMV}}{E_{SPC}}$$

Where:
- \text{mcf} = the TMV mass calibration factor;
- \( p \) = the pixel resolution (2.52 nm at 34000x);
- \( \Sigma \text{int} \) = the pixel intensity to be converted;
- \( SS_{\text{exp}} \) = the recorded small screen exposure time of that image;
- \( E_{TMV} \) = the recorded emulsion setting of TMV;
- \( E_{SPC} \) = the recorded emulsion setting of fibrillin microfibrils.

2.6.4 Measuring periodicity and flexion angles of fibrillin microfibrils imaged by either AFM or STEM using Angles B-B.

The program used for both AFM and STEM-acquired images was Angles B-B written by M. J. Sherratt. The program uses a combination of (x, y) coordinate trigonometry and vector calculation to give an angle between the centre-points of three beads and the distances between those points (Figure 2.10; Equation 2.5). Angles B-B allows the user to simultaneously measure flexion angles and periodicities simply by clicking on the centre of each bead in the microfibril (Figure 2.11).

Figure 2.10 Angles B-B uses coordinate trigonometry and the dot rule to calculate the vector lengths and central angle.

Equation 2.5

Where:
- \( a = (x_2 - x_1, y_2 - y_1) \)
- \( b = (x_2 - x_3, y_2 - y_3) \)

And:
- \( \theta = \cos^{-1}\left(\frac{a \cdot b}{|a||b|}\right) \)
Figure 2.11 Angles B-B allows the user to simultaneously measure flexion angles and periodicities simply by clicking on the centre of each bead in the microfibril. For periodicity and flexion angle measurements the user clicks on the centre of each bead.

2.6.4.1 Importing images into Angles-B-B.

Angles B-B was initially designed to allow the import of a standard NanoScope™ Multimode™ AFM ASCII export file. Therefore these files can be opened directly without the need for any conversions. However, the JPK® NanoWizard® 3 AFM and STEM exported images as .tiff files and .bin raw files respectively. Therefore, in order to open these in Angles B-B, they needed to be cropped to 512x512 .txt files and converted to an 8-bit grey scale using ImageJ.

2.6.5 Comparisons between microscopic techniques.

Measurements taken by either the NanoScope™ Multimode™ or JPK® NanoWizard® 3 are not significantly different and were therefore pooled (Appendix 8.1). Ultrastructural measurements taken from AFM and STEM will be kept separate since differences in surface chemistries of carbon and glass may lead to observed differences in fibrillin microfibrils by the two techniques (1.7.4.3).
2.7 Statistical analysis and data presentation.

2.7.1 Statistical analysis of both IHC and ultrastructural data.

There are multiple methods for analyses of data using statistics. The most appropriate for biological data is to pair the samples if they are from the same volunteer, since this reduces the influence of biological variation between individuals. However, a full raw data set cannot be paired so it must be a summary statistic (such as mean, median or variance) which is selected based on what is the ‘most appropriate’ descriptive of the data set. Once the summary statistics are paired a statistical test can be selected based on the sample size and whether the differences between the paired summaries are normally distributed (Campbell et al., 2007; Lowry, 2008; Motulsky, 2010).

In this investigation samples can be paired based on the effect of photoageing or retinoid treatment:

- Photoprotected paired to photoaged,
- Photoaged paired to retinoid treated photoaged,
- Photoprotected paired to retinoid treated photoprotected.

Data was paired like this for every parameter measured (photoageing grade, fibrillin microfibril grade and fibrillin microfibril ultrastructural measurement). Whilst this method of pairing samples was useful for an overall interpretation of the data and predictions for global trends, it had little power in small sample sizes and often masked changes which occur in individual volunteers. Similarly, in retinoid-treated populations volunteers were categorised according to retinoid response and therefore pooling different responders into one group is not appropriate.

In this investigation, differences between volunteers had a large impact on the results observed and overall paired statistical tests were not sufficient to fully describe the data, therefore statistical tests on full raw data sets for each volunteer were also performed.

2.7.1.1 Statistical testing

Choosing an appropriate statistical test should not be an automated process, deciding whether to use a parametric (Student’s t-test) or nonparametric (Mann-Whitney U-test or Kolmogorov-Smirnov two-sample test) statistical test was a
thoughtful process (Motulsky, 2010). Tests for normality, such as Shapiro-Wilks or D’Agostino-Pearson, helped make the decision, but in order to decide which statistical test should be used for each parameter measured, knowledge of the null hypothesis for the test was required ($H_0$). For example, for fibrillin microfibril ultrastructural data, histogram distributions were the most informative way to display the data and often the mean or median of the data was misleading – especially if one of the distributions was bimodal. For these datasets the most logical way to compare samples is to compare the distributions using a two-sample Kolmogorov-Smirnov test (not to be confused with the Kolmogorov-Smirnov one-sample test for normality). Like the Mann-Whitney U-test, the Kolmogorov-Smirnov test is a nonparametric method to compare two groups. The test works by comparing the two cumulative frequency distributions, and so has more power to detect small differences between the two distributions. The Mann-Whitney U-test is only useful when detecting changes in the median and similarly the parametric Student’s t-test is only useful when examining changes in the mean.

For parameters where the distribution was not the most useful way to display data, such as fibrillin microfibril grade, epidermal thickness and fibrillin microfibril abundance, a multi-step process was employed to help decide which test to use (Figure 2.12).

Do populations fit a Gaussian distribution?
Both by eye (bell shaped) and results from normality test.
(D’Agostino-Pearson $n \geq 8$, Kolmogorov-Smirnov $n \geq 5$)

- YES
- NO

Do populations have equivalent standard deviation or variance?
(equality of variance F-test)

- YES
- NO

Summary statistic to use;

- Mean
- Median

Conduct the following test for two samples;

- Student’s t-test
- Mann-Whitney U-test/Kolmogorov-Smirnov two-sample test

*Figure 2.12 Decision tree to determine which statistical test will be used to test each parameter.*
A D’Agostino-Pearson normality test was used for data sets greater than (or equal to) 8, whereas a Kolmogorov-Smirnov one-sample normality test was used for data sets greater than (or equal to) 5 using Prism 6® (© GraphPad Software Ltd). However, normality was also judged by eye since testing alone has many limitations:

- Data can be classed as non-normal due to the presence of outliers,
- In small data sets (n ≤ 20) normality tests have little power,
- In large data sets normality tests can be too sensitive (Motulsky, 2010).

An equality F-test for variance was also performed on each sample (using Prism 6®) since parametric tests require the two populations to be tested to have similar variance or standard deviations.

If the samples were not significantly different to a Gaussian distribution (as tested by a normality test), had a roughly bell-shaped distribution (viewed by eye) and an equal variance, a parametric two-sample Student’s t-test was used to compare the groups. If the samples failed any of these assumptions then a nonparametric two-sample Mann-Whitney U-test was used to compare the groups.

### 2.7.1.2 Pairing samples within each volunteer.

Once an appropriate summary statistic had been selected (either mean or median) a paired statistical test was performed. The choice between a parametric test (paired Student’s t-test) and nonparametric test (Wilcoxon signed rank test) was once again important, but as before, was not automated. A normality test was performed on the differences between the two paired samples, which can help with the decision. Both means and medians can be used in parametric tests, since it is the normality of the difference between the sample summaries which affects the tests, not the normality of the samples themselves. Furthermore, unlike unpaired parametric tests, a paired parametric test does not require the samples to have equal variance. Therefore, a normality test on paired differences was employed and the test was selected accordingly (normal; paired Student’s t-test, not-normal; paired Wilcoxon test). Unfortunately, a paired Student’s t-test performed on an n ≤ 3 has very little or no power, therefore when volunteers were categorised according to retinoid response, and two new groups were formed, paired analysis was obsolete (Lowry, 2008; Motulsky, 2010).
2.7.1.3 Presentation of data

In order to clearly represent the data collected in the investigation, data used in paired statistical tests were represented as a line graph (either mean or median plotted depending on summary statistic used) and the mean ± SEM was reported for the summary data of pooled means or medians.

For individual two-sample tests, data was represented according to the parameter measured. For fibrillin microfibril grades, epidermal thickness and abundance, box-whisker plots were drawn. For fibrillin microfibril ultrastructure analyses, histogram distributions were drawn. Significant statistical results were highlighted using the Michelin guide scale where; * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) and **** = (p < 0.0001, exact p-value not reported) (Motulsky, 2010).

A consistent colour scheme is used to represent results from the various sites and treatments in each volunteer:

- **Photoprotected**,  
- **Photoaged** (lines dashed to distinguish in black and white),  
- **Photoaged retinoid treated**,  
- **Photoprotected retinoid treated**.
Chapter 3

Does photoageing of the skin lead to changes in the ultrastructure of fibrillin microfibrils?
3 Does photoageing of the skin lead to changes in the ultrastructure of fibrillin microfibrils?

In human skin, fibrillin microfibrils are long-lived complex macromolecular structures which provide elasticity and resilience (Baldock et al., 2001; Kielty et al., 2002; Shapiro et al., 1991; Sherratt et al., 2003). With cumulative UVR exposure photoageing of the skin occurs (Kligman, 1969). Previous research demonstrated reduced staining of fibrillin microfibrils at the DEJ by IHC in photoaged skin (Watson et al., 1999).

Ultrastructural analysis of fibrillin microfibrils extracted and irradiated in vitro, revealed that at physiologically relevant doses of UVR, fibrillin microfibrils exhibited increased or decreased periodicities, increased flexibility and increased fragmentation, as well as a significant reduction in mass (Table 1.2) (Sherratt et al., 2010; Thurstan et al., 2012a, 2012b, 2013). Furthermore, fibrillin microfibrils extracted from intrinsically aged skin also had reduced mass per repeat, as well as a reduction in tensile strength (Table 1.2) (Langton et al., 2011). Therefore, it was proposed that due to their location in the upper papillary dermis, longevity and chromophore-rich amino acid content, in vivo changes in fibrillin microfibril ultrastructure may occur with photoageing (Sherratt et al., 2010).

Here, it was hypothesised that fibrillin microfibrils extracted from photoaged skin would exhibit a combination of changes described in in vitro UVR irradiated, intrinsically aged and enzyme-remodelled fibrillin microfibrils. In summary, a reduction in length, reduced mass per repeat, increased flexibility and increased or decreased periodicity (Hypothesis H1).

Here, ten healthy, but severely photoaged, volunteers were recruited and biopsies were taken in duplicate from sites on the upper inner arm (photoprotected) and the forearm (photoaged). One biopsy from each site was cryosectioned and used for histological stains and IHC, whilst the other was used to extract fibrillin microfibrils for ultrastructural analyses.

3.1 Clinical Evaluation of the Skin of Volunteers

Healthy volunteers were recruited under Salford and Trafford research ethics, study 10/H1014/02, by research nurse Sr. Gillian Aarons. Volunteers were asked to join the study if they matched the inclusion and exclusion criteria (section 2.1.1) and clinical notes were recorded (table 3.1).
Table 3.1 Ten volunteers were recruited into the study under ethics 10/H1014/02.

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<tr>
<th>Volunteer Code</th>
<th>Sex</th>
<th>D.O.B</th>
<th>Age at time of biopsy (in years)</th>
<th>Facial Grading 0-9</th>
<th>Forearm grading 0-4</th>
<th>Upper arm grading 0-4</th>
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<td>71.3</td>
<td>6.7</td>
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</tr>
</tbody>
</table>
3.1.1 Forearm photoageing grade was moderately correlated with volunteer age, but facial and upper inner arm photoageing grade were not.

Photoageing occurs with cumulative exposure to UVR and so it was hypothesised that the level and grade of photoageing could be linked to the age of the volunteer. To test this hypothesis, a correlation of photoageing grade to volunteer age was performed. For facial grading of photoageing, the grades were numbered 0 to 9, where 0 represents no visible signs of photoageing and 9 represents chronic photoageing (Griffiths et al., 1992a). For upper inner arm and forearm skin, an in-house 0 to 4 grading scale was used, where 0 represents no visible signs of photoageing and 4 represents chronic photoageing. Clinical signs of photoageing were assessed and graded in each volunteer by Sr. Gill Aarons.

Facial and upper inner arm photoageing grades were not correlated with age (n = 10 volunteers, $R^2 = 0.27$, $p = 0.13$ and $R^2 = 0$, $p = 1$ respectively), whereas forearm photoageing grades were correlated moderately with age (n = 10 volunteers, $R^2 = 0.57$, $p = 0.008$) (Figure 3.1).
Figure 3.1 Correlation of photoageing grade to volunteer age.

(A) Facial photoageing grades were not correlated to age ($n = 10$, $R^2 = 0.27$, $p = 0.13$). (B) Upper inner arm photoageing grades were not correlated with age ($n = 10$, $R^2 = 0$, $p = 1$).

(C) Forearm photoageing grades were correlated moderately with age ($n = 10$, $R^2 = 0.57$, $p = 0.008$).
3.1.2 Forearm skin was significantly photoaged compared to photoprotected upper inner arm skin of the same volunteer.

It was hypothesised that forearm skin would be significantly photoaged when compared to photoprotected upper inner arm skin of the same volunteer. To test this hypothesis, photoageing grades for the forearm were plotted alongside photoageing grades for the upper inner arm.

A paired Student’s t-test showed that there was a significant difference in the clinical grading of upper inner arm skin (2.3 ± 0.13 SEM) as compared to forearm skin (3.2 ± 0.15 SEM) (n=10 volunteers, p = 4.89 x 10^-5) (Figure 3.2). Hence, the forearm skin was photoaged compared to photoprotected upper inner arm skin from the same volunteer.

![Figure 3.2 Forearm skin was significantly photoaged compared to upper inner arm skin of the same volunteer. Photoageing grades (clinical signs of photoageing based on a 0 to 4 in-house scale) of both upper inner arm and forearm skin. (*) A paired Student’s t-test shows that there was a significant increase in the grades of the photoaged skin (n = 10, p = 4.89 x 10^-5).](image-url)
3.2 Fibrillin microfibrils were significantly reduced in the papillary dermis of photoaged skin.

Although it had been previously reported that fibrillin microfibrils were reduced in the papillary dermis in photoaged skin (Watson et al., 1999), it was important to confirm this for this specific cohort since biological variance can occur (Ginsburg and Willard, 2009; Hong and Oh, 2010; Issa, 2007). Sections were stained for fibrillin microfibrils and graded using a 0 to 4 scale where 0 represents no visible fibrillin microfibrils (a completely unstained papillary dermis) and 4 represents a cascade of fibrillin microfibrils in the papillary dermis connecting to the lower reticular dermis (Watson et al., 1999). Unfortunately, due to an underlying skin condition in volunteer RSR03, only 9 volunteers were taken forward for immunohistochemical analyses.

A paired Student’s t-test showed that there was a significant reduction in the fibrillin microfibril grades of photoaged skin (1.92 ± 0.19 SEM) as compared to photoprotected skin (3.02 ± 0.21 SEM) (n=9 volunteers, p = 0.004) (Figure 3.3).

Individual box-whisker plots of fibrillin microfibril grades showed that there was a significant reduction in fibrillin microfibril grade in eight of the nine volunteers as identified by two-sample Student’s t-tests (n = 9 grade measurements; RSR01, p = 1.11 x 10⁻⁴; RSR02, p = 0.035; RSR04, p = 2.31 x 10⁻⁷; RSR06, p = 0.019; RSR07, p = 3.41 x 10⁻¹⁰; RSR08, p = 6.11 x 10⁻⁶; RSR09, p = 0.009, RSR10, p = 1.85 x 10⁻⁶). However, the extent of reduction in fibrillin microfibril staining varies greatly from volunteer to volunteer, highlighting the biological variance between individuals (Figure 3.4).
Figure 3.3 Fibrillin microfibrils were reduced in photoaged skin. Arrows highlight either presence or lack of fibrillin microfibrils. Photoprotected upper inner arm of RSR07 (78 year old male) and photoaged forearm from the same individual, scale bar below. Semi-quantitative grades for fibrillin microfibril staining at the DEJ on a 5 point ordinal scale (n = 9). (*) A paired Student’s t-test shows that there was a significant reduction in papillary dermal fibrillin microfibrils in photoaged skin (n = 9, p = 0.004).
Figure 3.4 Fibrillin microfibril staining was significantly reduced in the papillary dermis of photoaged skin. Individual box-whisker plots. (Box: inter-quartile range and median, point: mean, whiskers; minimum and maximum values) of fibrillin microfibril grades showed that there was reduction in fibrillin microfibril grade in all volunteers, which was significant in eight of the nine volunteers as tested by Student’s t-tests (n = 9 measurements per bar; RSR01, p = 1.11 x 10^{-4}; RSR02, p = 0.035; RSR04, p = 2.31 x 10^{-7}; RSR06, p = 0.019; RSR07, p = 3.41 x 10^{-10}; RSR08, p = 6.11 x 10^{-3}; RSR09, p = 0.009, RSR10, p = 1.85 x 10^{-6}).
3.3 Fibrillin microfibrils can be successfully extracted from photoaged forearm skin.

Intact fibrillin microfibrils had previously been extracted from cell culture fibroblasts and photoprotected tissues. However, they had not before been extracted from photoaged skin. Previous research and results from this thesis showed that fibrillin microfibrils are reduced at the papillary dermis in photoaged skin (figure 3.3). Therefore, it was not known if intact fibrillin microfibrils could be extracted. It was hypothesised that they could, but that they would exhibit the changes found in extracted fibrillin microfibrils irradiated *in vitro*, i.e. their structure would be severely compromised.

Intact fibrillin microfibrils were successfully extracted from photoaged skin and were imaged by both STEM and AFM with no additional steps as compared to extracting and imaging fibrillin microfibrils from photoprotected skin (Figure 3.5).

*Figure 3.5* Intact fibrillin microfibrils were successfully extracted from photoaged skin. The fibrillin microfibrils required no additional steps during the extraction or imaging process as compared to fibrillin microfibrils from photoprotected skin. Fibrillin microfibrils were imaged by both STEM and AFM and appeared as the characteristic 'beads-on-a-string'. Scale bars = 200 nm.
3.4 Fibrillin microfibril abundance was unaffected by photoageing.

Abundance of fibrillin microfibrils on carbon-coated STEM grids was measured at an instrumental magnification of x17000 with a measured sampling frequency of 2.52 nm². Fibrillin microfibril abundance was expressed relative to the abundance of type VI collagen microfibrils, which are thought to remain unchanged in both photoaged and retinoid-treated skin (section 2.6.1).

It was hypothesised, due to reduced staining of fibrillin microfibrils in the papillary dermis of photoaged skin, there would be a significant reduction in the abundance of extracted fibrillin microfibrils. Due to technical difficulties, five volunteers were taken forward for fibrillin microfibril analyses: RSR05, RSR07, RSR08, RSR09 and RSR10.

A paired Student’s t-test was performed on the mean ratios of each population, there was no significant difference in abundance of fibrillin microfibrils extracted from photoaged skin (0.50 ± 0.11 SEM) as compared to photoprotected (0.35 ± 0.08 SEM) skin (n = 5, p = 0.052) (Figure 3.6).

Individual box-whisker plots highlighted the wide variation in fibrillin microfibril abundance between volunteers, which was consistently unaffected by photoageing (Figure 3.7).

![Figure 3.6 Fibrillin microfibril abundance was not significantly affected by photoageing. Box-whisker plot for all volunteers together. A paired Student’s t-test performed on the mean ratio for each population showed no significant difference (n = 5, p = 0.052).](image-url)
Figure 3.7 Fibrillin microfibril abundance was not significantly affected by photoageing (individual box-whiskers). Box-whisker plots (box; inter-quartile range and median, cross; mean, whiskers; minimum and maximum values) for each volunteer, showed a wide variation in fibrillin microfibril abundance between volunteers, which was unaffected by photoageing. n = 10 abundance ratios per bar.
3.5 Length of fibrillin microfibrils was unaffected by photoageing.

The length of fibrillin microfibrils was measured by counting the number of beads in each microfibril. For each population, 50 fibrillin microfibrils were measured using both STEM and AFM. It was hypothesised that photoageing would lead to increased fragmentation of fibrillin microfibrils (shorter lengths).

3.5.1 Length of fibrillin microfibrils imaged by STEM was unaffected by photoageing.

A paired Student’s t-test was performed on the median fibrillin microfibril lengths for each site in each volunteer which were measured from STEM generated images. There was no significant difference in length of fibrillin microfibrils extracted from photoaged skin (8.6 beads ± 2.3 SEM) as compared to photoprotected skin (9.3 beads ± 2.4 SEM) (n = 5 volunteers, p = 0.080) (Figure 3.8).

Individual histogram plots showed variation between fibrillin microfibril lengths of each volunteer. RSR05 and RSR07 had much longer fibrillin microfibrils overall as compared to RSR08, RSR09 and RSR10. However, there was no change in length with photoageing in any of the volunteers as tested by two-sample Kolmogorov-Smirnov tests (Figure 3.9).

Figure 3.8 Length of fibrillin microfibrils (as measured by STEM) was not significantly affected by photoageing.
A paired Student’s t-test indicates no significant difference between photoprotected and photoaged fibrillin microfibrils (n = 5, p = 0.080).
Figure 3.9 Length of fibrillin microfibrils (as measured by STEM) was not significantly affected by photoageing (individual basis). Individual histograms with the median value for each volunteer show variation especially between RSR05, RSR07 and RSR08, RSR09, RSR10.
3.5.2 Length of fibrillin microfibrils imaged by AFM was unaffected by photoageing.

A paired Student's t-test was performed on the median fibrillin microfibril lengths for each site in each volunteer which was measured on AFM generated images. There was no significant difference in the length of fibrillin microfibrils extracted from photoaged skin (8.4 beads ± 2.4 SEM) as compared to photoprotected skin (9.7 beads ± 3.4 SEM) (n = 5 volunteers, p = 0.271) (Figure 3.10).

As with the STEM-imaged fibrillin microfibrils, individual histogram plots showed variation between fibrillin microfibril lengths of each volunteer. RSR05 and RSR07 had much longer fibrillin microfibrils compared to RSR08, RSR09 and RSR10, yet there was no change in length with photoageing in any of the volunteers as tested by Kolmogorov-Smirnov tests (Figure 3.11).

*Figure 3.10* Length of fibrillin microfibrils (as measured by AFM) was not significantly affected by photoageing. A paired Student’s t-test indicates no significant difference between photoprotected and photoaged fibrillin microfibrils (n = 5, p = 0.271).
Figure 3.11 Length of fibrillin microfibrils (as measured by AFM) was not significantly affected by photoageing (individual basis). Individual histograms with the median value for each volunteer show variation especially between RSR05, RSR07 and RSR08, RSR09, RSR10.
3.6 Mass per repeat of fibrillin microfibrils was significantly reduced with photoageing.

A paired Student’s t-test was performed on the mean fibrillin microfibril mass per repeat for each site in each volunteer. There was a significant reduction in mass per repeat of fibrillin microfibrils extracted from photoaged skin (2290.49 kDa ± 42.49 SEM) as compared to photoprotected skin (2577.59 kDa ± 58.42 SEM) (n = 5 volunteers, p = 0.039) (Figure 3.12).

Individual histogram plots for each volunteer showed that none of the volunteers exhibited a bimodal distribution or a subpopulation of repeats with increased mass. In four of the five volunteers (RSR05, RSR08, RSR09 and RSR10) there was a distinctive shift in the histogram distribution to a lower mass per repeat. Individual Kolmogorov-Smirnov tests confirmed that in four of the five volunteers there was a significant reduction in mass after photoageing (n = 250 mass measurements, RSR05, p < 0.0001; RSR08, p < 0.0001; RSR09, p = 0.0004; RSR10, p < 0.0001). However, in volunteer RSR07, fibrillin microfibrils extracted from photoprotected skin already had a reduced mass per repeat compared to photoprotected fibrillin microfibrils from the other four volunteers (Figure 3.13) and a Kolmogorov-Smirnov test indicated no further mass loss with photoageing (n = 250; RSR07, p = 0.24).

*Figure 3.12 Mass per repeat of fibrillin microfibrils from photoaged skin was significantly reduced compared to photoprotected skin. (*) A paired Student’s t-test of population means indicates a significant reduction in mass per repeat in photoaged as compared to photoprotected (n = 5, p = 0.039).
Figure 3.13 Mass per repeat of fibrillin microfibrils from photoaged skin was significantly reduced compared to photoprotected skin (individual basis). RSR05, RSR08, RSR09 and RSR10 fibrillin microfibrils had significantly reduced mass per repeat as tested by Kolmogorov-Smirnov tests (RSR05, p < 0.0001; RSR08, p < 0.0001; RSR09, p = 0.0004; RSR10, p < 0.0001). RSR07 had a lower average mass per repeat of fibrillin microfibrils extracted from photoprotected skin, compared to that of the other volunteers.
3.6.1 Reduction in mass per repeat of fibrillin microfibrils was localised to the centre of the bead.

In order to localise the reduction in mass per repeat, mass matrices were plotted. It was hypothesised that mass loss would be primarily from the centre region of the bead.

Average mass maps of fibrillin microfibrils extracted from photoprotected skin showed how each population had a roughly circular-shaped bead, with the majority of the mass located at the centre of the bead. Similarly, fibrillin microfibrils extracted from photoaged skin also had a roughly circular-shaped bead with the majority of the mass at the centre-bead. In four of the five volunteers (RSR05, RSR08, RSR09 and RSR10) the maximum mass at the centre-bead was reduced compared to photoprotected. In contrast, the mass maps of RSR07 were similar in maximum mass of the centre-bead (Figure 3.14). Once again, there was a high level of variability between volunteers, with the mass in beads from RSR08 seemingly more condensed to the centre-bead. However, the consistent trend of mass loss is still dominant.

Mass difference maps showed that in volunteers RSR05, RSR08, RSR09 and RSR10 there was a localised mass loss at the centre of the bead repeat. Whereas RSR07 had no specific region which had a significant mass loss or gain (Figure 3.15).

Average mass maps containing data from all volunteers showed that there was a clear reduction in mass which was localised to the centre-bead (Figure 3.16).
Figure 3.14 Average bead repeat from each population in each volunteer (n = 5 beads). All beads orientated left to right (shoulder region on left, greatest mass on right).
Figure 3.15 Difference between photoprotected and photoaged average beads. Red depicts a reduction in mass of the region in photoaged as compared to photoprotected, green depicts an increase in mass in the area.
Figure 3.16 Average contour plots of 21-pixel repeats show that mass loss was localised to the centre of the bead-repeat. Average contour plots created from all volunteer data (n = 5). 'Difference' was calculated by photoprotected minus photoaged.
3.7 Flexibility of fibrillin microfibrils was generally unaffected by photoageing.

Flexibility of fibrillin microfibrils was measured by calculating angles between 3 beads (flexion angle). A smaller angle indicated an increased flexibility of the microfibril. For each microfibril population, 250 angles were measured using both the STEM and the AFM. It was hypothesised that photoageing would lead to increased flexibility of fibrillin microfibrils (reduced flexion angle).

3.7.1 Flexibility of fibrillin microfibrils imaged by STEM was not significantly affected by photoageing in four volunteers.

A paired Student’s t-test was performed on the median fibrillin microfibril flexion angle for each site in each volunteer as measured from STEM generated images. There was no significant difference in flexibility of fibrillin microfibrils extracted from photoaged skin (154.34° ± 3.29 SEM) as compared to photoprotected skin (157.76° ± 1.58 SEM) (n = 5 volunteers, p = 0.278) (Figure 3.17).

Individual histogram plots and Kolmogorov-Smirnov tests for each volunteer showed that in RSR05, RSR07, RSR09 and RSR10 the flexibility of fibrillin microfibrils was unaffected by photoageing (n = 250 flexion angle repeats; RSR05, p = 0.83; RSR07, p = 0.29; RSR09, p = 0.055; RSR10, p = 0.40). However, in volunteer RSR08 there was a significant increase in flexibility (n = 250, RSR08, p = 0.0017) (Figure 3.18).

Figure 3.17 Flexibility of fibrillin microfibrils does not significantly change with photoageing as measured by STEM. A paired Student’s t-test indicates no significant difference between photoprotected and photoaged fibrillin microfibrils (n = 5, p = 0.278).
Figure 3.18: Flexibility of fibrillin microfibrils does not significantly change with photoageing as measured by STEM (individual histograms). Histograms for flexion angle data in each volunteer for each population labelled with the median. RSR08 and RSR09 had a significant increase in flexibility as tested by a Kolmogorov-Smirnov test ($n = 250$, RSR08, $p = 0.0017$). RSR05, RSR07, RSR09 and RSR10 fibrillin microfibrils were unaffected by photoageing.
3.7.2 Flexibility of fibrillin microfibrils imaged by AFM was unaffected by photoageing.

A paired Student’s t-test was performed on the median fibrillin microfibril flexion angle for each site in each volunteer measured from AFM generated images. There was no significant difference in flexibility of fibrillin microfibrils extracted from photoaged skin (151.77° ± 3.05 SEM) as compared to photoprotected skin (149.11° ± 3.80 SEM) (n = 5 volunteers, p = 0.141) (Figure 3.19).

Somewhat similar to STEM-generated images, individual histogram plots for each volunteer showed that in all volunteers fibrillin microfibril flexibility was unaffected by photoageing as tested by Kolmogorov-Smirnov test (n = 250 flexion angle repeats; RSR05, p = 0.54; RSR07, p = 0.89; RSR08, p = 0.61; RSR09, p = 0.34; RSR10, p = 0.069) (Figure 3.20).

Figure 3.19 Flexibility of fibrillin microfibrils does not significantly change with photoageing as measured by AFM. A paired Student’s t-test indicates no significant difference in flexibility between photoprotected and photoaged fibrillin microfibrils (n = 5, p = 0.141).
Figure 3.20 Flexibility of fibrillin microfibrils does not significantly change with photoageing as measured by AFM (individual histograms). Histograms for flexibility data in each volunteer for each population labelled with the median.
3.7.3 Differences in flexion angle measurements are observed when comparing STEM and AFM generated images.

As discussed previously (1.7.4.3) surface chemistry difference between the STEM and AFM sample surfaces can lead to differences in how fibrillin microfibrils adsorb and perhaps differentially influence their ultrastructure (Sherratt et al., 2004, 2005, 2007).

Results presented here show that this may be the case. Individual plots of the mean ± SEM flexion angle in each population measured by both STEM and AFM show that in four out of five volunteers there were differences observed between the two techniques (Figure 3.21). In RSR05 the flexion angle measurements were very similar, with some slight differences following photoageing, whilst in RSR08 the shift was in the same direction. Interestingly, in RSR07, RSR09 and RSR10 the shift in flexibility following photoageing went in opposing directions, suggesting a high level of variability in the fibrillin microfibril response to surface chemistry.
Figure 3.21 Surface chemistry difference between STEM and AFM led to different flexion angle measurements.
3.8 Periodicity of fibrillin microfibrils was significantly affected by photoageing in some volunteers.

Periodicity of fibrillin microfibrils was measured by calculating bead-to-bead distance in nanometres. For each microfibril population, 250 periodicities were measured using both the STEM and AFM. It was hypothesised that photoageing would lead to both increases and decreases in periodicity of fibrillin microfibrils.

3.8.1 Periodicity of fibrillin microfibrils imaged by STEM was significantly affected by photoageing in four of five volunteers.

A paired Student’s t-test was performed on the median fibrillin microfibril periodicity for each site in each volunteer measured from STEM generated images. There was no significant difference in periodicity of fibrillin microfibrils extracted from photoaged skin (53.85 nm ± 2.04 SEM) as compared to photoprotected skin (53.89 nm ± 1.56 SEM) (n = 5 volunteers, p = 0.980) (Figure 3.22).

Individual histogram plots and Kolmogorov-Smirnov tests for each volunteer showed that RSR09 fibrillin microfibrils were unaffected by photoageing (n = 250 periodicity repeats; RSR09, p = 0.13). However, in fibrillin microfibrils from RSR05 and RSR08 there was a significant reduction in periodicity (n = 250, RSR05, p < 0.0001; RSR08, p = 0.015). In opposition, RSR07 and RSR10 had a significant increase in periodicity with photoageing (n = 250, RSR07, p = 0.0003; RSR10, p = 0.033) (Figure 3.23).

Figure 3.22 Periodicity of fibrillin microfibrils imaged by STEM was significantly affected by photoageing in four of five volunteers. A paired Student’s t-test indicated there was no significant difference in periodicity of fibrillin microfibrils extracted from photoaged skin compared to photoprotected skin (n = 5, p = 0.980).
Figure 3.23 Periodicity of fibrillin microfibrils imaged by STEM was significantly affected by photoageing in four of five volunteers (individual histograms). Histograms for periodicity data in each volunteer labelled with the median. RSR05 and RSR08 had a significant reduction in periodicity as tested by Mann-Whitney U-tests (n = 250, RSR05, p < 0.0001; RSR08, p = 0.015). In opposition, RSR07 and RSR10 had a significant increase in periodicity with photoageing (n = 250, RSR07, p = 0.0003; RSR10, p = 0.033).
3.8.2 Periodicity of fibrillin microfibrils imaged by AFM was significantly affected by photoageing.

A paired Student’s t-test was performed on the median fibrillin microfibril periodicity for each site in each volunteer measured from AFM generated images. There was no significant difference in periodicity of fibrillin microfibrils extracted from photoaged skin (61.96 nm ± 3.74 SEM) compared to photoprotected skin (56.32 nm ± 1.78 SEM) (n = 5 volunteers, p = 0.200) (Figure 3.24).

Similar to STEM-generated images, individual histogram plots for each volunteer showed that in all volunteers there was a significant difference in periodicity as tested by Kolmogorov-Smirnov tests, but once again there was a high level of individual variation. In RSR05 there was a significant decrease in periodicity (n = 250 periodicity measurements, RSR05, p < 0.0001). RSR07, RSR08, RSR09 and RSR10 all had a significant increase in periodicity with photoageing (n = 250, RSR07, p < 0.0001; RSR08, p = 0.0015; RSR09, p = 0.026; RSR10, p < 0.0001) (Figure 3.25).

![Figure 3.24 Periodicity of fibrillin microfibrils does not significantly change with photoageing as measured by AFM.
A paired Student’s t-test indicated there was no significant difference in periodicity of fibrillin microfibrils extracted from photoaged skin (62.06 nm, IQR 9.73) compared to photoprotected skin (57.75 nm, IQR 2.68) measured by AFM (n = 5, p = 0.200).](image-url)
Figure 3.25 Periodicity of fibrillin microfibrils did not significantly change with photoageing as measured by AFM (individual histograms). Histograms for periodicity data in each volunteer labelled with the median. RSR07, RSR08, RSR09 and RSR10 had significant increases in periodicity with photoageing ($n = 250$, RSR07, $p < 0.0001$; RSR08, $p = 0.0015$; RSR09, $p = 0.026$; RSR10, $p < 0.0001$). RSR05 had a significant decrease in periodicity with photoageing ($n = 250$, RSR05, $p < 0.0001$).
3.8.3 Differences in periodicity measurements are observed when comparing STEM and AFM generated images.

As discussed previously (1.7.4.3; 3.7.3) the differences in surface chemistry between the STEM and AFM samples may influence their ultrastructure (Sherratt et al., 2004, 2005, 2007).

Individual plots of the mean ± SEM periodicity in each population measured by both STEM and AFM show that in all volunteers there were differences observed between the two techniques (Figure 3.26). In both RSR05 and RSR09 the changes in periodicity with photoageing were roughly parallel, whilst in RSR07 the shift was not parallel but was in the same direction. Interestingly, in RSR08 and RSR10 the shift in periodicity following photoageing went in opposing directions, suggesting a high level of variability in the fibrillin microfibril response to surface chemistry.
Surface chemistry difference between STEM and AFM led to different periodicity measurements.

Figure 3.26
3.9 The ability to measure fibrillin microfibril tensile strength was affected by their short length.

The purpose of AFM in this investigation was to assess the tensile strength of fibrillin microfibrils extracted from photoaged skin. Fibrillin microfibrils were adhered to glass coated specimen discs and subjected to strain in a process known as molecular combing (Figure 1.7). Previous work had shown how fibrillin microfibrils could be extended by this procedure, with their periodicity greatly elongated with distances up to 140 nm (Langton et al., 2011; Sherratt et al., 2003). It was hypothesised that the decline in skin elasticity and recoil associated with photoageing could be associated with a decline in fibrillin microfibril tensile strength (caused by ultrastructural damage by UVR). In other words, photoaged fibrillin microfibrils would be extended more by molecular combing than photoprotected fibrillin microfibrils from the same individuals. However, compared to previous fibrillin microfibril extractions the microfibrils extracted from the current cohort of five volunteers were much shorter, with a mean length of 8 to 10 beads compared to previously reported ~30 beads (Sherratt et al., 2003, 2010). The mechanism of molecular combing requires partial adsorption of the fibrillin microfibril, with a portion suspended in buffer which will have a force applied to it. It was therefore hypothesised that the microfibrils in this investigation were too short to exhibit this behaviour, instead the whole microfibril adsorbed and no portion was subjected to the applied force.

Periodicity of combed fibrillin microfibrils was measured by calculating bead-to-bead distance in nanometres. For each microfibril population (combed and control), 250 periodicities were measured using the AFM. There was no increase in periodicity in any of the combed samples when compared to control samples of the same site (Appendix 8.2).
3.10 Photoageing of the skin leads to changes in the ultrastructure of some fibrillin microfibrils.

It was hypothesised (H1) that due to their location in the upper papillary dermis, longevity in human tissues and chromophore-rich content, fibrillin microfibrils could be particularly susceptible to the effects of photoageing in vivo (Sherratt et al., 2010). The aim of this chapter was therefore to produce a detailed characterisation of the structure of fibrillin microfibrils extracted from volunteers with photoaged skin, with the photoprotected (intrinsically aged) site of the same individual serving as an internal control.

The results of this investigation showed that in eight of the nine volunteers there was a reduction in the staining and grade of fibrillin microfibrils adjacent to the DEJ in photoaged skin. Yet, in spite of this, intact fibrillin microfibrils were successfully extracted from the photoaged biopsies of all five volunteers. In terms of ultrastructural analyses, this chapter clearly demonstrated that fibrillin microfibrils extracted from photoaged skin had a lower mass per repeat than those extracted from photoprotected skin of the same volunteer; this was localised to the centre of the bead. Furthermore, all volunteers displayed a significant alteration in periodicity (both increases and decreases were observed). However, there were no significant changes in length or flexion angle with photoageing.
Chapter 4
Assessment of retinoid response by epidermal thickness and fibrillin microfibril grade measurement.
4 Assessment of retinoid response by epidermal thickness and fibrillin microfibril grade measurement.

The last chapter demonstrated that photoageing leads to a significant reduction in the mass of fibrillin microfibrils and more specifically, a localised mass loss from the centre of the bead. Topical application of 0.025% t-RA has been shown to result in the deposition of fibrillin microfibrils in the papillary dermis of photoaged skin, proximal to the DEJ (Watson et al., 2001a) and to increase epidermal thickness (Griffiths et al., 1993).

It was hypothesised that changes in fibrillin microfibril architecture and abundance following topical t-RA treatment lead to the new synthesis or repair of structurally and functionally competent fibrillin microfibrils. To test this hypothesis, photoprotected, intrinsically aged (upper inner arm) and photoaged (forearm) skin of 10 volunteers were subjected to a four-day occluded application (patch test) of t-RA. Biopsies were taken in duplicate from both sites to allow histological and ultrastructural analyses of resident fibrillin microfibrils.

It is well-established that as many at 15-20% of people treated with t-RA will never respond (in terms of epidermal thickening or ECM deposition), whilst some respond immediately (Griffiths et al., 1992b; Tsoureli-Nikita et al., 2006; Weiss et al., 1988). Therefore, to help stratify the results of t-RA-treatment, volunteers were examined for both changes is epidermal thickness and deposition of fibrillin microfibrils in histological sections from skin treated with t-RA. A method for categorising volunteers according to their response was then implemented. A ‘retinoid-responder’ showed increases in both epidermal thickness and fibrillin microfibril deposition, a ‘partial responder’ showed either an increase in epidermal thickness or fibrillin microfibril deposition and a ‘non-responder’ showed no increase in either epidermal thickness or fibrillin microfibril deposition.

4.1 Clinical Evaluation of the Skin of Volunteers.

Clinical signs of retinoid effect were recorded by Sr. Aarons since there are irritant effects associated with t-RA treatment such as erythema, blanching and skin flaking (Table 4.1).
Table 4.1 Ten volunteers were recruited under ethics, study 10/H1014/02. Clinical notes were recorded by Sr. Gillian Aarons.

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<tr>
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<td>3</td>
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<td>-</td>
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<tr>
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<td>Mild (UA)</td>
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<tr>
<td><strong>Mean</strong></td>
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<td><strong>6.6</strong></td>
<td><strong>2.0</strong></td>
<td><strong>3.2</strong></td>
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</tbody>
</table>
4.2 Epidermal thickening following four-days topical t-RA treatment occurs in both photoaged and photoprotected (intrinsically aged) skin.

It had previously been reported that topical application of 0.025% t-RA results in a thickened epidermis of both intrinsically aged and photoaged skin (Griffiths et al., 1993). Therefore, it was hypothesised that in the current cohort of volunteers, topical t-RA treatment would significantly increase epidermal thickness.

From the images of skin sections stained for fibrillin microfibrils, epidermal thickness measurements were calculated using ImageJ.

4.2.1 Epidermal thickening occurs in the photoaged skin of four volunteers.

Individual two-sample Student's t-tests were performed on epidermal thickness measurements to identify whether there was a variability in response of photoaged skin to t-RA. Of the nine volunteers included in the study, four individuals responded to the t-RA treatment by exhibiting a thickened epidermis (n = 30 measurements; RSR02, p = 3.11 x 10⁻¹⁰; RSR04, p = 1.33 x 10⁻¹¹; RSR07, p = 0.0303; RSR10, p = 4.32 x 10⁻¹⁰). A further three individuals showed no change in epidermal thickness following t-RA treatment (RSR01, RSR04, RSR05 and RSR07), whilst two individuals exhibited a significant thinning of the epidermis after t-RA treatment (n = 30; RSR05, p = 1.23 x 10⁻⁴; RSR06, p = 3.40 x 10⁻⁶) (Figure 4.1 and Figure 4.2).
Figure 4.1 Individual variability to t-RA treatment in photoaged skin using epidermal thickness as a marker. (A) RSR06 had thinning of the epidermis following t-RA treatment. (B) RSR08 had no change in epidermal thickness following t-RA treatment. (C) RSR10 had a thickened epidermis following t-RA treatment.
Increased epidermal thickness with t-RA treatment

No change in epidermal thickness with t-RA treatment

Decreased epidermal thickness with t-RA treatment

Figure 4.2 Mixed responses to t-RA treatment using epidermal thickness as a marker (individual-by-individual). RSR02, RSR04, RSR07 and RSR10 had a significant thickening as tested by Student’s t-tests ($n = 30$; RSR02, $p = 3.11 \times 10^{-10}$; RSR04, $p = 1.33 \times 10^{-11}$; RSR07, $p = 0.0303$; RSR10, $p = 4.32 \times 10^{-10}$) Conversely, RSR05 and RSR06 exhibited a significant thinning of the epidermis after t-RA treatment ($n = 30$ measurements per bar; RSR05, $p = 1.23 \times 10^{-4}$; RSR06, $p = 3.40 \times 10^{-4}$). RSR01, RSR08 and RSR09 showed no alteration in epidermal thickness over the four-day treatment period.
4.2.2 Epidermal thickening occurs in the photoprotected (intrinsically aged) skin of four volunteers.

Individual two sample Student’s t-tests were also performed on epidermal thickness measurements of photoprotected (intrinsically aged) skin following t-RA treatment in each volunteer. Of the nine volunteers included in the study, four individuals responded to the t-RA treatment by exhibiting a thickened epidermis (n = 30 measurements; RSR02, p = 3.69 x 10^{-8}; RSR04, p = 0.0156; RSR08, p = 8.12 x 10^{-11}; RSR10, p = 5.62 x 10^{-13}). A further four individuals showed no change in epidermal thickness following t-RA treatment (RSR01, RSR05, RSR06 and RSR07) and one individual exhibited a significant thinning of the epidermis after t-RA treatment (n = 30; RSR09, p = 2.54 x 10^{-14}) (Figure 4.3 and Figure 4.4).
Figure 4.3 Individual variability to t-RA treatment in photoprotected (intrinsically aged) skin, using epidermal thickness as a marker. (A) RSR09 had thinning of the epidermis following t-RA treatment. (B) RSR06 had no change in epidermal thickness following t-RA treatment. (C) RSR10 had a thickened epidermis following t-RA treatment.
Increased epidermal thickness with t-RA treatment

No change in epidermal thickness with t-RA treatment

Decreased epidermal thickness with t-RA treatment

Figure 4.4 Mixed responses to t-RA treatment of photoprotected (intrinsically aged) skin using epidermal thickness as a marker (individual-by-individual). RSR02, RSR04, RSR08 and RSR10 had a significant thickening as tested by Student’s t-tests (n = 30; RSR02, p = 3.69 x 10^{-8}; RSR04, p = 0.0156; RSR08, p = 8.12 x 10^{-11}; RSR10, p = 5.62 x 10^{-13}.) Conversely, RSR09 exhibited a significant thinning of the epidermis after t-RA treatment (n = 30 measurements per bar; RSR09, p = 2.54 x 10^{-14}). RSR01, RSR05, RSR06 and RSR07 showed no alteration in epidermal thickness over the four-day treatment period.
4.3 Increase in fibrillin microfibril grade following four-days topical \( t \)-RA treatment occurs in both photoaged and photoprotected (intrinsically aged) skin.

It had previously been reported that topical application of 0.025% \( t \)-RA results in the deposition of fibrillin microfibrils in the upper papillary dermis of photoaged skin (Watson et al., 2001a). Therefore, it was hypothesised that in the photoaged skin of the volunteers, which had a baseline of reduced fibrillin microfibril grades in the papillary dermis (section 3.3), topical \( t \)-RA treatment would significantly increase the fibrillin microfibril grade of the tissue. However, in the photoprotected skin of the same individuals, which already had a high baseline of fibrillin microfibril grades, there would not be a significant increase in grades following \( t \)-RA treatment.

Sections were stained for fibrillin microfibrils and graded from 0 to 4, where a grade of 0 represents no visible fibrillin microfibrils and a grade of 4 represents a cascade of fibrillin microfibrils in the papillary dermis connecting to the lower reticular dermis (Watson et al., 1999).

4.3.1 Increase in fibrillin microfibril grade occurs in the photoaged skin in three of nine volunteers.

Individual two sample Student’s t-tests were performed on fibrillin microfibril grade of photoaged skin and \( t \)-RA treated photoaged skin. Of the nine volunteers included in the study, three individuals responded to the \( t \)-RA treatment by significantly increasing fibrillin microfibril grade (\( n = 9 \) grade observations; RSR08, \( p = 0.006 \); RSR09, \( p = 1.02 \times 10^{-4} \); RSR10, \( p = 0.002 \)). A further four individuals did not change following \( t \)-RA treatment under occlusion for 4-days (RSR01, RSR04, RSR05 and RSR07), whilst two individuals exhibited a significant reduction in the grade of fibrillin microfibrils in the papillary dermis (\( n = 9 \); RSR02, \( p = 0.005 \); RSR06, \( p = 5.01 \times 10^{-4} \)) (Figure 4.5 and Figure 4.6).
Figure 4.5 Individual variability to retinoid treatment in photoaged skin using fibrillin microfibril staining as a marker. (A) RSR06 had a decrease in fibrillin microfibril grade following t-RA treatment. (B) RSR05 had no change in fibrillin microfibril grade following t-RA treatment. (C) RSR08 had an increase in fibrillin microfibril grade following t-RA treatment.
Increased fibrillin microfibril grade  
with t-RA treatment

No change in fibrillin microfibril grade  
with t-RA treatment

Decreased fibrillin microfibril grade  
with t-RA treatment

Figure 4.6 Individual variability to retinoid treatment in photoaged skin using fibrillin microfibril staining as a marker (individual-by-individual). Semi-quantitative grades for fibrillin microfibril staining at the DEJ on a 5 point ordinal scale (n = 9 grades per bar). RSR08, RSR09 and RSR10 had a significant increase in fibrillin microfibril grade as identified by Student’s t-tests (RSR08, p = 0.006; RSR09, p = 1.02 x 10^{-4}; RSR10, p = 0.002). Conversely, RSR02 and RSR06 had a significant reduction in fibrillin microfibril grade in the upper papillary dermis RSR02, p = 0.005; RSR06, p = 5.01 x 10^{-4}), whilst volunteers RSR01, RSR04, RSR05 and RSR07 showed no alteration in fibrillin microfibril grade over the four-day treatment period.
4.3.2 Increase in fibrillin microfibril grade occurs in the photoprotected (intrinsically aged) skin of three volunteers.

Individual two-sample Student’s t-test were performed on fibrillin microfibril grades on photoprotected (intrinsically aged) skin before and after t-RA treatment for four-days. Of the nine volunteers, three had a significant increase in fibrillin microfibril grade following t-RA treatment (n = 9 grade observations; RSR02, p = 0.002; RSR04, p = 0.041; RSR05, p = 7.14 x 10^{-5}). A further four had no change in fibrillin microfibril grade (RSR06, RSR08, RSR09 and RSR10), whilst two volunteers exhibited a significant reduction in fibrillin microfibril grade (n = 9; RSR01, p = 1.44 x 10^{-4}; RSR07, p = 3.23 x 10^{-6}) (Figure 4.7 and Figure 4.8).
Figure 4.7 Individual variability to retinoid treatment in photoprotected (intrinsically aged) skin using fibrillin microfibril staining as a marker. (A) RSR07 had a decrease in fibrillin microfibril grade following t-RA treatment. (B) RSR06 had no change in fibrillin microfibril grade following t-RA treatment. (C) RSR02 had an increase in fibrillin microfibril grade following t-RA treatment.
Figure 4.8 Individual variability to retinoid treatment in photoprotected (intrinsically aged) skin using fibrillin microfibril staining as a marker (individuals). Semi-quantitative grades for fibrillin microfibril staining at the DEJ on a 5 point ordinal scale (n = 9 grades per bar). RSR02, RSR04 and RSR05 had a significant increase in fibrillin microfibril grade as tested by Student’s t-tests (n = 9; RSR02, p = 0.002; RSR04, p = 0.041; RSR05, p = 7.14 x 10⁻⁴). Conversely, RSR01 and RSR07 had a significant reduction in fibrillin microfibril grade in the upper papillary dermis (n = 9; RSR01, 1.44 x 10⁻⁴; RSR07, p = 3.23 x 10⁻⁴). RSR06, RSR08, RSR09 and RSR10 showed no change in fibrillin microfibril grade over the four-day treatment period.
4.4 The current cohort of nine volunteers contains four retinoid responders, three partial responders and two non-responders.

Fibrillin microfibril deposition in the upper papillary dermis and epidermal thickness measurements allowed the volunteers to be categorised into 3 categories; retinoid responders (in which there was both epidermal thickness increase and fibrillin microfibril deposition), partial responders (either epidermal thickness increase or fibrillin microfibril deposition) or non-responders (no epidermal thickness increase and no fibrillin microfibril deposition).

The average change in epidermal thickness and fibrillin microfibril grade following t-RA treatment was calculated from the two body sites (Figure 4.9). The volunteers were then categorised according to the position within the scatter plot. In the current cohort of nine volunteers there were four retinoid responders, three partial responders and two non-responders.

The following chapters used this information to sort volunteers into categories and allowed the link between histological data and ultrastructural analyses to be extrapolated.

Figure 4.9 Fibrillin microfibril deposition in the upper papillary dermis and epidermal thickness measurements allow the volunteers to be categorised into 3 categories. Retinoid responders (both epidermal thickness increase and fibrillin microfibril deposition), partial responders (either epidermal thickness increase or fibrillin microfibril deposition) or non-responders (no epidermal thickness increase and no fibrillin microfibril deposition).
Chapter 5

Does short-term topical t-RA treatment of photoaged skin lead to changes in the ultrastructure of fibrillin microfibrils?
5 Does four-days topical t-RA treatment of photoaged skin lead to changes in the ultrastructure of fibrillin microfibrils?

Chapter three demonstrated how photoageing of the skin can lead to a loss of mass per repeat of fibrillin microfibrils, localised to the central bead region. Topical application of 0.025% t-RA is known to partially restore fibrillin microfibrils in the upper papillary dermis and up-regulate the production of Fbn1 mRNA in keratinocytes in photoaged skin (Watson et al., 2001a). However, it is not known whether any newly deposited or repaired fibrillin microfibrils have the same ultrastructure (length, mass, flexion angle, periodicity etc.,) as those originally found in the tissue.

This chapter examines the ultrastructure of fibrillin microfibrils extracted from retinoid treated photoaged skin. Biopsies were taken from the photoaged forearm skin of 10 volunteers. One biopsy was left untreated (baseline) and another was occluded with 0.025% t-RA. Fibrillin microfibrils were extracted from 5 volunteers (RSR05, 07, 08, 09 and 10) for detailed ultrastructural analyses. Data was assessed according to the results from Chapter four which demonstrated how volunteers respond differently to four-days topical application of t-RA; definite retinoid responder, partial retinoid responder or retinoid non-responder (Table 5.1).

Table 5.1 Fibrillin microfibril deposition in the upper papillary dermis and epidermal thickness measurements allow the volunteers to be categorised into 3 categories. The current cohort of five volunteers has three partial-responders (RSR05, RSR07 and RSR09) and two responders (RSR08 and RSR10).

<table>
<thead>
<tr>
<th>Volunteer</th>
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<tr>
<td>RSR05</td>
<td>Partial-responder</td>
</tr>
<tr>
<td>RSR07</td>
<td>Partial-responder</td>
</tr>
<tr>
<td>RSR08</td>
<td>Responder</td>
</tr>
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<td>RSR09</td>
<td>Partial-responder</td>
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<tr>
<td>RSR10</td>
<td>Responder</td>
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It was hypothesised that t-RA treatment of photoaged skin for four-days would normalise the ultrastructure of extracted fibrillin microfibrils (in terms of length, mass, flexion angle and periodicity) to values observed in extracted populations of fibrillin microfibrils from photoprotected skin.
5.1 Fibrillin microfibrils were successfully extracted from t-RA treated photoaged forearm skin.

Intact fibrillin microfibril assemblies had previously been extracted from photoaged skin; however, it was not known whether t-RA treatment would affect the extraction process by for example, increasing the level of crosslinks at both the molecular and microfibril level by activation of TGF-β and LOX/LOXL proteins (Kubiczkova et al., 2012; Massague, 1990; Sethi et al., 2011) or retinoid-induced tissue transglutaminases (Griffiths et al., 1992c; Ohtake et al., 2006, 2008; Ou et al., 2000; Qian and Glanville, 1997).

Intact fibrillin microfibril assemblies were successfully extracted from t-RA-treated photoaged skin and were imaged by STEM and AFM with no additional steps to their preparation as compared to extracting and imaging fibrillin microfibrils from photoprotected or photoaged skin (Figure 5.1).

Figure 5.1 Intact fibrillin microfibrils were successfully extracted from t-RA-treated photoaged skin. The fibrillin microfibrils appear as the characteristic beads-on-a-string with no noticeable differences between images of the populations. Scale bars = 200 nm.
5.2 Fibrillin microfibril abundance was unaffected by t-RA treatment of photoaged skin.

In chapter four, we confirm that topical treatment with t-RA leads to an improvement in the architecture of photoaged skin. It was therefore hypothesised that there would be a significant increase in the abundance of fibrillin microfibrils extracted from t-RA-treated photoaged skin, as compared to the amount obtained from photoaged samples.

Individual box-whisker plots highlighted the wide variation in fibrillin microfibril abundance between volunteers, which was consistently unaffected by t-RA treatment (as tested by individual two-sample Student’s t-tests) and was not correlated with retinoid response category (Figure 5.2).

As before fibrillin microfibril abundance was expressed as a ratio to collagen VI abundance, which is thought to remain unchanged in human tissues (Watson et al., 2001b).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 5.2 Fibrillin microfibril abundance was not significantly affected by t-RA treatment in photoaged skin. Box-whisker plots for each volunteer showed a wide variation in fibrillin microfibril abundance between volunteers, which was unaffected by t-RA treatment (as tested by Student’s t-tests).
5.3 Length of fibrillin microfibrils was generally unaffected by t-RA treatment of photoaged in all volunteers.

In chapter three it was shown that photoageing did not alter the length of the extracted microfibril populations, as compared to photoprotected sites. Therefore it was hypothesised that length of individual microfibrils from the extracted population here (photoaged but t-RA-treated) would not significantly alter. To address this hypothesis, the length of fibrillin microfibrils was measured by counting the number of beads in each. For every sample 50 fibrillin microfibrils were measured using STEM and AFM.

5.3.1 Length of fibrillin microfibrils imaged by STEM was unaffected by t-RA treatment of photoaged skin in all volunteers.

Histogram plots showed that individual variation between the volunteers, in terms of fibrillin microfibril length, still remains: RSR05 and RSR07 had much longer fibrillin microfibrils overall as compared to RSR08, RSR09 and RSR10.

Two-sample Kolmogorov-Smirnov tests showed that in all volunteers, fibrillin microfibrils had no significant difference in length following t-RA treatment (n = 50 microfibrils; RSR05, p = 0.39; RSR07, p = 0.99; RSR08, p = 0.18; RSR09, p = 0.86; RSR10, p = 0.71) (Figure 5.3).
Partial retinoid responders

Retinoid responders

Figure 5.3 Individual frequency histograms of lengths of photoaged baseline and photoaged retinoid treated fibrillin microfibrils (as measured by STEM) with the median value for each population.

Data showed individual variation especially between RSR05, RSR07 and RSR08, RSR09, RSR10. Kolmogorov-Smirnov tests showed that fibrillin microfibrils had no significant increase in length following t-RA treatment.
5.3.2 Length of fibrillin microfibrils imaged by AFM was both increased and decreased by t-RA treatment in photoaged retinoid responder skin.

Length was also assessed by quantification of AFM images. Individual histogram plots confirmed the variation in length of fibrillin microfibrils between volunteers: RSR05 and RSR07 had much longer fibrillin microfibrils overall as compared to RSR08, RSR09 and RSR10. This is in agreement with the observations made by STEM.

However, using AFM, both retinoid responders (RSR08 and RSR10) had significant differences in length following t-RA treatment; RSR08 had a significant increase in length following t-RA treatment (n = 50; p = 0.039) whereas RSR10 had a significant decrease in length following t-RA treatment (n = 50; p = 0.039) as tested by two-sample Kolmogorov-Smirnov tests. RSR05, RSR07 and RSR09 fibrillin microfibrils had no significant difference in length following t-RA treatment (n = 50; RSR0, p = 0.39; RSR07, p = 0.54; RSR09, p = 0.39) (Figure 5.4).
Individual frequency histograms of fibrillin microfibril length (as measured by AFM) from photoaged baseline and photoaged retinoid-treated skin with the median value for each population.

Individual variation between volunteers especially RSR05, RSR07 to RSR08, RSR09, RSR10 was observed. Kolmogorov-Smirnov tests showed that RSR08 had a significant increase in length $(n = 50, p = 0.039)$ and RSR10 had a significant decrease in length following t-RA treatment $(n = 50, p = 0.039)$. RSR05, RSR07 and RSR09 fibrillin microfibrils had no significant difference in length following t-RA treatment.
5.4 Mass per repeat of fibrillin microfibrils from t-RA treated photoaged skin was significantly different as compared to untreated photoaged skin in two of five volunteers.

Photoageing of the skin can lead to a reduction of mass per repeat of fibrillin microfibrils, localised to the central bead region. Topical t-RA treatment of photoaged skin is known to partially restore fibrillin microfibrils in the papillary dermis and up-regulate the production of fib1 mRNA in keratinocytes in photoaged skin (Watson et al., 2001a). Therefore it was hypothesised that t-RA treatment would restore the mass loss observed in photoaged fibrillin microfibrils so that their mass per repeat was more similar to the photoprotected fibrillin microfibrils of that volunteer.

Individual two-sample Kolmogorov-Smirnov tests showed that fibrillin microfibril mass per repeat varied across volunteers. RSR10 (retinoid responder), had a dramatic increase in mass per repeat of t-RA treated photoaged fibrillin microfibrils (3314.13 kDa ± 60.53 SEM) as compared to baseline photoaged fibrillin microfibrils (2324.90 kDa ± 48.94 SEM) (n = 250 repeats per population, p < 0.0001). RSR07 (partial retinoid responder), also had a significant increase in mass per repeat of t-RA treated photoaged fibrillin microfibrils (2608.58 kDa ± 34.90 SEM) as compared to baseline photoaged fibrillin microfibrils (2406.61 kDa ± 32.11 SEM) (n = 250, p < 0.0001) (Figure 5.5). However, in both cases the increase in mass per repeat following t-RA treatment was not comparable to the mass per repeat of fibrillin microfibrils from photoprotected skin of the same individual (Figure 5.6). RSR07 had photoprotected fibrillin microfibrils with a relatively low mass per repeat (compared to the other four volunteers; 2366 kDa compared to 2578 kDa for rest of cohort), therefore the mass of t-RA treated photoaged fibrillin microfibrils of RSR07 were more comparable to photoprotected fibrillin microfibrils from the rest of the cohort. RSR10 had t-RA treated photoaged fibrillin microfibrils almost 700 kDa higher than photoprotected fibrillin microfibril (2676 kDa), however the distribution appears bimodal, with subpopulations of roughly 2400 kDa and 3500 kDa. RSR05, RSR08 and RSR09 showed no significant difference in mass per repeat following t-RA treatment as tested by Kolmogorov-Smirnov tests (n = 250; RSR05, p = 0.34; RSR08, p = 0.47; RSR09, p = 0.20).
Figure 5.5 Individual frequency histograms for mass per repeat data in each volunteer, along with the mean for each population. Kolmogorov-Smirnov tests showed that RSR07 and RSR10 both had a significant increase in mass per repeat following t-RA treatment (n = 250; RSR10, p < 0.0001; RSR07, p < 0.0001). RSR05, RSR09 and RSR08 showed no significant difference in mass per repeat after four-days t-RA treatment.
Figure 5.6 Increase in mass per repeat following t-RA treatment was not comparable to the mass per repeat of fibrillin microfibrils from photoprotected skin of the same individual.
RSR07 had photoprotected fibrillin microfibrils with a relatively low mass per repeat (2366 kDa), but the mass of t-RA treated photoaged fibrillin microfibrils (2609 kDa) was comparable to photoprotected fibrillin microfibrils from the rest of the cohort (n = 5; overall photoprotected 2578 kDa). RSR10 had t-RA treated photoaged fibrillin microfibrils almost 700 kDa higher than photoprotected fibrillin microfibril (3314 kDa compared to 2676 kDa).
5.4.1 Increase in mass per repeat following t-RA treatment is localised to the outer-bead region.

Mass matrices were plotted for volunteers RSR07 and RSR10 so as to localise the observed increase in mass per repeat. In fibrillin microfibrils extracted from photoaged skin, loss of mass occurred at the centre region of the bead. Hence, it was hypothesised that gain mass would be primarily in this region.

Average mass maps of fibrillin microfibrils extracted from photoaged skin identified a roughly circular-shaped bead, with the majority of the mass central to the bead. Similarly, fibrillin microfibrils extracted from t-RA treated photoaged skin had a roughly circular-shaped bead with the majority of the mass in the centre-bead.

In RSR07 the maximum mass at the centre-bead does not increase, but the area containing greater mass is increased following t-RA treatment and the bead becomes more rounded. In RSR10 fibrillin microfibrils the maximum bead area also increased as well as an increased bead diameter following t-RA treatment (Figure 5.7).

Mass difference maps showed that in volunteers RSR05, RSR08 and RSR09 there was no specific region of mass change within the bead following t-RA treatment. However, RSR07 and RSR10 had areas with a relatively large increase in mass at the bead edge which shifted and enlarged the centre-bead region (Figure 5.8).
No change in mass per repeat following t-RA treatment

Increase in mass per repeat following t-RA treatment

Figure 5.7 Mass contour maps of average (n=5) photoaged baseline bead (left) next to average (n=5) t-RA-treated photoaged bead (right). Kolmogorov-Smirnov tests showed that RSR07 and RSR10 both had a significant increase in mass per repeat following t-RA treatment (n = 250; RSR07, p < 0.0001; RSR10, p < 0.0001). RSR05, RSR09 and RSR08 showed no significant difference in mass per repeat after four-days t-RA treatment. All beads orientated left to right (shoulder region on left, greatest mass on right).
No change in mass per repeat following t-RA treatment

Increase in mass per repeat following t-RA treatment

Figure 5.8 Difference between photoaged and t-RA-treated photoaged average beads. Red depicts a reduction in mass of the region in photoaged as compared to t-RA treated photoaged, green depicts an increase in mass in the area. Arrows indicate regions where there was a relatively large increase in mass.

[Diagram showing mass per repeat changes with RSR05, RSR08, RSR09, RSR07, RSR10, and two color scales for +2.6 kDa/nm² and -2.6 kDa/nm²]
5.5 Flexibility of fibrillin microfibrils was mainly unaffected by $t$-RA treatment of photoaged skin.

Flexibility of fibrillin microfibrils was measured by calculating angles between three beads (flexion angle). A smaller angle indicated an increased flexibility of the microfibril. For each microfibril population, 250 angles were measured using both STEM and AFM. Results from chapter three revealed that photoageing did not affect flexibility of fibrillin microfibrils. Only one volunteer (RSR08) exhibited an increase in flexibility when measured by STEM. Therefore, it was hypothesised that in those samples unaffected by photoageing, flexibility would be unchanged by $t$-RA treatment, but in those where the flexibility was significantly altered by photoageing, there would be a normalisation in (i.e. a reduction) so that it was comparable to photoprotected fibrillin microfibrils.

5.5.1 Flexibility of fibrillin microfibrils imaged by STEM was significantly different following $t$-RA treatment in two volunteers.

Individual two-sample Kolmogorov-Smirnov tests showed that fibrillin microfibrils extracted from the photoaged skin of volunteers RSR07, RSR08 and RSR09 had no significant difference in flexibility following $t$-RA treatment ($n = 250$ flexion angle repeats; RSR07, $p = 0.29$; RSR08, $p = 0.087$; RSR09, $p = 0.24$). However, RSR05 and RSR10 both showed a significant increase in flexibility following treatment with $t$-RA ($n = 250$; RSR05, $p = 0.033$; RSR10, $p = 0.0004$) (Figure 5.9).

RSR08 fibrillin microfibrils had significantly increased flexibility with photoageing. Here, fibrillin microfibrils from $t$-RA-treated photoaged skin had decreased flexibility, but not significantly, leading to an intermediate flexibility between photoaged and photoprotected ($n = 250$; Kolmogorov-Smirnov, Bonferroni correction, $p = 0.40$) (Figure 5.10).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 5.9 Flexion angle of fibrillin microfibrils imaged by STEM was significantly affected by t-RA treatment of photoaged skin (in two of five volunteers). Two-sample Kolmogorov-Smirnov tests showed RSR05 and RSR10 both had a significant decrease in flexion angle following t-RA treatment (n = 250 flexion angle repeats; RSR05, p = 0.033; RSR10, p = 0.0004) making them more flexible, whereas RSR08, RSR07 and RSR09 photoaged fibrillin microfibrils had no significant difference in flexion angle following t-RA treatment.
Figure 5.10 The distribution of flexion angles of fibrillin microfibrils extracted from t-RA-treated photoaged skin (155.29°, IQR 42.75) was not significantly different to that of those extracted from photoprotected skin (158.13°, IQR 40.07) as tested by a Kolmogorov-Smirnov test with Bonferroni correction.
5.5.2 Flexibility of fibrillin microfibrils imaged by AFM was increased in one retinoid responder following t-RA treatment.

Histogram distributions showed that RSR05, RSR07, RSR08 and RSR09 photoaged fibrillin microfibrils had no significant difference in flexibility following t-RA treatment as tested by Kolmogorov-Smirnov tests (n = 250 flexion angle repeats; RSR05, p = 0.11; RSR07, p = 0.83; RSR08, p = 0.69; RSR09, p = 0.89). However, the retinoid responder RSR10 had a significant increase in flexibility following t-RA treatment (n = 250; RSR10, p = 0.043) (Figure 5.11).

Once more, there are differences in results observed by both STEM and AFM analysis, which may be due to different surface chemistries in sample preparation of the two microscopic methods (1.7.4.3) (Sherratt et al., 2004, 2005).
Figure 5.11 Flexion angle of fibrillin microfibrils extracted from photoaged skin treated with t-RA as imaged by AFM.

RSR05, RSR07, RSR08 and RSR09 showed no significant difference in flexion angle following t-RA treatment. However, RSR10 had a significant decrease in flexion angle following t-RA treatment ($n = 250$ flexion angle repeats, $p = 0.043$)
5.6 Periodicity of fibrillin microfibrils was significantly affected by t-RA treatment of photoaged skin in some volunteers.

Periodicity of fibrillin microfibrils from extracted populations was established by measuring bead-to-bead distances (in nanometres). For each microfibril population, 250 periodicities were measured using both STEM and AFM. Results from chapter three revealed that fibrillin microfibril periodicity was both increased and decreased by photoageing. Therefore, it was hypothesised that in those significantly altered by photoageing, a change in periodicity would be observed, returning the microfibril population to one comparable to those extracted from photoprotected skin.

5.6.1 Periodicity of fibrillin microfibrils imaged by STEM was significantly different following t-RA treatment of photoaged skin in four volunteers.

Individual two-sample Kolmogorov-Smirnov tests showed that there was a large variation in response to t-RA with regards to periodicity. In volunteer RSR08, fibrillin microfibrils extracted from photoaged skin showed no difference in periodicity following t-RA treatment (p = 250 periodicity repeats; p = 0.69); RSR07 and RSR10 both had a significant decrease in periodicity following t-RA treatment (n = 250; RSR07, p < 0.0001; RSR10, p < 0.0001), whereas RSR05 and RSR09 had a significant increase in periodicity following t-RA treatment (n = 250; RSR05, p = 0.026; RSR09, p = 0.015) (Figure 5.12).

In all four cases t-RA treatment did not reverse the effects of photoageing with regard to periodicity as measured by STEM (Figure 5.13).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 5.12 Periodicity of fibrillin microfibrils imaged by STEM was significantly affected by t-RA treatment of photoaged skin in four out of five volunteers. Individual Kolmogorov-Smirnov tests showed that only RSR08 did not have a significant change in periodicity; RSR07 and RSR10 both showed a significant decrease in periodicity following t-RA treatment (n = 250 periodicity repeats: RSR07, p < 0.0001; RSR10, p < 0.0001), whereas RSR05 and RSR09 had a significant increase in periodicity following t-RA treatment (n = 250; RSR05, p = 0.026; RSR09, p = 0.015).
Figure 5.13 Periodicity of fibrillin microfibrils imaged by STEM was significantly affected by t-RA treatment of photoaged skin. In four cases, t-RA treatment did not reverse the effects of photoageing with regards to periodicity as measured by STEM.
5.6.2 Periodicity of fibrillin microfibrils imaged by AFM was significantly affected by t-RA treatment of photoaged skin in three volunteers.

Periodicity was also measured from AFM images. Quantification showed that for volunteers RSR05 and RSR08, fibrillin microfibrils had unchanged periodicity following t-RA treatment of photoaged skin (n = 250 periodicity repeats; RSR05, p = 0.052; RSR08, p = 0.69). However, RSR07, RSR09 and RSR10 all showed a significant decrease in periodicity following treatment with t-RA (n = 250 repeats; RSR07, p < 0.0001; RSR09, p = 0.033; RSR10, p < 0.0001) (Figure 5.14).

RSR07 and RSR09 t-RA-treated photoaged fibrillin microfibrils were not significantly different (as tested by Kolmogorov-Smirnov test with Bonferroni correction) to those extracted from photoprotected skin, indicating a reversal of the effects of photoageing (n = 250; RSR07, p = 0.32; RSR09, p = 0.066). Whilst t-RA treated photoaged fibrillin microfibrils from RSR10 had a decreased periodicity as compared to baseline photoaged, they were still significantly different (as tested by Kolmogorov-Smirnov test with Bonferroni correction) from photoprotected fibrillin microfibrils (n = 250; RSR10, p < 0.0001) (Figure 5.15).
Figure 5.14 Periodicity of fibrillin microfibrils imaged by AFM was significantly affected by t-RA treatment of photoaged skin (in some volunteers). RSR05 and RSR08 photoaged fibrillin microfibrils had no significant difference in periodicity following t-RA treatment in AFM images. RSR07, RSR09 and RSR10 fibrillin microfibrils had a significant decrease in periodicity following t-RA treatment (n = 250 periodicity repeats; RSR07, p < 0.0001; RSR09, p = 0.033; RSR10, p < 0.0001).

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Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

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Figure 5.15 Periodicity of fibrillin microfibrils imaged by AFM was significantly affected by t-RA treatment of photoaged skin (in some volunteers). RSR07 t-RA treated photoaged fibrillin microfibrils were not significantly different (as tested by Kolmogorov-Smirnov test) to those extracted from photoprotected skin, indicating a reversal of the effects of photoageing. RSR09 and RSR10 fibrillin microfibrils had a decreased periodicity as compared to baseline photoaged, but they were still significantly different from photoprotected fibrillin microfibrils.
5.7 t-RA treatment of photoaged skin leads to changes in the ultrastructure of some fibrillin microfibrils.

It was hypothesised (H2) that t-RA treatment of photoaged skin of retinoid responders would lead to a population of extracted fibrillin microfibrils which were similar at the ultrastructural level to those extracted from photoprotected skin. Therefore the aim of this chapter was to characterise the ultrastructure of fibrillin microfibrils extracted from volunteers with t-RA-treated photoaged skin, with the untreated photoaged site of the same individual serving as an internal control.

The results of this investigation showed that fibrillin microfibrils were not increased in abundance following t-RA treatment. In terms of ultrastructural analyses, there were no conclusive results for the effect of t-RA treatment on fibrillin microfibrils extracted from photoaged skin; in all results presented, there was a high individual variation in fibrillin microfibrils parameters between volunteers. There was no trend following t-RA-treatment of changes in length (just one from five volunteers had an increase in length). In two of the five volunteers, t-RA treatment induced an increase in average mass per repeat, localised to the outer-bead region. However, in one volunteer this increase appeared excessive compared to photoprotected populations here and in the literature (Kielty et al., 2005). Flexibility was found to increase in two of the five volunteers following t-RA treatment, whilst t-RA-treatment was shown to alter periodicity in four of the five volunteers examined (again, both increases and decreases in this parameter were observed). However, this was only found to be comparable to photoprotected populations (and literature) when analysed by AFM, not STEM, suggesting a role in the interaction with surface chemistry influencing fibrillin microfibril ultrastructure and its subsequent measurement.
Chapter 6

Does short-term topical t-RA treatment of photoprotected, intrinsically aged, skin lead to changes in the ultrastructure of fibrillin microfibrils?
6 Does four-day topical t-RA treatment of photoaged skin lead to changes in the ultrastructure of fibrillin microfibrils?

Chapter five demonstrated how retinoid treatment of photoaged skin can lead to some ultrastructural changes observed in extracted fibrillin microfibrils, but that the effects were highly variable. Whilst photoprotected skin of volunteers in this study was used as a baseline for ultrastructural changes, the average age of the cohort (71.3 years) may suggest that intrinsic ageing has occurred. Therefore, it is not known whether retinoid treatment of the photoprotected (intrinsically aged) skin of these volunteers will result in any ultrastructural changes of fibrillin microfibrils.

Chapter four suggested that, histologically, t-RA treatment had a positive effect on the architecture of the skin, which increases in epidermal thickening, but that since fibrillin microfibril grade was already high in photoprotected baseline skin, there was no significant increase following retinoid treatment.

This chapter examines the ultrastructure of fibrillin microfibrils extracted from retinoid treated photoprotected (intrinsically aged) skin. Biopsies were taken from the photoprotected upper inner arm of 10 volunteers. One biopsy was left untreated (baseline) and another was occluded with 0.025% t-RA. Fibrillin microfibrils were extracted from 5 volunteers (RSR05, 07, 08, 09 and 10) for detailed ultrastructural analyses. Data was assessed according to the results from chapter four which demonstrated how volunteers respond differently to four-days topical application of t-RA; definite retinoid responder, partial retinoid responder or retinoid non-responder (Table 5.1).

It was hypothesised that t-RA treatment of photoprotected skin for four-days would not significantly influence the ultrastructure of extracted fibrillin microfibrils (in terms of length, mass, flexion angle and periodicity) from photoprotected skin, but may have some slight changes.
6.1 Fibrillin microfibrils were successfully extracted from t-RA treated photoprotected (intrinsically aged) skin.

Intact fibrillin microfibril assemblies were successfully extracted from t-RA-treated photoprotected skin (Figure 6.1).

![Image of STEM and AFM images showing fibrillin microfibrils](image)

Figure 6.1 Intact fibrillin microfibrils were successfully extracted from t-RA-treated photoprotected skin. The fibrillin microfibrils appear as the characteristic beads-on-a-string with no noticeable differences between images of the populations. Scale bars = 200 nm.
6.2 Fibrillin microfibril abundance was unaffected by t-RA treatment of photoaged skin in four of five volunteers.

In chapter four, we confirm that topical treatment with t-RA did not lead to increased fibrillin microfibril grade in photoprotected (intrinsically aged) skin, since it was high to start with. However, the initial hypothesis was that abundance would be significantly increased by retinoid treatment.

Individual box-whisker plots highlighted the wide variation in fibrillin microfibril abundance between volunteers. As before fibrillin microfibril abundance was expressed as a ratio to collagen VI abundance.

Student's t-tests showed that one volunteer had a significant decrease in fibrillin microfibril abundance. The partial-responder RSR05 has a significantly lower abundance of fibrillin microfibrils in retinoid treated photoprotected skin (0.16 ± 0.05 SEM) as compared to baseline photoprotected skin (0.54 ± 0.10 SEM) (n = 10 measurements, p = 0.003). However, in four of the five volunteers abundance was unaffected by t-RA treatment (RSR07, p = 0.057; RSR08, p = 0.099; RSR09, p = 0.42 and RSR10, p = 0.14) (Figure 6.2).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 6.2 Fibrillin microfibril abundance was not significantly affected by t-RA treatment in photoprotected skin. Plots for each volunteer showed a wide variation in fibrillin microfibril abundance between volunteers, which was unaffected by t-RA treatment (as tested by Student’s t-tests) in volunteers RSR07, RSR08, RSR09 and RSR10. In RSR05 there was a significant decrease in fibrillin microfibril abundance (n = 10 measurements, p = 0.003)
6.3 Length of fibrillin microfibrils from photoprotected (intrinsically aged) skin was normalised by t-RA treatment.

Chapter five showed that retinoid treatment of photoaged skin did not alter the length of the extracted fibrillin microfibrils. It was therefore hypothesised that length of fibrillin microfibrils from t-RA-treated photoprotected (intrinsically aged) skin would also not be significantly increased.

6.3.1 Length of fibrillin microfibrils from photoprotected (intrinsically aged) skin was normalised by t-RA treatment, as imaged by STEM.

Histogram plots of photoprotected fibrillin microfibrils showed wide individual variation between the volunteers, more specifically that RSR05 and RSR07 had much longer fibrillin microfibrils overall as compared to RSR08, RSR09 and RSR10.

Two-sample Kolmogorov-Smirnov tests showed that in four of the five volunteers, t-RA treatment had a significant normalising effect on the length of fibrillin microfibrils. In RSR05 and RSR07, which had relatively long fibrillin microfibrils in photoprotected skin, t-RA treatment reduced the length. In RSR05 there was a significant reduction in length following treatment (n = 50 microfibrils, p = 0.0003) but whilst there was a reduction in length in RSR07, this was not significant (p = 0.11). In RSR08, RSR09 and RSR10, which had relatively short fibrillin microfibrils, there was a significant increase in length following t-RA treatment of photoprotected skin (n = 50; RSR08, p = 0.0001; RSR09, p < 0.0001; RSR10, p < 0.0001) (Figure 6.3).
Figure 6.3 Individual frequency histograms of lengths of photoprotected (intrinsically aged) and t-RA-treated photoprotected (intrinsically aged) fibrillin microfibrils with the median value for each population. Kolmogorov-Smirnov tests showed that RSR05 had a significant reduction in length ($n = 50$ microfibrils, $p = 0.0003$) and RSR08, RSR09 and RSR10 had significant increases in length (RSR08, $p = 0.0001$; RSR09, $p < 0.0001$; RSR10, $p < 0.0001$). RSR07 did not significantly change following t-RA treatment.
6.3.2 Length of fibrillin microfibrils from photoprotected (intrinsically aged) skin was normalised by t-RA treatment, as imaged by AFM.

Length was also assessed by measurement of fibrillin microfibrils imaged by AFM. Individual histogram plots confirmed the results observed by STEM.

Two-sample Kolmogorov-Smirnov tests showed that in all five volunteers, t-RA treatment had a normalising effect on the length of fibrillin microfibrils. In RSR05 and RSR07, which had relatively long fibrillin microfibrils from photoprotected skin, t-RA treatment significantly reduced length (n = 50 microfibrils; RSR05, p < 0.0001; RSR07, p < 0.0001). In RSR08, RSR09 and RSR10, which had relatively short fibrillin microfibrils, there was a significant increase in length following t-RA treatment (n = 50; RSR08, p = 0.022; RSR09, p < 0.0001; RSR10, p < 0.0001) (Figure 6.4).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 6.4 Individual frequency histograms of lengths (measured by AFM) of photoprotected (intrinsically aged) and t-RA-treated photoprotected (intrinsically aged) fibrillin microfibrils with the median value for each population. Two-sample Kolmogorov-Smirnov tests showed that RSR05 and RSR07 had significantly reduced length following t-RA treatment (n = 50 microfibrils; RSR05, p < 0.0001; RSR07, p < 0.0001). Whilst RSR08, RSR09 and RSR10, had significant increases in length following t-RA treatment (n = 50; RSR08, p = 0.022; RSR09, p < 0.0001; RSR10, p < 0.0001)
6.4 Mass per repeat of fibrillin microfibrils from t-RA treated photoprotected skin was significantly altered in four volunteers.

Previous investigations found that fibrillin microfibrils extracted from photoprotected (intrinsically aged) skin had reduced mass per repeat (Langton et al., 2011), however only one of the five volunteers here had reduced mass in the photoprotected site compared to the other volunteers and literature. Furthermore, chapter five showed that t-RA-treatment had no significant increase in mass of photoaged fibrillin microfibrils. Therefore it was hypothesised that there would be no difference in mass per repeat following t-RA treatment of photoprotected (intrinsically aged) skin.

Individual two-sample Kolmogorov-Smirnov tests showed that in four of the five volunteers there was a significant difference in mass per repeat following retinoid treatment. Both retinoid responders, RSR08 and RSR10 had a significant decrease in mass per repeat following t-RA treatment, as did RSR05 (n = 250 measurements; RSR05, p < 0.0001; RSR08, p = 0.005; RSR10, p < 0.0001). Conversely, RSR09 had a significant increase in mass per repeat following t-RA treatment (n = 250, p < 0.0001). RSR07 had no significant change in mass per repeat following t-RA treatment, but there was a trend towards an increase in mass (n = 250, p = 0.069) (Figure 6.5). In RSR05 there was a very dramatic decrease in mass per repeat from baseline (2593 kDa ± SEM) to t-RA-treated photoprotected skin (1565 kDa ± SEM).
Partial retinoid responders

Retinoid responders

Figure 6.5 Individual frequency histograms for mass per repeat data in each volunteer, along with the mean for each population. Kolmogorov-Smirnov tests showed that RSR05, RSR08 and RSR10 had a significant decrease in mass per repeat (n = 250; RSR05, p < 0.0001; RSR08, p = 0.005; RSR10, p < 0.0001). Conversely, RSR09 had a significant increase in mass per repeat following t-RA treatment (n = 250, p < 0.0001). RSR07 had no significant change in mass per repeat following t-RA treatment, (n = 250, p = 0.069).
6.4.1 t-RA-treated photoprotected (intrinsically aged) fibrillin microfibrils exhibited mass reduction at the centre-bead and mass increase at the bead edge.

Mass map matrices were plotted so as to localise the observed changes in mass per repeat. In RSR05, RSR08 and RSR10 the maximum mass at the centre-bead decreases and the overall size of the bead appears to be smaller following t-RA treatment. In RSR09 fibrillin microfibrils the maximum bead does not increase, instead the bead area and apparent diameter increases following t-RA treatment (Figure 6.6).

Mass difference maps showed that in volunteers RSR05, RSR08 and RSR10 mass was reduced specifically at the centre-bead following t-RA treatment. RSR09 had areas with a relatively large increase in mass at the bead edge which shifted and enlarged the centre-bead region (Figure 6.7).
Figure 6.6 Mass contour maps of average (n=5) photoprotected baseline bead (left) next to average (n=5) t-RA-treated photoprotected bead (right). Kolmogorov-Smirnov tests showed that RSR05, RSR08 and RSR10 had a significant decrease (n = 250; RSR05, p < 0.0001; RSR08, p = 0.005; RSR10, p < 0.0001), RSR09 had a significant increase (n = 250, p < 0.0001) and RSR07 had no significant change in mass per repeat following t-RA treatment. Beads orientated left to right.
Decrease in mass per repeat following t-RA treatment

RSR05
****

RSR08
**

RSR10
****

Increase in mass per repeat following t-RA treatment

RSR09
****

No change in mass per repeat following t-RA treatment

RSR07

Figure 6.7 Difference between photoprotected and t-RA-treated photoprotected average beads. Red depicts a reduction in mass of the region in photoaged as compared to t-RA treated photoaged, green depicts an increase in mass in the area. Arrows indicate regions where there was a relatively large change in mass.
6.5 Flexibility of fibrillin microfibrils was mainly unaffected by t-RA treatment of photoaged skin.

Results from chapter five revealed that retinoid treatment of photoaged skin did not significantly affect flexibility of fibrillin microfibrils in the majority of samples. Therefore it was hypothesised that t-RA treatment of photoprotected (intrinsically aged) skin would also not significantly affect fibrillin microfibril flexibility.

6.5.1 Flexibility of fibrillin microfibrils imaged by STEM was significantly different following t-RA treatment in two volunteers.

Individual two-sample Kolmogorov-Smirnov tests showed that fibrillin microfibrils extracted from t-RA-treated photoprotected skin of volunteers RSR07, RSR08 and RSR10 showed no significant difference in flexibility (n = 250 flexion angle repeats; RSR07, p = 0.61; RSR08, p = 0.83; RSR10, p = 0.16). However, RSR05 had a significant increase (n = 250; p < 0.0001) and RSR09 had a significant decrease (n = 250, p = 0.043) in flexibility of fibrillin microfibrils extracted from t-RA-treated photoprotected skin (Figure 6.8).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 6.8 Flexion angle of fibrillin microfibrils imaged by STEM was significantly affected by t-RA treatment of photoprotected skin (in two of five volunteers). Two-sample Kolmogorov-Smirnov tests showed that RSR07, RSR08 and RSR10 had no significant difference in flexion angle following t-RA treatment. However, RSR05 had a significant decrease (n = 250; p < 0.0001) and RSR09 had a significant increase (n = 250, p = 0.043) in flexion angle of fibrillin microfibrils extracted from t-RA-treated photoprotected skin.
6.5.2 Flexibility of fibrillin microfibrils imaged by AFM was significantly different following t-RA treatment in four volunteers.

Kolmogorov-Smirnov tests performed on distributions showed that RSR08 fibrillin microfibrils from t-RA-treated photoprotected skin had no significant difference in flexibility (n = 250 flexion angle repeats; p = 0.61). However, RSR05, RSR07, RSR09 and RSR10 all had a significant difference in flexibility following t-RA treatment of photoprotected skin. RSR05 and RSR07 both had a significant increase in flexibility (n = 250; RSR05, p = 0.0003; RSR07, p = 0.026), whereas RSR09 and RSR10 had significant decreases in flexibility following t-RA treatment of photoprotected skin (n = 250; RSR09, p < 0.0001; RSR10, p = 0.0062) (Figure 6.9).
Figure 6.9 Flexion angle of fibrillin microfibrils extracted from photoprotected skin treated with t-RA as imaged by AFM. RSR05 and RSR07 both had a significant decrease in flexion angle (n = 250; RSR05, p = 0.0003; RSR07, p = 0.026), whereas RSR09 and RSR10 had significant increases in flexion angle following t-RA treatment (n = 250; RSR09, p < 0.0001; RSR10, p = 0.0062). RSR08 had no significant difference in flexion angle following t-RA treatment.
6.6 Periodicity of photoprotected fibrillin microfibrils was significantly decreased by t-RA treatment.

Results from chapter five revealed that periodicity was both increased and decreased in photoaged skin following t-RA treatment. Here, in photoprotected (intrinsically aged) skin, it was hypothesised that periodicity of fibrillin microfibrils would be unchanged by t-RA treatment.

6.6.1 Periodicity of fibrillin microfibrils imaged by STEM was decreased by t-RA treatment of photoprotected skin in four of five volunteers.

Individual two-sample Kolmogorov-Smirnov tests showed that in volunteers RSR05, RRS07, RSR08 and RSR10, fibrillin microfibrils from t-RA-treated photoprotected skin had decreased periodicity (p = 250 periodicity repeats; RSR05, p < 0.0001; RSR07, p = 0.011; RSR08, p < 0.0001; RSR10, p < 0.0001). Conversely, RSR09 fibrillin microfibrils had a significant increase in periodicity following t-RA treatment of photoprotected skin (n = 250; p < 00001) (Figure 6.10).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 6.10 Periodicity of fibrillin microfibrils imaged by STEM was significantly decreased by t-RA treatment of photoprotected skin in four out of five volunteers. Individual Kolmogorov-Smirnov tests showed that only RSR09 had a significant increase in periodicity ($n = 250$; $p < 0.0001$), whereas RSR05, RRS07, RSR08 and RSR10, fibrillin microfibrils all had decreased periodicity following t-RA treatment ($p = 250$; RSR05, $p < 0.0001$; RSR07, $p = 0.011$; RSR08, $p < 0.0001$; RSR10, $p < 0.0001$).
6.6.2 Periodicity of fibrillin microfibrils imaged by AFM was decreased by t-RA treatment of photoprotected skin in four of five volunteers

Periodicity was also measured from AFM images and showed the same trend as observed in STEM images. Again, fibrillin microfibrils extracted from volunteer RSR09 t-RA-treated photoprotected skin had increased periodicity (n = 250 periodicity repeats; RSR09, p = 0.033) compared to fibrillin microfibrils extracted from volunteers RSR05, RSR07, RSR08 and RSR10 t-RA-treated photoprotected skin which all showed a significant decrease in periodicity (n = 250; RSR05, p < 0.027; RSR07, p < 0.0001; RSR08, p < 0.0001; RSR10, p < 0.0001) (Figure 6.11).
Partial retinoid responders

RSR05

RSR07

RSR09

Retinoid responders

RSR08

RSR10

Figure 6.11 Periodicity of fibrillin microfibrils imaged by AFM was significantly decreased by t-RA treatment of photoprotected skin. RSR09 fibrillin microfibrils had increased periodicity following t-RA treatment (n = 250 periodicity repeats; RSR09, p = 0.033) compared to fibrillin microfibrils extracted from volunteers RSR05, RSR07, RSR08 and RSR10 photoprotected skin which all showed a significant decrease in periodicity following treatment with t-RA (n = 250; RSR05, p < 0.027; RSR07, p < 0.0001; RSR08, p < 0.0001; RSR10, p < 0.0001).
6.7 t-RA treatment of photoprotected skin leads to changes in the ultrastructure of fibrillin microfibrils.

It was hypothesised (H3) that t-RA treatment of photoprotected (intrinsically aged) skin of retinoid responders would lead to a positive effect on fibrillin microfibrils. Fibrillin microfibrils were successfully extracted from photoprotected (intrinsically aged) skin t-RA-treated for four-days, before detailed ultrastructural characterisation. The fibrillin microfibrils were compared to those extracted from untreated photoprotected (intrinsically aged) skin. Length of fibrillin microfibrils following t-RA treatment was shown to be effectively normalised. In the volunteers with longer length fibrillin microfibrils (RSR05 and RSR07), microfibrils were significantly shorter following treatment (before treatment; 15.9 beads ± 2.4 SEM, after treatment; 8.4 beads ± 0.9 SEM) and in volunteers with short fibrillin microfibrils (RSR08, RSR09 and RSR10), longer microfibrils were observed following treatment (before treatment; 5.6 beads ± 0.5 SEM, after treatment; 8.7 beads ± 0.5 SEM). The resulting effect was a global population of fibrillin microfibrils of roughly equal lengths. In three of the five volunteers, the fibrillin microfibril populations following t-RA treatment had a decrease in mass per repeat which was localised to the centre-bead; however, one volunteer had an increase in mass localised to the bead edge. Interestingly, the only volunteer with a low average mass per repeat before treatment showed a significant increase in mass following short-term t-RA treatment. Flexibility appeared not to be significantly affected by retinoid treatment, but the effects as imaged by AFM compared to STEM were different, again highlighting the differences between the two microscopic technique surface chemistries. In STEM-imaged fibrillin microfibrils, only two volunteers had a difference in flexibility following t-RA treatment, whereas in AFM-imaged fibrillin microfibrils, all volunteers exhibited a difference. Periodicity measurements also appeared to be normalised, where, in four of five, there was a significant reduction in periodicity following t-RA treatment, which resulted in a very narrow range population across all volunteers.
Chapter 7

Discussion and future work
Discussion and future work.

This thesis aimed to characterise the architecture, abundance and ultrastructure of fibrillin microfibrils extracted from photoprotected (intrinsically aged) and photoaged skin, and to assess whether t-RA treatment of either intrinsically aged or photoaged skin resulted in the repair of the fibrillin microfibril system at both histological and ultrastructural levels.

There were four results chapters, the first of which (chapter three) characterised the ultrastructure of fibrillin microfibrils extracted from both photoprotected (intrinsically aged) and photoaged skin of otherwise healthy volunteers. The photoprotected (intrinsically aged) site of each volunteer was the internal control for the comparison to the photoaged site. It was hypothesised that:

**H1.** Photoageing of the skin will lead to ultrastructural changes observed in extracted fibrillin microfibrils.

Chapter four addressed which of the volunteers included in the investigation could be categorised as retinoid responders. The experiments then investigated the ultrastructure of fibrillin microfibrils extracted from retinoid-treated photoaged skin (chapter five) and retinoid-treated photoprotected (intrinsically aged) skin (chapter six) of these volunteers. In both cases, the untreated site was the internal control and volunteers were stratified according to their retinoid response as described in chapter four. It was hypothesised that:

**H2.** t-RA treatment of photoaged skin of retinoid responders would have a positive effect on fibrillin microfibril ultrastructure. In partial-responders there would be some positive effects, but no effects in non-responders.

**H3.** t-RA treatment of photoprotected, intrinsically aged skin of retinoid responders would have a positive effect on fibrillin microfibril ultrastructure. There would be some positive effects in partial-responders, but no effects in non-responders.

7.1 Photoageing significantly affected both fibrillin microfibril architecture and ultrastructure in human skin.

These studies demonstrate that - as previously described (Watson et al., 1999) - fibrillin microfibril grade was significantly reduced in photoaged skin adjacent to the DEJ as compared to photoprotected skin. This is a well-known characteristic of
photoaged skin and is often used as a marker for photoageing (Farwick et al., 2007; Watson et al., 2008, 2001a). However, there was also an apparent increase in staining of fibrillin microfibrils in the deeper dermis as previously described (Bernstein et al., 1994; Watson et al., 1999) (figure 7.1). This may either be a result of reorganisation of fibrillin microfibrils due to a loss of structural integrity caused by damage to ultrastructure, or a complete loss of microfibrils adjacent to the DEJ coupled with enhanced deposition and synthesis in the deeper dermis (1.4.2.1). The most likely explanation is that it is a combination of both, since previous research has shown both a reduction (Watson et al., 1999) and an increase (Bernstein et al., 1994) in abundance of fibrillin-1 mRNA in photoaged dermis. The semi-quantitative grading scale was designed to measure fibrillin microfibrils adjacent to the DEJ and does not take into account staining in the deeper dermis (Watson et al., 1999, 2001a).

Fibrillin microfibrils were successfully extracted from photoaged skin. This is despite their loss adjacent to the DEJ, but, as discussed, this does not mean fibrillin microfibrils are reduced throughout the whole dermis. The extraction procedure for fibrillin microfibrils was to digest a full-thickness skin biopsy, which would contain both the upper papillary dermis and the deeper reticular dermis (Kielty et al., 1991). Abundance of extracted fibrillin microfibrils was not significantly altered by photoageing, which could be due to the extraction procedure (7.5), however there was a wide variation of fibrillin microfibril abundance between volunteers.
Focussing on ultrastructural results, this investigation did not find any significant effect on length of fibrillin microfibrils with photoageing. However there was wide individual variation, especially between the longer microfibrils of volunteers RSR05 and RSR07, and the much shorter microfibrils extracted from the biopsies procured from RSR08, RSR09 and RSR10. It is difficult to explain this result, as it may be partly due to genetic differences, lifestyle differences or another external influence. However, age, or any other parameter measured here, did not appear to correlate with length, so perhaps this variation in microfibril length is not an effect of intrinsic ageing directly. Furthermore, previous analyses of intrinsically aged microfibrils showed long structures, with no effect on length, however these observations were on fibrillin microfibrils extracted from photoprotected buttock skin (Sherratt et al., 2006 and unpublished observations (personal communication)).

Results showed that photoaged fibrillin microfibrils had a significantly reduced mass per repeat. Based on in vitro investigations, it was hypothesised that fibrillin microfibril mass would be significantly reduced (Sherratt et al., 2010). It was also found that the loss of mass per repeat in fibrillin microfibrils from photoaged skin was localised to a specific region central to the bead. In the folding arrangement of fibrillin microfibrils, mass is maximal at the centre of the bead (Baldock et al., 2001; Kielty et al., 2005; Sherratt et al., 1997). Hence, although damage to fibrillin microfibrils occurs randomly throughout the repeat, the chance of it occurring in the centre bead is highest. Previous investigations suggested that there could also be a reduction in mass per repeat of intrinsically aged fibrillin microfibrils (Langton et al., 2011; Sherratt et al., 2006). However, there was no additional control for comparison to the photoprotected (intrinsically aged) upper inner arm.

It was also found that photoageing leads to both increases and decreases in periodicity. This variability between volunteers may be due to random damage throughout the entire fibrillin microfibril rather than a consistent area or region which is always damaged. Due to the nature of a folded fibrillin microfibril, damage to individual amino acid residues and subunits could increase or decrease domain flexibility and either cause periodicities to reduce, or increase, but would not be predictable. Finally, there was found to be no change in flexibility of fibrillin microfibrils with photoageing, except in one volunteer. This may be due to individual variability, or it may be due to a combination of effects in photoageing. Previous investigations in vitro have found that MMP-3 selectively degraded fibrillin microfibrils with increased flexibility due to UVA irradiation (Thurstan et al., 2011).
UV irradiation is a known to trigger an increase in the activation of MMPs and neutrophil elastase (Fisher et al., 1999; Muthusamy and Piva, 2010), so perhaps enzyme-mediated degradation following UV irradiation in vivo may play a role in removing damaged fibrillin microfibrils.

To conclude, this investigation confirmed that fibrillin microfibril abundance and architecture adjacent to the DEJ is severely affected by photoageing as examined by IHC. However, it demonstrated for the first time that fibrillin microfibrils extracted from photoaged skin have multiple ultrastructural differences compared to photoprotected intrinsically aged microfibrils. Fibrillin microfibrils are therefore susceptible to remodelling and damage in vivo as well as in vitro, which is likely mediated by a combination of direct UVR absorption by chromophores, ROS-mediated damage and enzyme-mediated remodelling.

However, it is not known how these ultrastructural changes in the fibrillin microfibrils affect their functions within the dermal ECM and there could be multiple consequences. Furthermore, it needs to be addressed whether these ultrastructural changes can be repaired, and the functions of fibrillin microfibrils restored by topical treatment with t-RA.

7.1.1 Potential consequences of ultrastructural changes in photoaged fibrillin microfibrils.

Changes in fibrillin microfibril ultrastructure may affect skin stiffness and compliance (Escoffier et al., 1989; Kielty et al., 2002; Sherratt et al., 2003; Smalls et al., 2006; Takema et al., 1994), as well as tissue homeostasis and regulation of TGF-β, elastic fibre elastogenesis and cell motility and communication (Bax et al., 2003; Chaudhry et al., 2007; Koenders et al., 2009; Mecham, 1991; Pfaff et al., 1996; Robb et al., 1999; Sengle et al., 2008; Trask et al., 1999).

Many examples of functional consequences of ultrastructural changes can be seen in the array of genetic mutations of the fibrillin-1 gene (FBN1) that have been reported. To date there are over 1000 mutations of FBN1 which lead to ultrastructural or functional changes in fibrillin microfibrils. Most mutations lead to Marfan syndrome (MFS), an autosomal dominant condition which can result in skeletal and ocular defects such as abnormally long limbs, lens dislocation (ectopia lentis) and life threatening cardiovascular conditions (Collod-Beroud et al., 2003; Hollister et al., 1989; Kielty et al., 1995). The mutations which cause MFS are highly
variable and spread widely throughout the gene with only a handful being familial recurrent (Collod-Beroud et al., 2003). Most of the mutations that cause MFS change a single amino acid in fibrillin-1 protein. Mutations have been found to disrupt the binding of calcium exposing a cleavage site for MMPs (Hewett et al., 1993; Liu et al., 1996; Reinhardt et al., 2000a) or disrupt the binding of TGF-β increasing its bioavailability (Neptune et al., 2003). Furthermore, fragments of fibrillin-1 may contribute to the development of MFS by upregulating MMPs (Booms et al., 2006). MFS patients also have reduced fibrillin microfibrils in the ECM which are characterised ultrastructurally by increased and irregular periodicities and a frayed appearance (Figure 7.2).

Other mutations within FBN1 can lead instead to a completely different connective tissue disorder, Weill-Marchesani syndrome (WMS), often described as having an almost opposite phenotype to MFS. WMS is characterised by ocular defects, short stature and thickened skin (Faivre et al., 2003; Kutz et al., 2011; Sengle et al., 2012). Dominant deletion mutations have been found to produce fibrillin-1 lacking a region surrounding the proline–rich domain. This leads to ultrastructural changes such as fibrillin microfibril fragmentation and reduced and irregular periodicities (Sengle et al., 2012). Other mutations can lead to ectopia lentis, a disease which affects the fibrillin microfibril-pure ciliary zonules of several species leading to displacement of the eye's lens from its normal location (Balock et al., 2001; Kielty et al., 1995). Fibrillin microfibrils extracted from canine ciliary zonules with this condition show irregular and increased periodicity, leading to weaker structures and increased stiffness (Balock et al., 2001) (Figure 7.2). Other mutations in FBN1 can lead to scleroderma or stiff skin syndrome (SSKS), characterised by hard, thick skin, usually over the entire body, which limits joint mobility and causes flexion contractures and muscle weakness (Fleischmajer et al., 1991b; Loeys et al., 2010; Siracusa et al., 1996). Furthermore, a duplication mutation can lead to a tight skin phenotype in mice (TSK) which is characterised by thickened skin, increased skin stiffness and an accumulation of extracellular matrix. Extracted fibrillin microfibrils have diffuse interbeads, longer periodicity, increased mass and a tendency to aggregate (Kielty et al., 1998; Siracusa et al., 1996) (Figure 7.2).
Figure 7.2 Extracted fibrillin microfibrils from tissues with genetic disorders show profound changes in ultrastructure and hence function. (a) Rotary shadowing electron micrographs of fibrillin microfibrils isolated from dermal fibroblast cell layers of patients with Marfan’s syndrome. The image shows how mutations in FBN1 lead to irregular periodicity of the beads of the fibrillin microfibril as well as a ‘frayed’ appearance (Kielty et al., 1995). (b) Dark field STEM image of a fibrillin microfibril extracted from the ciliary zonule of a canine with ectopia lentis (Baldock et al., 2001). Bead periodicity of the microfibril has increased from the average value of 50-60nm. (c) Rotary shadowing analysis of fibrillin microfibrils from TSK mice skin have diffuse interbead and increased periodicity (arrows) (Kielty et al., 1998). Scale bars = 100nm.

It is unknown why different mutations in the FBN1 gene cause such a variety of disorders, or why genotype is seemingly not correlated to phenotype, since mutations in the same gene can cause opposite effects (shortened bone growth or lengthened bone growth) – this again highlights individual variation (Faivre et al., 2003; Hollister et al., 1989; Kielty et al., 1995; Neptune et al., 2003; Robinson et al., 2006; Sengle et al., 2012; Wirtz et al., 1996). It was suggested that ultrastructural differences in fibrillin microfibrils may be the underlying factor for the observed differences following genetic mutation (Baldock et al., 2001; Kielty et al., 1998; Sengle et al., 2012). However, since many of the mutations lead to abnormal TGFβ signalling, it could be this which leads to the observed phenotypes (Chaudhry et al., 2007; Koenders et al., 2009; Kubiczkova et al., 2012; Neptune et al., 2003). Or perhaps it is the disruption of regions within fibrillin-1 is known to bind to associated proteins - which in turn regulate the bioavailability of TGFβ (Hubmacher and Apte, 2011; Kuo et al., 2007; Kutz et al., 2011; Sengle et al., 2012). Mutations can also lead to disruption of the integrin binding sites (RGD site) of fibrillin-1 deregulating cell adhesion, motility and mobility (Bax et al., 2003; Loeys et al., 2010; Mariko et al., 2010).

Genetic disorders of fibrillin-1 occur by a wide range of mutations within FBN1, which lead a range of unpredictable phenotypes. Fibrillin microfibrils are macromolecular structures that are chromophore-rich throughout their entire molecule, susceptible to non-specific ROS and enzyme-mediated damage, meaning
that there is a potential for damage to occur anywhere within the protein complex (Jensen et al., 2009; Naylor et al., 2010; Reinhardt et al., 2000b; Sherratt et al., 2010; Watson et al., 2013). Therefore, as with genetic mutation which can affect any region of FBN1 and hence fibrillin-1, damage to fibrillin microfibrils via photoageing may not produce a predictable ultrastructural or functional consequence and the effect may be varied depending on the location and severity of damage.

7.2 t-RA treatment of photoaged skin does not significantly alter the ultrastructure of fibrillin microfibrils.

It was hypothesised (H2) that t-RA-treatment of photoaged skin would lead to an extracted population of fibrillin microfibrils which were comparable to those extracted from photoprotected skin, thereby indicating repair or new synthesis of fibrillin microfibrils to repair or replace those lost in photoageing. Results from this investigation showed a wide variation between fibrillin microfibrils extracted from t-RA-treated photoaged skin. However, the wide variations observed at the molecular level did not appear to correlate with variations in histological retinoid response. Fibrillin microfibril abundance was found not to increase following t-RA treatment, despite a significant increase in the IHC staining in three of the five volunteers. This could suggest a repair of existing fibrillin microfibrils, with an addition of immunoreactive fibrillin but not intact microfibrils. Conversely, it could point to new synthesis of fibrillin microfibrils which have been detected by the antibody but are not mature enough to be visible by STEM or AFM. Once again, there was a high amount of variation between volunteers.

Ultrastructurally, whilst there was found to be no change in length of fibrillin microfibrils with t-RA treatment, this does not necessarily mean there is no repair or new synthesis. Newly synthesised fibrillin microfibrils (such as those in developmental maturation) had been found to increase in length over time (Kielty et al., 1993). However, the fact that the fibrillin microfibrils already resident in photoaged skin were short to begin with, could mask any newly synthesised short fibrillin microfibrils following t-RA treatment. Furthermore, the length of treatment (four-days) may not be a long enough time to influence length of fibrillin microfibrils. It is still not clear in the literature how fibrillin microfibrils elongate during microfibril assembly (Kielty et al., 2005).

In three of the five volunteers, mass was not found to be significantly different, but in two volunteers there was an increase in mass per repeat, to different extents,
again raising the issue of individual variation. This may be due to the effect of retinoid on increasing fibrillin-1 mRNA (Watson et al., 2001a) (and hence fibrillin-1 protein for repair) or it may be addition of fragments of fibrillin-1 to each repeat, perhaps by increased transglutaminase activity induced by the action of retinoids (Griffiths et al., 1992c; Ohtake et al., 2006; Ou et al., 2000; Qian and Glanville, 1997). The increase in mass per repeat, in both instances, was localised to the outer bead exterior, perhaps because it is more accessible than the centre-bead.

It was hypothesised that periodicity would be normalised by t-RA treatment, in other words, it would do the opposite of the effect of photoageing and revert the fibrillin microfibrils back to how they were in photoprotected skin. This was the case in AFM-imaged fibrillin microfibrils, but STEM-imaged fibrillin microfibrils, whilst affected by t-RA treatment, were not comparable to photoprotected fibrillin microfibrils of the same volunteer. This once again highlights the differences observed between fibrillin microfibrils imaged by STEM and AFM, and could be due to an underlying interaction with the surface substrate (1.7.4.3) (Sherratt et al., 2004, 2005, 2007).

To conclude, this investigation found no improvement or significant differences in fibrillin microfibrils extracted from t-RA-treated photoaged skin. Perhaps this is due to the short length of the patch-test study; a length in which fibrillin microfibril staining is increased in abundance as observed by IHC, perhaps is not long enough to impact on the complex ultrastructure. Perhaps individual variation in retinoid response could have played a part, with only five volunteers and only two retinoid-responders, it is difficult to put the results in context with a wider population.

7.3 t-RA treatment of photoprotected (intrinsically aged) skin does not significantly alter the ultrastructure of fibrillin microfibrils.

It was hypothesised (H3) that t-RA-treatment of photoprotected (intrinsically aged) skin would lead to the extraction of fibrillin microfibrils comparable to those from photoprotected young skin, indicating a repair or new synthesis of fibrillin microfibrils.

Results from this part of the investigation once again showed a wide individual variation which did not correlate with variations in histological retinoid response highlighting the need for ultrastructural characterisation. Fibrillin microfibril abundance was found not to increase following t-RA treatment, nor was IHC
staining increased in four of the five volunteers. However, in the photoprotected (intrinsically aged) skin, fibrillin microfibril grade was high to start with, therefore it could be argued that there is no need for any retinoid-mediated repair, and since there is no other internal control skin for each volunteer (volunteer is intrinsically aged) then there is no knowing whether the photoprotected (intrinsically aged) upper inner arm site is undamaged (7.5).

In contrast to t-RA-treated photoaged skin, there appeared to be a significant normalisation in lengths of fibrillin microfibrils from t-RA-treated photoprotected (intrinsically aged) skin. This normalisation was also observed in periodicity measurements. The normalisation effect of retinoids has been reported before, such as in t-RA-treatment of psoriatic skin. Usually t-RA-treatment increases the thickness of the epidermis, but in patients with psoriasis, the epidermis of plaque-skin is already very thick. Following treatment the epidermis becomes a thickness equivalent to that of non-plaque skin in the same volunteer (Jean et al., 2011; Reichrath et al., 2007).

Again, in contrast to fibrillin microfibrils from t-RA-treated photoaged skin, in photoprotected t-RA-treated skin the majority of fibrillin microfibril populations had a decrease in mass per repeat which was localised to the centre-bead. It is difficult to explain this result, since a loss of mass (as observed in photoaged fibrillin microfibrils) could suggest damage to fibrillin microfibril ultrastructure. However, it cannot be ruled out that lower mass fibrillin microfibril repeats may be a result of repair to fibrillin microfibrils or indeed, new synthesis of lower mass immature microfibrils as observed in development (Sherratt et al., 1997, 2010).

Finally, fibrillin microfibril flexibility following t-RA treatment was found to both increase and decrease, with the effects varying depending on imaging techniques, highlighting the effects of individual variation and differing surface chemistries in each of the two microscopic techniques (1.7.4.3; 7.4).

Previous investigations utilising immunohistochemical detection have shown that fibrillin microfibrils are only minimally affected by intrinsic ageing compared to photoaged skin (El-Domyati et al., 2002; Langton et al., 2011; Watson et al., 1999), with ultrastructural changes being a decrease in mechanical strength and mass per repeat (Langton et al., 2011; Sherratt et al., 2006). However, the volunteers characterised here had no apparent ultrastructural changes in the photoprotected (intrinsically aged) fibrillin microfibrils when compared to the literature for young,
adult photoprotected microfibrils (Ballock et al., 2001; Kielty et al., 2005; Sherratt et al., 2010; Thurstan et al., 2011). Therefore, there appears to be no need to repair existing, or synthesise new, fibrillin microfibrils which is perhaps why there is no trend throughout the five volunteers, or even the two retinoid-responders alone. Furthermore, perhaps as in photoaged t-RA-treated skin, the patch-test is simply not long enough to observe any major changes in fibrillin microfibril ultrastructure, or that the sample-size is not large enough (especially with only two retinoid-responders).

7.4 Individual variation was found to be high, even in photoprotected skin.

In all results presented here there was a high individual variation in fibrillin microfibrils from volunteer to volunteer, even in baseline photoprotected microfibrils. All volunteers varied in age, sex and clinical photoageing grading, with no two volunteers with the same clinical evaluation, therefore it may be a result of differential effects of intrinsic ageing (unique genotype and lifestyle) in each volunteer leading to increased variability across the cohort. Previous ultrastructural analyses performed on young healthy adult fibrillin microfibrils reported a relatively low amount of variation; between individuals, tissue location and even species (Ballock et al., 2001; Handford et al., 2000; Hubmacher and Reinhardt, 2011; Jensen et al., 2009; Keene et al., 1991; Kielty et al., 2005; Ramirez and Sakai, 2010, 2010; Sherratt et al., 2010). However, it has been known for over a century that individual variation is not only part of our appearance and personality (Harris, 2010), but also an innate part of our genomics, affecting our biochemistry, disease manifestations and response to drugs (Ginsburg and Willard, 2009; Hong and Oh, 2010; Issa, 2002, 2007; Osler, 1903; Teng and Eng, 2012).

Innate individual variation coupled with the macromolecular, long-lived properties of fibrillin microfibrils, along with their susceptibility to an accumulation of damage with time due to intrinsic ageing, photoageing and glycation, leads to an unpredictable phenotype in aged or photoaged individuals (Atanasova et al., 2009; Sherratt et al., 2006; Watson et al., 1999). In order to reveal any trends within a population a large sample size is required, highlighted by the numerous clinical trials which often recruit well over one hundred volunteers (UK Clinical Research Network Study Portfolio; 180 studies on human skin currently in database, 76 recruited below 100 volunteers, 77 recruited between 100 and 1000 volunteers, 27
recruited 1000 or above volunteers). Yet with the onset of ‘personalised medicine’, averaging the population to a trend seems inappropriate, especially when considering potential treatments and interventions (Ginsburg and Willard, 2009; Issa, 2002, 2007; Teng and Eng, 2012). Furthermore, variable responses to drug treatments are also well-known (see Issa 2002 and 2007 for reviews) and variable response to retinoid-treatment has been noted before (Griffiths et al., 1992b; Tsoureli-Nikita et al., 2006; Weiss et al., 1988). In this investigation volunteers were categorised and results were stratified according to retinoid-response where ‘retinoid-responders’ showed increases in both epidermal thickness and fibrillin microfibril deposition, ‘partial responders’ showed either an increase in epidermal thickness or fibrillin microfibril deposition and ‘non-responders’ showed no increase in either epidermal thickness or fibrillin microfibril deposition (chapter four). It was hypothesised that 15-20% of volunteers treated with t-RA would not respond. Here there were no non-responders, but only two full responders. However, the method of stratifying volunteers into one of three categories is crude since there can be many degrees of response due to individual variation. It was therefore difficult to put any results into context since the sample size of retinoid responders was so small. In future investigations it may be wise to have a method of elucidating if a volunteer is a responder before inclusion in the study and full analysis.

As well as inter-individual variation (between volunteers) in skin there is also high intra-individual variation between anatomical sites (Bailey et al., 2012; Craven et al., 1997; Cua et al., 1990; El-Domyati et al., 2002; Farage et al., 2007, 2008; Greene et al., 1970; Igarashi et al., 2007; Kadoya et al., 2005; Talwar et al., 1995; Waller and Maibach, 2005; Watson et al., 1999). For example, in young photoprotected skin there is a difference in skin elasticity of upper inner arm skin compared to dorsal forearm skin and multiple other anatomical sites (Cua et al., 1990; Smalls et al., 2006). Furthermore, in studies on the photoageing of skin, often buttock or hip (photoprotected) skin is used as a control for dorsal forearm (photoaged) skin with upper inner arm (photoprotected) skin as an anatomical site control. In these studies, upper inner arm skin is often regarded as an intermediate for the staining of fibrillin microfibrils (Watson et al., 1999), procollagens I and III (Talwar et al., 1995) and collagen VII (Craven et al., 1997). Perhaps this is due to anatomical site variation intra-individual, or perhaps a result of the effects of photoageing on the upper inner arm. Mild effects of photoageing may further explain the wide inter-
individual variations observed in the photoprotected (intrinsically aged) upper inner arm site controls.

7.5 Strengths and limitations of the procedure for extracting and characterising fibrillin microfibrils.

Previous investigations utilised the method of immunohistochemical detection of fibrillin microfibrils in skin sections. The need for ultrastructural analyses of extracted fibrillin microfibrils is necessary to detect changes that would otherwise be undetectable using IHC. For example, in WMS, IHC showed no discernible differences in abundance or architecture of fibrillin microfibrils compared to disease-free skin, but differences were obvious at the ultrastructural level when imaged by electron microscopy (Sengle et al., 2012). Ultrastructural analyses were also important to address the effects of t-RA on fibrillin microfibrils. Although previous studies showed increases in staining of fibrillin microfibrils following treatment, ultrastructural analyses could elucidate the mechanisms of action of t-RA.

Whilst this investigation employed bacterial collagenase IA digestion of a full thickness skin biopsy as previously published (Kielty et al., 1991), there are other methods of fibrillin microfibril extraction which could have been used. Incubation with another type of bacterial collagenase could have been used (such as type VII collagenase (Cain et al., 2006)), or tissue homogenisation followed by 6 M guanidine hydrochloride (GuHCl) denaturation and centrifugation steps (Gibson et al., 1989; Keene et al., 1991; Kuo et al., 2007). The different methods are known to differently influence the ultrastructure that is observed and fibrillin microfibrils incubated with GuHCl were found to have increased flexibility and were more extendable compared to collagenase-extracted ones (Kielty and Shuttleworth, 1997; Thurmond and Trotter, 1996). Furthermore, mass spectrometry analysis of trypsin-digested fibrillin microfibrils revealed that GuHCl-extracted microfibrils were not found associated with MAGP-1, whereas collagenase-extracted microfibrils were (Cain et al., 2006). This, along with a higher yield of fibrillin microfibrils extracted using bacterial collagenase (Kuo et al, 2007) was why the bacterial collagenase extraction method was chosen. However, one investigation reported that bacterial collagenase digestion resulted in fibrillin-1 cleavage and loss of specific IHC epitopes (Kuo et al., 2007). Therefore, it cannot be discounted that the current extraction procedure may have affected fibrillin microfibril ultrastructure and it may be useful for future
investigations to look at the effects of intrinsic ageing and photoageing on GuHCl-
extracted microfibrils.

The fibrillin microfibril extraction procedure may also have been affected by
increased cross-linking with age and photoageing severity, due to the inability to
extract heavily cross-linked complexes. Both enzymatic [lysyl oxidase (LOX) or lysyl
oxidase-like (LOXL)] and non-enzymatic [glycation (Atanasova et al., 2009;
Gkogkolou and Bohm, 2012; Verzijl et al., 2000)] cross-links are correlated with age
and photoageing and could have increased fibrillin microfibril crosslinking in these
tissues (Chen et al., 2000; Ichihashi et al., 2011; Langton et al., 2012a, 2012c, 2013;
Yamauchi et al., 1988). Furthermore, retinoid treatment is known to increase the
activity of tissue transglutaminases (Griffiths et al., 1992c; Ohtake et al., 2006, 2008;
Ou et al., 2000; Rosenthal et al., 1992) which are known to cross-link fibrillin
microfibrils during maturation and assembly (Kojima et al., 1993; Qian and
Glanville, 1997). Increased cross-links by any mechanism could lead to a population
of fibrillin microfibrils not fully representative of those resident in the tissue. Cross-
linking may be linked to the short lengths of fibrillin microfibrils observed or to
results observed in retinoid-treated tissue extracts. Future studies may wish to
employ a dual step extraction process involving collagenase digest and
centrifugation to extract non-cross-linked fibrillin microfibrils (as was employed
here), followed by further digestion of the leftover pellet from the first stage by the
GuHCl to break cross-links and extract any more microfibrils which may be
particularly bound within the ECM.

7.6 Future work.

Future work may build on the findings reported here and could include functional
studies on fibrillin microfibrils, a longer patch test and a larger sample size of
volunteers. Future studies may wish to address the potential question of the upper
inner arm as a photoaged intermediate between photoaged forearm and
photoprotected buttock skin by acquiring biopsies from all three sites. This would
eliminate any potential doubts of photoageing occurring in the upper inner arm.

Abundance measurements by STEM was normalised to the abundance of fibrillin
microfibrils to collagen VI, which is known to remain unchanged in photoaged skin
(Watson et al., 2001b) but has yet to be examined in retinoid-treated skin. Future
investigations could either examine the immunohistochemical abundance of
collagen VI in retinoid-treated skin (as a complementary study to Watson et al., 2001b), or they could implement a total protein extraction and polyacrylamide gel electrophoresis (PAGE) and western blot. If collagen VI is significantly affected by retinoid treatment, other normalisation methods could be utilised, such as normalising using the ÅKTA absorbance spectra to calculate total protein content of each biopsy.

7.6.1 Volunteer recruitment and ethics application

This investigation applied for ethics to recruit ten volunteers with inclusion and exclusion criteria listed in 2.1.1. Volunteers were not required to answer any lifestyle questions and were not known tested for retinoid-response before recruitment. Future studies would greatly benefit from a lifestyle questionnaire which includes collecting information like use of sunbeds, sunbathing habits, use of sunscreen (including sun protection factor and frequency of reapplication), cigarette smoking habits, use of dietary supplements and comments on diet in general (high in carotenoids/vitamin A?). It is not known whether the volunteers in this study were smokers, sunbed users or had diets high in vitamin A, which could have biased results and lead to variation. Specific skin type information was also not collected, although it is likely that volunteers here were Fitzpatrick types I to III (Fitzpatrick, 1988); future studies would be wise to collect this information. Furthermore, this investigation had no knowledge of whether a volunteer was a retinoid responder before biopsy and IHC staining. Perhaps a future study could address whether there are any clinical manifestations of retinoid response, or non-invasive genetic profiling which could help to identify a retinoid-responder perhaps based on single nucleotide polymorphisms in retinoid receptor genes.

Future studies could also look to standardise recruitment and biopsy methods, such as recruiting only one gender to remove any hormonal differences and variation and to recruit volunteers of a more similar age (perhaps younger but still photoaged to remove any influences from intrinsic ageing). Shaving of the biopsy site before patches are applied also needs to be standardised one way or another, since shaving of the skin can lead to inflammation (increase in pro-inflammatory cytokines), erythema, irritation, loss of ‘squame-rafts’ (areas of stratum corneum which are removed by the mechanical force) and reduced epidermal barrier function (Evans et al., 2012; Marti et al., 2003; Turner et al., 2007); which could influence fibrillin microfibrils or the effectiveness of t-RA.
7.6.2 t-RA treatment of skin for a longer patch test.

It has been well documented that whilst fibrillin microfibril staining at the DEJ may be increased in a four-day t-RA patch test, there are no other dermal ECM proteins known to respond this quickly. However, in longer patch tests (twelve-days), or a prolonged clinical study of weeks to months, there are observed improvements in a large array of ECM proteins (Bhawan et al., 1996; El-Domyati et al., 2004; Griffiths et al., 1993b; Kligman et al., 1993; Varani et al., 1994; Watson et al., 2001a; Weiss et al., 1988; Woodley et al., 1990a), indicating that some mechanisms of t-RA can take longer to act than others. Furthermore, investigations on fibrillin microfibrils in development and wound healing suggest that whilst they may be visible from an early stage, their development into a mature microfibril or network can take months or even years (Kielty et al., 1993; Raghunath et al., 1996). This suggests that the four-day patch test employed here was perhaps not a long enough treatment period to observe full ultrastructural repair or assembly of fibrillin microfibrils into mature, functional complexes. Future investigations could employ either a long-term patch test of twelve-days, with t-RA cream re-applied every four-days; this method is often used in occluded patches of over-the-counter ‘anti-ageing’ products (Watson et al., 2008). Or perhaps a much longer term clinical study of daily t-RA cream application at a lower concentration - which has been done, but future studies could include fibrillin microfibril extraction (Watson et al., 2001a). It may be even more useful to take multiple biopsies at regular time points to assess whether any time-dependent maturation or repair occurs. Furthermore, inter-individual variation and retinoid-response may be the reason for the delay in observations of ultrastructural improvements, and future investigations could first examine individual retinoid-response before inclusion into the study. Either this, or a larger sample size should be used (such as in clinical trials) so as to reduce the influence of non-responders on the results.

7.6.3 Investigating changes could occur in fibrillin microfibrils.

This investigation found that fibrillin microfibrils extracted from photoaged skin had a reduced mass per repeat. However, it is not known what was lost from the repeat. It may be small fragments of fibrillin-1 caused by breakage of peptide bonds within the protein, or it may be dissociation of microfibril associated proteins which are no longer able to cross-link to the microfibril. To address this, future studies could use gold-conjugated secondary antibodies and STEM or TEM analysis to
identify potential areas of damage. Using primary antibodies specific against, for example, MAGP-1 or LTBP5, followed by a gold-conjugated secondary antibody could pinpoint locations within the repeat where associated proteins bind (Henderson et al., 1996; Isogai et al., 2003). Following photoageing or retinoid treatment the same experiment may show differences in binding sites, or a lack of binding indicating a loss of the associated protein. Fibrillin-1 epitope mapping could also help to elucidate how changes in periodicity occur with photoageing, and if t-RA treatment effects fibrillin microfibril folding and assembly (Baldock et al., 2001).

7.6.4 Functional studies on fibrillin microfibrils.

This investigation was able to successfully characterise the ultrastructure and immunohistochemical distribution of fibrillin microfibrils from photoaged skin and retinoid treated skin. However, it is still not known how these structural changes affect the functions of fibrillin microfibrils in the dermal ECM.

The technique of molecular combing was used in this investigation to examine the mechanical strength of fibrillin microfibrils both before and after photoageing and following retinoid treatment. However the short length of the fibrillin microfibrils meant that this method was not suitable. Therefore, in order to test mechanical strength in future investigations several other methods may be explored such as force spectroscopy and lateral force spectroscopy (Carrion-Vazquez et al., 2000; Clausen-Schaumann et al., 2000; Liu et al., 2005; Rief et al., 1997). Furthermore, force spectroscopy could be useful in determining the folding arrangement of an intact fibrillin microfibril due to the varying strength of molecular interactions within the microfibril (Clausen-Schaumann et al., 2000; Evans, 1998).

Fibrillin microfibrils also function in tissue homeostasis via cellular binding, motility and communication via integrin binding sites. Previous investigations have examined cell attachment and spreading on extracted fibrillin microfibrils adsorbed to glass coverslips (Bax et al., 2003). Human dermal fibroblasts were able to attach and spread on fibrillin microfibrils extracted from foetal calf aortas, as well as fibrillin-1 recombinant peptides either including or excluding the RGD site (Bax et al., 2003). If photoageing or t-RA treatment disrupted the RGD site or any other region required for cell attachment and spreading, an assay like this would be able to identify it.
Fibrillin microfibrils also play an important role in controlling the bioavailability of TGF-β. Disruptions to fibrillin microfibril structure caused by photoageing or t-RA treatment may enhance the abundance of TGF-β or downstream signalling molecules associated with enhanced TGF-β activity. For example, the signalling molecule SMAD2 is phosphorylated due to TGF-β activity (pSMAD2) and can be detected by either polyacrylamide gel electrophoresis (PAGE) and Western blots (Sengle et al., 2012) or IHC staining (Loeys et al., 2010). TGF-β activity can also lead to expression of α-smooth muscle actin (α-SMA) by myofibroblast infiltrates which can be detected by IHC (Loeys et al., 2010; Neptune et al., 2003; Sengle et al., 2012) (Figure 7.3). Furthermore, TGF-β can also be detected directly via IHC (Koenders et al., 2009; Sengle et al., 2012) and via suction blister fluid (Leivo et al., 2000) and enzyme-linked immunosorbent assays (ELISA) (Sengle et al., 2012).

Figure 7.3 IHC detection of TGF-β signalling. 
(A,B) Control skin stained for aSMA (upregulated by enhanced TGF-β signalling), (C,D) the same stain in stiff skin syndrome patients shows increased staining. (E,F) control skin stained for pSMAD2 (upregulated by enhanced TGF-β signalling), (G,H) the same stain in stiff skin syndrome patients shows increased staining. 
Images from (Loeys et al., 2010). Scale bars; (A,C) 50 µm, (B, D) 20 µm, (E,F,G,H) 100 µm.
7.6.5 Other potential future work.

Other research projects could investigate the effects of t-RA on cell cultures or isolated cell-matrix *in vitro*. Human keratinocytes (KCs) or human dermal fibroblasts (HDFs) in monolayers in culture media are known to manufacture intact fibrillin microfibrils (Baldock et al., 2001; Haynes et al., 1997). *t*-RA could be added to the liquid cell media before fibrillin microfibril extraction and purification. The most logical would be to use a KC and HDF co-culture, or a 3D skin equivalent, as this would serve as a more skin-like model.

7.7 Final conclusions.

To conclude, this investigation demonstrated for the first time that fibrillin microfibrils are susceptible to photoageing-related remodelling and damage *in vivo*. This is likely mediated by a combination of direct UVR absorption by chromophores, ROS-mediated damage and enzyme-mediated remodelling. The consequences of these ultrastructural alterations is currently unknown, but may include changes in skin elasticity and recoil, dysregulation of TGF-β activity, alterations in elastic fibre formation and reduced cell motility and communication. The investigation then addressed if topical *t*-RA treatment could repair (or induce new synthesis of) fibrillin microfibrils in photoaged skin and intrinsically aged (photoprotected) skin. *t*-RA was found not to significantly alter the ultrastructure of fibrillin microfibrils, perhaps due to the short length of the patch-test study, the complex macromolecular nature of fibrillin microfibrils, or individual variation in retinoid response. Future investigations have been suggested which could build upon the results presented here: Firstly, to examine the functional consequences of ultrastructural damage of fibrillin microfibrils in photoageing; secondly, to continue investigations of the effect of *t*-RA on repair (or new synthesis) in a larger, and longer, study.
Chapter 8
Appendix
8 Appendix.

8.1 Fibrillin microfibrils measured by NanoScope™ Multimode™ and JPK® NanoWizard® 3 are comparable.

In order to be confident that fibrillin microfibrils imaged and analysed by the two different AFM instruments employed here, 500 periodicity measurements were made on the same sample (RSR05 photoprotected upper inner arm) by both microscopes. Results confirmed that there was no significant difference between the periodicity measurements using the two microscopes (two sample Kolmogorov-Smirnov test, n = 500, p = 0.17) (Figure 8.1). Therefore, measurements taken on the two microscopes could be pooled for analysis and statistical testing.

Flexibility of fibrillin microfibrils was also found to be comparable between the two AFM microscopes as tested by a Kolmogorov-Smirnov test (n = 500, p = 0.49) (Figure 8.2).
There were no significant differences between flexion angle measurements on images captured on either the JPK NanoWizard or the NanoScope Multimode. As tested by Kolmogorov-Smirnov test, $p = 0.49$.

8.2 The ability to measure fibrillin microfibril extension by molecular combing was affected by their short length.

Periodicity of combed fibrillin microfibrils was measured by calculating bead-to-bead distance in nanometres. For each microfibril population (combed and control), 250 periodicities were measured using the AFM. There was no increase in periodicity in any of the combed samples when compared to control samples of the same site (Figure 8.3 and Figure 8.4).
Figure 8.3 Molecular combing data for fibrillin microfibrils extracted from photoprotected (upper inner arm) skin. There was no significant increase in periodicity in any of the volunteers. It was suspected that this was due to the length of fibrillin microfibrils in these volunteers. Short fibrillin microfibrils would not be successfully extended by molecular combing.
There was no significant increase in periodicity in any of the volunteers. It was suspected that this was due to the length of fibrillin microfibrils in these volunteers. Short fibrillin microfibrils would not be successfully extended by molecular combing.
Literature cited
Literature cited


Elizabeth Naylor PhD Thesis: Literature cited


