The Nuclear Hormone Receptor,

Liver X Receptor β, In Skin Ageing

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Contents

Abstract 11

Declaration 11

Copyright statement 12

Acknowledgements and dedication 13

The author 14

List of abbreviations.....15

1. Introduction 19

1.1 Ageing.....19

1.2 Evolutionary theories of ageing.....21

1.3 The free radical theory of ageing.....23

1.4 Stochastic non-enzymatic protein modification and ageing.....25

1.5 Autophagy and ageing 28

1.6 Telomeres and ageing 31

1.7 Progeroid conditions 32

1.8 Endocrine aspects of ageing 36

1.9 Calorie restriction (CR) 38

1.10 Common biology of long-lived humans 42

1.11 Skin anatomy 44

1.12 Skin ageing.....48

1.13 Ageing in the epidermis 49

1.14 Dermal collagen networks in skin ageing.....52

1.15 Dermal elastic fibres in skin ageing.....53

1.16 Nuclear hormone receptors **56**

1.17 Liver X receptors **58**

1.18 Retinoids.....**62**

1.19 NHR expression in ageing.....**64**

1.20 Project hypotheses **65**

1.21 Statement of hypotheses **66**

2. Methods and materials **67**

2.1 Volunteer recruitment **67**

2.1.1 Skin biopsy procedure.....**68**

2.2 Tissue processing **71**

2.2.1 Tissue processing for *in situ* hybridisation, immunohistochemistry and immunofluorescence **71**

2.2.2 Tissue processing for qPCR.....**71**

2.2.3 Tissue processing for Affymetrix® microarrays **72**

2.3 Cell culture.....**72**

2.3.1 Thawing frozen cells to culture.....**72**

2.3.2 Maintenance of cell cultures **73**

2.3.3 Freezing cells for long term storage **73**

2.4 Fixing cells and tissues **73**

2.5 Immunohistochemistry **74**

2.5.1 Immunohistochemistry in frozen sections of human skin **75**

2.6 Immunofluorescence **75**

2.6.1 Immunofluorescence in frozen sections of human skin **76**

2.7 Protein extraction **76**

2.7.1 Formulation of protein extraction buffer.....**77**

2.7.2 Protein extraction from cells **77**

2.8 Protein quantification **77**

2.8.1 Quantification of protein extracts using a relative standard curve **78**

2.9 Western blot **79**

2.9.1 Separation of proteins by gel electrophoresis **79**

2.9.2 Detection of bound antibody during Western blot **79**

2.9.3 Western blot for LXRβ in soluble protein extracts from human skin **81**

2.10 Oligonucleotide design **82**

2.10.1 Oligonucleotide probe design for *in situ* hybridisation **82**

2.10.2 Physical and chemical considerations of *in situ* hybridisation probes **83**

2.10.3 Use of commercially designed probe sets for qPCR **84**

2.11 Working with RNA **85**

2.12 RNA extraction **85**

2.12.1 Extraction of total RNA **86**

2.13 Quantitative polymerase chain reaction (qPCR) **87**

2.13.1 qPCR to determine relative LXRβ mRNA expression levels in human skin **.... 88**

2.14 *In situ* hybridisation **88**

2.14.1 Pretreatment **89**

2.14.2 Hybridisation **89**

2.14.3 Stringency washes and detection **89**

2.14.4 Controls **90**

2.14.5 Pretreatment of frozen skin sections to fix RNA **91**

2.14.6 Oligonucleotide probe biotinylation **91**

2.14.7 Hybridisation of oligonucleotide probe to RNA in frozen skin sections **91**

2.14.8 Stringency washes **92**

2.14.9 Detection of bound probe following hybridisation in frozen skin sections....**92**

2.14.10 Quantification of *in situ* hybridisation staining in frozen skin sections **92**

2.14.11 Quantification of epidermal thickness in frozen skin sections.....**92**

2.15 Affymetrix® microarray analysis **93**

2.16 Comparison of Affymetrix® microarray datasets **93**

3. Development and validation of methodologies.....**95**

3.1 *In silico* analysis of *in situ* hybridisation oligonucleotide probes specificity.....**95**

3.2 Verification of oligonucleotide biotinylation for *in situ* hybridisation **95**

3.3 LXRβ mRNA is expressed in human skin **95**

3.4 Confirmation of LXRβ antibody specificity by Western blot. **99**

3.5 LXRβ protein is expressed in human skin.....**99**

3.6 Epidermal fluorescence scores obtained by image analysis and by manual estimation are highly correlated. **103**

4. Liver X receptor β in intrinsically aged skin.....**105**

4.1 Epidermal thickness in young and aged photoprotected hip skin **105**

4.2 Quantitative polymerase chain reaction (qPCR) shows that LXRβ mRNA is stable in young and intrinsically aged human skin **105**

4.3 *In situ* hybridisation shows that LXRβ mRNA expression is stable between young and aged photoprotected human skin **105**

4.4 Immunofluorescent staining for LXRβ in young and aged photoprotected skin **111**

4.5 LXRβ protein expression does not significantly differ between young and aged human skin **111**

4.6 Comparison of Affymetrix® microarray datasets shows overlapping gene regulation between an LXR agonist diet and i) ageing; ii) CR in mice **114**

5. Liver X receptor β expression in photoaged skin **116**

5.1 Histological assessment of photoageing **116**

5.2 Quantitative polymerase chain reaction (qPCR) shows that LXRβ mRNA is stable in photoprotected and photoaged human skin **116**

5.3 *In situ* hybridisation shows that LXRβ mRNA is stable in photoprotected and photoaged human skin **120**

5.4 Epidermal thickness in photoprotected hip, upper inner arm and photoaged forearm **120**

5.5 Immunofluorescent staining for LXRβ protein in photoprotected and photoaged human skin **125**

5.6 LXRβ protein expression is stable in photoprotected and photoaged human skin **125**

5.7 LXRβ expression varies significantly between individuals **125**

5.8 LXRβ expression in retinoid-treated photoaged forearm skin.....**129**

5.9 Histological assessment of retinoid repair **129**

5.10 Epidermal thickness in baseline and retinoid-treated photoaged forearm **129**

5.11 Quantitative polymerase chain reaction (qPCR) shows that LXRβ mRNA is stable in baseline and retinoid-treated human skin **130**

5.12 *In situ* hybridisation shows that LXRβ mRNA is stable in baseline and retinoid-treated human skin.....**130**

5.13 LXRβ protein expression does not significantly differ between baseline and retinoid-treated photoaged skin **130**

6. Discussion **139**

Bibliography.....**150**

Appendix 1. Microarray comparisons shared genes: LXR agonists vs i) ageing and ii) calorie restriction.....**189**

Appendix 2. Publications arising from this thesis **199**

List of figures

Figure 1-1. Death rates in the UK by decade of life. **19**

Figure 1-2. Schematic of the biomolecular damage hypotheses of ageing. **27**

Figure 1-3. Diagram of skin anatomy **45**

Figure 1-4. Clinical photograph to illustrate photoageing.....**50**

Figure 1-5. Schematic of potential interactions between LXR signalling and ageing. **61**

Figure 2-1. Illustration of the skin biopsy procedure. **69**

Figure 2-2. Illustration of the skin biopsy procedure, second part. **70**

Figure 2-3. Example BCA assay standard curve. **78**

Figure 2-4. Diagram of enzyme-catalysed chemiluminescence.....80

Figure 3-1. Graphical output from BLASTn search algorithm showing antisense probe complementarity with NCBI database RNA sequences.....**96**

Figure 3-2. Graphical output from BLASTn search algorithm showing sense probe complementarity with NCBI database RNA sequences.....**97**

Figure 3-3. Dot blot to verify the successful biotinylation of the oligonucleotide probes used for in situ hybridisation experiments.....**98**

Figure 3-4. Western blot for LXRβ in fibroblastic (COS-1) and keratinocytic (HaCaT) cell line protein extracts.....**100**

Figure 3-5. Immunohistochemistry for LXRβ in 10 µm sections of frozen human skin. **101**

Figure 3-6. Immunofluorescence for LXRβ in 10 µm sections of frozen human skin. **102**

Figure 3-7. Regression analysis comparing manual and automated methods of scoring LXRβ fluorescence.....**104**

Figure 4-1. Box and whisker plot of epidermal thickness data for the intrinsic ageing in situ hybridisation cohort. **106**

Figure 4-2. Box and whisker plots showing intrinsic ageing qPCR data **107**

Figure 4-3. In situ hybridisation for LXRβ mRNA in photoprotected human skin from young (18-30 years; a) and aged (70-75 years; b) volunteers. **109**

Figure 4-4. Box and whisker plot showing LXRβ mRNA^{+ve} cells (a) per high power microscope field and (b) normalised for epidermal thickness **110**

Figure 4-5. Immunofluorescence for LXRβ protein in photoprotected human buttock skin sections from young (a; 18-30 years) and aged (b; 70-75 years) volunteers. ..**112**

Figure 4-6. Box and whisker plots of LXRβ protein expression data derived from immunofluorescence staining. **113**

Figure 5-1. Immunohistochemistry for fibrillin-1 in human skin cryosections.....**117**

Figure 5-2. Box and whisker plots showing qPCR data (a) for the ratio of LXRβ to β-actin mRNA expression in photoprotected hip, upper inner arm and photoaged forearm. **118**

Figure 5-3. In situ hybridisation for LXRβ mRNA.....**121**

Figure 5-4. Box and whisker plot showing epidermal thickness data for the photoageing in situ hybridisation study cohort. **122**

Figure 5-5. Box and whisker plots showing LXRβ mRNA^{+ve} cell counts.....**123**

Figure 5-6. Immunofluorescent staining for LXRβ protein in three anatomical sites **126**

Figure 5-7. Box and whisker plot of epidermal LXRβ protein expression data derived from immunofluorescence staining. **127**

Figure 5-8. Individual plots of LXRβ expression in epidermis of skin from three anatomical sites; photoprotected hip, photoprotected upper inner arm and photoaged extensor forearm. **128**

Figure 5-9. Fibrillin-1 staining in baseline (a) and 4 day retinoid-treated (b) photoaged forearm skin sections. **131**

Figure 5-10. Box and whisker plots showing epidermal thickness data for retinoid-treated photoaged in situ hybridisation cohort. **132**

Figure 5-11. Box and whisker plots showing LXRβ to β-actin qPCR gene expression ratios **133**

Figure 5-12. In situ hybridisation for LXRβ mRNA **134**

Figure 5-13. Box and whisker plots showing LXRβ mRNA^{+ve} cell counts **135**

Figure 5-14. Immunofluorescent staining for LXRβ protein in untreated (a) and retinoid-treated (b) photoaged forearm skin. **137**

Figure 5-15. Box and whisker plots of epidermal LXRβ immunofluorescence data in baseline and retinoid-treated photoaged forearm skin. **138**

List of tables

Table 1-1. Details of interventions reported to increase maximum lifespan in mammals. **43**

Table 4-1. Statistical analysis of logarithm-transformed qPCR data for LXRβ to β-actin mRNA expression ratios in photoprotected skin from young (18-30 years old) and aged (65-75 years old) volunteers. **108**

Table 4-2. Summary of comparisons between Affymetrix® microarray gene expression datasets from LXR agonist treatment, ageing and CR. **115**

Table 5-1. General linear model comparison of LXRβ:β-actin mRNA expression ratios quantified by qPCR in photoprotected hip, upper inner arm and photoaged forearm. **119**

Table 5-2. RM-ANOVA with Bonferroni multiple comparisons post-hoc test of logarithm-transformed normalised LXRβ mRNA^{+ve} cell counts between photoprotected hip, upper inner arm (UIA) and photoaged forearm (FA). **124**

Table 5-3. SPSS output for Student's paired two-tailed t-test comparison of logarithm-transformed normalised LXRβ mRNA^{+ve} cell counts between baseline and retinoid-treated photoaged forearm skin.....**136**

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Abstract

This thesis, “The Nuclear Hormone Receptor, Liver X Receptor β, In Skin Ageing” is submitted to The University of Manchester by Christopher Thomas Ford for the degree of Doctor of Philosophy (PhD) in the year of 2010.

The nuclear hormone receptor (NHR) liver X receptor β (LXRβ) has been highlighted as a possible candidate for involvement in ageing by several recent findings. LXRβ is the closest human homologue to the longevity-associated gene *daf-12* in the nematode worm and LXRα haplotypes have been associated with longevity at old ages in a longitudinal human genetic study. Whilst LXRα is primarily responsible for mediating the effects of LXR oxysterol ligands throughout most of the body, LXRβ is the primary mediator of these effects uniquely in the skin. In this thesis studies are presented on the expression of LXRβ mRNA and protein in human skin, comparing young vs intrinsically (chronologically) aged skin, photoprotected vs photoaged (due to ultraviolet radiation exposure) skin and untreated vs retinoid-treated photoaged skin, retinoid treatment being a primary clinical intervention for photoageing. *In situ* hybridisation and quantitative polymerase chain reaction (qPCR) were used to identify LXRβ mRNA and immunofluorescence was used to identify LXRβ protein. These comparisons revealed that both the mRNA and protein expression of LXRβ are highly stable throughout the ageing, photoageing and retinoid treatment of human skin. Previous authors have identified overlap between microarray gene expression datasets in the LXRβ$^{-/-}$ mouse and in normal human skin ageing. In these studies comparisons of different microarray datasets have been conducted with the finding that LXR agonist treatment of mice produces gene regulation patterns with significant overlap to that seen in both ageing and calorie restriction in mice (binomial test; $p<0.001$). Furthermore, when considering the genes commonly regulated in LXR agonist treatment and ageing, 73% of these genes are regulated in opposite directions. Conversely, when considering genes commonly regulated in LXR agonist treatment and calorie restriction, 70% of these genes are regulated in the same direction. These findings suggest that LXR agonists have possible benefit as ageing therapies, perhaps due to stimulating a calorie restriction-like response. Further work would be necessary to confirm these properties of LXR agonists and to define the roles of LXRβ in the ageing and normal function of human skin.

Declaration

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Acknowledgements and dedication

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List of abbreviations

9-*cis*-RA	9-*cis*-retinoic acid
A	Adenosine
ABCG	ATP-binding cassette G
AGE	Advanced glycation end-product
AMPK	Adenosine monophosphate-activated protein kinase
AP-1	Activating protein-1
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLASTn	Basic local alignment search tool for nucleotides
BSA	Bovine serum albumin
C	Cytosine
cAMV	Cloned avian myeloblastosis virus
CMA	Chaperone-mediated autophagy
CR	Calorie restriction
DAB	3,3'-diaminobenzidine
DEJ	Dermal-epidermal junction
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water

DNA	Deoxyribonucleic acid
ECL$^+$	Enhanced chemiluminescent reagent$^+$
EDTA	Ethylenediaminetetraacetic acid
FAM	6-carboxyfluorescein
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FOX	Forkhead box
FXR	Farnesoid X receptor
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	Growth hormone
GHR/BP	Growth hormone receptor / binding protein
GHRH	Growth hormone releasing hormone
HGPS	Hutchinson-Gilford progeria syndrome
HRP	Horseradish peroxidise
IGF-1	Insulin-like growth factor 1
IIS	Insulin / insulin-like signalling pathway
IκB	Nuclear factor κ-light-chain enhancer of activated B cells inhibitor
JNK	c-Jun N-terminal kinase
LAMP-2a	Lysosome-associated membrane protein-type- 2a
LPS	Lipopolysaccharide

LXR	Liver X receptor
MAPKK4	Mitogen-activated protein kinase-kinase-4
MAPKKK5	Mitogen-activated protein kinase-kinase-kinase-5
MGB	Minor groove binder
MKP-1	Mitogen-activated protein kinase-phosphatase-1
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information (USA)
NCoR	Nuclear receptor co-repressor
NFκB	Nuclear factor κ-light-chain enhancer of activated B cells
NHR	Nuclear hormone receptor
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PFA	Paraformaldehyde
PGC-1	Peroxisome proliferator activated receptor γ coactivator-1
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
RAR	Retinoic acid receptor
RIPA	Radioimmunoprecipitation assay
RM-ANOVA	Repeated measures analysis of variance

RNA	Ribonucleic acid
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SIRT-1	Sirtuin-1
SPSS	Statistical package for the social sciences
Src-1	Steroid receptor co-activator-1
ssDNA	Single-stranded deoxyribonucleic acid
T	Thymine
TBS	Tris-buffered saline
TEMED	Tetramethyleneethylenediamine
Th1/Th17	T-helper-1 / T-helper-17
TIMP-1	Tissue inhibitor of metalloproteinase enzymes
TLR4	Toll-like receptor 4
T_m	Melting temperature
TR	Triiodothyronine receptor
***t*-RA**	All-*trans*-retinoic acid
tRNA	Transfer ribonucleic acid
UVB	Ultraviolet B
UVR	Ultraviolet radiation
WRN	Werner protein

1. Introduction

1.1 Ageing

All organs of the body are affected by the endogenous process of biological ageing, which manifests as a progressive decline in biological function with advancing chronological age. The risk of all-cause mortality dramatically increases in old age (Gompertz, 1825; Office For National Statistics (UK), 2001; fig.1-1) and advancing age increases the probability of developing most non-congenital diseases.

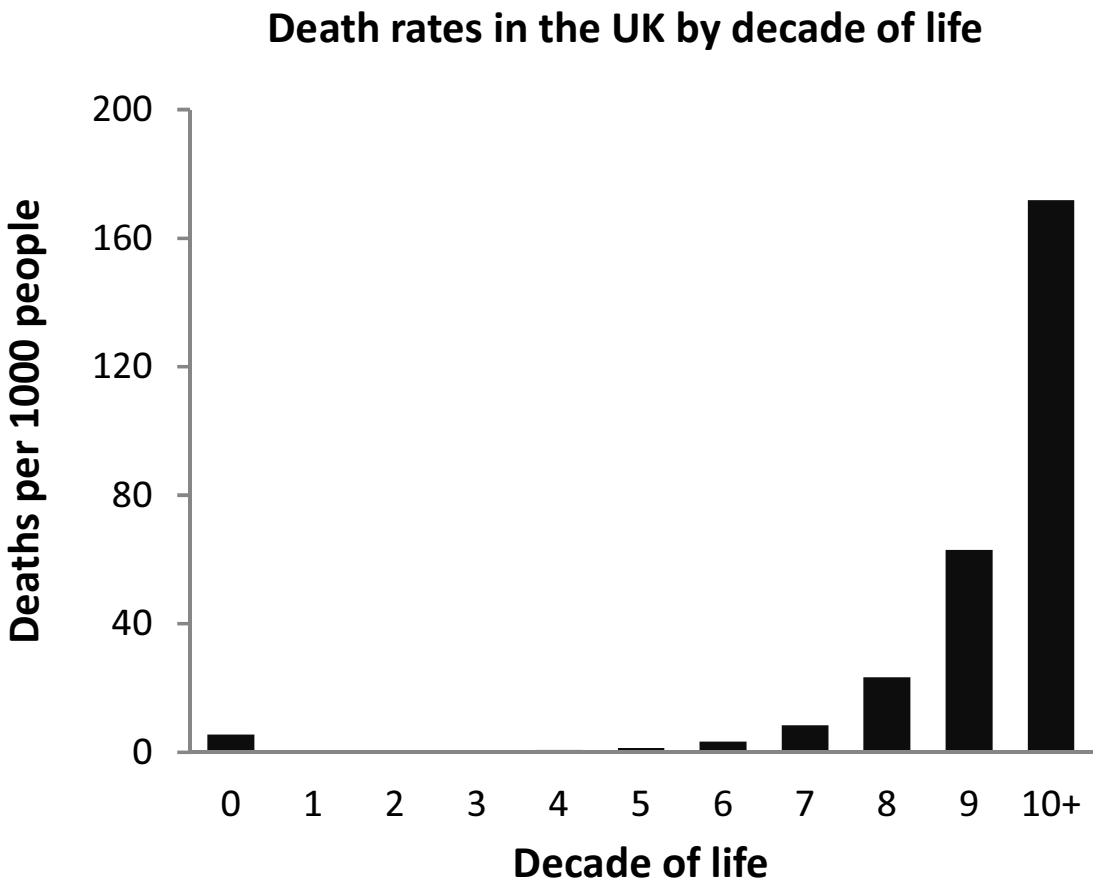


Figure 1-1. Death rates in the UK by decade of life. *The risk of death dramatically increases from the 6th decade onwards compared to earlier adulthood. Graph drawn from Office For National statistics (UK) dataset "Age specific death rates: by sex, 2001: Regional Trends 38".*

Plotting average human lifespan for the longest-lived geographical population at any given time, a striking and constant increase in longevity is clear from the first available records to the most recent. Female average life expectancy at birth increased from 45 years in Sweden in 1840 to 85 years in Japan in 2000, following an almost perfectly linear trend with a gradient of 3 months increase in mean life expectancy at birth per year (Oeppen and Vaupel, 2002).

It is not known for certain why this is the case; however increasing standards of living, education, sanitation, nutrition and medicine are likely contributors. Women live longer than men; in England the gender gap in average life expectancy is approximately 4.4 years (Office for National Statistics (UK), 2009). The longest-lived woman for whom good documentation is available was Jeanne Calment (1875-1997) who lived for 122 years and 164 days. The longest-lived man, Christian Mortensen (1882-1998), lived for 115 years and 252 days.

Almost all areas of human physiology decline with age following the age of potential peak function. The age of potential peak function varies substantially between individuals and different physiological parameters. Actual peak function depends on lifestyle and environment, factors that commonly vary throughout life, so that the age at which an individual experiences actual peak function does not necessarily correspond with the age of potential peak function.

The best indications for the age of potential peak function come from longitudinal studies of professional athletes. These studies imply that the potential peak for athletic performance tends to occur between 20-30 years of age (Schulz and Curnow, 1988; Ericsson, 1993). Following the potential peak, the potential function of physiological parameters gradually declines. With advancing age, individuals eventually become unable to maintain normal function. Notable deteriorations in the advanced stages of ageing include sarcopenia, immunosenescence, hearing loss, cataracts, macular degeneration, lowered fertility and impaired memory.

Sarcopenia is the loss of skeletal muscle mass and function with age. Over 30% of women and over 50% of men aged 80 years or more had significantly lower muscle mass than young adults in a US cohort (Iannuzzi-Sucich et al., 2002). Low strength is a major risk factor for all-cause mortality at advanced ages (Metter et al., 2002).

With age, the adaptive immune system undergoes complex dysregulations leading to increased susceptibility to pathogens, an increased risk of autoimmune problems and heightened systemic inflammation (Hakim and Gress, 2007). These changes, collectively termed immunosenescence, are thought to be linked to thymic involution, the gradual diminishment of thymic epithelial space that occurs throughout life (Gruver et al., 2007). Thymic epithelial space is lost at a rate of approximately 3% per year until 35-45 years of age, then 1% per year until death (Steinmann et al., 1985).

As yet, no unified theory of ageing has been validated or commonly accepted. Whilst a number of theories hold traction (section 1.2-1.8), it has not yet been possible to clearly identify the primary, initiating causes of ageing. It is abundantly clear that ageing features the accumulation of several forms of biomolecular damage and altered cell behaviours. The difficulty in identifying the root cause arises from the highly interconnected nature of the systems underlying ageing theories; for the most part any one as a primary driver of ageing could reasonably produce the other phenomena as secondary amplifying events. When the terms ‘primary’ and ‘secondary’ damage or pathology are used in regard to the theories of ageing explained in the following sections, this is the sense in which they are meant.

1.2 Evolutionary theories of ageing

A full understanding of why organisms age would be an invaluable guide to the direction of research into ageing and age-related diseases. At present there is an unresolved conflict between two major paradigms of ageing in animals; firstly that ageing is genetically programmed and secondly that ageing is imposed by endogenous processes or the environment and is actively delayed by biological longevity assurance mechanisms.

Lifespan is highly variable between species. Amongst mammals, maximum lifespans range from 2 years in the American water shrew *Sorex palustris* to at least 170 years in the bowhead whale *Balaena mysticetus* (George et al., 1999; Gusztak and Campbell, 2004). Interestingly, the age-associated functional declines seen towards the end of life are pathophysiologically similar in both short- and long-lived mammals. Sarcopenia, cataract formation, immunosenescence, thymic involution, hormonal signalling and calorie restriction (CR) responses are highly conserved in mammals (Hindle et al., 2009; Shanley et al., 2009; Wolf, 2010).

Early 20th century studies correlating lifespan and body size in mammals led to the 'rate of living' theory of ageing, which proposed a linear relationship between metabolic rate and lifespan (Rubner, 1908; Benedict, 1938). As further research catalogued the longevity of a wider range of species, it became apparent that the correlation between lifespan and body size is not as strong as initially thought (R^2 = 0.34; AnAge database build 8; n = 1701 species; de Magalhaes and Costa, 2009). Strikingly, several species of bats can live for much longer than non-flying rodents of similar body size and metabolic rate (Austad and Fischer, 1991).

Examination of large datasets of species lifespans suggests an alternative interpretation; that animals under a high threat of predation tend to be short-lived. The rationale for this is that if the vast majority of individuals in a wild population are killed by predation or other environmental factors before a certain age, there is no selective advantage in having a higher maximum lifespan than would ever be practically realised. This theory is borne out in a island population of opposums isolated for 3000-4000 years, which face lower predation pressure than their mainland relatives and appear to age more slowly (Austad, 1993). However, at least one exception to this theory has been observed in a fish population, suggesting that other environmental factors such as nutrient availability may also be influential in the evolution of animal lifespan (Reznick et al., 2006).

Due to the lack of active selection beyond wild lifespans, genes beneficial during advanced age are not likely to be selectively favoured while deleterious genes are not selected against. Furthermore, mutations that confer a fitness advantage in youth but that are deleterious at older ages would be likely to confer an overall reproductive advantage (Williams, 1957). As an alternative perspective, in a dangerous environment it is not evolutionarily optimal to divest resources in offspring during gestation in order to confer long term self maintenance capabilities (Kirkwood, 1977).

There is a general correlation between gestation period and maximum lifespan, however there are exceptions and the sample sizes used for these estimations have been small to date (Kanungo, 1975). In humans, average remaining life expectancy at age 50 varies with birth month by up to one year. In the Northern Hemisphere, lifespan is on average shortest for those born in May and longest for those born in September, a trend which is reversed in the Southern Hemisphere (Doblhammer and Vaupel, 2001). In rats a lower birth weight within normal ranges is predictive of longer lifespan, whilst a low-protein maternal diet lowers the average lifespan of offspring without significantly affecting birth weight (Aihie Sayer et al., 2001; Langley-Evans and Sculley, 2006).

1.3 The free radical theory of ageing

The free radical theory of ageing was first published in the mid-1950s (Harman, 1956). Free radicals are highly reactive atoms and molecules lacking a full complement of electrons, which can oxidise biomolecules; nucleic acids, proteins, lipids and carbohydrates. Well-characterised free radicals include certain reactive oxygen species and reactive nitrogen species (Hensley et al., 2000; Drew and Leeuwenburgh, 2002). Most free radicals affecting animals are endogenous byproducts of metabolism generated at the mitochondrial electron transport chain (Cadenas and Davies, 2000).

Cumulative free radical damage has received considerable attention as a possible cause of ageing. Free radicals have been implicated in all age-related diseases and functional declines where they have been studied to date (Birch-Machin, 2000; Finkel and Holbrook, 2000; Behrend et al., 2003; Barnham et al., 2004; Ferrari et al., 2004; Valko et al., 2004).

Given the demonstrated central role of free radicals in ageing biology, it might be expected that antioxidants, which nullify free radicals, should have an anti-ageing effect. In animal models, some supplementation experiments have shown increases in mean or median lifespan, suggesting delays in disease related mortality (Clapp et al., 1979). However, dietary supplementation with antioxidants has never been shown to extend maximum lifespan in mammals. Certain experimental antioxidant regimes have produced unanticipated side effects in humans, forcing clinical trials to be stopped before completion (Omenn et al., 1996; Bjelakovic et al., 2008).

The failure of dietary antioxidants to delay ageing led researchers to hypothesise that endogenous antioxidants may be more effective than exogenous antioxidants. To test this possibility, genetic manipulation has been extensively used to increase the expression of cellular antioxidants in rodents. Of the many genetic manipulations conducted, only one study has shown any increase in maximum lifespan. This increase in longevity was shown by a mouse strain engineered to express peroxisomal catalase targeted to mitochondria (Schriner et al., 2005). Compared to wild type controls, the maximum lifespan of these mice was increased by nearly 20%. This data seems promising and warrants further study, however criticism has suggested that the effect may be specific to the strain of mice used in this experiment (Lapointe and Hekimi, 2010).

The deletion of key antioxidant genes in mice increases oxidative stress, but out of five single and six double loss of function mutants created by the leading group in the field, only the manipulation targeting superoxide dismutase 1 caused decreased lifespan (Pérez et al. 2009). Furthermore, mice expressing an error-prone version of mitochondrial DNA polymerase develop premature ageing phenotypes without increased free radical production (Trifunovic et al., 2005).

Taken together, the many observations on the relationship of antioxidants to lifespan present a serious challenge to the free radical theory of ageing (Lapointe and Hekimi, 2010). Despite this challenge, a great number of the observations of ageing researchers are consistent with the accumulation of biomolecular damage driving primary ageing processes. Whether or not free radicals are influential in the primary pathology of ageing, they do appear to be important contributors to the secondary progression of ageing and many age-related diseases.

It may be that, if a form of biomolecular damage is key to the primary pathology of ageing as observations suggest, this damage might be caused by an agent other than free radicals (e.g. sections 1.4-1.8). Alternatively, primary biomolecular damage may be caused by free radicals in a way that cannot be prevented by antioxidant interventions so far studied, with the possible exception of mitochondria-targeted catalase. For example, less well studied free radicals such as those produced via lipid peroxidation (Spiteller, 2007; Spiteller, 2010) or localised free radical activity in mitochondria or lysosomes may be exclusively influential in ageing. Further study is necessary to robustly confirm or exclude these scenarios.

1.4 Stochastic non-enzymatic protein modification and ageing

Following ribosomal translation, a variety of post-translational modifications may be applied to proteins through enzyme-catalysed, gene-directed reactions, altering protein structure, function or interactions (Wold, 1981; Walsh et al., 2005). In addition to the wide spectrum of functional post-translational modifications, stochastic chemical reactions produce non-coordinated protein modifications. These randomly sited modifications may disrupt protein structure or function or produce undesirable crosslinks between proteins (Harding et al., 1989).

Widespread damage to proteins is one possible primary cause of ageing. Proteins are the effectors of cellular processes including DNA repair and replication, antioxidant defense and metabolism. Decreased efficiency in any of these systems would create the conditions for further protein damage or error, beginning a vicious cycle of biomolecular damage of the sort widely believed to typify ageing (fig. 1-2).

Stochastic modifications that have received particular attention in the ageing literature include glycation, glycoxidation and carbonylation. Glycation occurs when reducing sugars interact with proteins. The resulting modification, which forms via several intermediate steps (Maillard, 1912), is known as an advanced glycation end-product (AGE). Free radicals may oxidise glycation products, resulting in glycoxidative modifications. Carbonylation is also an oxidative process; carbonyls may be formed directly via the metal catalysed oxidation of amino acid side chains or through oxidative reactions with AGEs or glycoxidation products (Nyström, 2005).

Glycoxidation, AGEs and carbonylation are irreversible modifications (Dalle-Donne et al., 2003). Modified proteins are selectively targeted for degradation (Bota and Davies, 2002; Grune et al., 2004). However, heavily modified proteins can be resistant to degradation, resulting in their accumulation in postmitotic tissues with age (Handa et al., 1999; Stadtman, 2006; Terman and Brunk, 2006).
Some degradation-resistant modified proteins form aggregates which impair cellular function; particularly those functions related to protein and organelle turnover due to aggregate 'clogging' of proteasomes and lysosomes (Grune et al., 2004; Terman and Brunk, 2006). In human skin autofluorescence at 375 nm is reported to increase with age at sun protected sites at a rate of approximately 2% per year between ages 20-70 years (Na et al., 2001). Age-related autofluorescence in skin is thought to be emitted by AGEs, primarily of collagen (Dyer et al., 1992).

In addition to endogenous AGE production, exogenous dietary AGE intake influences systemic AGE accumulation and ageing. Mice fed a low AGE diet, achieved via reduced heat processing of food, showed reduced serum and tissue AGE accumulation and 7% extension of maximum lifespan compared to mice on a regular diet (Cai et al., 2007).

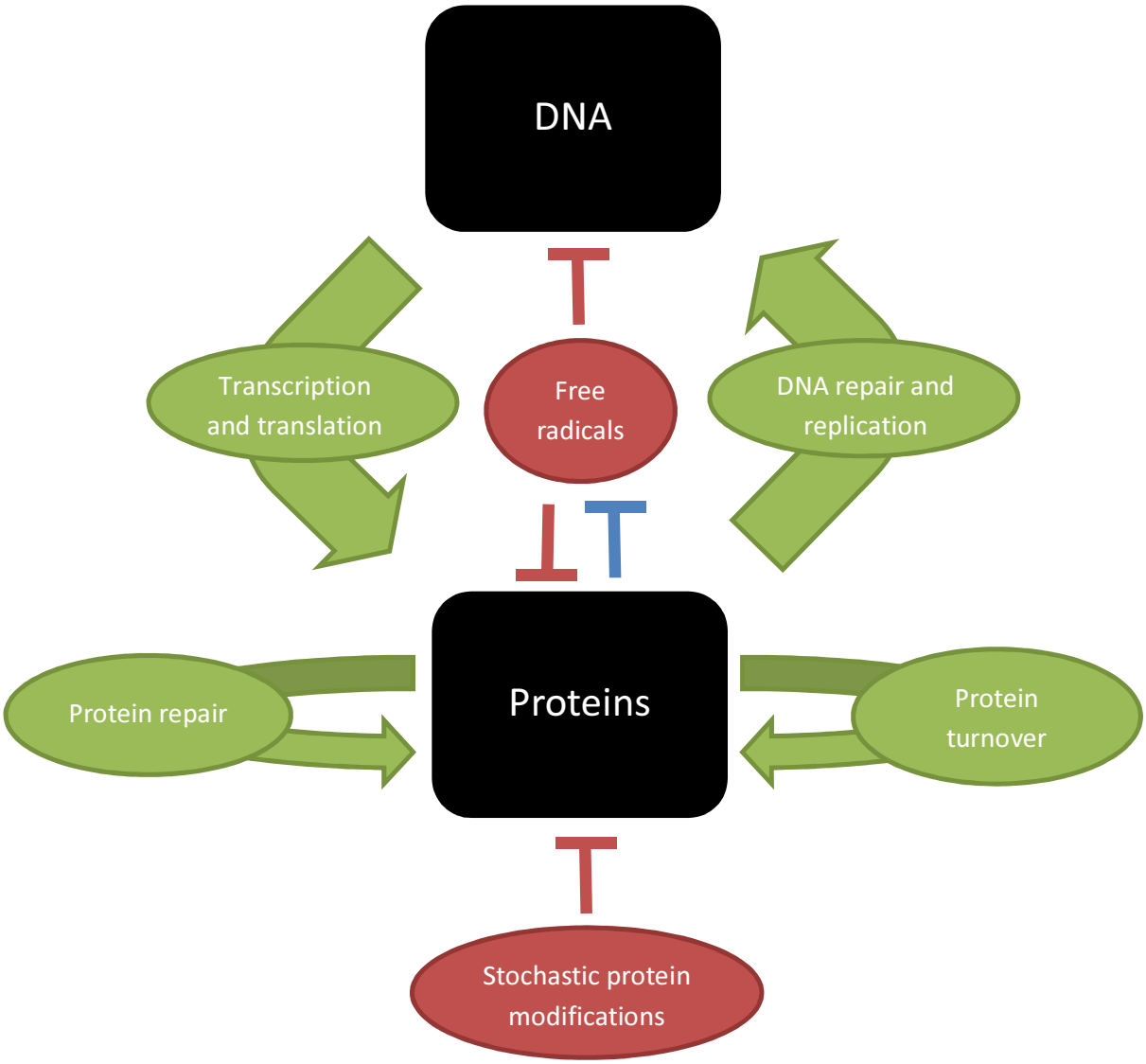


Figure 1-2. Schematic of the biomolecular damage hypotheses of ageing. *Biomolecules (proteins and DNA; other biomolecules not shown for simplicity) execute biological functions (green) necessary for the maintenance of organismal viability. Agents that stochastically damage biomolecules (red) produce functional impairments. As biomolecular functions include defense (blue) against damaging agents, repair and self-replication, a 'vicious cycle' can be conceptualised, as damage will increase subsequent damage and replication errors. A major goal of ageing research is to dissect this process and identify the primary events that initiate the cyclical generation of damage and errors thought to underlie biological ageing. Candidates for this primary event include biomolecular damage and replication errors, although there are other possibilities that could not be clearly depicted in this diagram.*

1.5 Autophagy and ageing

Autophagy is a process by which damaged proteins, organelles, protein aggregates or foreign bodies are transported to lysosomes, broken down and recycled into their component molecules (Klionsky and Emr, 2000; Todde et al., 2009). Autophagic activity declines in ageing (Vittorini et al., 1999; Cuervo and Dice, 2000; Cuervo 2008), resulting in decreased removal and recycling of damaged cellular components. A prime factor in this decline is thought to be the accumulation of undigestible materials in the lysosome. Lipofuscin or 'age pigment', an autofluorescent material rich in oxidised lipids and proteins (Terman and Brunk, 2004) builds up in the lysosomes of slowly dividing and non-dividing cells with age, inhibiting lysosome function (Brunk and Terman, 2002; Terman and Brunk, 2006).

Three distinct forms of autophagy exist in humans; macroautophagy, microautophagy and chaperone-mediated autophagy (CMA; Dice, 2007). In macroautophagy, vesicles form *de novo* to transport larger structures to lysosomes, whilst in microautophagy the lysosome membrane invaginates to envelope substances proximal to the lysosome. In CMA, chaperone proteins bind to individual damaged proteins and target them to the lysosome. Chaperones with bound proteins interact with lysosome-associated membrane protein type 2a (LAMP-2a) in the lysosomal membrane. LAMP-2a protein levels and consequently CMA are reduced in ageing (Cuervo and Dice, 2000; Dice, 2007).

The decline in LAMP-2a protein with age is related to protein stability at the lysosomal membrane rather than decreased transcription (Dice, 2007; Orenstein and Cuervo, 2010). Using a transgenic mouse with inducible expression of LAMP-2a in liver, Cuervo and colleagues were able to show that transcriptional up-regulation of liver LAMP-2a in ageing mice corrected much of the age-related decline in CMA, reduced lipofuscin accumulation and improved liver function in aged (22 month old) mice (Zhang and Cuervo, 2008).

Autophagy appears to be a highly promising area for ageing research. Genetic manipulations have shown that autophagy is necessary for life extension achieved via insulin/insulin-like signalling (IIS) knockouts, CR, target of rapamycin (TOR) deficiency, p53 orthologue knockout, calcineurin knockout, *sir2.1* over expression or decreased mitochondrial respiration in the nematode worm *Caenorhabditis elegans* (Melendez et al., 2003; Jia and Levine, 2007; Tavernarakis et al., 2008; Tóth et al., 2008; Morselli et al., 2010). In addition, the inhibition of autophagy shortens lifespan, hastens lipofuscin and protein aggregate accumulation and leads to the early onset of ageing pathologies in both *C. elegans* worms and *Drosophila* fruitflies (Juhász et al., 2007; Tóth et al., 2008).

In light of the emerging crucial role of autophagy in organismal longevity, a number of recent studies have experimented with promoting autophagy in model organisms. The genetic enhancement of autophagy in the nervous system of *Drosophila* fruitflies was found to increase mean lifespan by 54% (Simonsen et al., 2008). Spermidine pharmacologically induced autophagy and increased longevity in yeast, *C. elegans* and *Drosophila* by 20-50%. Mice beginning spermidine treatment at 12-16 weeks were found to have increased serum free thiol group concentrations, a measure of decreased oxidative stress, at 1 year of age (Eisenberg et al., 2009). Mice in this study were sacrificed for serum measurements, however survival at earlier ages did appear to be enhanced by spermidine treatment.

A separate study found that mice treated with combined spermine and spermidine supplementation showed lower mortality and reduced glomulerosclerosis towards the end of their lifespans (Soda et al., 2009). This study also finished before the maximum lifespan of the experimental mouse strain was reached; mice were sacrificed at 88 weeks of age. No measurement has yet been made of the effect of spermidine or any other polyamine treatment on maximum mammalian lifespan.

Concordant with findings in spermidine and spermine treatment of model animals, polyamine depletion in yeast leads to increased oxidative stress, early necrotic death and histone H3 hyperacetylation (Eisenberg et al., 2009). *In vitro* data suggest that glucosamine is also an activator of autophagy (Shintani et al., 2010).

Interestingly, spermidine inhibits histone acetylases, drawing some authors to speculate on whether it may share a mode of action with the longevity-associated histone deacetylase sirtuin-1 (SIRT-1; Eisenberg et al., 2009; Morselli et al., 2009).

Indeed, transient SIRT-1 up-regulation has been shown to stimulate autophagy in mice, where it complexes with and directly deacetylates several key autophagy genes and proteins (Lee et al., 2008). SIRT-1 is required for the induction of autophagy by the longevity-associated polyphenol resveratrol or dietary restriction, but is independent of rapamycin or p53 knockout-induced autophagy in human cells and *C. elegans* (Morselli et al., 2010).

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is, among its other functions, a potent repressor of autophagy. Inhibiting mTOR function with the drug rapamycin induces autophagy (Blommart et al., 1995; Díaz-Troya et al., 2008) and produces profound immunosuppression (Thomson et al., 2009). Treatment of mice with rapamycin in a sterile environment delayed the time until 90% mortality by 9-14% compared to an untreated control group (Harrison et al., 2009). Relatively long-lived, genetically heterogeneous mice were used for this study. Rapamycin is one of the few interventions definitively shown to increase maximum lifespan in mammals to date (table 1-1).

Deletion of ribosomal protein S6 kinase 1, a downstream target of mTOR and IIS (section 1.8) increases lifespan by approximately 14% in female mice, although only minor benefit was seen for males (Selman et al., 2009). Ribosomal protein S6 phosphorylation is inhibitory for autophagy *in vitro* (Blommart et al., 1995).

Converse to the trend of autophagy induction correlating with increased longevity, one study found that the RNA interference-mediated suppression of certain autophagy genes extends, rather than decreasing lifespan in *C. elegans* (Yasufumi et al., 2009). The authors did not measure autophagic activity in their treated animals, so it is possible that a compensatory increase in total autophagic activity might occur in response to the suppression of individual genes.

1.6 Telomeres and ageing

Telomeres are nucleoprotein structures formed from repeated 5'-TTAGGG-3' DNA hexamers that cap the ends of chromosomes, enabling DNA repair mechanisms to differentiate chromosome ends from broken double stranded DNA. The loss of telomeres results in genomic instability and induces a sustained DNA damage response causing cells to enter apoptosis or senescence (Deng et al., 2008).

During cell division telomeres are imperfectly replicated, resulting in the erosion of telomere length. Compensating for telomere erosion, telomere lengths may be extended by the enzyme telomerase and in some circumstances by a form of recombination known as alternative lengthening of telomeres (Gilson and Geli, 2007; Cesare and Reddel, 2010).

Telomerase expression is low in most adult human somatic cells as measured at any single time point, although reports have shown that telomerase may be transiently up-regulated during proliferation and tissue repair (Hiyama et al., 1995; Bodnar et al., 1996; Hiyama et al., 2001; Minamino and Kourembanas, 2001; Klapper et al., 2003; Parsch et al., 2004). Based on these observations and the apparently telomere-dependent replicative senescence of many cell types *in vitro* (Hayflick and Moorhead, 1961; Allsopp et al., 1992; Bodnar et al., 1998), telomere theories of ageing hypothesised that telomere attrition led to catastrophic levels of apoptosis and senescence, impairing tissue function late in life (de Lange et al., 1990; Harley, 1991; Shay, 1995; Sedivy 1998).

Telomere length has been found to decrease with age in multiple human tissues and in genetic diseases of accelerated ageing and tissue dysfunction (Blasco, 2007; Armanios, 2009; Decker et al., 2009). The first longitudinal epidemiological study on telomere length appeared to confirm a positive association between telomere length and survival in the elderly (n=143; Richard et al., 2003). However, two subsequent larger studies (Bischoff et al., 2006, n=812; Njajou et al., 2009, n=3,075) failed to find an association between telomere length and lifespan or any individual cause of death in elderly cohorts.

A comparative biology study in 15 species of rodents found that telomerase activity co-evolved in this group with body mass but not with lifespan (Seluanov et al., 2007). This study suggests that tight temporal regulation of telomerase activity in somatic tissues is a necessary adaptation to limit tumourigenesis in large animals (Gorbunova and Seluanov, 2009).

From current knowledge, a causal link between telomere attrition and organismal ageing is not fully clear. Future work may yet reveal such a connection; it is possible that telomere attrition may specifically contribute to the diminishment of tissue stem cell compartments in ageing (Blasco, 2007; Sharpless and DePinho, 2007; Aubert and Lansdorp, 2008). There also is growing evidence that telomerase has multiple functions in addition to its role in telomere maintenance (Geserick and Blasco, 2006).

Cancer resistant mice over expressing telomerase have an extended maximum lifespan but similar mean telomere lengths in late life as compared to wild type mice (Gonzalez-Suarez et al., 2005). Telomerase over expression on a normal genetic background causes increased tumour incidence. In a human genetic study, both positive and negative associations were found with the frequency of specific telomerase haplotypes in centenarians compared to the general population (Atzmon et al., 2010).

1.7 Progeroid conditions

A number of rare monogenic conditions have been described that produce phenotypes resembling accelerated ageing in multiple tissues. Many of these conditions, termed progerias, are caused by mutations in genes involved in DNA repair.

Progerias that particularly resemble normal ageing include Hutchinson-Gilford progeria syndrome (HGPS), estimated to affect around 1 in 4 million live births (Raoul, 2006) and Werner syndrome, with an estimated incidence of around 1 in 1 million live births (Cohen, 2008). Progerias produce clinical pathologies that are not found in ageing, but there is significant overlap between their symptoms and certain physical and molecular changes characteristic of normal ageing (Martin, 1978; Ramírez et al., 2007).

Hutchinson-Gilford progeria syndrome is most commonly caused by point mutations in the lamin A gene that cause the production of an abnormal splice form, resulting in a protein that lacks an endopeptidase cleavage recognition site (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). Lamin A protein is a major component of the nuclear lamina, a filamentous network in the peripheral nucleus which is important in maintaining the structure of the nuclear membrane (Sullivan et al., 1999). Lamin A interacts with both the nuclear membrane and chromatin; HGPS lamin A mutations perturb DNA synthesis, chromatin organisation, chromosomal segregation during anaphase and nuclear reassembly during telophase (Cao et al., 2007; Dechat et al., 2007; Dechat et al., 2008).

Symptoms of HGPS include delayed growth, micrognathia (small jaw), loss of subcutaneous fat, alopecia, osteolysis, prominent eyes and vasculature, premature atherosclerosis, skin sclerosis (thickening), joint contractures and hearing loss (Merideth et al., 2008). Symptoms are progressive and HGPS sufferers normally die of acute cardiovascular failure in their teenage years.

Werner syndrome is normally caused by mutations in Werner protein (WRN). WRN is a DNA helicase involved in DNA repair and topological remodelling of DNA in homologous recombination, replication and telomere maintenance (Prince et al., 2001; Cox and Faragher, 2007; Bohr, 2008; Aggarwal et al., 2010; Kusumoto-Matsuo et al., 2010). Approximately 20% of patients diagnosed with Werner syndrome have wild type WRN. A subset of these ‘atypical’ patients instead carry mutations in the lamin A gene (Lishan et al., 2003).

Patients with Werner syndrome are not usually diagnosed until their third decade of life, when symptoms begin with skin atrophy, graying hair and progressive alopecia. Following these initial symptoms, patients develop abdominal subcutaneous fat deposits, osteoporosis, cataracts and sarcopenia particularly affecting the limbs. Chronic skin ulcers on the lower extremities are common (Cohen and Shelley, 1963; Yeong and Yang, 2004).

In the further progression of the condition patients commonly develop various age-related diseases including type II diabetes, atherosclerosis, hypogonadism and neoplasms, with an elevated incidence of certain rare tumours including soft tissue sarcomas and benign meningiomas (Goto et al., 1996; Muftuoglu et al., 2008). The average age of death for Werner syndrome sufferers is 47 years, most often due to cancer or cardiovascular disease (Goto, 2000).

In addition to the physiological overlap between Werner syndrome and normal ageing, gene expression patterns in response to ionising irradiation have been reported to be similar between primary fibroblasts from aged donors and Werner syndrome donors but different in fibroblasts from young donors (Kyng et al., 2005).

Progerias are pathological conditions, however their symptoms suggest that they may hold insights into normal ageing. The nature of molecular defects in progeroid conditions highlights the importance of genomic stability in ageing. One possibility within the biomolecular damage theories of ageing (fig. 1-2) is that that ageing originates from damage to DNA, causing loss of gene function, altering gene expression and stimulating DNA damage responses, senescence and apoptosis.

Senescence, along with apoptosis, is one of the mechanisms via which a cell undergoing a sustained damage response ceases proliferation. Senescence is thought to have evolved primarily to limit tumourigenesis and also appears to have other physiological benefits, having been recently reported to curtail liver fibrosis (Krizhanovsky et al., 2008; Prieur and Peeper, 2008). In contrast to apoptosis, a form of gene-directed suicide, cells entering senescence irreversibly exit the cell cycle but remain active in the tissue environment (Freund et al., 2010).

Senescent cells acquire a pro-inflammatory secretory phenotype that may contribute to ageing and age-related diseases (Campisi and d'Adda di Fagagna, 2007; Freund et al., 2010). Senescent cells accumulate *in vivo* during normal ageing (Herbig et al., 2006; Jeyapalan et al., 2007) and are implicated in the pathogenesis of certain age-related diseases (Burton, 2009; Loeser, 2009). Werner syndrome cells are prone to early senescence *in vitro* (Salk, 1982; Faragher et al., 1993; Kill et al., 1994; Benson et al., 2010).

While the study of cellular senescence in organismal ageing is an emerging field, interesting observations have been made in experimental systems. Human dermal cell populations have been shown to produce dermal fragility and subepidermal blistering correlating with the number of *in vitro* population doublings of the cell population prior to incorporation into a reconstituted human skin equivalent (Funk et al., 2000). Telomerase expression was able to rescue the tissue viability of late passage cultures in this system (Funk et al., 2000). In a separate study, the induction of senescence by ras oncogene overexpression into balloon catheter-injured rat carotid arteries produced inflammation and changes consistent with the development of atherosclerosis (Minamino et al., 2003), suggesting a potential link between senescence and human ageing (Cox and Faragher, 2007).

Interestingly, the splice variant produced by the HGPS lamin A mutation is also expressed in the skin of healthy individuals at low levels and the mutant protein accumulates in a subset of fibroblasts and keratinocytes during ageing (McClintock et al., 2007). A recent study found that an uncleaved precursor of lamin A similar to the mutant protein produced in HGPS accumulates in aged human vascular smooth muscle, where it causes DNA damage, genomic instability, nuclear morphology defects and cellular senescence (Ragnauth et al., 2010).

1.8 Endocrine aspects of ageing

Over the preceding few decades numerous studies have succeeded in extending the life of model organisms by genetic or surgical manipulations targeting endocrine pathways. While some longevity-associated endocrine pathways are species specific and not well conserved, interventions converging on the IIS pathway have been shown to increase lifespan in nematodes, fruitflies, mice and rats.

Initial indications of the importance of IIS in ageing came from mice and rats with surgically removed pituitary glands, which displayed delayed molecular ageing and an extended lifespan (Everitt and Cavanagh, 1965; Everitt et al., 1980; Powers et al., 2006). The up to 68% longer-lived than wild type Ames dwarf mice and up to 40% longer-lived Snell dwarf mice are now known to carry mutations preventing pituitary development (Flurkey et al., 2002; Bartke et al., 2004).

The pituitary gland produces a range of hormones, including growth hormone (GH) and insulin-like growth factor-1 (IGF-1), two components of the IIS pathway. These two hormones are thought to be responsible for most of the effects of decreased pituitary function in increasing lifespan (Brown-Borg, 2009).

The Laron dwarf mouse has a targeted homozygous loss of function mutation in the growth hormone receptor/binding protein (GHR/BP) gene and lives up to 40% longer than wild type or GHR/BP heterozygote mice. Laron dwarf mice are unresponsive to GH and have very low serum IGF-1 levels throughout life (Coschigano et al., 2000; Coschigano et al., 2003).

Further mouse studies consistent with the proposed functions of the IIS pathway in regulating longevity are the GH releasing hormone (GHRH) knockout (up to 25% longer-lived on a low fat diet), IGF-1 receptor heterozygote (up to 33% longer-lived), insulin receptor substrate 1 knockout (females are up to 12.5% longer-lived) and fat-specific insulin receptor knockout (up to 14% longer-lived) mice (Flurkey et al., 2001; Bluher et al., 2003; Holzenberger, 2004; Selman et al., 2008). However, whilst these perturbations of the IIS pathway can extend life in the mouse, humans with

congenital GHRH deficiencies do not appear to have an advantage in life span (Aguiar-Oliveira et al., 2010).

Perhaps not all findings in the rodent IIS pathway are directly translatable to humans but the IIS pathway does appear to be important in human ageing. Three independent human genetic studies recently found a strong association between FOXO3A gene haplotypes and longevity in geographically disparate populations (Willcox et al., 2008; Anselmi et al., 2009; Flachsbart et al., 2009). A separate study found that haplotypes of both FOXO3A and the closely related gene FOXO1A are associated with mortality and age-related diseases in very elderly humans (Kuningas et al., 2007).

The FOXO transcription factors are strongly repressed by the IIS pathway (Tissenbaum, 2010), so it seems probable that the IIS pathway affects longevity via regulation of the FOXO transcription factors in addition to other components. The IIS pathway is also a strong repressor of autophagy (Meijer and Codogno, 2006).

To better define the downstream effects of IIS pathway signalling, microarray analysis has been used to search for genes showing similar changes in expression in long-lived mutants of nematodes, fruit flies and mice (McElwee et al., 2007). In these experiments, IIS pathway downstream components were found to be poorly conserved between species at the level of individual genes. However, functional gene clusters showed similar patterns of expression; sugar metabolism and cellular detoxification systems (type I, type II and xenobiotic) were up-regulated whilst protein synthesis was down-regulated.

Cellular detoxification has previously been linked to longevity in long-lived mouse models (Daniel et al., 2007; Zimniak, 2008) and down-regulation of protein synthesis extends longevity in nematode worms (Kally et al., 2007; Malene et al., 2007). These systems are therefore prominent candidate mediators of the anti-ageing effects of the IIS pathway.

1.9 Calorie restriction (CR)

The pro-longevity effects of CR have been known since the mid-1930s, when it was shown that supplying less daily calories to rats than they would have eaten *ad libitum* extended their mean and maximum lifespans (McCay and Crowell, 1934; McCay et al., 1935). Since these seminal experiments, CR has been reported to extend longevity and delay age-related changes in multiple species including yeast and other fungi, water fleas, nematode worms, fruit flies, spiders, fish, mice, hamsters, rats, and dogs, while an ongoing primate study is producing promising results (Ingle et al., 1937; Comfort, 1963; Austad, 1989; Masoro, 2005; Messaoudi et al., 2010; van Diepeningen et al., 2010).

House flies, medflies, some rotifer species and certain strains of mice do not however respond favourably to CR (Kirk, 2001; Carey et al., 2002; Cooper et al., 2004; Sohal et al., 2009). Mice lacking a pro-longevity response to CR include the genetically heterogeneous offspring of wild caught mice (Harper et al., 2006). This raises the possibility that the pro-longevity effects of CR may be partly an artefact of obesogenic laboratory conditions or traits of inbred laboratory mouse strains.

In addition, restriction of the essential amino acid methionine has been shown to extend longevity in some species (Orentreich et al., 1993). The lifespan extension seen in some CR studies may have been partly due to a methionine restriction effect (Masoro, 2006; Pamplona and Barja, 2006). CR is well documented to be associated with reduced mitochondrial free radical generation in rodents (Merry and Ash, 2010). This reduced free radical production was reproduced in rats by a similar degree of methionine restriction to that incurred in CR experiments (Caro et al., 2008; López-Torres and Barja, 2008; Caro et al., 2009).

Restricting all amino acids except methionine did not affect mitochondrial free radical production in rats but increased SIRT-1 expression and reduced markers of mitochondrial lipoxidation, protein carbonylation and glycoxidation (Caro et al., 2009). Whether this diet affects lifespan is not yet known.

Although these caveats may temper the interpretation of CR study findings, CR experiments have documented a wealth of molecular changes associated with longevity extension. Mitochondrial free radical generation is lowered in CR rodents, which may be attributable to a methionine restriction effect; biomolecular damage including oxidative protein and lipid modifications (Forster et al., 2000; Teillet et al., 2000; Pamplona et al., 2002; Lambert et al., 2004; Hulbert, 2005; Chakravarti and Chakravarti, 2007) and markers of mitochondrial DNA damage (López-Torres et al., 2002; Gómez et al., 2007) is reduced in CR rodents, while nuclear DNA repair mechanisms are up-regulated (Heydari et al., 2007; Kisby et al., 2010).

Calorie restriction reduces visceral fat in rats (Barzilai et al., 1998). Surgical removal of visceral fat in young rats extends maximum lifespan by approximately 11%, suggesting that altered visceral fat mass is a primary component of CR life extension (Muzumdar et al., 2008). Humans on 14-day, 6-month or 1 year CR diets also showed significant reductions in visceral fat mass (Li et al., 2003; Racette et al., 2006; Redman et al., 2007). In both rodents and humans, CR lowers fasting insulin and IGF-1 levels while increasing insulin sensitivity and IGF-1 receptor expression (Smith et al., 1995; Heilbronn et al., 2006; Redman and Ravussin, 2009; Fontana et al., 2010). Fasting serum glucose, cholesterol and triglyceride levels as well as the inflammatory biomarker C-reactive protein were found to be reduced by 6 years of CR in humans (Fontana et al., 2004).

Microarray profiling of young, aged and CR aged skeletal muscle in mice showed that most age-related gene expression changes were limited by CR. Functional analysis showed that gene clusters altered in CR but not ageing were involved in defense against biomolecular damage and increased protein turnover (Lee et al., 1999; Weindruch et al., 2001). A separate study conducted a similar analysis in 10 different CR murine tissues. Tissues showed varying responses and only a small subset of genes had a common response between tissues; these genes were involved in nitric oxide signalling, zinc homeostasis, circadian rhythms, heat response, protein refolding and chemical/xenobiotic responses (Swindell, 2008). An analysis of microarray studies on CR animals from various species found no single

gene whose expression was consistently altered, but functional pathways including metabolism, immune responses, cell growth and transcriptional regulation were modulated by CR in all species (Han and Hickey, 2005).

Under conditions of nutrient deprivation, autophagy (section 1.5) is up-regulated, increasing protein and organelle recycling (Mizushima and Klionsky, 2007). CR up-regulates baseline autophagy and prevents its attenuation with age (Cuervo, 2008).

Increased organelle and protein turnover may limit the accumulation of damaged molecules with age. In nematode worms, knockout of key autophagy genes in CR animals abrogates lifespan extension (Jia and Levine, 2007). However, knockout of the FOXO homologue *daf-16* also prevents the longevity response to CR in nematodes, suggesting that raised autophagy alone is not sufficient to increase lifespan (Hansen et al., 2008).

Given the wide conservation in lifespan extension by CR, speculation has arisen that it may be a viable mechanism for longevity in humans. While a controlled experiment to assess lifespan extension by CR in humans would be impractical, short term CR studies have shown similar physiological responses to those seen in model animals.

Human CR responses documented so far in studies up to 2 years of length include decreased visceral fat mass, serum fasting glucose, triglycerides and cholesterol as described above, reduced blood pressure, decreased circulating insulin and thyroid hormone, increased insulin sensitivity, reduced body temperature, increased skeletal muscle mitochondrial biogenesis, increased SIRT-1 expression and reduced DNA damage markers (Walford et al., 2002; Heilbronn et al., 2006; Civitarese et al., 2007; Redman et al., 2008).

In addition, the diet of the population of the Japanese island Okinawa has been proposed to represent a natural model of human CR. A survey in the 1970s found that Okinawans ate on average only 83% of the calories eaten elsewhere in Japan (Kagawa, 1978). Recent studies have confirmed a lifetime low calorie intake in older

Okinawans, although this calorie deficit has narrowed in the past few decades (Willcox et al., 2007). Interestingly, the mean age at death of the longest- lived 99th percentile in the Okinawan population was approximately 3 years higher than either the general Japanese or USA populations as determined from 1995 datasets (Willcox et al., 2006).

These findings suggest that CR may be effective in humans, however the CR diet requires discipline to adhere to and is unlikely to ever be a popular lifestyle choice. The ideal solution would be to pharmaceutically mimic a CR response using a 'CR mimetic' drug (Ingram et al., 2006). A prominent target for CR mimetics is the NAD-dependent deacetylase enzyme SIRT-1. CR up-regulates SIRT-1 protein in many tissues and species (Cohen et al., 2004; Chen et al., 2008; Cantó and Auwerx, 2009). Transgenic SIRT-1 (or homolog) over-expression extends life in yeast, nematodes, fruit flies and mice, producing phenotypes similar to CR (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004; Bordone et al., 2007).

Activators of the SIRT-1 pathway have also produced CR-like effects in wild type mice. The polyphenol resveratrol, the effects of which are thought to be partly mediated through SIRT-1 activation (Howitz et al., 2003; Wood et al., 2004; Lagouge et al., 2006; Cantó and Auwerx, 2009; Smith et al., 2009) in addition to other targets (Pirola and Fröjdö, 2008) has produced CR-like effects in yeast, nematode worms, fruit flies and mice, although no lifespan extension has been observed in resveratrol-treated rodents (Pearson et al., 2008).

Among resveratrol's reported targets is the energy-sensing enzyme adenosine monophosphate-activated protein kinase (AMPK; Pirola and Fröjdö, 2008). SIRT-1 is a downstream target of AMPK (Canto et al., 2009), suggesting one mechanism for the pro-longevity functions of AMPK in model organisms (Apfeld et al., 2004). The AMPK activators phenformin and metformin have been linked to a CR-like response and longevity extension in mice (Anisimov et al., 2003; Dhahbi et al., 2005). Rapamycin also extends life by pathways that overlap with CR and may be considered a CR mimetic (Kaeberlein, 2010).

1.10 Common biology of long-lived humans

In developed countries, approximately 1 in 10000 people live to ages over 100 years. The study of these exceptionally long-lived people, termed centenarians, has revealed some shared traits associated with human longevity. Compared to the general population, centenarians tend to spend a low proportion of their lives in a state of frailty and have a remarkably low incidence of age-related diseases and cancer (Hitt et al., 1999). Health in centenarians in correlated with high serum levels of retinol and α-tocopherol, low levels of albumin and cholesterol and negligible serum 25-hydroxyvitamin D (Mecocci et al., 2000; Passeri et al., 2003). The pro-inflammatory factors C-reactive protein and interleukin 6 (IL-6) were found to be high in Tokyo centenarians, suggesting a chronic inflammatory state. The centenarians in this study also tended to be mildly anaemic (Hirose et al., 2004).

Longevity has a strong genetic basis; a recent study determined a battery of 150 single nucleotide polymorphisms (SNPs) reported to be 77% accurate in predicting an individual's potential to live beyond 100 years (Sebastiani et al., 2010). Mitochondrial DNA polymorphisms have also been associated with centenarians in various geographical populations (Salvioli et al., 2008). As would be expected from these findings, longevity is a heritable trait (Perls et al., 1998; Atzmon et al., 2005; Willcox et al., 2006; Adams et al., 2008). The genetic basis to longevity may be interpreted as a genetic upper limit to human lifespan. However, such a limit has historically been proposed and exceeded several times (section 1.1) so this inference should perhaps be regarded with caution.

Intervention	Initial report(s)	Current state of knowledge
Calorie restriction	McCay and Crowell, 1934; McCay et al., 1935	30-60% reduction in total calories extends life by 20-50% in most but not all strains of mice and rats. Ongoing tests are promising in rhesus monkeys and humans.
Methionine restriction	Orentreich et al., 1993	80% reduction in methionine extends rodent lifespan by 15-45%.
Rapamycin	Harrison et al., 2009	Validated in genetically heterogeneous mice. 14% lifespan extension in females, 9% in males when treatment begun at age 600 days.
Hypophysectomy	Everitt et al., 1980 Powers et al., 2006	Hypophysectomy of rats at 7 months extended lifespan by approx. 15%. Hypophysectomy of mice at 9 months extended life span by 21%.
Visceral fat removal	Muzumdar et al., 2008	Surgical removal of visceral fat in 5-month old rats increased maximum lifespan by approx. 11%.
Phenformin or phenytoin	Dilman and Anisimov, 1980; Anisimov et al., 2003	23-26% extension of maximum life span in female C3H/Sn mice.
Low AGE diet	Cai et al., 2007	7% extension of maximum life span in male C57BL mice

Table 1-1. Details of interventions reported to increase maximum lifespan in mammals. *It should be noted that various interventions have been documented with large impacts on mean but not maximum lifespan in humans; for example a balanced diet and regular exercise. Whether such interventions represent modulations of biological ageing is unclear. Genetic models of extended lifespan do not yet offer practical postnatal interventions for mammals and are not listed here.*

1.11 Skin anatomy

Skin is an important organ. It is instrumental in the maintenance of bodily water and temperature homeostasis and an active frontline barrier to external pathogens and ultraviolet radiation (UVR); skin health is also crucial for social and psychological fitness and individual recognition. Skin is the largest organ of the body by weight and furthermore plays a wide ranging role in endocrine secretion (Slominski and Wortsman, 2000).

The skin is a layered tissue (fig. 1-3). The surface layer, the epidermis, is highly cellular. Approximately 90% of epidermal cells by number are keratinocytes. Other cells in the epidermis include nerve fibers, mechanosensors (Merkel cells), dendritic Langerhans cells and pigmentary melanocytes.

Following their geneses from a sparse population of basal epidermal stem cells, keratinocytes set upon a regimented process of differentiation. As they progress through a series of discrete phenotypes, keratinocytes also migrate towards the epidermal surface. These processes together result in the formation of stratified layers within the epidermis, where each layer comprises keratinocytes at a particular stage of differentiation (fig. 1-3). In healthy skin, the full maturation of newly generated keratinocytes into corneocytes typically takes 3-4 weeks.

Clonal units of keratinocytes emanate and expand from the basal layer in conical formations and typically comprise 16 ± 6 cells in mouse dorsal skin (Allen and Potten, 1974; Zhang et al., 2001; Strachan and Ghadially, 2008). The basal layer of the epidermis comprises a self-perpetuating population of progenitor cells, newly generated keratinocytes and pigmentary melanocytes. The stem cell population of mouse epidermis has been estimated at approximately one stem cell per thousand epidermal cells (Schneider et al., 2003).

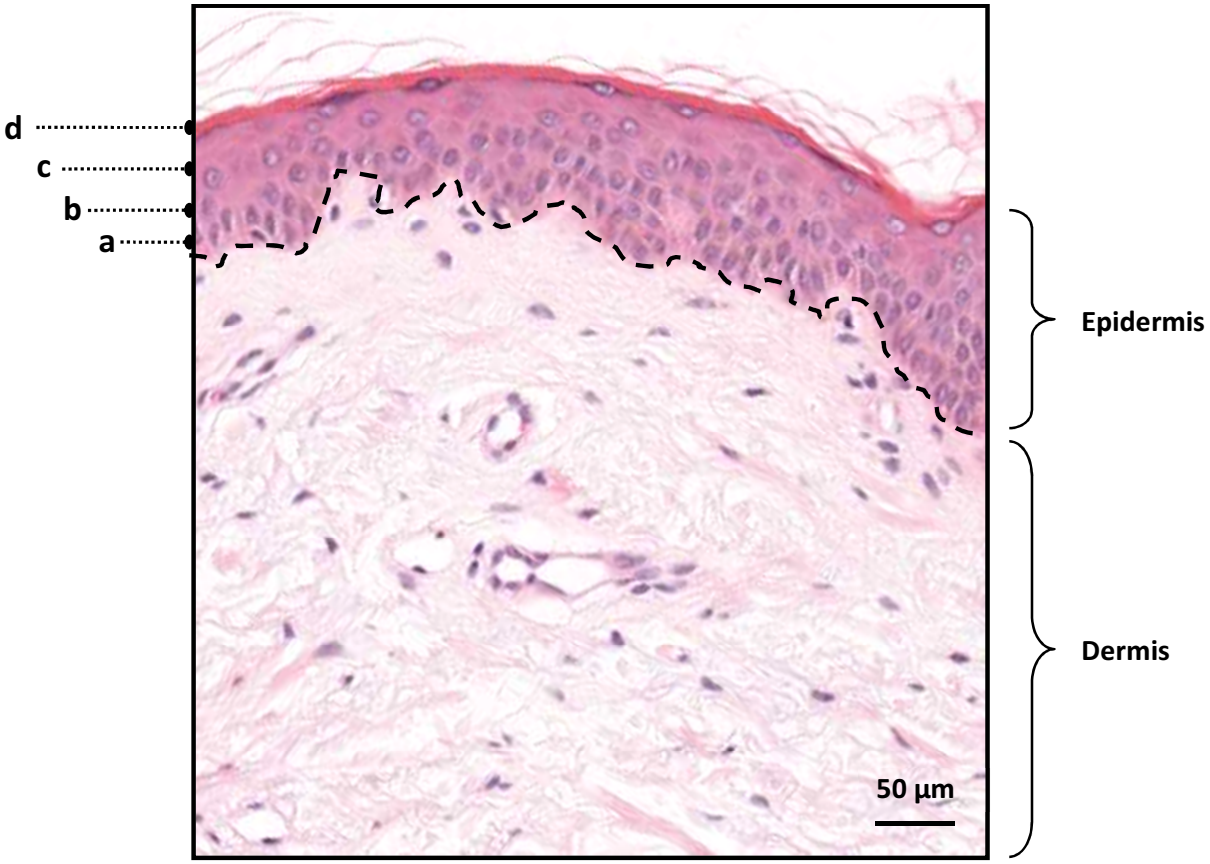


Figure 1-3. Diagram of skin anatomy: *The major histological features of the skin are labelled in this haemotoxylin-eosin stained frozen section of healthy skin. The dashed line marks the dermal-epidermal junction (DEJ).* ***a****) marks the basal layer,* ***b****) the spinous layer,* ***c****) the granular layer and* ***d****) the stratum corneum. Cell nuclei are stained purple, primarily keratinocytes in the epidermis, fibroblasts and endothelial cells in the dermis.*

Compared to mouse skin, human skin has a greater number of cell layers and more pronounced undulation of the DEJ. Despite these morphological differences, the fundamental arrangement of the epidermis as clonal proliferative units is similar, as shown by lentiviral fluorescent labelling in human skin grafted to mice (Ghazizadeh and Taichman, 2005).

Slow cycling epidermal stem cells have a high replicative potential but a low rate of division (Pellegrini et al., 2001; Papini et al., 2003). Two models have been proposed to describe epidermal stem cell function. The longest standing model states that epidermal stem cells can divide to produce either daughters with a stem cell phenotype or 'transit amplifying cells', which have a much higher rate of proliferation but limited proliferative potential (Senoo et al., 2007; Blanpain and Fuchs, 2009). Generating highly proliferative intermediates by asymmetric division would allow the high cell turnover of the epidermis to be sustained whilst preserving the genomic integrity of stem cells.

An alternative model derived from mathematical analysis of clone sizes *in vivo* postulates that the epidermis is maintained by a single type of quickly proliferating progenitors with high replicative potential (Clayton et al., 2007). However, it is unclear how this model explains the behaviour of proliferative epidermal cells isolated *in vitro*; after 7 days in culture whole epidermal cell isolates form a mixture of holoclones (large, still growing and <5% of cells terminally differentiated), intermediary (meroclones) and paraclones (small, <15 cell doublings, all cells terminally differentiated; Barrandon and Green, 1987). Expression of the epidermal stem cell marker p63 is high in holoclones, low in meroclones and undetectable in paraclones (Pellegrini et al., 2001).

Keratinocytes in the spinous layer extrude thin cytoplasmic extensions that interconnect via desmosomes. Migration to the spinous layer is associated with downregulation of α6β4 integrin, which binds basal cells to laminin of the DEJ (Sonnenberg et al., 1991). In the upper spinous layer, cells build lipid-rich lamellar granules (Matoltsy and Parakkal, 1965; Odland and Holbrook, 1981). These organelles persist until their exocytotic release in the upper granular layer.

Lamellar granules contain lipids and acid hydrolases that form a hydrophobic 'cement' which fills intracellular space in the stratum corneum, presenting a firm barrier to the passage of water between the internal and external environments. Granular layer keratinocytes also form cytoplasmic non-membranous keratohyalin granules that represent aggregations of cornified envelope precursor material (Matoltsy and Matoltsy, 1970).

There are no blood vessels in the epidermis, so epidermal cells rely entirely on nutrient diffusion from the dermal microvasculature. Nutrient availability declines towards the superficial epidermis, where keratinocytes become flatter and less metabolically active. In their final stages of differentiation, keratinocytes undergo the coordinated autophagy of many cellular organelles (Morioka et al., 1999). This process produces corneocytes; tight-fitting, flattened anuclear cells connected by corneodesmosomes that possess a strong envelope of crosslinked proteins with an outer hydrophobic ceramide layer (Serre et al., 1991). Corneocytes are typically layered ten to twelve cells thick on the skin surface, providing physical protection and a highly effective barrier to water and exogenous microbes. The outermost layer of corneocytes continually sloughs off during desquamation, mediated by the enzymatic degradation of corneodesmosomes (Caubet et al., 2004).

The dermis is made up by a connective tissue matrix that is largely produced, maintained and inhabited by fibroblasts. Other cell types routinely found in the dermis include endothelial cells and pericytes of the dermal microvasculature, cutaneous nerves, myelinating Schwann cells, mast cells and macrophages. The heavily vascularised, lipid rich subcutaneous tissues beneath the dermis are important for nutrient storage, thermal insulation and endocrine secretion. The skin also comprises special structures that traverse multiple skin layers. Major structures are eccrine sweat glands and hair follicles, with their associated arrector pili muscles and apocrine sebaceous glands.

1.12 Skin ageing

Young adult skin is smooth, elastic, supportive and resilient. However, the functionality of skin decreases progressively with advancing years following maturity (Sehl and Yates, 2001). Skin ageing occurs through two pathways; intrinsic ageing and extrinsic ageing, which produce overlapping spectra of symptoms but develop under the influence of distinct processes.

Skin, like all other organs, is subject to the intrinsic processes of biological ageing. As the interface between organisms and the external environment, skin is conjointly affected by extrinsic factors. Extrinsic skin ageing broadly resembles intrinsic ageing clinically, but may be grossly accelerated in onset and increased in severity depending on the types and doses of extrinsic factors an individual's skin is exposed to. Causes of extrinsic ageing are both physical and chemical, primarily UVR from the sun and artificial sources such as tanning beds (Spencer and Amonette, 1998; Young, 2004) and tobacco smoke (Lahmann et al., 2001; Leung and Harvey, 2002).

Intrinsic ageing becomes prominent only in late life. Varani et al. (2000) studied a panel of skin function markers in human cohorts, finding that significant functional decline was detectable in some markers amongst their 60-79 year old group and pronounced in all markers amongst their 80+ year old group in sun protected skin compared to their young (18-29 year old) and intermediate (30-59 year old) age groups.

Intrinsically aged skin is typically finely lined and somewhat friable compared to young skin, with a moderately thinner dermis and epidermis. In contrast, a high level of exposure to extrinsic ageing factors may produce profound changes at relatively young ages. Extrinsically aged skin is typified by deep wrinkles, dryness and a leathery character (fig. 1-4). Intrinsic and extrinsic ageing can both impair functional and cosmetic aspects of skin including elasticity, immune responses, hydration and pigmentation.

Extrinsic ageing is accepted to be the cause of most serious clinical and cosmetic problems associated with skin ageing (Leyden, 1990; Taylor et al., 1990). The most prevalent and best studied cause of extrinsic ageing is UVR exposure. Skin ageing due to UVR (fig. 1-4) is termed photoageing, an important subset of extrinsic ageing.

Skin ageing is a universally recognisable visual cue for estimating the chronological age of an individual.Throughout much of the world, youth is perceived as the aesthetic ideal and a great number of individuals with discernable signs of ageing take active measures to appear younger. This interest supports a thriving multi-billion pound international cosmeceutical industry (Freedonia, 2006). A wide range of compounds have been identified as being effective in improving the function and appearance of aged skin. Amongst these compounds, retinoids are notable for their widespread usage and range of effects (section 1.18).

1.13 Ageing in the epidermis

In both sun protected and photoaged skin, changes to the gross structure of the epidermis are subtle (Lavker et al., 1989; Tagami, 2008). Several groups have reported epidermal thinning in intrinsically aged skin and flattening of the youthfully sinuous DEJ is well established (Marks, 1981; Lock-Andersen et al., 1997; Kirsten et al., 2002; Gambichler et al., 2006). Decreases in epidermal thickness in intrinsic ageing are reported to be gradual and essentially linear following maturity at age 25-30 years (Branchet et al., 1990; Kirsten et al., 2002). However, some studies have reported no change in epidermal thickness with intrinsic ageing, whilst others have found divergent responses with gender and anatomical location (Whitton and Everall, 1973; El-Domyati et al., 2002; Sandby-Moller et al., 2003). It therefore appears that whilst there is a net trend towards decreasing epidermal thickness with intrisic ageing, it is not uniformly consistent. Epidermal thickness has a variable response in photoageing, usually exhibiting atrophy in white Caucasian skin (Fitzpatrick skin type I-II), but hypertrophy in darker skin types (Fitzpatrick skin type III-IV; Yaar and Gilchrest, 2007).

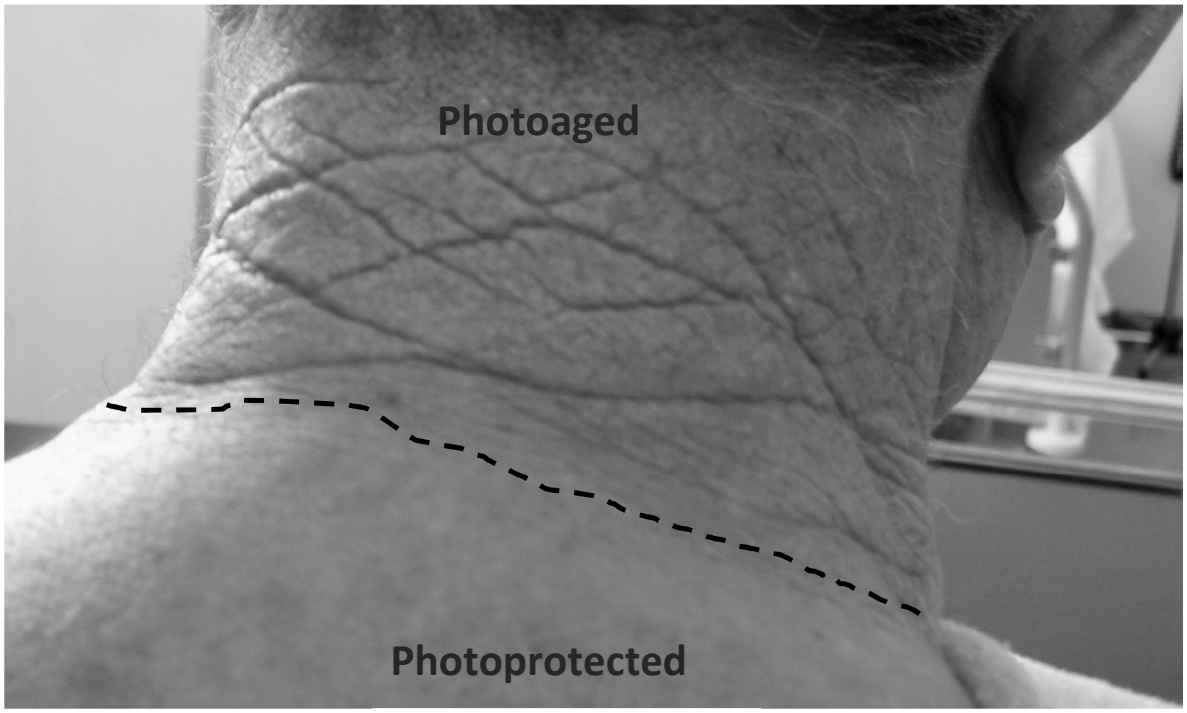


Figure 1-4. Clinical photograph to illustrate photoageing. *The regularly sun exposed neck of an older male subject presents extensive deep wrinkles, while photoprotected lower neck and upper back skin is much less wrinkled. Image courtesy of Dr. Tsoureli-Nikita, Athens.*

In sun protected epidermis, proliferation is reduced and the keratinocyte maturation process lengthens from approximately 20 days on average in young volunteers to approximately 30 days in aged skin (Grove and Kligman, 1983; Engelke et al., 1997; Giangreco et al., 2008). The rate of decline in keratinocyte genesis is most pronounced following young adulthood until age 50 years, stabilising thereafter.

Interestingly, despite lower proliferation in the intrinsically aged epidermis, stem cell frequency does not appear to alter with age in either mouse or human epidermis (Engelke et al., 1997; Giangreco et al., 2008). Epidermal stem cells are however reported to be depleted in photoaged human skin (Kwon et al., 2008). Epidermal stem cell function is stable between young adult and aged mice; with no significant changes in telomere length or expression of cell cycle markers *in vivo,* or differentiation of clones *in vitro* (Stern and Bickenbach, 2007). Whether the replicative capacity or proliferation rate of murine epidermal stem cells show any decrease with age is not clear, with contrasting reports (Stern and Bickenbach, 2007; Charruyer et al., 2009). In mouse models of photoageing, cyclobutane pyrimidine dimers and (6-4) photoproducts accumulate in stem cells, which represent UVR-induced lesional alterations to DNA structure (Nijhof et al., 2007).

Array analysis of young adult and aged murine stem cells revealed no significant differences, however transit amplifying cells show differential gene expression with age (Liang et al., 2004; Stern and Bickenbach, 2007). Greater numbers of transit amplifying cells with decreased proliferation rates and extended cell cycle durations are found in aged, compared to young adult murine skin (Charruyer et al., 2009).

Uneven pigmentation and localised pigmented lesions frequently develop in aged and particularly in photoaged skin, where hyperpigmented solar lentigines or ‘age spots’ are common (Hodgson, 1963).

Dendritic antigen-presenting Langerhans cells decrease in number in both intrinsic ageing and photoageing in human epidermis (Gilchrest et al., 1982; Thiers et al., 1984). Intrinsically aged skin is at higher risk of viral and fungal infection as well as tumours and is reduced in sensitivity to contact allergens, evidence for a less effective skin immune system in the aged (Thivolet and Nicolas, 1990; Sunderkotter et al., 1997).

1.14 Dermal collagen networks in skin ageing

The fibrillar collagens I and III are strong triple-helical macromolecules that comprise 70-80% of the dry weight of the healthy dermis, granting tensile strength and resilience (Oikarinen et al., 1985). Collagen content decreases gradually during intrinsic ageing, but more severely in chronically photoaged skin, which has been estimated to show 20-32% decreases in collagen (Schwartz et al., 1993; Kligman et al., 2000). Only 2% of amino acids in collagen show strong absorbance in the UVR spectrum (Reilly et al., 2007) and UVR degrades collagen slowly when compared with heat or enzymes (Cooper and Davidson, 1965; Menter et al., 2001). This implies that direct physical damage has a relatively minor role in depleting collagen. Instead, signalling mechanisms direct decreased synthesis (Griffiths et al., 1993; Talwar et al., 1995; Chung et al., 2001) and increased degradation (Fligiel et al., 2003) of collagen with increasing age.

UVR is known to induce matrix metalloproteinase (MMP) enzymes capable of degrading collagen in skin (Scharffetter-Kochanek et al., 1991; Fisher and Voorhees, 1996; Kahari and Saarialho-Kere, 1997; Ohnishi et al., 2000). Activating protein 1 (AP-1) is a transcription factor influential in the control of collagen synthesis and degradation. AP-1 is upregulated by UVR via the induction of c-Jun (Ryseck and Bravo, 1991). Phosphorylation of c-Jun at two serine residues by c-Jun N-terminal kinase (JNK) is required to fully activate AP-1 (Hibi et al., 1993).

UVR causes activation of Src and Ras kinases, triggering the phosphorylation and activation of JNK (Binetruy et al., 1991; Smeal et al., 1991; Devary et al., 1992). UVR therefore has a two-pronged effect in facilitating transcription of AP-1 responsive genes by inducing c-Jun and activating JNK. In the skin, AP-1 is involved in the induction of MMP-1 and other MMPs (Angel et al., 1987; Han et al., 2001), and repression of collagen I expression (Chung et al., 1996).

1.15 Dermal elastic fibres in skin ageing

Elastic fibres are highly conserved biopolymers responsible for elasticity, flexibility and resilience in skin and other organs (Thurmond and Trotter, 1996; Kielty et al., 2002). Elastic fibres are composed of amorphous elastin within a microfibrillar sheath, associated with proteins including emilin (Bressan et al., 1993) and microfibril-associated glycoprotein (Gibson et al., 1986; Midwood and Schwarzbauer, 2002). Mature elastic fibres are approximately five times as extensible as rubber bands of the same cross-sectional area (Rosenbloom et al., 1993).

Three distinct components of the dermal elastic fibre network can be demarcated (Cotta-Pereira et al., 1976). Oxytalan fibres (Fullmer and Lillie, 1958) composed of fibrillin-rich microfibrils extend from the upper papillary dermis to the DEJ. In the lower papillary dermis oxytalan fibres connect to elaunin fibres, which are more elastin-rich than oxytalan fibres (Schwartz and Fleischmajer, 1986) but contain less elastin than the deeper elastic fibres. Elaunin fibres may represent a sparser, less mature version of the dense elastic fibre band of the reticular dermis (Montes, 1996).

During intrinsic ageing the dermal elastic network gradually diminishes, oxytalan fibres recede and the density of deeper fibres is reduced. Elastic fibres in aged skin are less compact with a higher mean length and surface area (Robert et al., 1988; Frances et al., 1990), which may contribute to reduced skin tone.

The histochemical appearance of elastin in intrinsically aged skin can be mimicked by digestion with trypsin or elastase enzymes (Braverman and Fonferko, 1982). Elastin has a long molecular lifetime and turns over slowly *in vivo* (Shapiro et al., 1991; Ritz-Timme et al., 2003), perhaps leading to the accumulation of stochastic enzyme degradation. Elastin synthesis is normally low but constant throughout life in sun protected skin. It has been reported that dermal fibroblasts from donors aged over 70 years synthesise elastin less actively than fibroblasts from young adults (Sephel and Davidson, 1986).

A characteristic feature of photoaged skin is the formation of a dense conglomerate of elastic fibre-rich dermal material termed a solar elastosis (Chen et al., 1986; Mera et al., 1987; Calderone and Fenske, 1995; El-Domyati et al., 2002). Paradoxically, photoaged skin has a reduced elastic response despite having an overabundance of elastic fibres. This incongruity is unravelled by electron microscopy, which reveals that elastin in UVR-exposed rat skin is tangled and slack compared to the taut, linear fibres in non-UVR-exposed young rat skin (Imokawa et al., 1995).

Interestingly, the antioxidant system of the upper dermis is weak compared to the epidermis and lower dermis and shows a different profile in ageing. In both photoageing and intrinsic ageing, catalase activity increases in the epidermis but decreases in the dermis (Rhie et al., 2001). Glutathione reductase activity is increased in intrinsically aged epidermis, but not in the dermis. Other antioxidants show universal declines in ageing and not enough is known to predict the system as a whole. It is however clear that the upper dermis accumulates oxidatively-modified proteins during photoageing (Sander et al., 2002) and there is evidence that peroxidised lipids and glycation products localise to the upper dermis in photoaged skin (Jeanmaire et al., 2001; Tanaka et al., 2001).

UVR directly stimulates the elastin promoter via free radical production (Bernstein et al., 1995; Kawaguchi et al., 1997; Bernstein, 2002) and elastin is highly expressed in chronic photoageing at the mRNA level (Bernstein et al., 1994). In photoageing, degradation as well as synthesis of elastin becomes aberrant (Sellheyer, 2003).

Elastase enzymes are induced in human dermal fibroblasts by UVR (Fimiani et al., 1995) and mice deficient in elastase do not develop elastosis in response to ultraviolet B (UVB) irradiation (Starcher and Conrad, 1995). Neutrophils also produce elastase enzymes and are stimulated to migrate into the skin by UVR (Rijken et al., 2005). However, lysozyme is deposited in solar elastosis, where it appears to limit or prevent the enzymal degradation of elastin (Seite et al., 2006). NFκB is a transcription factor that regulates several MMPs (Sato and Seiki, 1993; Borghaei et al., 2004) including MMP-9 (Ricca et al., 2000), which is capable of breaking down elastin and fibrillin. UVR causes NFκB to dissociate from its inhibitory partner IκB and become active.

The second major structural component of elastic fibres, the fibrillin-rich microfibril, is regionally decreased in photoageing by lowered synthesis and increased degradation in oxytalan fibres. Watson et al. (1999) found that in photoaged, compared to photoprotected skin, epidermal fibrillin-1 mRNA transcription is reduced, whilst fibrillin-2 transcription is unaltered. Total dermal fibrillin-1 mRNA in the lower dermis increases in severely photodamaged skin (Bernstein et al., 1994). The specific loss of oxytalan fibres in the upper papillary dermis is supported by histological studies (Dahlback et al., 1990; Bernstein et al., 1994).

What happens to fibrillin in mid and lower dermal elastic fibres during photoageing is less clear. Some studies report decreased fibrillin immunostaining in dermal elastosis (Dahlback et al., 1990; Suwabe et al., 1999). Others have found increased staining (Chen et al., 1986; Werth et al., 1996).

Fibrillin may be subject to epitope masking by proliferating elastin, causing immunostaining techniques to underestimate its abundance in photoaged skin (Schwartz et al., 1993; Kligman et al., 2000). MMPs capable of degrading fibrillin (MMPs 2, 9, 12, 13 and 14; Ashworth et al., 1999) are known to be increased in photoageing, as are serine proteases produced by infiltrating neutrophils (Kielty et al., 1994).

1.16 Nuclear hormone receptors

Nuclear hormone receptors (NHRs) are protein transcription factors that bind to responsive DNA elements to activate or repress gene transcription. A total of 48 human NHRs have been identified (www.nursa.org), which may be broadly subdivided into two classes. In the absence of ligand, type I NHRs bind to heat shock proteins in the cytoplasm. Ligand binding causes heat shock proteins to dissociate from type I NHRs, allowing the receptors to form homodimers and translocate to the nucleus.

Type I NHRs include the estrogen, progesterone, androgen, glucocorticoid and mineralocorticoid receptors. Type II NHRs include retinoic acid receptors (RAR; α, β and γ), retinoid X receptors (RXR; α, β and γ), triiodothyronine receptor (TR; α and β), peroxisome proliferator-activated receptor (PPAR; α, β, δ and γ), vitamin D receptor, farnesoid X receptor (FXR) and liver X receptor (LXR; α and β). Type II receptors, usually either nucleoplasmic or bound to genetic response elements when ligands are absent, bind co-repressors (Chen and Evans, 1995; Robyr et al., 2000) and exchange these for co-activators upon binding to ligands in the nucleus (Perissi and Rosenfeld, 2005). Inhibiting co-repressors increases type II NHR activity (Nagy et al., 1997).

Ligand binding induces type II NHRs to form either heterodimers with RXR or less commonly homodimers (Zhang et al., 1992; Kliewer et al., 2002). 9-*cis*-retinoic acid (9-*cis*-RA), a ligand with high affinity for RXR (Heyman et al., 1992; Mangelsdorf et al., 1992; Allenby et al., 1993) induces the formation of RXR homodimers in addition to RAR/RXR heterodimers (Zhang et al., 1992). Some synthetic retinoids specifically induce RXR homodimers (Lehmann et al., 1992). LXRs, however, are thought to require an RXR partner for efficient action (Lehmann et al., 1997).

Different NHR/RXR heterodimers may bind to a range of response elements with variable affinities, whilst over 300 NHR coregulators have been identified to date that act to promote or repress the transcriptional activity of NHRs (Lonard and O'Malley, 2007). Such diverse regulatory input allows a limited number of NHRs to execute complex transcriptional activities.

Microarray analysis found 319 loci that were significantly up- or down-regulated in wild type, but not in LXR$\alpha^{-/-}$/LXR$\beta^{-/-}$ mice after 7 day dietary supplementation with LXR agonists (Stulnig et al., 2002). Approximately the same number of loci are estimated to be regulated by RARs in mice (Glozak et al., 2003). Comparative bioinformatic analyses of the mouse and human genomes detect that the number of protein coding genes is similar in each species. Many NHR gene targets are themselves transcription factors, enabling NHRs to potentially initiate broad, cascading effects throughout the transcriptome and proteome.

Nuclear hormone receptors and particularly PPARγ have been implicated in CR responses, where NHR activity is modulated by the common regulators PPARγ coactivator 1 (PGC-1) α and β. The PGC-1 proteins are convergent downstream targets of FOXO transcription factors, SIRT-1 and other longevity-associated signalling pathways. PGC-1 α and β are NHR co-factors capable of regulating the activity of multiple NHRs to modify diverse functions including metabolism, xenobiotic detoxification and stress resistance (Corton and Brown-Borg, 2005). Interestingly, significant overlap has been shown in microarray profiling of mouse liver between CR and agonists of the NHRs PPARα, LXR and RXR (Corton et al., 2004).

1.17 Liver X receptors

The nematode worm *Caenorhabditis elegans* has been a successful platform for the identification of various genes and proteins involved in the control of lifespan (Kenyon, 2005). The longevity-associated gene *daf-12* encodes an NHR (Antebi et al., 2000) involved in regulating developmental transitions. Comparative studies suggest that *daf-12* is homologous to several human NHRs, the closest relatives being LXR α and β, Pregnane X Receptor (PXR) and Vitamin D Receptor (Mooijaart et al., 2005).

In the nematode, *daf-12* is expressed in the epidermis during early life. In adult worms *daf-12* expression is low or undetectable. Upstream regulators of *daf-12* reside in two major pathways homologous to the mammalian transforming growth factor-β and insulin systems (Snow and Larsen, 2000). Genetic manipulation of *daf-12* can substantially extend the mean and maximum lifespan of nematodes carrying specific mutant alleles for *daf-2*, an insulin/IGF-1 receptor homologue (Larsen et al., 1995; Gems et al., 1998). Additionally, lifespan extension of genetically normal nematodes by laser germline ablation requires functional *daf-12* (Hsin and Kenyon, 1999; Gerisch et al., 2001).

Both LXR and PXR form heterodimers with RXR and are involved in sensing and regulating the metabolism and transport of lipids (Peet et al., 1998; Venkateswaran et al., 2000; Chawla et al., 2001), processes that *daf-12* also mediates (Motola et al., 2006). LXR ligands include oxysterols such as: 22(R)-hydroxycholesterol; 27-hydroxycholesterol; 24(S),25-epoxycholesterol (Janowski et al., 1996; Lehmann et al., 1997; Fu et al., 2001); cholesterols and other products of mevalonate metabolism (Forman et al., 1997). PXR has a wide range of ligands, many of which are xenobiotic (Kliewer et al., 2002). Glucose has been reported to interact with LXR and modify its activity both by direct binding with high affinity as a ligand (Mitro et al., 2007) and via glycosylation (Anthonisen et al., 2010).

LXR targets include a range of genes involved in lipid and glucose metabolism (Stulnig et al., 2002; Laffitte et al., 2003). LXR and PXR are both implicated in cutaneous development from studies in rat foetal epidermis, where LXR and PXR agonists induce differentiation and cornification (Hanley et al., 1999). LXRα/RXR heterodimers bind to LXR response elements. These elements are activated by LXRα/RXR in response not only to LXR-specific ligands, but also to 9-*cis*-RA and possibly other retinoids (Willy et al., 1995). MMP-9 is repressed by LXR in macrophages (Castrillo et al., 2003; Joseph et al., 2003), so LXR may further influence extracellular matrix turnover.

Interestingly, activation of LXR decreases amyloid β peptide secretion by neural cell lines engineered to overexpress amyloid precursor protein. Drawing from these results, LXR activation was speculated to perhaps be useful in reducing the risk of age-related Alzheimer's disease (Sun et al., 2003). However, further study showed that this effect of the LXR agonist used (T0901317) was independent of LXR activation and only relevant at much higher agonist concentrations than are necessary to stimulate LXR activity (Czech et al., 2006).

LXRα and LXRβ are both expressed in human skin and have been reported at both the mRNA and protein level (Russell et al., 2005; Ford et al., 2009). LXR activation is anti-proliferative in human keratinocytes (Russell et al., 2005) and anti-inflammatory in mouse models of inflammation (Fowler et al., 2003). In human keratinocytes *in vitro*, oxysterol or non-steroidal agonist activation of LXR induces the AP-1 proteins c-Fos, Jun-D and Fra-1. This has the effect of increasing involucrin levels, promoting differentiation and stimulating tissue transglutaminase cross-linkage of cornified layer constituent proteins (Schmuth et al., 2004). In human skin, LXR activation stimulates the synthesis and extracellular processing of lipids, implicating LXR as a possible regulator of barrier function homeostasis (Man et al., 2006).

The broad spectrum NAD-dependent deacetylase SIRT-1 promotes the deacetylation of LXRβ and increases its transcriptional activity (Li et al., 2007). LXRα is transcriptionally up-regulated by the SIRT-1 agonist resveratrol in macrophages *in*

vitro (Sevov et al., 2006). LXRα has been associated with human lifespan in a longitudinal genetic study of a cohort aged 85 years at baseline who were monitored for 6–8 years (Mooijaart et al., 2007). One LXRα haplotype was found in this study to be moderately protective against mortality from all causes and highly protective against mortality caused by infectious disease.

Human keratinocytes UVR-irradiated *in vitro* display down-regulated LXR signalling; whilst an albino hairless UVR-irradiated mouse exhibits dose-dependent decreases in skin thickness in response to LXR agonists (Chang et al., 2008). Microarray analyses have revealed that alterations in gene expression between normal and LXRβ$^{-/-}$ mouse skin notably resemble changes between young and aged human skin (Ly et al., 2000; Chang et al., 2008). These findings give rise to the hypothesis that LXRβ signalling may be reduced in ageing skin. Interestingly, a low protein maternal diet in mice produces pups with prenatal hypermethylation at the LXRα promoter in liver and intestine and reduced expression of the LXR target genes ATP-binding cassette G (ABCG)-5 and 8 (van Straten et al., 2010).

LXRα is the dominant LXR subtype throughout most of the mammalian body. LXRα$^{-/-}$ mice and LXRα$^{-/-}$/LXRβ$^{-/-}$ mice are similarly impaired in cholesterol and bile acid metabolism, while LXRβ$^{-/-}$ mice show a less severe phenotype (Peet et al., 1998; Schultz et al., 2000). In murine skin, however, LXRβ is the primary mediator of oxysterol responses. Topical oxysterol treatment produces a pro-differentiative, anti-proliferative response in mouse skin that is abolished in LXRβ$^{-/-}$ mice but remains in LXRα$^{-/-}$ mice (Komuves et al., 2002).

LXRβ$^{-/-}$ mice have a notable epidermal phenotype, with a thinner epidermis, reduced basal layer proliferating cell nuclear antigen (PCNA) expression and decreased expression of the differentiation markers loricrin and fillagrin compared to both wild type and LXRα$^{-/-}$ mice (Komuves et al., 2002). For these reasons, the work reported herein focused on LXRβ rather than LXRα. As well as their skin phenotype, LXRβ^{-}/$^{-}$ mice develop adult-onset central nervous system motor defects associated with lipid accumulation in the spinal cord (Andersson et al., 2005). A theoretical model of LXRs and ageing is shown in fig. 1-5.

Figure 1-5. Schematic of potential interactions between LXR signalling and ageing. *In this theoretical model, LXR acts as a nutritional sensor that influences resource allocation to defence and repair mechanisms dependent on food intake and nutritional status. 'Other exogenous factors' includes microbes, UVR and inhaled substances. 'Physiological defence and repair mechanisms' may include autophagy, DNA repair, hormonal responses, extracellular matrix remodelling, antioxidant systems, mitochondrial regulation, protein refolding, metabolic adjustments and the modulation of immune responses. For the purposes of this model, green objects and connections are broadly considered beneficial, orange neutral and red detrimental in the context of ageing. This model represents one interpretation of the literature at present and may be refined by future studies.*

1.18 Retinoids

Retinoids are small hydrophobic molecules related to vitamin A (retinol), which are widely used to treat skin ageing. All-*trans*-retinoic acid (*t*-RA) is used topically in the treatment of acne vulgaris, parakeratosis and hyperkeratosis (Roos et al., 1998) and is considered the clinical 'gold standard' for the treatment of photoaged skin (Samuel et al., 2005). The first indications of *t*-RA's action against ageing came from a swathe of anecdotal reports from facial acne patients, who noticed reduced wrinkling after using such prescribed creams.

These observations spurred scientific investigation. Kligman et al. (1984) examined skh-1 hairless mice, irradiating them for ten weeks with sun lamps to mimic photoageing. Irradiated mice treated topically with 0.05% *t*-RA possessed more intact collagen networks and more numerous, active fibroblasts than vehicle treated controls. Weiss et al. (1988) found in humans that daily use of *t*-RA for four months improved the histological appearance of photoaged skin in >95% of their subjects.

Kafi et al. (2007) treated the photoprotected, intrinsically aged skin of volunteers aged ≥80 years with 0.4% retinol for 24 weeks. Fine wrinkling and tactile roughness were significantly lower after 8 weeks compared to a vehicle treatment control and continued to improve until 24 weeks treatment. Measures returned to baseline 24 weeks after the cessation of treatment. Extracellular matrix production is stimulated by retinoid application to cultured primary human fibroblasts from both sun protected and sun exposed skin (Varani et al., 1998).

Retinoids promote collagen turnover, replacing damaged molecules (Kligman et al., 1984). Treatment of photoaged mouse models for 10 weeks with *t*-RA increased mRNA for collagen I and procollagen III by 200% (Schwartz et al., 1991; Kim et al., 1992). In a human trial, collagen I levels were up-regulated 80% by 10-12 months *t*-RA treatment in photodamaged skin. Collagen I levels in skin treated with vehicle alone for the same time decreased by 14% (Griffiths et al., 1993).

All-*trans*-RA application reduces AP-1 activity and suppresses MMP induction following UVB irradiation of human skin (Fisher and Voorhees, 1996). This appears to be due to suppression of MAPKK4 by transcriptional upregulation of MKP-1 (MAP kinase-phosphatase-1; fig. 1-5; Lee et al., 1999). In sun protected older skin, retinoids are active in suppressing the collagen degrading enzymes MMP-1 and MMP-9, although MMP-2 expression is unaffected (Varani et al., 2000).

In vitro, *t*-RA and 9-*cis*-RA reduce MMP-1 transcription in normally cultured rabbit fibroblasts (Pan et al., 1995). Pre-treatment of human skin with topical *t*-RA prior to UVB irradiation strongly represses declines in procollagen synthesis following acute UVB exposure (Fisher et al., 2000). Transcription of c-Jun is also repressed in skin treated with *t*-RA before UVB irradiation (Fisher et al., 1998; Fisher et al., 2000).

Retinoids stimulate the repair of photoaged elastic fibres. Photoaged skin shows distinct improvements after retinoid treatment; amorphous elastin content decreases and elastic fibres become more structurally defined (Feldman et al., 1991; Tsukahara et al., 1999; El-Domyati et al., 2004).

All-*trans*-RA inhibits upregulation of elastin transcription in cultured human fibroblasts *in vitro* following UVB exposure (Lee et al., 1998). Systemic application of *t*-RA has been found to reduce plasma levels of the elastin degrading enzyme MMP-9 and increase tissue inhibitor of metalloproteinase-1 (TIMP-1) levels in patients with chronic respiratory disorders (Frankenberger et al., 2001; Mao et al., 2003). MMP-9 protein expression is reduced to youthful levels by retinoid treatment in intrinsically aged skin from subjects ≥80 years of age (Varani et al., 2000).

Transfecting RARα into a fibroblastic cell line either transiently (Ratnayaka et al., 2003; Watson et al., 2004) or stably (Ratnayaka et al., 2004) increases the expression of MMP-1; the major fibrillar-collagenolytic enzyme in skin. This effect was observed in cells grown in delipidated medium, which is depleted in retinoids. RARα is therefore implicated as a driver of extracellular matrix degradation in ageing, with ligand independent activity. Addition of *t*-RA to RARα-over expressing

fibroblastic cell line cultures reduces RARα and MMP-1 expression (Ratnayaka et al., 2004; Watson et al., 2004).

1.19 NHR expression in ageing

The expression of a number of type II NHRs alters with ageing in various organs. RARγ and TR mRNA are decreased in peripheral blood mononuclear cells from aged subjects (Feart et al., 2005). RARβ, RXRβ, RXRγ and TR mRNAs are decreased in aged rat brain (Enderlin et al., 1997; Enderlin et al., 1997). The expression of neurogranin and the activity of tissue transglutaminase also decreases. Gastric intubation of elderly rats with *t*-RA 24 hours before sacrifice up-regulates mRNA for all three NHRs and returns neurogranin expression and tissue transglutaminase activity to levels found in the young (Enderlin et al., 1997; Enderlin et al., 1997).

RARβ and TR mRNA expression levels and the maximum binding capacity and ligand affinity of these receptors decrease in the liver of aged rats. The activities of malic enzyme and tissue transglutaminase, transcriptional targets of TR and RARβ, also decrease. UVB causes an acute, severe depletion of RARγ and RXRα mRNAs and proteins in human skin *in vivo* (Wang et al., 1999) and keratinocytes *in vitro* (Boudjelal et al., 2000). This depletion can be limited by pre-treatment with *t*-RA and is due in part to proteasomal degradation of the NHR proteins.

RARα expression in epidermal keratinocytes increases almost twofold in the sun protected skin of 65-75 year old individuals as compared to 20-28 year old subjects (Watson et al., 2004).

1.20 Project hypotheses

It was decided to study LXRβ in skin ageing because of its relationship to the longevity regulator *daf-12* in the nematode worm (Mooijaart et al., 2005), the association between LXRα and mortality at old ages in humans (Mooijaart et al., 2007), the dominance of LXRβ as opposed to LXRα in skin (Komuves et al., 2002) and the reported overlap between gene expression patterns in LXRβ$^{-/-}$ mice and models of human skin ageing (Chang et al., 2008). Skin photoageing represents a model of accelerated ageing and alterations in the expression of other nuclear hormone receptors having previously been reported to be similar in both intrinsic skin ageing and photoageing (Watson et al., 2004). LXRβ$^{-/-}$ mice show epidermal thinning (Komuves et al., 2002) similar to that seen during intrinsic ageing and, for many lighter skinned individuals, in photoageing of human skin. The hypothesis driving these aspects of the study was therefore that LXRβ expression would be decreased in human skin ageing and photoageing.

Retinoids exert powerful corrective effects on aged skin, while nuclear hormone receptor signalling pathways are thought to show extensive cross-talk including the direct transcription of other family members. A further hypothesis was that LXRβ expression would increase following the retinoid treatment of photoaged skin. The fourth and final hypothesis underlying this study was that, given the reported overlaps in gene expression patterns between LXRβ$^{-/-}$ mouse skin and models of human ageing, that significant overlaps would also exist between LXR agonist treatment, ageing and CR in mice.

1.21 Statement of hypotheses

1. LXRβ expression will be decreased in intrinsically aged human skin.

2. LXRβ expression will be decreased in photoaged human skin.

3. LXRβ expression will be increased after *t*-RA treatment of photoaged human skin

4. Gene expression patterns will significantly overlap between LXR agonist treatment and **i)** ageing; **ii)** CR in mice

2. Methods and materials

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK).

2.1 Volunteer recruitment

To assess LXRβ expression in human skin, volunteers were recruited for skin biopsies. All studies were approved by The Salford and Trafford Research Ethics Committee and volunteers gave written informed consent. Biopsies were obtained under 1% lignocaine local anaesthesia. Volunteers were recruited under ethics study codes 09/H1006/23 (photoageing study for all experiments and intrinsic ageing study for qPCR and *in situ* hybridisation); 09/H1004/68 (retinoid treatment study for all experiments); and 07/Q1404/7 (intrinsic ageing study for *in situ* hybridisation and immunofluorescence). Volunteers for the Affymetrix® study described in this chapter were also recruited under ethics code 09/H1006/23. Volunteers were Fitzpatrick skin types I-III.

To assess the impact of intrinsic ageing on LXRβ expression, volunteers were recruited in two age groups; 18-30 years (n=19) and 65-75 years (n=19). Punch biopsies (6 mm diameter) were taken from the photoprotected buttock to isolate intrinsic ageing changes from extrinsic ageing. These volunteers were allocated to experiments as follows: immunofluorescence and *in situ* hybridisation n=14; quantitative polymerase chain reaction (qPCR) n=18; Affymetrix® n=6.

To study photoageing, volunteers were recruited aged 52-76 years (n=9; 4 men and 5 women). The use of elderly individuals was possible because results from the intrinsic ageing studies showed that LXRβ mRNA expression does not differ significantly between young and aged individuals. Photoageing of volunteers was assessed using the 9 point photonumeric scale (Griffiths et al., 1992). Applicants graded >5 on this scale at the forearm were accepted to the study. 6 mm punch biopsies were taken from the photoprotected buttock, upper inner arm and photoexposed forearm.

The upper inner arm was included as an anatomical site control, allowing any differences between buttock skin and arm skin unrelated to photoexposure to be identified.

Recruitment and biopsies for these studies were performed by research nurse Gillian Aarons, with the exception of the tissue used for intrinsic ageing *in situ* hybridisation and immunofluorescence experiments, which was kindly donated by Dr Michael Sherratt (Regenerative Biomedicine, The University of Manchester).

To study the effects of topical retinoid treatment on LXRβ expression, 2 male and 7 female photoaged volunteers were recruited aged 42-79 years. Applicants with photoageing of the forearm graded >5 on the 9 point photonumeric scale (Griffiths et al., 1992) were accepted to the study. Baseline (occluded but untreated) or 0.025% *t*-RA (Retin-A®; Janssen-Cilag Ltd., Beerse, Belgium) was applied to photoaged extensor forearm under 6 mm diameter Finn chambers on Scanpor tape (Epitest, Tuusula, Finland). The study was 4 days in length. On day 4 Finn chambers were removed and 3 mm punch biopsies were taken from each site. Clinical assessment of photoageing, recruitment and biopsies were performed by research nurses June Bowden and Jean Bastrilles.

2.1.1 Skin biopsy procedure

After verifying that the volunteer understood the procedure and obtaining written consent, lignocaine anaesthetic was administered to the biopsy site using a syringe (fig. 2-1a). After 2-3 minutes, when the volunteer reported that they were unable to feel pain on pricking the area with a needle, a 6 mm diameter biopsy punch was employed to produce a circular section of tissue (fig. 2-1b). The tissue was lifted with forceps and excised using surgical scissors (fig. 2-2a). Finally, the wound was closed using stitches (fig. 2-2b).

Figure 2-1. Illustration of the skin biopsy procedure. *Lignocaine local anaesthetic was first administered to the biopsy site by syringe (**a**). A few minutes later, the site was tested using a sharp implement and the volunteer reported whether they could feel pain or only pressure. When the volunteer could no longer sense pain at the biopsy site, a 6 mm diameter punch biopsy was used to cut around the tissue (**b**).*

Figure 2-2. Illustration of the skin biopsy procedure, second part. *After applying a punch biopsy to cut around a section of tissue (fig. 2-1), forceps were used to lift the biopsy section above the skin surface and the biopsy was excised from underlying tissue using scissors (**a**). Finally, the wound was closed using stitches (**b**).*

2.2 Tissue processing

Biopsied tissue may be processed in various ways depending on the end application. The processing methods used in this study are outlined below:

2.2.1 Tissue processing for *in situ* hybridisation, immunohistochemistry and immunofluorescence

Tissue and epitope integrity are progressively compromised by microbial digestion and endogenous processes after removal from the body. Tissue is commonly preserved for *in situ* hybridisation and immunostaining techniques by either rapid freezing, or formalin fixation and wax embedding. Both techniques serve to harden the tissue so that it can be effectively cut into thin sections. Wax embedded tissue tends to have better preservation of tissue morphology. However wax embedding often impedes immunological staining by masking epitopes and commonly mandates antigen retrieval procedures.

Frozen sections were used throughout this project for *in situ* hybridisation, immunohistochemistry and immunofluorescence. Following biopsy, tissue samples were embedded in optimal cutting temperature compound (Tissue-Tek®; Miles Laboratories, Elkhart, IN, USA) and snap frozen in liquid nitrogen. Biopsies were cut to 7-10 µm thick sections by cryostat microtome (Bright Instruments, Huntigdon, UK) and fixed to glass microscope slides. Slides and remaining biopsy tissue were stored at -70°C.

2.2.2 Tissue processing for qPCR

RNA is exceptionally labile among cellular constituents. To prevent rapid degradation of RNA following excision, biopsies were immediately embedded in optimal cutting temperature compound (Tissue-Tek®; Miles Laboratories, Elkhart, IN, USA) and snap frozen in liquid nitrogen.

2.2.3 Tissue processing for Affymetrix® microarrays

The following work was performed by Dr. Abigail Langton (Dermatological Sciences, The University of Manchester, UK). Immediately following excision, biopsies for RNA extraction for Affymetrix® microarrays were submerged in RNA*later* solution (Invitrogen) to stabilise RNA against degradation. Biopsies were transported on dry ice and in <1 hour were frozen at -70°C.

2.3 Cell culture

The propagation of cells using *in vitro* culture systems is a fundamental mainstay of molecular biology, allowing a range of experimental manipulations that would be difficult or impossible to achieve using other models. Primary cells derived directly from animal or human tissue can be cultured but present practical difficulties, being subject to slow growth and the phenomenon of replicative senescence (Hayflick and Moorhead, 1961), whereby cells cease to divide after a finite number of population doublings. It is therefore more convenient to work with immortalised cell lines that have been oncogenically transformed to produce rapid, indefinite growth. In this project, HaCaT (keratinocyte) and COS-1 (fibroblast) cell lines were used. Both cell lines are fast growing and thrive in a standard culture medium at 37°C with 5% CO_2. To limit the possibility of microbial contamination, cell culture work was carried out in a class II laminar airflow biological safety cabinet and all equipment was sterilised with 70% ethanol.

2.3.1 Thawing frozen cells to culture

Frozen 1 ml aliquots of HaCaT (originally supplied by Dr NE Fusenig, German Cancer Research Center, Heidelberg, Germany) or COS-1 (European Collection of Cell Cultures, Porton Down, UK) cells were thawed from liquid nitrogen storage rapidly in a water bath at 37°C. Aliquots were added to a 25 cm^2 base surface area cell culture vessel with 4 ml of medium (Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 584 mg/L glutamine and 110 mg/L pyruvate (PAA Laboratories, Austria) and 10% foetal bovine serum (FBS; Invitrogen)) prewarmed to 37°C.

2.3.2 Maintenance of cell cultures

Cultures were incubated at 37°C with 5% CO_2 in a humidified incubator with twice weekly changes of medium. When 80% confluent, cultures were passaged 1:3 as follows: medium was aspirated and replaced with trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.5 g/L trypsin, 0.53 mM EDTA•4Na in phosphate buffered saline (PBS)), then cultures were incubated at 37°C for 5 minutes to detach adherent cells. Trypsin-EDTA solution was inactivated by the addition of medium to a final volume three times that of the original culture volume. The vessel contents were then transferred to a sterile vessel or vessels with three times the base surface area of the original vessel.

2.3.3 Freezing cells for long term storage

To store cell stocks for future use, active cultures were grown to 80% confluency and adherent cells were detached by replacing medium with trypsin-EDTA solution and incubating for 5 minutes at 37°C. Freezing medium (DMEM with 4.5 g/L glucose, 584 mg/L glutamine and 110 mg/L pyruvate (PAA Laboratories) and 30% FBS (Invitrogen)) prewarmed to 37°C was added at the standard culture volume. Vessel contents were then distributed to cryovials (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) as 1 ml aliquots. Cryovials were placed into an isopropanol bathed vessel and frozen gradually in a -80°C freezer overnight before transfer to liquid nitrogen storage.

2.4 Fixing cells and tissues

The choice of fixative greatly influences techniques including immunohistochemistry, immunofluorescence, immunocytochemistry and flow cytometry. Tissues are fixed to stabilise biochemical activity, inactivate degradative enzymes and protect against microbial degradation during the procedures. Two broad classes of fixative are in general use. Crosslinking fixatives such as paraformaldehyde (PFA) create covalent bonds between lysine residues of proteins (Gustavson, 1956). Coagulating fixatives such as ethanol, methanol and acetone precipitate proteins and denature tertiary structures (Ramos-Vara, 2005).

Coagulating fixatives compromise membrane integrity sufficiently to allow access to antibodies and other molecules. However, when using crosslinking fixatives it is necessary to subsequently permeabilise membranes to ensure intracellular accessibility.

Optimisation is necessary to determine the most suitable fixation protocol for any given antibody. Too little fixation can insufficiently stabilise the biochemical processes of the cell and lead to molecular alterations or structural damage throughout the staining procedure. Alcohol fixatives need time to fully permeabilise cell membranes, especially those bounding subcellular compartments. Too much fixation can damage epitopes or render them inaccessible to the antibody and increase background staining. In this project, tissue sections were fixed in PFA for 10 minutes or equivolumetric methanol:acetone solution for 5 minutes. Cells used in immunocytochemistry and flow cytometry are not as matrix-rich as tissue and were fixed for approximately 30% less time.

2.5 Immunohistochemistry

Immunohistochemistry is a versatile technique that enables the visualisation of proteins and other antigens *in situ*. Antibodies are employed to specifically bind antigens of interest following tissue fixation and membrane solubilisation. Antibodies are either directly conjugated to a label or indirectly labelled using a conjugated secondary antibody. Horseradish peroxidase (HRP) is a common label due to its ability to catalyse a reaction that results in the formation of a localised precipitate in chromogenic reagents. Endogenous peroxidases are highly expressed in skin (Coulomb et al., 1983). An excess of hydrogen peroxide is applied when staining skin to exhaust inherent peroxidase activity and prevent nonspecific staining.

2.5.1 Immunohistochemistry in frozen sections of human skin

Whole skin cryosections (7 µm) were thawed and briefly air dried. Sections were fixed in 50% v/v methanol:acetone solution and incubated with 0.6% (v/v) hydrogen peroxide in methanol for 30 minutes to inhibit endogenous peroxidase activity. Sections were blocked for 60 minutes in 1% bovine serum albumin (BSA)/1% normal goat serum in tris-buffered saline (TBS), then 5, 10 or 20 µg/ml mouse anti-human LXRβ monoclonal antibody (Perseus Proteomics, Tokyo, Japan) was applied overnight at 4°C in block. Sections were washed 2 x 5' in TBS and then incubated with 2° biotinylated antibody (Vectastain Elite ABC kit, Vector Laboratories, CA, USA) for 30 minutes followed by avidin-biotin complex reagent (Vectastain® Elite ABC mouse kit, Vector Laboratories) for 30 minutes. SG chromogen (Vector Laboratories) was used to visualise bound antibody complexes. Sections were briefly counterstained with nuclear fast red (Vector Laboratories), dehydrated through serial alcohols and cleared in xylene. Glass cover slips were mounted using Pertex mounting medium (Histolab, Gothenburg, Sweden) and photographs were acquired under brightfield microscopy (Keyence BZ-8000; Osaka, Japan).

2.6 Immunofluorescence

Immunofluorescence and immunohistochemistry (section 2.5) allow the assessment of the distribution of proteins or other molecules in tissue sections. Immunofluorescence is highly similar to immunohistochemistry, using a fluorescent label to localise antigens rather than a chromogenic reaction. Immunofluorescence is well suited to quantification by image analysis and so was chosen for use during this project. During immunofluorescence, tissue sections are fixed, membranes are solubilised and a primary antibody specific to the protein of interest is applied. This is followed by a secondary fluorochrome-conjugated antibody that can be visualised using a fluorescent microscope.

2.6.1 Immunofluorescence in frozen sections of human skin

Whole skin cryosections were thawed and briefly air dried. Tissue was fixed in 50% v/v methanol:acetone solution, washed 2 x 30 minutes in TBS then blocked in 1% BSA/1% normal goat serum for 60 minutes. 20 µg/ml mouse anti-human LXRβ monoclonal antibody (Perseus Proteomics) was applied overnight at $4^{o}C$ in block. Following 2 x 5 minute TBS washes, sections were incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated anti-IgG antibody (Dako, Denmark) diluted 1:10 from stock for 30 minutes and washed 2 x 5 minutes in TBS. Slides were mounted with glass cover slips using aqueous mounting medium and photographed immediately under a fluorescent microscope under constant camera conditions (Keyence BZ-8000). Protein expression was quantified by image analysis (ImageJ; NIH, Bethesda, MD, USA). Negative controls were performed omitting primary antibody.

2.7 Protein extraction

A variety of downstream applications rely on the efficient extraction of proteins from cells and tissues. The majority of proteins can be extracted using a detergent solubilisation method. Certain proteins such as resilient components of the cytoskeleton are resistant to detergent solubilisation and may require more aggressive extraction methods. However, the detergent buffer used should have been efficient in solubilising the cytoplasmic and nucleoplasmic proteins examined in this project. High molecular weight contaminants including extracellular matrix and cytoskeletal components were removed by centrifugation following protein extraction.

2.7.1 Formulation of protein extraction buffer

The extraction buffer used in this project is a form of modified radioimmunoprecipitation assay (RIPA) buffer. RIPA buffer is a common term in the literature; however a great deal of variation exists between RIPA buffer formulations used by different groups. The formulation used in this project comprises salt (NaCl) and a buffering agent (HEPES) to maintain physiological tonicity and pH, detergents for membrane and protein solubilisation (sodium dodecyl sulfate (SDS) and Triton X-100) and protective agents (aprotinin, NaF and $NaVO_4$) to prevent enzymatic modification or degradation of proteins during extraction.

2.7.2 Protein extraction from cells

Adherent cells were grown at 37°C under 5% CO_2 until 80% confluent. Medium was aspirated and ice cold RIPA buffer (120 mM NaCl; 25 mM HEPES; 1% (v/v) Triton X-100; 25 mM NaF; 1 mM NaVO4; 0.2% (w/v) SDS; 1 KU/ml aprotinin (Calbiochem, CA, USA) was added at 24 µl/cm^2 culture vessel area. A cell scraper was used vigorously to detach adherent cells and the vessel contents were transferred to a 1.5 ml Eppendorf® tube. The solution was incubated on ice for 30 minutes, briefly inverting the tube at 10 minute intervals. Reactions were centrifuged at 12,000 x g for 15 minutes at 4°C to pellet large cell debris. The supernatant containing soluble proteins was transferred to a fresh 1.5 ml Eppendorf® tube and frozen at -20°C.

2.8 Protein quantification

To allow the normalisation of sample loading in Western blots, it is essential to determine the concentration of total protein in solutions. Several assays are commercially available to determine protein quantification, most of which produce a colorimetric change with a linear relationship to protein concentration, measured using an absorbance spectrophotometer. The bicinchoninic acid (BCA; Pierce, IL, USA) assay was found to produce consistent results for the purposes of this project.

2.8.1 Quantification of protein extracts using a relative standard curve

A series of standards were made from BSA at 0, 0.25, 0.5, 0.75, 1, 1.5 and 2 mg/ml. Standards and samples were pipetted to a 96-well plate in triplicate and incubated with BCA solution (Pierce). Absorbances were read at 540 nm using a Labtech LT-4000 plate reader (Labtech, East Sussex, UK). Total protein concentrations of samples were determined from the standard curve trendline (example shown in fig. 2-3) by rearrangement of the linear equation *y=mx+c*.

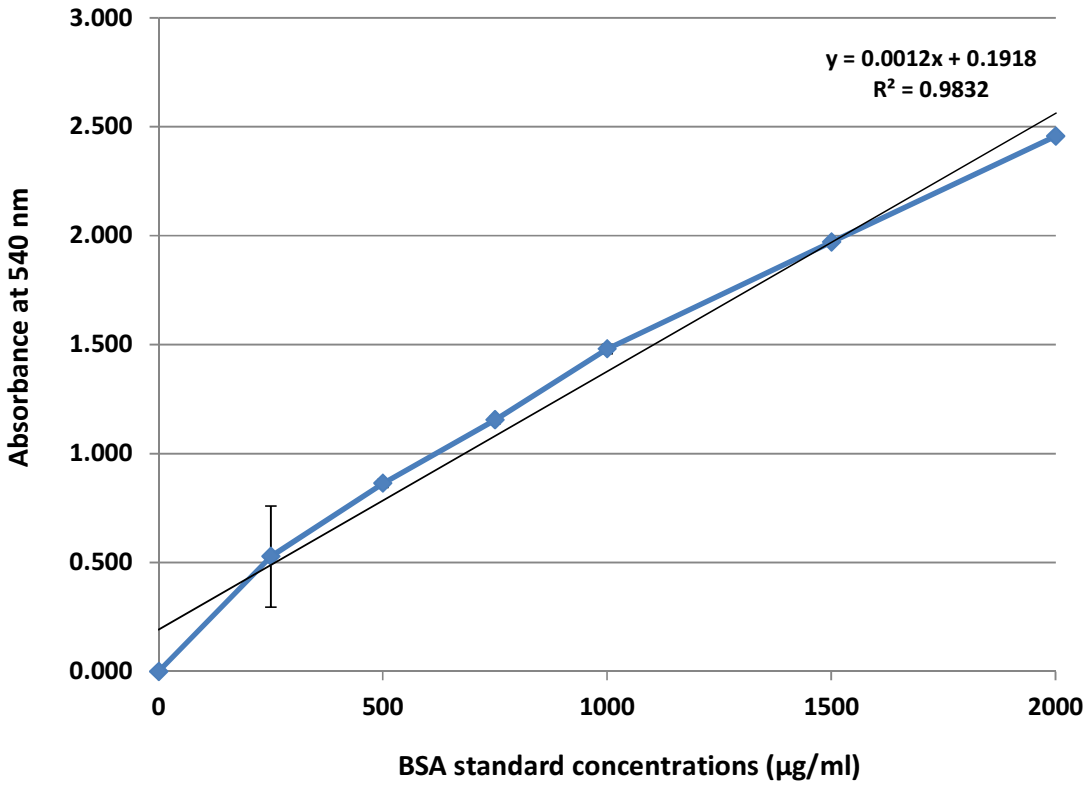


Figure 2-3. Example BCA assay standard curve. *The graph shows a trendline, the linear equation of the trendline in the form* y=mx+c *and the square of the correlation coefficient (R^2).*

2.9 Western blot

Western blots combine electrophoretic separation of proteins with immunological detection. This technique allows the qualitative assessment of whether a protein of interest is present in any given sample and can be used to compare relative levels of protein expression between samples.

2.9.1 Separation of proteins by gel electrophoresis

To separate proteins, an electric field is produced that applies a constant force to propel negatively charged proteins through a polyacrylamide gel from cathode to anode. Polyacrylamide gels form pores of an approximately constant size. Proteins with a higher frictional coefficient, i.e. those that are larger or less compact move more slowly than proteins with a low frictional coefficient. To limit the impact of protein conformation on migration speed, samples are denatured by boiling. Protein size is therefore the major determinant of migration speed.

2.9.2 Detection of bound antibody during Western blot

Following separation, proteins are transferred to a protein-binding membrane and probed using protein-specific antibodies. Bound antibodies are commonly visualised using chemiluminescence or fluorescence. In the reported work, a secondary HRP-conjugated antibody was employed to catalyse a chemiluminescent reaction (fig. 2-4). Light was detected with autoradiographic film.

The molecular weight of target proteins can be estimated by running samples alongside commercially available protein ladders, mixtures of a small number of proteins with a range of known sizes. To ensure equal loading of gels, a housekeeping control protein is probed alongside the proteins of interest. A separate gel can be run for this purpose. It is also possible to reprobe or simultaneously dual-probe a single membrane with very little loss of signal or increase in background staining. Reprobing or dual probing provides a more accurate loading control and conserves samples. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control throughout this project.

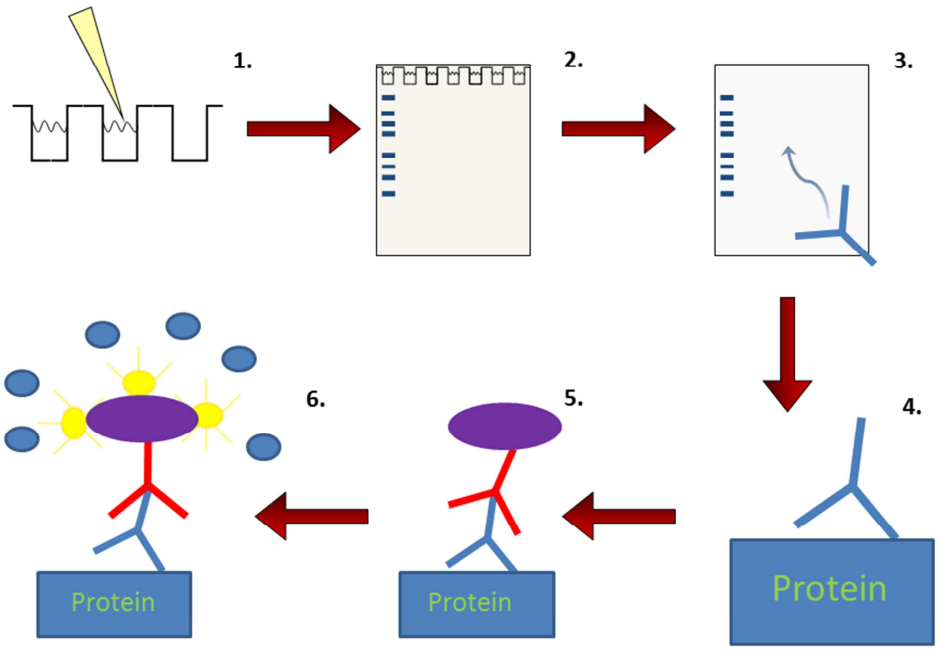


Figure 2-4. Diagram of enzyme-catalysed chemiluminescence. 1: *Samples are loaded to a polyacrylamide gel.* ***2:*** *The gel is run from cathode to anode to separate proteins according to size.* ***3:*** *Proteins are transferred to a polyvinyldifluoride membrane and the primary antibody (blue) is applied.* ***4:*** *The primary antibody binds to the protein of interest.* ***5:*** *The secondary antibody (red) is applied to the membrane, and binds to the primary antibody. The secondary antibody is conjugated to an enzyme (purple). 6: The chemiluminescent reagent is applied. The enzyme catalyses a localised reaction that causes the chemiluminescent reagent to produce light. The membrane is exposed to autoradiographic film to visualise chemiluminescence and show the location of the target protein on the membrane.*

2.9.3 Western blot for LXRβ in soluble protein extracts from human skin

12% polyacrylamide resolving mini gels (40% (v/v) ProtoGel (National Diagnostics, Atlanta, GA, USA); 375 mM Tris; 0.1% (w/v) SDS; 0.05% (w/v) ammonium persulfate (APS); 0.05% (v/v) tetramethyleneethylenediamine (TEMED)) were cast at 0.75 mm thickness. Once set, resolving gels were topped with 4% 'stacker' polyacrylamide gels (13.8% (v/v) ProtoGel (National Diagnostics); 125 mM Tris; 1% (w/v) SDS; 0.1% (w/v) APS; 0.08% (v/v) TEMED). Samples were mixed with an equal volume of Laemmli buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8; Laemmli, 1970), then samples and weight markers (prestained protein marker, broad range (6-175 kDa); New England Biolabs, Herts, UK) were boiled for 2 minutes. Gels were loaded with samples normalised to the same amount of total protein (3-12 µg) and weight markers, then run at 150 volts for 60 minutes in running buffer (25 mM Tris; 192 mM glycine; 0.1% SDS).

Proteins were transferred to PVDF membrane (Hybond-P; GE Life Sciences, Amersham, UK) at 100 volts for 60 minutes in transfer buffer (25 mM Tris; 192 mM glycine; 15% (v/v) methanol). Membranes were blocked overnight in 5% (w/v) casein / TBS (20 mM Tris; 137 mM NaCl) at 4°C. LXRβ mouse anti human monoclonal (Perseus Proteomics; 1:100 dilution) and GAPDH rabbit anti human polyclonal (AbCam, Cambridge, UK; 1:1000 dilution) antibodies were applied for 60 minutes in 5% (w/v) casein / TBS with 0.1% (w/v) NaN_3. Membranes were washed 3 x 5 minutes with TBS-T (5% (w/v) casein; 20 mM Tris; 137 mM NaCl; 0.5% (v/v) Tween-20). HRP-conjugated anti-IgG secondary antibodies (BioRad, Hertfordshire, UK) were applied at 1:5000 in TBS-T for 25 minutes. After 3 x 5 minutes TBS-T and 2 x 5 minute TBS washes, enhanced chemiluminescent reagent (ECL^+; GE Life Sciences; Amersham, UK) was applied for 90 seconds and membranes were exposed to autoradiographic films (Hyperfilm; GE Life Sciences). Films were processed using developer and fixer (Kodak, Hertfordshire, UK).

2.10 Oligonucleotide design

Nucleic acid binding is sequence dependent. Hydrogen bonds form between guanine (G) and cytosine (C), and between adenine (A) and thymine (T; DNA) or uracil (U; RNA). This specificity makes it possible to target a sequence of interest through the synthesis of a complementary sequence. An active commercial industry supports the synthesis of short (<160 bases) nucleic acid sequences termed oligonucleotides.

The National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) curates online databases of nucleic acid sequences. A large number of DNA and RNA sequences can be found by using the database search functions at http://www.ncbi.nlm.nih.gov/. This database is vital in designing oligonucleotides specific to target sequences.

This project utilised oligonucleotides for two purposes, *in situ* hybridisation and qPCR. Each technique poses distinct considerations for oligonucleotide design as detailed below.

2.10.1 Oligonucleotide probe design for *in situ* hybridisation

In situ hybridisation (section 2.14) utilises a labelled oligonucleotide probe to target RNA or DNA sequences in whole tissue sections. Several criteria constrain the design of an oligonucleotide probe for *in situ* hybridisation. The probe must be complementary to the target nucleic acid sequence, and must also have physical, chemical and sequence properties conducive to hybridisation. Probes must be specific, without crossreactivity to non-target RNA or DNA molecules. The NCBI website hosts algorithms to perform *in silico* hybridisations against databased sequences, allowing any undesired complementarity of a probe with non-target sequences to be identified.

This project utilised *in situ* hybridisation to identify RNA sequences. Any region of target RNA can be used as the template for probe design. However, regions of low sequence conservation are less probable to be highly similar to non-target sequences. The 5' and 3' untranslated regions of mRNA are not often highly conserved, although they are often prone to problematic repetitive sequence. The probe used in this project was designed against exon 8 of LXRβ mRNA. Targeting this region allowed a probe to be designed with low similarity to other sequences and minimal secondary structure.

It is important to control for non-specific binding in *in situ* hybridisation. This is achieved by using a sense oligonucleotide in addition to the sequence-specific antisense probe. Sense oligonucleotides are identical to the complementary RNA sequence (with substitution of thymine for uracil) so will not bind via sequence complementarity. Sense oligonucleotides should exhibit similar levels of nonspecific binding due to physical and chemical similarity to the antisense probe. Increased staining with the antisense probe compared to the sense oligonucleotide is therefore evidence for specificity. *In silico* analysis was conducted to check for cross-reactivity against NCBI database human RNA sequences using the BLASTn search algorithm (Altschul et al., 1990).

2.10.2 Physical and chemical considerations of *in situ* hybridisation probes

The melting temperature (T_m) of a probe is the equilibrium temperature at which 50% of probe is bound to a complementary nucleic acid sequence and 50% is dissociated. Determining the T_m value of a probe is important for hybridisation, as the highest rate of probe binding to target sequence occurs at approximately 15°C below T_m. Factors that influence T_m include probe length, GC content and characteristics of the hybridisation solution such as salt concentration. T_m is commonly manipulated for the purposes of hybridisation by altering the concentration of formamide in the hybridisation buffer; each added 1% (v/v) of formamide causes T_m to decrease by approximately 0.75°C (Farrell, 1998). T_m is altered during stringency washes by altering salt concentration (Britten and Davidson, 1985).

T_m can be determined for an oligonucleotide 14-70 bases long by the following formula (Sambrook et al., 1989):

$$T_m = 81.5 - 16.6(\log[Na^+]) + 41(\%GC) - (600 \div N) - 0.75(\%F) - (\%Mismatch)$$

Where Na^+ is the solution sodium concentration in molarity, %GC is the percentage of G and C bases in the oligonucleotide sequence, N is the length of the oligonucleotide in bases, %F is the percentage of formamide in the hybridisation solution and %mismatch is the number of non-complementary bases between the sequences within the annealing region. During the design of probes for this project, the software program NetPrimer (Premier Biosoft International, CA, USA) was used to highlight internal secondary structures that may inhibit binding to target sequences.

Probe length is important due to its influence on T_m and because large probes may be less able to move freely within fixed tissues. Longer probes are also more prone to forming secondary structure. Short probes, on the other hand, generally require inconveniently low hybridisation and wash temperatures. 30-36 bases is an optimal probe length for *in situ* hybridisation.

The GC content of the probe must also be considered. A greater GC content results in stronger binding of the probe to target sequence, however the frequency of non-specific binding also increases. As a general guideline, *in situ* hybridisation probes should be designed with 45-55% GC content (Rattray and Michael, 1998).

2.10.3 Use of commercially designed probe sets for qPCR

Oligonucleotide probes can be manually designed for use in qPCR. The considerations for this process are broadly similar to those described above for *in situ* hybridisation. However, in this study an alternative commercial solution was chosen for qPCR. TaqMan® probe and primer sets sold by Applied Biosystems (Warrington, UK) are pre-optimised and highly specific. Although these probes are significantly more expensive than self-designed probes, value is recovered by obviating the necessity for optimisation.

2.11 Working with RNA

Compared to proteins and DNA, RNA is much less stable and can be rapidly degraded by both endogenous RNases in tissue and those produced by ambulatory microbes. Contamination must therefore be rigorously avoided when working with RNA in the laboratory. A common route for introduction of microbial nucleases into a reaction is via contaminated water. Although autoclaving water kills the vast majority of microbial species and denatures most proteins, it is insufficient to activate RNases. During this project, all RNA work utilised either i) water treated with diethyl pyrocarbonate (DEPC) and autoclaved, or ii) injection grade water (B. Braun Medical Ltd., Sheffield, UK). Surfaces and containers were cleaned using RNase*Zap®* spray (Ambion, TX, USA).

2.12 RNA extraction

The extraction and purification of ribonucleic acids (RNA) from cells and tissues is a precursor to a number of downstream techniques including Affymetrix® microarrays, qPCR and *in situ* hybridisation. A wide choice of systems are available for the extraction of total RNA. All systems follow similar basic considerations. Cell membranes are solubilised to release RNA into solution and RNA is isolated from protein, DNA and other contaminants. During this process, effective inhibition of nuclease activity must be maintained, otherwise RNA would be rapidly degraded following cellular lysis.

In this study total steady-state RNA was extracted from skin using Trizol® reagent in conjunction with silica spin columns (Purelink™ RNA kit; Invitrogen). Trizol® reagent, based on the work of Chomczynski and Sacchi, 1987, contains chaotropic guanidinium salts for cellular lysis and nuclease inactivation. DNA is removed by acidic phenol-chloroform phase separation and RNA is isolated from other contaminants using silica spin columns. Silica spin columns contain a silica membrane that reversibly binds to RNA under low-pH, high salt conditions. Non-RNA molecules are removed by centrifuging wash solutions through the membrane. Finally, RNA is eluted from the membrane into RNase-free water.

A methodology developed by Dr. Abigail Langton (Dermatological Sciences, The University of Manchester, UK) was used to extract RNA for qPCR from frozen skin sections. Compared to RNA extraction from whole biopsies, this method conserves sample and increases the proportional yield of RNA due to an increased surface area:volume ratio and lower resistance to homogenisation. Accuracy can be achieved with qPCR using a relatively low amount of RNA. Whole biopsies were used in total RNA extractions for Affymetrix® microarrays.

2.12.1 Extraction of total RNA

The following work was performed by Dr. Abigail Langton. To extract total RNA from skin biopsies for Affymetrix® analysis, biopsies were thawed on ice and RNA*later* solution was discarded. Biopsies were transferred to a RNAse free sterile 2ml Eppendorf® tube and 800 µl Trizol® (Invitrogen, Carlsbad, CA) was added. Biopsies were homogenised for 60 seconds using an Ultra-Turrax T10 Basic homogeniser (IKA® Werke GmbH & Co. KG., Staufen, Germany) fitted with a dispersing element (S 10 N – 5 G; IKA® Werke GmbH & Co. KG). The lysate was incubated for a further 5 minutes at room temperature and then processed using a Purelink™ RNA micro to midi kit (Invitrogen). Briefly, 160 µl chloroform was added to each lysate and tubes were shaken vigorously by hand for 15 seconds then incubated for 3 minutes at room temperature.

Phase separation was completed by centrifugation at 12,000 x g for 15 minutes. 350 µl of the colourless upper phase was transferred to a fresh tube and an equal volume of 70% ethanol was added. Samples were then transferred to silica spin columns in collection tubes and centrifuged at 12,000 x g for 15 minutes. Flow-through liquid was discarded and 350 µl 'wash buffer I' (Invitrogen Purelink™ RNA micro to midi kit) was added before centrifuging at 12,000 x g for 15 seconds. The spin columns were transferred to fresh collection tubes and 80 µl DNase mix (8 µl DNase reaction buffer, 10 µl DNase, 62 µl RNase-free water; Invitrogen) was added.

Samples were incubated at room temperature for 15 minutes, then 350 µl 'wash buffer I' was added and columns were centrifuged at 12,000 x g for 15 seconds. Flow-through liquid was discarded, 500 µl 'wash buffer II' was added and columns were centrifuged at 12,000 x g for 15 seconds. Flow-through liquid was discarded, 500 µl 'wash buffer II' was added and columns were again centrifuged at 12,000 x g for 15 seconds, discarding the flow-through liquid. Spin columns were centrifuged for a further 1 minute to dry the membrane then inserted into fresh tubes. 30 µl RNase-free water was added and columns were centrifuged at 12,000 x g for 60 seconds to elute RNA. The quality of extracted RNA was verified using a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

To extract total RNA for qPCR, ten frozen 20 µm sections were cut per biopsy using a cryostat (Bright Instruments). The ten sections were transferred to a RNAse free sterile 2ml Eppendorf® tube and 800 µl Trizol® (Invitrogen, Carlsbad, CA) was added. Sections were homogenised for 60 seconds using an Ultra-Turrax T10 Basic homogeniser (IKA® Werke GmbH & Co. KG., Staufen, Germany) fitted with a dispersing element (S 10 N – 5 G; IKA® Werke GmbH & Co. KG). The lysate was incubated for a further 5 minutes at room temperature and then processed using a Purelink™ RNA mini kit (Invitrogen) following a process identical to that described above.

2.13 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) allows the quantification of relative or absolute levels of a specific DNA sequence in a sample. The method employs the polymerase chain reaction to amplify the DNA template while monitoring in real time fluorescence produced by a reporter dependent on the amount of the target nucleic acid sequence. The expression level is taken as the first PCR cycle at which clear linear amplification can be detected. By combining qPCR with a reverse transcription reaction, the expression levels of specific RNA molecules in a biological sample can be determined. In this project relative quantitation of LXRβ mRNA was performed by calculating the ratio of LXRβ expression to the expression of β-actin, a housekeeping control gene.

2.13.1 qPCR to determine relative LXRβ mRNA expression levels in human skin

Cloned DNA was prepared from RNA samples by reverse transcription using a cloned avian myeloblastosis virus (cAMV) first-strand cDNA synthesis kit (Invitrogen). Briefly, 150 ng random oligonucleotide hexamer primers were mixed with approx. 270-540 ng of sample RNA and a final concentration of 1.7 mM deoxyribonucleotide triphosphates. This mixture was incubated for 5 minutes at 65°C to denature RNA and primers then cDNA synthesis mix was added (final concentrations 12.5 mM DTT, 1 x 'cDNA synthesis buffer', 120 units RNaseOUT™ and 45 units cAMV). Samples were incubated on a Hybaid PCRsprint thermal cycler (Thermo Fisher Scientific; MA, USA) at 25°C for 10 minutes for primer annealing followed by reverse transcription at 50°C for 50 minutes. The reaction was terminated by a final 5 minute incubation at 85°C.

Following cDNA synthesis, cDNA samples were loaded to a 96-well plate mixed as follows in each well: 2 µl cDNA, 10 µl TaqMan® fast universal PCR master mix (Applied Biosystems), 1 µl LXRβ TaqMan® 6-carboxyfluorescein/minor groove binder (FAM/MGB) labelled probe and primer set (Applied Biosystems; sequence Hs00173195_m1) or 1 µl β-actin TaqMan® FAM/MGB labelled endogenous control probe and primer set (Applied Biosystems) and 7 µl dH_2O. cDNA was omitted and replaced with dH_2O for negative control wells. After loading, plates were sealed and analysed over 40 cycles on a StepOne qPCR machine (Applied Biosystems).

2.14 *In situ* hybridisation

In situ hybridisation offers a powerful tool for the identification of nucleic acid sequences in tissue sections. In this project *in situ* hybridisation has been utilised to target RNA sequences, providing information on the localisation and quantity of steady-state RNA *in situ*. An oligonucleotide probe is designed to bind to the sequence of interest. The probe may be conjugated to either fluorescent tags or enzymes to catalyse a chromogenic reaction. The system used in this project employed a biotin tag, which was bound by a HRP-conjugated avidin reagent to

produce a localised chromogenic precipitate from 3,3'-diaminobenzidine (DAB) detection reagent.

2.14.1 Pretreatment

RNA is labile and easily degraded by endogenous nucleases, so tissue must be fixed by pretreatment to stabilise total RNA and increase the accessibility of tissue to probes (Höfler et al., 1998). Acetic anhydride confers a negative charge to nucleic acids by acetylation, which may help to reduce non-specific binding (Hayashi et al., 1978). During pretreatment, tissue is dehydrated and delipidated, increasing the penetration of probe into cells.

2.14.2 Hybridisation

The composition of the hybridisation buffer is vital to successful hybridisation. The formamide composition of the buffer and hybridisation temperature should be prepared so that the probe T_m value (section 2.10.2) is approximately 15-25°C greater than the hybridisation temperature. Hybridisation buffers contain reagents such as dextran sulfate, ficoll and polyvinylpyrrolidine to limit the aqueous fraction of the buffer volume and increase rates of hybridisation. A further function of hybridisation buffers is to block non-specific reactions between probe and tissue by saturation with molecules of similar reactivity prior to and during hybridisation. The buffer used in this project incorporated three blocking reagents; polyadenylic acid potassium salt, sonicated salmon sperm single-stranded DNA (ssDNA) and transfer RNA (tRNA).

2.14.3 Stringency washes and detection

Following hybridisation, unbound probe must be removed before specifically bound probe can be detected. This is achieved by washes in salt solution. The stringency of washes can be increased by increasing the temperature or lowering the salt concentration. Optimal stringencies for the removal of unbound probe may vary between probes. The detection methods used for the identification of bound probe depends on the oligonucleotide label. In this project, DAB chromogen solution with

nickel was used to produce a positive grey-black reaction. Skin has a high expression of endogenous peroxidases. It was therefore necessary to block peroxidase activity by saturation with hydrogen peroxide prior to detection.

2.14.4 Controls

To ensure that reactivity seen is due to specific binding of probe to target RNA molecules, it is necessary to perform control experiments for comparison. Several negative controls are possible for *in situ* hybridisation. The first *in situ* hybridisation experiment in the intrinsic ageing cohort included four negative controls (*i-iv* below). These controls produced highly similar results. To conserve tissue only the sense strand control was used following this initial optimisation step.

i) Sense strand control

A probe complementary to the specific probe, *i.e.* with the same sequence as the target RNA (except for the substitution of thymine for uracil) has similar chemical characteristics to the specific, antisense probe, but lacks complementarity to the target. This control shows that binding is due to a specific sequence effect rather than other chemical processes.

ii) No probe control

Probe is omitted from the hybridisation reaction. This control would help to identify non-specific reactivity of the enzyme complex or chromogen.

iii) RNase treated control

Enzymatically degrading tissue RNA prior to hybridization highlights any non-specific binding of probe to molecules other than RNA in the tissue.

iv) Excess unlabelled probe control

Tissue is incubated with a 10 fold higher amount of unlabelled probe prior to hybridisation, saturating binding so that the labelled probe has no or very few specific targets. This control also shows that binding is due to complementary sequence rather than other processes.

2.14.5 Pretreatment of frozen skin sections to fix RNA

Frozen sections (7-10 µm) were fixed in 4% (w/v) PFA for 5 minutes, rinsed in PBS and then submerged in acetylation solution (100 mM triethanolamine, 150 mM NaCl, 0.25% (v/v) acetic anhydride) for 10 minutes. After rinsing in PBS, sections were dehydrated sequentially in 70%, 80%, 95% and 100% (v/v) ethanol for 2 minutes each. Sections were delipidated in chloroform for 10 minutes and partially rehydrated in 100% and 95% (v/v) ethanol for 2 minutes each.

2.14.6 Oligonucleotide probe biotinylation

A Photoprobe® biotin kit (Vector Laboratories, CA, USA) was used to biotinylate oligonucleotides. Briefly, 40 µl of Photoprobe® biotin (Vector Laboratories) was added to 40 µl of 0.5 µg/µl oligonucleotide probe (Sigma Genosys, Cambridge, UK) and incubated at 95°C for 30 minutes. 64 µl dH_2O, 16 µl Tris buffer and 160 µl sec-butanol were added. The reaction was vortexed thoroughly, centrifuged at 1000 x g for 1 minute and the upper aqueous phase was discarded. The reaction was centrifuged at 1000 x g for 1 minute one further time, again discarding the upper aqueous phase. 150 µl 95% (v/v) ethanol and 2.5 precipitant mix from the Photoprobe® biotin kit were added and mixed thoroughly, then the reaction was centrifuged at 10,000 x g for 15 minutes.

The supernatant was replaced with 75% (v/v) ethanol and centrifuged again at 10,000 x g for 5 minutes. The supernatant was removed, and the pellet was resuspended in tris-ethanol buffer.

2.14.7 Hybridisation of oligonucleotide probe to RNA in frozen skin sections

Hybridisation buffer (0.6 M NaCl, 60 mM sodium citrate, 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 0.4% (w/v) ficoll, 0.4% (w/v) polyvinylpyrrolidine, 0.4% BSA, 0.02% (w/v) sonicated salmon sperm ssDNA, 0.01% (w/v) polyadenylic acid potassium salt, 0.01% (w/v) tRNA) was boiled for 10 minutes at 100°C, quenched on ice and then brought to 26°C. Sections were treated with either i) 100 µg/ml RNase at 37°C for 30 minutes followed by 200 ng/ml antisense

probe (5' TCCTTGCTGTAGGTGAAGTCCTTCAAGAAG 3') ii) 2 µg/ml unlabelled antisense probe overnight at 26°C followed by 10 minutes wash in SSC buffer then 200 ng/ml biotinylated antisense probe iii) hybridisation buffer only, iv) 200 ng/ml sense strand probe (5' CTTCTTGAAGGACTTCACCTACAGCAAGGA 3') or v) 200 ng/ml biotinylated antisense probe diluted in hybridisation buffer overnight at 26°C (calculated T_m - 25°C).

2.14.8 Stringency washes

Washes were performed in 1 x SSC at room temperature briefly, 1 x SSC for 60 minutes at 48°C replacing twice and then 1 x SSC for 60 minutes at room temperature.

2.14.9 Detection of bound probe following hybridisation in frozen skin sections

Sections were treated with 0.6% H_2O_2 in methanol for 30 minutes, replacing once, and washed in 1 x SSC for 10 minutes, replacing once. Avidin-Biotin reagent (Vector Laboratories) was applied for 30 minutes, sections were then washed in 1 x SSC for 10 minutes, replacing once. DAB chromogen with nickel (Vector Laboratories) was applied for 15 minutes and sections were washed in 1 x TBS for 10 minutes and mounted using Citifluor™ mounting reagent (Citifluor Ltd., London, UK).

2.14.10 Quantification of *in situ* hybridisation staining in frozen skin sections

LXRβ mRNA^{+ve} epidermal cells were counted in three high power fields per tissue section for a total of up to 18 fields per volunteer.

2.14.11 Quantification of epidermal thickness in frozen skin sections

The mean epidermal thickness of each section was measured to allow the accurate normalisation of cell counts. ImageJ (NIH) was used to measure epidermal thickness perpendicular to the plane of the DEJ from the most superficial point of the DEJ to the most superficial point of the stratum granulosum. Epidermal thicknesses were averaged from five measurements per photograph in three photographs per section.

2.15 Affymetrix® microarray analysis

Microarray experiments were performed by Dr. Abigail Langton using the human genome U133A oligonucleotide array (Affymetrix Inc., High Wycombe, UK) following the manufacturer's instructions. 100 ng total RNA from male volunteers aged 18-30 years (n=3) and >70 years (n=3) was processed with a two-cycle cDNA synthesis kit (Affymetrix Inc.; one sample hybridised per array). Technical quality control was performed using dChip (Li and Wong, 2001). Background correction, quantile normalisation and gene expression analyses were performed using gene chip robust multiarray averaging. Differential expression between the two age groups was tested statistically with CyberT (Baldi and Long, 2001) on logarithmic scale data. False-discovery correction was performed with Q-value software (Storey, 2002). Significance was defined by p-value <0.05 and expression level >50.

2.16 Comparison of Affymetrix® microarray datasets

A previously published microarray dataset on LXR agonist-treated mice (liver, white adipose tissue and brown adipose tissue; Stulnig et al., 2002) was compared with published datasets on mouse ageing (heart; Lee et al., 1999), mouse CR (heart; Lee et al., 1999), rhesus monkey ageing (skeletal muscle; Weindruch et al., 2001) and rhesus monkey CR (skeletal muscle; Weindruch et al., 2001), as well as an unpublished dataset on human skin ageing (Langton et al., unpublished data).

Datasets were chosen for inclusion on the criteria of representing the biological states of ageing or CR in each species and the accessibility of the data in a public database. Methodologies for published microarray experiments can be found in the applicable references. The methodology for the unpublished human skin ageing dataset is described in sections 2.2.3, 2.12.1 and 2.15 above. A systematic manual analysis was used to compare datasets. Only genes surpassing the fold-change threshold suggested by each dataset's authors were considered. The fold-change threshold for inclusion was +/- 1.5 in all datasets except for monkey ageing and monkey CR, where it was +/- 2. Comparisons were made between the LXR agonist treated mouse dataset and each other dataset only. Each gene regulated by LXR

agonist-treatment in mice in at least one of the three tissues examined in that study was manually searched for in each of the other datasets by both gene name and Genbank (NCBI) number.

Shared genes (listed in appendix 1) were scored in each dataset as either being regulated in the same or opposite direction to the LXR agonist-treated mouse dataset. In cross-species comparisons, close gene homologues were considered, where they could be identified, as equivalent to shared genes. Binomial analysis was used to statistically analyse the degree of overlap between the LXR agonist treated mouse dataset and: i) the mouse ageing dataset; ii) the mouse CR dataset. Cross-species comparisons were performed but due to the inherent difficulty of these comparisons they were not statistically analysed. Genes that were regulated in different directions in different tissues in the LXR agonist-treated mouse dataset were ignored as non-conserved changes for the purposes of statistical analysis.

3. Development and validation of methodologies

3.1 *In silico* analysis of *in situ* hybridisation oligonucleotide probes specificity

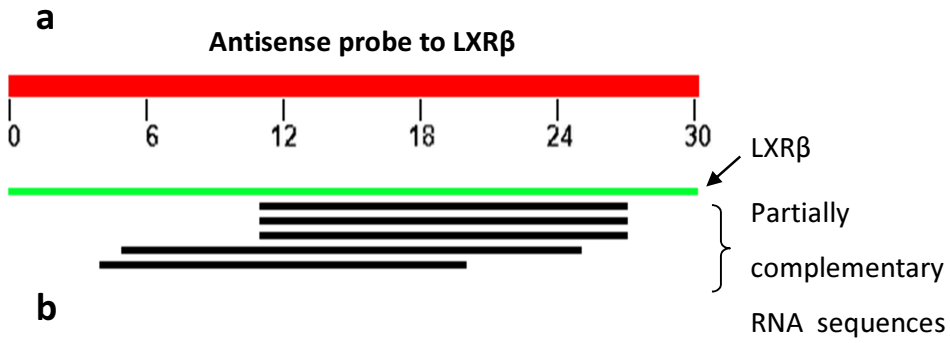
To assess potential cross reactions between LXRβ antisense and sense probes and non-target cellular RNA sequences, *in silico* bioinformatic analysis was conducted (figs. 3-1 and 3-2). No non-target human RNA sequences were identified on this database with high complementarity to either antisense or sense LXRβ oligonucleotide probes. Sequences with partial complementarity were identified but complementarity was sufficiently low to exclude these sequences as confounding factors, as their interactions with the probes have substantially lower T_m values (section 2.10.2) than LXRβ.

3.2 Verification of oligonucleotide biotinylation for *in situ* hybridisation

For *in situ* hybridisation experiments a thermal biotinylation procedure was used to label oligonucleotide probes. To verify successful probe biotinylation, dot blots were carried out with titrated dilutions of labelled probe. Reactivity was observed in labelled probes comparable to or in excess of the pre-labelled kit control (fig. 3-3).

3.3 LXRβ mRNA is expressed in human skin

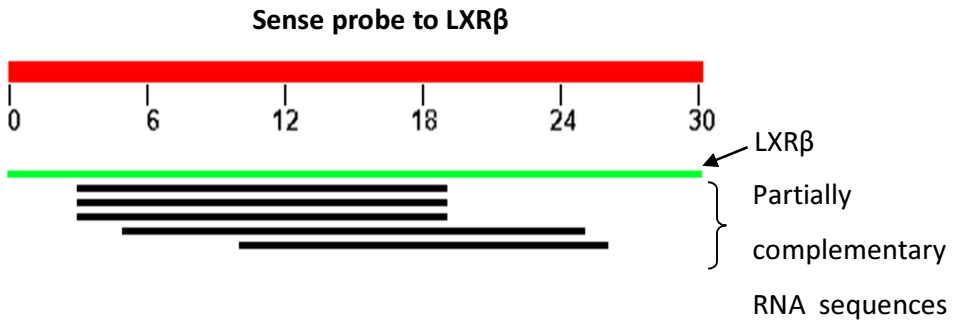
In this study, LXRβ mRNA expression in human skin cryosections was analysed using two different techniques; qPCR and *in situ* hybridisation. Using pre-optimised TaqMan® probe sets, LXRβ mRNA was detected in whole skin extracts, confirming previous work (Russell et al., 2007), where LXRβ mRNA was detected by qPCR in primary skin keratinocytes and fibroblasts cultured *in vitro*. *In situ* hybridisation analysis of LXRβ mRNA expression in human skin cryosections was performed using an oligonucleotide probe targeting exon 8 of LXRβ mRNA. LXRβ mRNA expression was consistently present in the epidermis. In the dermis, the identification of $mRNA^{+ve}$ cells was confounded by non-specific reactivity to extracellular matrix and lower cellularity.



Antisense RNA identity	Complementarity	T_m
LXRβ	30/30 bases	51.6°C
Similar to ADAMTS-like 2	16/30 bases	35.48°C
ADAMTS-like 2 variant 2	16/30 bases	35.48°C
ADAMTS-like 2 variant 1	16/30 bases	35.48°C
PRDM2	19/30 bases, one gap	37.22°C
MON2 homolog	16/30 bases	35.48°C

Figure 3-1. Graphical output from BLASTn search algorithm showing antisense probe complementarity with NCBI database RNA sequences. *The red bar (a) represents the 30-base antisense probe used for* in situ *hybridisation against LXRβ. Green bars represent fully complementary sequences, black bars represent partially complementary sequences. b) lists the identities, degree of complementarity and* T_m *values of identified RNA sequences.* T_m *values for partially complementary sequences were substantially lower than for LXRβ mRNA. Probe annealing to these non-target sequences should therefore have been removed by stringency washes.*

a



b

Antisense RNA identity	Complementarity	T_m
MON2 homolog	16/30 bases	35.48°C

Figure 3-2. Graphical output from BLASTn search algorithm showing sense probe complementarity with NCBI database RNA sequences. *The red bar (a) represents the 30-base sense probe used for in situ hybridisation against LXRβ. Green bars represent fully complementary sequences, black bars represent partially complementary sequences. b) lists the identities, degree of complementarity and* T_m *values of identified RNA sequences.* T_m *values for partially complementary sequences were substantially lower than for LXRβ mRNA. Probe annealing to these non-target sequences should therefore have been removed by stringency washes. Sequences shown in a) that are not listed in b) are similar in the sense rather than antisense orientation and would not have bound to the probe.*

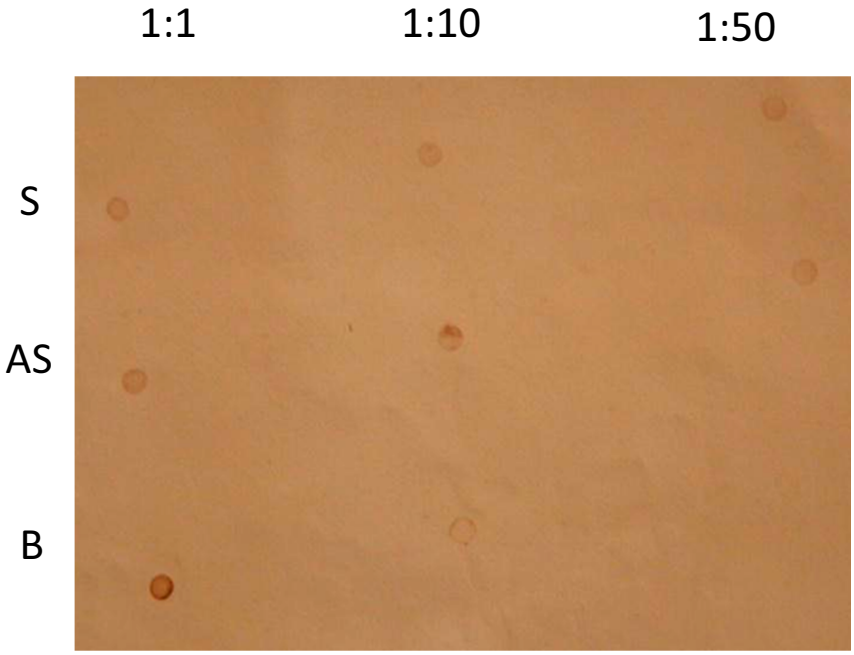


Figure 3-3. Dot blot to verify the successful biotinylation of the oligonucleotide probes used for in situ hybridisation experiments. *Dilutions of 1:1, 1:10 and 1:50 are shown of labelled sense (S) and antisense (AS) probes, with biotinylated DNA kit control (B) for comparison. Reactivity was detectable at all assayed dilutions down to 1:50 for both antisense and sense probes, indicating a high labelling efficiency.*

3.4 Confirmation of LXRβ antibody specificity by Western blot.

During this study a monoclonal antibody against human LXRβ protein (Perseus Proteomics, Tokyo, Japan) was used to immunochemically stain tissue sections. At the time of conducting the experiments this was the only commercially available antibody that specifically targeted LXRβ but not LXRα. Recently two groups have reported an inability to produce specific immunostaining in frozen and formalin fixed tissue using this antibody (Russell et al., 2007; Takegoshi et al., 2009). In this project, by contrast, abundant immunoreactivity was observed in frozen human skin sections using fluorescent and peroxidase labelled methods.

Taking into consideration this disagreement with previous reports it was necessary to verify the specific nature of the LXRβ immunoreactivity observed in this study. To achieve this the LXRβ antibody was used to probe extracts from fibroblastic (COS-1) and keratinocytic (HaCaT) cell lines by Western blot. These experiments confirmed that the antibody produces a single band of the correct molecular weight with no undesirable cross-reactivity to non-LXRβ proteins in these extracts (fig. 3-4).

3.5 LXRβ protein is expressed in human skin

LXRβ protein has previously been demonstrated to be expressed in human skin cells *in vitro* by Western blots (Russell et al., 2007). Here immunohistochemistry (fig. 3-5) and immunofluorescence (fig. 3-6) were used to identify LXRβ protein in frozen sections of human skin.

Unlike Western blots, which are performed using homogenised protein extracts, immunostaining techniques allow the distribution of protein expression to be visualised in whole tissue sections *in situ*. Immunohistochemistry and immunofluorescence produced similar distributions of expession within the epidermis. LXRβ staining was abundant throughout the basal, spinous and granular layers of the interfollicular epidermis, whilst dermal staining was less frequent and difficult to reliably discern from background staining. Unexpectedly these approaches showed that LXRβ immunofluorescence staining is striking in the peripheral cytoplasm of epidermal cells, but absent from cell nuclei.

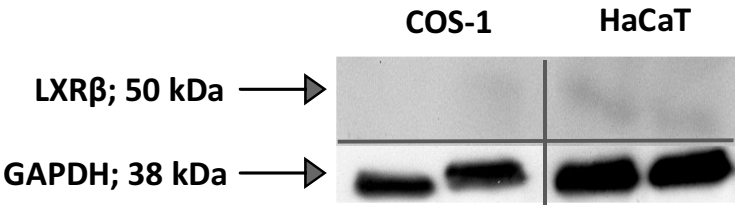


Figure 3-4. Western blot for LXRβ in fibroblastic (COS-1) and keratinocytic (HaCaT) cell line protein extracts. *The constitutively expressed metabolic housekeeping protein GAPDH was probed in the same samples as an internal reference standard. Reactive bands for LXRβ were clearly visible in HaCaT extracts and could be discriminated by eye in COS-1 extracts with the aid of a back light. Bands co-migrated with weight markers at approximately 50 kDa (LXRβ) and 38 kDa (GAPDH). A molecular weight of 50 kDa is consistent with previous reports for LXRβ (Russell et al., 2007) and prediction from LXRβ mRNA sequence NM_009473.*

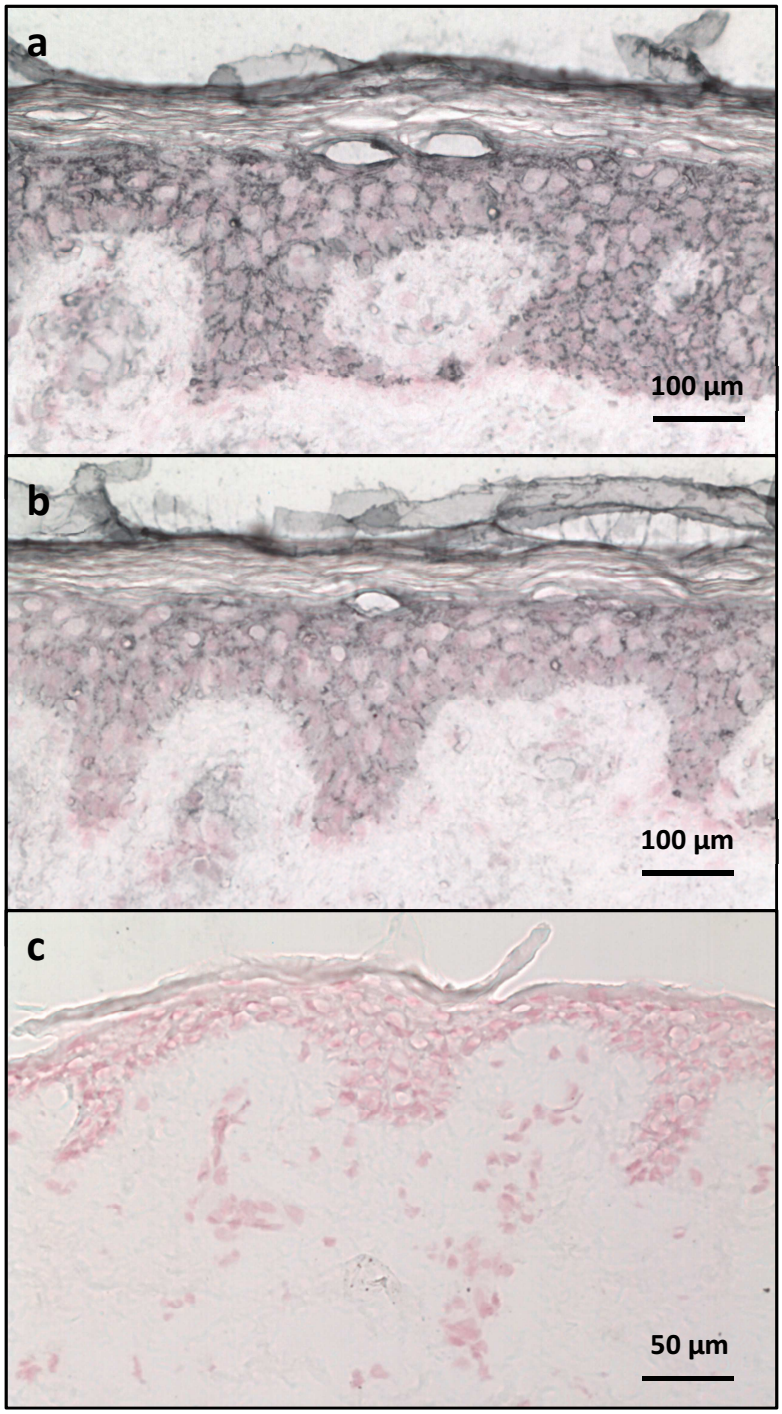


Figure 3-5. Immunohistochemistry for LXRβ in 10 µm sections of frozen human skin. *Reactivity is represented by dark grey/black staining. Cells are counterstained using nuclear fast red (pink). LXRβ reactivity is prominent at the periphery of epidermal cells, while specific staining in the dermis is minimal. Two titrations are shown; 10 µg/ml LXRβ antibody (a) and 5 µg/ml (b). The no antibody negative control (c) shows only minor background staining.*

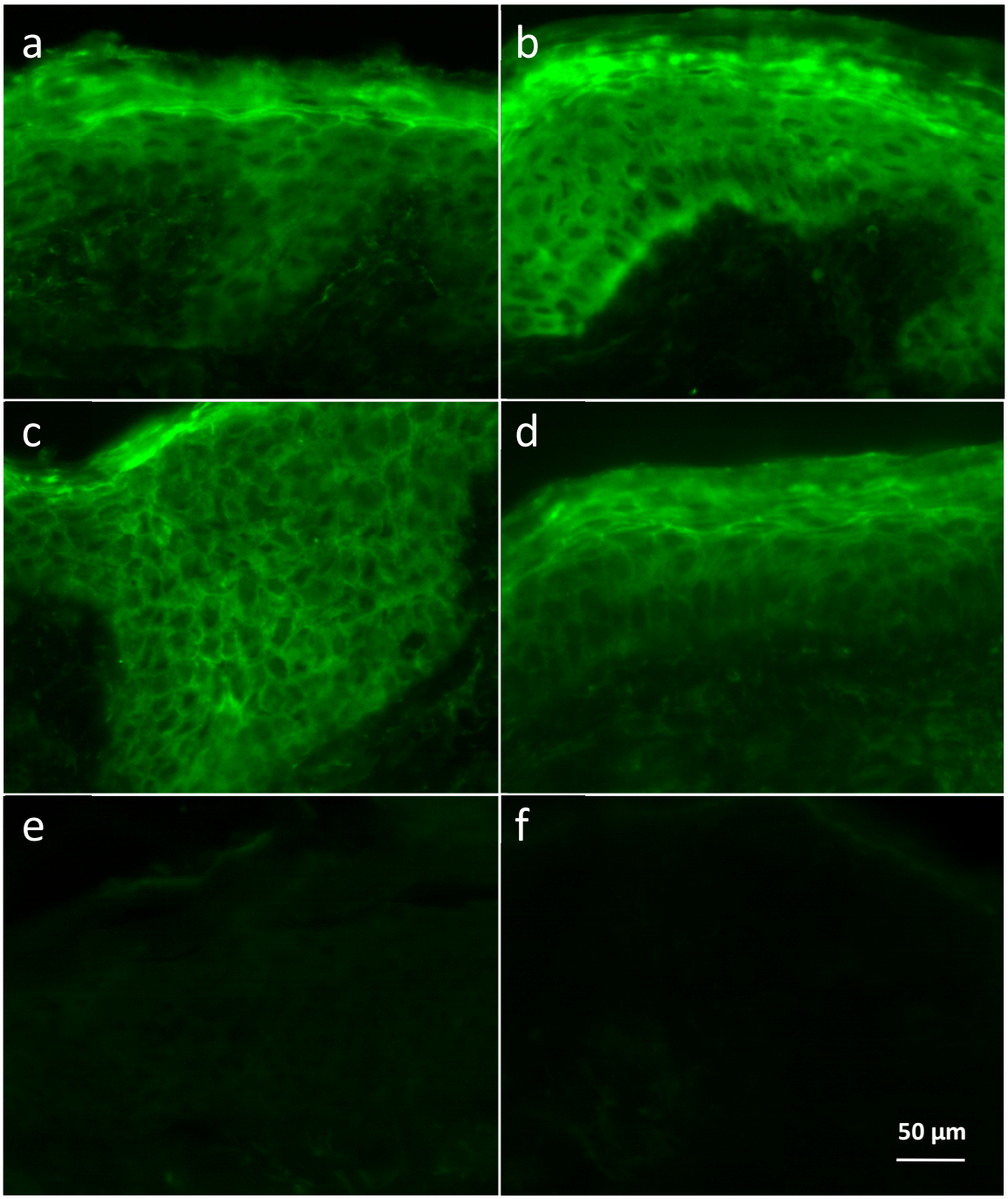


Figure 3-6. Immunofluorescence for LXRβ in 10 µm sections of frozen human skin. *Immunofluorescent reactivity against LXRβ antibody (a-d) shows a clear and abundant pattern of staining in the epidermis, with minimal staining in the dermis. In no antibody negative controls (e-f), only minor background staining is visible. Figure adapted from Ford et al., 2009.*

3.6 Epidermal fluorescence scores obtained by image analysis and by manual estimation are highly correlated.

Quantification of immunofluorescent staining was performed by manual visual scale scoring and image analysis. These approaches were compared by quantifying a panel of 90 images using both methods. Linear regression showed a significant correlation (R^2=0.811; $p<0.0001$) between the two datasets (fig. 3-7). In further work image analysis was preferred due to processing speed, continuous rather than discrete scale data output and a lower possibility of operator bias.

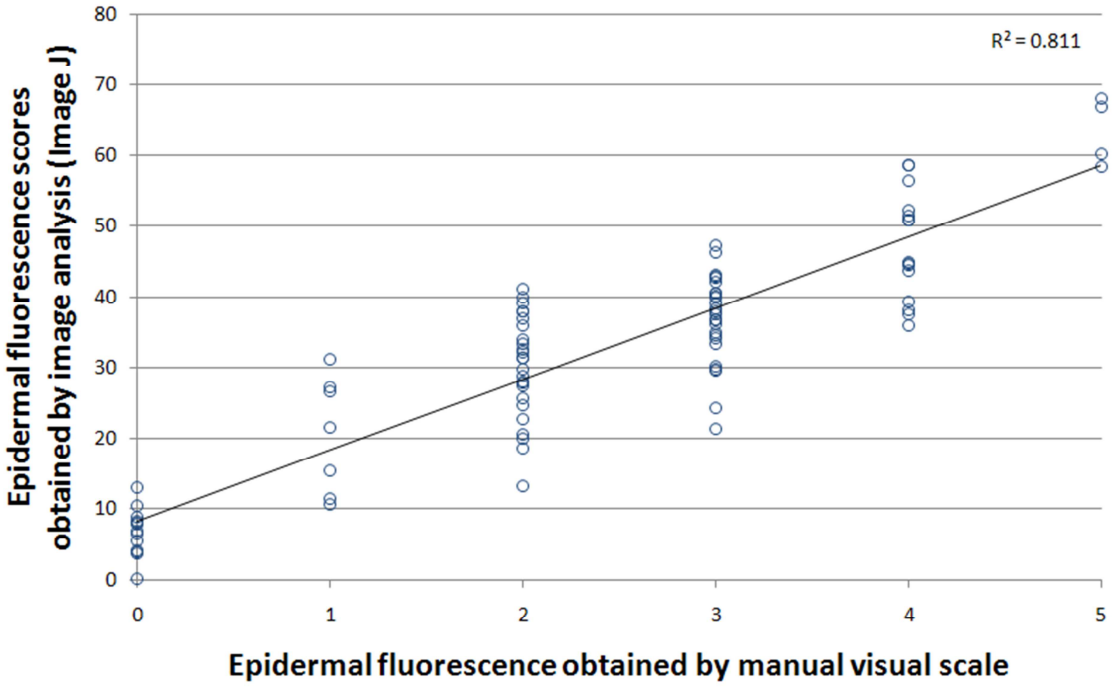


Figure 3-7. Regression analysis comparing manual and automated methods of scoring LXRβ fluorescence. *Epidermal fluorescence scores obtained by software image analysis (Image J; NIH, Bethesda, MD) are presented on the y axis, whilst scores obtained by manual semiquantitative scoring are on the x axis. Images were randomised and blinded for both scoring methods. The two datasets are significantly correlated (R=0.811; p<0.0001).*

4. Liver X receptor β in intrinsically aged skin

Liver X receptor β has not previously been studied in the context of ageing at either mRNA or protein levels. Here qPCR, *in situ* hybridisation and immunofluorescence were used to compare the expression and distribution of LXRβ between photoprotected skin from young and aged volunteers.

4.1 Epidermal thickness in young and aged photoprotected hip skin

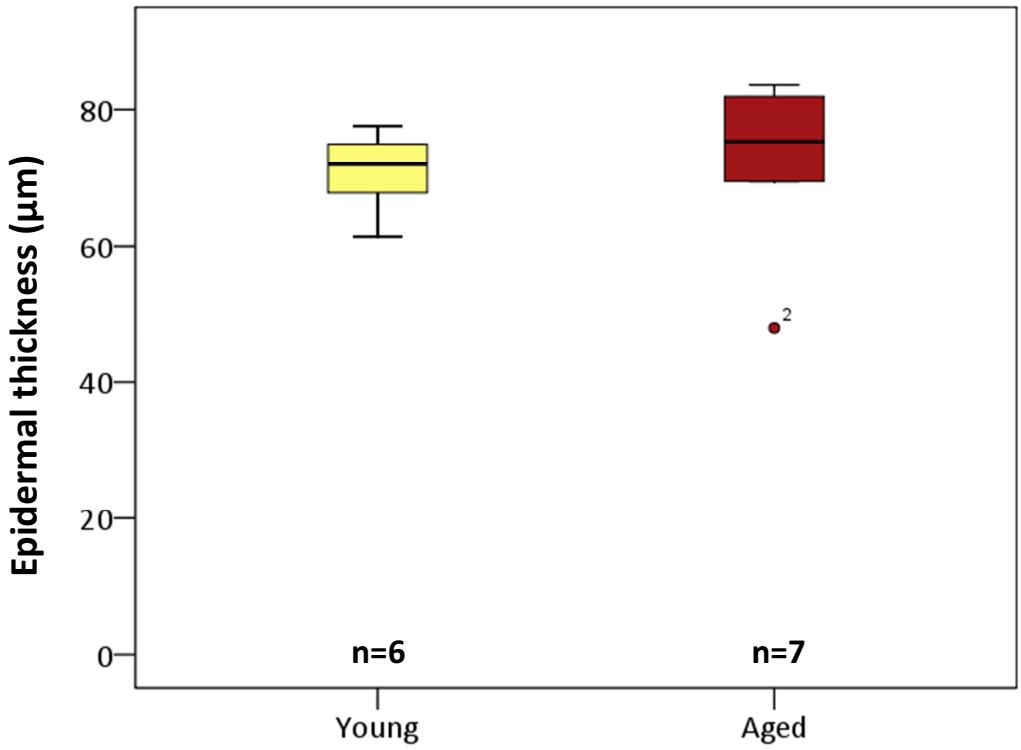
Epidermal thickness has been reported both to vary and to not vary with age (Whitton and Everall, 1973; Branchet et al., 1990; El-Domyati et al., 2002; Kirsten et al., 2002; Sandby-Moller et al., 2003). No significant effect of age could be identified on epidermal thickness in the volunteer cohorts studied here (fig. 4-1; p=0.96).

4.2 Quantitative polymerase chain reaction (qPCR) shows that LXRβ mRNA is stable in young and intrinsically aged human skin

Quantitative polymerase chain reaction was used to quantify LXRβ to β-actin mRNA expression ratios in young (18-30 years old) and aged (65-75 years old) volunteers. Due to negative skew, the raw data were logarithm-transformed prior to statistical analysis (fig 4-2). Statistical analysis revealed no significant differences in LXRβ mRNA expression between the young and aged volunteers groups (Student's two-tailed unpaired t-test; p=0.21).

4.3 *In situ* hybridisation shows that LXRβ mRNA expression is stable between young and aged photoprotected human skin

A normalised measure of LXRβ mRNA expression (fig. 4-4) was derived from *in situ* hybridisation staining (fig. 4-3) by dividing the number of LXRβ mRNA^{+ve} cells in a 400x microscope field by epidermal thickness in photoprotected skin sections from young and aged volunteers. Statistical comparison revealed no significant differences in normalised LXRβ mRNA expression between young and aged human epidermis (Student's two-tailed unpaired t-test; p = 0.90; Ford et al., 2009).



	N	Mean	Std. Deviation	Std. Error Mean
Young	6	70.9667	5.76139	2.35208
Aged	7	71.2357	12.38654	4.68167

Student's two-tailed unpaired t-test

	t	df	**Sig. (2-tailed)**	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Equal variances assumed	-0.04	11	**0.96**	-0.26	5.52	-12.43	11.90
Equal variances not assumed	-0.05	8.743	**0.96**	-0.26	5.23	-12.17	11.63

Figure 4-1. Box and whisker plot of epidermal thickness data for the intrinsic ageing* in situ *hybridisation cohort. *No significant difference could be identified in epidermal thicknesses between the young (18-30 years) and aged (70-75 years) groups (Student's two tailed unpaired t-test; p=0.96; SPSS output shown).*

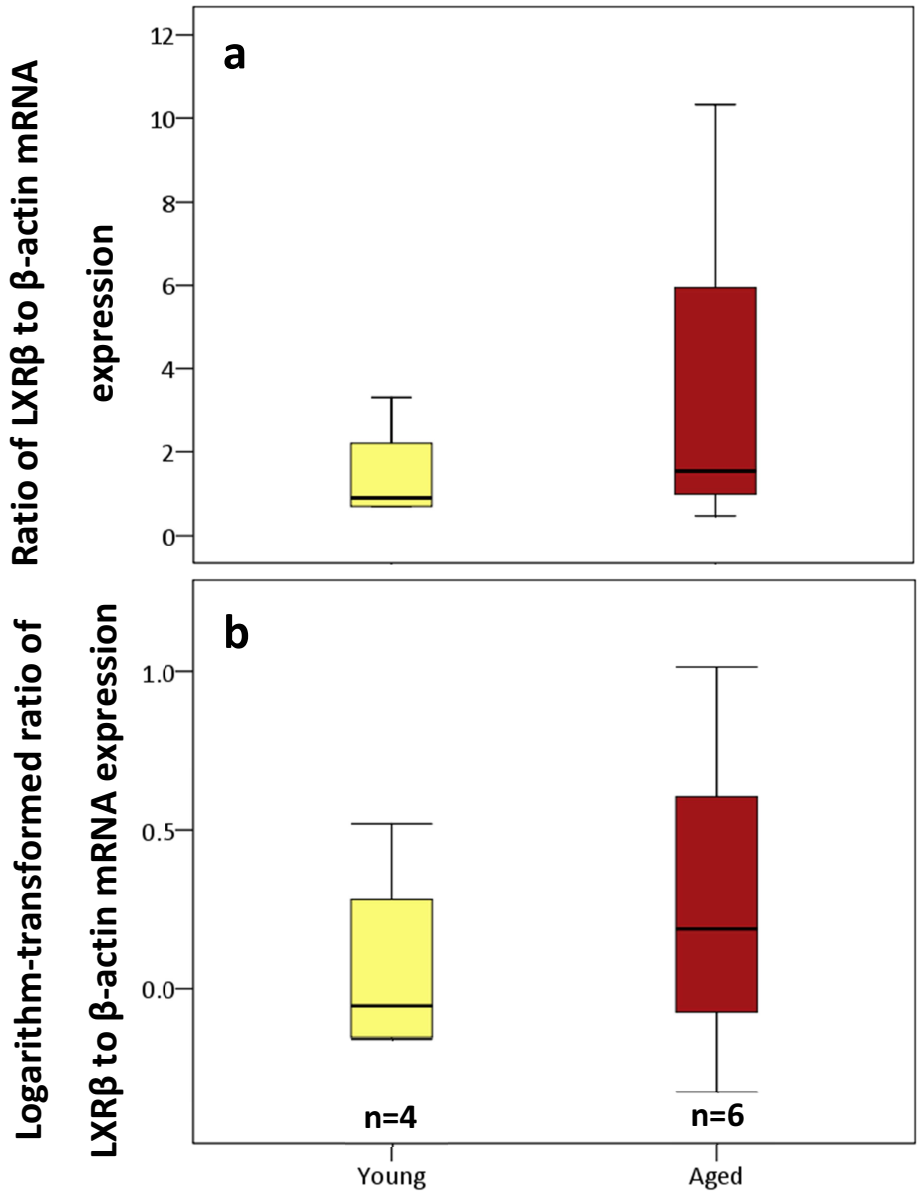


Figure 4-2. Box and whisker plots showing intrinsic ageing qPCR data for the ratio of LXRβ to β-actin mRNA expression in photoprotected hip from young (18-30 years old) and aged (65-75 years old) volunteers. *Due to negative skew the raw data (a) was logarithm-transformed (b) prior to parametric analysis. Analysis revealed no significant differences in LXRβ mRNA expression between age groups (Student's two-tailed unpaired t-test; p=0.21).*

Descriptive statistics

	N	Mean	Std. Deviation	Std. Error Mean
Young	4	.0633	.31743	.15872
Aged	6	.4873	.55076	.22485

Student's two-tailed unpaired t-test

	t	df	**Sig. (2-tailed)**	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Equal variances assumed	-1.378	8	**.206**	-.42404	.30780	-1.13381	.28574
Equal variances not assumed	-1.541	7.939	**.162**	-.42404	.27522	-1.05955	.21148

Table 4-1. Statistical analysis of logarithm-transformed qPCR data for LXRβ to β-actin mRNA expression ratios in photoprotected skin from young (18-30 years old) and aged (65-75 years old) volunteers. *Analysis revealed no significant differences in LXRβ mRNA expression between age groups (Student's two-tailed unpaired t-test; p=0.21).*

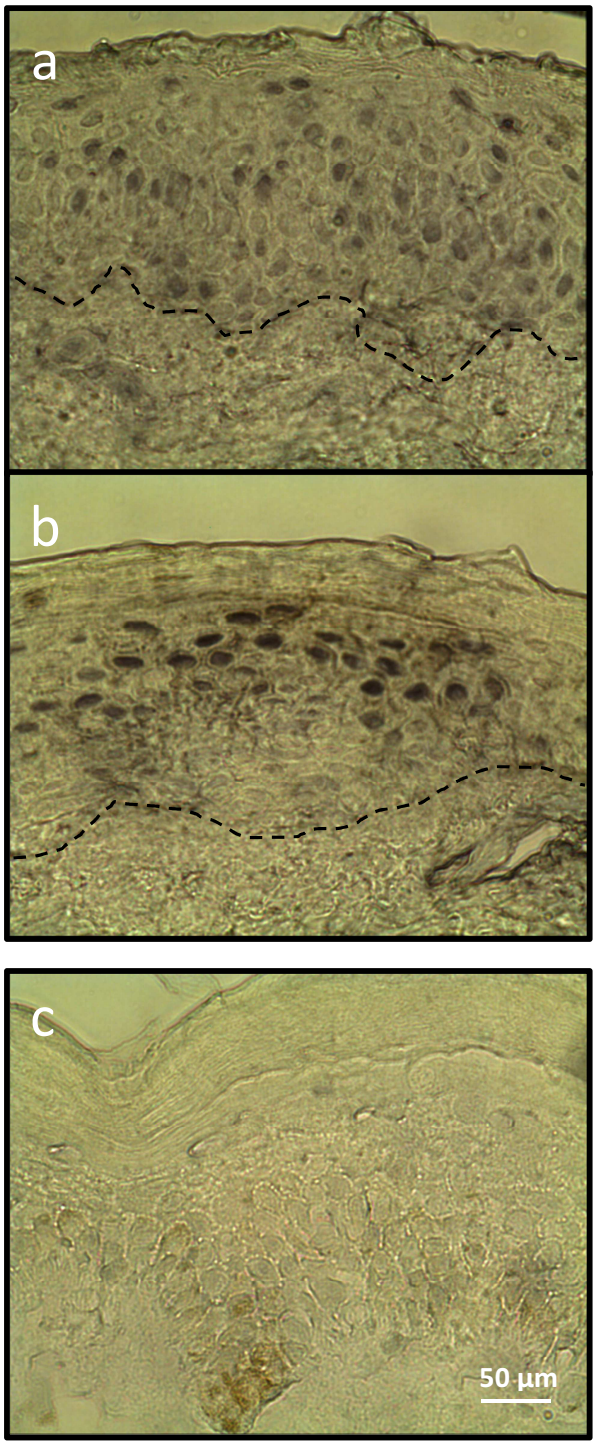
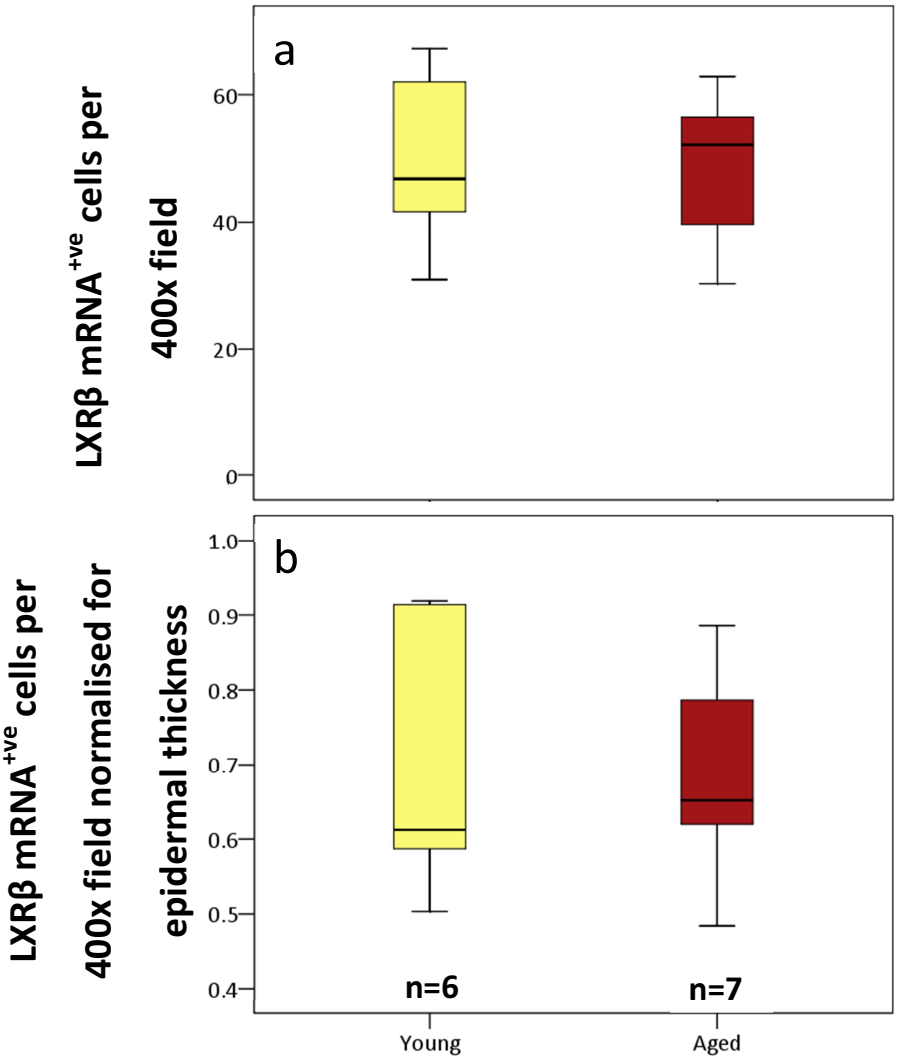


Figure 4-3. **In situ** ***hybridisation for LXRβ mRNA in photoprotected human skin from young (18-30 years; a) and aged (70-75 years; b) volunteers.*** *The sense strand control (c) shows only minor background staining. Dashed lines mark the DEJ; figure adapted from Ford et al., 2009.*



Two-tailed unpaired Student's t-test

	t	df	**Sig. (2-tailed)**	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Equal variances assumed	-0.13	11	**0.90**	-0.01	0.08	-0.20	0.18
Equal variances not assumed	-0.12	9.49	**0.90**	-0.01	0.09	-.021	0.19

Figure 4-4. Box and whisker plot showing LXRβ mRNA^{+ve} cells (a) per high power microscope field and (b) normalised for epidermal thickness *in young (18-30 years) and aged (70-75 years) photoprotected buttock skin. Analysis of normalised data determined no significant difference between the photoprotected buttock skin of young (18-30 years) and aged (70-75 years) volunteers (Student's two-tailed unpaired t-test; p = 0.90; SPSS output shown).*

4.4 Immunofluorescent staining for LXRβ in young and aged photoprotected skin

LXRβ protein has previously been reported to be expressed in human skin (Russell et al., 2007). The NHR RARα has been documented to alter in expression during intrinsic skin ageing (Watson et al., 2004), but prior studies have not considered whether LXRβ expression alters during ageing. Here LXRβ protein expression was investigated in the intrinsic ageing of skin using immunofluorescence in cryosections of photoprotected buttock skin from young (18-30 years) and aged (70-75 years) cohorts (fig. 4-5).

4.5 LXRβ protein expression does not significantly differ between young and aged human skin

Immunofluorescence was used to identify LXRβ protein expression in 10 µm cryosections of human buttock skin from young (18-30 years) and aged (70-75 years) volunteers. Image analysis was used to quantify epidermal expression, producing a value representing fluorescent intensity divided by area. Statistical analysis revealed no significant differences in LXRβ expression between the sun protected epidermis of young and aged volunteers (Student's unpaired two-tailed t-test; $p = 0.27$; fig. 4-6; Ford et al., 2008; Ford et al., 2009).

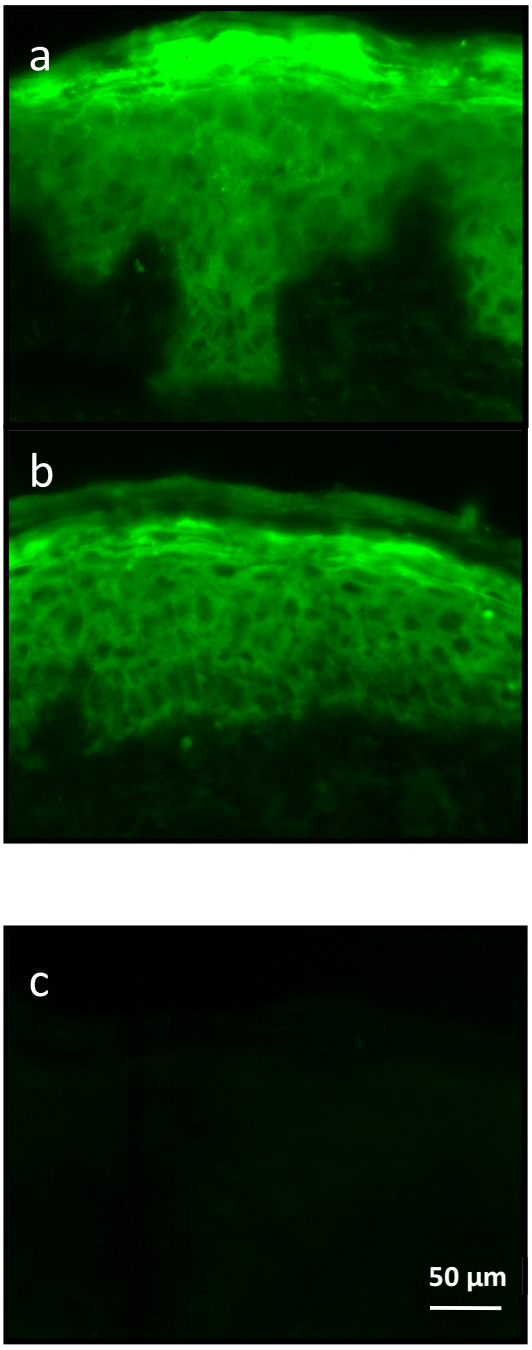
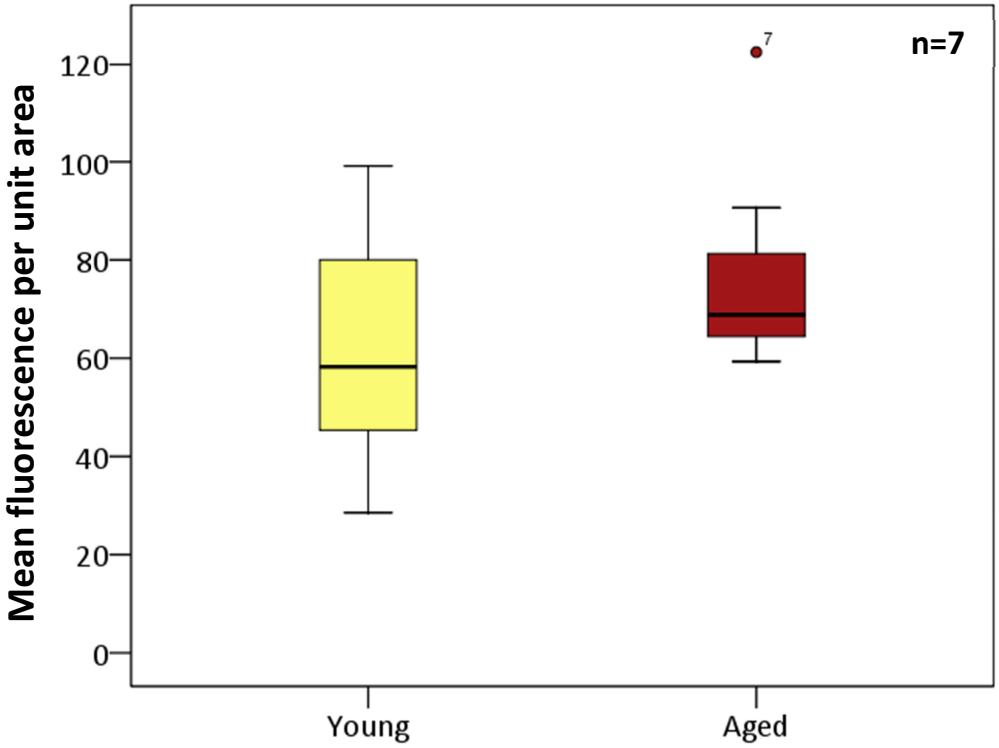


Figure 4-5. Immunofluorescence for LXRβ protein in photoprotected human buttock skin sections from young (a; 18-30 years) and aged (b; 70-75 years) volunteers. *The negative control (c) shows background staining only. Figure adapted from Ford et al., 2009.*



	N	Mean	Std. Deviation	Std. Error Mean
1	7	62.3671	25.80794	9.75448
2	7	77.4529	22.30739	8.43140

Two-tailed unpaired Student's t-test

	t	df	**Sig. (2-tailed)**	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						Lower	Upper
Equal variances assumed	-1.170	12	**.265**	-15.08571	12.89335	-43.17791	13.00649
Equal variances not assumed	-1.170	11.754	**.265**	-15.08571	12.89335	-43.24333	13.07190

Figure 4-6. Box and whisker plots of LXRβ protein expression data derived from immunofluorescence staining. *No significant differences were found in LXRβ protein expression between the photoprotected buttock skin of young (18-30 years) and aged (70-75 years) volunteers (Student's unpaired two-tailed t-test; p = 0.27; SPSS output shown).*

4.6 Comparison of Affymetrix® microarray datasets shows overlapping gene regulation between an LXR agonist diet and i) ageing; ii) CR in mice

Published microarray data on mice treated with dietary LXR agonists (Stulnig et al., 2002) was compared with published datasets from mouse ageing (Lee et al., 1999), mouse CR (Lee et al., 1999), rhesus monkey ageing (Weindruch et al., 2001) and rhesus monkey CR (Weindruch et al., 2001), as well as an unpublished dataset on human ageing (Langton et al., unpublished data). The aims of this investigation were to assess whether, given highlighted LXR roles in ageing and its roles in nutritional sensing and metabolism, there might be any substantial overlap between LXR agonist-regulated, ageing-regulated and/or CR-regulated gene expression. A systematic manual searching methodology was used to identify genes shared between the compared datasets. Summary data is shown in table 4-2. The genes identified as shared between LXR agonist treatment and one or more of the other datasets in the comparison are fully listed in appendix 1.

A significant overlap with genes regulated by LXR agonist treatment of mice was found for the mouse ageing (binomial test; $p<0.0001$) and mouse CR (binomial test; $p<0.0001$) datasets. Statistical analysis is unreliable for cross-species comparisons and was not conducted for the rhesus monkey or human datasets. In the mouse, it is striking to observe that 73% of genes regulated in both LXR agonist treatment and ageing are regulated in opposite directions, while 71% of genes regulated in both LXR agonist treatment and CR are regulated in the same direction. A similar trend of lesser magnitude is sustained in cross-species comparisons.

Dataset	Mouse LXR agonists	Mouse ageing	Mouse CR	Monkey ageing	Monkey CR	Human ageing
Array size (number of probes/genes)	50,000	9977	9977	7070	7070	22,000
Number genes over threshold[a] and percentage up-regulated	523 53% up	312 57% up	831 39% up	449 67% up	199 53% up	671 53% up
Number genes shared with LXR agonist dataset	525	56[b]	68[b]	16	9	24
Percentage genes regulated in same direction as LXR agonist dataset	100%	27%	71%	44%	67%	42%
Percentage genes regulated in opposite direction to LXR agonist dataset	0%	73%	29%	56%	33%	58%

a: Threshold fold-changes for inclusion were +/-1.5 for mouse and human datasets and +/-2.0 for monkey datasets. b: The overlap is statistically significant (binomial test; p<0.0001).

Table 4-2. Summary of comparisons between Affymetrix® microarray gene expression datasets from LXR agonist treatment, ageing and CR. *A significant overlap is observed between mouse LXR agonist treatment and i) mouse ageing; ii) mouse CR. A striking trend is apparent for opposite directionality of regulation between LXR agonist treatment and mouse ageing, but shared directionality between LXR agonist treatment and mouse CR. Cross-species comparisons were not statistically analysed, but show a lesser trend in the same directions.*

5. Liver X receptor β expression in photoaged skin

5.1 Histological assessment of photoageing

Immunohistological staining for fibrillin-1 is routinely carried out in this laboratory to validate the clinical assessment of photoageing in forearm and photoprotection in hip and upper inner arm. Fibrillin-1 is depleted by chronic photoexposure and serves as a sensitive histological marker for photoageing (Watson et al., 1999). Fibrillin-1 immunostaining in the cohort used for *in situ* hybridisation (fig. 5-1) verified the accuracy of clinical assessments and validated the use of this cohort to assess the effects of photoageing on LXRβ mRNA expression.

5.2 Quantitative polymerase chain reaction (qPCR) shows that LXRβ mRNA is stable in photoprotected and photoaged human skin

An established paradigm (e.g. Lavker et al., 1980; Watson et al., 1999) for the investigation of human photoageing is to compare photoprotected anatomical sites such as the hip and upper inner arm with photoexposed sites such as the extensor forearm. Biopsies were taken at these sites from volunteers aged 42-79 years with clinically and histologically assessed photoageing. Following the extraction of total RNA, LXRβ and the endogenous control β-actin expression were quantified by qPCR in photoprotected hip, upper inner arm and photoaged forearm skin.

Raw data were negatively skewed. The raw data were therefore subjected to logarithmic transformation prior to parametric analysis (fig. 5-2; table 5-1). Analysis revealed no significant difference in LXRβ mRNA expression between anatomical sites (Repeated measures analysis of variance (RM-ANOVA); $p=0.022$).

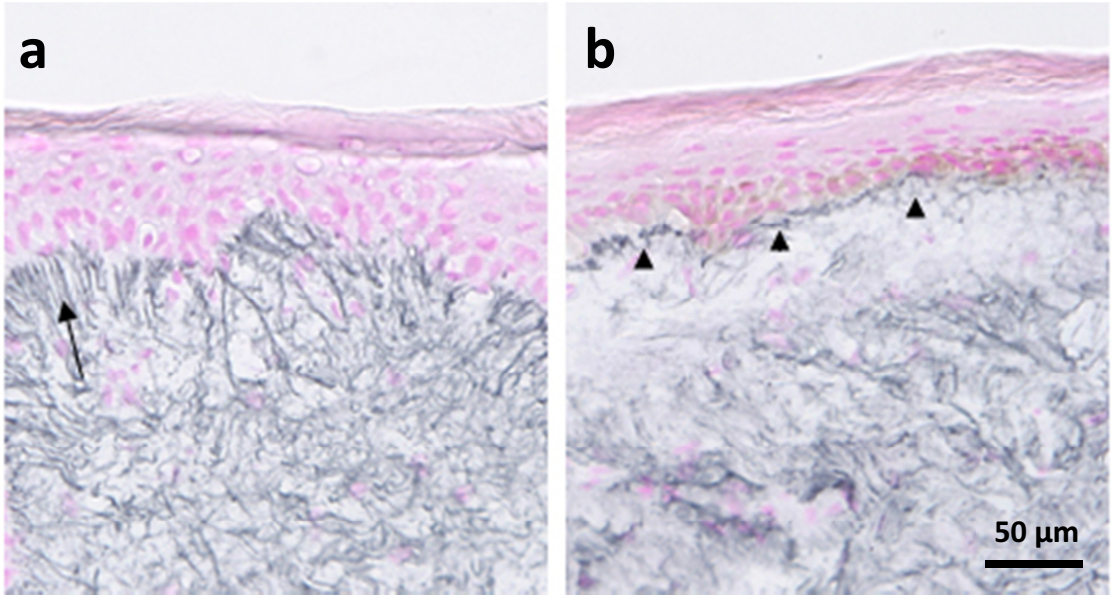


Figure 5-1. Immunohistochemistry for fibrillin-1 in human skin cryosections *of biopsies from (a) photoprotected, intrinsically aged skin and (b) photoaged skin. In intrinsically aged skin (a) the candelabra-like fibrillin-rich microfibrils (arrow, dark grey/black staining) near the DEJ are clearly defined and abundant, whilst in photoaged skin (b) the structure of the fibrillin microfibrils is largely reduced to a thin band (arrows) at the DEJ. The arrangement of fibrillin-containing elastic fibres in the deeper dermis is also perturbed in (b). These pictures are representative of the assessment performed to verify photoageing in each volunteer used in the study. All volunteers were confirmed to be photoaged. Figure adapted from Langton et al., 2010.*

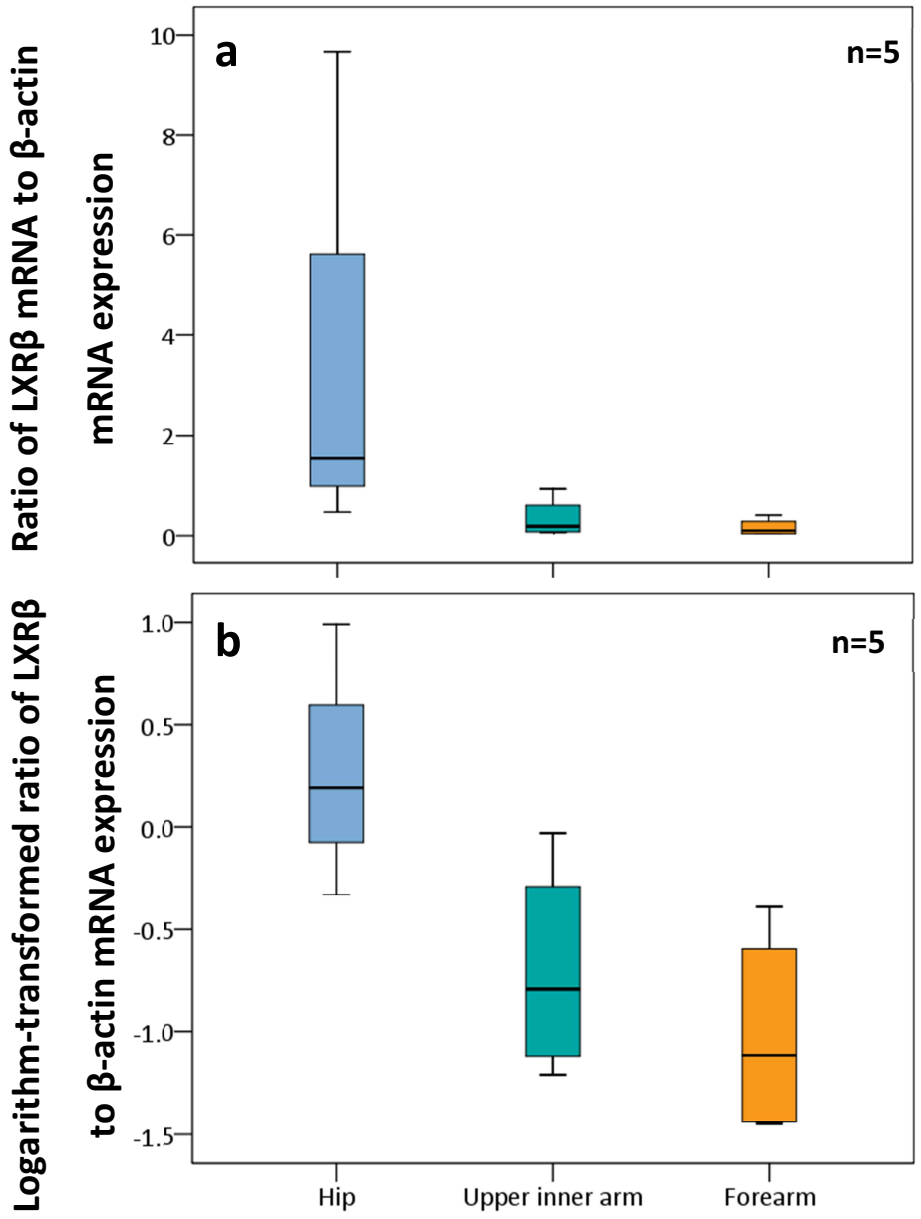


Figure 5-2. Box and whisker plots showing qPCR data (a) for the ratio of LXRβ to β-actin mRNA expression in photoprotected hip, upper inner arm and photoaged forearm. *Due to negative skew this data was logarithm-transformed (b) prior to parametric analysis. Analysis revealed no significant difference in LXRβ mRNA expression between anatomical sites (RM-ANOVA; p=0.74).*

Descriptive statistics

	Range	Minimum	Maximum	Mean	Std. Error	Std. Deviation
Hip (log)	1.32	-.33	.99	.3840	.24480	.54738
Upper inner arm (log)	1.43	-1.46	-.03	-.7783	.22191	.54356
Forearm (log)	1.06	-1.45	-.39	-1.0900	.17353	.42506

Tests of within-subjects contrasts

		Type III Sum of Squares	df	Mean Square	F	Sig.
Contrast	UA vs HIP	.158	1	.158	.227	.659
	FA vs HIP	.138	1	.138	.125	.741
Error	UA vs HIP	2.796	4	.699		
	FA vs HIP	4.394	4	1.099		

Tests of between-subjects effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	2.852	1	2.852	7.418	.072
Error	1.153	3	.384		

Table 5-1. General linear model comparison of LXRβ:β-actin mRNA expression ratios quantified by qPCR in photoprotected hip, upper inner arm and photoaged forearm. *Analysis using hip as a baseline comparator revealed no significant difference in LXRβ mRNA expression between anatomical sites (RM-ANOVA; p=0.74).*

5.3 *In situ* hybridisation shows that LXRβ mRNA is stable in photoprotected and photoaged human skin

In situ hybridisation was performed to assess any potential impact of chronic photoageing on LXRβ mRNA expression or distribution in skin. Following cryosectioning, photoprotected hip, upper inner arm and photoaged forearm tissue was probed with a biotinylated anti-LXRβ oligonucleotide (fig. 5-3). Microscopy counting of stained cells was used to describe LXRβ mRNA abundance in the epidermis. Raw cell count data were normalised for epidermal thickness (fig. 5-4) and log transformed for parametric analysis (fig. 5-5). No statistical differences were identified in LXRβ mRNA abundance between photoprotected and photoaged sites (table 5-2; RM-ANOVA; $p=0.08$).

LXRβ mRNA staining was equally frequent in the basal, spinous and granular epidermal layers and was equivalently distributed in each individual assessed. The intensity of cellular LXRβ mRNA staining varied between individuals and between sections from the same individual but did not correlate with anatomical site. Staining was also present in the dermis but the frequencies of positively stained cells could not be reliably quantified due to heavy non-specific staining of the dermal matrix.

5.4 Epidermal thickness in photoprotected hip, upper inner arm and photoaged forearm

Measurements were taken of epidermal thickness in cryosections used for *in situ* hybridisation (section 5.3) from the photoprotected hip, upper inner arm and photoaged forearm of 9 volunteers. In this cohort, upper inner arm was found to be significantly less thick than either hip or forearm (fig. 5-4; RM-ANOVA with Bonferroni multiple comparison post-hoc analysis; $p<0.05$). This difference is likely to reflect anatomical variability rather than an effect of photoexposure. No other differences between these three anatomical sites were identified as significant.

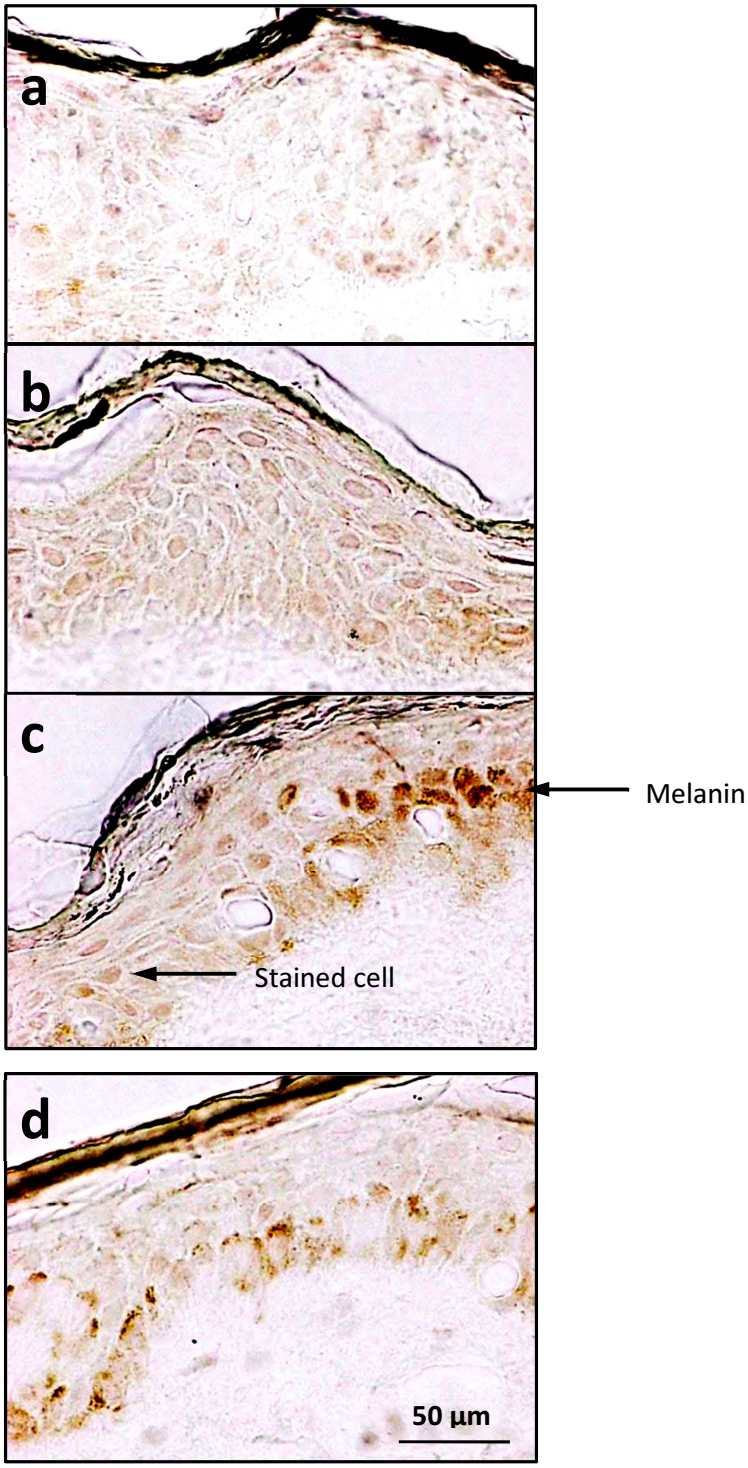
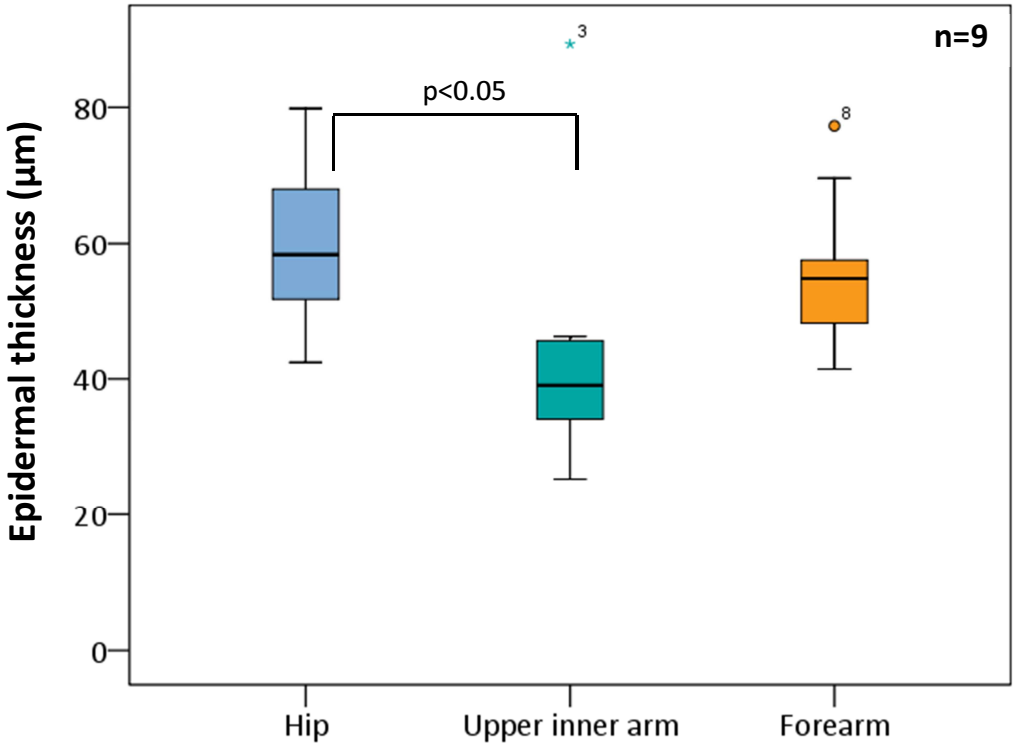


Figure 5-3. **In situ** ***hybridisation for LXRβ mRNA*** *in photoprotected hip (a), upper inner arm (b) and photoaged forearm (c). The sense strand control (d) shows background staining only. Photoexposed forearm often shows a protective melanin response. Annotation has been added to differentiate melanin from probe reactivity.*



Pairwise comparisons

(I) Site	(J) Site	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
HIP	vs UIA	16.455*	4.628	**.022**	2.498	30.412
	vs FA	4.092	5.003	**1.000**	-10.995	19.178
UIA	vs HIP	-16.455*	4.628	**.022**	-30.412	-2.498
	vs FA	-12.363	7.239	**.378**	-34.194	9.467
FA	vs HIP	-4.092	5.003	**1.000**	-19.178	10.995
	vs UIA	12.363	7.239	**.378**	-9.467	34.194

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Figure 5-4. Box and whisker plot showing epidermal thickness data for the photoageing* in situ *hybridisation study cohort. *Data are shown for mean epidermal thicknesses in photoprotected hip, upper inner arm and photoaged forearm. Upper inner arm was significantly thinner than both hip and forearm (RM-ANOVA with Bonferroni multiple comparisons post-hoc test; p<0.05). No other statistically significant differences in epidermal thickness were identified between sites (SPSS output shown). Interindividual differences were significant (p<0.001).*

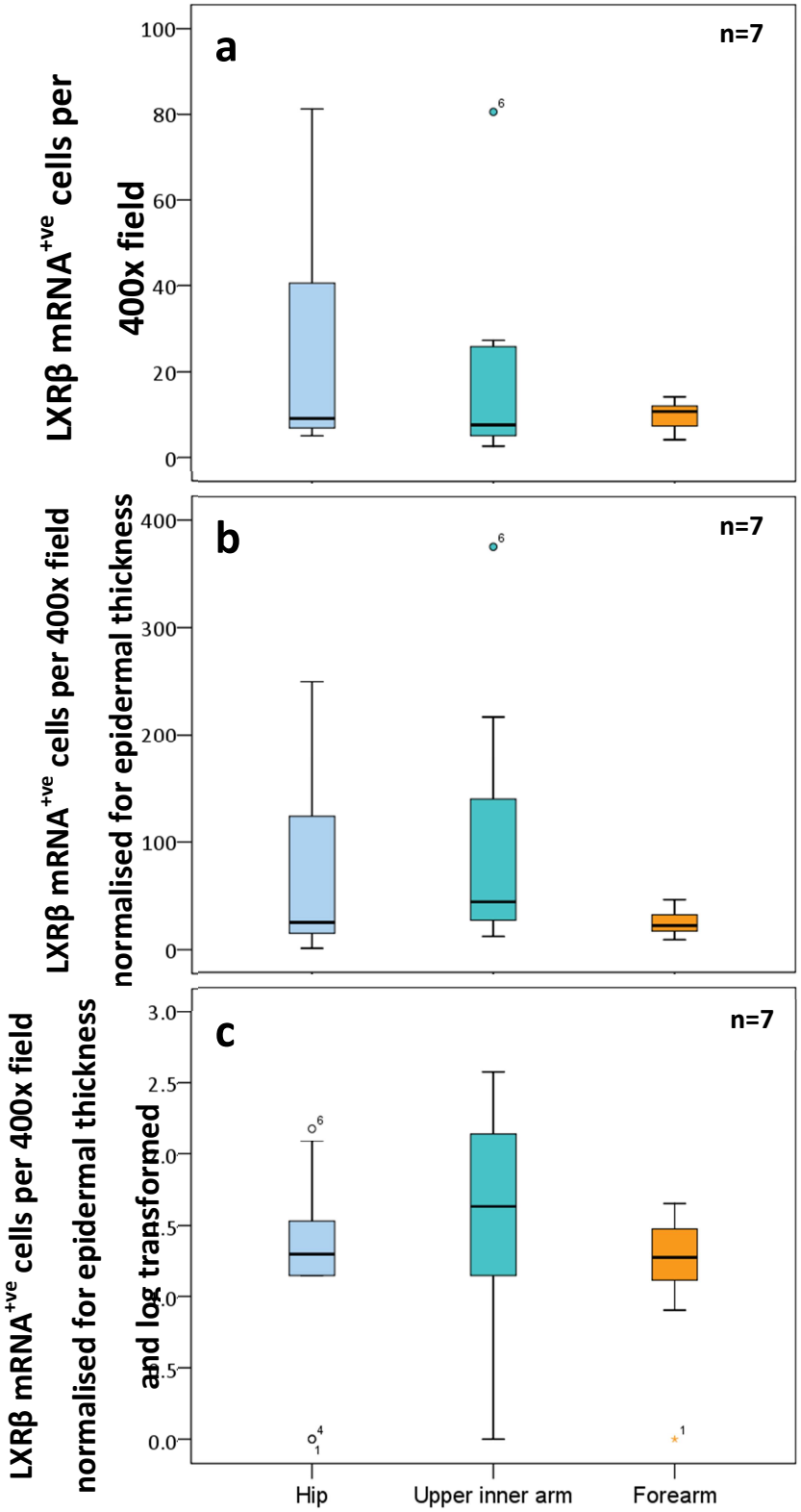


Figure 5-5. Box and whisker plots showing LXRβ mRNA^{+ve} cell counts *(a) per 400x field (b) normalised for epidermal thickness and (c) normalised then logarithm transformed to allow parametric analysis in photoprotected hip, upper inner arm and photoaged forearm (n=7).*

Pairwise Comparisons

(I) Site	(J) Site	Mean Difference (I-J)	Std. Error	**Sig.**[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
HIP	vs UIA	-.412	.239	**.369**	-1.133	.309
	vs FA	.028	.187	**1.000**	-.535	.591
UIA	vs HIP	.412	.239	**.369**	-.309	1.133
	vs FA	.440	.167	**.089**	-.063	.944
FA	vs HIP	-.028	.187	**1.000**	-.591	.535
	vs UIA	-.440	.167	**.089**	-.944	.063

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Table 5-2. RM-ANOVA with Bonferroni multiple comparisons post-hoc test of logarithm-transformed normalised LXRβ mRNA^{+ve} cell counts between photoprotected hip, upper inner arm (UIA) and photoaged forearm (FA). *This analysis did not identify any significant differences in the frequency of LXRβ mRNA^{+ve} cells between anatomical sites (p=0.08). Interindividual variation was highly significant (p<0.001).*

5.5 Immunofluorescent staining for LXRβ protein in photoprotected and photoaged human skin

Photoageing induces multiple alterations to the molecular composition of skin. The protein expression of the NHR RARα has been reported to change in photoageing (Tsoureli-Nikita et al., 2004). Immunofluorescent staining was conducted to ascertain whether LXRβ protein expression is similarly affected by chronic UVR exposure (fig. 5-6).

5.6 LXRβ protein expression is stable in photoprotected and photoaged human skin

LXRβ protein was identified by immunofluorescence (fig. 5-6) in the photoprotected hip, photoprotected upper inner arm and photoaged extensor forearm of nine volunteers. Image analysis was used to quantify epidermal expression, producing a value representing mean fluorescence divided by area. Statistical analysis by repeated analysis general linear model revealed no significant differences in epidermal LXRβ protein expression between the sun protected and photoaged anatomical sites (fig. 5-7; $p=0.61$). There were however significant differences between individuals in LXRβ protein expression (fig. 5-8; $p<0.01$).

5.7 LXRβ expression varies significantly between individuals

Plotting the results by individual for LXRβ protein expression at photoprotected buttock, upper inner arm and photoaged forearm clearly illustrates the interindividual variability in LXRβ expression. Statistical analysis revealed variability between individuals to be significant (fig. 5-8; RM-ANOVA; $p < 0.01$). Interindividual variation was also significant for LXRβ mRNA as measured by *in situ* hybridisation (RM-ANOVA; $p<0.001$), but was marginally below significance as measured by qPCR (RM-ANOVA; $p=0.07$). No relationship was found for the interindividual variability in these experiments with age, gender, Fitzpatrick scale skin type or the clinical severity of photodamage.

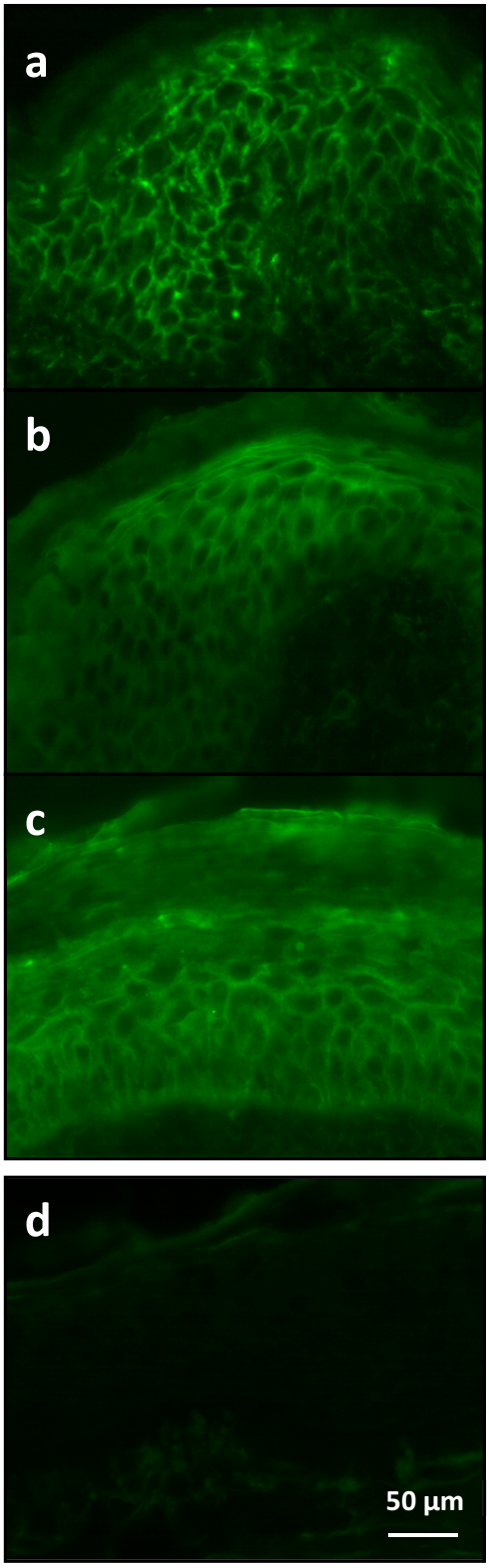
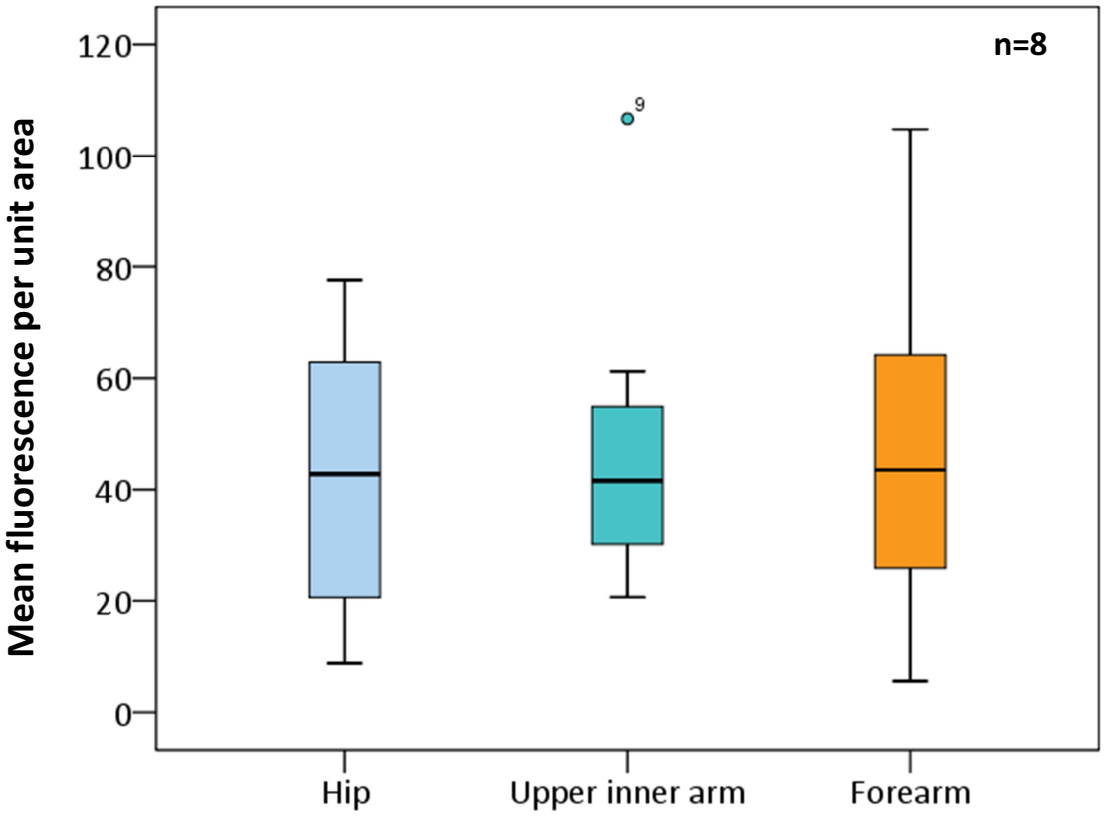


Figure 5-6. Immunofluorescent staining for LXRβ protein in three anatomical sites*; photoprotected hip (a); photoprotected upper inner arm (b) and photoaged extensor forearm (c). The no antibody negative control (d) shows background autofluorescence. Consistent photographic conditions were maintained.*

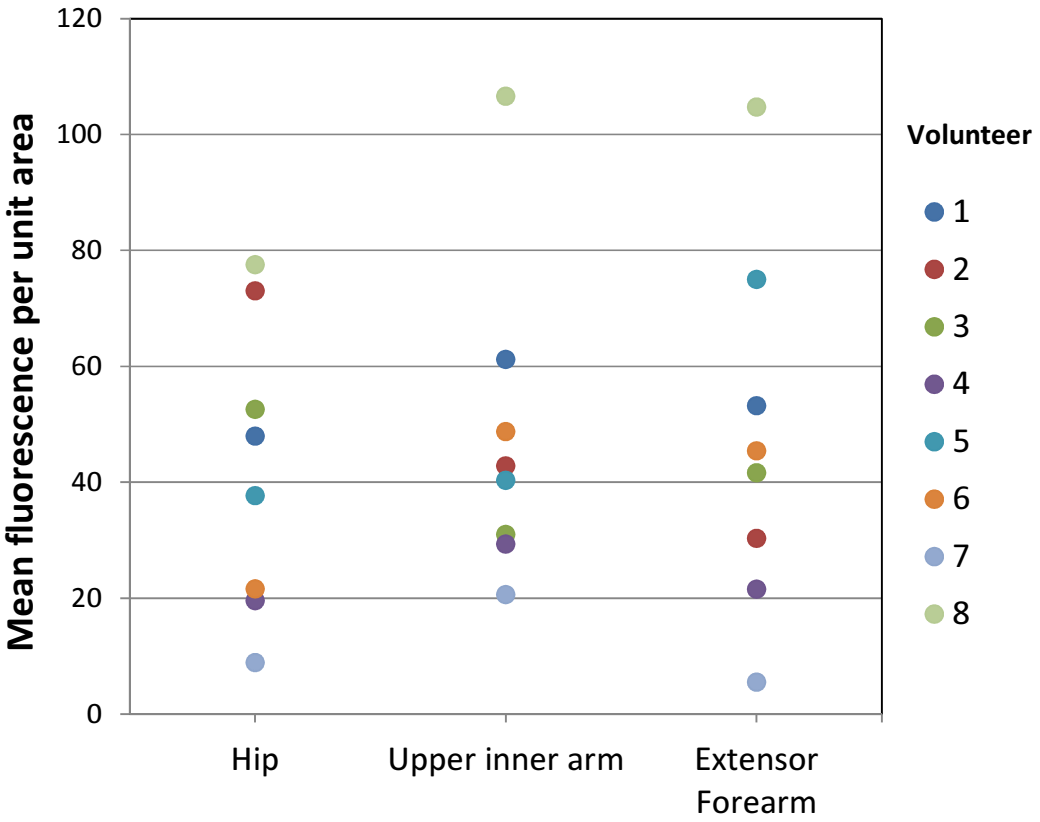


Pairwise Comparisons

(I) Site	(J) Site	Mean Difference (I-J)	Std. Error	**Sig.**[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
1	2	-5.236	7.508	**1.000**	-28.717	18.245
	3	-4.819	8.963	**1.000**	-32.852	23.214
2	1	5.236	7.508	**1.000**	-18.245	28.717
	3	.417	5.723	**1.000**	-17.481	18.314
3	1	4.819	8.963	**1.000**	-23.214	32.852
	2	-.417	5.723	**1.000**	-18.314	17.481

a. Adjustment for multiple comparisons: Bonferroni.

Figure 5-7. Box and whisker plot of epidermal LXRβ protein expression data derived from immunofluorescence staining. *No significant difference in LXRβ protein expression was detected between photoprotected buttock, photoprotected upper inner arm and photoaged extensor forearm (RM-ANOVA with Bonferroni multiple comparisons post-hoc test; p = 0.75; SPSS output shown). Interindividual differences were significant (fig 5-8; p<0.01).*



Tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	Mean Square	F	**Sig.**
Intercept	47.410	1	47.410	42.701	**.000**
Error	8.882	8	1.110		

Figure 5-8. Individual plots of LXRβ expression in epidermis of skin from three anatomical sites; photoprotected hip, photoprotected upper inner arm and photoaged extensor forearm. *Each line represents a single volunteer. This representation shows that expression varies between individuals to a greater degree than between anatomical sites. The interindividual differences are statistically significant (RM-ANOVA; p<0.01).*

5.8 LXRβ expression in retinoid-treated photoaged forearm skin

Extensive crosstalk is known to occur between NHR signalling pathways. A host of complex interactions occur directly between receptor proteins (Zhang et al., 1992; Kliewer et al., 2002) and indirectly via cofactors (Chen and Evans, 1995; Robyr et al., 2000; Perissi and Rosenfeld, 2005). Retinoids are the most effective known agents for the repair of photoaged skin (Samuel et al., 2005). To determine whether retinoid receptors and LXRβ signalling pathways may interact or collaborate in the repair of photoageing, we assessed LXRβ mRNA and protein expression in biopsies taken from baseline and retinoid-treated sites of photoaged forearm skin.

5.9 Histological assessment of retinoid repair

Immunohistological staining for fibrillin-1 is routinely carried out in this laboratory to verify that a retinoid-mediated repair response is present in retinoid-treated photoaged individuals, as a small subset of people do not show a response. Fibrillin-1 is depleted by chronic photoexposure and is deposited in the early stages of retinoid-mediated dermal repair. Fibrillin-1 deposition is detectable after 4 days retinoid treatment in responsive individuals (Watson et al., 2001). Fibrillin-1 immunostaining (fig. 5-9) verified that all nine volunteers in the cohort used for *in situ* hybridisation and immunofluorescence were responsive to retinoid treatment.

5.10 Epidermal thickness in baseline and retinoid-treated photoaged forearm

Measurements were taken of epidermal thickness in cryosections used for *in situ* hybridisation from baseline and 4-day retinoid-treated photoaged forearm skin. Analysis by Student's paired two-tailed t-test revealed that epidermal thicknesses were significantly greater in retinoid-treated photoaged forearm skin compared to baseline photoaged forearm skin (fig. 5-10; $p<0.005$).

5.11 Quantitative polymerase chain reaction (qPCR) shows that LXRβ mRNA is stable in baseline and retinoid-treated human skin

Quantitation of mRNA expression was performed by qPCR in baseline and retinoid-treated biopsies from the photoaged forearm skin of volunteers aged 42-79 years. This quantitative approach revealed a high stability of LXRβ mRNA expression in untreated and retinoid-treated skin. No significant difference was statistically detected between baseline and retinoid-treated sites (fig. 5-11; Student's paired two-tailed t-test; p=0.95).

5.12 *In situ* hybridisation shows that LXRβ mRNA is stable in baseline and retinoid-treated human skin

LXRβ mRNA was detected by *in situ* hybridisation (fig. 5-12) and compared between baseline and retinoid-treated biopsies from the photoaged forearm skin of volunteers aged 42-79 years (n=8). LXRβ mRNA^{+ve} cell count data were normalised for epidermal thickness and logarithm-transformed to permit parametric analysis (fig. 5-13). Statistical analysis of the data revealed no significant difference in LXRβ mRNA expression between baseline and retinoid-treated sites (table 5-3; p=0.22).

5.13 LXRβ protein expression does not significantly differ between baseline and retinoid-treated photoaged skin

LXRβ protein was identified by immunofluorescence (fig. 5-14) in biopsies from two sites on photoaged forearm; one occluded with no treatment (12 days) and the other with 0.025% *t*-RA (4 days). Area-normalised epidermal fluorescence was quantified by image analysis and statistically analysed by Student's paired two-tailed t-test. No significant differences were found in epidermal LXRβ expression between the baseline untreated and retinoid-treated photoaged sites (fig. 5-15; Student's paired two-tailed t-test; p = 0.77; Ford et al., 2009).

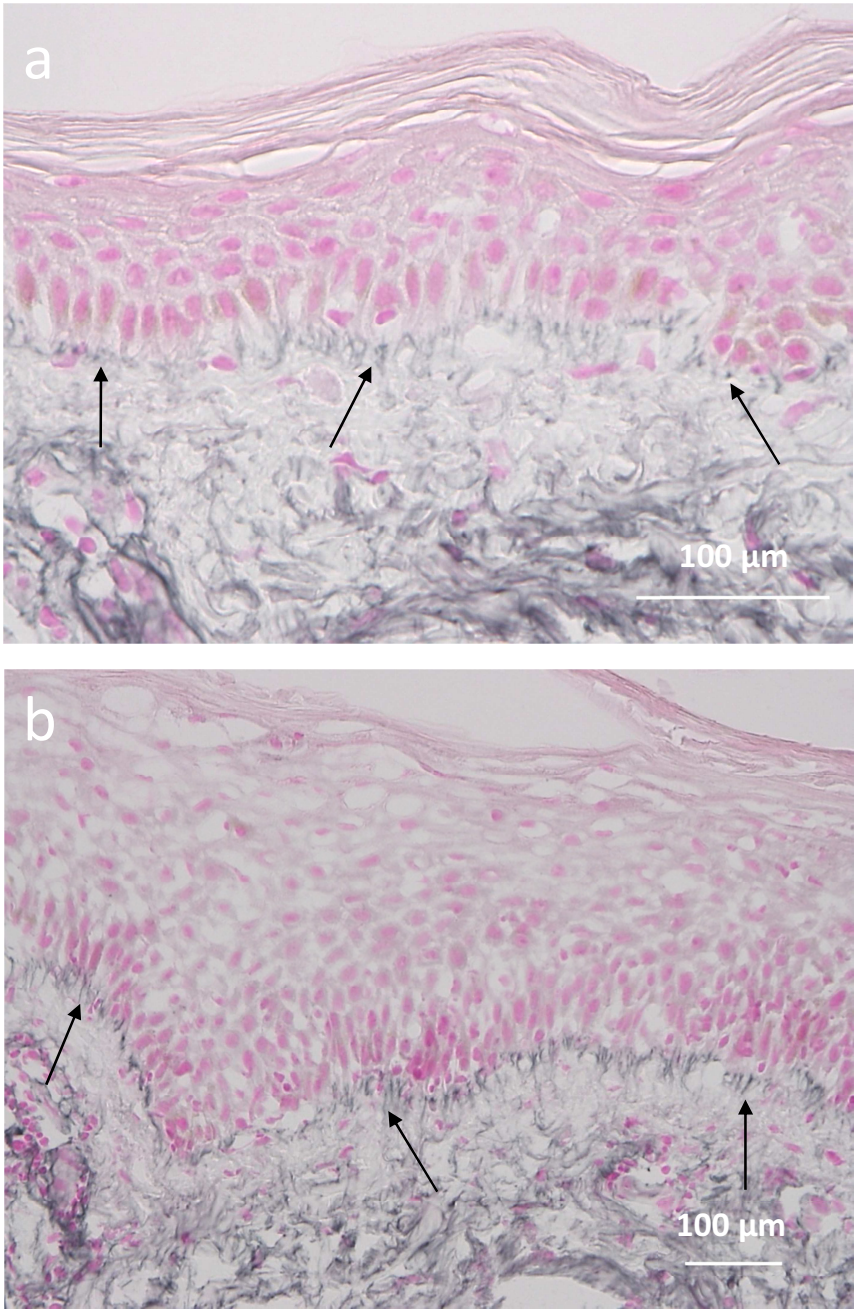
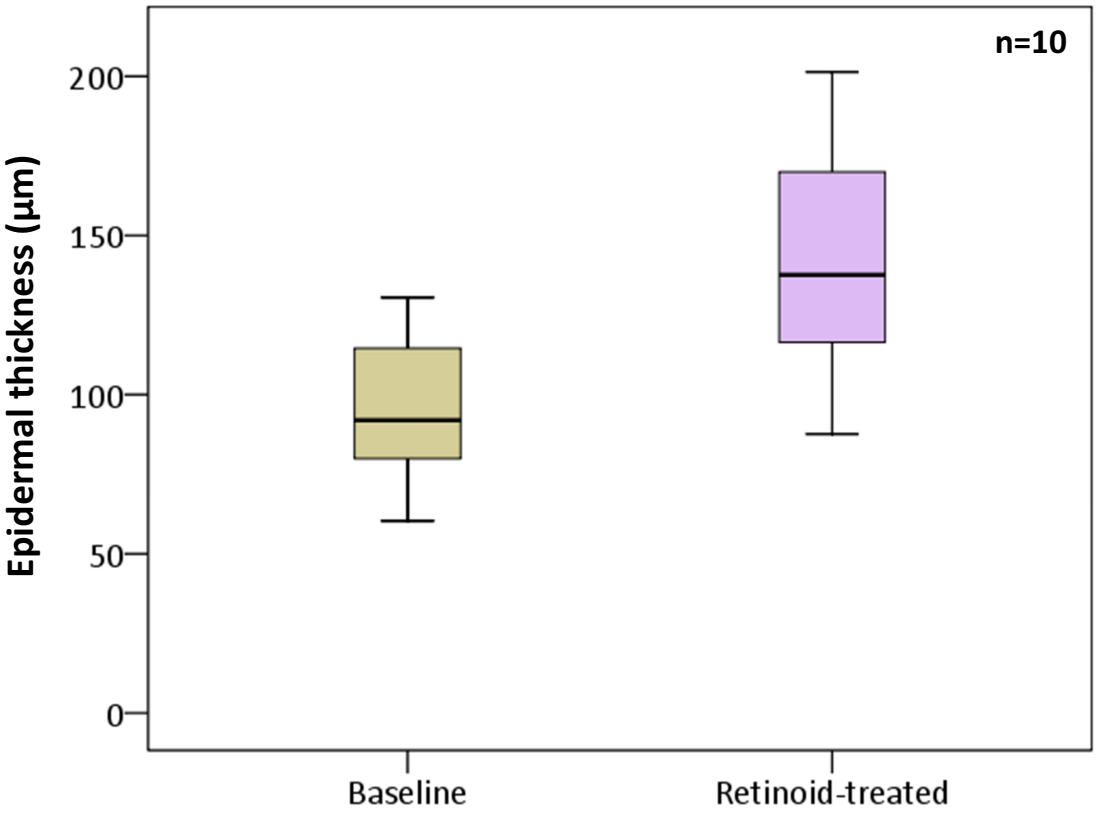


Figure 5-9. Fibrillin-1 staining in baseline (a) and 4 day retinoid-treated (b) photoaged forearm skin sections. *Arrows show fibrillin-1 staining in oxytalan fibres subjacent to the DEJ, which is visibly more prominent and well-defined in the retinoid-treated (b) compared to the baseline samples (a). The retinoid-treated section (b) also shows epidermal hyperplasia characteristic of a retinoid response.*

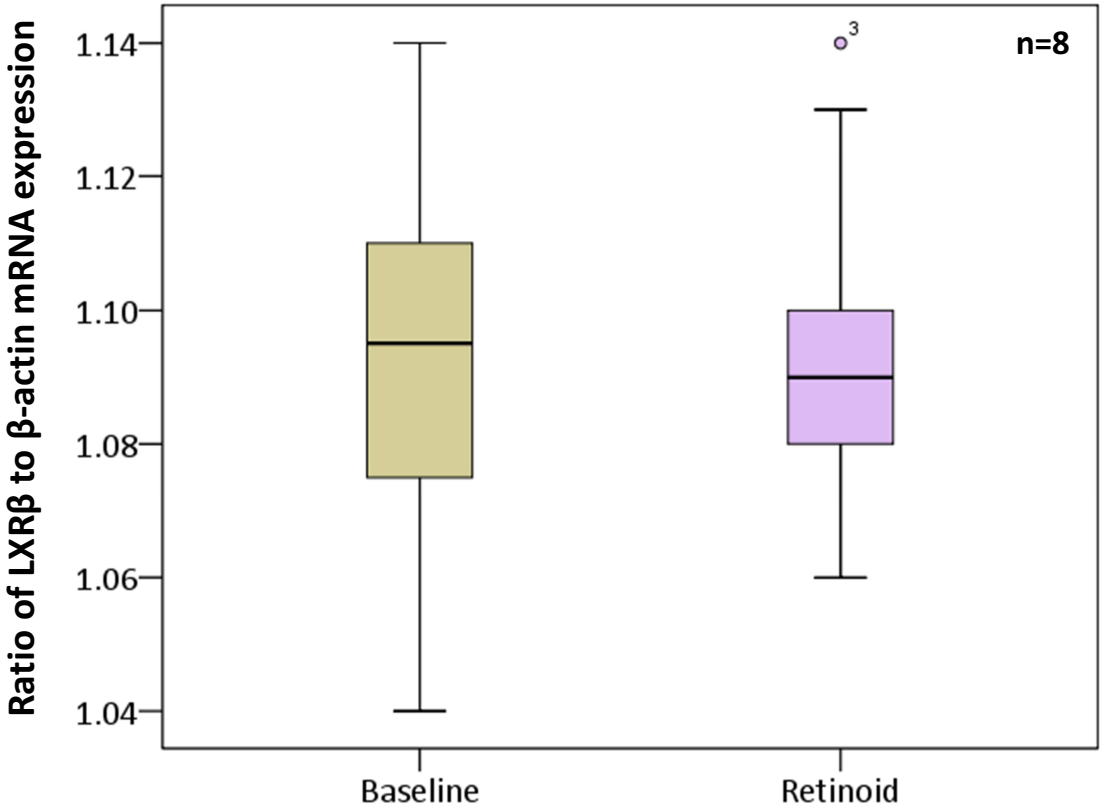


	Mean	N	Std. Deviation	Std. Error Mean
Baseline	95.0337	10	22.61269	7.15076
Retinoid-treated	142.4836	10	36.19800	11.44681

Student's paired two-tailed-test

			95% Confidence Interval of the Difference				
Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	**Sig. (2-tailed)**
-47.44983	38.54902	12.19027	-75.02613	-19.87352	-3.892	9	**.004**

Figure 5-10. Box and whisker plots showing epidermal thickness data for retinoid-treated photoaged* in situ *hybridisation cohort. *Data shown for mean epidermal thicknesses in baseline and retinoid-treated photoaged forearm skin. Mean epidermal thickness was significantly greater in retinoid-treated photoaged forearm than in baseline photoaged forearm (Student's paired two-tailed t-test; p<0.005).*



	Mean	N	Std. Deviation	Std. Error Mean
Baseline	1.0944	16	.02707	.00677
Retinoid	1.0950	16	.02129	.00532

Student's paired two-tailed-test

Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	**Sig. (2-tailed)**
			Lower	Upper			
-.00063	.03623	.00906	-.01993	.01868	-.069	15	**.946**

Figure 5-11. Box and whisker plots showing LXRβ to β-actin qPCR gene expression ratios *in baseline and retinoid-treated photoaged forearm (n=8). Statistical analysis revealed no significant differences between sites (Student's paired two-tailed t-test; p=0.95; SPSS output shown).*

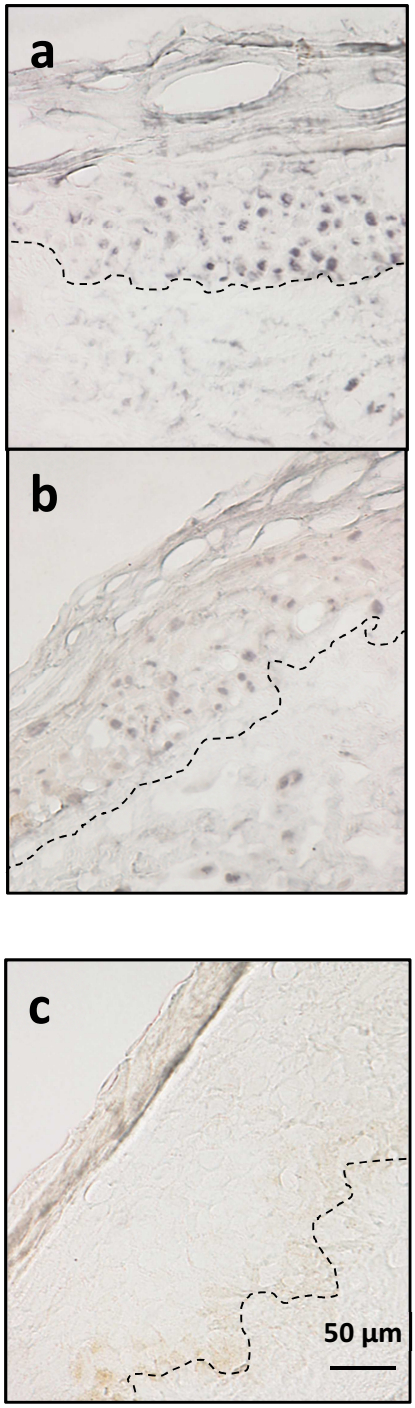


Figure 5-12. **In situ *hybridisation for LXRβ mRNA*** *in (a) baseline photoaged forearm skin and (b) retinoid-treated photoaged forearm skin. (c) shows a sense probe control treatment in retinoid-treated photoaged forearm skin. Dashed lines indicate the DEJ.*

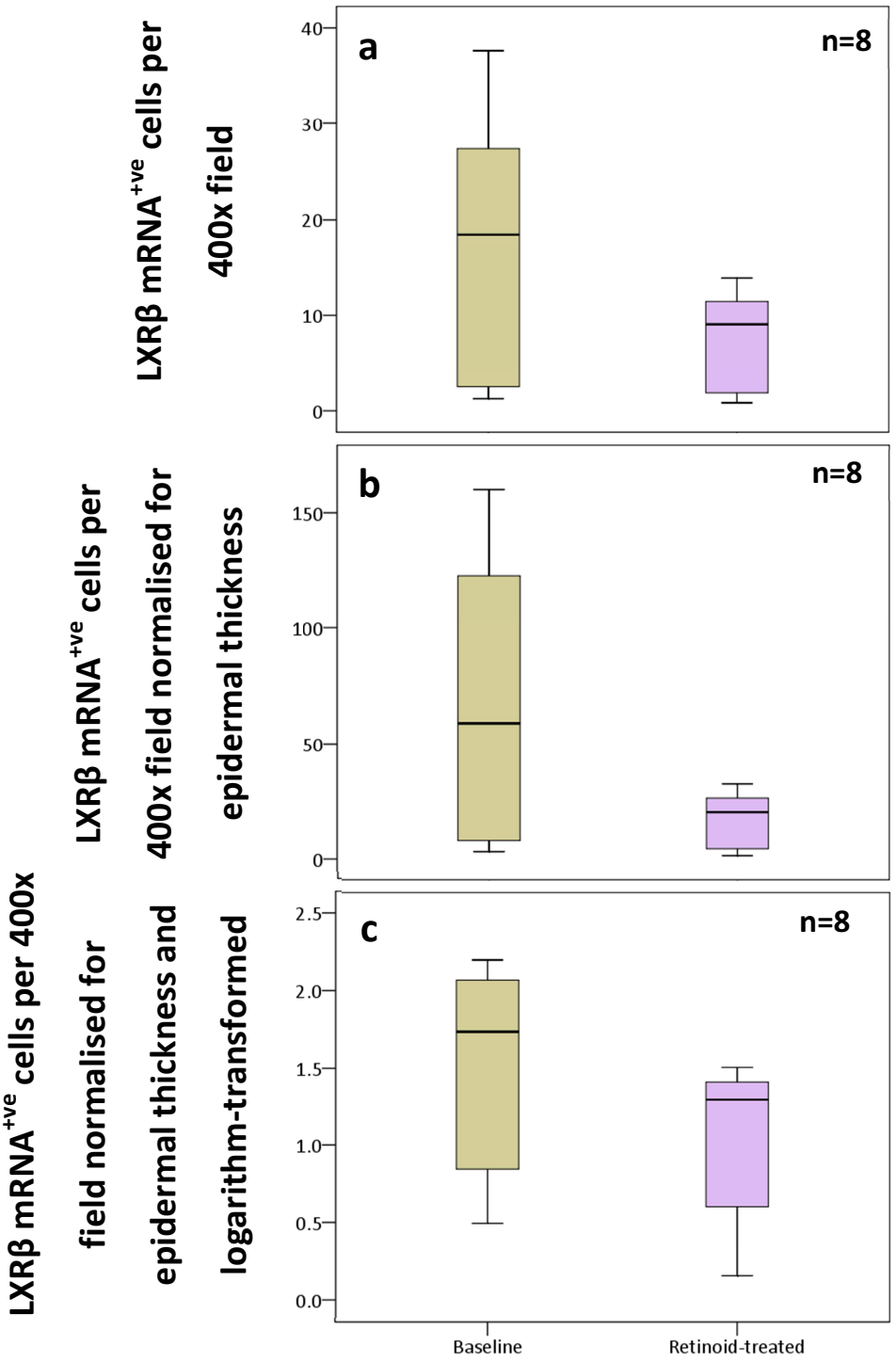


Figure 5-13. Box and whisker plots showing LXRβ mRNA^{+ve} cell counts *(a) per 400x field (b) normalised for epidermal thickness and (c) normalised then logarithm transformed to allow parametric analysis in untreated and retinoid-treated photoaged forearm (n=8).*

Descriptive statistics

	Mean	N	Std. Deviation	Std. Error Mean
Baseline	1.5062	8	.68256	.24132
Retinoid-treated	1.0434	8	.51506	.18210

Student's paired two-tailed-test

Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference		t	df	**Sig. (2-tailed)**
			Lower	Upper			
.46281	.96665	.34176	-.34533	1.27095	1.354	7	**.218**

Table 5-3. SPSS output for Student's paired two-tailed t-test comparison of logarithm-transformed normalised LXRβ mRNA^{+ve} cell counts between baseline and retinoid-treated photoaged forearm skin. *This analysis did not identify any significant differences in the frequency of LXRβ mRNA^{+ve} cells between baseline and retinoid-treated sites (Student's paired two-tailed t-test; p=0.22).*

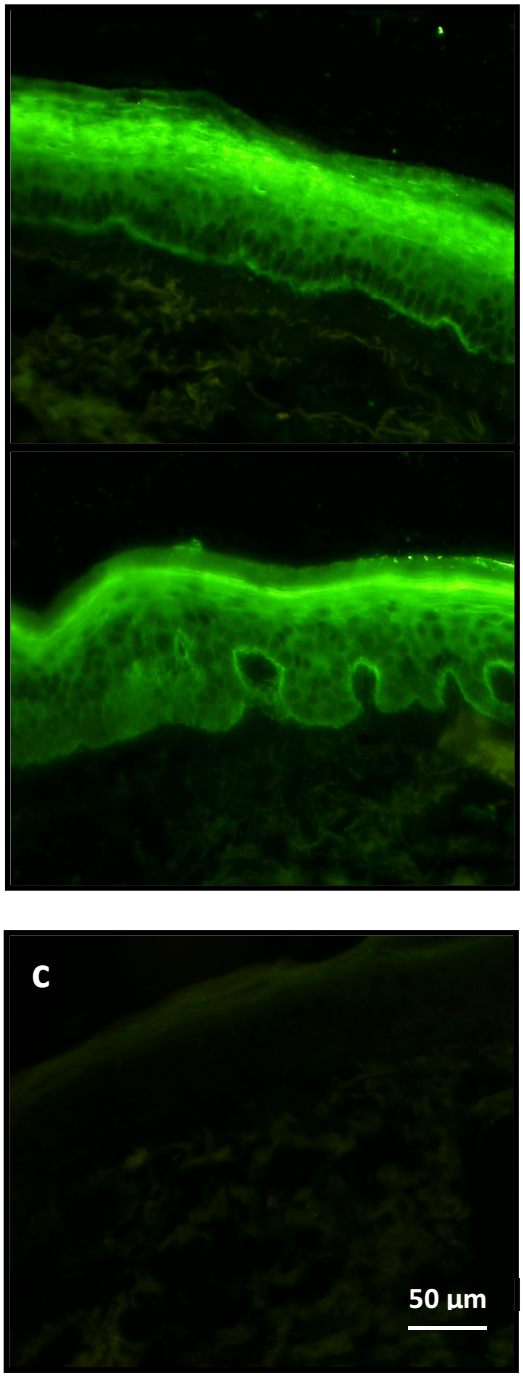
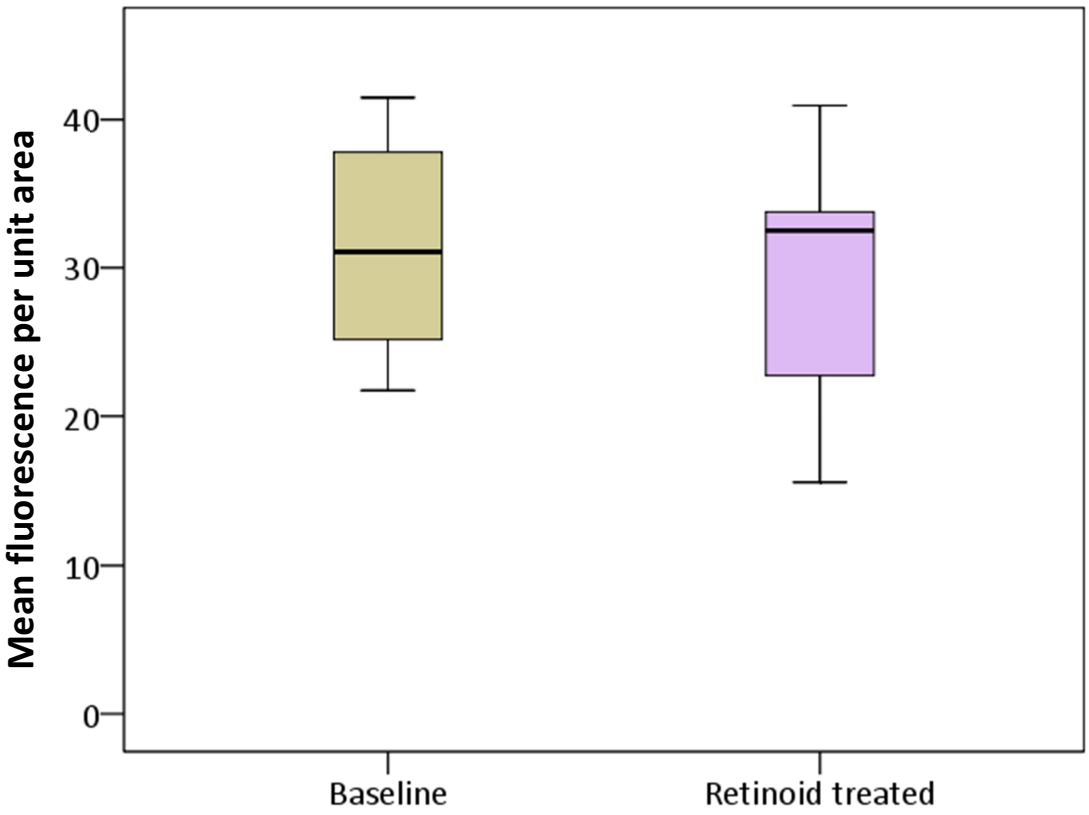


Figure 5-14. Immunofluorescent staining for LXRβ protein in untreated (a) and retinoid-treated (b) photoaged forearm skin. *The no antibody negative control (c) show background autofluorescence only.*



	Mean	N	Std. Deviation	Std. Error Mean
Baseline	30.7425	9	7.42812	2.47604
Retinoid-treated	29.6315	9	8.36658	2.78886

Student's paired two-tailed-test

			95% Confidence Interval of the Difference				
Mean	Std. Deviation	Std. Error Mean	Lower	Upper	t	df	**Sig. (2-tailed)**
1.11102	10.82165	3.60722	-7.20724	9.42928	.308	8	**.766**

Figure 5-15. Box and whisker plots of epidermal LXRβ immunofluorescence data in baseline and retinoid-treated photoaged forearm skin. *No significant difference was found between baseline and retinoid-treated photoaged forearm sites. (Student's paired two-tailed t-test; p = 0.77).*

6. Discussion

In these studies the expression of LXRβ, an NHR that mediates lipid and glucose homeostasis, was examined in intrinsically aged, photoaged and retinoid-treated photoaged human skin. Immunofluorescence was used to identify LXRβ protein, whilst LXRβ mRNA was identified by qPCR and *in situ* hybridisation. In agreement with a previous study (Russell et al., 2007), LXRβ mRNA and protein were abundantly expressed by keratinocytes of the intrafollicular epidermis. In contrast, dermal LXRβ signal could not be reliably discriminated for either mRNA and protein, due to low cellularity and a tendency of the dermal matrix to produce non-specific staining. The low signal-to-noise ratio of dermal staining prevented robust quantification by immunofluorescence and *in situ* hybridisation. These studies therefore focused on LXRβ expression in the epidermis.

To verify the specificity of the antibody used for immunofluorescence staining of LXRβ, Western blots were conducted using samples from keratinocyte and fibroblast cell lines. A single reactive band co-migrated with weight markers at the predicted molecular weight of LXRβ (≈50 kDa), supporting the specific nature of the immunofluorescence staining. Reactivity was detected in keratinocyte and fibroblast cell line extracts at approximately equal intensity, corresponding with previous findings by Russell and co-workers (2007).

Whilst the localisation of LXRβ mRNA and the tissue distribution of LXRβ protein were as expected, protein reactivity was observed in the cell periphery in immunofluorescence experiments. This is unusual; type II NHRs are normally confined to the nucleus. However, there are precedents for the cytoplasmic expression of LXRβ specifically amongst type II NHRs. A study utilising fluorescent LXR fusion proteins showed that while LXRα is only expressed in the nucleus, LXRβ is partially exported to the cytoplasm under low ligand conditions (Prüfer and Boudreaux, 2007).

As additional support for the cytoplasmic expression of LXRβ in keratinocytes, a pan-LXR polyclonal antibody used by Russell et al. (2007) for immunostaining in whole skin sections showed both nuclear and cytoplasmic reactivity.

In Western blots using the same monoclonal LXRβ antibody as used in these studies, Russell and co-workers (2007) found that while a single reactive band was present in other differentiated skin cells and cell lines, primary human keratinocytes showed two bands at approximately 50 and 55 kDa. These bands might represent two distinct isoforms of LXRβ produced by alternative exon splicing or post-translational modification. If these isoforms localise differentially within the cell, the epitope for the antibody used in this study might speculatively be present and accessible only in the non-nuclear location.

Alternative splicing to produce an mRNA variant has been reported in the mouse LXRβ gene. The splice variant so far discovered, which is preferentially expressed in the pituitary, encodes a non-translated RNA regulator of LXRβ protein (Hashimoto et al., 2009; Hashimoto et al., 2010). However, as yet undiscovered, translated splice variants may exist with tissue specificity. NHR genes including LXRα, PXR, FXR, RARα, β and γ and PPARγ and δ have been reported to each express several splice variants, some with tissue specificity (Huber et al., 2002; Chen et al., 2005; Germain et al., 2006; Lundell et al., 2007; Bruedigam et al., 2008).

In this study, LXRβ mRNA and protein expression were compared between photoprotected skin from young (18-30 years) and intrinsically aged (65-75 years) volunteers by qPCR, *in situ* hybridisation and immunofluorescence. The 18-30 year group were chosen in this age range to represent the life period following most development but before the onset of most ageing processes, whilst the 65-75 year old group were chosen to represent moderate intrinsic ageing.

The use of older volunteers was excluded for two reasons; firstly that recruitment and clinic visits are difficult for very old persons. The second reason for using volunteers 65-75 years old i.e. moderately aged individuals is that most functional deteriorations in ageing are thought to be linear until the last few years of life, when declines in tissue function become exponential due to the failure of linked systems (Crevecoeur, 2001; Gavrilov and Gavrilova, 2001; Sehl and Yates, 2001; Laird and Sherratt, 2009). Studies of these two stages arguably address different biological questions; during the late, exponential phases of decline many secondary events may confound identification of primary ageing processes. Finally, individuals at the oldest ages represent by definition a high longevity demographic unrepresentative of the general population.

The results of the experiments reported here show that LXRβ mRNA and protein expression are stable in intrinsic skin ageing. This contrasts with several other type II NHRs, which are altered in expression in intrinsic ageing in other organs (RARβ, RXRβ, RXRγ and TR; Enderlin et al., 1997; Enderlin et al., 1997) and in epidermis (RARα; Watson et al., 2004).

One factor affecting NHR expression is ligand availability (Enderlin et al., 1997; Pallet et al., 1997). For instance, it is suggested that the increase in RARα expression during ageing may be an adaptive response to reduced ligand availability in aged epidermis (Watson et al., 2004). Total serum oxysterol levels do not appear to alter with age. One endogenous LXR ligand, 24-hydroxycholesterol, peaks in serum shortly after birth and steadily decreases until age 16-18, then is stable thereafter up to at least the 9th decade of life (Janowski et al., 1996, Lutjohann et al., 1996). It is not however known how serum concentrations of LXRβ ligands are related to concentrations within cells and tissues or whether these may alter with age.

In skin, chronic UVR exposure produces an accelerated ageing phenotype. These studies examined the effects of photoageing on LXRβ protein expression by comparing skin biopsies from photoprotected sites (hip and upper inner arm) and photoaged forearm of otherwise healthy adults. Comparing these photoprotected and photoaged sites in nine volunteers did not reveal any significant differences in LXRβ mRNA or protein expression.

Although LXRβ mRNA and protein expression appeared similar between photoprotected and photoaged anatomical sites, expression did vary significantly between individuals. This variance may represent genetic variation in LXRβ expression at baseline or perhaps the effect of an unidentified factor in this cohort; for example serum LXR ligand concentrations related to diet or inter-individual metabolomic variation.

To identify whether retinoids influence LXRβ expression via signalling pathways crosstalk, LXRβ expression was assayed in photoaged human skin following treatment with *t*-RA. Retinoids have profound molecular effects in skin, stimulating widespread transcriptional changes (Nelson et al., 2009). However, these results show that LXRβ mRNA and protein expression are stable following topical *t*-RA treatment of photoaged skin.

The results of these studies show that LXRβ mRNA and protein expression is highly stable in ageing, photoageing and *t*-RA treatment of photoaged human epidermis. One question arising from these observations is whether LXRβ expression is actively maintained within a tight range due to a key role in epidermal biology, or whether the pathways controlling LXRβ expression are unaffected by ageing.

A number of NHRs are altered in expression during ageing at both the mRNA and protein levels, whilst global protein synthesis has been reported to decline in advanced age (Makrides, 1983; Syntichaki and Tavernarakis, 2006; Tavernarakis, 2008). There is evidence to suggest that LXR signalling throughout the body is likely to be evolutionarily tightly optimised due to LXR effects on immune function and inflammation. A multi-year tracking study of individuals over 85 years of age illuminated an association between LXRα gene haplotypes and human longevity (Mooijaart et al., 2005). Individuals with the most protective haplotype showed a 32% reduction in all-cause mortality over the course of the study, and 62% less mortality from infectious diseases.

These human data suggest a strong link between LXRs and immunity that is also supported by studies in models. Mice infected with *Klebsiella pneumonia* show reduced survival when treated with synthetic LXR agonists compared to untreated controls (Madenspacher et al., 2008). This is associated with impaired neutrophil motility and altered chemokine spectra (Smoak et al., 2008). The response of primary human macrophages to lipopolysaccharides (LPS) is potentiated by dietary LXR agonist treatment *in vitro*, up-regulating toll-like receptor 4 (TLR4) expression and increasing free radical generation (Fontaine et al., 2007). Interestingly, treatment of mouse macrophages with LXR agonist mutes their response to LPS and *Escherichia coli* infection and reduces the expression of several macrophage immune response genes (Joseph et al., 2003).

Whilst these two studies used similar experimental designs, they assessed different markers. It appears therefore either that LXR agonist modulation of the macrophage immune response differs between mice and humans, or that some aspects of the macrophage immune response are accentuated and others diminished in both species by LXR agonist treatment.

The importance of LXRs in innate immunity is further cemented by observations of LXRα$^{-/-}$/LXRβ$^{-/-}$ mice infected with *Listeria monocytogenes*, which are impaired in bacterial clearance and show increased rates of macrophage apoptosis compared to wild type mice. These findings were recapitulated in bone marrow transplants from LXRα$^{-/-}$/LXRβ$^{-/-}$ mice to wild type mice, demonstrating a specific fault in macrophage function in mice lacking LXR signalling (Joseph et al., 2004).

Reduced bacterial resistance was also reported in LXRα$^{-/-}$/LXRβ$^{-/-}$ mice infected with *Mycobacterium tuberculosis*, where the function of Th1 and Th17 cells was found to be compromised. In this study, LXR agonist treatment was immunostimulatory in wild type mice, eliciting a pronounced reduction in the bacterial burden on the lungs and stimulating Th1/Th17 cell function (Korf et al., 2009).

No studies have examined LXRβ in the skin immune system. However given the strong ties between LXRs and the broader immune system, it is possible that LXRβ expression may be stabilised in skin partly to fulfil immune functions. In addition to modulating the macrophage immune response, LXR agonists inhibit *in vivo* inflammatory gene induction and suppress skin thickness increases by approximately 50% in chemically-induced mouse models of contact dermatitis (Fowler et al., 2003; Joseph et al., 2003).

LXR ligand treatment has been shown to both inhibit the development of atherosclerotic lesions in mouse models of atherosclerosis (Joseph et al., 2002) and to decrease the size of existing lesions (Levin et al., 2005). This action of LXR ligands in atherosclerosis is dependent on macrophage LXR competence (Tangirala et al., 2002; Levin et al., 2005). LXR signalling benefits atherosclerosis via enhanced lipid clearance additional to lowered inflammation (Nomiyama and Bruemmer, 2008). Low grade chronic inflammation is a characteristic of ageing and is widely regarded as instrumental in driving ageing processes and age-related disease development (Franceschi et al., 2000; Chung et al., 2001; Brink et al., 2009; Chung et al., 2009).

From theoretical standpoints it could be deduced that activating LXR signalling using agonists should be beneficial in ageing. However, murine infection studies suggest that while LXR agonism reduces inflammation, it also lowers resistance to some pathogens. Abrogation of LXR signalling by genetic knock out similarly produces deficits in pathogen resistance. An LXRα haplotype is strongly associated with reduced mortality from infectious disease at old age in humans (Mooijaart et al., 2007), which suggests that severe agonistic or antagonistic perturbation of LXR signalling may carry risks in a non-sterile environment.

Further research into the LXRα haplotypes identified as protective against mortality in aged humans (Mooijaart et al., 2005) to determine how LXRα signalling is altered compared to the non-protective haplotypes would help to ascertain whether nutritional or drug modulation of LXRs might be protective against infectious disease at old age.

The literature to date is insufficient to estimate whether LXRβ influences the skin immune system. The roles of LXRβ in skin may be very different to LXR roles in other organs as NHR signalling is thought to be highly tissue-specific; for example the related receptor FXR shares only 11% of target genes between mouse liver and intestine (Thomas et al., 2009).

The primary functions of LXRβ in human skin are not yet clear, but this receptor is involved in several roles that may require its expression to be stabilised in the skin. LXRβ$^{-/-}$ mice have a skin phenotype that includes a thinner epidermis and a reduced expression of proliferation and differentiation markers (Komuves et al., 2002). Treatment of human keratinocytes *in vitro* with oxysterols or synthetic non-steroidal LXR agonists induces AP-1 components Fra-1, JunD and c-Fos, which are key to keratinocyte differentiation and epidermal barrier formation (Schmuth et al., 2004). AP-1 is also a primary inducer of enzymes capable of degrading the collagens and elastic fibers forming the dermal matrix (Benbow and Brinckerhoff, 1997; Chakraborti et al., 2003). This AP-1 effect can be functionally detrimental, damaging and degrading the matrix, or positive, stimulating matrix turnover and removing damaged molecules.

LXR agonists have been further shown to aid barrier homeostasis by modulating lipid synthesis and upper epidermal lamellar body formation, processing and excretion (Man et al., 2006). These LXR roles in barrier homeostasis may be tied to observations on LXR roles in innate immunity, as the barrier is an important seal against external microbes. Functions in immunity, barrier homeostasis or both may predicate the tight regulation of LXRβ expression in skin.

The data shown in the studies reported herein reveal that LXRβ expression appears remarkably stable in intrinsic and extrinsic skin ageing. This may be because selective constraints balancing immune function, inflammation and/or other functions have produced mechanisms to tightly regulate LXR expression and signalling. Alternatively, LXRβ signalling but not expression might alter during ageing.

The complexity of NHR regulation presents a number of avenues by which LXRβ signalling might be altered other than direct changes in protein expression. During ageing, the pattern of epigenetic modifications to chromatin is altered, influencing the accessibility of genes to transcription factors (Fraga and Esteller, 2007; Sedivy et al., 2008). Epigenetic modification of LXRβ target genes is therefore one possible point of regulation for LXRβ signalling.

Like all type II NHRs, LXRβ is also influenced by the expression and activity of numerous cofactors, which are instrumental in dictating tissue specific activities of NHRs (Gronemeyer et al., 2004; Perissi and Rosenfeld, 2005). Little is known about patterns of NHR cofactor activity in ageing, however the complexity of this regulatory system offers multiple points of regulation by which LXRβ signalling could potentially be modified during ageing. The few studies conducted to date on NHR cofactors in ageing have found that the expression of steroid receptor co-activator-1 (src-1) decreases in rodent and human white adipose tissue with age (Miard et al., 2009); while sirtuin-1 (SIRT-1), a putative longevity-associated histone deacetylase, interacts with nuclear receptor co-repressor (NCoR) at two binding sites (Picard et al., 2004). This field is open to further study.

SIRT-1 also interacts directly with LXRβ and SIRT-1-mediated deacetylation of LXRβ protein increases the transcriptional activity of LXRβ (Li et al., 2007). Post-translational modifications of LXRβ protein such as acetylation could potentially present a further point of regulation for LXRβ signalling activity during ageing.

Whilst this thesis presents data sufficient to rule out any major alterations in LXRβ expression during ageing, photoageing or following retinoid treatment of photoaged skin, the links between LXRs and ageing remain intriguing. The first direction recommended for future work would be to assay for alterations to LXRβ (and LXRα) signalling in ageing. Given the overlap between LXR-regulated and CR-regulated genes identified by microarray in mice, it would also be interesting to measure LXR transcriptional activity in CR mice at baseline and in response to dietary LXR ligands for comparison with controls fed *ad libitum.*

At present, there is no literature concerning LXRβ signalling in ageing. An overview of LXRβ signalling activity could be obtained by using an assay that either determines LXRβ DNA binding in an enzyme-linked immunosorbent assay (ELISA) style configuration or that employs a cell reporter system to measure LXRβ-driven transcription. Quantitative PCR against a panel of LXR regulated genes from different functional pathways may also constitute a somewhat reliable assay.

The unusual localisation of LXRβ immunofluorescence antibody reactivity in the peripheral cytoplasm rather than the nucleus in skin is interesting. Recently, new antibodies have become commercially available specific to LXRβ, which are raised against different epitopes to the antibody used in these studies. If another LXRβ antibody could be used for immunofluorescence in human skin, comparison with the results presented herein may be useful in defining the intracellular localisation of LXRβ protein in epidermal keratinocytes.

In conclusion, the results of these studies suggest that LXRβ expression is remarkably stable in human skin, being unchanged in intrinsic ageing, photoageing and *t*-RA treatment of photoaged skin.

Larger sample sizes may allow the confident identification of subtle changes in expression as significant, though the largest difference between any two group means in the reported studies was less than 20%. For comparison, a 200% up-regulation of the related NHR RARα was observed in aged and photoaged epidermis (Tsoureli-Nikita et al., 2004; Watson et al., 2004).

Like other NHRs, LXRβ is nested in complex signalling pathways with a number of possible points of regulation to modify the activity of the receptor without affecting its expression. The specifics of LXRβ's role in epidermal biology and skin ageing are open to elucidation by future studies.

In this study, comparisons of microarray datasets revealed a significant overlap between genes regulated by LXR agonists in mice and i) genes regulated by ageing; ii) genes regulated by CR in mice. A marginal trend was seen in the same directions in rhesus monkeys for ageing and CR and in humans for ageing. Cross-species comparisons are difficult to draw conclusions from as there is likely to be a high rate of false negatives in the identification of shared genes. The false negatives arise due to incomplete standardisation of gene naming between species and biological differences; not all mouse genes exist or have easily identifiable close equivalents in rhesus monkeys or humans. Nevertheless, the fact that the same trend is sustained in each dataset despite these technical difficulties is interesting.

It is possible to be more confident when analysing intraspecific comparisons in mice. Genbank (NCBI; Bethesda, MD, USA) numbers could be reliably used to search for genes, eliminating the problem of alternate gene names producing false negatives. Statistical analysis of these intraspecific comparisons showed that a significantly higher number of genes were commonly regulated between LXR agonist treatment and both ageing and CR than would be expected by chance. Furthermore, 73% of genes shared between LXR agonists and ageing in mice were regulated in opposite directions, while 70% of genes shared between LXR agonists and CR in mice were regulated in the same direction.

The microarray experiments analysed were performed in different tissues: liver, white adipose tissue and brown adipose tissue for LXR agonist treatment in mice and heart tissue for ageing and CR in mice. The observed degree of overlap in gene number and directionality of regulation may be lower than would be seen in comparisons between the same tissues.

These observations build on previous work (Chang et al., 2008) where a microarray of gene regulation in LXR$\beta^{-/-}$ skin was observed to overlap with a human fibroblast cell based model of intrinsic ageing (Ly et al., 2000). The observations reported here complement these prior findings, showing that not only do LXR signalling deficits partially mimic gene regulation in ageing, but that LXR agonist stimulation partially opposes gene regulation in ageing and furthermore that LXR agonist stimulation partially mimics CR. These findings suggest that LXR agonists may be interesting to examine as anti-ageing treatments and putative mimetics of the CR response.

No previous work has directly studied LXR agonists as a treatment for intrinsic ageing in whole organism models. Oxysterols and synthetic LXR agonists can be provided as dietary supplements, so such experiments should be relatively simple to perform, either in invertebrates or mammalian models. Human studies could also be performed to assess the effect of topical LXR agonists on the accelerated ageing phenotype produced by chronic UVR exposure. Previous work has found that topical LXR agonist application inhibits UVR-induced wrinkling and skin thickening in murine models (Chang et al., 2008).

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Appendix 1. Microarray comparisons shared genes: LXR agonists vs i) ageing and ii) calorie restriction

Legend: ***LIV****=Liver,* ***WAT****=White adipose tissue and* ***BAT****=brown adipose tissue from mice treated with LXR agonists.* ***MA****=Mouse ageing;* ***MCR****=mouse calorie restriction.* ***RMA****=Rhesus monkey ageing;* ***RMCR****=rhesus monkey calorie restriction.* ***HSA****=Human skin ageing. Numbers represent fold changes in mRNA expression. Please see sections 2.16 and 4.1 for background information on these data.*

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
Adhesion	Cadherin 13		2.2				-2.1		
	Connexin 43 (α-1 gap junction)			-1.95	1.6	-2.1			
Amino acid synthesis/metabolism	Histidine ammonia lyase		-2.6	4.4					-1.65
Apoptosis	E1B 19K/Bcl-2-binding protein homolog (Bnip3)	1.9	-1.6		-1.9	1.8			
Carbohydrate metabolism - Glycogen	Homologous to human glucan (1,4-α-), branching enzyme 1 (glycogen branching enzyme)	1.6	-1.9	-2	1.6				
Carbohydrate metabolism - Glycolysis	Phosphofructokinase, B-type		-1.5	-2.7	1.5	-1.5			
	Pyruvate dehydrogenase kinase 4		3.8		-8.5	-1.7		5.3	
Carbohydrate metabolism - Other	α-amylase-1	1.5				1.6			
	GLUT4		1.7				3.4	2.8	

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
	Transketolase	1.6							-1.96
Cytoskeleton - Actins	Actin, α 1, skeletal muscle		2			-2.3			
Cytoskeleton - Tubulin	Tubulin, β2			-1.8		-1.6			
Cytoskeleton - Others	Cofilin	-1.8				-3.1			
	Microtubule-associated protein tau	-1.7						2.7	
Detoxification	Flavin-containing monooxygenase 1 (Fmo1)	1.7			2.4	1.7			
Endocrinology - Cytokines	Leptin		-1.95						3.35
	Leukemia inhibitory factor receptor (mouse) / leukemia inhibitory factor receptor α (human)			-3.85					2.12
Endocrinology - Eicosanoids	Leukotriene C4 synthase		-1.7	1.7			2.9		
Endocrinology – Growth hormone	Growth hormone receptor			-1.5		-3.1	-1.9		
	Insulin like growth factor binding protein 4			-1.7		2.7			
	Insulin-like growth factor binding protein 3			1.6				1.6	-1.89
Endocrinology - Other	Catechol-O-methyltransferase			-2.9	-2.5	1.5	-4.4		-1.57

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
Endocrinology - Steroid hormones	17-β-hydroxysteroid dehydrogenase 11			1.9	-2	3.1			
Endocrinology – Thyroid hormones	Thyroid hormone receptor α			1.7			1.7	1.5	-1.87
Energy metabolism	Cytochrome C, somatic (mouse) / Cytochrome C1 (monkey)			-1.5				-22.8	
Extracellular matrix	Connective tissue growth factor (Ctgf)		1.9		-1.7	3.3			
	Glypican 4	-1.6				2.4			
	Latent transforming growth factor β binding protein 2			-1.6			2.2		1.58
	Prolyl 4-hydroxylase α(I)-subunit			-2.2		-2			
	Secreted acidic cysteine rich glycoprotein (Sparc)			-1.6		-1.5			
	Procollagen type I α-1		-1.6			-2			
	Procollagen type V α-2			-1.6		-2.1			
	Procollagen type VI α-2		-1.6			-3.3			-1.56
Heterotrimeric G-protein signalling	G protein β-subunit	2.2	2	2.8	-1.7				
	G protein signalling regulator RGS2 (rgs2)			-1.7		2.3			
Heavy metal binding	Metallothionein 1	5.1			-2				
	Metallothionein 2	2.1			-1.9	1.7			

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
Heme synthesis	Amino levulinate synthase (ALAS1, ALAS-H)	2.3			2.4				
Hemostasis	Thrombospondin 1		3						1.74
Inflammation	Interferon γ inducible protein 30		1.6			-2.3			
	Monocyte chemoattractant protein-2		1.7		3.4				
	Pre-B-cell colony-enhancing factor	2.9				2.3			
Lipid synthesis/metabolism – Fatty acid oxidation	Carnitine acetyltransferase	2.5			-1.6				
	Dodecenoyl-CoA δ-isomerase	1.7			-1.5				
Lipid synthesis/metabolism - Fatty acid synthesis	Fatty acid synthase	4.1			8.6				
	Malic enzyme	3.9	-2.7		1.9				
	Acyl CoA thioesterase 1, cytosolic	6.3		4.6	-5.1	3.9			
Lipid synthesis/metabolism - Lipoproteins	Apolipoprotein CI		1.6	5.4		-2.5			

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
	Apolipoprotein D		13.1	15.9					2.2
	CD36 antigen			-1.6	-1.8				1.88
	Phospholipid transfer protein	6.7	1.6	1.6		-3.6			
Nuclear proteins - Histones	H1 histone family member 2 (H1f2)			-2.2	-1.6				
	Histone 2A			-1.8	1.7				
	Histone 2B	-1.6		-2			-4.5		
Peroxisomes	L-specific multifunctional β-oxidation protein	8.9	1.8	6.4		2.4			
	Peroxisomal biogenesis factor	1.6				2.3			
	Peroxisomal δ3, δ2-enoyl-Coenzyme A isomerase	1.6			-1.6	1.9			
Proliferation – Cell cycle related	Cyclin G2			-1.8		1.8			
	Cyclin-dependent kinase inhibitor 1A (P21)		-1.7				4.1		
	Growth arrest and DNA-damage-inducible 45 α (Gadd45a)	2.2	2.1			-3			

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
	Homologous to rat CDK5 activator-binding protein C53			-1.9	-2.8	-4.8			
Proliferation - EGFR	Epidermal growth factor receptor (EGFR)	-5.6							1.68
Proliferation - Oncogenes	Kras oncogene-associated (mouse) / sarcospan (human)			1.7					1.5
	Necdin			-1.7	2				
Proliferation - Other	Prohibitin			-1.6		-10.3			
Proteasomes	Proteasome (prosome, macropain), α 7	1.5				1.5			
	Ubiquitin-specific protease 2			1.6		1.9			
Protein synthesis	Elongation factor Tu, mitochondrial precursor			-1.9		-1.6			
Protein/vesicle traffic	Homologous to human sorting nexin 2	2.1				-4.2			
	Sec61 α subunit homolog (mouse) / Sec61 α 1 subunit (human)	-1.8							-1.65
	RAB9, member RAS oncogene family (Rab9)	1.7				2			
Proteinase inhibitors	Cathepsin Z			-2.2		-4.3			

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
	Spi2 protease inhibitor 2-2	-1.6	-1.8		-6.5				
Purin synthesis/metabolism	Xanthine dehydrogenase	1.8	1.5		-2.2	1.9			
Redox metabolism – Pentose phosphate shunt	Transaldolase			-1.6	1.9				
Serum proteins – Complement components	Complement component 4 binding protein	-1.8					2.6		
Serum proteins – Kinine formation	Kallikrein-binding protein	-1.6	-1.7		-4.5				
Serum proteins - Other	Acid phosphatase 5, tartrate resistant			-2.5					-1.6
	α-albumin	-1.6						2.8	
	Major urinary protein 1	-1.8				-3.2			
Signal transduction – Calcium signalling	FK506 binding protein 5 (FKBP5)	2.8				16.7			
	Inositol 1,4,5-triphosphate receptor 1 (ITPR1)			-1.9					1.58
Signal transduction - Kinases	MAP kinase-interacting serine/threonine kinase 2	3	1.5		-1.5				
Sterol biosynthesis/metabolism – Sterol biosynthesis	Sqaulene epoxidase	1.5			-1.8		2.8		1.5

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
	ATP binding cassette 1 (ABCA1)		2.8	2.9	-2.3				
Sterol biosynthesis/metabolism – Bile acids	Cytochrome C oxidase, subunit VIIIb (mouse) / subunit VIII (monkey)			-1.5			-2.2	-2.4	
Transcription - Regulation	B-cell leukemia/lymphoma 6 (Bcl6)	-4		-1.7				2.2	
	CCAAT/enhancer binding protein (C/EBP), β	1.5			-2.2	3.6			
	D site albumin promoter binding protein		2.6						-2.04
	Mrg1, Cbp/p300-interacting transactivator	-1.7				2			
	TGFβ inducible early growth response (Tieg)		1.9			2			
Translation - Chaperones	DnaJ HSP40 homolog subfamily B member 9	-1.5		-1.8		-1.7			1.5
	Heat shock protein (HSP) 47			-2	1.5	-4			
	Heat shock protein (HSP) 70		1.7	-4			3.4		
	Heat shock protein, 105 kDa			-2.9		-2			

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
Other functions	Aquaporin 1		1.6	2.1	-1.8				
	Basigin			-1.6		-2.1			
	Carbonic anhydrase-like sequence 1 (Cals1)	-1.8			1.7				
	Endogenous murine leukemia virus modified polytropic	-1.7			-2.5	-2.3			
	Fragile X mental retardation syndrome 1 homolog		-2.1						1.57
	Heme-binding protein 1			1.6		1.7			
	Hemoglobin, β adult major chain	-1.6		-1.9			4.3		
	Homocysteine-inducible, endoplasmic reticulum	-1.7				2.5			
	Inhibin β-C	-1.7			-1.5				
	Lumican		-1.7					2.8	
	NAD(P)+ arginine ADP-ribosyltransferase			2	-1.6	2.2			
	Neuronatin (Nnat)		1.8		-2.7				
	Odz, odd Oz/ten-m homolog 4 (Drosophila)		-1.75						-1.51
	Pale ear			1.9	-1.5	2.2			
	Poly A binding protein, cytoplasmic 1 (Pabpc1)			1.5		1.5	-2.4		

Function	Gene name	LIV	WAT	BAT	MA	MCR	RMA	RMCR	HSA
	Testosterone-regulated RP2 protein	1.5			1.7				
	Transmembrane 4 superfamily member 7		1.6		-8.3				
Unknown	Unknown	2.8			-2.6				
	Unknown	2.1		1.8	-2.5	2.7			
	Unknown	1.8			-1.6	2			
	Unknown	1.7	2.3		1.5	-2			
	Unknown	1.7				-2			
	Unknown			-2	1.9	-4.3			
	Unknown			-1.7	1.5				
	Unknown		2.8	3.5	1.5				
	Unknown			-1.6	1.5				
	Unknown			-1.5	2				
	Unknown			-1.5	2.5	-3.7			
	Unknown		1.7	1.8	-1.9				
	Unknown		1.8	1.6	-1.5	-2.1			
	Unknown			-1.6		-1.6			
	Unknown			-1.6	1.6				
	Unknown	1.7			-2.6	2.3			
	Unknown	1.5				1.6			
	Unknown		1.5			-1.6			
	Unknown			1.6		1.7			

Appendix 2. Publications arising from this thesis

Ford, C. T., M. J. Sherratt *et al.* (2009). Liver X receptor β: maintenance of epidermal expression in intrinsic and extrinsic skin aging. ***AGE*** **31**(4): 365-72.