# Characterisation of 11beta Hydroxysteroid Dehydrogenase 1 in Ocular and Orbital Tissues

By

Claire Uchechukwu Onyimba

Presented to

University of Birmingham

For the Degree of Doctor of Philosophy

Academic Unit of Ophthalmology

Division of Immunity and Infection

And

Department of Endocrinology

Division of Medical Sciences

University of Birmingham

United Kingdom

# Summary

The eye is a glucocorticoid target tissue which orchestrates expression of target genes through the glucocorticoid receptor (GR). The classical function of GR involves its interaction with glucocorticoid to influence transcription of genes involved in numerous physiological processes which include inflammation. The first line of defence in the ocular tissues includes the mucosal barrier and expression of receptors that recognise pathogen. These mechanisms activate the innate immune response during inflammation, however, in the 'normal' eye, immunomodulatory components exist to promote immune privilege. 11beta hydroxysteroid dehydrogenase 1 (11β-HSD1) regulates cortisol locally in tissues and has already been localised to to some ocular surface and intraocular tissues. The aim of this thesis is to evaluate the functional role of 11β-HSD1 in the eye and the orbit and whether the 11β-HSD1 can be targeted to modify various disease processes in the eye. An animal model was used to characterise the pre-receptor regulation of glucocorticoids and this was further characterised in human ocular and orbital tissues and cells. The results showed that 11β-HSD1 is functional in certain ocular (corneal epithelial, fibroblast and conjunctival fibroblast) and orbital (orbital preadipocyte) cells. The data therefore emphasises the putative role of 11β-HSDs in the ocular and orbital microenvronment.

**To my parents**

# Acknowledgements

I would like to express my gratitude to Miss Saaeha Rauz and Dr Elizabeth Walker for their supervision and guidance during the course of this PhD.

I would like to thank Mr Omar Durrani, Dr Areeb Moosavi and Mr Tristan Reuser for providing orbital adipose tissue samples that were so precious and valuable to my work. I would also like to thank Mrs Susan Hughes, Miss Pamela Khosla and Dr Alexandra Sinclair for their unequivocal support in the lab. I would like to thank Joseph Abbott for his role in devising the automated histomorphometric method for studying adipocyte cell characteristics.

I am very grateful to Mr Charlie Shaikh who spent a lot of time helping with tissue sectioning and immunohistochemistry techniques in the labs of the Academic Unit and who welcomed me into his lab and kept me company at times. I should also thank Dr Iwona Bujalska, Dr Jeremy Tomlinson, Dr John Curnow and Dr Graham Wallace for their advice on different aspects of my PhD.

I would also like to thank Professor Philip Murray for his support and for making me feel like part of the group. I will also thank Professor Paul Stewart for allowing me to perform the vast majority of my experiments in his labs at the Institute of Biomedical Research (IBR). I will also extend my gratitude to the people I have come to know in the lab at the IBR who have given me advice and have helped to make my experience enjoyable in the lab.

Finally, I am very grateful to my parents, for their much valued and continued support.

# List of Figures and Tables

# List of Abbreviations

| | |
|---|---|
| $^{3}$HE | Tritiated cortisone |
| 6GP | glucose 6 phosphate |
| 5α THF | 5 alpha tetrahydrocortisol |
| ACAID | Anterior chamber-associated immune deviation |
| ACE | Angiotensin converting enzyme |
| ACTH | Adrenocorticotrophin hormone |
| ADSC | Adipose tissue derived stromal cells |
| Allo THF | 5α tetrahydrocortisol |
| AME | Apparent mineralocorticoid excess |
| APC | Antigen presenting cells |
| ARVO | Association for Research in Vision and Ophthalmology |
| ASC | Adipose stromal cell |
| ASP | Acylation-stimulation protein |
| BMEC | Birmingham Midland Eye Centre |
| BSA | Bovine serum albumin |
| C/EBP (beta) | CCAAT/enhancer binding protein |
| CA | Carbonic anhydrase |
| CALT | Conjunctival associated lymphoid tissue |
| CAS | Clinical activity-scoring |
| CB | Ciliary body |
| CCL | Chemokine (C-C motif) ligand |
| CCR | Chemokine (C-C motif) receptor |
| CD | Cluster of differentiation |
| CHO | Chinese hamster ovary |
| CK | Cytokeratin |
| $Cl^{-}$ | Chloride ion |
| $CO_2$ | Carbon dioxide |

| | |
|---|---|
| CRD | Cortisone reductase deficiency |
| CRH | Corticotrophin releasing hormone |
| Ct | Cycle threshold |
| CT | Computed tomography |
| CTLA | Cytotoxic T lymphocyte-associated antigen |
| CV | Central vein |
| CYP | Cytochrome P450 |
| DAB | 3, 3’-Diaminobenzidine |
| DC | Dendritic cell |
| dCt | Difference in cycle threshold |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| dNTP | Deoxyribonucleotide |
| DPX | Dibutyl-polystyrene-xylene |
| E | Cortisone |
| EGF | Epidermal growth factor |
| ENaC | Epithelium sodium channel |
| ERK | Extracellular signal-regulated kinase |
| F | Cortisol |
| FABP | Fatty acid binding protein |
| Fas | Fatty acid synthase |
| Fas-L | Fatty acid synthase ligand |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FRAME | Fund for the Replacement of Animals in Medical Experiments |
| G3PDH | Glyceraldehyde-3-phosphate dehydrogenase |
| G6P | Glucose 6 phosphate |
| GAG | Glycosaminoglycan |
| GE | Glycyrrhetinic acid |

| | |
|---|---|
| GR | Glucocorticoid receptor |
| GRE | Glucocorticoid response element |
| H & E | Haematoxylin and Eosin |
| H6PDH | Hexose 6 phosphate dehydrogenase |
| hBD2 | Human beta defensin 2 |
| HCEC | Human corneal epithelial cell-line |
| HCF | Human conjunctival fibroblast |
| $HCO_3$ | Hydrogen carbonate |
| HCoF | Human corneal fibroblast |
| HGF | Hepatocyte growth factor |
| HLA | Human leukocyte antigen |
| HOAT | Human orbital adipose tissue |
| HML | Human mucosal lymphocyte antigen-1 |
| HPA | Hypothalamic-pituitary-adrenal axis |
| HSD | Hydroxysteroid dehydrogenase |
| HSL | Hormone sensitive lipase |
| IBMX | 3-Isobutyl-1-methylxanthine |
| ICAM | Intracellular adhesion molecule |
| ICC | Immunocytochemistry |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IHC | Immunohistochemistry |
| iKK | inhibitory-binding protein kappa B kinase |
| IL | Interleukin |
| IOI | Idiopathic orbital inflammation |
| IOP | Intraocular pressure |
| IRAK | IL-1R associated protein kinase |
| IRF | Interferon regulatory factor |
| IκB | Inhibitory kappa B |

| | |
|---|---|
| $K^-$ | Potassium ion |
| KGF | Keratinocyte growth factor |
| KTEC | Kidney tubule epithelial cell |
| LBP | Lipopolysaccharide binding protein |
| LPL | Lipoprotein lipase |
| LPS | Lipopolysaccharide |
| LREC | Local research ethics committee |
| MAMP | Microbial associated molecular pattern |
| MAPK | Mitogen-activated protein kinase |
| MCP | Monocyte chemotactic protein |
| MD2 | Myeloid differentiation 2 |
| MEH | Moorfields Eye Hospital |
| mAb | Monoclonal antibody |
| mFas-L | Membrane bound fatty acid synthase ligand |
| $MgCl_2$ | Magnesium chloride |
| MHC | Major histocompatibility complex |
| MMP | Matrixmetalloproteinase |
| MR | Mineralocorticoid receptor |
| MRI | Magnetic resonance imaging |
| MyD88 | Myeloid differentiation factor 88 |
| $Na^+$ | Sodium ion |
| $Na^+$ $K^+$ ATPase | Sodium/potassium ATP enzyme |
| NAD | Nicotinamide adenine dinucleotide |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Reduced nicotinamide adenine dinucleotide phosphate |
| NDS | Normal donkey serum |
| NEMO | NFκB Essential. Modulator |
| NFκB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NIDDM | Non-insulin dependent diabetes mellitus |

| | |
|---|---|
| NLR | NOD-like receptor |
| NOD | Nucleotide-binding and oligomerisation domain |
| NPE | Non-pigmented epithelium |
| NSS | Normal swine serum |
| NZWAR | New Zealand white Albino rabbit |
| $O_2$ | Oxygen |
| OM | Omental |
| P/S | Penicillin and Streptomycin |
| PA | *Pseudomonas aeruginosa* |
| PAI | Plasminogen activator inhibitor |
| PBS | Phosphate buffered saline |
| PCOS | Polycystic ovary syndrome |
| PCR | Polymerase chain reaction |
| PDGF | Platelet-derived growth factor |
| PE | Pigmented epithelium |
| PHCEC | Primary corneal epithelial cell |
| PMN | Polymorphonuclear |
| PPAR | Peroxisome proliferators-activated receptor |
| RANTES | Regulated on activation normal T cell expressed and secreted |
| RT | Reverse transcription |
| SC | Subcutaneous |
| SDR | short chain alcohol dehydrogenase superfamily |
| SEM | Standard error of the mean |
| sFas-L | Soluble fatty acid synthase ligand |
| SGK | Serum and glucocorticoid induced kinase |
| $T_3$ | Triiodothyronine |
| $T_4$ | Thyroxine |
| TAK | Transforming growth factor-β-activated kinase |
| TAO | Thyroid associated ophthalmopathy |

| | |
|---|---|
| TBK | TANK-binding protein kinase |
| TBS | Tris buffered saline |
| TGF | Transforming growth factor |
| Th | T helper |
| THE | Tetrahydrocortisone |
| THF | Tetrahydrocortisol |
| TLC | Thin layer chromatography |
| TLR | Toll-like receptor |
| TM | Trabecular meshwork |
| TNF | Tumour necrosis factor |
| TNFαIP | Tumour necrosis factor alpha induced protein |
| TRH | Thyrotropin-releasing hormone |
| TSAB | Thyroid stimulating autoantibodies |
| TSH | Thyroid stimulating hormone |
| UFAW | Universities federation for Animal Welfare |
| UFF | Urinary free cortisol |
| US | Ultra Sound |
| UV | Ultraviolet |
| v/v | volume (of solute) per volume (of solvent) |
| WAT | White adipose tissue |
| WG | Wegner’s granulomatosis |

# Table of Contents

# 1. Chapter 1

# General Introduction

## *1.1. Introduction*

## *1.2. Human Eye in the Orbit*

The human eye is a complex structure which acts as our sensory receptor to focus light via the cornea, pupil and lens and onto the retina. The iris can alter the size of the pupil for entry of light depending on the intensity of the light in the surrounding environment. The ciliary muscles contract to alter the shape of the lens to influence the refraction of light on to the retina (Figure 1-1) and the ciliary processes are responsible for aqueous humour production. The aqueous humour production is known to have important roles in nourishing and removing waste materials from the various processes of the eye which include the cornea and lens which do not have access to local blood supply. The conjunctiva is a mucosal surface which is able to produce mucous to lubricate and keep the eyeball clean while the vitreous fluid, a jelly-like fluid in the posterior part of the eye provides a pressure to keep the globe spherical.

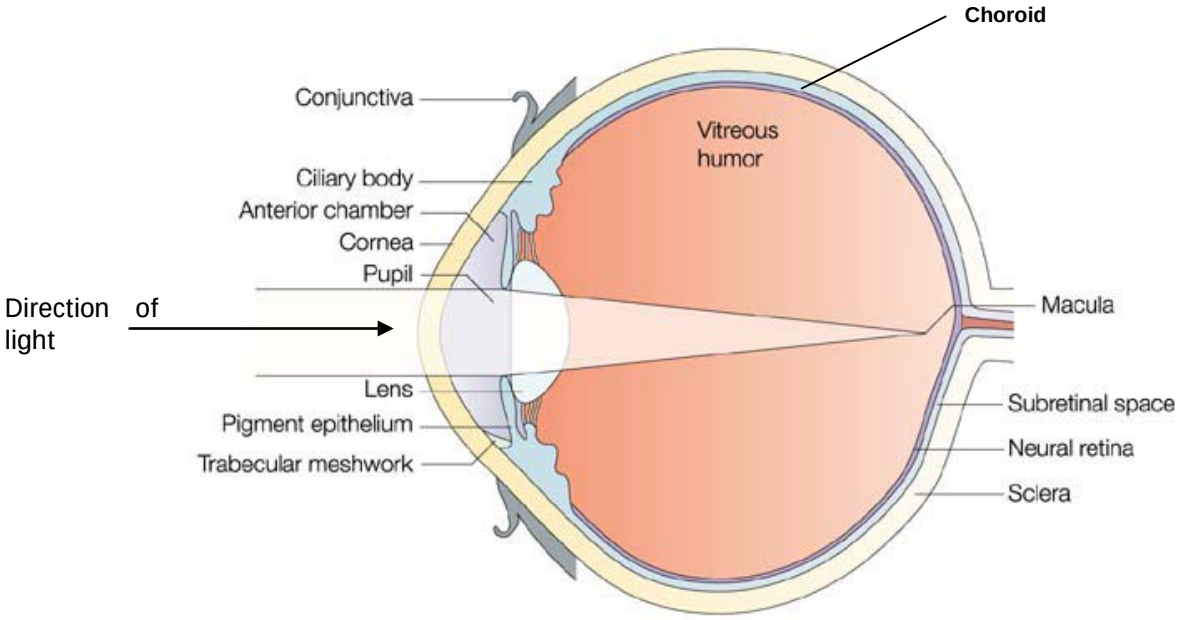



*Figure 1-1: Diagram of the human eye depicting essential anatomy for correct eye vision.*
*Picture taken from Streilein, 2003a*

The human eye is anchored in the orbit by the attachment of extraocular muscles which consist of the lateral, medial, inferior and superior rectal muscles and superior and inferior oblique muscles from the sclera to the bony orbit (Davson, 1990). Any spaces within the orbit which do not contain the globe, muscles, nerves or vessels are occupied by adipose tissue which anatomically can be subdivided into the retrobulbar fat, located posterior to the eyeball and peribulbar fat that surrounds the eyeball and acts to protect the structures of the eye.

### *1.3. Anatomy of Ocular Surface Defence Barriers*

The ocular surface consists of tear film, the conjunctiva, cornea, and the area of transition known as the limbus.

### 1.3.1. Conjunctiva

The conjunctiva is a vascularised mucosal surface which is a continuous membrane but comprises of three main sections (Figure 1-2):

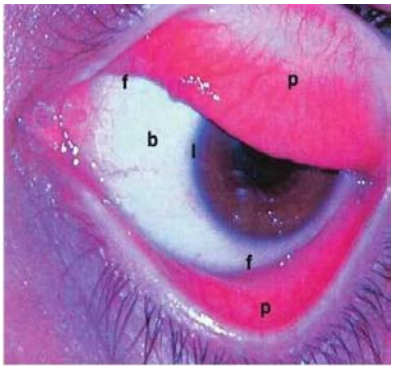
(1) The palpebral conjunctiva, where the cells are stratified squamous in appearance and cover the inside of the eyelid. This consists of three main parts, which are the mucocutaneous junction which is located behind the meibornian gland. It possesses conjunctival associated lymphoid tissue (CALT) which consist of lymphocytes, dendritic cells and plasma cells located near small vessels and high endothelial venules (HEV), (Knop and Knop, 2000, Ono and Abelson, 2005, Rosellini et al., 2007).

(2) The conjunctival cul-de-sac or fornix consists of the superior fornix, located at level of the orbital rim near the limbus; the inferior fornix and the lateral fornix where the cells become less squamous at the junction between the posterior of the eyelid and the eyeball.

(3) The bulbar is composed of the scleral part which extends from the fornix to the limbus and the limbal part which form a ring at the junction between the conjunctival and corneal epithelium (Hoang-Xuan, 2001). The cells of the bulbar conjunctiva are typically stratified columnar (Forrester et al., 2001, Calonge and Stern, 2004).

The conjunctival epithelium also possesses goblet cells, cytokeratin 19 and is negative for cytokeratin 3 (CK3). Cytokeratins are cytoskeletal proteins which form intermediate filaments during epithelial development and differentiation (Rosellini et al., 2007, Elder et al., 1997, Boehlke et al., 2004). The tear film, which is present to protect the eye and provide nourishment for ocular surface, is composed of lipid, aqueous layers and mucin which is produced from the meibornian gland in the eyelids, lacrimal gland and conjunctival goblet cells.

**A**




**B**

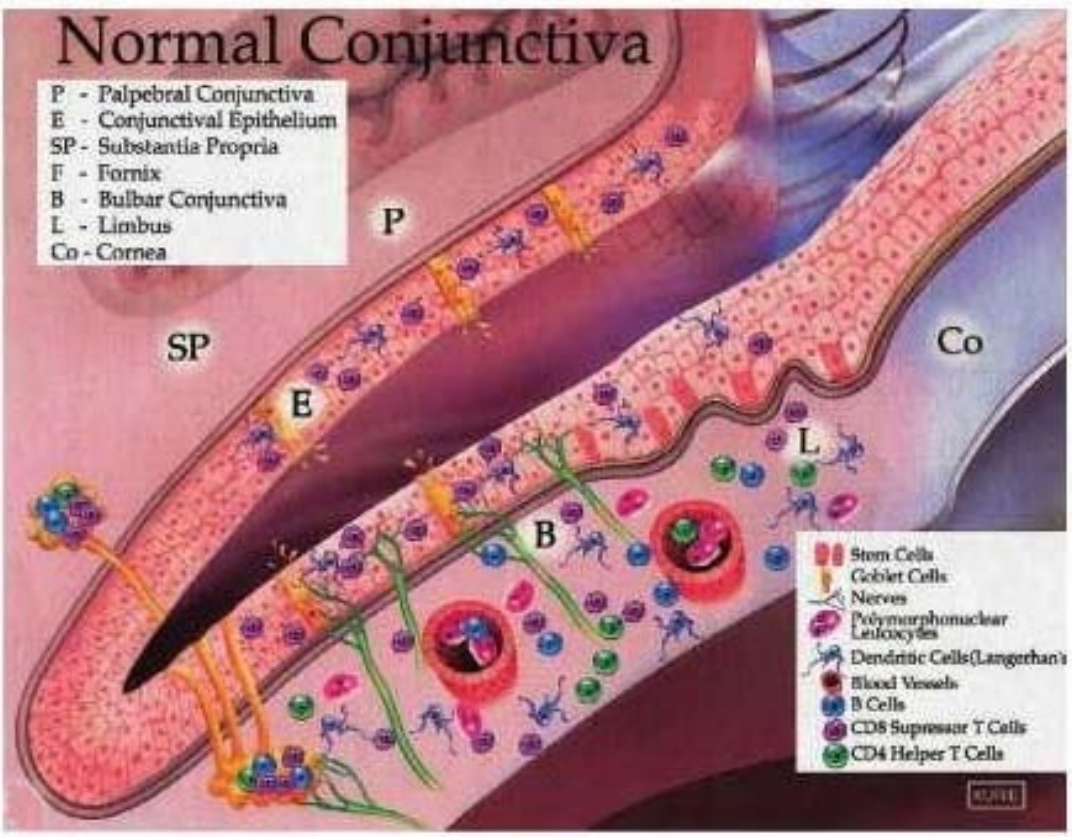



*Figure 1-2: The Human eye and the human conjunctiva.*

*(A) Photograph of the human eye. Picture shows the bulbar (b), palpebral (p) and forniceal (f) conjunctiva zones. The limbus (l) is the area of transition between the conjunctiva and the cornea.*
*(B) Schematic of the normal conjunctiva showing the epithelium (E), substantia propria (SP) and the three parts of the conjunctiva: palpebral (P), forniceal (F) and bulbar (B). The cornea (Co) and the limbus (L) are in close proximity. Immune cells are present in the conjunctiva which include dendritic cells, B cells and T cells. Taken from Pflugfelder et al., 2004*

### 1.3.2. Cornea

The cornea is avascular and therefore needs to be nourished by the aqueous humour internally and externally by a thin tear film. The epithelium is non-keratinising and is composed of five layers consisting of basal cells which are cuboidal to columnar cells found in the differentiated central cornea compartment. The corneal epithelial cells become flattened and form wing cells which are two to three layers thick. The corneal epithelium is continuous with a highly specialised area called the limbus which houses stems cells vital for corneal perpetuation. The five layers of the cornea (Figure 1-3) which is about 500μm in thickness are: (1) the outermost epithelium, (2) the Bowman's membrane, (3) the stroma, (4) the Descemet's membrane and (5) and the endothelium.

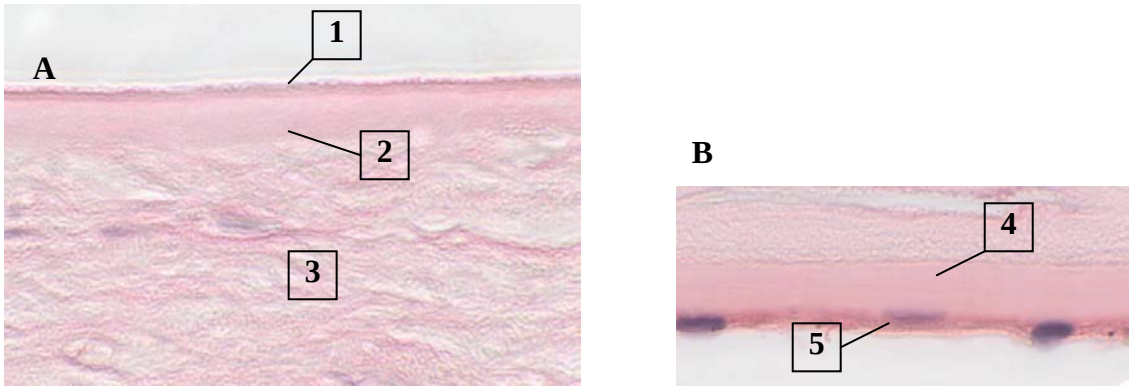



*Figure 1-3: Haematoxylin and Eosin staining of the human cornea.*

*(A) consists of (1) corneal epithelium (2), Bowman's capsule (3), stroma at x40 magnification and (B) consists of (4) Descemet's capsule and (5) endothelium at x 100 magnification.*

The corneal epithelium expresses a genotype which includes connexion 43 (Cx43), gap junction protein; cytokeratin 3 (CK3) marker for corneal epithelial cell differentiation; cytokeratin 5 (CK5); cytokeratin 12 (CK12); cytokeratin 14 (CK14); cytokeratin 19 (CK19), marker for intermediate filaments; p63, marker for morphogenesis; Ki-63, marker for corneal epithelial cell differentiation and vimentin, and marker for

intermediate filaments, all of which are found in the intracytoplasmic cytoskeleton of the epithelium (Papini et al., 2005, Ma et al., 2007, Joseph et al., 2004, Zhao et al., 2008).

The Bowman's membrane is an acellular matrix which lies between the basal lamina and the corneal stroma and it acts as a resistant barrier to trauma and bacterial invasion (Gordon et al., 1994, Stocum, 2006).

The stroma or substantia propria is intricately formed and composed of mostly tightly bound sheets of collagen fibres orientated in a tightly regular manner in an extracellular matrix which includes glycosaminoglycans, type I, V and VI collagens and mucopolysaccharides. Regulation of fluid movement throughout this layer is critical for normal transparency. Interspersed within the stroma are fibrocytes known as keratocytes. These cells proliferate during corneal wound healing and are therefore crucial to the process. Posterior to the stroma is the Descemet's membrane which is a basement membrane of hyaline produced by the endothelial layer. The endothelium itself is a single layer of flattened cuboidal endothelial cells which fluid transport is important for regulating the hydration of the corneal stroma (Fischbarg, 1997, Fischbarg and Diecke, 2005).

### 1.3.3. Limbus

The limbus is found in the peripheral part of the cornea, and represents the junction between the cornea, the conjunctiva and the sclera. There is strong evidence suggesting that corneal limbal epithelial stem cells are located in the limbus but there is still a need for confirmation of specific markers for the localisation of limbal epithelial stem cells that reside in the limbus (Lyngholm et al., 2008, Shortt et al., 2007).

The possibility that the basal epithelium of the limbus contains limbal epithelial stem cells, leads to the potential for cell renewal after corneal injury or infection. Figure 1-4 shows the migration of P63 positive limbal epithelial stem cells towards the central cornea over a period of 72 hours after 4A4 pan-63 mAb immunofluorescence staining. Limbal epithelial stem cells are useful because they have a high capacity for self renewal with the potential for error-free cell proliferation and division and the cells have a long life span. The location for stem cells in the limbus is supported by the fact that the limbus contains the least number of differentiated cells. The limbus also contains proliferative characteristics of stem cells and experimental observations have shown poor corneal wound healing and infiltration of conjunctival epithelial cells, vascularisation and chronic inflammation when the limbal epithelium is partly or completely removed (Chen et al., 1991, Chen et al., 1990, Huang et al., 1991, Kruse et al., 1990).

Limbal epithelial stem cells are more resistant to solar damage, heavily pigmented and resistant to shearing forces compared to corneal epithelial cells which indicates that limbal epithelial stem cells are in the ideal location to regenerate corneal epithelial cells (Dua et al., 2000).

The limbus expresses the presumed limbal epithelial stem cell association markers ABCG2 (a ATP-binding cassette (ABC) family of cell surface transport proteins) and p63 (Sudha et al., 2006, De Paiva et al., 2005, Reidy et al., 2004). The limbal cells are distinguished from corneal epithelial cells because corneal epithelial cells do not express ABCG2. The limbal cells also express CK3, CK14 and CK19 (Schlötzer-Schrehardt and Kruse, 2005). However, the corneal epithelium does not express the putative haematopoietic stem cell markers CD34 and CD133 (Dua et al., 2003).

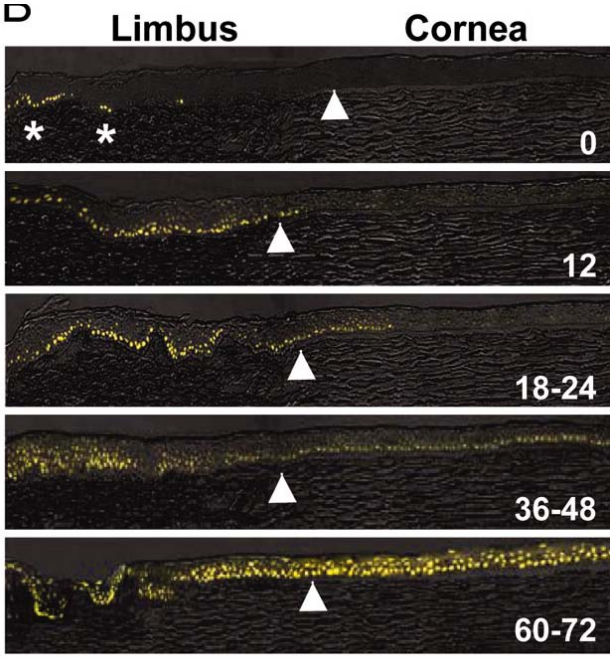



*Figure 1-4: 4A4 pan-p63 mAb immunofluorescence staining performed on human resting corneas.*

*Corneas were taken within 4 hours of death. The basal limbal layer of the resting limbus is indicated by the asteriks. The 4A4 pan-p63 mAb immunofluorescence staining for p63 positive cells is shown at basal limbal layer at 0 hour only. The arrows show the cornea-limbus border. At differention incubation periods of 0, 12, 18-24, 36-48 and 60-72 hours shows the migration of p63 positive cells from the peripheral cornea to the central cornea. Taken from Di Iorio et al., 2005*

### 1.3.4. Anatomy of Intraocular Barriers

#### 1.3.4.1. Ciliary Body

The ciliary body is important in aqueous humour production for providing nourishment to intraocular structures and immunological defences. The ciliary processes that extend from the scleral spur to the retina and choroid at the ora serrata (Figure 1-5) are part of the vascular coat, which consists of a mass of capillary vessels making this the most vascularised area of the eye (Davson, 1990, Duane and Jaeger, 1985).

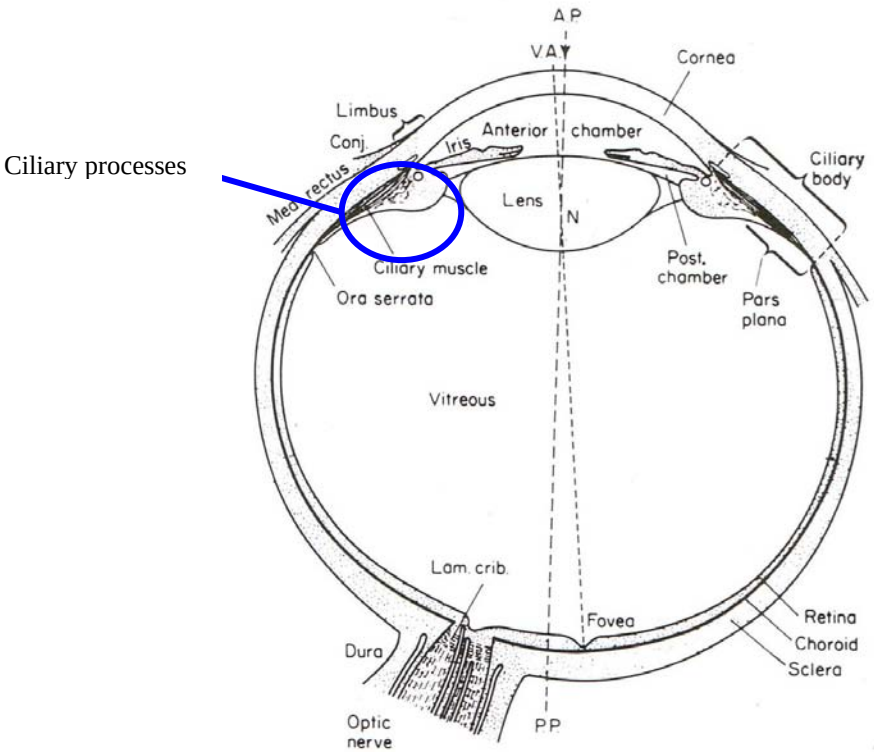



*Figure 1-5: Human eye showing the location of the ciliary processes in respect to the rest of the globe.*

*The ciliary processes extend from the ora serrata to the retina and choroid. Picture taken from Davson, 1990*

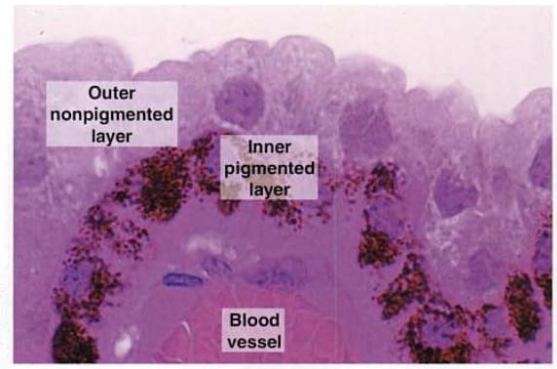
The ciliary body is composed of muscle, nerve fibres, connective tissue, capillaries and epithelia. Ciliary muscles are specialised smooth muscles which can be divided into three main types; meridonal (longitudinal) fibres which attach anteriorly from the scleral spur to the connective tissue of the choroid; radial (oblique) fibres which are continuous with the corneo-scleral trabeculae and the circular (Müller's muscle) fibres which are the inner most component (Forrester et al., 2001, Ishikawa, 1962). It is the contraction of these muscles that causes the inward and forward movement of the two-thirds of the ciliary body to create tension in the zonule during accommodation (Forrester et al., 2001, Duane and Jaeger, 1985). The ciliary muscles have also been linked to preventing the Schlemm's canal from collapsing, and distend the trabecular meshwork (Forrester et al.,

2001, Duane and Jaeger, 1985). Innervations of the ciliary body include sympathetic, parasympathetic as well as sensory nerve fibres can be both of unmyelinated or myelinated forms. The ciliary epithelium contains two epithelia (the pigmented and non-pigmented) (Figure 1-6), joined at their apices and which derived from the neuroectoderm of the optic cup (Duane and Jaeger, 1985).

#### 1.3.4.2. Pigmented Ciliary Epithelium

The pigmented ciliary epithelium (PE) is continuous with the retinal pigmented epithelium posteriorly, and the iris stroma anteriorly and its basal parts are in contact with the ciliary stroma. The PE cells are held to adjacent cells with gap junctions, desmosomes and puncta adhaerentia (Raviola and Raviola, 1978) (Figure 1-6). As a result, different macromolecules can pass from the fenestrated capillaries of the ciliary body stroma into the posterior chamber (Forrester et al., 2001, Duane and Jaeger, 1985). The pigmented epithelial cells contain many ribosomes and a small number of mitochondria (Figure 1-6). It is considered to have minimal barrier function (Duane and Jaeger, 1985). Evidence suggests that the PE maybe involved in anti-inflammatory responses where increased IL-6 secretion from the PE is found in intraocular fluids (Fleisher et al., 2000).

**A**




**B**

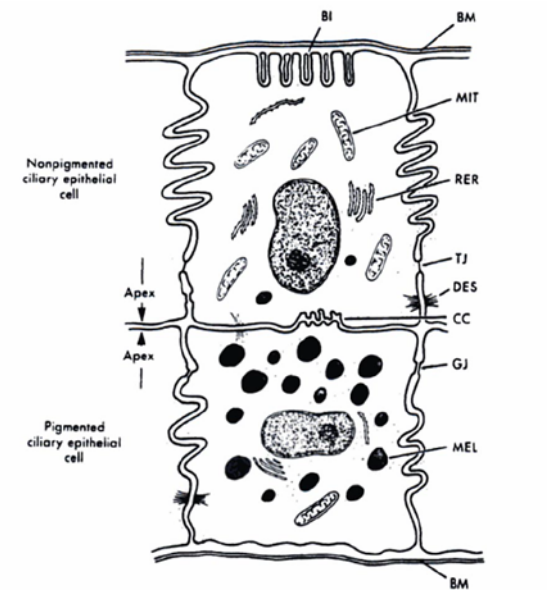



*Figure 1-6: The ciliary epithelium.*

*(A) Layers of the ciliary epithelium. Outer layer is the non-pigmented epithelium and the inner layer is the pigmented epithelium. A blood vessel is visible alongside the layer. Taken from Junqueira, 2005. (B) schematic diagram of ciliary epithelium bilayer. The non-pigmented epithelial cell contains mitochondria (MIT), rough endoplasmic reticulum (RER) and basal infoldings (BI) facing towards aqueous humour. The pigmented epithelial cell contains fewer mitochondria and rough endoplasmic reticulum but contains many melanosomes (MEL) that give layer pigmentation. Both pigmented and non-pigmented epithelial cells possess tight junctions (TJ) gap junctions (GJ), desmosomes (DES) and ciliary channels (CC) between the two layers. There is also a basement membrane (BM) at the basal side of each cell. Picture A taken from Junqueira and Carneiro, 2005 and Picture B taken from Burnstock and Sillito, 2000*

### 1.3.4.3. Non-pigmented Ciliary Epithelium

The non-pigmented epithelium (NPE) starts from the root of the iris and is continuous with the neural retina at the ora serrata and pigmented epithelium of the iris (Duane and Jaeger, 1985). Between the double layer ciliary epithelium, there are many gap junctions and some puncta adhaerentia and desmosomes (Raviola and Raviola, 1978). At the apical side of the NPE, there are a distribution of gap junctions, zonula adherens and zonula occludens (tight junctions), (Figure 1-6) which are important in the blood aqueous barrier function in the ciliary body (Duane and Jaeger, 1985). In the cytoplasm of the NPE, there are numerous mitochondria and endoplasmic reticulum. This layer has close relations with zonule fibre formation where discovery of the NPE has been found to (Figure 1-6) secrete fibrillins in mammalian zonular microfibril bundles (Hanssen et al., 2001). The main function of the NPE is in aqueous humour secretion.

#### *1.3.4.4. Aqueous Humour Production*

The formation of aqueous humour requires blood plasma to be ultra filtrated by fenestrated capillaries and the uveoscleral outflow pathway. The filtrate that contains amino acids, ions, lactate, pyruvate, ascorbate, urea and glucose is then able to be actively taken up by the NPE and PE and secreted with water into aqueous humour. One of the main transport mechanisms involved in this process is the enzyme $Na^+$, $K^+$-ATPase which is located along the basolateral membranes of human NPE and between the PE and NPE layers (Holthöfer et al., 1991).

Evidence is now emerging to confirm the presence of specific sodium channels that maybe involved in aqueous humour production. An example of this is the Amiloride sensitive sodium epithelial channel (ENaC) which has been linked to aqueous

reabsorption into the NPE and regulated by serum- and glucocorticoid-regulated kinase isoform 1 (SGK1), (Watsky et al., 1991, Civan et al., 1996, Rauz et al., 2003c).

#### 1.3.4.5. Aqueous Humour Composition

Aqueous has very specialised properties. It has the ability to nourish the cornea, trabecular meshwork and the lens with nutrients and oxygen while removing toxic and waste substances, mediating inflammation, providing the right refractive index for light and creating the internal pressure in the eye known as intraocular pressure (IOP). The composition of aqueous humour consists of electrolytes such as sodium, chloride, potassium and hydrogen carbonate; organic solutes such as ascorbate, hydrogen peroxide, urea, lactate and amino acids; active compounds such as prostaglandins, catecholamines, cortisol and fibroblast growth factor; proteins such as albumin; enzymes such as plasminogen activator and immunoglobulins which include IgG and IgE and cytokines which include IL-1β, IL-2, IL-4, IL-6, IL-10, TNFα and TGFβ (Curnow et al., 2005, Ooi et al., 2006, Berman, 1991). It was discovered in the early 1920s by Seidel, that aqueous humour was a dynamic process. Formation occurs from the NPE (and drainage through the posterior and anterior chambers) and flows past the iris, lens and cornea before draining into the trabecular meshwork through the Schlemm's canal and through the uveoscleral outflow routes (Seidel, 1920, Johnson, 2006). A revolutionary method for measuring flow rate was developed by Maurice in the 1960s who used topically applied fluorescein on the cornea and showed that it flows into aqueous with less than 10% minimal loss with a rate of between 2-3µl/min (Brubaker, 1991, Jones and Maurice, 1966).

### 1.3.5. Mucosal Barriers to Defence

#### 1.3.5.1. Mechanical and Chemical

The structures that are involved in mechanical defences in the eye are the lacrimal gland, tear film and the eyelids. These anatomical structures are considered to be involved in the first line of defence in innate immunity. The lacrimal gland is located above and to the side of the eye and functions to secrete and drain tears. The tears flow from the lacrimal gland and under the eyelids before being drained through the drainage pathways (Pandit et al., 1999). The tear film is composed of lipid, water and mucous and works with the eyelids to wash away foreign material and cell debris from the ocular surface. Tears also contain defensins which have antimicrobial activity and phospholipase and lysozyme which are non-specific antibacterial factors responsible for destabilising bacterial membranes (Aho et al., 1996, Fleiszig et al., 2003).

Mucin is produced from the goblet cells of the conjunctival and corneal epithelial surfaces and is also present in tear film. Its role is to lubricate the eye during blinking and form a protective layer in addition to binding to microorganisms and immune cells to prevent microorganisms binding to the cornea (Round et al., 2002).

Mucin is a molecule composed of carbohydrate and tandem repeats of amino acids rich in serine, proline and threonine which serve as O-glycosylation sites (Moniaux et al., 2001). The genes encoding the mucin are designated the prefix of MUC, however the numbering system does not necessarily represent the order in which they are found as MUC21 is the latest human mucin to be found and there are over 21 mucins found to date (Itoh et al., 2008).

The corneal and conjunctival epithelia have shown to express the membrane-associated mucins MUC1, -4 and -16 on their glycocalyx (outer component of cell membrane containing acidic carbohydrate). The goblet cells of the conjunctiva also express the large gel forming mucin MUC5A. The secreted mucins move freely over the membrane and the glycocalyx due to the negative charge of the glycosylated mucins which are highly hydophillic (Gipson and Gipson, 2004). This facilitates the smooth movement to prevent epithelial-epithelial adherence (Gipson and Gipson, 2004).

#### 1.3.5.2. Immunological

The ocular mucosal surface can produce many immune components for immune protection. For example, IgA is not only produced from the plasma cells of lacrimal glands but has recently been found to be produced from the conjunctiva and is also found in tear film (Erich Knop, 2005). Secretory IgA (SIgA) is the major immunoglobulin found to bind antigen but there is also limited production of IgG and IgM (Bennett and Weissman, 2004).

There are several mechanisms for immunological protection that exist on the ocular mucosal surface. One of these is the conjunctival associated lymphoid tissue (CALT) which is rich in bone marrow derived cells and is distributed within the epithelium as intra-epithelial lymphocytes and in the subepithelial lamina propria (Chan et al., 2008). The conjunctiva also expresses cell surface adhesion molecule, human mucosal lymphocyte antigen-1 (HML-1), (Dua et al., 1994b). Lymphocyte cells from the CALT system are one of the main immune cells which take part in functions which include anti-microbial defence, hypersensitivity, allo-graft-rejection and immune tolerance mechanisms (Dua et al., 1994a, Zhong et al., 2007). $CD8^+$/HML-$1^+$ and $CD45Ro^+$ and $CD25^+$ T lymphocytes are also expressed in human conjunctival epithelium, lacrimal

gland, and corneoscleral limbus and may act mainly as suppressor cells to promote immunosuppression (Dua et al., 1994b, Cepek et al., 1994, Hingorani et al., 1997). Antigen presenting cells are expressed by the conjunctiva which include $CD68^+$ macrophages, dendritic cells, mast cells, neutrophils in the normal conjunctiva and eosinophils only in the event of an inflammatory condition such as ocular allergy (Hingorani et al., 1997, Razzaque et al., 2004). The cornea expresses antigen presenting cells such as dendritic cells and macrophages while being infiltrated by neutrophils and eosinophils during inflammation (Hall et al., 1999, Hamrah et al., 2003b, Hamrah et al., 2002).

Antigen presentation to lymphocytes occurs when antigen is captured by the antigen presentation cells on the surface epithelial cell-types for example. All antigen presentation cells use specialised mechanisms in which to take up antigen. In the case of macrophages, it is possible that the expression of mannose receptors facilitates in this process which is also true for dendritic cells (Marttila-Ichihara et al., 2008, Caux and Dubois, 2001). This leads to internalisation of the antigen which is a process known as macropinocytosis. The antigen processing forms stable peptide-MHC class I molecules, which are presented to lymphocytes. Antigen-MHC class II molecules are typically brought to the cell surface for presentation to $CD4^+$ T cells while antigen-MHC class I molecules are presented to $CD8^+$ cytotoxic T cells in the peripheral lymphoid tissue (Boes et al., 2002, Johnstone and Del Val, 2007). Antigen presenting cells such as macrophages, neutrophils and dendritic cells expressing pathogenic recognition receptors (PRR), (Chapter 3.1.2), can facilitate activation of the innate immune response which induces the expression of various pro-inflammatory cytokines, chemokines, adhesion molecules while activating effector functions of innate immune cells such as phagocytosis. This in turn can lead to activation of the adaptive immune response where

there is clonal expansion of lymphocytes which is antigen specific and mediated by antigen receptors on B and T lymphocytes that leads to immunological memory (Chang et al., 2006).

In the normal substantia propria of the conjunctiva and the cornea, there is expression of $CD45^+$ cells (Yamagami et al., 2006). In conjunctival substantia propria, these are mainly of $CD68^+$ phenotype which is indicative of macrophage like cells while in the corneal substantia propria there is predominantly $CD11c^+/CD16^-$ dendritic cells (Yamagami et al., 2006, Yamagami et al., 2005). These cells may be involved in the innate immunity that can occur during inflammation in the substantia propria.

The presence of cytokines on the ocular surface is vital for responses to different immunological events. For example, epidermal growth factor (EGF) in tears, increases proliferation of corneal epithelial cells as well as keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) synthesised by the stroma. In addition to this, research has shown that transforming growth factor (TGF) regulates wound healing by antagonising proliferation of migrating cells (Zieske et al., 2001).

The expression of inflammatory cytokines such as Interleukin 1 alpha (IL-1α), IL-6 and IL-8 in the corneal epithelium and tears, increases after corneal injury, thereby facilitating wound healing processes such as epithelial cell migration, proliferation, expression of matrixmetalloproteinases (MMP) and collagenases from keratocytes and the migration and differentiation of inflammatory cells to the site of injury for the removal of cell debris and associated pathogens (Sotozono et al., 1997, Wilson et al., 2001b, Tran et al., 1996, Agrawal and Tsai, 2003). Conversely, TNFα is thought to suppress the migration of corneal epithelial cells and increase ocular surface inflammation (Okada et al., 2007, Saika, 2007).

The conjunctiva expresses cytokines that include IL-1α, IL-2, IL-6, IL-8, TNFα, IFNγ, TGFβ, platelet-derived growth factor (PDGF) and basic fibroblast factor (bFGF), (Bernauer et al., 1993, Pflugfelder et al., 1999). The expression of these cytokines is vital to cell trafficking and mediating immune responses.

There are several events which are important in corneal wound healing which are crucial for the cornea to retain its refractive and barrier properties. The epithelium must be able to 'cross-talk' with fibroblast cells to enable removal of dead or destroyed cells to be replaced by newly proliferated epithelial cells, which adhere to the extracellular matrix through the formation of integrins. The limbus area of the cornea at the corneo-scleral junction is responsible for providing these migrating, proliferating and differentiating cells to the injury. The cells in this region are epithelial progenitor cells and have been postulated to be stem cells by the use of a variety of markers such as ABCG2 and p63 (Davanger and Evensen, 1971, Schermer et al., 1986a, Cotsarelis et al., 1989, Polisetty et al., 2008, Lyngholm et al., 2008, Pellegrini et al., 1999, Dravida et al., 2005, Reidy et al., 2004). An intact basement membrane is also important to epithelium renewal as erosion may trigger the need for the formation of more adhesion complexes such as anchoring fibrils, laminin, fibronectin, and Types IV and VII collagen in addition to modifying intracellular signalling pathways that may result in persistent epithelial defect (Agrawal and Tsai, 2003, Gipson et al., 1987, Duan and Sheardown, 2007, Chen et al., 2006b).

Depending on the type of corneal injury, different types of ophthalmic solutions and procedures can be used to treat corneal wound healing. There is much controversy with the use of corticosteroids and the regulation of inflammation as inflammation and glucocorticoids have been associated with delayed wound healing. Clinical and animal research has shown in some cases that corticosteroids affect keratocytes and delay stromal

wound healing, however, more recent data show that glucocorticoids such as dexamethasone have beneficial effects when used with antibiotics and show little interference on corneal wound healing and minimise inflammation (Ashton and Cook, 1951, McDonald, 1970, Wu et al., 2006, Yulek et al., 2006, Sarchahi et al., 2008, Russo et al., 2007, Bourcier et al., 2000).

### 1.3.6. Bacteria on the Ocular Surface

#### 1.3.6.1. Commensal Bacteria

Commensal bacteria or non-pathogenic bacteria exist on all our surface tissues such as the skin and mucosal surfaces and have a symbiotic relationship where the commensal bacteria and our body may benefit from the interaction. The non-pathogenic bacteria may play a role in maintaining health and normal function; however they are not essential for life. Examples of commensal bacterial function include synthesising vitamin K and aiding absorption of nutrients from the intestinal tract (Andrew J. Macpherson, 2005, Brooks, 2004). Commensal bacteria also colonise the surface in great numbers and therefore compete with pathogenic bacteria for access to the surface and remain non-invasive until the surface is breached. The ocular commensal bacteria can commonly include *staphylococcus epidermis*, *Neisseria* species, *corynebacteria* and *propionibacterium acnes* (Ueta et al., 2007b, Amy Li, 2000). *Staphylococcus epidermis* are mostly commonly isolated as normal flora in the conjunctival sac and eyelid edge (Ueta et al., 2007a).

#### 1.3.6.2. Pathogenic Bacteria

There are many bacteria that can cause ocular disease and they can do this because they possess virulence factors that can include toxins, adhesins and invasins. Armed with

these apparatus, they cause disease when the ocular surface has been compromised by injury for example. Some of these ocular pathogenic bacteria can include *Pseudomonas aeruginosa, Streptococcus pyogenes, Staphylococcus pneumoniae, Staphylococcus aureus* and *Chlamydia eubacteria*, viral (e.g. *herpes simplex* and *varicella zoster*), fungi (e.g. *fusarium* species) and single-cell organisms such as A*canthamoeba* (Tabbara et al., 2000, Al-Shakarchi, 2007, Basak et al., 2005, Goldblum et al., 2008, Magone et al., 2005, Agarwal, 2006, Thompson et al., 2008). The common diseases of the cornea include keratitis where the cornea becomes infected with a micro-organism which causes the cornea to become inflamed and for the conjunctiva; the common disease experienced is conjunctivitis where micro-organisms and allergies may cause the inflammation of this tissue.

### 1.3.7. Intraocular Defence Barriers

#### 1.3.7.1. Immune Privilege

The anterior chamber in addition to the vitreous cavity and subretinal cavity is considered to be immune privilege (Jiang and Streilein, 1991, Wenkel et al., 1999). Inflammation to the eye can have serious effects which can disrupt its primary optical function. The immune privilege status enables the eye to provide immune protection against the majority of pathogens without the need of intense inflammation.

There are many factors which contribute to an immune privilege state. These include the absence of blood vessels from the cornea and the iris stroma; the absence of lymphatic drainage from the anterior chamber except for the uveoscleral outflow routes; the production of soluble immunomodulatory factors from aqueous humour which are secreted by the ciliary epithelium and released from the surrounding tissues and cells such

as transforming growth factor beta 2 (TGFβ2), α-melanocyte stimulating hormone (α–MSH), vasoactive intestinal peptide (VIP), calcitonin gene related-peptide (CGRP), somatostatin and thrombospondin (TSP)-1 (Novak et al., 2003).; expression of immunomodulatory factors on the corneal endothelium and pigmented epithelium and provision of tolerance-promoting antigen-presenting cells in the peripheral and central cornea, iris and possibly the trabecular meshwork and outflow routes (Hamrah et al., 2003a, Streilein, 2003b).

There are different ways in which the immune privilege status of the eye can be investigated. One way is the study of the anti-inflammatory and immunosuppressive microenvironment in the eye such as the effects of TGFβ, VIP and α–MSH. Another example is that the corneal epithelial cells, endothelial cells and ciliary body can express Fas ligand (FasL) which can inhibit complement activation and promote apoptosis of $CD95^+$ cells that come into contact with them (Griffith et al., 1995).

Immune privilege sites are also able to produce tolerance to eye-derived antigens. This occurs by a process known as anterior chamber-associated immune deviation (ACAID). This is defined by the ability of the anterior chamber to eliminate pathogens with the aid of T and B cells, in the absence of inflammation. Studies have found that ACAID can be induced in mice, rats, rabbits and monkeys and there is some evidence to suggest humans have ACAID (Lu et al., 2008, Saban et al., 2008, Zheng et al., 2000, Eichhorn et al., 1993, Takeuchi et al., 2006).

Fas-Ligand (Fas-L) is an important molecule involved in maintaining immune privilege in the eye. It is a type II transmembrane protein from the TNF family and is responsible for inducing apoptosis of $Fas^+$ cells (Nagata and Golstein, 1995).

TGFβ is found in aqueous humour in a quantity which is enough to elicit a biological effect and promote immune privilege (Granstein et al., 1990, Konstas et al., 2006, Chen et al., 1998, D'Orazio and Niederkorn, 1998, Knisely et al., 1994). There have been three isoforms of TGFβ in aqueous humour found to date (TGFβ 1, 2 and 3), (Yoneda et al., 2007). TGFβ has been found to modulate proliferation of epithelial cell, endothelial cells, and induces T regulatory (Treg) function (Horwitz et al., 1999, Andersson et al., 2008, Zelenka and Arpitha, 2008, Chen et al., 1999). Treg function is important for immunotolerance and therefore maybe involved in maintaining the immune privilege states in the eye (Stein-Streilein and Taylor, 2007, Zhu et al., 2007).

## *1.4. Orbital Adipose Tissue in the Orbit*

### 1.4.1. Adipose Tissue

The realisation that adipose tissue is much more than an energy store but also acts as an endocrine organ able to secrete compounds that regulate other cells, has implicated its role in diseases such as obesity.

Adipose tissue is a specialised connective tissue organ capable of endocrine function. There are two types of adipose tissue: brown and white. The distribution of adipose tissue depends on the species in question but interestingly, brown adipose exists among human neonates to generate heat during development but into adulthood, a very small quantity of brown adipose exists. The distribution of white adipose tissue throughout the body is vast and is considered to be one of the largest organ tissues of the body.

The main types of adipose tissue that have recently gained much attention are adipose tissues from the omentum and subcutaneous adipose. The reason for this is that both fat depots have been implicated in the increased risk of obesity and the metabolic syndrome

which comprises of insulin sensitivity, glucose intolerance, hypertension and dyslipidemia (Tulloch-Reid et al., 2004, Hayashi et al., 2003, Hayashi et al., 2004, Wajchenberg, 2000, Fox et al., 2007). Distribution and proportion of adipose tissue is generalised and different in each individual and is very much gender and genetic specific (Katzmarzyk et al., 1999, Bouchard et al., 1993).

### 1.4.2. Morphology of Adipose

White adipose tissue (WAT), which is the main tissue discussed in this chapter, can be characterised as a group of lipid storing cells called adipocytes. WAT contains a collagen fibre network with fibrous connective tissue, endothelium, resident monocytes/macrophages, vascular smooth muscle cells and preadipocytes that are part of the stromal vascular cell fraction (Dardick et al., 1976). Pluripotent adipose-tissue derived stromal cells (ADSC) within this fraction have the potential to be differentiated into other cell types in-vitro (Figure 1-7) such as bone (medium includes dexamethasone and ascorbic acid), cardiomyocytes (medium includes TGF-β1), cartilage (medium includes dexamethasone and ascorbate-2 phosphate), neurons (medium includes valproic acid, hydrocortisone and insulin), endothelial cells (medium includes FGF2) and adipose cells (medium includes dexamethasone and insulin) when cultured under specific conditions (Dardick et al., 1976, Zhang, 1999, Schaffler and Buchler, 2007, Gerhardt et al., 2001, Weisberg et al., 2003, Shoelson et al., 2006, Zuk et al., 2002, Noël et al., 2008, So-Jung et al., 2009, Huang et al., 2007, Hongxiu et al., 2009).

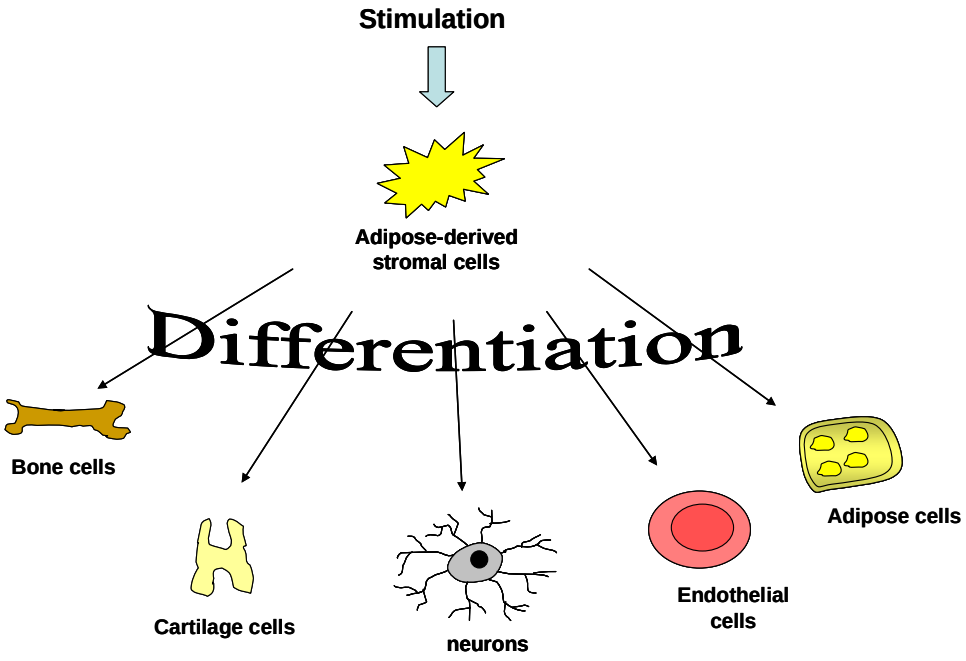



*Figure 1-7: Adipose-derived stromal cells (ADSC) and their pluripotent potential to differentiate into different cell types.*

*These include bone cells (influenced by ascorbic acid), cartilage cells (influenced by ascorbate-2 phosphate), neurons (influenced by valproic acid), endothelial cells (influenced by FGF2) and adipose cells (influenced by dexamethasone).*

During adulthood, the WAT becomes more unilocular in morphology, with a colour that varies from white to dark yellow. The adipocytes have a very thin ring of cytoplasm with a flattened nucleus at the periphery, and a large central lipid droplet surrounded by smaller lipid droplets. The cytoplasm around the nucleus has a golgi apparatus, mitochondria, rough endoplasmic reticulum and free polyribosomes while the thin ring of cytoplasm contains the small lipid droplets containing smooth endoplasmic reticulum and pinocytotic vesicles (Junqueira and Carneiro, 2005). The sympathetic nervous system innervations of adipose tissue are via the capillaries and it is thought that these release the neurotransmitter, norepinephrine that activates hormone sensitive lipase which is necessary for the mobilisation of lipids (Bowers et al., 2004, Niijima, 1989, Cantu and Goodman, 1967, Bartness and Bamshad, 1998).

### 1.4.3. Metabolism of Adipose Tissue

The lipids stored in adipose tissue are mostly triglycerides stored in very low density lipoprotein (VLDL) or chylomicrons.

Free fatty acids are synthesised from glucose in adipose tissue, (a process which mitochondria and the smooth endoplasmic reticulum are actively involved in) and are stored in adipose (Junqueira and Carneiro, 2005). This process is increased by insulin which regulates uptake of glucose and increases lipoprotein lipase synthesis (Yao et al., 1999, Rosato et al., 1997, Appel and Fried, 1992).

Triglycerides can travel from the liver to the adipose via the bloodstream in the form of chylomicrons. These triglycerides can be hydrolysed by adipose synthesised lipoprotein lipase (LPL) which transports triglycerides to the lumen of capillaries to release free fatty acids and glycerol. The fatty acids and glycerol are taken up by the adipocytes and resterified for storage (Reynisdottir et al., 1997, Kolehmainen et al., 2002). Hormone sensitive lipases (HSL) in adipocytes are the rate limiting step in releasing free fatty acids and glycerol stored as triglycerides in adipose (Kolehmainen et al., 2002). The free fatty acids bound to albumin can then be transported to other tissues of the body (Reynisdottir et al., 1997).

As adipose tissue distribution is thought to be regulated by neuronal and hormonal factors. The discovery of the obesity (ob) gene product, leptin, a 164 amino acid protein which signals to the brain to influence food intake, has been of increasing interest in obesity (Baskin et al., 1999). Other hormonal factors involved in adipose tissue metabolism, in particular lipolysis, include growth hormone, thyroid stimulating hormone, parathyroid hormone and sex steroids (Moller et al., 1990, Müller et al., 1990, Taniguchi et al., 1987,

Mattsson and Olsson, 2007, Turgeon et al., 2006). The effects that glucocorticoids have on adipose tissue are less well characterised, however, it is believed they are involved in influencing LPL and HSL action thereby modulating lipid uptake and turnover at tissue specific sites (Macfarlane et al., 2008). Furthermore, glucocorticoids are responsible for promoting differentiation of preadipocytes to mature adipocytes (Hauner et al., 1987, Tomlinson et al., 2006). There is increasing evidence for the role of 11beta hydroxysteroid dehydrogenase 1 (11β-HSD1) activating cortisone from cortisol (described further in chapter 1.7) in adipose tissue to induce preadipocytes to differentiate into mature-like adipocytes (Bujalska et al., 2008).

### 1.4.4. Adipose Tissue Function

The classical functional roles of adipose tissue include mechanical cushioning of internal organs, heat insulation and as an energy source through lipolysis when carbohydrates are not readily available.

Adipose tissue expresses endocrine cytokines known as adipokines which include leptin, adiponectin (a protein exclusively produced from adipocytes that is involved in sensitising insulin to repress glucose production in the liver), angiotensinogen, PAI-I, ASP, IL-6, TNFα, and resistin (Table 1-1). This enables adipose tissue to signal to distant tissue systems thereby influencing immune function, metabolism and neuroendocrine functions (Kershaw and Flier, 2004).

*Table 1-1: Examples of some adipokines produced and secreted by adipose tissue*

| **Adipokine** | **Function** |
|---|---|
| **Angiotensinogen** | Increased levels may be contributable to increased WAT mass and nutrition appears to relate angiotensinogen to adipose tissue as levels of angiotensinogen decrease during fasting and increase with food intake (Frederich et al., 1992, Saint-Marc et al., 2001). |
| **PAI-I**<br>**(Plasminogen activator inhibitor I)** | Primary inhibitor of fibrinolysis. High levels are found after a myocardial infact and plasma levels increase with visceral adiposity (Hamsten et al., 1985, Shimomura et al., 1996). |
| **ASP (Acylation-stimulation protein)** | Derived from complement C3 interaction with adipsin but not known to be involved in immune function. However, its role is considered in stimulating triglyceride storage in adipose tissue (Cianflone et al., 1999, Van Harmelen et al., 1999, Maslowska et al., 2006). |
| **IL-6** | Large amounts are secreted by adipose tissue and is highly correlated with increased body mass and decreased insulin sensitivity (Bastard et al., 2000, Bastard et al., 2002). |
| **TNFα** | Overexpressed in WAT in obesity and causes decreased insulin sensitivity. Its effects on adipose tissue maybe more of an autocrine/paracrine fashion rather than endocrine (Ruan and Lodish, 2003, Guerre-Millo, 2004). |
| **Resistin** | Also known as adipose tissue specific secretory factor (ADSF) is associated with insulin resistance and thought to play role in the link between type II diabetes and obesity (Ribot et al., 2008, Berger, 2001). |
| **Leptin** | Protein that regulates food uptake by acting on the brain and influences body weight, energy expenditure and neuroendocrine functions (Friedman and Halaas, 1998, Ahima and Flier, 2000a, Ahima and Flier, 2000b). |
| **Adiponectin** | An effective sensitiser of insulin in the liver (Scherer, 2006, Maeda et al., 1996, Weyer et al., 2001). |

### 1.4.5. Orbital Adipose Tissue

Orbital adipose tissue is a type of WAT originally derived from the neuroectoderm. It is found to occupy the space in the bony orbit cavity surrounding other orbital tissues which include muscles, blood vessel nerves and the globe of the eye. The tissue can be split into two main parts depending on where it is located in the orbit; either in the retrobulbar area or the peribulbar area (Figure 1-8), (R. Wolfram-Gabel, 2002, Bremond-Gignac et al., 2004).

The retrobulbar adipose is located posteriorly where it envelopes the extraocular muscles (mainly the four rectus muscles) and the optic nerve (R. Wolfram-Gabel, 2002). It also accounts for the majority of adipose in the orbit. The peribulbar adipose surrounds the globe of the eye superiorly, inferiorly, medially and laterally (R. Wolfram-Gabel, 2002). This tissue acts to support the structures of the eye including muscles, nerves and blood vessels in addition to aiding the slide mechanism of the eye as it rotates on its axis (R. Wolfram-Gabel, 2002). In addition to this, orbital adipose tissue can also be described as an endocrine organ as will be discussed later in the chapter.

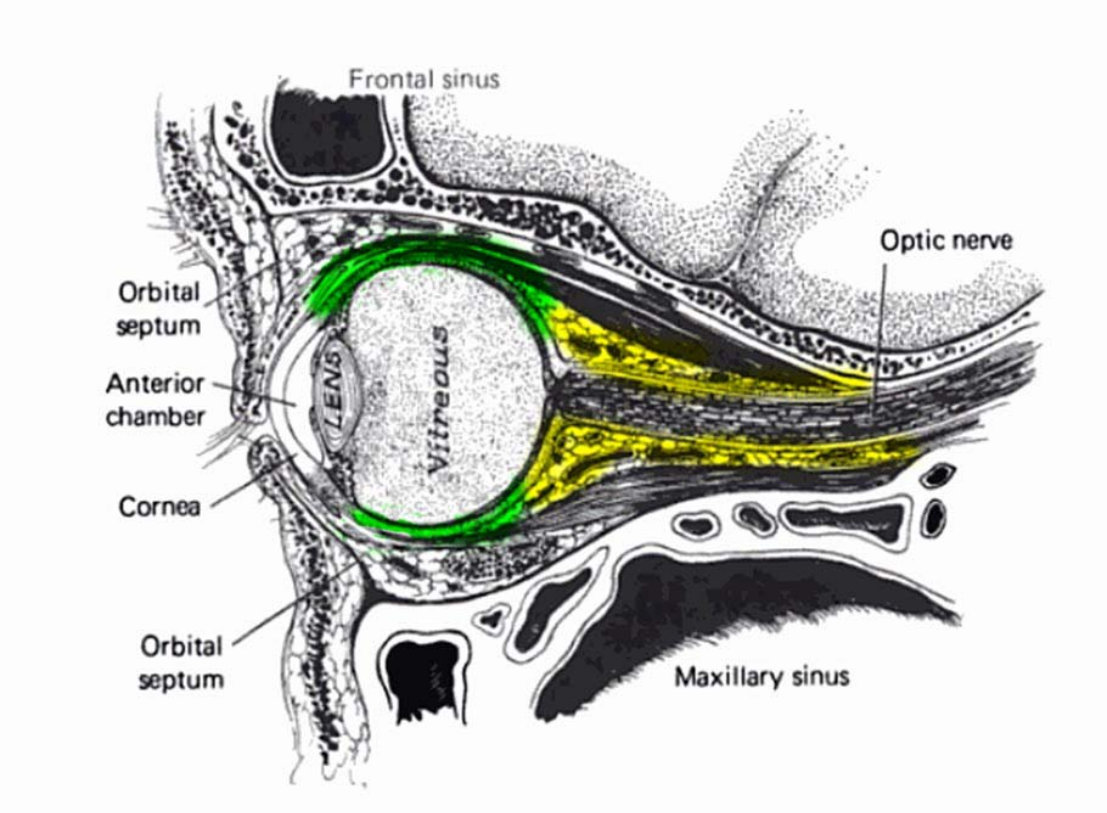



*Figure 1-8: Sketch drawing of the eyeball in the orbit.*

*The areas which are green coloured represent some spaces of the orbit which may contain peribulbar fat around the eyeball while the yellow coloured areas represent the spaces that contain retrobulbar fat situated in the posterior part of the eyeball. Picture taken from Corn and Koenig, 1996*

### *1.5. Glucocorticoids in Ophthalmology*

The eye is a glucocorticoid target tissue which orchestrates expression of target genes through the glucocorticoid receptor (GR). The ability for the eye to utilise glucocorticoids is important as they have been shown to have a function in ocular inflammation and ocular autoimmune disease (Galor and Thorne, 2007). The classical action of glucocorticoids is to bind to the GR or the mineralocorticoid receptor (MR). In doing so, the receptor-ligand-complex is then available to translocate to the nucleus to bind to the

glucocorticoid response element (GRE) and elicit the transcription of genes such as MCP-1 (Monocyte chemotactic protein-1) , SGK (Serum- and glucocorticoid-inducible kinase), HSP70 (Heat shock protein 70) and CCK (Cholecystokinin) (Gupta et al., 2005, Náray-Fejes-Tóth et al., 2000, Rauz et al., 2003b).

The GR is expressed in several ocular structures of the eye (Table 1-2). The cornea which consists of the corneal epithelium, basement membrane, stroma, endothelium and descemet membrane has some expression of GR in the corneal epithelium, stroma and endothelium (Rauz et al., 2001, Suzuki et al., 2001, Mirshahi et al., 1997). GR expression is found in the NPE (non-pigmented epithelium), PE (pigmented epithelium) and stroma (Stokes et al., 2000, Suzuki et al., 2001). The sclera, trabecular meshwork (TM) and the lens have expression of the GR (Stokes et al., 2000, Suzuki et al., 2001). In addition, expression of the GR was found in the retinal cell layers (e.g. PE and ganglion cell layer), (Suzuki et al., 2001).

*Table 1-2: Expression of glucocorticoid and mineralocorticoid receptors in human ocular tissues*[a]

| | GR | | MR | |
|---|---|---|---|---|
| **Conjunctiva** | | Ref. | | Ref. |
| - Epithelium | ? | | ? | |
| - Stroma | ? | | ? | |
| **Cornea** | | | | |
| - Epithelium | +/- | 2,3,4 | +/- | 2,3 |
| - Stroma | + | 2, 4 | - | 2 |
| - Endothelium | +/- | 2,3,4 | +/- | 2,3 |
| **Ciliary Body** | | | | |
| - NPE | + | 2,3 | + | 2,3 |
| - PE | + | 2 | - | 2 |
| - Stroma | + | 2 | - | 2 |
| **TM** | + | 1,3 | - | 1,3 |
| **Lens** | + | 2,3 | - | 2,3 |
| **Retina** | + | 2 | - | 2,4 |
| **Optic Nerve** | + | 2 | - | 2 |
| **Sclera** | +/- | 2,3 | - | 2,3 |
| **Orbital Adipose Tissue** | ? | | ? | |

[a]*Immunoreactivity and/or mRNA expression is classified as +; present, -; not present, +/- ; conflicting data; ?, not known. References 1; (Rauz et al., 2001), 2; (Suzuki et al., 2001), 3; (Stokes et al., 2000), 4; (Mirshahi et al., 1997).*

### 1.5.1. Therapeutic Uses of Glucocorticoids in Ophthalmology

Glucocorticoids are known to have many physiological effects and the most frequently studied are their effects on metabolism such as gluconeogenesis and effects on immune function and inflammation. Due to their anti-inflammatory and immunosuppressive effects, glucocorticoids are used abundantly in ophthalmic practice for the treatment of

ocular ailments such as orbital diseases for example in thyroid associated ophthalmopathy and idiopathic orbital inflammation (section 4.1.2) and ocular inflammatory diseases such as uveitis, diabetic macular oedema, and ocular surface inflammation such as allergic conjunctivitis and peripheral ulcerative keratitis (Leibovitch et al., 2007, Toker et al., 2002, Grenga et al., 2008, Arcangelo and Peterson, 2005, Wahid et al., 2008). Glucocorticoids used systemically in thyroid associated ophthalmopathy include methylprednisolone acetate given intravenously and prednisone given orally (Marcocci et al., 2001). They are presumed the appropriate course of treatment in moderate to severe thyroid associated ophthalmopathy due to the improvement in symptoms such as neuropathy and a reduction in acute inflammation. The mechanism of action of glucocorticoids in thyroid associated ophthalmopathy are not well known, however it is postulated, that glucocorticoids may interfere with lymphocyte recruitment and inhibit inflammatory mediators such as cytokines (Heufelder and Bahn, 1994).

Idiopathic orbital inflammation is inflammation usually confined to the orbit. The condition is characterised by the infiltration of inflammatory cells and fibrous tissue into the soft tissues of the orbit. Glucocorticoid treatment is the first line of treatment for this condition and can include oral prednisolone or intraorbital injections of corticosteroid or in more severe cases, intravenous methylprednisolone (Leibovitch et al., 2007, Zborowska et al., 2005).

Uveitis is inflammation of part of the uvea which is made of different parts of the eye which include the iris, choroid and ciliary body. The symptoms include pain, photophobia, and decreased visual acuity and when the specific aetiology of uveitis is not known, there is the need for the use of immunomodulatory agents which reduce the signs and symptoms of inflammation and prevent long term sequelae (Van Gelder and Kaplan,

1999). The use of glucocorticoids is particular useful in uveitis and can be oral steroids or intravenous steroids such as methylprednisone for more severe cases (Van Gelder and Kaplan, 1999, Toker et al., 2002).

During diabetic macular oedema, individuals with diabetes mellitus have a 10 percent chance of developing swelling in the retina with retinal thickening (Sander et al., 2002). This can be of two types: (1) focal macular oedema occurs due to dilated capillaries and leakage of microaneurysms and (2) diffuse macular oedema occurs after a breakdown in the generalised blood-retinal barrier with the formation of large cystoid spaces (Fernando and Pandit, 2005). It is in diffuse diabetic macular oedema in which treatments including intravitreal corticosteroid, sub-tenons capsule injection of corticosteroid or retrobulbar injection of corticosteroid have promising effects (Jonas et al., 2005, Grenga et al., 2008, Cardillo et al., 2005, Knudsen, 2004).

Allergic conjunctivitis can be described as inflammation of the bulbar or palpebral conjunctiva which is the clear membrane which covers the white part of the eye. The condition can be of bacterial or viral in origin and is characterised by the symptom of itchy eye. Low dose topical corticosteroids such as medrysone, prednisolone or dexamethasone can be used to treat inflammation but only used for the short term due to adverse side effects which include cataracts and increased intraocular pressure leading to steroid induced glaucoma (Arcangelo and Peterson, 2005, Mohan and Muralidharan, 1989).

Peripheral ulcerative keratitis is a condition that can occur in rheumatoid arthritis and results in a corneal ulcer that can perforate the eye or cause loss of vision. Rapid treatment is most important and although glucocorticoids are not the preferred treatment in this case, oral corticosteroid may be given in severe cases and prednisolone can be

given peri-operatively and post-operatively (before and after surgery) (Squirrell et al., 1999, Wahid et al., 2008).

### 1.5.2. Adverse Effects of Therapeutic Glucocorticoids

Although the exogenous (or endogenous) use of glucocorticoids can be beneficial, they can also have adverse side effects. Osteoporosis is a disease where the bones become weakened and fragile and increase the risk of fractures in areas which include the spine, wrist and hips. In glucocorticoid-induced osteoporosis the risk of fractures is increased as the glucocorticoids are thought to affect bone absorption and formation. The adverse effects include an overall decrease in bone formation and density with osteoid thickening, reduced rate of bone mineralization and decreased osteoblast activity (Dalle Carbonare et al., 2001). The cellular effects involved in bone reabsorption can vary considerably, however, in bone formation the cellular effects include altered osteoblast cell differentiation, inhibition of osteoblast matrix synthesis, an increase in osteoblast apoptosis, decrease in gene expression that code for proteins such as osteoclacin, type I collagen, and several matrix proteins and an increase in osteoclast formation (Nishimura and Ikuyama, 2000, Bland, 2000, Manolagas et al., 1999). Prolonged use of glucocorticoids in this case can rapidly have detrimental effects on bone and even after cessation of treatment there is only partial recovery of bone that has been lost.

Another condition of which glucocorticoids can have adverse effects on the bone is in osteonecrosis. This is when glucocorticoid-induced osteonecrosis occurs due to lipid blocking the microvasculature of the bone and involves apoptosis of a bone cell type known as osteocytes (Weinstein et al., 2000).

Another effect caused by the use of glucocorticoids is shown in glucocorticoid-induced diabetes mellitus. Diabetes Mellitus is a condition which the pancreas inadequately produces and or loss of insulin sensitivity occurs. The mechanisms involved in glucocorticoid-induced diabetes mellitus involve (1) effects on hepatic gluconeogenesis where glucocorticoids are responsible for the upregulation of regulatory proteins such as glucose-6-phosphatase and phosphoenolpyruvate carboxylase (PEPCK) which contributes to hyperglycaemia (Vander Kooi et al., 2005, McCurdy and Friedman, 2006), (2) in pancreatic cells, glucocorticoids may suppress insulin release from the pancreas (In-Kyung et al., 2001), (3), glucocorticoids may provoke insulin resistance by inhibiting glucose transporter production in adipose cells and muscle cells (Buren et al., 2002, Weinstein et al., 1998) and (4) glucocorticoids are shown to raise blood glucose levels after fasting and after a meal (Levetan and Magee, 2000).

Prolonged use of glucocorticoids has also been associated with obesity, a feature of Cushing's disease and the metabolic syndrome. Excess glucocorticoid such as that experienced during chronic stress is thought to play a role in the redistribution of abdominal fat in these diseases (Rosmond and Bjorntorp, 2000, Adam and Epel, 2007).

Glucocorticoids are shown to delay wound healing, for example glucocorticoids inhibit keratinocyte growth factor (KGF) which is important for epithelial cell proliferation (Chedid et al., 1996). It is the anti-inflammatory nature of glucocorticoids which contribute to inhibition of wound healing as glucocorticoids are able to regulate proteins involved in the repair process such as pro-inflammatory cytokines (e.g. IL-1α, IL-1β, IL-6, and TNF-α), growth factors e.g IGF-1 and TGF-β) and matrix proteins (e.g. tenascin-C and stromelysin-2) (Beer et al., 2000, Grose et al., 2002, Werner and Grose, 2003).

The risk of high dose glucocorticoid use is also associated with dyslipidaemia and atherosclerosis (Da Silva et al., 2006). Dyslipidaemia is the result of abnormal concentrations of lipid or lipoprotein in the blood and atherosclerosis involves a chronic inflammatory response which results in fatty deposits called plaques on the walls of arteries. Although high dose glucocorticoids have effects on these diseases, there is no evidence to suggest that low dose glucocorticoids have the same effect (Klippel, 2007).

Due to the potential adverse effects glucocorticoids can have on physiology, there is the need to search for regulation of glucocorticoid dependent disease processes at a tissue specific level to avoid these effects. The regulation of glucocorticoids in this case is performed by two short-chain alcohol dehydrogenases known as 11beta hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11beta hydroxysteroid dehydrogenase type 2 (11β-HSD2) which will both be discussed later in this chapter.

In order for this to be achieved, the study of autocrine regulators of glucocorticoids in ocular and orbital tissues is attractive. The potential of this concept will rely upon the understanding of the classical corticosteroid regulatory mechanisms and the hypothalamic-pituitary-adrenal axis (HPA).

## *1.6. Classical Corticosteroid Regulatory Mechanisms*

### 1.6.1. Corticosteroid Biosynthesis

The main site for corticosteroid production and secretion in humans is from the adrenal gland which can be divided into the adrenal medulla and the adrenal cortex (Figure 1-9). The adrenal cortex is responsible for the majority of corticosteroids secreted into

circulation whereas the adrenal medulla's function is for the production and secretion of catecholamines.

The adrenal cortex can be divided into three further sections (Figure 1-9) which consist of: (i) the zona glomerulosa which is responsible for synthesising the mineralocorticoids from cholesterol such as aldosterone under the control of the renin-angiotensin system (ii) the zona fasiculata responsible for synthesising glucocorticoids such as cortisol which is under the control of the hypothalamopituitary axis and (iii) the zona reticularis which is responsible for the synthesis of sex steroids (Brook and Marshall, 2001).

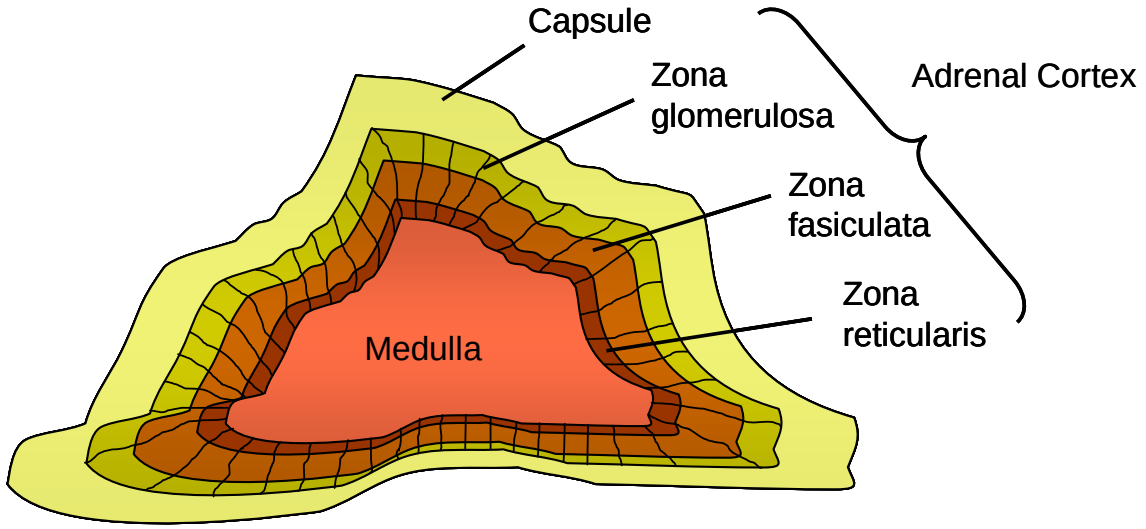



*Figure 1-9: Simplified diagram of the adrenal gland.*

*The adrenal gland is separated into the adrenal medulla and the adrenal cortex. The adrenal cortex consists of the zona glomerulosa responsible for mineralocorticoid production, zona fasiculata which produces glucocorticoids and zona reticularis which synthesises sex steroids. Adapted from Endocrinology: an integrated approach, pg 123 (Nussey and Whitehead, 2001).*

Corticosteroids originate from the biosynthetic pathway that exists in the adrenal cortex. Cholesterol is a precursor to all corticosteroids (Figure 1-10) which are released into the

peripheral blood stream, ready to interact with their cognate receptors within target tissues instigating a physiological response.

### 1.6.2. Cortisol and Aldosterone Metabolism and Effects on Physiology

Cortisol was first characterised from urine in the early 1950s and was found to be the main corticosteroid secreted (Amelung et al., 1953). Relatively large amounts of glucocorticoids are secreted into the circulation in comparison with mineralocorticoids (15mg/d vs. 15μg/d respectively), (Cope and Black, 1958, Esteban et al., 1991, Jones et al., 1959).

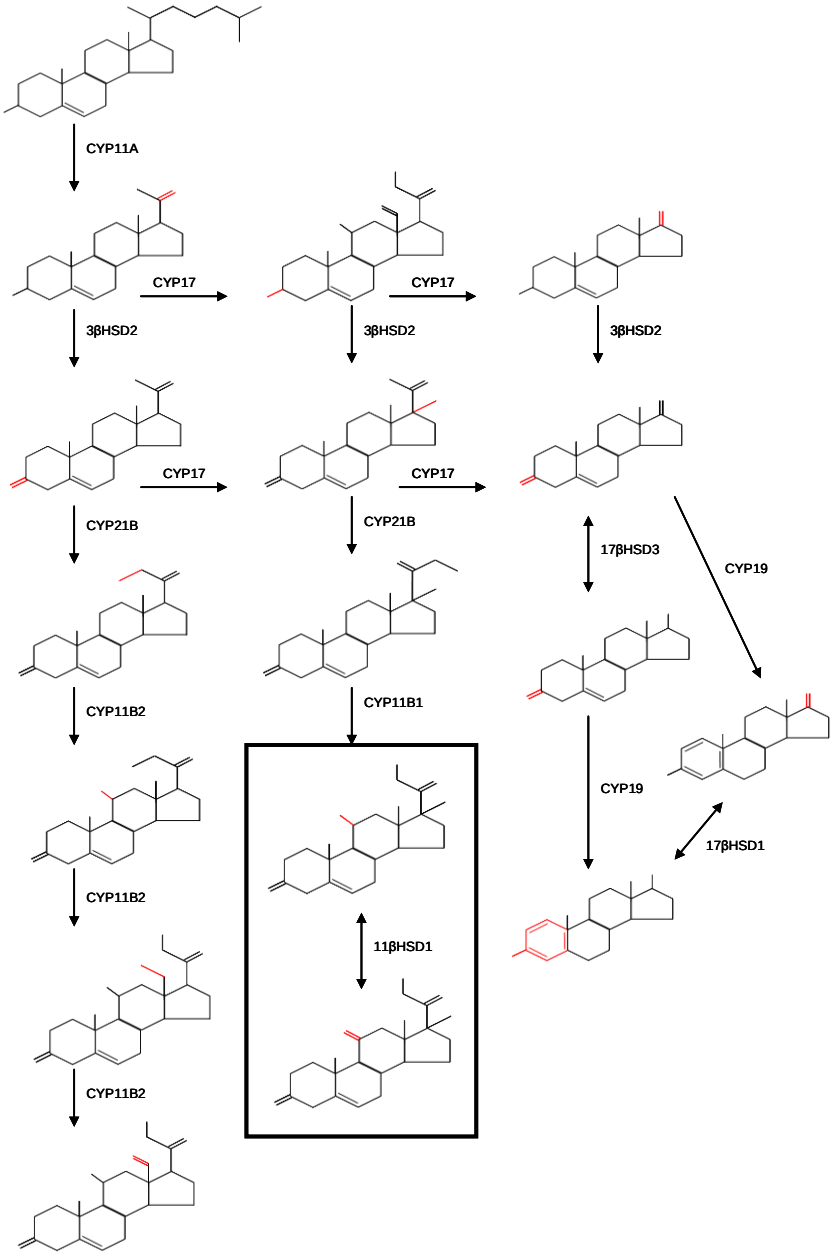



*Figure 1-10: Steroid biosynthesis pathway.*

*This pathway occurs in the adrenal cortex of the adrenal gland. The pathway starts with cholesterol and the parts of the diagram in red represent the change in molecular structure from the previous molcule. (CYP11A, Cytochrome P450 cholesterol side chain cleavage; CYP11B1, 11β-hydroxylase; CYP11B2, Aldosterone synthase; CYP17, 17α-hydroxylase; CYP19, Aromatase; CYP21, 21-hydroxylase; 3β HSD 2, 3beta hydroxysteroid dehydrogenase 2; 17β HSD 1, 17beta hydroxysteroid dehyrogenase 1; 17β HSD 2, 17beta hydroxysteroid dehyrogenase 2; 11β-HSD1, 11beta hydroxysteroid dehydrogenase 1). Adapted from Essential Endocrinology, 4th edition, Blackwell Publishing, (Brook and Marshall, 2001).*

At least 90% of circulatory cortisol is bound to cortisol binding globulin while a small faction of cortisol is bound to serum globulin. The remaining unbound cortisol is 'biologically' active and excreted as as urinary free cortisol (UFF) through the kidneys in humans which is only about 1% of the total cortisol excretion rate (Tomlinson et al., 2004).

Cortisol is metabolised to dihydrocortisol and dihydrocortisone by reduction of the C4-5 double bond by steroid 5β or 5α reductase (Figure 1-11) and is then hydroxylated on the 3-oxo group to form 5β or 5α tetrahydrocortisol (5β or 5α/allo THF) and 5β or 5α tetrahydrocortisone (5β or 5α THE) (Figure 1-11) where 5β THF predominates in normal physiology (Cope, 1972, Jamieson et al., 1999). At the same point, cortisol and cortisone can also be reduced by 20α or 20β HSD on the 20-oxo group to produce α or β cortols or cortolones respectively (Figure 1-11 and Figure 1-12) (Shackleton, 1993, Szymanski and Furfine, 1977). In addition to this, the C6 position is hydroxylated to give 6β hydroxycortisol or 6β-hydroxycortisone (Figure 1-11 and Figure 1-12) while cleaving the THF and THE can produce $C_{19}$ steroids. Oxidation of the C21 position or cortols or cortolones yields cortolic or cortolonic acids (Figure 1-11 and Figure 1-12). Traditionally, THF, allo THF and THE is conjugated to glucuronic acid and excreted in urine (Monder and Bradlow, 1980).

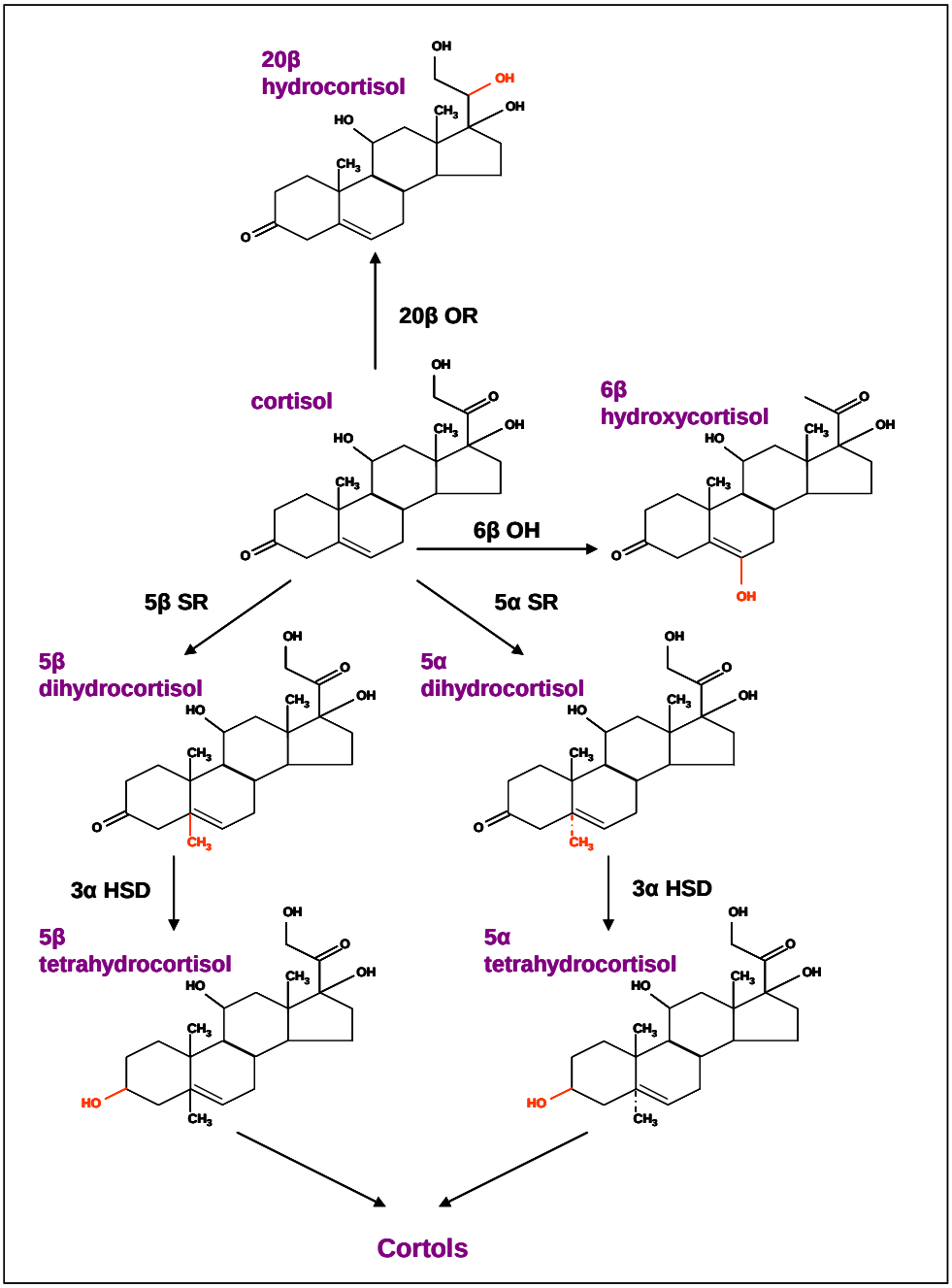



*Figure 1-11: Metabolism of cortisol.*

*Cortisol can be reduced to 20β hydrocortisol by 20β OR and hydroxylated to 6β hydroxycortisol by 6β OH. Cortisol can also be reduced to 5β dihydrocortisol and 5α dihydrocortisol by 5β/5α SR respectively. This in turn can be hydroxylated by 3α HSD to produce 5β/5α tetrahydrocortisol. The tetrahydrocortisols are oxidised to produce cortols. 20β OR, 20β oxoreductase; 6β OH, 6β hydroxylase; 5β/α SR, steroid 5β/α reductase; 3α HSD, 3α hydroxysteroid dehydrogenase.*

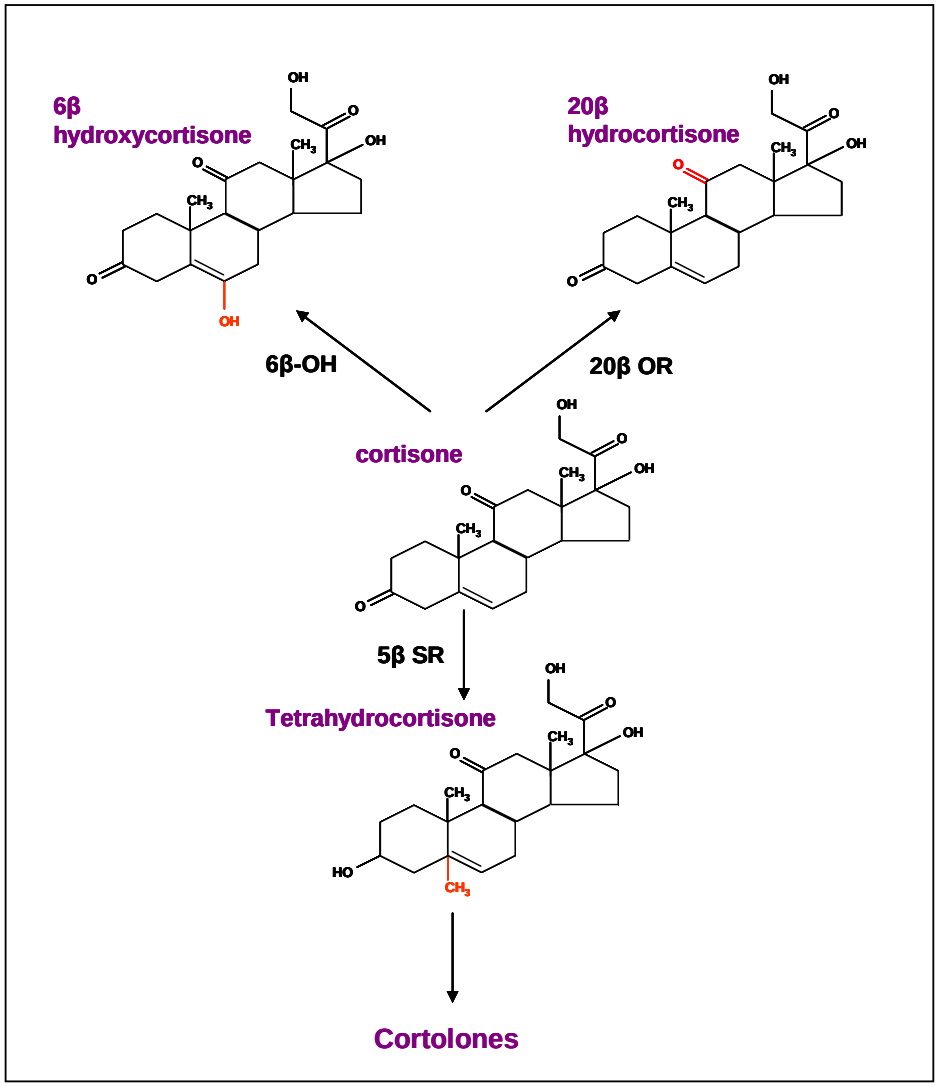



*Figure 1-12: Metabolism of cortisone.*

*Cortisone is hydroxylated to 6β hydroxycortisone by 6β OH and oxidised to 20β hydrocortisone by 20β OR. In addition, cortisone is reduced to tetrahydrocortisone by 5β SR. This compound is then oxidised to cortolones. 20β OR, 20β oxoreductase; 6β OH, 6β hydroxylase; 5β SR, steroid 5β reductase.*

Aldosterone, which has a rapid turnover, is metabolised to a variety of different compounds and cleared through the liver and urine. These metabolites include tetrahydroaldosterone glucuronide and 21-deoxytetrahydroaldosterone (Morris, 1981, Luetsche.Ja et al., 1965).

Cortisol and aldosterone action are known to have various effects within the human body which include a role in glucose metabolism and blood pressure regulation (Table 1-3). These effects are conveyed through cognate receptors: the glucocorticoid receptor and mineralocorticoid receptor (GR and MR).

*Table 1-3: Examples of the physiological role of cortisol and aldosterone in humans*

| | Cortisol | Aldosterone |
|---|---|---|
| **Kidney** | Increase in urinary calcium secretion (Leclerc et al., 2004) | $Na^+$ transport in epithelia for water reabsorption (McCormick et al., 2005). |
| **Liver** | Stimulation of gluconeogenesis (Tayek and Katz, 1997) | Physiological effects not yet estalished in humans |
| **Adipose Tissue** | Regulates adipose tissue differentiation, function and distribution (Bujalska et al., 1997) | Physiological effects not yet estalished in humans |
| **Bone** | Increases bone reabsorption by stimulating osteoclasts to regulate bone density (Bockman and Weinerman, 1990) | Physiological effects not yet estalished in humans |
| **Brain** | Feedback loop regulation via effects on hypothalamus and pituitary glands (Tasker et al., 2006) | Regulates sodium and potassium levels (Stier et al., 2002). |
| **Inflammation** | Reduces inflammation by influencing the inhibition of leukocytes and the apoptosis of lymphocytes (Tuckermann et al., 2005) | Acts as a proinflammatory mediater of inflammation via MR in cardiovascular tissue (Funder, 2004, Pitt et al., 2003) |

### 1.6.3. Corticosteroid Receptors and their Function

Glucocorticoid and mineralocorticoid receptors (GR and MR) belong to the nuclear receptor superfamily which shares a common structure. The unbound receptors can exist in the nucleus or the cytoplasm and form complexes with chaperone proteins such as heat shock proteins 90 and 70 (Pratt, 1987). The main domains of nuclear receptors include a

$NH_2$ terminal domain for activation of transcriptional activity and confers specificity of the action of the receptor, a DNA binding domain, a hinge region and a ligand binding domain (Aranda and Pascual, 2001). Glucocorticoids interact with both the GR and MR but mineralocorticoids are generally known to act specifically at the MR.

These membrane bound receptors interact with their cognate ligand, they enable translocation to the nucleus and subsequent transcription of target genes mediated through coactivators and corepressors (Table 1-4) evoking a physiological response. It is also important to add that glucocorticoid and mineralocorticoids are also involved in non-genomic effects which require no gene transcription. For example, corticosteroids are believed to affect proteins in a yeast model known as *Schizosaccharomyces pombe* (Zöllner et al., 2008). In this model, steroid receptors are not present, however, it does contain regulatory mechanisms that are similar to mamamals (Böhmer et al., 2006, Zöllner et al., 2008). As a consequence, aldosterone was found to have regulatory effects on proteins involved in osmotic regulation (NAD-dependent malic enzyme and glycerol-3-phosphate-dehydrogenase) and organisation of the cytoskeleton (VIP1 and glyceraldehyde-3-phosphate dehydrogenase) which are also affected in the human HCT116 cells, an immortalised colorectal carcinoma cell-line (Böhmer et al., 2006) .

Another example is of the rapid effects of glucocorticoids which are not affected by MR and GR blockade and/or protein synthesis inhibition is on membrane proteins such as $Ca^{2+}$ trafficking and cytoplasmic proteins which include mitogen-activated protein kinase (MAPK) and phospholipases (Haller et al., 2008, Sze and Iqbal, 1994, Ai-Qun et al., 2005).

*Table 1-4: Glucocorticoid and mineralocorticoid receptor cofactors*

| | Nuclear receptor target | Function |
|---|---|---|
| **Coactivators** | | |
| SRC-1/NCoA-1 | GR[1, 5, 6, 7], MR[8] | Acetyltransferase activity<br>Interacts with CBP |
| TIF2/ NCoA-2/ GRIP1 | GR[3,4], MR[9, 10] | Mediates AF-2 activation of NR |
| P300 | GR[12], MR [9] | |
| CBP | GR[2, 11] , MR[9] | Inhibits NFκB activity<br>Inhibits AP-1 activity |
| **Corepressor** | | |
| NCoR/SMRT | GR[13], MR[10] | Binds to N terminus of NR for full antagonist activity |

*Regulatory proteins found to influence GR or MR activity in mammalian species. 1: (Collingwood et al., 1999); 2: (Kamei et al., 1996); 3: (Voegel et al., 1996); 4: (Hong et al., 1999); 5: (Li et al., 2003); 6: (Ding et al., 1998); 7: (Yao et al., 1996); 8: (Li et al., 2005); 9: (Fuse et al., 2000); 10: (Wang et al., 2004); 11: (Sheppard et al., 1998); 12: (Kino et al., 1999); 13: (Schulz et al., 2002)*

### 1.6.4. Synthesis and Release of Glucocorticoids and Mineralocorticoids

#### 1.6.4.1. Cortisol

Glucocorticoids are regulated systemically by a negative feedback loop known as the hypothalamus-pituitary-adrenal axis. The hypothalamus is activated by an external stimulus to produce and release corticotrophin releasing hormone (CRH) (Figure 1-13). CRH stimulates the anterior pituitary to synthesise and release adrenocorticotrophin (ACTH) which in turn stimulates the adrenal cortex to release cortisol from the zona fasiculata. The increasing levels of cortisol feedback to the hypothalamus decreasing

CRH release and on the pituitary to decrease ACTH release (Tasker et al., 2006) (Figure 1-13).

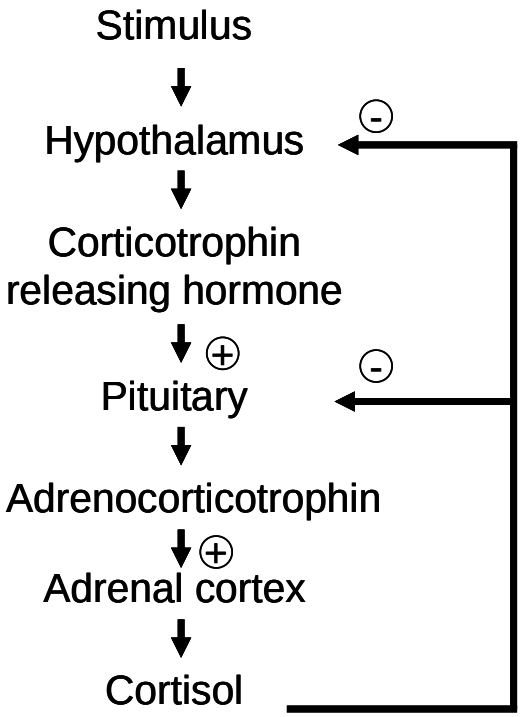



*Figure 1-13: Hypothalamus-pituitay-adrenal cortex axis.*

*The hypothalamus is stimulated to produce corticotrophin releasing hormone (CRH) which acts on the pituitary to produce adrenocorticotrophin (ACTH). ACTH then acts on the adrenal cortex to produce cortisol. Cortisol negatively feeds back on the hypothalamus and pituitary to decrease CRH and ACTH production respectively.*

#### 1.6.4.2. Aldosterone

Mineralocorticoids are synthesised in the zona glomerulosa under the control of the renin-angiotensin system. Stimuli such as decreased sodium in the blood, hypotension and sympathetic innervations, stimulate the juxtaglomerular cells of the kidney to release renin into the bloodstream. Renin proteolytically cleaves angiotensinogen to angiotensin I which in turn is cleaved by angiotensin converting enzyme (ACE) to produce angiotensin II. Active angiotensin II stimulates the adrenal cortex to produce aldosterone which

serves to regulate sodium transporting systems such as those within the kidney and the salivary glands (Figure 1-14). Natriuretic peptide produced from the heart and vasculature and somatostatin principally produced from the hypothalamus and cells of the intestine are able to inhibit angiotensin II induced aldosterone synthesis (Liang et al., 2007a, Aguilera et al., 1981)

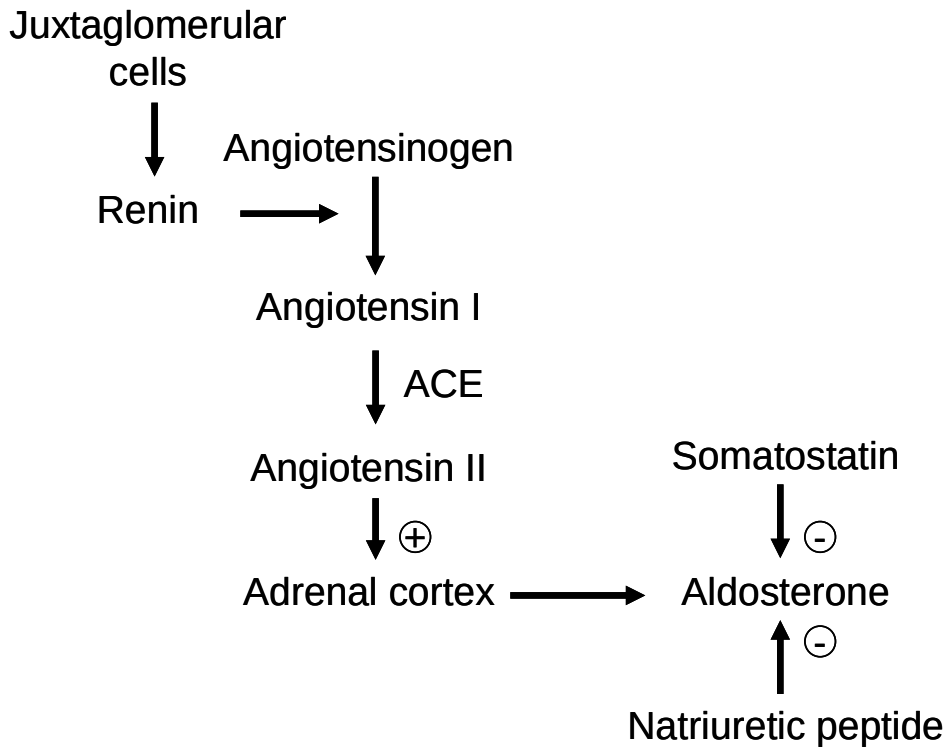



*Figure 1-14: Renin-angiotensin system.*

*Renin released from the juxtaglomerular cells of the kidney cleaves angiotensinogen to angiotensin I. Angiotensin I is converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II stimulates the adrenal cortex to produce aldosterone. In addition to this, aldosterone can be inhibited by somatostatin and natriuretic peptide.*

In addition to systemic regulation of corticosteroids, there is a need for the regulation of corticosteroids at a tissue specifc level so that the effects can be controlled locally. In the case of cortisol this is performed by 11beta hydroxysteroid dehydrogenase enzymes.

## *1.7. Tissue Specific Regulator of Glucocorticoids*

### 1.7.1. Pre-receptor Regulation of Glucocorticoids and Mineralocorticoids

Glucocorticoid and mineralocorticoid activity are regulated locally in tissues by an autocrine feedback loop (Figure 1-15). In the case of cortisol, this regulation is mediated by the 11beta hydroxysteroid dehydrogenases which are part of the short chain alcohol dehydrogenase superfamily (SDR). There are two isozymes that have been characterised: 11beta hydroxysteroid dehydrogenase 1 (11β-HSD1) and 11beta hydroxysteroid dehydrogenase 2 (11β-HSD2).

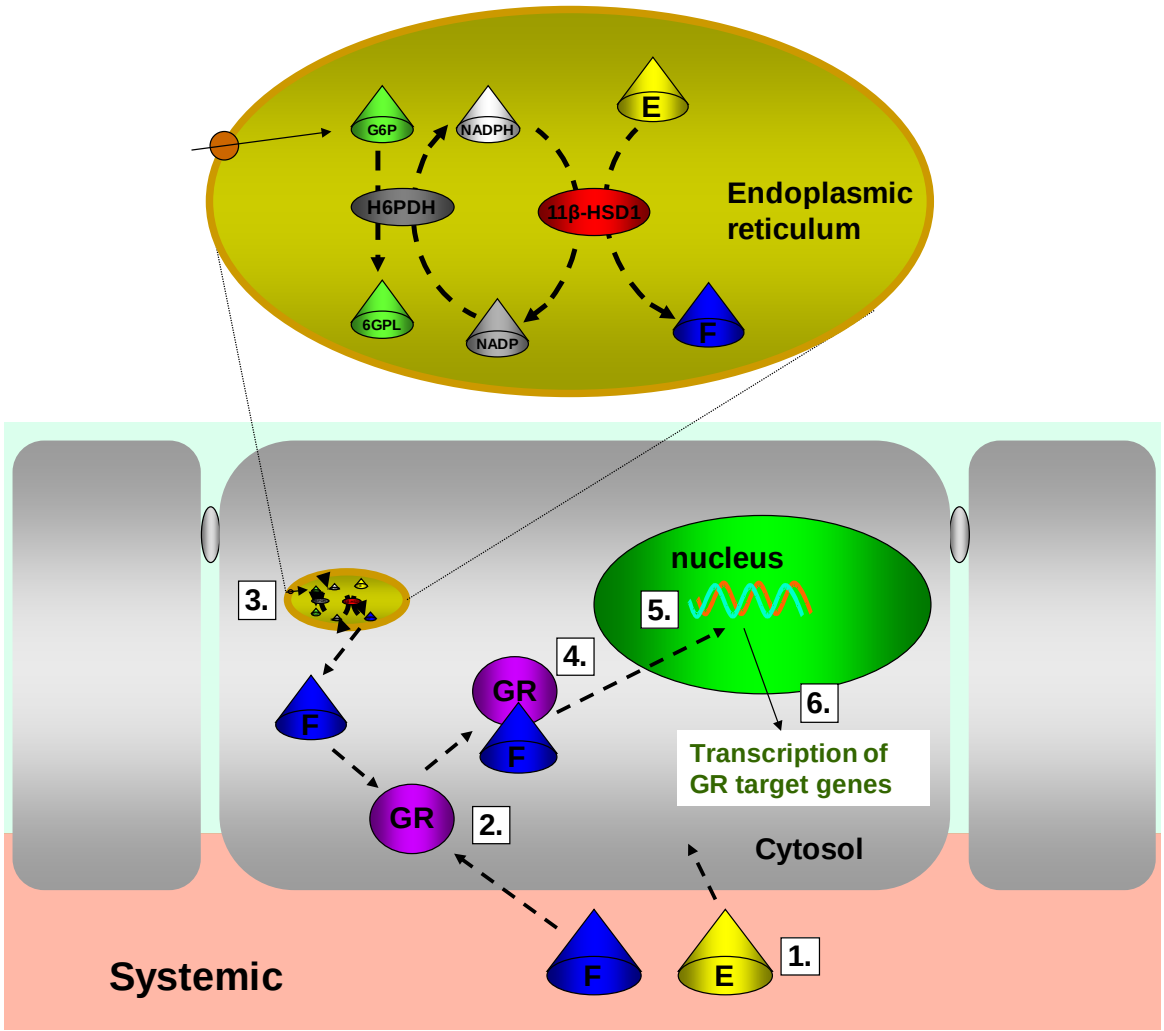



*Figure 1-15: Pre-receptor regulation of glucocorticoids.*

*Cortisol and cortisone enter the cell from the circulation. 2. The active cortisol from circulation can bind directly to the glucocorticoid receptor or 3. 11β-HSD1 can reactivate cortisol from cortsone using NADPH provided for by H6PDH. H6PDH utilises G6P for this reaction. G6P is transported into the cell by G6PT. 4. Activated GR can translocate to the nucleus. 5. Transcription is mediated by GR. 6. This leads to transcription of specific target genes. (E, cortisone; F, cortisol; GR, glucocorticoid receptor; 11β-HSD1, 11beta hydroxysteroid dehydrogenase; H6PDH, hexose 6 phosphate; G6P, glucose 6 phosphate; 6GPL, 6 phosphogluconate; G6PT, glucose 6 phosphate transporter)*

When cortisol enters the cell from the circulation or cortisone is activated to cortisol intracellularly, it can bind to the glucocorticoid receptor and upon GR activation can allow the GR-cortisol complex to translocate to the nucleus to influence transcription of genes. Glucocorticoid activation specifically takes place in the endoplasmic reticulum by membrane bound 11β-HSD. Hexose-6-phosphate dehydrogenase (H6PDH) utilises the substrate glucose-6-phosphate (G6P), (Kimura et al., 1979, Stegeman and Klotz, 1979, Ozols, 1993) in the endoplasmic reticulum lumen to govern the directionality of 11β-HSD1 (Figure 1-16) by providing the cofactor NADPH (Sherbet et al., 2007, Bujalska et al., 2005).

### 1.7.2. 11β-HSD1 Expression and Distribution

11β-HSD1, which was the first isoform discovered, was characterised in the late 1980s and cloned from rat liver (Lakshmi and Monder, 1988, Agarwal et al., 1989). The enzyme is located in the lumenal side of the endoplasmic reticulum (Ozols, 1995) and is found to be bidirectional, capable of catalysing the interconversion between hormonally inactive cortisone and active cortisol (Figure 1-16) with low affinity (Amelung et al., 1953, Bush et al., 1968, Lakshmi and Monder, 1988);

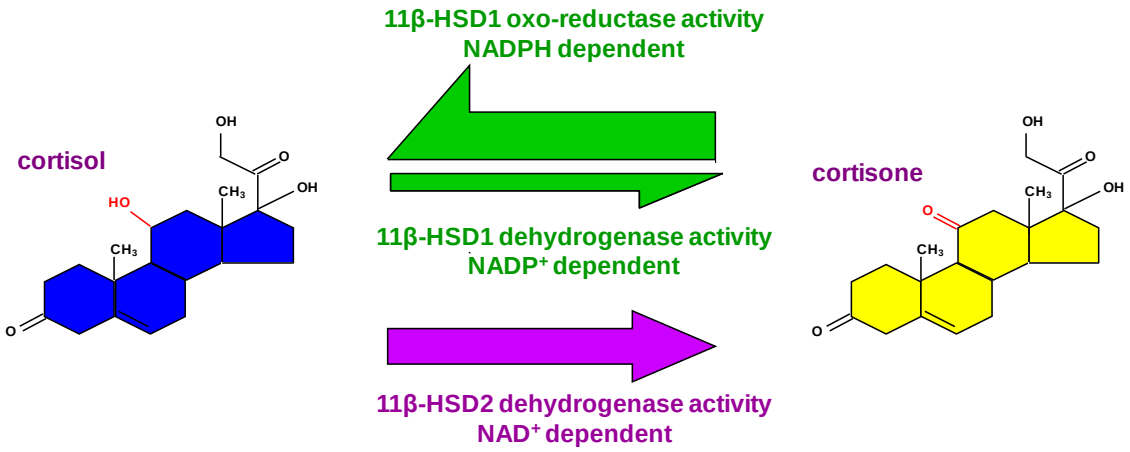



*Figure 1-16: Interconversion of cortisol and cortisone by 11beta hydrosteroid dehydrogenase enzymes (11β-HSD).*

*11β-HSD1 predominantly converts cortisone to cortisol in intact cells while converting cortisone to cortisol in disrupted cells. 11β-HSD2 only converts cortisol to cortisone.*

Previous research has shown that 11β-HSD1 acts predominantly in intact cells as a reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent oxo-reductase which in-vivo or in intact cells, converts inactive cortisone to active cortisol (Tomlinson et al., 2004, Nikkila et al., 1993, Jamieson et al., 1995, Brem et al., 1995, Bujalska et al., 1997). However, some tissue homogenates and microsomes exhibit a nicotinamide adenine dinucleotide phosphate ($NADP^+$) dependent dehydrogenase reaction where cortisol is converted to cortisone (Abramovitz et al., 1982, Bernal et al., 1982, Lakshmi and Monder, 1988) (Figure 1-16).

Kinetic analysis of 11β-HSD1 shows that a Michaelis constant of 17.3 ± 2.24μM can be seen for cortisol in microsomes which, is much lower than its dehydrogenase form (Lakshmi and Monder, 1988, Monder and Lakshmi, 1989, Koerner, 1969).

The 11β-HSD1 gene has been mapped to chromosome location 1q32.2 using rat 11β-HSD cDNA to probe a human testis cDNA library and is found to consist of 6 exons and 5

introns (Tannin et al., 1991). The 34kDa protein is found to be highly expressed in a variety of tissues regarded as glucocorticoid target tissues including human liver, adipose, gonads and placenta (Table 1-5).

In human adipose tissue, 11β-HSD1 is expressed in subcutaneous (SC) and omental (OM) adipose tissue (Table 1-5) with predominantly has greater oxo-reductase activity in OM compared to SC (Bujalska et al., 1997, Bujalska et al., 2002b) and has been linked to the complex pathogenesis of obesity (De Sousa Peixoto et al., 2008, Tomlinson et al., 2002). In the human adrenal gland, 11β-HSD1 is expressed mainly in the zona reticularis of the adrenal cortex, although there is some expression within the zona glomerulosa and zona fasiculata (Table 1-5). The role of this enzyme at this site remains unclear (Ricketts et al., 1998a).

11β-HSD1 expression in human bone (Table 1-5) was found in both osteoclasts and osteoblasts (Bland et al., 1999, Cooper et al., 2000). The function of glucocorticoids in this tissue is to facilitate osteoblast differentiation, but can also adversely promote osteoblast apoptosis and decrease proliferation at high doses causing osteoporosis and bone metabolism (Cooper et al., 1999, Delany et al., 1995, Hong et al., 2008, Kim et al., 2007). Fibroblasts from the synovium have a higher expression of 11β-HSD1 in synovial fibroblasts compared to dermal fibroblasts and may have an important role in autocrine activation of glucocorticoids (Hardy et al., 2006).

11β-HSD1 has also been characterised to the human colon where it has been localised to the lamina propria and mucosal epithelium (Whorwood et al., 1994) and recent data has suggested a role for induction of 11β-HSD1 in colonic inflammation (Zbankova et al., 2007).

*Table 1-5: 11β-HSD1 expression and activity in physiology*

| Tissue | 11β-HSD1 Expression and or Activity | References |
|---|---|---|
| **Adipose** | + | (Yang et al., 1997, Bujalska et al., 1997, Balachandran et al., 2008, Bujalska et al., 2006, Bujalska et al., 2002b) |
| **Adrenal** | Adrenal cortex: +; adrenal medulla: +/- | (Ricketts et al., 1998a, Albertin et al., 2002) |
| **Bone** | Osteoblast: +; osteoclast: +; synovial fibroblasts | (Bland et al., 1999, Cooper et al., 2000, Hardy et al., 2006) |
| **Colon** | Rectal colon: -; surface epithelia; +; lamina propria: + | (Whorwood et al., 1994) |
| **CNS** | Cerebellum: + | (Whorwood et al., 1995) |
| **Eye** | NPE: +,;corneal endothelium: +; TM: +/-; Lens: +/-, Corneal epithelium: +/- | (Stokes et al., 2000, Rauz et al., 2001, Rauz et al., 2003, Suzuki et al., 2001, Mirshahi et al., 1997) |
| **Gonad** | Testis: +; ovary: + | (Ricketts et al., 1998a, Tannin et al., 1991) |
| **Heart** | Aortic smooth muscle cells: + | (Hatakeyama et al., 2001) |
| **Kidney** | Medulla: + | (Albiston et al., 1994) |
| **Liver** | + | (Ricketts et al., 1998a) |
| **Lung** | Lung epithelial cell: -; foetal lung tissue: - | (Garbrecht et al., 2006) |
| **Immune Cell** | Macrophage: + | (Makkonen et al., 2007) |
| **Lymphoid** | Spleen: + | (Whorwood et al., 1995, Thieringer et al., 2001b) |
| **Pancreas** | Islets of Langerhan: + | (Davani et al., 2000) |
| **Pituitary** | Folliculo-stellate cells: +; corticotrophs: - | (Korbonits et al., 2001) |
| **Placenta** | + | (Bernal et al., 1980) |
| **Skin** | Epidermis: +; fibroblasts: + | (Hardy et al., 2006, Teelucksingh et al., 1990) |
| **Decidua** | + | (Arcuri et al., 1996, Ricketts et al., 1998a) |

*+: present; -: absent; +/-: conflicting*

There have been several studies into the expression of 11β-HSD1 in the rat brain (Moisan et al., 1990) and rabbit brain (Sinclair et al., 2007) but very little in the human brain except for mRNA expression in the human cerebellum (Whorwood et al., 1995). Gene

expression for MR and GR can be found in the foetal human brain suggesting glucocorticoids can bind to these receptors during development and may have an impact on HPA activity (C.W. Noorlander, 2006). However, 11β-HSD1 activity has been established in foetal hippocampal cells showing predominant oxo-reductase activity (Rajan et al., 1996).

In human gonads, the testis and the ovaries express 11β-HSD1 (Table 1-5), (Ricketts et al., 1998a, Tannin et al., 1991). Both reductase and dehydrogenase can be found in these regions and it has been shown that in the mouse testis, glucocorticoids reduce testosterone production (Payne and Sha, 1991) and in the mouse ovaries, glucocorticoids can inhibit FSH-stimulated aromatase activity (Hsueh and Erickson, 1978). Prolactin and growth hormone secreting cells of the pituitary as well as folliculo-stellate cells have been shown to express 11β-HSD1 but 11β-HSD1 is absent from ACTH and FSH secreting cells, suggesting that the isozyme is not important for the regulation of the hypothalamus-pituitary-axis by glucocorticoids (Korbonits et al., 2001).

In addition, 11β-HSD1 has been established in human aortic smooth muscle cells (Table 1-5), (Hatakeyama et al., 2001) and this can be affected by inflammatory stimuli indicating a role in inflammatory conditions such as atherosclerosis (Cai et al., 2001). This may have physiological significance as glucocorticoids are known to increase vascular tone and therefore local action of 11β-HSD1 may influence this function (Hatakeyama et al., 2001).

11β-HSD1 was the first isozyme to be characterised in the kidney (Table 1-5) and was shown to have little expression which is restricted to the medulla and functions as a dehydrogenase (Albiston et al., 1994, Gong et al., 2008, Stewart et al., 1994, Brem et al., 1997).

The highest expression of 11β-HSD1 is found in the liver (Table 1-5) which predominantly converts cortisone to cortisol and also possesses a high population of glucocorticoid receptors (GR). This was established after it was found that hepatic venous blood has a higher F/E ratio compared to renal venous blood (Walker et al., 1993). The 11β-HSD1 isozyme is localised maximally and radially from the central vein in the liver (Ricketts et al., 1998a). A major role for the liver is to provide glucose during fasting and 11β-HSD1 increases gluconeogenic enzymes and therefore enhances glucose output (Walker et al., 2007, Kotelevtsev et al., 1997). Elevated 11β-HSD1 is involved in the metabolic syndrome and has been linked to fatty liver and insulin resistance (Johansson et al., 2001, Paterson et al., 2004).

Expression of 11β-HSD1 has also been localised to lymphoid tissue (Table 1-5) such as the spleen (Whorwood et al., 1995) and 11β-HSD1 is induced after differentiation of monocytes to macrophages and after treatment with IL-4 and IL-13 (Thieringer et al., 2001b). A previous study shows the expression of 11β-HSD1 in the islets of Langerhan of the pancreas (Table 1-5) and it is thought to have a role in glucocorticoid mediated inhibition of insulin release (Davani et al., 2000).

Placental tissue express 11β-HSD1 but shows negligible reductase activity and predominant dehydrogenase activity suggesting that its role may be to regulate cortisol excess to the foetus (Bernal et al., 1980). In addition to this, the decidua expresses 11β-HSD1 which may be important in preventing immune rejection of the foetus (Arcuri et al., 1996).

The skin epidermis (Teelucksingh et al., 1990) and skin fibroblasts (Hardy et al., 2006) express 11β-HSD1 (Table 1-5) and cortisol decreased IL-6 expression suggesting that

skin fibroblasts may have the capacity to respond to inflammatory stimulation such as that in leprosy (Andersson et al., 2007, Hardy et al., 2006).

### 1.7.3. Regulation of 11β-HSD1 Activity

There are many regulators of 11β-HSD1 in humans and these are summarised and described briefly in Table 1-6. Inflammatory stimuli can also regulate 11β-HSD1 for example IL-1α, IL-1β, IL-6, TNFα, IL-4 and IL-13 can up-regulate oxo-reductase activity and thus locally promote availability of cortisol in the tissues such as the liver, adipose and bone (Yong et al., 2002, Escher et al., 1997a, Thieringer et al., 2001a, Evagelatou et al., 1997, Hardy et al., 2006). GH treatment of hypopituitary male and females found that urinary THF/THE ratios decreased while being treated with a particular course of hydrocortisone (Gelding et al., 1998). Moreover, it has emerged that IGF-I may be mediating effects on 11β-HSD1 via GH after in-vitro studies on omental preadipocytes showed a decrease in cortisone to cortisol conversion from dose dependent IGF-I and not GH (Moore et al., 1999)

Other factors such as insulin have differential effects on 11β-HSD1 oxo-reductase activity depending on the cell type. For example insulin increases or has no effect on 11β-HSD1 oxo-reductase activity in human adipose (Wake et al., 2006, Handoko et al., 2000) and decrease activity in skin fibroblasts (Hammami and Siiteri, 1991). Glucocorticoids such as cortisol and dexamethasone can increase 11β-HSD1 expression in adipose and amnion fibroblasts respectively (Li et al., 2006, Sun and Myatt, 2003). This occurs indirectly through C/EBP(beta) (Sai et al., 2008). The sex steroids medroxyprogesterone acetate and estradiol can increase 11β-HSD1 expression while progesterone decreases 11β-HSD1

expression in ovarian epithelium or has no effect on 11β-HSD1 expression in human hepatocytes (Arcuri et al., 1996, Fegan et al., 2008, Ricketts et al., 1998b).

*Table 1-6: Regulators of 11β-HSD1*

| Regulatory Factor | Effect on Human 11β-HSD1 | Ref. |
|---|---|---|
| **IL-1** | ↑ (e.g. ovary) | (Yong et al., 2002) |
| **IL-6** | ↑ (e.g. adipose) | (Tomlinson et al., 2001b) |
| **TNFα** | ↑ (e.g. aorta)<br>← (e.g. monocytes) | (Thieringer et al., 2001b, Cai et al., 2001) |
| **IL-4** | ↑ (e.g. monocytes)<br>← (e.g. synovium) | (Hardy et al., 2006, Thieringer et al., 2001b) |
| **IL-13** | ↑ (monocytes) | (Thieringer et al., 2001b) |
| **GH** | ↓ (e.g. adipose) | (Paulsen et al., 2006, Sigurjonsdottir et al., 2006, Tomlinson et al., 2001b) |
| **IGF-I** | ↓ (adipose) | (Moore et al., 1999, Tomlinson et al., 2001b) |
| **Insulin** | ↑ (e.g. adipose)<br>← (e.g. adipose)<br>↓ (e.g. skin fibroblasts) | (Handoko et al., 2000, Wake et al., 2006, Hammami and Siiteri, 1991) |
| **Cortisol** | ↑ (e.g. adipose) | (Tomlinson et al., 2002, Li et al., 2006) |
| **Dexamethasone** | ↑ (e.g. amnion fibroblasts) | (Sun and Myatt, 2003) |
| **Estradiol** | ↑ (e.g. ovary) | (Arcuri et al., 1997) |
| **Medroxyprogesterone Acetate** | ↑ (e.g. ovary) | (Arcuri et al., 1996) |
| **Progesterone** | ↓ (e.g. ovary)<br>← (e.g. hepatocytes) | (Fegan et al., 2008, Rae et al., 2004, Ricketts et al., 1998b) |
| **Thyrotropin** | ← (e.g. hepatocytes) | (Ricketts et al., 1998b) |

*Effects on 11β-HSD1 are classified as ↑, increase; ↓, decrease and ←, no effect.*

Thyrotropin ($T_3$) has been shown not to have an effect on 11β-HSD1 expression in human hepatocytes (Ricketts et al., 1998b).

### 1.7.4. 11β-HSD2 Expression and Distribution

The 11β-HSD2 isoform is a unidirectional nicotinamide adenine dinucleotide ($NAD^+$) dependent dehydrogenase with high affinity for cortisol. The gene for this enzyme was cloned in the mid 1990s and is mapped to chromosome 16q22 (Albiston et al., 1994).

11β-HSD2 is commonly expressed in tissues rich in mineralocorticoid receptors such as the kidney, placenta (Krozowski et al., 1995, Brown et al., 1993, Stewart et al., 1995), salivary gland (Smith et al., 1996) and colon (Whorwood et al., 1994, Hirasawa et al., 1997). In the human kidney, 11β-HSD2 is localised to the collecting ducts of the cortex and medulla as well as the distal convoluted tubules. The primary role of this isoform is to protect the mineralocorticoid receptor from cortisol excess, thereby allowing aldosterone to genomically mediate transcription. The two genes known to be genomically regulated by aldosterone are the epithelium sodium channel (ENaC) and serum and glucocorticoid induced kinase (SGK) which are involved in the transport of sodium across the epithelia, permits water reabsorption from the kidney tubule to the capillary bed (Figure 1-17). SGK is postulated to modulate ENaC activity by interacting with accessory proteins such as Nedd4-2 which is known to interact with and downregulate ENaC (Snyder et al., 2002, Fejes-Toth et al., 2008).

Similar mechanisms exist in the salivary glands (Rauh et al., 2006) and colon (Matos et al., 2007, Coric et al., 2004) where expression of the enzyme is restricted to the intercalating and striated ducts of the salivary glands, and columnar epithelial cells of the distal colon. In the placenta, localisation of 11β-HSD2 within the syncytial trophoblast cells (Krozowski et al., 1995) protects the developing foetus from high levels of circulating maternal cortisol (Krozowski, 1999, Sun et al., 1998, Driver et al., 2003). 11β-HSD2 is also important during pregnancy, maintaining the foetus and facilitating

maturation (Sato et al., 2008b). However, this is thought to be regulated in part by progesterone, which decreases 11β-HSD2 expression.

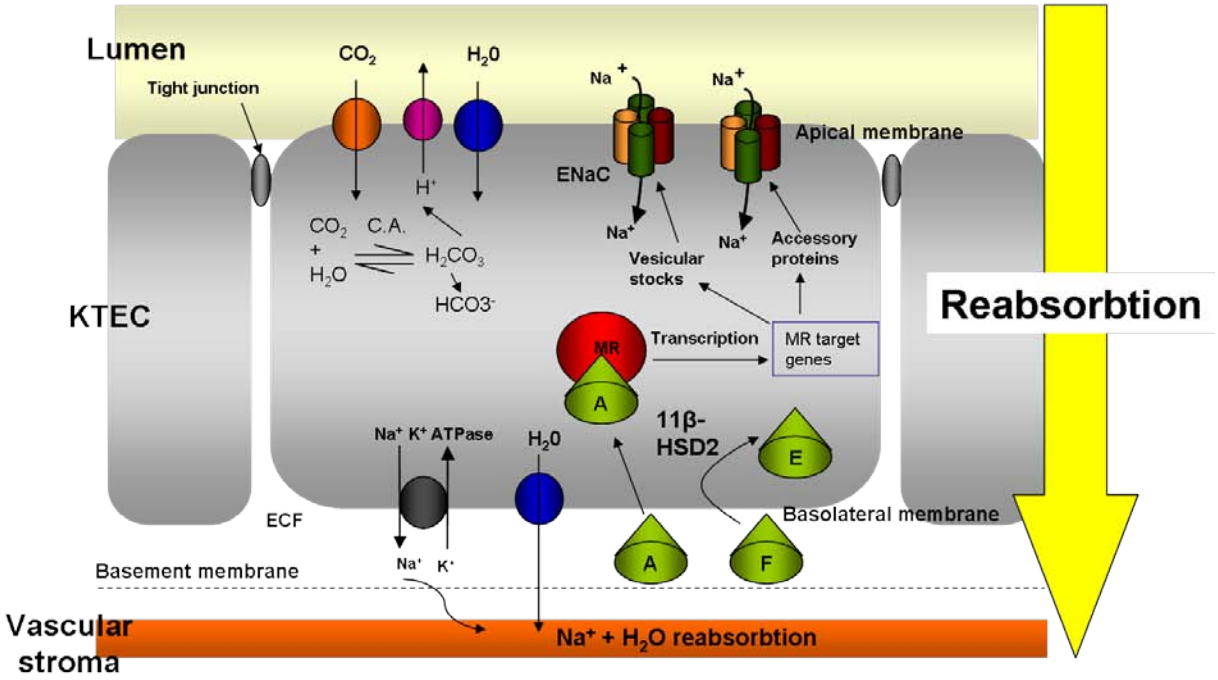



*Figure 1-17: Aldosterone effects on sodium/water movement in the kidney tubule epithelial cell (KTEC).*

*Cortisol(F) from the vascular bed is inactivated to cortisone (E) by 11β-HSD2 in the cell allowing aldosterone A) to bind to the mineralococrticoid receptor (MR) and initiate transcription of a series of genes involved in sodium transport e.g. epithelial sodium channel (ENaC). This ultimately results in water reabsorbtion.*

### 1.7.5. Regulation of 11β-HSD2

Circulating sex steroids can regulate 11β-HSD2 expression and activity. For example, progesterone inhibits 11β-HSD2 activity in human placental trophoblast cells (Sato et al., 2008a) and estradiol increases 11β-HSD2 activity in the human endometrial cell-line (Darnel et al., 1999). Other factors include EGF which causes a decrease in 11β-HSD2 activity in the human endometrial cell-line (Darnel et al., 1999)

### *1.8. 11β-HSD in the Eye and the Orbit*

In the human eye, 11β-HSD1 has been localised to several anatomical areas of the eye which are briefly reported in Table 1-7.

*Table 1-7: Expression of 11beta hydroxystroid dehydrogenase 1 and 2 in human ocular tissues*[a]

| | **11β-HSD1** | | **11β-HSD2** | |
|---|---|---|---|---|
| **Conjunctiva** | | Ref. | | Ref. |
| **Epithelium** | ? | | ? | |
| **Stroma** | ? | | ? | |
| **Cornea** | | | | |
| **Epithelium** | +/- | 1,3,4 | - | 3, 4 |
| **Stroma** | - | 3 | - | 3 |
| **Endothelium** | +/- | 3,4 | +/- | 1,3,4 |
| **Ciliary Body** | | | | |
| **NPE** | +/- | 1,2,3,4 | +/- | 1,3,4 |
| **PE** | - | 1,3,4 | - | 1,3 |
| **Stroma** | - | 3 | - | 3 |
| **TM** | +/- | 1,4 | - | 1,4 |
| **Lens** | +/- | 1,3,4 | - | 1,3,4 |
| **Retina** | - | 3 | - | 3 |
| **Optic Nerve** | - | 3 | - | 3 |
| **Sclera** | - | 3,4 | - | 3,4 |
| **Orbital Adipose Tissue** | ? | | ? | |

[a]*Immunoreactivity and/or mRNA expression is classified as +; present, -; not present, +/- ; conflicting data; ?, not known. References 1; (Rauz et al., 2001), 2; (Rauz et al., 2003), 3; (Suzuki et al., 2001), 4; (Stokes et al., 2000).*

The cornea which consists of the corneal epithelium, Bowman's capsule, stroma, endothelium and descemet's membrane has some expression of 11β-HSD1. Expression of 11β-HSD1 is found in the corneal epithelial cells (Rauz et al., 2001, Stokes et al., 2000)

but there are some conflicting reports (Suzuki et al., 2001). There are no reports of 11β-HSD1 expression in the stroma or endothelium (Rauz et al., 2001, Suzuki et al., 2001, Stokes et al., 2000).

The expression of 11β-HSD1 can be found in the non-pigmented epithelium (NPE) of the ciliary body (Rauz et al., 2003a, Rauz et al., 2001, Stokes et al., 2000). There is no expression of 11β-HSD1 expression in the pigmented epithelium (PE) of the ciliary body (Stokes et al., 2000, Rauz et al., 2001, Suzuki et al., 2001) and no expression in the ciliary body stroma (Suzuki et al., 2001). There are also conflicting reports of 11β-HSD1 expression in the NPE (Stokes et al., 2000, Suzuki et al., 2001). MR expression is found in the NPE (Suzuki et al., 2001, Stokes et al., 2000) but not in the PE or stroma of the ciliary body (Suzuki et al., 2001). The regeneration of the glucocorticoid, cortisol in these systems (the cornea and NPE) by 11β-HSD1 may be involved in stimulating apical sodium channels and basolateral $Na^+$-$K^+$-ATPase in the regulation of water and solute movement and in immunoprotection.

The trabecular meshwork (TM) has expression of 11β-HSD1 (Rauz et al., 2001) but no expression of 11β-HSD2 and MR. There is some evidence of 11β-HSD1 expression in the lens epithelium (Stokes et al., 2000) (Stokes et al., 2000, Rauz et al., 2001, Suzuki et al., 2001). Expression of 11β-HSD1 (Suzuki et al., 2001) was not found in any of the retinal cells. The optic nerve also showed no evidence of expression for 11β-HSD1 (Suzuki et al., 2001). Within the sclera there is no expression of 11β-HSD1 (Suzuki et al., 2001, Stokes et al., 2000). With respect to orbital adipose tissue, it has recently been found that 11β-HSD1 was expressed and was able to actively produce cortisol from cortisone (Bujalska et al., 2007).

11β-HSD2 expression in the cornea is conflicting. No expression of this enzyme has been found in corneal endothelial cells (Rauz et al., 2001, Stokes et al., 2000) or the stroma (Rauz et al., 2001, Suzuki et al., 2001), however, there is conflicting evidence suggesting expression in the corneal epithelium and corneal epithelial cells (Stokes et al., 2000). There is no expression of 11β-HSD2 and MR in the lens (Suzuki et al., 2001, Stokes et al., 2000). 11β-HSD2 (Suzuki et al., 2001) and MR (Suzuki et al., 2001) were not found to be expressed in any of the retinal cells or optic nerve. The sclera had no expression of 11β-HSD2 or MR (Suzuki et al., 2001, Stokes et al., 2000) and no expression of 11β-HSD2 can be found in orbital adipose tissue (Bujalska et al., 2007).

Little is known about 11β-HSD in the eye, especially in the orbit. There are few studies detailing the distribution and expression of 11β-HSD in the eye and there have been no reports on 11β-HSD in the orbit but there is the incidence of 11β-HSD in orbital adipose tissue from the orbit (section 4.3.3.1), (Bujalska et al., 2007).

11β-HSD1 may have a beneficial role for local tissue specific regulation of glucocorticoids in inflammation, however, overexpression of 11β-HSD1 may up-regulate glucocorticoid-induced adverse effects such as those seen in glaucoma.

### 1.8.1. Potential Applications of 11β-HSD1

The establishment of 11β-HSD1 in disease may provide a potential target for different therapeutic interventions. These may include manipulation of 11β-HSD1 for the regulation of glucocorticoids which have effects on ocular surface disease, intraocular immune privilege and orbital disease states.

### 1.8.2. Ocular and Orbital Disease States

Regulation of 11β-HSD on ocular surfaces which include the cornea and intraocular environments such as the ciliary epithelium may have influences upon regulating inflammation and cell repair which is vital for normal eye function. The significance of the specific regulation of 11β-HSD1 in the corneal epithelium and conjunctival epithelium could suggest a role in the regulation of ocular surface inflammatory diseases such as infective keratitis where inhibition of 11β-HSD1 could provide a therapeutic target for reduced inflammation especially in autoimmune eye disease. For example, a specific 11β-HSD1 inhibitor, compound 544 was successfully used to inhibit 11β-HSD1 within insulin resistant mice and improved their insulin sensitivity (Hermanowski-Vosatka et al., 2005).

Cortisol is produced in human and rabbit aqueous humour in the anterior chamber of the eye (Rauz et al., 2001, Obenberger et al., 1971, Stárka et al., 1975). 11β-HSD1 involved in the generation of glucocorticoids may potentially be regulating local production of cortisol from the ciliary NPE which may be important for intraocular immunoprotection.

Immune privilege sites in the eye exist for example where there is no blood supply such as that found in the central cornea and where there are vascular barriers such as that in the ciliary body (Koevary, 2000). It is hypothesised that immune privilege is achieved because antigens are isolated from being presented to cells of the immune system. Manipulation of 11β-HSD1 for the generation of glucocorticoids in immune privilege sites may protect these sites from immune intervention and promote other factors essential to maintaining the immune privilege state (chapter 1.3.7.1).

Glucocorticoid therapy for thyroid associated ophthalmopathy can cause adverse effects when used systemically. Local generation of glucocorticoids by 11β-HSD1 in inflamed orbital adipose tissue may taper inflammation without severe side effects and could provide a novel therapeutic target for treatment of thyroid associated ophthalmopathy.

### *1.9. Hypothesis and Aims*

This thesis was designed to evaluate the functional role of 11β-HSD in the eye and the orbit and whether the 11β-HSD1 can be targeted to modify various disease processes in the eye (Figure 1-18).

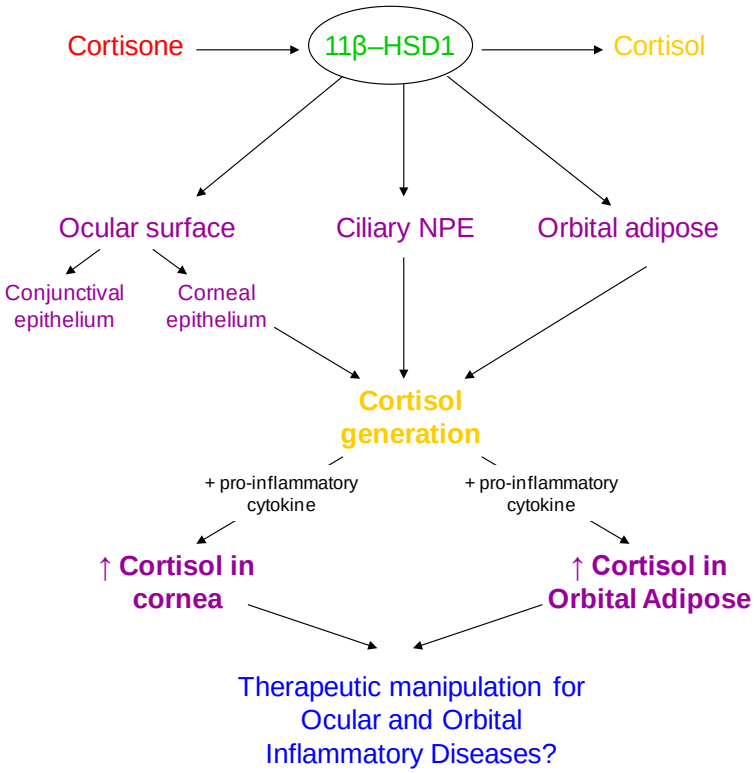



*Figure 1-18: Hypothesis diagram.*

*Cortisol levels are regulated by 11β-HSD1. 11β-HSD1 is hypothesised to be involved in the local generation of cortisol in corneal and ciliary epithelium and orbital adipose tissue. Cortisol generation further increased from corneal epithelium and orbital adipose by pro-inflammatory cytokines. The inflammatory insult together with the increased local cortisol production in tissue may contribute to the pathology of ocular surface and orbital inflammatory disease. 11β-HSD1 and pro-inflammatory cytokines may provide therapeutic targets in ocular and orbital inflammatory diseases. 11β-HSD1, 11 beta hydroxysteroid dehydrogenase 1.*

The first part of the results in this thesis will focus on 11β-HSD1 on the ocular surface and intraocular environment and will include:

- The animal model, New Zealand White Albino Rabbit (NZWAR), used to confirm expression of 11β-HSD1 in the corneal epithelium, conjunctival epithelium and ciliary NPE.
- Evaluation of intraocular generation of cortisol from ciliary body and epithelium.
- Evaluation of the human ocular surface which will include study of the cornea to confirm 11β-HSD1 expression and activity in corneal epithelium and other cell-types that include fibroblasts from the conjunctiva and cornea.
- Studying the effects of cytokines on 11β-HSD1 activity in corneal epithelial cells which may have significance in ocular surface defence.
- Consideration of corneal epithelial cell biology and studying the effects of glucocorticoids on cell proliferation and what implication this may have on ocular physiology.

The last part of the results will focus on 11β-HSD1 in orbital adipose tissue of the orbit and will include:

- Detailed analysis of the human orbital adipose tissue depot and comparing this to well characterised adipose tissue depots, namely, omental (OM) and subcutaneous (SC) adipose.

- Evaluation of 11β-HSD1 expression in orbital adipose tissue.

- Evaluation of the pre-receptor regulation of glucocorticoids in ‘control’ orbital adipose preadipocytes compared to ‘thyroid’ orbital adipose preadipocytes.

- Cytokine profiling of orbital adipose will be compared to OM and SC and the effects of pro-inflammatory cytokines on the orbital preadipocyte 11β-HSD1 oxo-reductase activity will be shown.

## 2. Chapter 2

# General Material and Methods

## *2.1. Introduction*

The general chemicals and solutions used during the course of this research were obtained from Sigma-Aldrich Company (Sigma-Aldrich Company Ltd, Dorset, UK) and Fisher Scientific UK Ltd (Loughborough, Leicestershire, UK). Phosphate buffered saline (PBS) and distilled water were autoclaved before using for cell culture stock solutions. Solutions of unlabelled cortisol (F), cortisone (E) and glycyrrhetinic acid (GE) were all from Sigma-Aldrich Company Ltd, Dorset, UK, were made in absolute ethanol in stock solutions of $10^{-2}$ M and stored at -20ºC. The radiolabelled hydrocortisone (cortisol) [1,2,6,7-3 H (N)] – 2.6 TBq/mmol (70.00 Ci/mmol) was obtained from New England Nuclear-Dupont Ltd (Herts, UK) and was stored at -20ºC.

## *2.2. Tissue Ethics Approval*

### 2.2.1. Animal Legislation

Ocular tissues were harvested from the NZWAR in accordance with the FRAME Guidelines on research involving the use of laboratory animals, the UFAW Handbook on the care and management of laboratory animals and the ARVO Statement for the use of animals in ophthalmic and vision research. The rabbits which were female were housed in pens in groups of two or three. Their approximate age was 5 months and their weight was up to 1.5kg. The rabbits were fed with a standard pelleted diet which was given at a rate of about 60-80g/kg body weight/day and given water daily. They were provided with hay which they ate and used for bedding. The rabbits were kept in recommended lighting requirements of 12h: 12h light/dark cycle.

### 2.2.2. Human Tissue Ethics

The collection of human corneoscleral discs, eye, liver, kidney and orbital adipose tissues for in-vitro investigation was approved by the local research ethics committee (LREC). Informed consent was obtained from all patient volunteers or their relatives.

All human orbital adipose tissue (HOAT) specimens (n=87) were obtained from BMEC and Moorfields Eye Hospital (MEH) with informed consent and following ethical approval.

### 2.2.3. Preparation and Storage of Human and Rabbit Tissues

#### *2.2.3.1. New Zealand White Albino Rabbit (NZWAR) Tissue Collection, Preparation and Storage*

All NZWARs (n=6) were housed in the Biomedical Services Unit (BMSU), University of Birmingham, UK. Liver and kidney sections were taken and fixed and the remaining tissue stored in RNA-later (Ambion - Europe Ltd, Cambridgeshire, UK) at -80ºC and fixed in 10% formalin (Sigma-Aldrich Company Ltd, Dorset, UK). A section of conjunctiva was surgically dissected from the NZWAR globes (n=12) and fixed in formalin or stored in a sterile container for primary culture. One globe of each rabbit was fixed in formalin. The remaining globes were bisected in the coronal plane separating the anterior and posterior segments of the eye. The ciliary body and corneal scleral rim were carefully dissected from the anterior segment with the aid of a dissecting stereomicroscope.

### *2.2.3.2. Human Tissue Collection, Preparation and Storage*

Human corneoscleral discs, central corneal buttons (transplant waste), whole eye, liver and kidney were obtained from the Birmingham Midland Eye Centre (BMEC). Human eye tissue was also obtained from either healthy anterior segments of human eyes being enucleated for pathology of the posterior part of the eye or corneoscleral discs and sentral corneal buttons (transplant waste) from penetrating and lamellar keratopathy surgical procedures. The human corneoscleral disc, whole eye, liver and kidney tissues were kept in organ culture medium at room temperature before placing in either 10% formalin for fixing or primary culture media for corneal fibroblasts, conjunctival fibroblasts and corneal epithelial cells.

The HOAT specimens were obtained from patients undergoing routine surgery which included orbital decompression surgery and eyelid surgery (blepharoplasty). The qHOAT from patients were free from inflammation and had not used topical or systemic glucocorticoids for three months prior to surgery. No patients were on systemic GC-sparing immunosuppression, and none had undergone previous orbital radiotherapy. Subcutaneous and omental tissue were obtained from patients undergoing elective laparotomy. These samples were either fixed in 10% formalin before sectioning or placed in RNA later and stored at -20ºC (Ambion - Europe Ltd, Cambridgeshire, UK).

HOAT were placed in a sterile container and transported at room temperature to the laboratory after harvesting. A proportion of the HOAT samples were divided into two or three pieces (depending on the size of the specimen) and one piece placed in RNA later and stored at -20ºC (Ambion - Europe Ltd, Cambridgeshire, UK), while the other section

was cultured within 24 hours. If the specimen was too small, it was used either in its entirety for cell culture, RNA extraction or placed in formalin for tissue sectioning.

## *2.3. Cell Culture*

Cell culture medium was obtained in 1x solution form in 500ml bottles from Sigma-Aldrich Company Ltd, Dorset, UK or Gibco-Life Technologies, Invitrogen Paisley, UK. Heat inactivated foetal calf serum was acquired from Sigma-Aldrich Company Ltd, filtered through a 0.2μm sterile filter and stored as 50ml aliquots at -20°C. Trypsin (stock concentration of 0.25% v/v) was prepared by diluting 20ml trypsin (10x concentration) in 480ml 1x PBS/0.5M EDTA and aliquots were stored at -20°C.

### 2.3.1.1. Human and NZWAR Corneal Epithelial Primary Cell Culture

Corneoscleral buttons were incubated with Dispase (Invitrogen Ltd, Paisley, UK) at 37ºC for two hours to dislodge cells. Cells transferred to 48 well plates were maintained in keratinocyte–SFM medium with L-glutamine, EGF-1, bovine pituitary extract (Invitrogen Ltd, Paisley, UK), 10% fetal bovine serum (FBS) and 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen Ltd, Paisley, UK) at 37ºC in a humidified atmosphere containing 5% $CO_2$ and 95% $O_2$. Cells were maintained to approximately 80-90% confluency (controlled under microscope for characteristic hexahedral architecture) for approximately 7-14 days to obtain the maximal amount of cells until 11β-HSD enzyme assay was performed. After 11β-HSD enzyme assay was performed for both 11β-HSD1 and 2, the cells were stored in PBS at -80ºC while the assay solution was processed through thin layer chromatography (TLC).

#### 2.3.1.2. Human and NZWAR Corneal Fibroblast Primary Culture

Primary cultures were established by incubating central corneal buttons (dissected away from the remnant corneal–scleral disc used for deriving primary corneal epithelial cells) in serum-free conditions consisting of DMEM with glutamax, 1000 mg/ml glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin and sodium pyruvate (Invitrogen Ltd, Paisley, UK) in a humidified atmosphere set at 37°C with 5% $CO_2$ (Lee et al., 2008). Corneal fibroblasts were distinguised from corneal epithelial cells due to their distinctive elongated morphology. Confluent corneal fibroblast cultures were established after 14 days and were subcultured as required. All experiments were carried out on passages 1 and 2. After 11β-HSD enzyme assay was performed, cells were stored in PBS at -80ºC.

#### 2.3.1.3. Human and NZWAR Conjunctival Fibroblast Primary Cell Culture

Surgically removed human and NZWAR conjunctiva tissues were placed in 6 well plates and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Ltd, Paisley, UK) containing 10% FBS and 100 IU/ml penicillin, 100 µg/ml streptomycin at 37ºC in a humidified atmosphere containing 5% $CO_2$ and 95% $O_2$. The medium of the cells was changed every 2-3 days and the cells were maintained to approximately 90% confluency before passaging. After 11β-HSD enzyme assay was performed, cells were stored in PBS at -80ºC.

#### 2.3.1.4. Storage and Recovery of Human SV40 Immortalised Corneal Epithelial Cells

Human SV40 immortalised corneal epithelial cell-line (HCEC) was obtained as a gift from Graham Wallace. The cells were trypsinised when approximately 80-90% confluent. Cells were washed with 1x PBS before using 1ml 0.25% trypsin for a 25cm$^2$

flask. Cells were then centrifuged at 1000 x *g* before washing in 1x PBS. Cells were resuspended in 1ml foetal calf serum containing 10% (v/v) dimethylsulphoxide (DMSO) and transferred to a cryovial. Vials were kept in an ice cold methanol bath at -80°C for 24 hours before transferring to liquid nitrogen storage at -140°C.

Cell-lines in 1ml vials were thawed gently in a 37°C waterbath for less than 5 minutes before being transferred to 50ml centrifuge tube. In a drop wise manner, 5ml of culture media were added to the cells and gently mixed. The cells were centrifuged at 1000 x *g* before the supernatant was discarded and the cell pellet was resuspended in 5ml culture media and placed in a 25cm$^2$ flask.

#### 2.3.1.5. Primary Culture of Human Orbital Adipose Tissue (HOAT)

HOAT samples were finely chopped and treated with collagenase digestion solution (Worthington Biochemical Corporation, Lakewood, New Jersey). The method was adapted from the paper by Crisp and collegues (Crisp et al., 2000). The HOAT samples were disaggregated in a shaking 37ºC water bath for approximately 1 hour or until the sample appeared digested. The resulting HOAT suspension was centrifuged at 1500 rpm for 5 minutes at room temperature to separate the preadipocytes from the lipid and the adipocytes (Figure 2-1). The lipid, adipocyte and collagenase layers were removed using a pipette to leave the preadipocytes in the stromal pellet. The pellet was resuspended in growth medium consisting of DMEM nutrient mixture HAM F-12 1:1 (Invitrogen Ltd, Paisley, UK) containing 10% FBS and 100 IU/ml penicillin, 100 µg/ml streptomycin and plated into two or four wells in 24 or 48 well plates depending on the size of the sample. The cells were then incubated at 37ºC in the presence of humidified atmosphere containing 5% $CO_2$ and 95% $O_2$ and the medium was changed at intervals of every 2 days. The cells were maintained to about 80-90% confluency before the 11β HSD

enzyme assay was performed. After the 11β-HSD enzyme assay was performed, the cells adhered to the plates were stored at -80ºC.

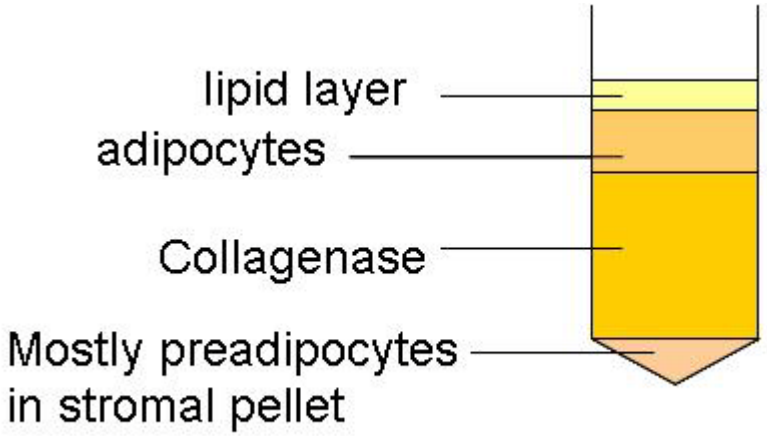



*Figure 2-1: Tissue fractionates formed after centrifugation of HOAT with collagenase.*
*Preadipocytes were collected in the stromal pellet.*

#### 2.3.1.6. Preadipocyte Differentiation

Once the cells had reached 80-90% confluency, the medium was removed from the orbital preadipocytes before washing in PBS. Chemically defined medium (DMEM nutrient mixture HAM F-12 1:1; 10% FBS; 100 IU/ml penicillin, 100 µg/ml streptomycin; 3-isobutyl-1-methylxanthine (IBMX); $10^{-4}$M cortisol; 0.2M insulin) was placed on the cells and incubated at 37ºC in humidified atmosphere containing 5% $CO_2$ and 95% $O_2$. The medium was changed on day 5 to the above medium but without IBMX. The cells were then maintained for a further 9 days until an 11β-HSD enzyme assay was performed on what was termed ‘day 14’ of differentiation.

## *2.4. RNA Extraction and Reverse Transcription*

### 2.4.1. RNA Extraction

Primary human orbital adipose tissue, human primary corneal epithelial cells, corneal fibroblast cells, conjunctival fibroblast cells and human SV40 immortalized corneal epithelial cell-line were washed in 1x PBS before extracting with Tri-reagent. Human tissue was either snap frozen in liquid nitrogen and stored at -80°C or stored in RNA later (Ambion - Europe Ltd, Cambridgeshire, UK) and left to equilibrate in the fridge for 24 hours before long term storage at -20°C. Tissue samples on ice were homogenised using a rotor-stator blade in a class 2 laminar flow cell culture hood. The homogenate was centrifuged at 12,000 x *g* for 10 minutes to remove insoluble material leaving the supernatant that contains the RNA and protein.

Isolation of RNA from samples was performed using a 200µl of chloroform per ml of tri-reagent to create an aqueous interface (Sigma-Aldrich Company Ltd, Dorset, UK,) after shaking vigorously for 15 seconds and allowed to stand for 10 minutes. Centrifugation was carried out at 12,000 x *g* for 30 minutes at 4°C and the aqueous phase was removed. The inorganic phase was centrifuged again at 12,000 x g for 15 minutes and the aqueous phase removed. The RNA was precipitated using 500µl of isopropanol per ml of tri-reagent (Sigma-Aldrich Company Ltd, Dorset, UK,). The supernatant was kept at room temperature for 1 hour before centrifuging at 12,000 x *g* for 30 minutes at 4°C. The supernatant was removed using a speed vac to leave a resultant RNA pellet which was washed in 1ml of 70% ethanol per ml of tri-reagent before removing the supernatant and leaving to dry for 5 minutes. The pellet was resuspended in 20-50µl of nuclease free water and stored at -80ºC. RNA quantification was analysed by using a spectrophotometer (Spectronic Unicam, Cambridge, UK) and RNA quality was visually

verified following electrophoresis of a 1% agarose gel containing ethidium bromide. Visualisation was by means of a UV camera. Pictures taken were checked for bands, usually, a distinct 28s and a 18s band can be seen without smearing suggest less degradation of the RNA.

### 2.4.2. Reverse Transcription

Reverse transcription was carried out in 20µl final volume according to manufacturer's protocol (Applied Biosystems, Foster City, CA) where up to 1µg total RNA was combined with random hexamers (2.5µM final), 10x Taqman RT buffer (1x final concentration), 25mM Magnesium Chloride ($MgCl_2$, 5.5mM final), dNTP (500µM final), RNase inhibitor (0.4U/µl final concentration), 50U/µl Multiscribe Reverse Transcriptase (1.25U/µl final concentration) and made up to a total volume of 50µl with nuclease free water. Conditions used for the RT cycle were 25ºC for 10 minutes, 37ºC for 60 minutes, 48ºC for 30 minutes, 95ºC for 5 minutes and cooled to 4 ºC. The resultant cDNA was kept for long term storage at -20 ºC.

## *2.5. Conventional and Real-Time PCR*

### 2.5.1. Conventional PCR

Reactions were performed using 1X (final concentration) PCR buffer containing 2.5mmol/l dNTP, 2mmol/l $MgCl_2$, 0.5pmol/µl each of forward and reverse primers for gene of interest and 1U Taq polymerase made up to a final volume of 20µl using nuclease free water. Samples were amplified using cycle conditions specific to each gene primer. Gene expression was compared to the housekeeping gene 18s rRNA supplied by Ambion, Austin, TX. The cycle conditions for 18s were an initial denaturation cycle of 94ºC for 5

minutes, 30 cycles of 94ºC (30 seconds), 60ºC (30 seconds), 72ºC (30 seconds) followed by a final elongation step at 72ºC for 7 minutes. PCR products were visualised following electrophoresis of a 1% agarose gel containing ethidium bromide. Visualisation was by means of a UV camera.

### 2.5.2. Real-Time PCR

Real-time PCR was performed using an ABI 7700 system (Perkin-Elmer, Biosystems, Warrington, UK). PCR was performed in 25µl reactions on 96-well plates. Reactions contained 1x Taqman universal PCR master mix (Applied Biosystems, Warrington, Cheshire, UK) 10nM primers, 250nM Taqman probe and 100pg-50ng cDNA. Reactions were performed multiplex, using 18s rRNA as an internal reference. All target gene probes were labelled with fluorescent label FAM, and the 18s probe with fluorescent label VIC. The cycle conditions were 50ºC for 2 minutes, 95ºC for 10 minutes and then 44 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. Data was analysed according to the manufacturer's guidelines and were quantified using the Ct value which is the cycle number at which logarithmic plots cross a calculated threshold value. Ct value was used to determine dCt values (dCt = Ct of the target gene minus Ct of the internal reference, 18s). All of the oligonucleotide primers and probes except 11β-HSD1 and 11β-HSD2 were designed and made by Applied Biosystems of which the sequences are not known to the public domain. Primers for 11β-HSD1 and 11β-HSD2 were designed in house and made by Alta Bioscences, Birmingham UK.

## *2.6. Immunohistochemistry*

### 2.6.1. Tissue Section Preparation

Human eye, rabbit eye, conjunctiva, subcutaneous, omental and orbital adipose tissues fixed in 10% formalin (Sigma-Aldrich Company Ltd, Dorset, UK) and embedded in paraffin wax were obtained from the Academic Unit, Birmingham University, City Hospital. Human samples were acquired in accordance with the local ethical committee. Five microns thick sections of tissue were placed on microscope slides dried in a 60ºC oven before dewaxing in xylene (Genta Medical, York, UK), rehydrating in 100% ethanol, 95% ethanol and rinsed in water before the immunohistochemistry procedure.

### 2.6.2. Haematoxylin and Eosin Staining

Dewaxed and rehydrated sections were placed on a flat staining rack and incubated with Meyer's haematoxylin stain for 5 minutes then rinsed in water for several minutes. A few drops of 1% acid alcohol were added to the section and immediately removed and rinsed with water for several minutes. Eosin stain (Surgipath-Europe Ltd, Peterborough, UK) was then added to each section and incubated for about 2 minutes. The sections were then rinsed in water before processing through a series of washes consisting of 100% ethanol and xylene. The sections were then mounted on to coverslips using dibutyl-polystyrene-xylene (DPX) from BDH laboratory supplies, Poole, UK and stored at room temperature.

### 2.6.3. 11β-HSD1 Protocol

Tissues were fixed in 10% formalin before embedding in paraffin wax. Tissue cut to 3 microns thickness were placed on microscope slides coated with poly-lysine coated or X-tra adhesive (Surgipath, Peterborough, UK) The sections were then dried in a 60ºC oven before dewaxing in xylene (Genta Medical, York, UK), rehydrating in 100% ethanol, 95%

ethanol and rinsed in water ready for immunohistochemistry. Dewaxed and rehydrated sections were incubated with methanol hydrogen peroxidase (56.25:1 v/v) to block endogenous peroxidase activity. The sections were rinsed in water, washed with phosphate buffered saline (PBS) and incubated with 10% normal donkey serum (NDS) in PBS before incubation with primary antibody (1:100 or 1:50 dilution of polyclonal antibody raised against human 11β-HSD1) in 10% NDS/PBS for 90 minutes at room temperature. Negative control sections were incubated with 11β-HSD1 immunising peptide (Alta biosciences, Birmingham, UK) at 100 fold excess of antibody in 10% NDS in PBS. The washes and incubation of antibodies was performed using the Shandon sequenza (Thermo Scientific, Pittsburg, PA, US).

After incubation, slides were washed in PBS at least three times before incubation with secondary antibody (1:100 dilution of donkey anti-sheep IgG peroxidase conjugate from the Binding Site Limited, Birmingham, UK) for 30 minutes at room temperature. The sections were washed in water prior to chromagen visualisation with 3, 3'-Diaminobenzidine (DAB from Sigma-Aldrich Company Ltd, Dorset, UK) tablet dissolved in water (1mg/ml concentration). The DAB was left to develop for the desired time which was usually between 5 and 15 minutes before being washed in water and counterstained with Meyer's haematoxylin stain for 30 seconds and rinsed again. The sections were put through a series of washes consisting of 100% ethanol and xylene before mounting on coverslips using DPX.

### 2.6.4. 11β-HSD2 Protocol

Dewaxed and rehydrated sections were put through an antigen retrieval process by placing sections in 0.01M citrate buffer (pH 6) and heating to 95°C using a hot water bath. The sections were washed in water after cooling to room temperature. The slides were

incubated with methanol hydrogen peroxidase (56.25:1 v/v) to block endogenous peroxidase activity. The sections were rinsed in water, washed with phosphate buffered saline (PBS) and then incubated with primary antibody (1:100 dilution of polyclonal antibody raised against human 11β-HSD2) in 10% NDS/PBS for 60 minutes at room temperature. Negative control sections were incubated in 10% NDS in PBS without primary antibody. The washes and incubation of antibodies was performed using the Shandon sequenza. The sections were then rinsed in PBS and incubated with secondary antibody (1:100 dilution of donkey anti-sheep IgG peroxidase conjugate) for 30 minutes at room temperature. The sections were then washed in PBS before visualisation. Visualisation of the sections was achieved by using DAB for between 10 and 15 minutes before being washed in water and counterstained with Meyer's haematoxylin stain for 30 seconds. The sections were put through a series of washes consisting of 100% ethanol and xylene before mounting on coverslips using DPX.

### 2.6.5. CD68 Protocol

CD68 immunohistochemistry was also performed on inactive TAO, active TAO and control specimens. The procedure was as follows: dewaxed and rehydrated slides were blocked with endogenous biotin for 10 minutes using the Zymed avidin/biotin blocking kit (Invitrogen Ltd, Paisley, UK). All incubations and washes were performed using the Shandon sequenza (Thermo scientific, UK). Antigen retrieval was then performed using tris buffered saline (TBS) and EDTA at pH 7.8 and a microwave at full power (900W) for 15 minutes. The sections were then allowed to cool to room temperature before being rinsed in water and incubated with primary antibody (CD68 clone PG-M1 1:100 dilution; Dakocytomation, Carpinteria, California) in normal swine serum (NSS) in TBS buffer (pH 7.8) for 60 minutes. Negative control sections were incubated in TBS buffer without

primary antibody. The positive control sections used were human tonsil tissue. The sections were then washed in TBS buffer (pH 7.8) before incubating in Link reagent (Dakocytomation, Carpinteria, California) for 10 minutes. The sections were then rinsed again in TBS buffer (pH 7.8) and then incubated with streptavidin reagent (Dakocytomation, Carpinteria, California) for 10 minutes. Sections were then rinsed in TBS buffer (pH 7.8) before incubating with CheMate liquid DAB (DAB: substrate buffer, diluted 1:50; Sigma-Aldrich Company Ltd, Dorset, UK) for approximately 10 minutes. The sections were then washed in water before counterstaining with Meyer's haematoxylin (Sigma-Aldrich Company Ltd, Dorset, UK) for 45 seconds. The sections were washed again with water before processing through the last series of washes consisting of 100% ethanol and xylene. Sections were mounted on to coverslips using DPX (BDH laboratory supplies, Poole, UK) and viewed under a light microscope. Photographs of the immunoreactivity from all immunohistochemistries performed and control sections were captured using the Olympus BHR-RFCA microscope (London, UK) and a Microfire camera (Optronics).

## *2.7. Immunocytochemistry*

### 2.7.1. 11β-HSD1, 11β-HSD2 and Cytokeratin 3 Protocol

NZWAR and human corneal epithelial cell suspensions were seeded onto chamber slides coated with poly-D-lysine (ICN Biomedicals) and collagen IV (ATCC) and grown until 50% confluent. Cells were washed with PBS, and fixed in pre-cooled methanol by incubating for 10 min at -20°C followed by a further incubation in pre-cooled acetone at -20ºC for 1 min. Fixed corneal epithelial cells were washed in TBS [3 x 5 min]. Cells were blocked with 5% normal donkey serum in TBS for 30 minutes at 37ºC in a $CO_2$ incubator, and incubate with primary antibody (1:48 for NZWAR corneal epithelial cells and 1:24

for human corneal epithelial cells of mouse monoclonal anti-cytokeratin 3 (CK3) in 5% NDS (AE5 - ICN, Biomedicals); 1:24 for in-house generated human polyclonal anti-11β-HSD1 and 2) at 37ºC for 1 hour. Anti-CK3 is against specific 64K keratin found in corneal epithelial cells and limbal area of the cornea. It was used to distinguish corneal epithelial cultures form other cell types (Schermer et al., 1986b). Cells were washed in TBS [3 x 5 min] and incubated with secondary antibody (FITC labelled sheep anti-mouse (Binding site) for CK3 primary, donkey anti-sheep for 11β-HSD1 and 11β-HSD2 primary antibodies, all at 1:16 dilutions) at 37ºC for 30 minutes in a dark chamber. Finally, cells were washed in TBS [3 x 5 min], and counterstained with Prolong Gold anti-fade reagent with DAPI (Molecular Probes) which shows the cells nuclei as blue. The resulting slide was then covered in foil and stored in the refrigerator until visualization using appropriate filters for DAPI and FITC. Control sections consisted of the use of no antibody or antibody pre-treated with immunising peptide (1:1). Counterstained slides were viewed using an Olympus-BH2-RFC microscope (Olympus UK Ltd, Southall, Middx, UK) and a MicrofireTM - S99808 camera (Indigo Scientific, Baldock, Herts, UK).

### 2.7.2. CD90 Protocol

The fibroblasts (conjunctival and corneal) were seeded in chamber slides, with a two wells for positive and negative staining for each cell type. Once about 50% confluent, the cells were fixed by incubating cells with 100 µl ice cold methanol (Sigma, Poole, UK) at -20°C for 5 minutes. Following this, the methanol was removed and 100 µl ice cold acetone (Sigma, Poole, UK) was incubated with the cells for 1 minute. The acetone was evaporated and chamber slides stored at -20ºC until immunocytochemistry was performed.

At the time of staining, the chamber slide was allowed to equilibrate to room temperature. The fibroblasts were rehydrated with 100 µl PBS while shaking on an orbital shaker for 5 minutes. This was repeated three times. The PBS removed and 5% donkey serum (DS, GibcoTM, Invitrogen Corp., Paisley UK) in PBS was added for 10 minutes. The PBS was removed and 75-100 µl of CD90 (ASO2) primary antibody (Invitrogen, Paisley, UK) (1:100 dilution) was added to all the wells. The negative control well contained 5% DS in PBS. The chamber slides were kept at 4ºC for 24 h.

The supernatant was removed from all of the wells and washed in PBS three times. The secondary anti-mouse IgG1 antibody Alexa 633 anti-serum (75-100 µl) (Invitrogen, Paisley, UK), (1:100 dilution) was added to the wells of the chamber slide and incubated at room temperature on an orbital shaker. After incubation, the plastic wells of the chamber slide were removed to leave the cells on the glass slide. The cells were washed in PBS for 15 minutes. All excess PBS was removed and the fibroblasts were counterstained with Hoechst nuclear stain (Invitrogen, Paisley, UK) for 2 minutes at room temperature. The slide was washed again in PBS, for 15 minutes and after removing all excess PBS, a drop of 1, 4-diazabicyclo[2.2.2]octane (DABCO, Invitrogen, Paisley, UK) was added to each area of cells, and a coverslip was placed over the slide. The glass slide was stored at -20ºC in the dark until visualisation or visualised immediately on a confocal microscope.

### *2.8. Histomorphometric Analyses of Human Orbital Adipose Tissue (HOAT), SC and OM Tissue Explants*

Paired samples of SC and OM adipose tissue were obtained from 8 patients undergoing elective laparotomy. The control HOAT samples were taken from 14 patients undergoing lid ptosis or lid blepharoplasty, 15 quiescent HOAT (qHOAT) samples from patients undergoing procedures that include decompression surgery and 5 inflamed HOAT (iHOAT) samples. Sections were cut and stained with haematoxylin and Eosin (H&E) and photographed in triplicate using an Olympus- BH-2-RFC microscope and MicroFireTM - S99808 camera. The software package Image-ProR Plus (v4.0, Media Cybernetics) was used to analyse the adipocyte in the photograph. This was achieved by processing the images using the green channel of the software to enhance edge prominence. The software parameters were then optimized, in order to identify the visual edge of the cell membrane and generate an outline of the cell profiles for analysis. The scaling factor was calculated by phographing a micro-grid of known dimension. The descriptive variables that resulted were cell area, cell diameter, radius ratio (the ratio between the radius of the widest and the narrowest part of the cell), roundness, feret (dimensions are taken from a right angled rectangle drawn around the cell) and cell perimeter. The statistics performed consisted of comparisons between groups using a nested general linear model (GLM) with a p value $< 0.05$ being significant using the software package MINITAB©. The correlation function was used to calculate Pearson correlation coefficients.

## *2.9. Protein Assay*

### 2.9.1. Protein Extraction from Cells

Cells which had grown to confluency and adhered to wells within 24 or 48 well plates were used to extract protein from. Excess medium was removed from the cells and washed with PBS before extraction. Cells were collected using distilled water (50µl, 100µl, 150µl and 200µl for well surface areas of 0.75cm$^2$, 2 cm$^2$, 3.8 cm$^2$, 9.6 cm$^2$ respectively) and the rubber end of an insulin syringe or a suitable sized cell scraper (BD Becton Dickinson UK Ltd, Oxford, UK) before transferring to a micro-centrifuge tube and centrifuging briefly. The cell suspension was snap frozen in liquid nitrogen and thawed in a 37ºC water bath three times to disrupt the cells and release the protein. The cell suspension was then centrifuged again for 1 minute before the supernatant was transferred to a fresh tube. The protein was then used for a protein concentration determination assay before long-term storage at -80ºC.

### 2.9.2. Detergent compatible (DC) Protein Assay

Bovine serum albumin (BSA) standards (5µl each) obtained from Sigma-Aldrich Company Ltd (0mg/ml, 0.25mg/ml, 0.5mg/ml, 1.0mg/ml, 1.5mg/ml and 2mg/ml) were added in duplicate into a 96 well plate alongside of protein samples (5µl each). This range was used due to the nature of the protein found in the cells being of large concentration. Reagent A (25µl) (Bio-Rad Laboratories Ltd, Hertfordshire, UK) and Reagent B (200µl) (Bio-Rad Laboratories Ltd, Hertfordshire, UK) were added to each well using an automated pipette and incubated at room temperature for 10 minutes. The Bio-Rad protocol on the Victor3 1420 multilabel counter (Perkin Elmer, Wellesley, USA) was used to estimate the 680nm wavelength absorbance from protein standards and samples. A standard curve was then plotted to estimate the protein concentration of

protein samples (Figure 2-2). The equation derived from this standard curve was used to calculate the the protein concentration of the human and rabbit samples.

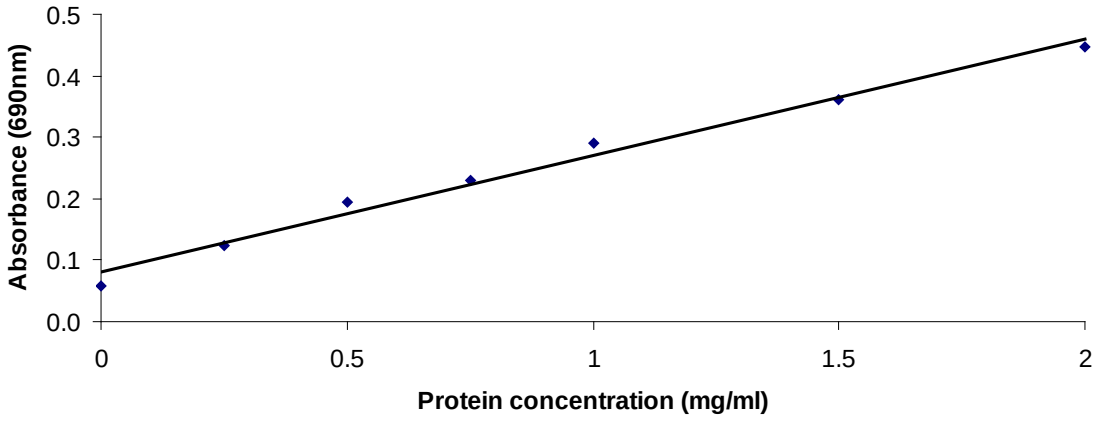



*Figure 2-2: Protein assay standard curve.*

*Graph displaying relationship between protein concentration and optical density at 690nm*

## *2.10. 11β-HSD Activity Assay*

### 2.10.1. Production of Tritiated Cortisone ($^3$HE)

Placental protein (1mg) was incubated with nicotinamide adenine dinucleotide (NAD) (10mM), $^3$HF (70Ci/mmol) and potassium phosphate buffer (0.1M) at 37ºC for 4 hours in a shaking waterbath. The organic phase was evaporated at 55ºC under air using a sample concentrator. The labelled steroids and unlabelled F and E standards used as markers were separated using a thin layer chromatography (TLC) plate (Fluka chemie GmbH, Buchs, Switzerland) and the mobile phase chloroform: ethanol (92:8). The plate was scanned using a BioScan system 200 imaging detector which showed conversion of F to E to be at least 95% and the appearance of two peaks (F preceding E).

The appropriate region containing labelled E was marked using the markers and ultraviolet light as a guide. The silica was then scraped and eluted in absolute ethanol before being vortexed, centrifuged and stored at 4ºC overnight. The resultant supernatant was transferred to a fresh borosilicate tube and evaporated at 55ºC under an airflow using a sample concentrator and re-dissolved in 500µl absolute ethanol. The synthesised 3H E was assessed for purity using BioScan imaging detector and diluted with ethanol as appropriate where 1µl gives approximately 2000 counts. $^{3}$HE was stored at -20ºC.

### 2.10.2. Basic 11β-HSD Activity Assay

Cells or tissue were placed in serum free medium for at least 2 hours before the assay. The assay mix for the assessment of oxo-reductase activity consisted of E (final concentration 100nM) and $^{3}$HE as a tracer in each well. The assay mix for the assessment of dehydrogenase activity consisted of F (final concentration 100nM) and $^{3}$HF as a tracer in each well. GE (5µM) was used in each well for the inhibition of 11β-HSD. All assays were either in duplicate or triplicate and performed for a period of 24 hours. Following the assay, steroid extraction from the assay medium was performed or the assay medium was stored at -20ºC.

## *2.11. Steroid Extraction and Separation by Thin Layer Chromatography*

A mobile phase of chloroform: ethanol (92: 8 v/v) was prepared in a tank and allowed to equilibrate for 1 hour before use. Steroid extraction of samples from 11β-HSD activity assay was carried out by the addition of 7ml dichloromethane per 1ml of sample. The samples were shaken on an orbital shaker for 15 minutes before being centrifuged at 2000 rpm for 5 minutes. The aqueous layer was removed by aspiration and the remaining organic layer containing the steroids was evaporated under air flow at 55ºC for between

40-45 minutes using a sample concentrator. The TLC plate was spotted with unlabelled cortisol and cortisone standards (1mg/ml solutions prepared in ethanol and stored at 4ºC) and was used as markers for the steroids extracted from samples. The samples were re-dissolved in dichloromethane (60µl) and applied to the TLC plate. The TLC plate was placed in the mobile phase for 90-120 minutes and once dry, they were scanned using the BioScan system 200 imaging detector. Figure 2-3 is an example of the TLC traces that are produced when plates are scanned by the Bioscan system 200 imaging detector.

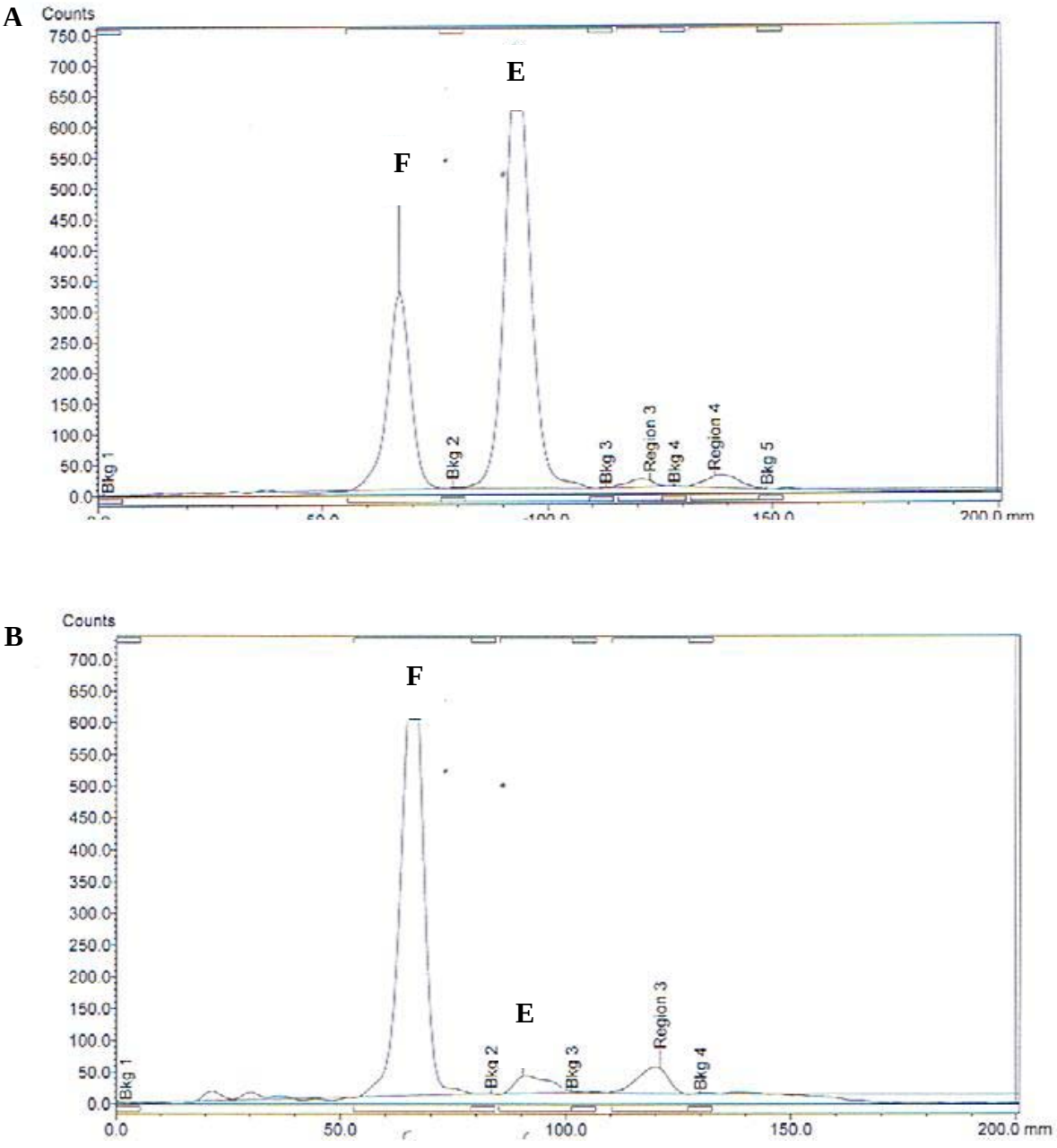



*Figure 2-3: TLC traces*

*Trace shows conversion of cortisone (E) to cortisol (F) (reductase activity), (A). The next trace shows conversion of cortisol to cortisone (dehydrogenase activity), (B).*

Substrate conversion rate (picomole/milligram of protein/hour or $pmol^{-1}$ $mg^{-1}$ $h^{-1}$) was calculated using the folloing equation:

$$\left(\left(\frac{\%\ Conversion}{100}\right) \times ([S] \times volume)\right) \div \left(\frac{protein}{time}\right) = subtrate\ conversion\ rate$$

The % conversion is derived from the TLC trace. The substrate concentration ([S]) in mmol/l is multiplied by the volume in millilitres (ml). The protein in mg is derived from the protein assay as described in chapter 2.9.2. The assay was performed over a 24 hour period.

### *2.12. Proliferation Assay*

Cells were seeded in a 96 well plate and using a commercially available colorimetric assay for determining the number of viable cells over a given time period (CellTiter 96® AQueousOne, Promega Madison, WI) according to the manufacturer's guidelines with appropriate control (no cells). The CellTiter 96® AQueousOne solution contains a tetrazolium compound and electron coupling reagents that can bind to living cells and form a formazan product. The absorbance reading of this fomazan product is directly proportional to the number of living cells, therefore dead or the existence of no cells will not produce absorbance readings. Cells were co-incubated with cortisone, cortisol and vehicle in addition with the reagent for 1 h at 37ºC in a humidified 5% CO2 atmosphere. The absorbance reading was measured at 490nm to represent the number of living cell using a 96 well- ELISA plate reader. Readings were performed in triplicate and the mean 'no-cell' control reading was subtracted from the other values.

### *2.13. DNA-Binding ELISA for Activated Glucocorticoid Receptor*

Glucocorticoid receptor (GR) DNA-binding activity was assessed using the TransAM GR transcription factor ELISA kit (Active Motif Europe, Rixensart, Belgium). Human corneal epithelial SV40 immortalized cells were incubated with dexamethasone or LPS with or without RU486 for 1 hour in duplicate. Nuclear extraction was performed using the manufacturer's instructions which consisted of washing cells with ice cold

PBS/Phosphatase inhibitor buffer (PIB). Cells were scraped into PBS/PIB before being transferred to a pre-cooled tube and centrifuged at 300 x *g* for 5 minutes. The supernatant was discarded and the pellet was re-suspended into ice cold hypotonic buffer. The cells were allowed to swell for 15 minutes before adding 10% Nonidet P-40 and vortexing for 10 seconds. Homogenate was centrifuged at 14,000 x *g* at 4°C for 30 seconds and supernatant discarded. The pellet was re-suspended in complete lysis buffer before shaking on ice for 30 minutes. The homogenate was centrifuged 14,000 x *g* for 10 minutes at 4°C before the supernatant (nuclear extract) was stored at -80°C.

The GR transcription assay was performed by incubating complete binding buffer and 20µg nuclear extract in a 96 well plate with gentle agitation for 1 hour at room temperature. HeLa nuclear extract (5µg) was used as a positive control and complete lysis buffer as a negative control. Wells were washed with 1 x wash buffer and then incubated with GR antibody (1:1000 dilution) for 1 hour at room temperature before a further wash and incubation with HRP-conjugated antibody (1:1000) at room temperature for an hour. Wells were washed again and developed with developing solution for between 15-20 minutes. Stop solution was applied and the absorbance was read at 450nm within 5 minutes on a Victor$^3$ 1420 multilabel counter (Perkin Elmer, Wellesley, USA).

### *2.14. Statistics*

Data sets were tested for normality using the Kolmogorov-Smirnov test. For data sets that were normally distributed, one-way ANOVA analysis was used and for data that was not normally distributed, non-parametric test such as Mann Whitney and Wilcoxon were used (SPSS for Windows Version 11.5.1, SPSS, Inc., Chicago, IL, USA, 2002 and Prism for

Windows Version 4.03c, GraphPad Software, Inc., San Diego, CA, USA). Significance equal to or above 0.05 is represented by one star (*). Significance equal to or above 0.01 is represented by two stars (**) while significance equal to or above 0.001 is represented by three stars (***).

## 3. Chapter 3

# Expression and Functional Role of 11beta Hydroxysteroid Dehydrogenase in the Rabbit and Human Eye

## *3.1. Introduction*

Regulation of immune processes in the eye is essential for maintenance of the the anatomical and biochemical structures needed for correct function. In light of this, understanding the pathogenesis of infectious diseases such as infectious keratitis and the immune processes that occur during immune privilege can assist in deciphering the role that 11β-HSD1 and cortisol may play in regulating inflammation.

### 3.1.1. Infective Keratitis

In infective keratitis, the corneal epithelial barrier is breached. This can be caused by many factors which include poor sanitation, dry eye, trichiasis (in-grown eyelashes), entropion (eyelids fold inwards) contact lens wear, topical corticosteroid and antiviral use and ocular trauma (Wong et al., 2003).

The actual prevalence of infectious keratitis is not known, however, the incidence of corneal ulceration per 100000 population per year is estimated to vary from 6.3 in Hong Kong and 11 in the USA with the incidence increasing six fold with contact lens wear (Lam et al., 2002, Erie et al., 1993, Thomas et al., 2007).

Clinical features of infective keratitis involve corneal ulceration where an erosion appears on the corneal surface, abscess formation, perforation and intraocular inflammation which can appear as corneal plaques and may include anterior chamber inflammation and conjunctival vascular reaction (Garcia-Valenzuela and Song, 2005). Without prompt treatment, the corneal scarring and endophthalmitis can lead to blindness.

### 3.1.2. Signalling Pathways for Pathogenic Bacteria

The main pathogenic recognition receptor (PRR) pathways are RIG-like (RLR) that are involved in antiviral defences (Creagh and O'Neill, 2006), NOD-like receptor (NLR) which recognise specific cell wall components of bacteria (Sirard et al., 2007) and toll-like receptor (TLR) pathways which recognise a whole host of pathogenic entities and will be discussed in further detail.

Bacterial pathogens are equipped to try and evade actions of the host's immune system and cause disease. One such way is survival of pathogenic bacteria in macrophages. This is achieved when the bacterium, mycobacterium tuberculosis, is able to produce a phosphatidylinositol analog to inhibit class III phosphoinositide 3-kinase which is involved in the maturation process of the phagosome for killing of the pathogen (Fratti et al., 2001). As a consequence, the bacterium is not phagocytosed and causes an enhancement in the inflammatory process which increases inflammatory cytokine production and leads to tissue damage as more immune cells are recruited to the infection site.

The cell wall of pathogenic bacteria can produce toxins which are pathogenic proteins that can damage or destroy the host cell when released. These factors remain in the bacterial cell wall and do not cause disease until the bacterium is ultimately destroyed and the bacterial toxins are released. This can result in septic shock as the toxins can mediate action of cytokines, complement factors and coagulation cascade factors (Wilson et al., 2002, Verhoef and Mattsson, 1995).

#### *3.1.2.1. Toll-Like Receptor (TLR) Signalling*

Gram-negative bacteria are first intercepted by the innate immune system via toll-like receptor (TLR) expression on the surface of the cornea. There are 13 TLRs in mammals, where 11 TLRs are expressed in humans to date of which 10 are expressed on the surface of the cornea (Figure 3-1) which include TLR1-10 (Jin et al., 2007, Li et al., 2007, Wu et al., 2008, Wu et al., 2007, Jin et al., 2008). TLR receptors are also expressed in other ocular tissues which include TLR4 in the bulbar conjunctiva, sclera and uvea, TLR1-5, 6 and 9 in the retina and TLR1-6 in the conjunctival epithelium (Li et al., 2007, Chang et al., 2006). Each receptor is assigned to recognise different microbial components for example TLR1 heterodimerises with TLR2 to recognise triacyl lipopeptide; TLR2 is important in the recognition of peptidoglycans from gram-positive bacteria; TLR3 recognises double stranded DNA from viruses; TLR5 recognises flagellin from flagellinated bacteria; TLR6 heterodimerises with TLR2 and can recognise di-acyl lipopeptides, TLR7 recognises synthetic compounds such as Imidazoquinoline; TLR8 recognises single stranded RNA; TLR9 interacts with CpG DNA from viruses and TLR10 has no known ligand to date (Takeda et al., 2003, Heil et al., 2004, Chang et al., 2006, Bell et al., 2007, Takeuchi et al., 2002).

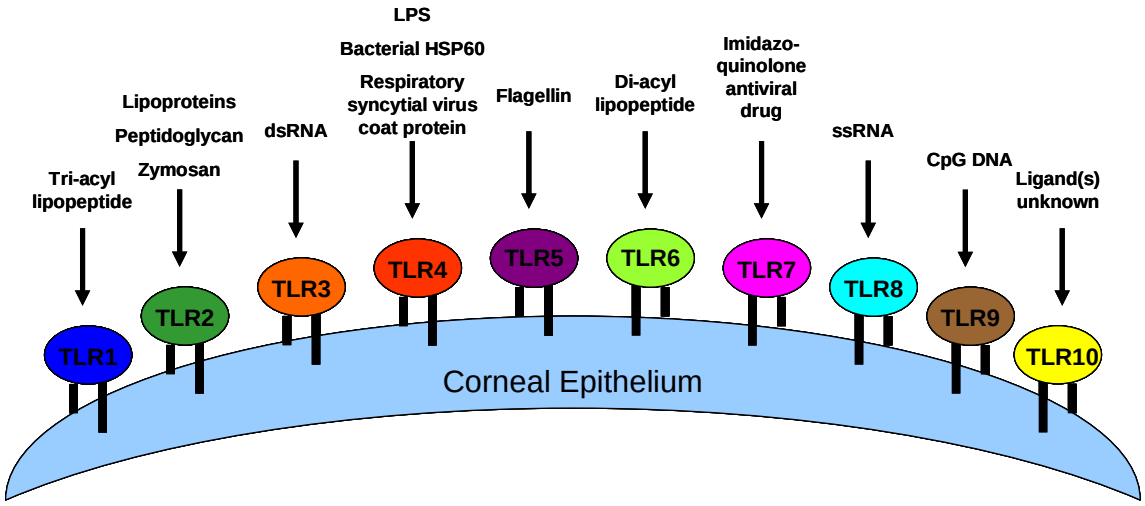



*Figure 3-1: Toll-like receptors expressed in the human corneal epithelium.*

*TLR1 recognises triacyl lipopeptide; TLR2 is important in the recognition of peptidoglycans from gram-positive bacteria; TLR3 recognises double stranded (ds) DNA from viruses; TLR5 recognises flagellin from flagellinated bacteria; TLR6 recognises di-acyl lipopeptides, TLR7 recognises synthetic compounds such as Imidazoquinoline; TLR8 recognises single stranded (ssRNA) RNA; TLR9 interacts with CpG DNA from viruses and TLR10 has no known ligand to date.*

TLR4 is important in the mechanism of interaction with *Pseudomonas aeruginosa* (PA), (Figure 3-2), since it recognises the lipopolysaccharide (LPS) component of gram negative bacteria. The initiation of the innate immune response to PA involves opsonisation of LPS by lipopolysaccharide binding protein (LBP) which is recognised by the opsonic receptor CD14 expressed on the surface of the cornea (Wright et al., 1990, Song et al., 2001). The LPS-LBP-CD14 complex is thought to activate TLR4. However, the exact mechanism by which this occurs is not known. Previous research has shown that expression of Myeloid differentiation 2 (MD2), a secreted glycoprotein which binds to the extracellular domain of TLR4, is thought to be important in the signalling pathway of TLR4 and binds to monophosphoryl lipid A of LPS (Shimazu et al., 1999, Fitzgerald and Golenbock, 2007, Nagai et al., 2002). However, others have shown that this appears not to be essential for activation of TLR4 (Ohnishi et al., 2007, Visintin et al., 2006).

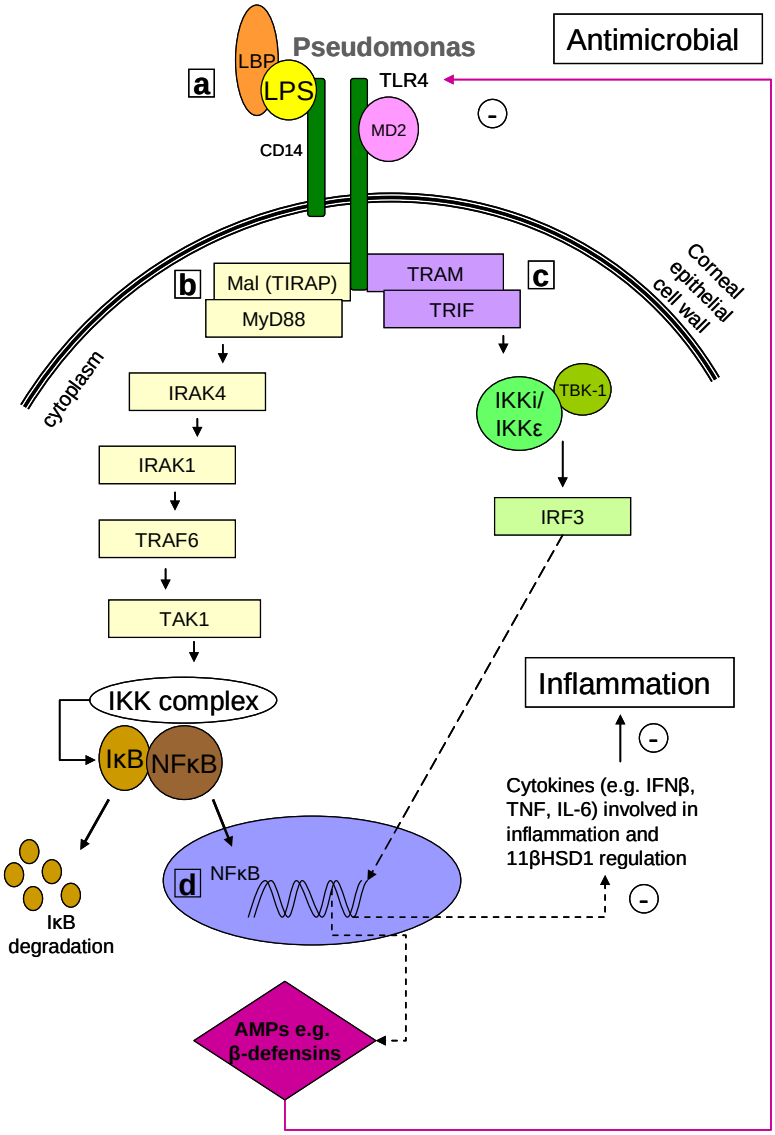



*Figure 3-2: LPS-TLR4 signalling pathway.*

*(a) LPS interaction with CD14 with the help of LBP allows signal transduction to TLR4 with the aid of MD2, however, how this occurs is still not fully understood. (b) Activation of the MyD88 dependent pathway leads to phosphorylation and activation of IRAK4 which in turn activates IRAK1 to activate MAPK, TAK1. TAK1 ultimately leads to activation and release of NFκB from IκB. (c) adaptor proteins TRAM and TRIF, associated with the intracellular domain of TLR4, activate IKKi/IKKε and TBK-1 kinases for the phosphorylation of IRF3 via the MyD88 independent pathway. This leads to immunoregulatory of gene expression. (d) translocation of activated NFκB to the nucleus influences transcription of immunoregulatory components.*

Following binding, the TLR4 can initiate two major intracellular signalling pathways: the MyD88-dependent and TRIF-dependent (MyD88-independent). The main role of the

TRIF-dependent pathway increases co-stimulatory molecules and type I interferons while in the MyD88-dependent pathway, there is increase in inflammatory cytokines such as IL-6, IL-12 and TNFα.

*3.1.2.1.1 MyD88-Dependent Pathway*

In the MyD88 (myeloid differentiation factor 88) -dependent early pathway, both adaptor proteins, MyD88 and Mal (MyD88 adaptor-like protein) contain a death domain able to interact with the death domain of IL-1R associated protein kinase 1 (IRAK1), a homologue of Drosophila kinase Pelle (Wesche et al., 1997). This kinase in turn hyperphosphorylates IRAK 1 whose activation leads to its interaction with another adaptor protein known as tumour necrosis factor associated factor 6 (TRAF6) (Medzhitov et al., 1998, Palsson-McDermott and O'Neill, 2004). This protein associated with adaptor protein TAK-1 binding protein 2 (TAB2), activates a Mitogen-Activated Protein Kinase (MAPK) known as Transforming growth factor-β-activated kinase 1 (TAK1) which is associated with TAB1. TAK1 activates other MAPK pathways which ultimately lead to the activation of nuclear factor kappa B (NFκB). In order for this to occur, there is the assembly of inhibitory-binding protein κB kinase (iKK) complex comprising iKKα, iKKβ and the scaffolding protein iKKγ (NEMO). This complex then phosphorylates the inhibitory-binding proteins κB (IκB) which causes its ubiquitination and degradation and the release of NFκB for its translocation to the nucleus for transcription of inflammatory mediators.

*3.1.2.1.2 MyD88-Independent Pathway (TRIF-Dependent Pathway)*

During the MyD88-independent late pathway, an adaptor protein TRAM, associated with the intracellular domain of TLR4, bridges TLR4 with TRIF to allow activation of IKKε and TANK-binding protein (TBK-1) kinases for the phosphorylation of interferon

regulatory factor 3 (IRF3) (Fitzgerald et al., 2003). IRF3 translocates to the nucleus and binds to interferon-sensitive response elements to allow transcription of inflammatory genes which include IFN-β (Fitzgerald et al., 2003).

### 3.1.3. Antimicrobial Products

The lack of immune cells in cornea during normal function causes the cornea to be reliant upon other components of the innate immune system present in tear film and surrounding tissues to evade attack from potential pathogens. Tears contain a range of potent antimicrobial products against Gram-negative and Gram-positive bacteria, fungi and some viruses. These antimicrobial products include iron metabolism protein such as hepcidin and lactoferrin; S100 family proteins such as psoriasin; elastase inhibitors such as elafin; granulysin produced by T-cells; chemokines such as CCL20 and CXCL9 and the classical cationic peptides such as cathelicidins and defensins which are the best characterised (Kolls et al., 2008).

#### 3.1.3.1. Cathelicidins

Cathelicidins are released from neutrophils, in addition to expression on the surface of the cornea and conjunctiva, and therefore are important the first line of defence against pathogens (Zhang et al., 2008). Cathelicidins contain a highly conserved N-terminal domain with a less conserved C-terminal antimicrobial region which varies among different species (Gordon et al., 2005). The only type of cathelicidin found in humans is hCAP18. The cathelicidin, hCAP18, is cleaved by serine proteases to produce the antimicrobial peptide LL37. In addition to microbial activity, LL37 acts as a chemoattractant for monocytes, T cells, neutrophils, and mast cells, stimulates mast cell histamine release, modulates dendritic cell differentiation, and stimulates IL-8 secretion

(Gordon et al., 2005). LL37 has several roles on the cornea and these may include regulating the ocular surface immune response, direct killing of invading pathogens and wound healing (Gordon et al., 2005).

#### 3.1.3.2. Defensins

Defensins are a group of small cationic peptides which is involved in the opsonisation of pathogens leading to their elimination. Defensins can be categorised into three main groups: α, β and θ according to their distribution and spacing between cysteine residues and disulfide bridges which typically consists of three disulfide bonds and six cysteine rich residues (Ganz and Lehrer, 1994, Chen et al., 2006a). Currently there have been six human alpha-defensins and at least twenty eight human beta-defensins found to be involved in innate immunity in humans (Chen et al., 2006a, Schutte et al., 2002). The alpha-defensins (α) were originally uncovered in human neutrophil cells (Selsted et al., 1985) which then lead to their identification in paneth cells (epithelial cells found in the intestinal gland) and other epithelia of the female reproductive tract (Chen et al., 2006a, Quayle et al., 1998). Theta (θ) defensins were first isolated from both leucocytes and bone marrow from rhesus monkeys, and although humans possess genes for these proteins, they are unable to be translated due to an unexpected codon that truncates the protein. However, the possibility has not been ruled out that some individuals may have the ability to produce these proteins (Tang et al., 1999, Yasin et al., 2004, Nguyen et al., 2003).

Human Beta-defensin 1 (HBD1) was first revealed and isolated from human plasma in the mid 1990s (Bensch et al., 1995) and has since been found to be constitutively expressed in many tissues. This was followed by the discovery of human beta defensin 2 in psoriatic scales (Harder et al., 1997) and other beta defensins 3-6 were exposed by the basic local

alignment search tool (BLAST) and lead to their detection in various tissues which included testis, heart, muscle, lung, kidney and epididymis (Chen et al., 2006a, Jia et al., 2001).

The defensins produced in the human eye include alpha and beta but theta expression has not been established. Beta-defensin 2 has increasingly become of interest in the corneal epithelia since its recent find in the late 1990 (McNamara et al., 1999, Haynes et al., 1999). LPS from PA has been implicated in the up-regulation of human beta defensin 2 in corneal epithelial cells (Figure 3-2) through the TLR4 signalling pathway (Maltseva et al., 2007) and its up-regulation may have importance in the re-epithelization of the corneal epithelium (McDermott et al., 2001) in addition to anti-bactericidal properties.

### 3.1.4. Gram-Negative Bacterial Keratitis and Contact Lens Wear

In the case of bacterial infections of the cornea (bacterial keratitis), contact wearers are the most susceptible where *Pseudomonas aeruginosa* (PA) (an aerobic, gram-negative bacterium), is responsible for at least 70% of cases (Liesegang, 1997). The condition is usually characterised by corneal infiltration of polymorphonuclear (PMN) cells leading to corneal ulceration and stromal destruction (Kessler et al., 1977). Diagnosis of this condition normally involves a comprehensive eye examination and diagnostic tests which include corneal cultures and biopsies.

Treatments for bacterial keratitis include antibiotics and antimicrobial agents (e.g. gentamicin, cefuroxime and ofloxacin) and corticosteroids for inflammation. In some cases, however, the intense inflammatory response mediated through chemokines and cytokines may not be responsive to these treatments with resultant corneal damage and loss of sight.

### 3.1.5. Structure of Gram-Negative Bacteria

The structure of a gram-negative bacterium (Figure 3-3) can consist of the cell wall which contains numerous peptidoglycans chains and contain lipopolysaccharide (LPS) chains in the outer membrane of the cell wall. Not all gram-negative have a cell wall but those that do include *Pseudomonas* and *Salmonella*. The cell membrane and the cell wall contain endotoxins and exotoxins that can damage host cells. Other features of the bacterium include the flagella, plasmid, the cytoplasm which contains the nucleic material and ribosomes.

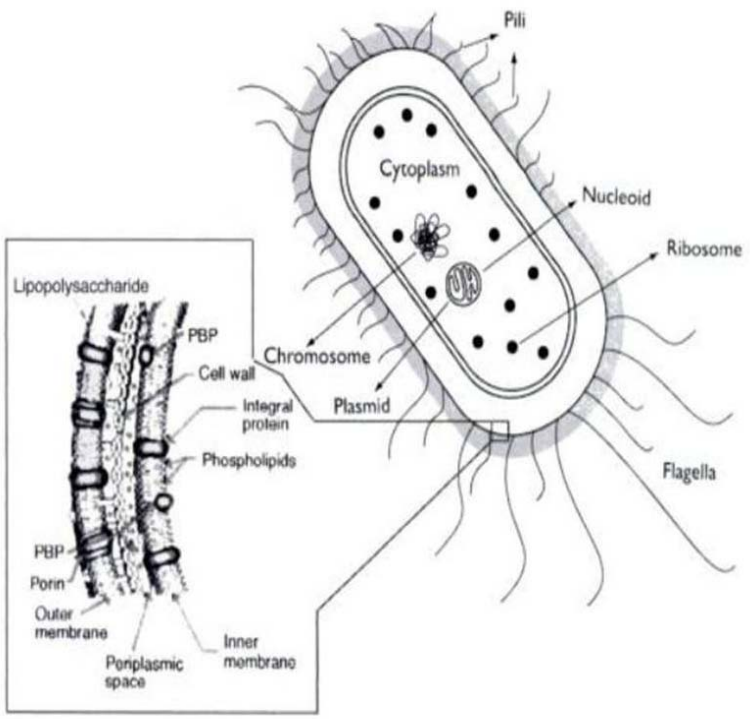



*Figure 3-3: Structure of gram-negative bacterium.*

*Structure shows the cell wall, which is associated with the outer membrane that contains LPS. The other features include flagella, plasmid, nucleic material and ribosomes. (LPS, lipopolysaccharide; PBP, penicillin-binding proteins). Picture taken frompaper by Mascaretti and collegues (Mascaretti, 2003).*

### 3.1.6. The Potential Role of 11β-HSD1 in the Regulation of Bacterial Infectious Keratitis

The toll-like receptor 4 (TLR4) signalling pathway involves interaction with foreign matter such as LPS which may lead to the up-regulation of immunoregulatory components such as NFκB. Many compounds can interact in this pathway to alter the outcome of events following pathogenic attack. Glucocorticoids such as dexamethasone have already been found to inhibit NFκB (Mukaida et al., 1994, Scheinman et al., 1995a), a transcription factor involved in inducing transcription of many inflammatory cytokines. Its inhibition is thought to be due to the glucocorticoids ability to increase the expression of inhibitory molecule IκBα which inhibits NFκB activation (Scheinman et al., 1995a). The LPS component of a pathogenic bacterium such as *pseudomonas* interacting with TLR4 on the surface of the cornea is already known to modulate the production of anti-bacterial products (MacRedmond et al., 2005, Caterina Romano et al., 2009) and could possibly lead to the induction of 11β-HSD1 to initiate the local regeneration of cortisol by 11β-HSD1 for anti-bacterial properties (Figure 3-4); however, this has not been proven as yet. Pro-inflammatory cytokines such as IL-6 and TNFα may also induce 11β-HSD1 to generate more cortisol. Both LPS and cytokines acting together may induce 11β-HSD1 to activate cortisol and in doing so, may contribute to the regulation of gene transcription of defensins such as human beta-defensin 2 (HBD-2) and repression of pro-inflammatory cytokines while increasing anti-inflammatory cytokine synthesis.

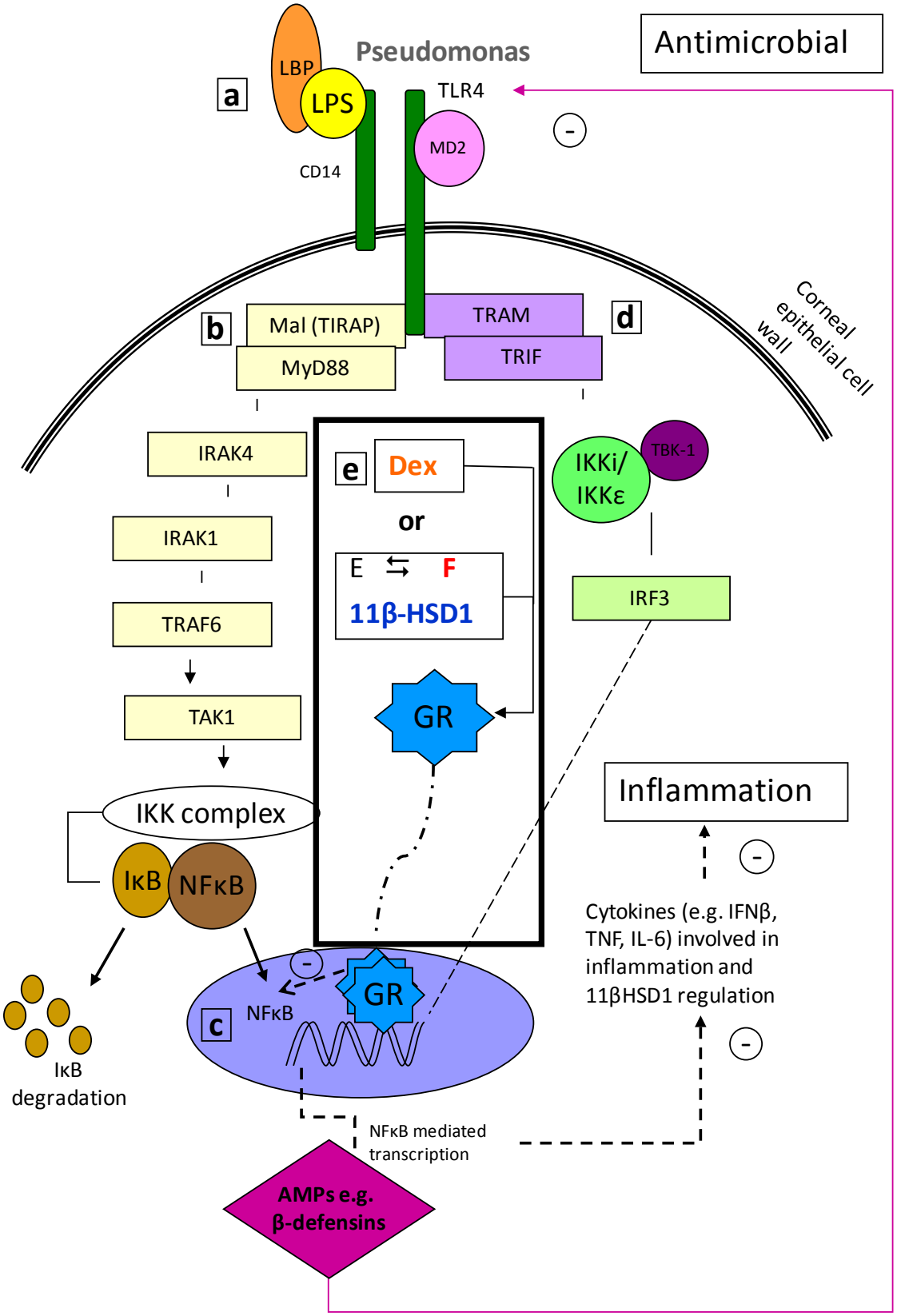



*Figure 3-4: Diagram showing the effect LPS may have on 11β-HSD1.*

*(a) LPS interaction with CD14 with the help of LBP allows signal transduction to TLR4 with the aid of MD2, however, how this occurs is still not fully understood. This leads to one of two main pathways. One is (b) activation of the MyD88-dependent pathway involving the adaptor proteins MyD88 and Mal activating a series of kinases which inturn activate the IKK complex. The IKK complex releases IκB for degradation and activates NFκB leading to (c) cytokine and antimicrobial peptide transcription. The other pathway, (d) MyD88-independent pathway or TRIF dependent pathway involves the adaptor proteins TRAM and TRIF to activate IKKi/IKKε and TBK-1 kinases for the phosphorylation of IRF3. This leads to immunoregulatory gene expression. We hypothesise that another pathway (e) involving LPS induction of 11β-HSD1 maybe involved in local generation of cortisol which binds to GR and the activated GR may regulate transcription of cytokines and other transcription factors such as NFκβ.*

Human studies have shown cortisol is present in aqueous humour (Stokes et al., 2003, Rauz et al., 2003a), but it is not determined whether this cortisol is ultrafiltrated from the blood stream or generated locally by 11β-HSD1. It is hypothesised that cortisol is generated locally and is one of the intraocular protection components.

### 3.1.7. The Potential Role of 11β-HSD1 in Immune Privilege

Immune privilege, a state of immune protection without intense inflammation is required to sustain current functioning of the eye components such as the ciliary body and the cornea. It is well publicised that cortisol and other immunomodulatory factors such as TGFβ contribute to immune privilege (Knisely et al., 1994, D'Orazio and Niederkorn, 1998). As previously mentioned (chapter 1.8.2), cortisol is found in aqueous humour in the anterior chamber of the eye. This cortsol produced may be under the control of 11β-HSD1 in the cilliary NPE.

### 3.1.8. Aim

The aims of this chapter will be:

- To evaluate the cortisol generating pathways in rabbit cells and tissues
- To evaluate the cortisol generating pathways in human cells and tissues

### *3.2. Methodology*

NZWAR liver, kidney, eye, and conjunctiva embedded in paraffin wax blocks were processed as described in chapter 2.6.1. Haematoxylin and eosin, 11β-HSD1 and 11β-HSD2 staining were performed as described in chapter 2.6.2, 2.6.3 and 2.6.4 respectively. Human corneoscleral tissue was processed as described in chapter 2.6.1. Dewaxed and rehydrated sections underwent immunohistochemistry for human 11β-HSD1 and 11β-HSD2 as described in chapter 2.6.3 and 2.6.4. Immunocytochemistry for 11β-HSD1 and 2 was performed on primary human and NZWAR corneal epithelial cells as described in chapter 2.7.1. Immunocytochemistry for CD90 was also performed on primary human corneal and conjunctival fibroblasts as described in chapter 2.7.2.

Primary NZWAR corneal epithelial cells, corneal fibroblasts, conjunctival fibroblasts, and primary human corneal epithelial cells, corneal fibroblasts, conjunctival fibroblasts and the immortalized corneal epithelial cell-line were cultured as described in chapter 2.3. Total RNA extraction and reverse transcription was carried as on human ocular cells described in chapter 2.4.1. Reverse transcription and PCR was carried out as described in chapter 2.4.2 and 2.5.1. PCR primers used for reaction are detailed in Table 3-1. Table 3-1 also shows the amplification stages for each gene. The primers for TLR4 produced four bands due to TLR4 consisting of many variants, the primers were designed by R and D to detect 4 of these variants which are specific to TLR4.

The positive cDNA control for 18s, GR, 11β-HSD1 and H6PDH was primary human liver, for MR and 11β-HSD2 was primary human placenta, for IL-6, TNFα, IL-1β, CD14 and TLR4 was LPS treated macrophages and the negative control was nuclease free water. All primers except for TLR-4 (R and D systems, Abingdon, UK) were obtained from Alta Biosciences, Birmingham, UK.

*Table 3-1: Primer sequences, annealing temperature, cycle number and product size for target genes*

| Gene | Primer sequence | Annealing temperature (°C) | PCR cycle | Product size (bp) |
|---|---|---|---|---|
| **11β-HSD1** | S: 5′-ACCAGAGATGCTCCAAGGAA-3′<br>A: 5′-ATGCTTCCATTGCTCTGCTT-3′ | 60 | 30 | 411 |
| **11β-HSD2** | S: 5′-TGGAGGTGAATTTCTTTGGC-3′<br>A: 5′-GGATTCTTTAGGCCAGGGTC-3′ | 55 | 30 | 774 |
| **H6PD** | S: 5′-AGAAGCGAGACAGCTTCCAC-3′<br>A: 5′-GCTGCTGGGAAAAGAACAAC-3′ | 60 | 30 | 603 |
| **GRα** | S: 5′-TCGACCAGTGTTCCAGAGAAC-3′<br>A: 5′-TTTCGGAACCAACGGGAATTG-3′ | 60 | 30 | 693 |
| **MR** | S: 5′-AACTTGCCTCTTGAGGACCAA-3′<br>A: 5′-AGAATTCCAGCAGGTCGCTC-3′ | 60 | 30 | 450 |
| **IL-6** | S: 5′-TCAATGAGGAGACTTGCCTG-3′<br>A: 5′-GATGAGTTGTCATGTCCTGC-3′ | 60 | 30 | 261 |
| **TNFα** | S: 5′-AGGCGCTCCCCAAGAAGACA-3′<br>A: 5′-TCCTTGGCAAAACTGCACCT-3′ | 60 | 30 | 556 |
| **IL-1β** | S: 5′-TTCTCTTCAGCCAATCTTCAT-3′<br>A: 5′-TAGAGTGGGCTTATCATCTTTC-3′ | 60 | 30 | 630 |
| **CD14** | S: 5′ CACAGGACTTGCACTTTCCA 3′<br>A: 5′ CTGTTGCAGCTGAGATCGAG 3′ | 51 | 30 | 507 |
| **TLR4** | Manufacturer's specification (R and D systems) | 55 | 30 | 529, 409, 362, 242 |
| **HBD-2** | S: 5′ TTTGGTGGTATAGGCGATCC 3′<br>A: 5′ GAGGGAGCCCTTTCTGAATC 3′ | 58 | 30 | 182 |

Human and NZWAR corneal epithelial cells, fibroblasts and corneal fibroblasts were assayed as described in chapter 2.10.2. Following the assay, steroid extraction and thin layer chromatography (TLC) were performed as described in section 2.11. Reductase

activity was measured by calculating the rate at which the cortisol is generated and in the case of dehydrogenase activity, the rate at which cortisone is produced. For studying the effects of cytokines and LPS on human ocular cells, the primary cultured cells were incubated with either tumour necrosis factor alpha (TNFα, 10ng/ml) or Interleukin-6 (IL-6, 10ngml) in serum-free media for 24 hours or 1μg/ml LPS for 48 hours. These doses were considered as they have previously been documented to elicit a response in other protein expression in corneal epithelial cells (Li et al., 2001, Burbach et al., 2001). The enzyme assay was then performed as described in 2.10.2 with addition of the following treatments in separate wells: TNFα (10ng/ml), IL-6 (10ng/ml), LPS (1μg/ml) and GE (5μM). All assays were performed in duplicate. Reductase activity was measured as mentioned in chapter 2.11.

Proliferation assays performed on primary NZWAR corneal epithelial cells are described as in chapter 2.12. Cells were assayed at 0h, 48h and 96h after co-incubation with 100nM cortisone or cortisol. The DNA binding ELISA was performed on the immortilized human corneal epithelial cell-line as described in chapter 2.13.

## *3.3. Results*

### 3.3.1. Rabbit Studies

#### 3.3.1.1. Immunohistochemistry of the NZWAR Corneal Epithelium and Conjunctival Epithelium

Initial experiments were carried out to confirm that the human specific 11β-HSD1 and 11β-HSD2 antibodies were able to identify respective rabbit proteins. The control tissues used for 11β-HSD1 and 11β-HSD2 immunoreactivity were the rabbit liver and the kidney respectively (Figure 3-5). These controls identified 11β-HSD1 staining around the central vein in the rabbit liver and 11β-HSD2 around the collecting ducts of the kidney cortex (Kyossev et al., 1996) in a similar pattern to that seen in humans and thus confirmed reactivity of the antibodies. When using the blast program (National Center for Biotechnology Information website), the human peptide (19-33 ammino acids) used to raise the 11β-HSD1 antibody in sheep was shown to have 100% homology with the rabbit.

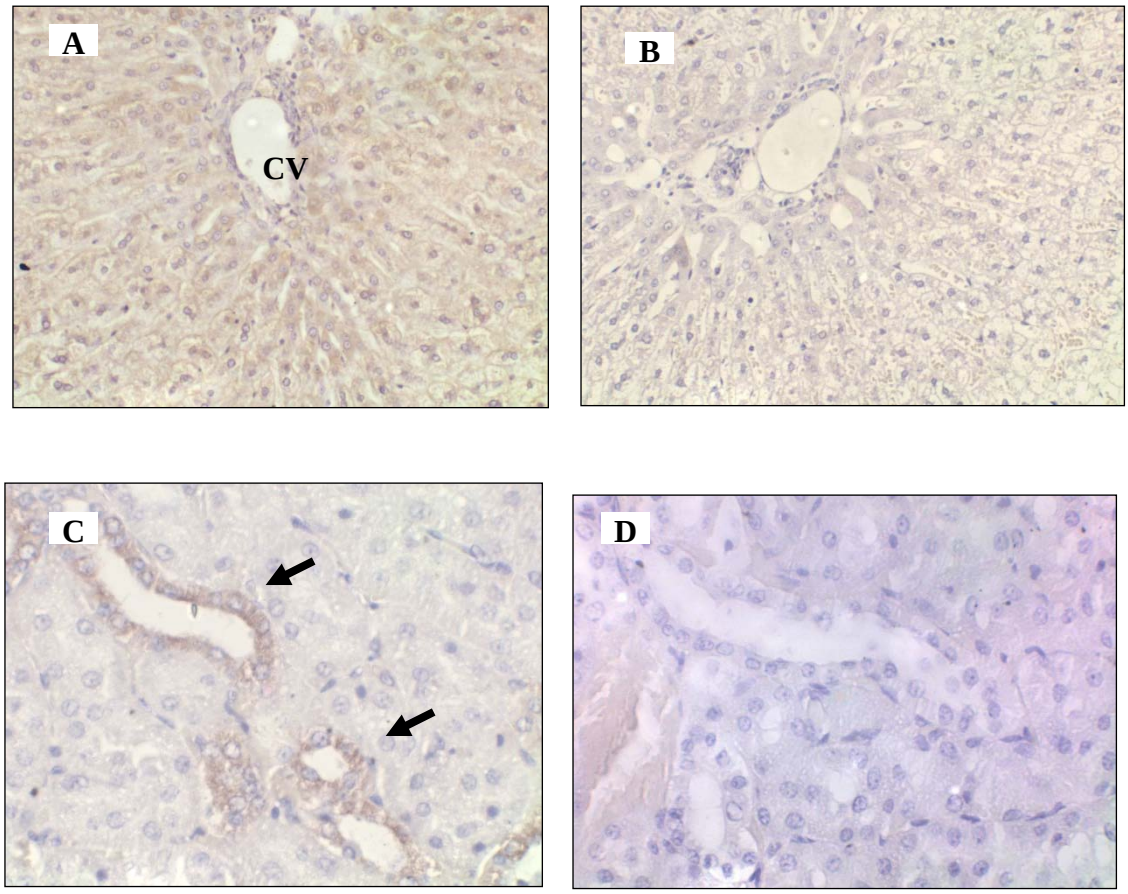



*Figure 3-5: 11β-HSD1 and 11β-HSD2 immunohistochemistry in human control tissues.*

*11β-HSD1 immunoreactivity in NZWAR liver, localised around the central vein (A), 11β-HSD1 preabsorbed with immunising peptide liver control section (B), 11β-HSD2 immunoreactivity with the cuboidal epithelium of the collecting tubules in the NZWAR cortex of the kidney indicated by arrows (C), no primary antibody control kidney section (D). All at x 40 magnification (CV, central vein).*

Once species reactivity had been confirmed, staining experiments were then carried out on the NZWAR tissues. Immunoreactivity was seen for 11β-HSD1 but not 11β-HSD2 in the NZWAR corneal epithelium and endothelium (Figure 3-6).

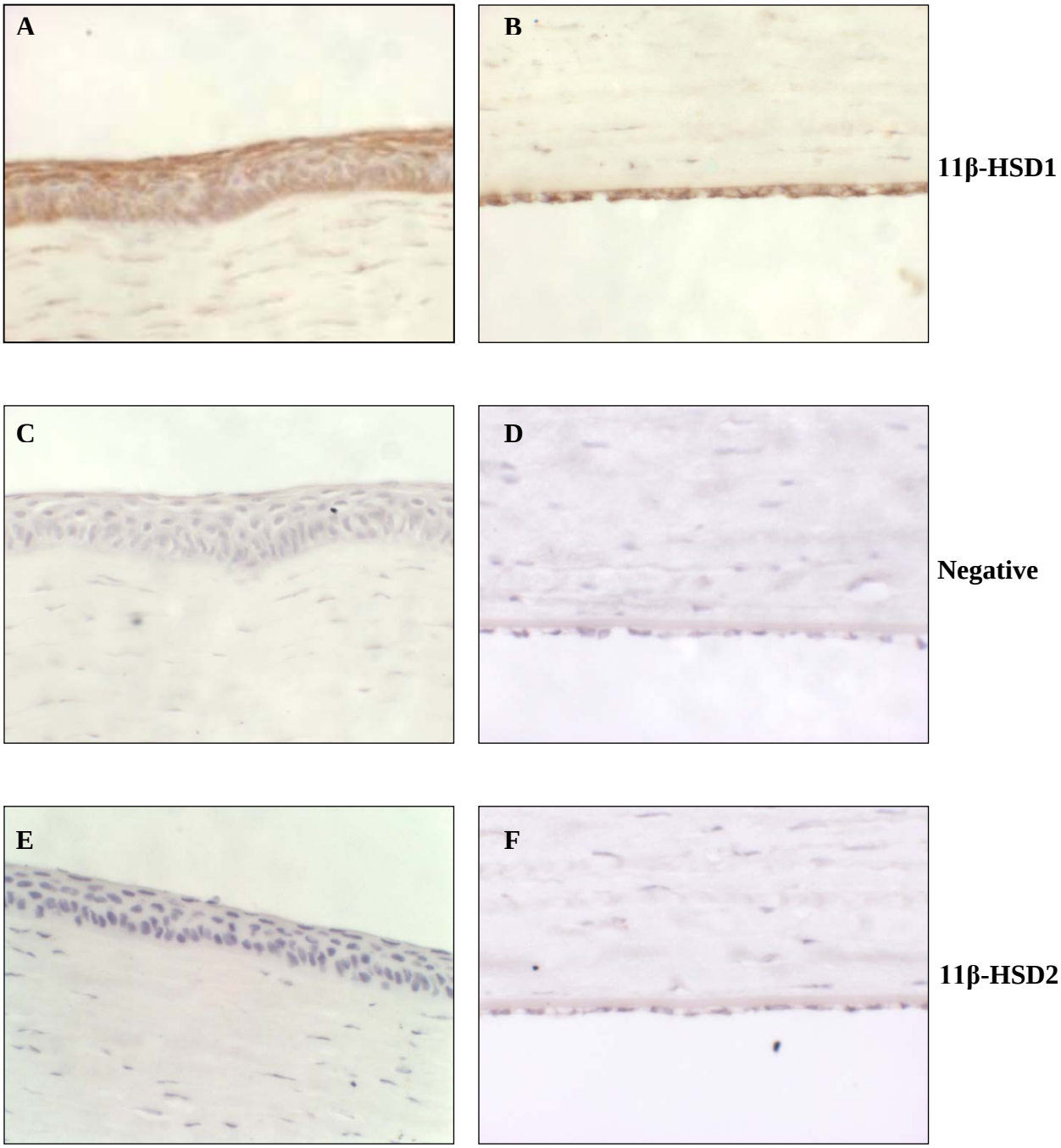



*Figure 3-6: Immunohistochemistry of the NZWAR cornea with 11β-HSD1 and 11β-HSD2.*

*Immunostaining for 11β-HSD1 in the NZWAR corneal epithelium (A) and NZWAR corneal endothelium (B). No primary was used as a negative control for the NZWAR corneal epithelium (C) and NZWAR corneal endothelium (D). No immunostaining for 11β-HSD2 was seen in the NZWAR corneal epithelium (E) or corneal endothelium (F). All at x 40 magnification*

11β-HSD1 but not 11β-HSD2 was also localised to the NZWAR conjunctival epithelium around the goblet cells (Figure 3-7).

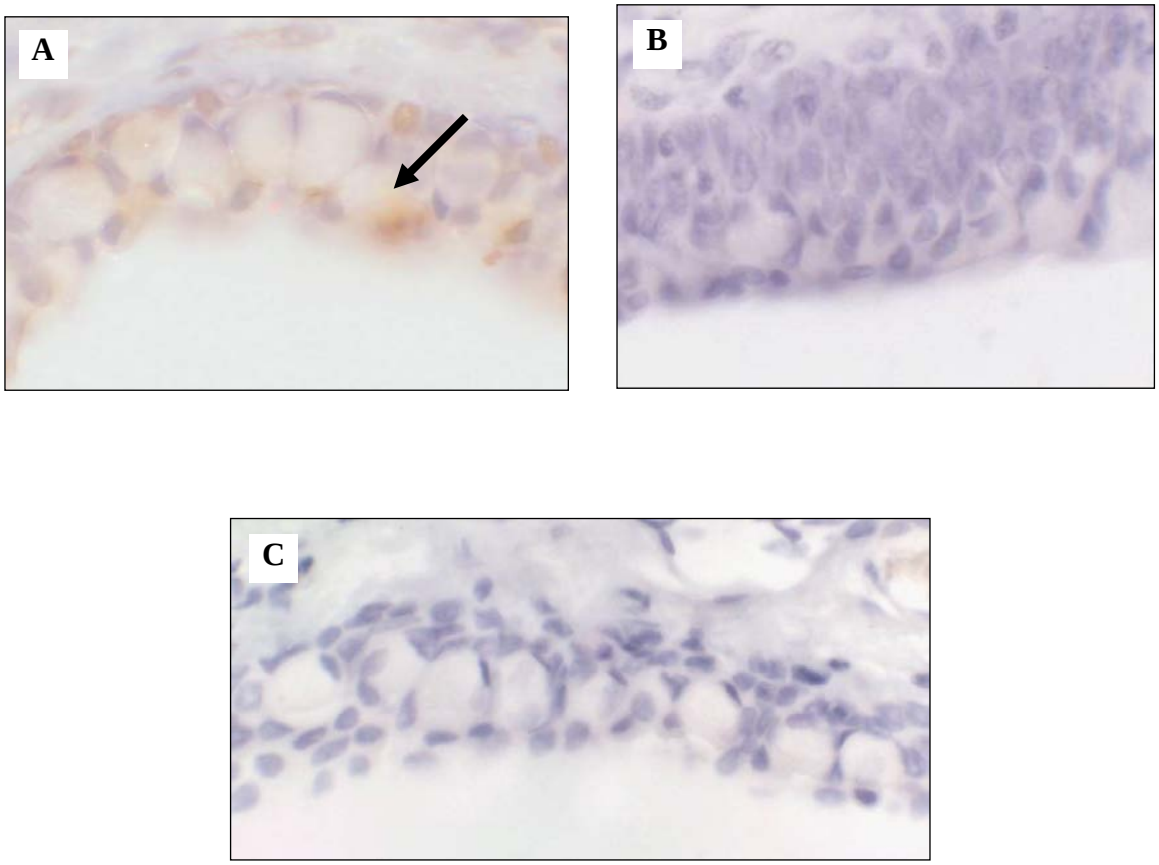



*Figure 3-7: Immunohistochemistry of the NZWAR conjunctival epithelium with 11β-HSD1 and 11β-HSD2.*

*11β-HSD1 immunoreactivity was seen in the NZWAR conjunctival epithelium around the arrowed goblet cells (A) No primary control section (B), 11β-HSD2 immunoreactivity was not localised to the NZWAR conjunctival epithelium (C). All at x 40 magnification.*

### 3.3.1.2. Primary Culture Analysis of NZWAR Corneal Epithelial Cells, Conjunctival Cells and Keratocytes

A confluent monolayer of corneal epithelial cells with distinctive hexahedral architecture was seen between 7 and 14 days of culture (Figure 3-8).

After approximately 14 days in culture, outgrowth of stellate shaped keratocytes could be observed from the central corneal explants (Figure 3-8). NZWAR conjunctival fibroblasts demonstrated characteristic, elongated morphology (Figure 3-8).

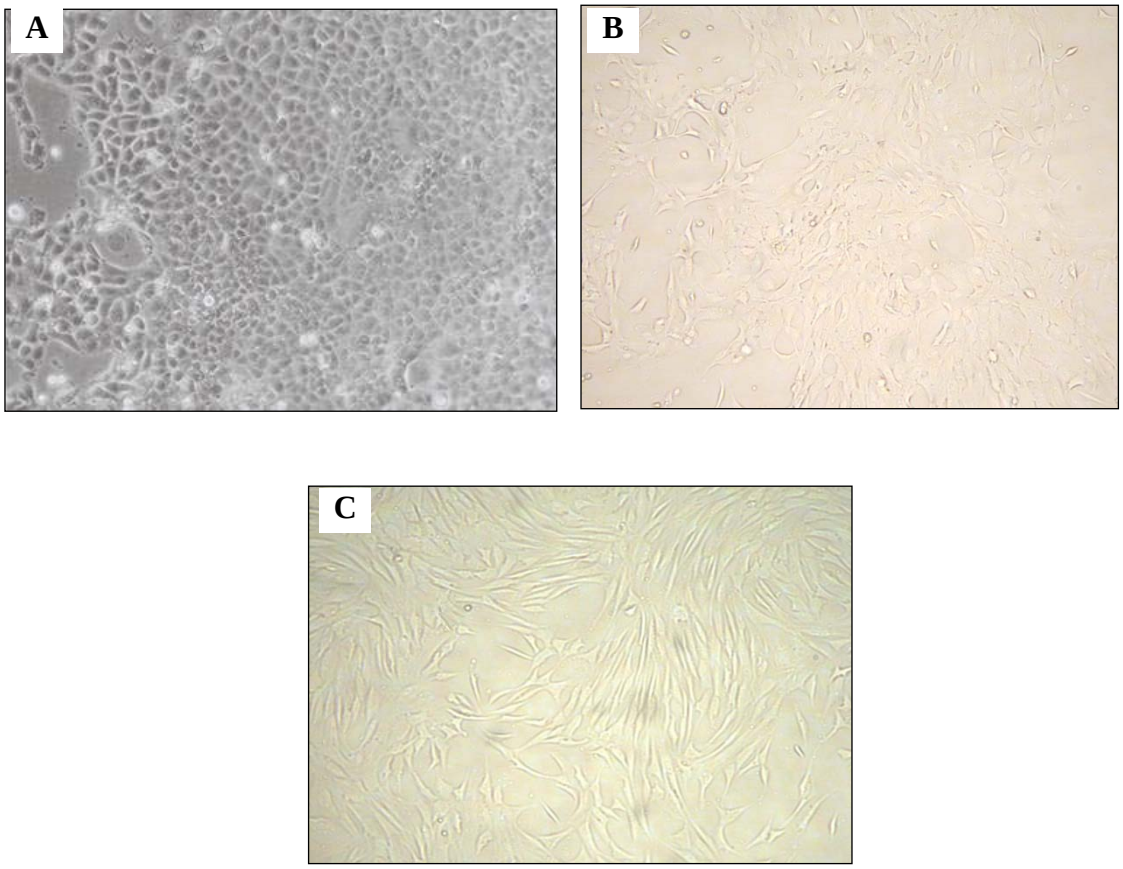



*Figure 3-8: Photographs of primary NZWAR ocular cells.*

*Established NZWAR corneal epithelial cells after approximately 7-14 days in cultre (A), NZWAR keratocytes after approximately 14 days (B) and NZWAR conjunctival fibroblasts after approximately 3 weeks (C).*

### 3.3.1.3. Immunocytochemistry Analysis of NZWAR Corneal Epithelial Cells

Corneal epithelial cell phenotype was confirmed by cytokeratin-3 immunofluorescence staining (Figure 3-9). These cells also expressed 11β-HSD1 (Figure 3-9) which was absent when 11β-HSD1 antibody was preincubated with immunising peptide (Figure 3-9).

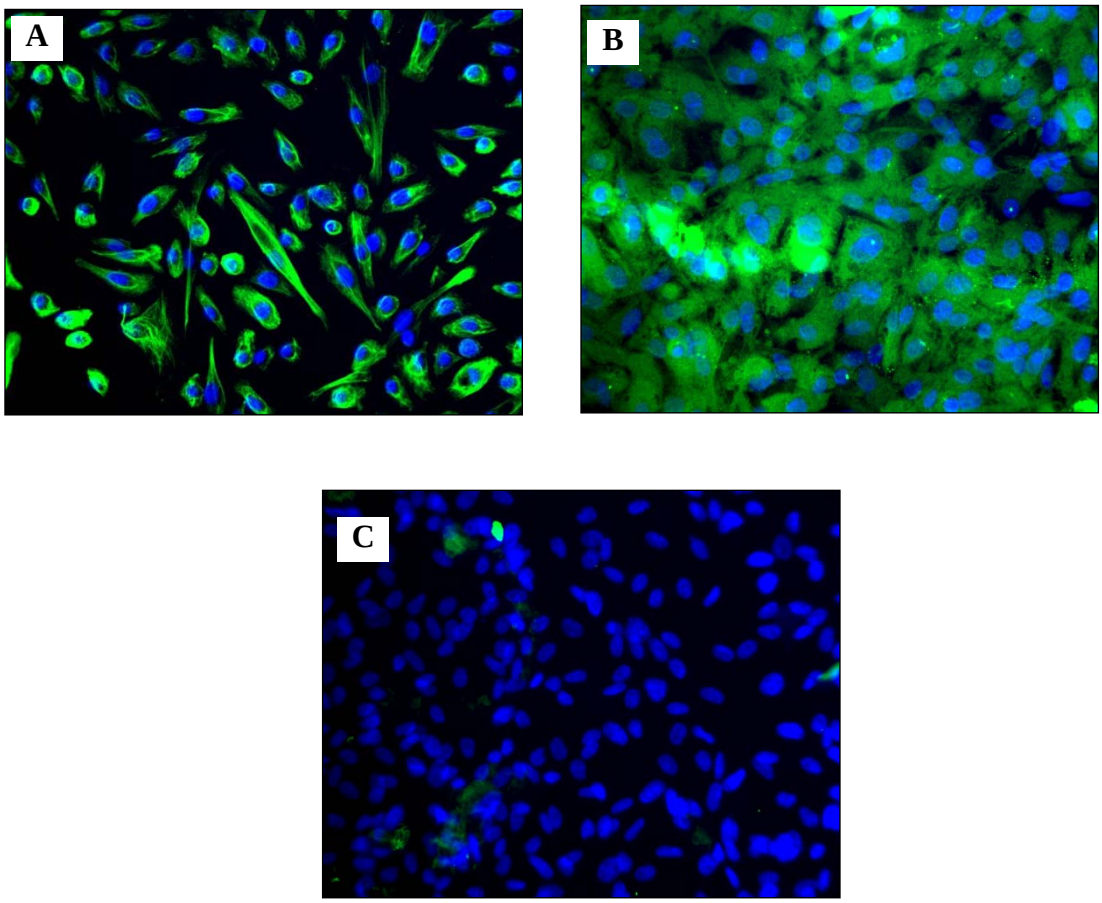



*Figure 3-9: Immunocytochemistry of primary NZWAR corneal epithelial cells with 11β-HSD1 and cytokeratin 3.*

*Corneal epithelial cell-type was confirmed in primary cultures of NZWAR corneal epithelial cells using anti-cytokeratin (A). 11β-HSD1 was immunoreactivity was established in NZWAR corneal epithelial cells (B) when compared to the 11β-HSD1 preabsorbed with immunising peptide control (C). The blue staining represents the nucleus of the cells. All pictomicrographs are taken at x 20 magnification.*

### 3.3.1.4. 11β-HSD1 Activity Assays on NZWAR Primary Cells

Specific enzyme assay performed on primary NZWAR corneal epithelial cells revealed a predominant 11β-HSD1 reductase activity ($p<0.05$) compared to dehydrogenase (Figure 3-10). The 11β-HSD1 reductase activity from NZWAR corneal epithelial cells was significantly reduced ($p<0.01$) by the non-specific 11β-HSD inhibitor, glycyrrhetinic acid (GE), however, GE did not reduce dehydrogenase activity significantly further (Figure 3-10). Conjunctival fibroblasts and corneal keratocytes showed zero conversion of cortisone to cortisol (reductase activity) or cortisol to cortisone (dehydrogenase activity).

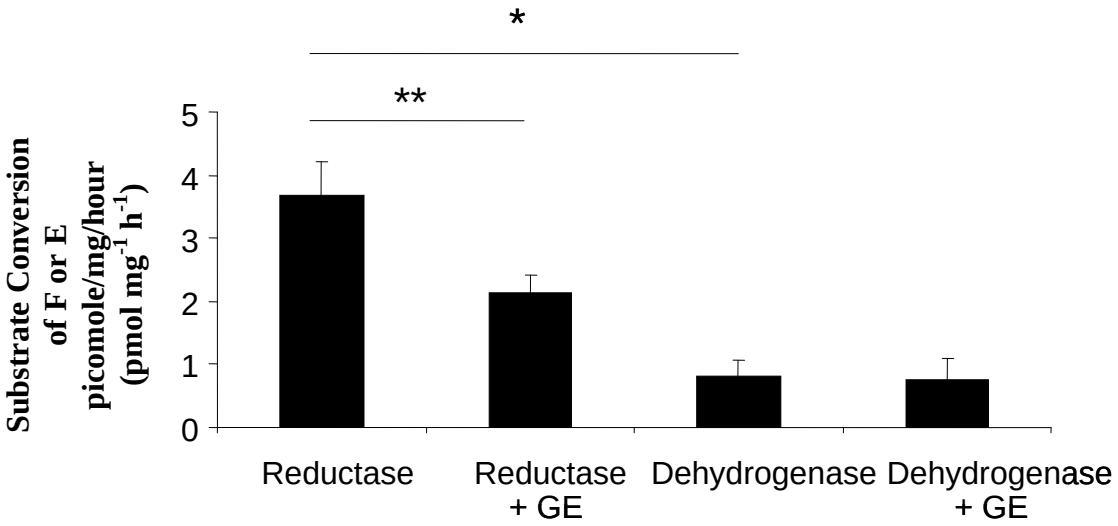



*Figure 3-10: Specific enzyme assays for 11β-HSD1 in NZWAR primary corneal epithelial cells.*

*11β-HSD1 reductase activity (3.7±0.5 pmol $mg^{-1}$ $h^{-1}$) was significantly higher ($p<0.01$) compared to dehydrogenase activity (0.82±0.2 pmol $mg^{-1}$ $h^{-1}$) in NZWAR corneal epithelial cells. This reductase activity was reduced sigficantly($p<0.05$) by GE (2.1±0.3). There was no significant change in dehydrogenase actvity by GE. Data are expressed as means and error bars as standard errors of the mean (SEM).*

### 3.3.1.5. NZWAR Corneal Epithelial Cell Proliferation

NZWAR corneal epithelial cells were shown to proliferate from baseline in medium without addition of steroids (Figure 3-11). However, there appeared to be at least a 50% reduction in cell divisions over a 96 hour period ($p<0.01$) when cells were co-incubated with either cortisone or cortisol.

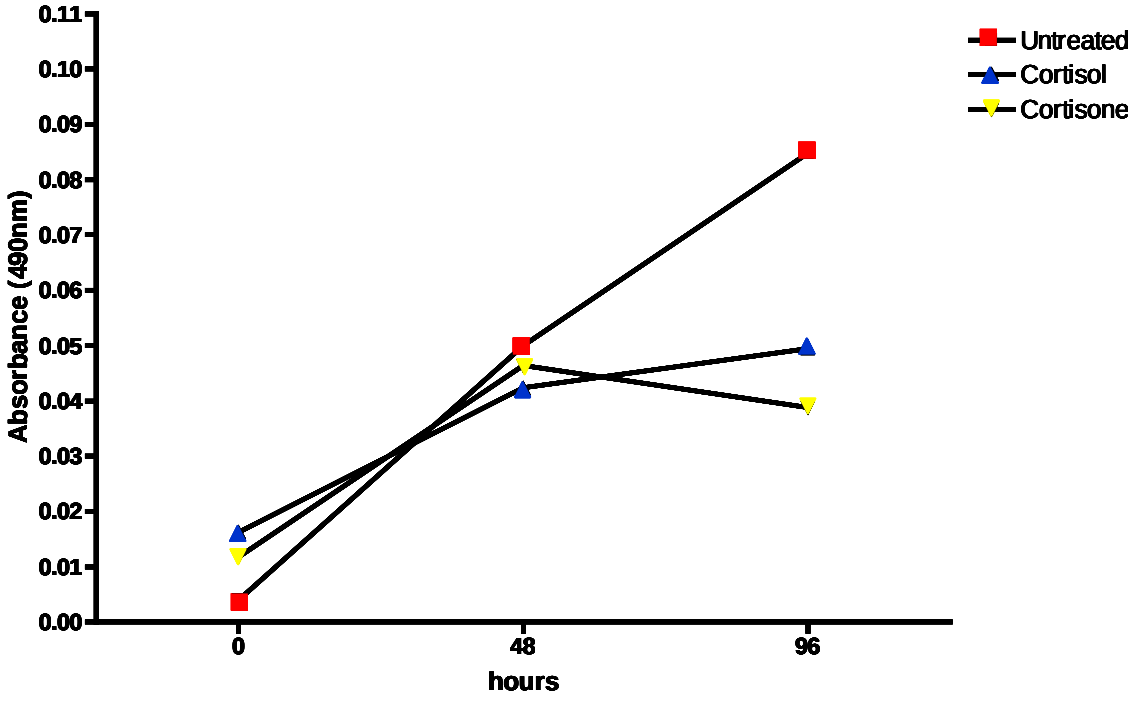



*Figure 3-11: NZWAR primary corneal epithelial cell proliferation assay.*

*Cells co-incubated with cortisol or cortisone elicited a 50% reduction in proliferation of corneal epithelial cells compared to baseline values (n=3 rabbits; p<0.01).*

### 3.3.1.6. Immunohistochemistry of the NZWAR Ciliary Body

11β-HSD1 but not 11β-HSD2 was localised to the non-pigmented epithelium (NPE) and not the pigmented epithelium (PE) of the NZWAR ciliary body (Figure 3-12).

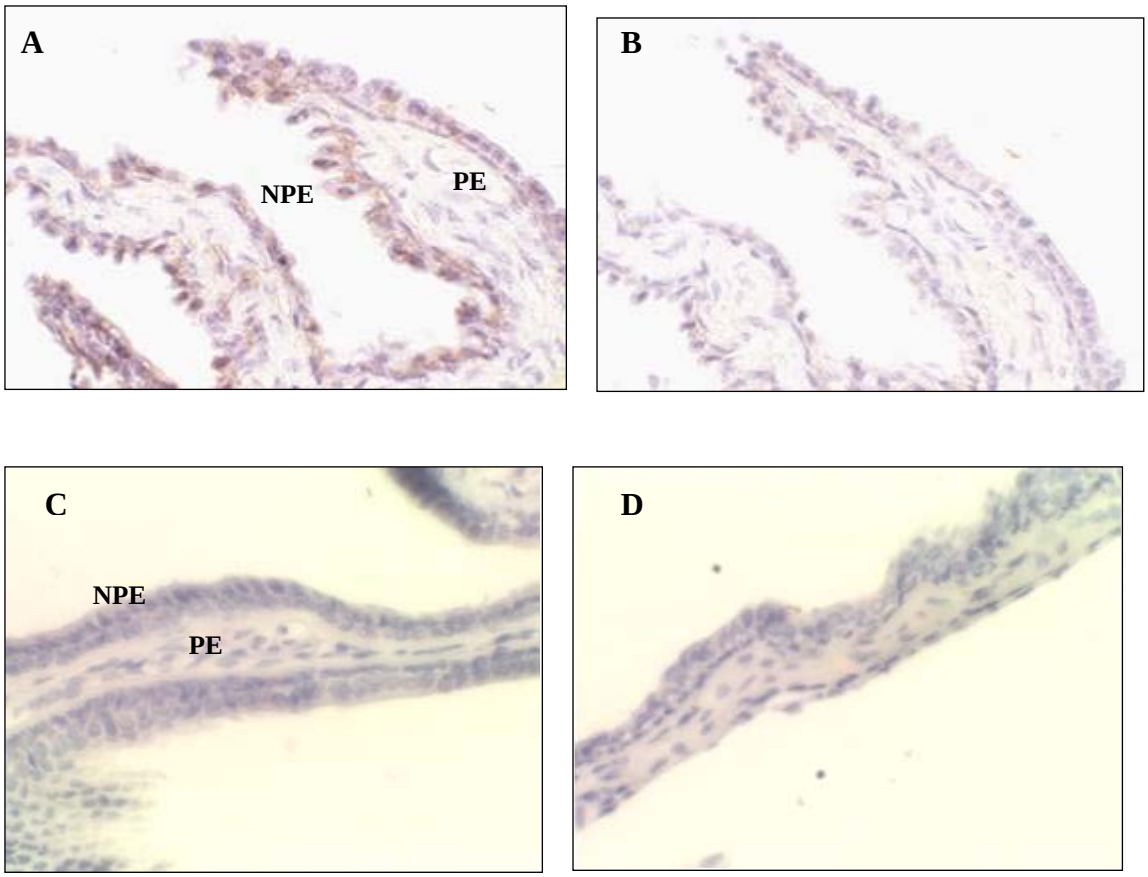



*Figure 3-12: Immunohistochemistry of the NZWAR ciliary body with 11β-HSD1 and 11β-HSD2.*

*Immunostaining for 11β-HSD1 in the NZWAR ciliary epithelium (A) and 11β-HSD1 preabsorbed with immunising peptide as a negative control (B). No immunoreactivity for 11β-HSD2 was seen in the NZWAR ciliary epithelium (C) negative control (D). All at x 40 magnification. (NPE, non-pigmented epithelium; PE pigmented epithelium).*

### 3.3.1.7. 11β-HSD1 Reductase Activity in NZWAR Ciliary Body Explants

NZWAR ciliary body explants showed predominantly 11β-HSD1 reductase activity and this was significantly higher ($p<0.01$) than 11β-HSD1 dehydrogenase activity from this tissue (Figure 3-13). 11β-HSD1 reductase activity was significantly reduced ($p<0.05$) with a non-specific 11β-HSD inhibitor, glycyrrhetinic acid (Figure 3-13).

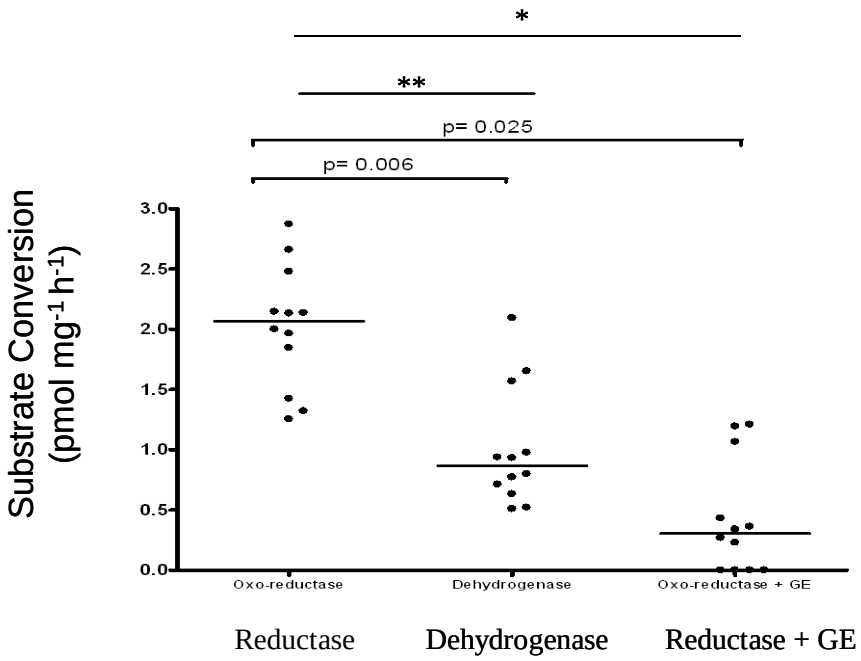



*Figure 3-13: Specific enzyme assay for 11β-HSD1 in NZWAR ciliary body.*

*11β-HSD1 reductase activity (median 2.1 pmol $mg^{-1}$ $h^{-1}$ and range 1.25-2.8 pmol $mg^{-1}$ $h^{-1}$) was significantly higher (n= 12 rabbits; p<0.01) when compared to dehydrogenase activity (median 0.87 pmol $mg^{-1}$ $h^{-1}$ and range 0.5-2 pmol $mg^{-1}$ $h^{-1}$ ) in NZWAR ciliary body explant. The reductase activity was reduced significantly (n=12 rabbits; p<0.05) by GE (median 0.4 pmol $mg^{-1}$ $h^{-1}$ and range 0.2-1.2 pmol $mg^{-1}$ $h^{-1}$). Data is expressed as medians with full range plots.*

### 3.3.2. Conclusion

These data suggest that the rabbit corneal epithelium is able to generate cortisol from cortisone and maybe involved in the autocrine regulation of cortisol which may be important in ocular surface renewal. The ciliary body generates intraocular cortisol via 11β-HSD1 and may be a pivotal defence mechanism protecting the intraocular environment.

After studying the pre-receptor regulation of glucocorticoids in the rabbit, I wanted to move on to look at human tissues and relate this to human ocular disease processes.

### 3.3.3. Human Studies

#### 3.3.3.1. Immunohistochemistry of the Human Cornea

The control tissues used for 11β-HSD1 and 11β-HSD2 immunoreactivity were the liver and the kidney respectively. Figure 3-14 shows the localisation of 11β-HSD1 in the liver and type 2 in the kidney compared to their negative controls.

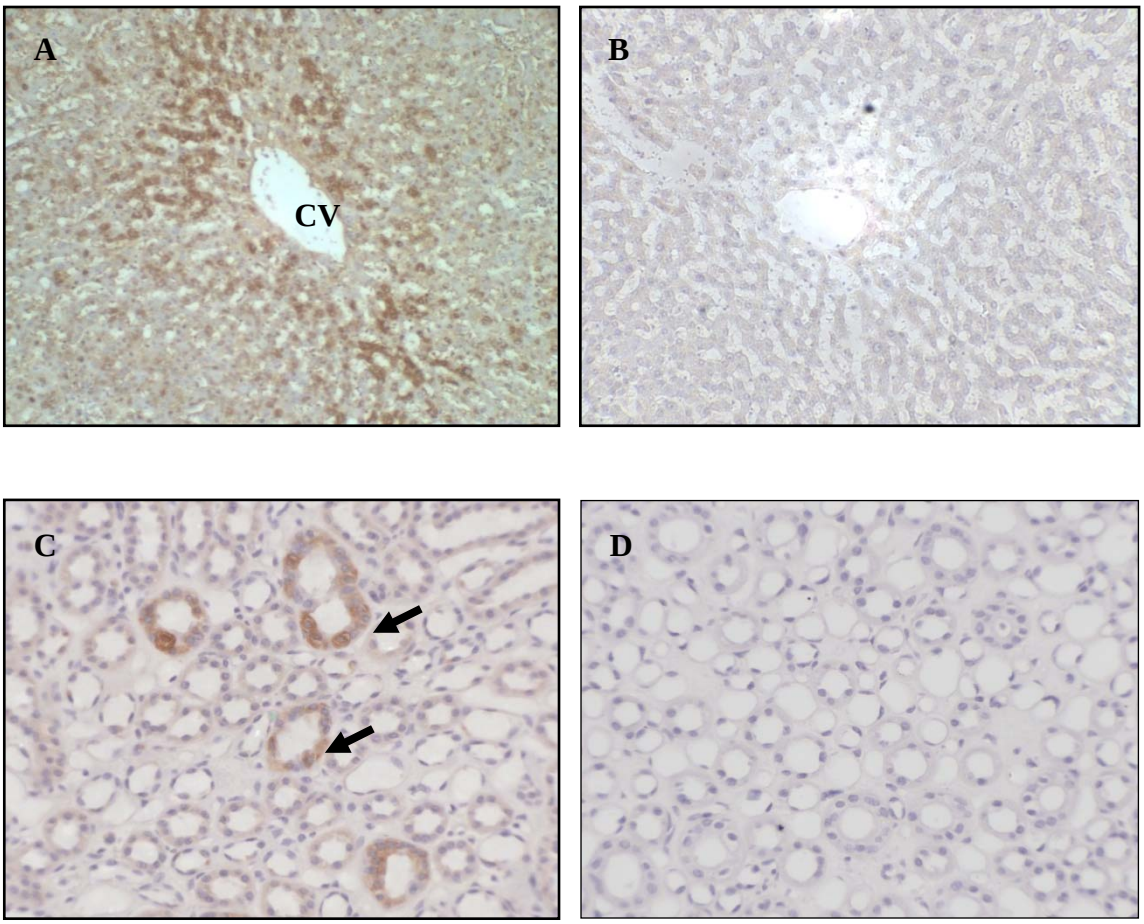



*Figure 3-14: Immunohistochemistry of the human control tissues with 11β-HSD1 and 11β-HSD2.*

*11β-HSD1 immunoreactivity of human liver around central vein (A), 11β-HSD1 preabsorbed with immunising peptide liver control section (B), 11β-HSD2 immunoreactivity with cuboidal epithelium of the collecting tubules in the human kidney medulla indicated by arrows(C), no primary antibody control kidney section (D) all at x 40 magnification (CV, central vein).*

Although immunostaining was successful for the liver and kidney, this was not true for the human cornea except for immunoreactivity of the human corneal endothelium with 11β-HSD1 (Figure 3-15). However, 11β-HSD1 expression has previously been confirmed in the basal cells of the human corneal epithelium (Rauz et al., 2001). However, some staining of the endothelium by 11β-HSD1 was shown (Figure 3-15) which was also found by Rauz and walker in 2001. No immunostaining for 11β-HSD2 was found in the human corneal epithelium (Figure 3-15).

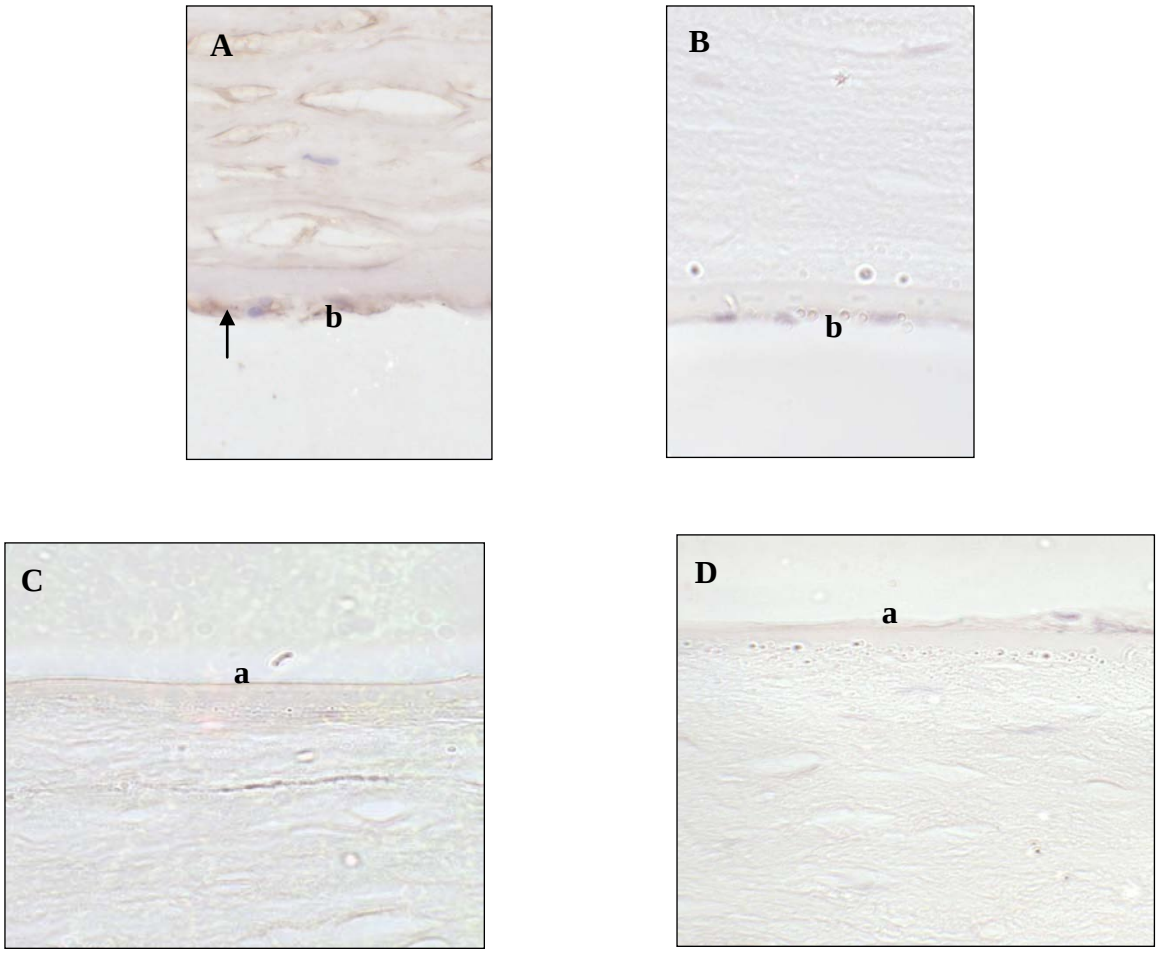



*Figure 3-15: Immunohistochemistry of the human cornea with 11β-HSD1 and 11β-HSD2.*

*Immunoreactivity for 11β-HSD1 was seen in the human corneal endothelium as shown by arrow (A) and compared to the 11β-HSD1 preabsorbed with immunising peptide as a negative control (B). No immunostaining for 11β-HSD2 was seen from the human epithelium (C) compared to the no primary negative control (D) Pictures taken at x 40 magnification. (a, corneal epithelium; b, corneal endothelium)*

### 3.3.3.2. Primary Culture Analysis of Human Corneal Epithelial Cell, Conjunctival Cell and Corneal Fibroblast Morphology

Primary human corneal epithelial cells possess a distinctive rounded shape with a centrally positioned nucleus which is in contrast to corneal fibroblasts which have a

stellate appearance and conjunctival fibroblasts which are elongated and spindled in shape (Figure 3-16). The differing characteristics qualitatively distinguish these cultures.

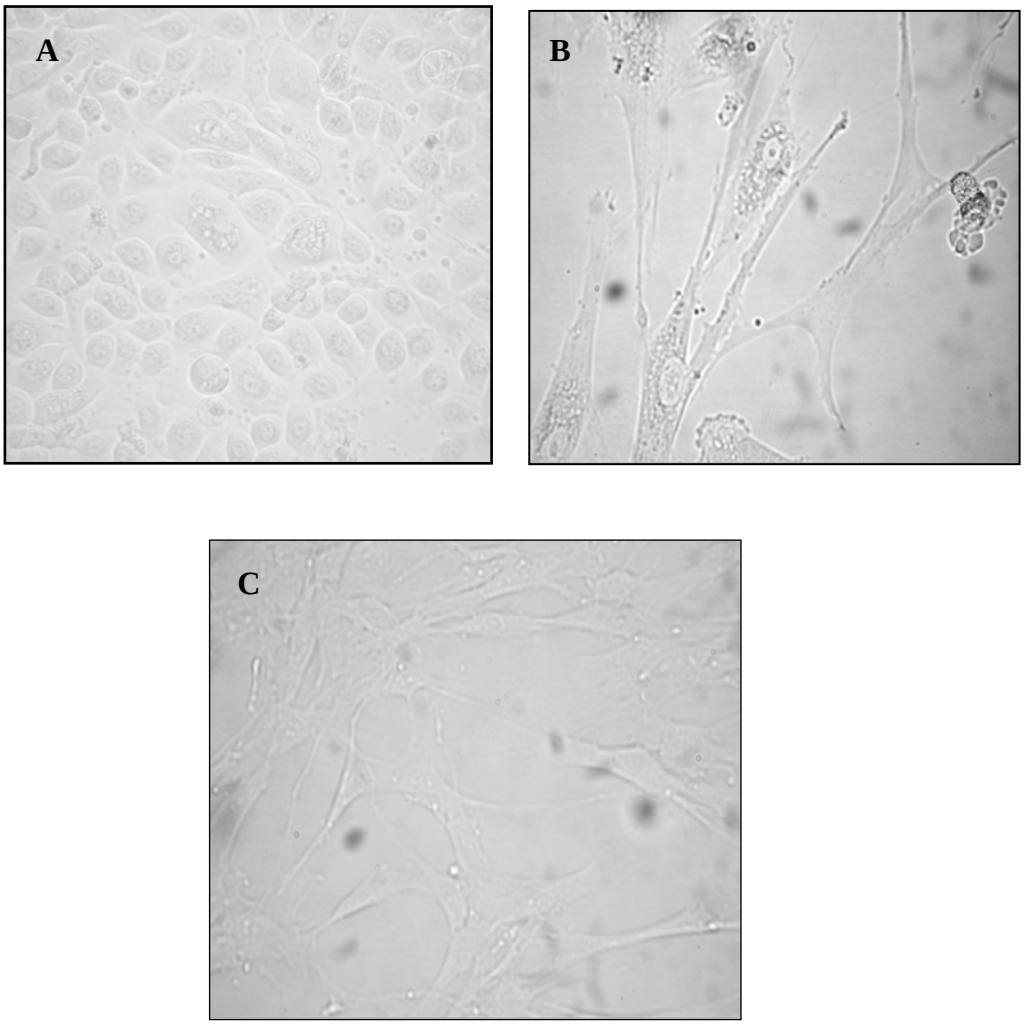



*Figure 3-16: Photographs of primary human ocular cells.*

*(A) Primary human corneal epithelial cells (x40 magnification) (B) Primary human corneal keratocyte (40 magnification), (C) Primary human conjunctival fibroblasts (x40 magnification).*

### 3.3.3.3. Identification of CD90 (ASO2) in Primary Human Conjunctival fibroblasts

The fibroblast marker, CD90 (ASO2) was used as a marker to distinguish fibroblast cells. Conjunctival fibroblasts were shown to express CD90 when compared to the negative control (Figure 3-17) but the technique used was not successful for corneal fibroblasts perhaps due to the concentration of the primary antibody (ASO2) not optimally found. A remedy for this could involve adjusting the final concentration of the primary antibody to a higher or lower concentration.

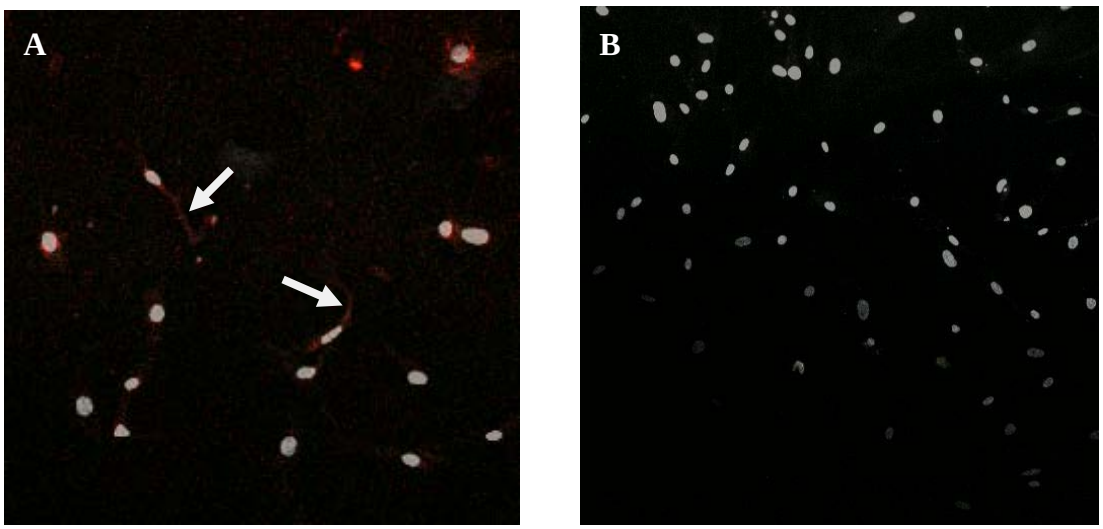



*Figure 3-17: Immunocytochemistry of primary human fibroblast cells with CD90 (ASO2).*

*(A) Conjunctival fibroblasts staining for fibroblast marker CD90 on the surface of the cell (arrowed) and (B) negative control.*

#### 3.3.3.4. Identification of Cytokeratin 3 and 11β-HSD1 in Primary Human Corneal Epithelial Cells

Anti-cytokeratin 3 antibodies were used to identify corneal epithelial cells in culture. 11β-HSD1 immunoreactivity was seen in primary corneal epithelial cells while 11β-HSD2 immunoreactivity was minimal when compared to negative controls (Figure 3-18).

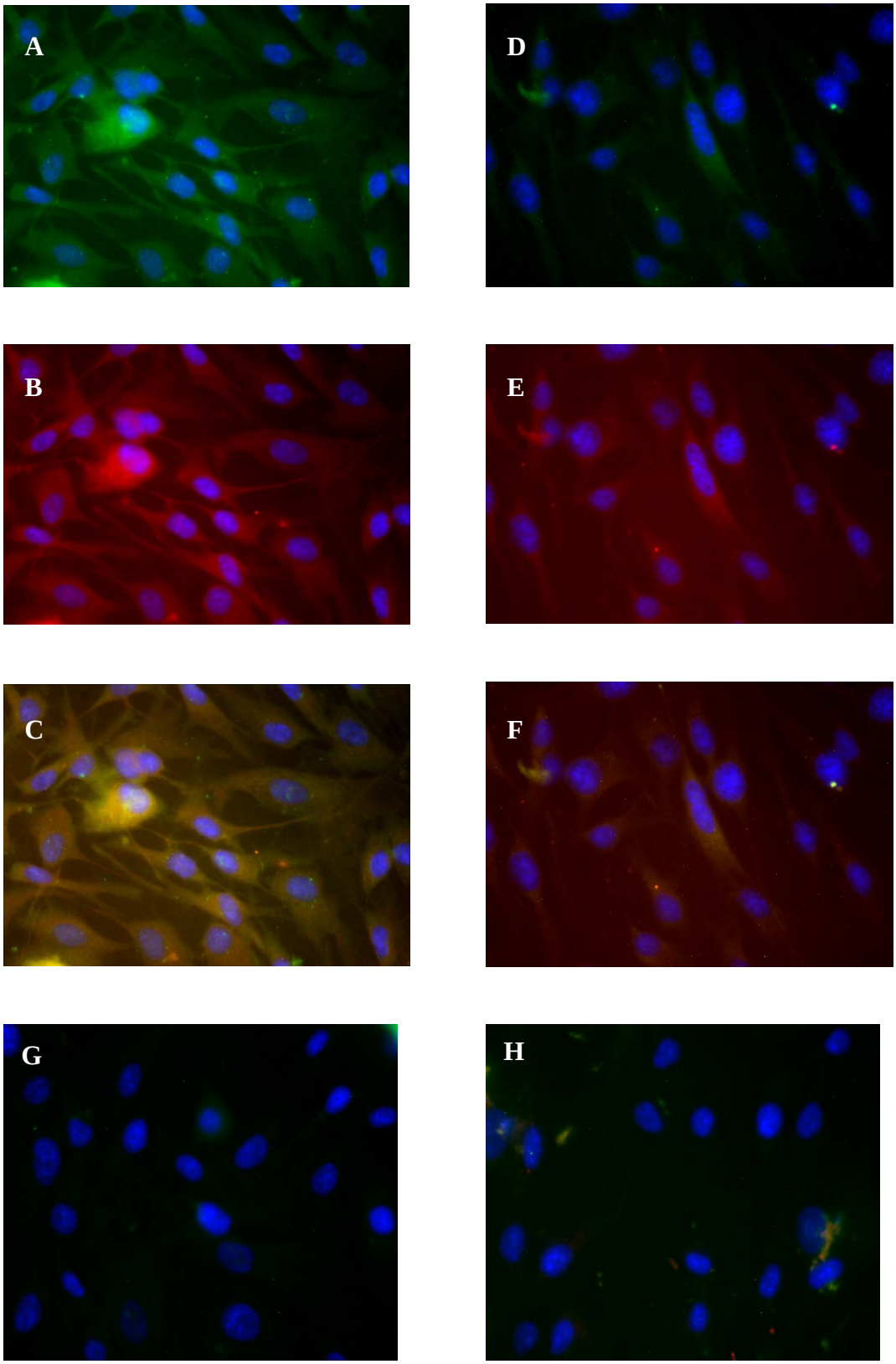



*Figure 3-18: Immunocytochemistry of primary human corneal epithelial cells with 11β-HSD1 and 11β-HSD2.*

*(A) Immunocytochemistry of PHCEC using an anti-cytokertin 3 conjugated to FITC, (B) Anti-11β-HSD1 conjugated to Texas red fluorescein (C) both combined (D) Anti-11β-HSD2 conjugated to FITC (E) Anti-cytokeratin conjugated to Texas red and (F) with both combined. Neagtive controls were no primary antibody (G) and 11β-HSD1 preabsorbed with immunising peptide (H). The same light density and background was used when comparing the negative controls with the stained samples.*

#### 3.3.3.5. Glucocorticoid-Related and Cytokine-Related Gene expression in Human Corneal Epithelial, Corneal Fibroblast and Conjunctival Fibroblast Cells

All the glucocorticoid related genes involved in the pre-receptor regulation and detection (GR) of glucocorticoids (11β-HSD1, H6PD and GR) and MR but not 11β-HSD2 were found to be expressed in primary human corneal epithelial cells (PHCEC), (Figure 3-19). Expression of H6PD and GR were also found in immortalised human corneal epithelial cell-line (HCEC) but no mRNA expression of 11β-HSD1 and MR were found in this cell-line (Figure 3-19). 11β-HSD1, H6PD and GR were also expressed in primary corneal and conjunctival fibroblasts (HCoF and HCF respectively) (Figure 3-19). IL-6 and IL-1β were expressed in PHCEC but not TNFα (Figure 3-19). IL-6 and IL-1β were expressed in the HCEC but no expression of TLR4 was evident. TLR4 was shown to express four gene variants in PHCEC (Figure 3-19). HBD-2 was found to be expressed in both PHCEC and HCEC (Figure 3-19). IL-1β and IL-6 were expressed in PHCEC and HCF (Figure 3-19) but TNFα was not expressed.

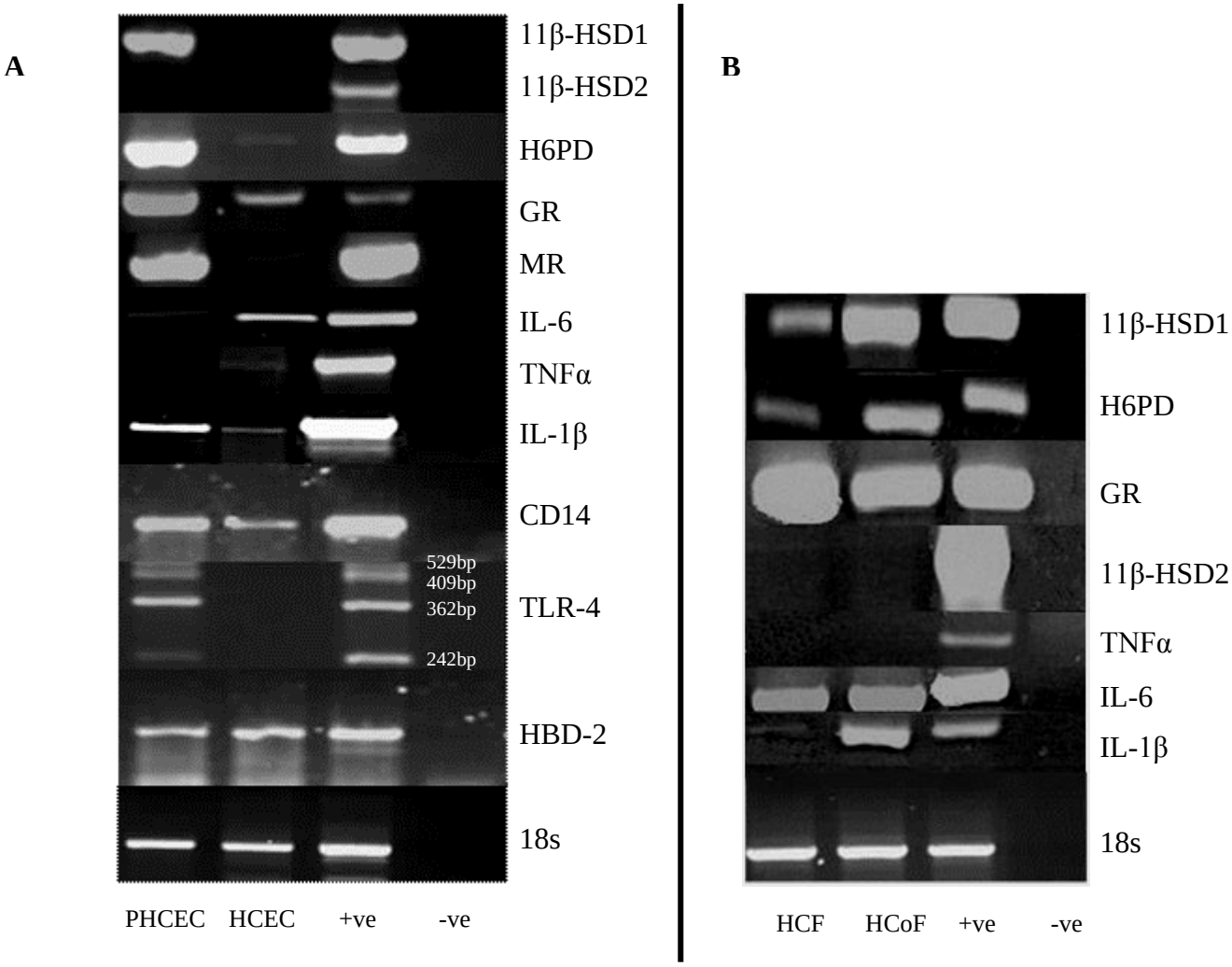



*Figure 3-19: Gene expression in human ocular cell-lines.*

*PCR panel showing (A) expression of genes in primary human corneal epithelial cells and the immortalized human corneal epithelial cell-line. TLR4 has four specific variants (B) expression of genes in primary human corneal fibroblasts and conjunctival fibroblasts. 18s was used as the housekeeping gene control for the PCR reaction. (11β-HSD1, 11beta-hydroxysteroid dehydrogenase 1; H6PD, Hexose-6-phosphate dehydrogenase; GR, Glucocorticoid receptor; 11β-HSD2, 11beta-hydroxysteroid dehydrogenase 2; MR, Mineralocorticoid receptor; IL-6, Interleukin 6; TNFα, Tumor Necrosis Factor alpha; IL-1β, Interleukin 1 beta; CD14, HBD-2, Human beta defensin 2; PHCEC, primary corneal epithelial cells; HCEC, immortalized human corneal epithelial cell-line; HCF, human conjunctival fibroblasts; HCoF, human corneal fibroblasts).*

### 3.3.3.6. 11β-HSD1 Reductase Activity in Human Corneal Epithelial, Corneal Fibroblast and Conjunctival Fibroblast Cells

Specific enzyme assay on primary human corneal epithelial cells revealed a predominant reductase activity ($P<0.001$) compared to dehydrogenase (Figure 3-20). Reductase activity was also found to exist in primary human corneal and conjunctival fibroblasts (Figure 3-20). Reductase activity from human conjunctival fibroblasts was highest but this was found not to be statistically significant.

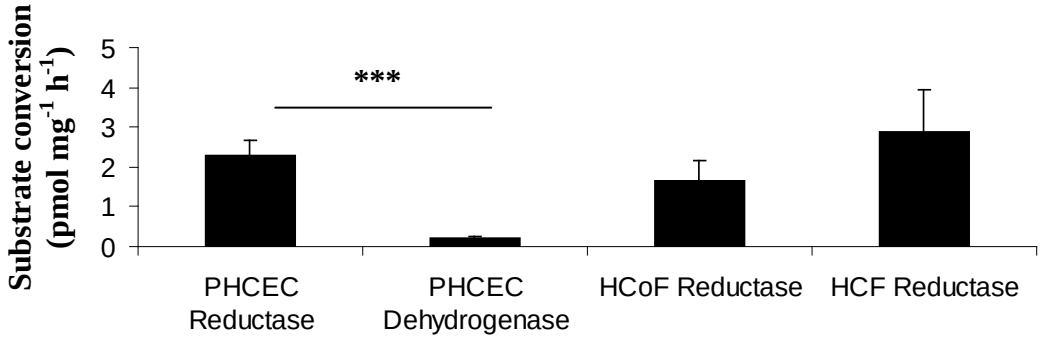



*Figure 3-20: Specific enzyme assay for 11β-HSD1 in primary human ocular cells.*

*11β-HSD1 reductase activity (n=3, 2.3 ±0.5 pmol $mg^{-1}$ $h^{-1}$) is significantly higher compared to dehydrogenase activity (n=3, 0.2±0.1 pmol $mg^{-1}$ $h^{-1}$) in human corneal epithelial cells (PHCEC). Reductase activity was also found in primary corneal fibroblasts (HCoF, n=3;1.66±0.9 pmol $mg^{-1}$ $h^{-1}$) and conjunctival fibroblasts (HCF, n=3; 2.86±1.5 pmol $mg^{-1}$ $h^{-1}$). There was zero 11β-HSD1 dehydrogenase activity from HCoF and HCF. Data are expressed as means and error bars as standard errors of the mean.*

### 3.3.3.7. 11β-HSD1 Reductase Activity in Human Corneal Epithelial Cells After LPS/Glycyrrhetinic Treatment

Treatment of primary human corneal epithelial cells with LPS mimicked the effect of pathogenic attack and showed a significant increase in 11β-HSD1 reductase activity

(P<0.01) which was inhibited (P<0.01) when the cells were co-incubated with the non-specific 11β-HSD inhibitor, glycyrrhetinic acid (Figure 3-21). LPS treatment of the other human ocular cell-lines was not performed.

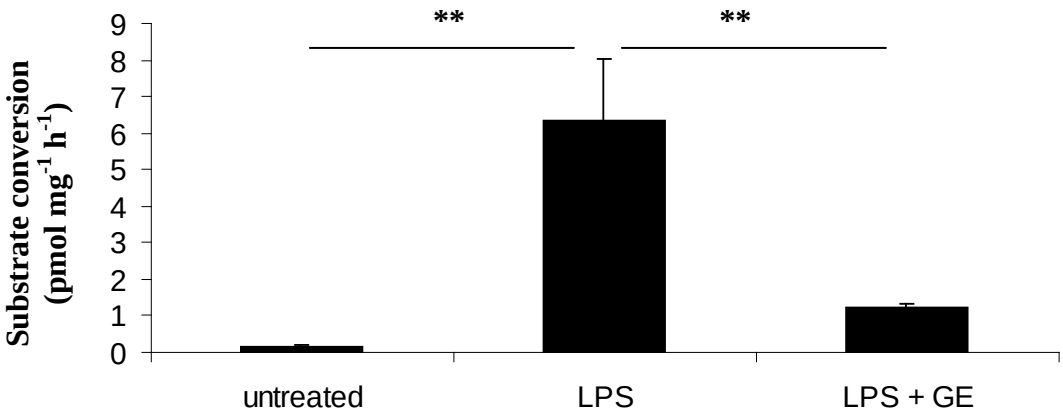



*Figure 3-21: Specific enzyme assay for 11β-HSD1 in primary human corneal epithelial cells after LPS treatment.*

*11β-HSD1 reductase activity (cortisone to cortsol conversion) in human corneal epithelial cells increases after LPS (n=3, 6.3±1.7 pmol $mg^{-1}$ $h^{-1}$) compared to untreated cells (n=3, 0.13±0.1 pmol $mg^{-1}$ $h^{-1}$). GE treatment significantly reduced LPS effect on increased reductase activity from PHCEC (n=3, 1.2±0.1 pmol $mg^{-1}$ $h^{-1}$). (LPS, Lipopolysaccharide; GE, glycyrrhetinic acid; PHCEC, primary human corneal epithelial cells). Data are expressed as means and error bars as standard errors of the mean.*

### 3.3.3.8. 11β-HSD1 Reductase Activity in Human Corneal Epithelial Cells After Pro-inflammatory Cytokine Treatment

Primary human corneal epithelial cells subjected to TNFα stimulation had no significant effect on increasing 11β-HSD1 reductase activity compared to untreated cells (Figure 3-22). However, IL-6 was able to significantly increase reductase activity (P<0.01) compared to untreated and TNFα stimulated cells (Figure 3-22). TNFα increased reductase activity from primary corneal and conjunctival fibroblasts while IL-6 was not able to produce this effect (Figure 3-22). The TNFα effect on primary human corneal

fibroblast reductase activity was significantly higher ($P<0.05$) than in primary human corneal epithelial cells (Figure 3-22).

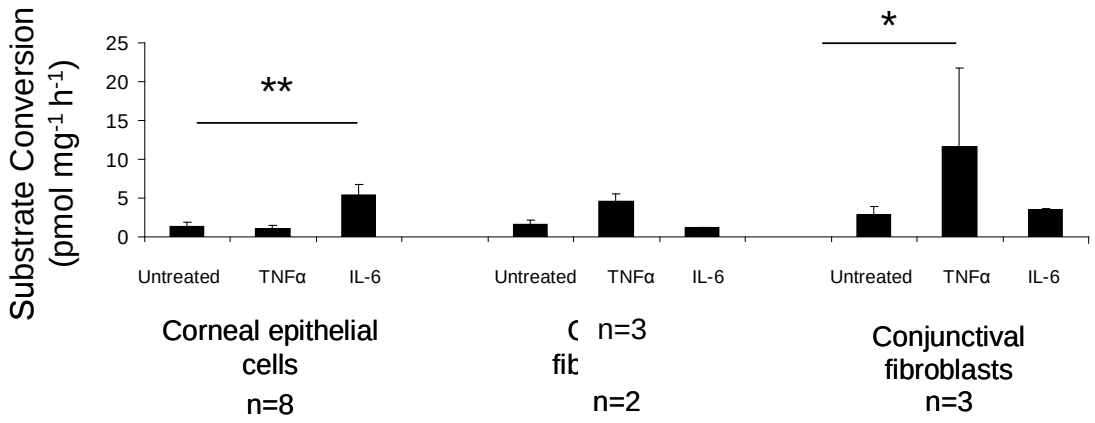



*Figure 3-22: Specific enzyme assay for 11β-HSD1 in primary human ocular cells after TNFα and IL-6 treatment.*

*Reductase actvity does not increase after treatment with TNFα (n=8, 1.1±0.4 pmol $mg^{-1}$ $h^{-1}$) but does significantly increase after IL-6 stimultion (n=8, 5.4±1.4 pmol $mg^{-1}$ $h^{-1}$, $p<0.01$) in primary human corneal epithelial cells compared to untreated (n=8, 1.4±0.5 1 pmol $mg^{-1}$ $h^{-1}$). Primary human corneal fibroblasts reductase activity is increased after TNFα treatment (n=3, 4.6±0.9 pmol $mg^{-1}$ $h^{-1}$) when compared to untreated cells (n=3, 1.7±0.5 pmol $mg^{-1}$ $h^{-}1$). IL-6 treatment of primary human corneal fibroblasts exhibts a reduced reductase activity (n=3, 1.1±0.1 pmol $mg^{-1}$ $h^{-1}$) when compared to untreated and TNF treated cells. A significant increase in reductase activity is shown by human conjunctival fibroblasts (n=3, 11.6±10.1 pmol $mg^{-1}$ $h^{-1}$, $p<0.05$) when compared to untreated cells (n=3, 2.9±1.1 1 pmol $mg^{-1}$ $h^{-1}$). There is a slight increase in IL-6 treatment when compared to untreated cells (n=3, 3.5±0.1 pmol $mg^{-1}$ $h^{-1}$). Data are expressed as means and error bars as standard errors of the mean.*

### 3.3.3.9. DNA-Binding of Activated GR Assay

The DNA-binding ELISA relies on specific double-stranded oligonucleotides binding to the activated transcription factor, which in this case is the GR. DNA-binding was shown to occur after dexamethasone treatment in immortalised human corneal epithelial cell-line (Figure 3-23). The cell-line was easily accessible compared to the primary cell-line and

the technique was used to confirm glucocorticoid interaction with the GR which maybe involved in negative regulation of pro-inflammatory cytokines (Scheinman et al., 1995b, Ray and Prefontaine, 1994, Auphan et al., 1995). To ascertain whether a component of pseudomonas could also activate GR, LPS was used. LPS did not significantly increase GR binding of DNA (Figure 3-23). The use of the GR inhibitor, RU486 reduced DNA binding of the activated GR by dexamethasone but not LPS (Figure 3-23).

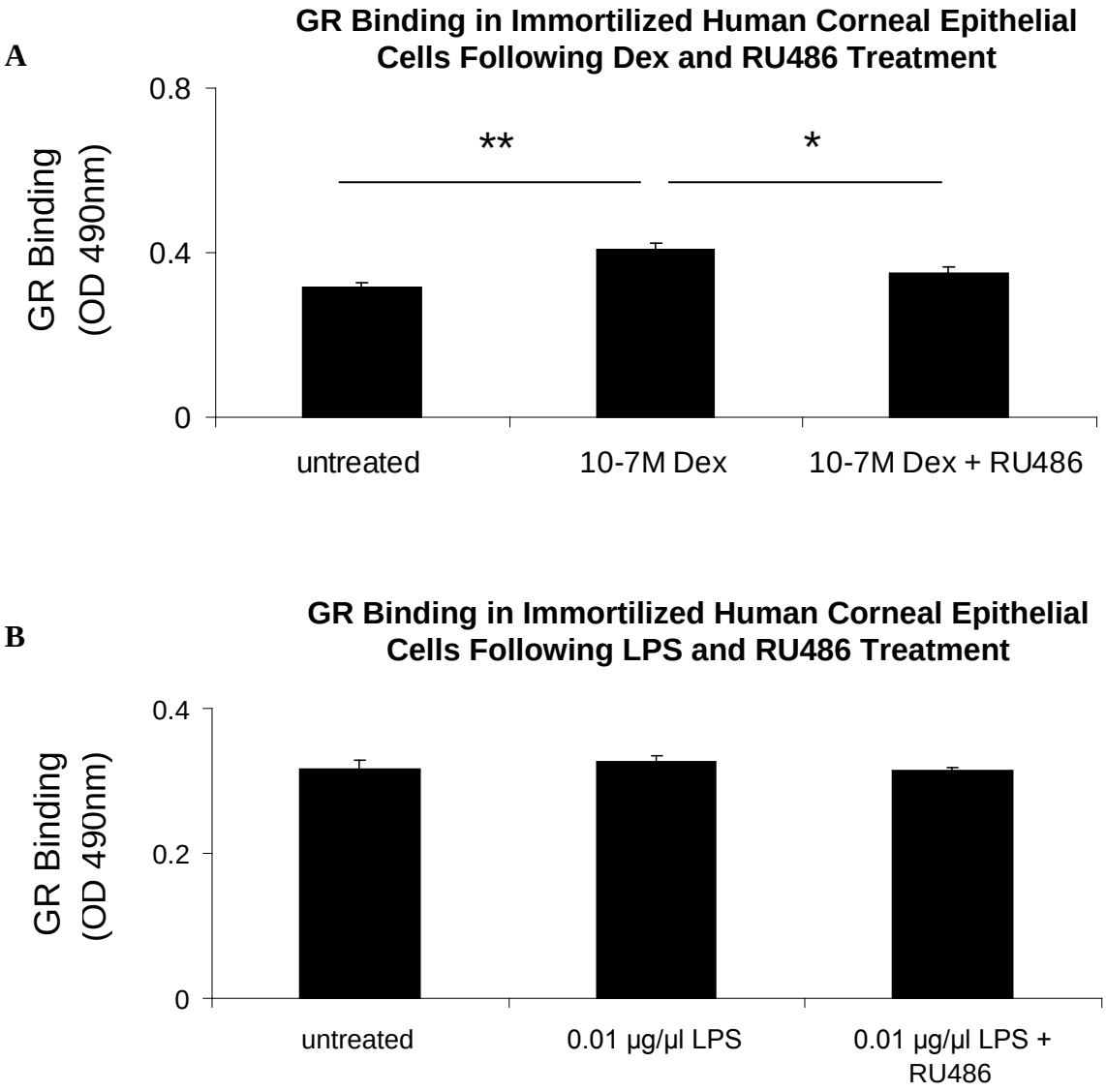



*Figure 3-23: GR binding in immortilized human corneal epithelial cells.*

*(A) The use of RU486 significantly decreased GR binding to baseline after dexamethasone treatment (n=3, 0.35±0.01. (B) LPS treatment had no effect on GR activity and RU486 also had no effect. (Dex, dexamethasone; LPS, Lipopolysaccharide). Data are expressed as means and error bars as standard errors of the mean.*

### 3.3.4. Conclusion

These data confirm that an autocrine regulation of cortisol exists in the human corneal epithelium and in human ocular fibroblasts. IL-6 and TNFα have great effects on ocular 11β-HSD1 and this may have implications for cortisol generation for modulating

inflammatory events. GR activity was also confirmed in corneal epithelial cells which is important in conferring glucocorticoid action in inflammatory settings.

I have characterised the pre-receptor regulation of glucocorticoids and the components involved in the process in human ocular surface (corneal epithelial cells, corneal fibroblasts, conjunctival fibroblasts) and rabbit ocular surface (corneal epithelial cells, corneal fibroblasts, conjunctival fibroblasts) and intraocular (non-pigmented and pigmented epithelium) tissues (Table 3-2). Results of studies yet to be determined are mostly from the rabbit and this is a result of time pressures in pursuing studies in the human further. The immunostaining results not obtained in the human ocular tissue and cells such as that in corneal fibroblasts and conjunctiva will need further optimization.

*Table 3-2: Summary table comparing 11β-HSD1 expression/activity in rabbit and human eyes*

| | Rabbit | Human |
|---|---|---|
| **Corneal Epithelial Cells** | | |
| **RT-PCR** | ? | + |
| **IHC 11β-HSD1** | + | + |
| **IHC 11β-HSD2** | - | - |
| **11β HSD 1 Activity Regulators** | | |
| **LPS** | ? | + |
| **IL-6** | ? | + |
| **TNFα** | ? | + |
| **Proliferation** | + | ? |
| **Corneal Fibroblasts** | | |
| **RT-PCR** | ? | + |
| **ICC CD90** | ? | ? |
| **11β-HSD1 activity** | - | + |
| **Conjunctiva** | | |
| **IHC 11β-HSD1** | + (epithelium) | ? |
| **ICC CD90 (fibroblasts)** | ? | + |
| **11β-HSD1 (fibroblasts)** | - | + |
| **Ciliary Body** | | |
| **IHC 11β-HSD1** | + | + |
| **11β-HSD1 activity** | + | + |
| **Quantification of cortisol** | + | + |

*+, positive; -, negative; ?, unknown; IHC, immunohistochemistry; ICC, immunocytochemistry; 11β HSD 1/2, 11 beta hydroxysteroid dehydrogenase 1/2; RT-PCR, reverse transcription polymerase chain reaction.*

### *3.4. Discussion*

The confirmation of active 11β-HSD1 reactivating cortisol from cortisone in corneal epithelial cells suggests a role in the local production of glucocorticoids to orchestrate transcription of glucocorticoid receptor genes that maybe involved in corneal repair in addition to anti-inflammatory action against unwanted pathogen. This must be tissue specifically controlled as too high a level of glucocorticoids have been shown to have adverse effect on their proliferation (chapter 3.3.1.5) when used at therapeutic levels. Synthetic glucocorticoids at high concentrations are known to inhibit the corneal wound healing process (Bourcier et al., 2000) and our data suggests that active glucocorticoid as well as reactivated glucocorticoid (cortisone to cortisol) reduces corneal epithelial cell proliferation.

There was no expression of 11β-HSD2 in the corneal epithelium, corneal endothelium and corneal epithelial cells of the NZWAR which is in parallel with the human corneal surface; however, there was expression of 11β-HSD1 in the NZWAR corneal epithelium and endothelium which was also comparable in the human (Rauz et al., 2001). This was achievable as the human peptide used to raise the 11β-HSD1 antibody possesses 100% homology to the rabbit.

The choice of using the New Zealand White Albino Rabbit (NZWAR) as a model is weighted by the fact that it has previously been used in different studies concerning the ocular surface and the intraocular environment. For example a study showing the use of rabbits in corneal wound healing involved using carboxymethyl cellulose to stimulate corneal epithelial wound healing on rabbit eye model in vivo (Garrett et al., 2008). The corticosteroid hormone profile of the rabbit is also similar to humans (i.e. cortisol rather than corticosterone in rodents), (Szeto et al., 2004, Fuentes and Newgren, 2008). Rabbits

have been used to study ocular mucosal surfaces, for example a study focused on the use of rabbit oral mucosal epithelial cells in the reconstruction of the corneal epithelium surface (Hayashida et al., 2005). Rabbit models are also used to study ocular surface diseases and conditions such as keratitis, dry eye and keratoconjunctivitis (Thomas et al., 2008, Oh et al., 2007, Guzek et al., 1998).

Expression of glucocorticoid related and certain pro-inflammatory cytokine genes suggest that the human corneal epithelium may have the necessary apparatus to respond to a potential inflammatory stimulus. Confirmation of CD14, HBD-2 and TLR4 gene expression in primary human corneal epithelial cells supports other literature on these findings that are involved in the regulation of inflammation (Song et al., 2001, Terai et al., 2004, Li et al., 2007).

Attempts to show localisation of 11β-HSD1 on the human corneal epithelium as confirmed by other reports (Rauz et al., 2001) failed and this maybe attributable to many factors which may include optimal staining parameters not being reached and human error. In contrast to this, 11β-HSD1 staining in cultured human corneal epithelial cells by immunocytochemistry confirmed expression of 11β-HSD1 enzyme protein. Confirmation of 11β-HSD1 in NZWAR corneal and conjunctival epithelium with human anti-11β-HSD1 antibody reconfirmed the cross-reactivity between these two models and highlighted the potential for these cell types to locally generate cortisol.

To check whether the 11β-HSD1 protein is active, specific enzyme assays were used to assess activity in cultured NZWAR and human corneal epithelial cells, corneal fibroblasts, conjunctival fibroblasts and NZWAR ciliary epithelium tissue (NPE and PE). Predominant activity was present in NZWAR NPE compared to PE. This suggests that the NPE is responsible for the local generation of cortisol in this tissue which can have

intracrine and paracrine effects to regulate aqueous humour production possibly by regulating SGK and ENaC expression (Rauz et al., 2003b). This is important because aqueous humour provides the components for immunoprotection in the eye and cortisol may aid in these processes.

The predominant reductase activity from NZWAR and human corneal epithelial cells suggests the ability of these cells to take part in the pre-receptor regulation of glucocorticoids in intact cells at least in-vitro. Other studies have confirmed predominant reductase activity in intact cells, for example, from liver, lung, and adipose tissue (Jamieson et al., 1995, Hundertmark et al., 1995, Bujalska et al., 1997) and therefore highlights the role 11β-HSD1 plays in regulating local cortisol levels in these tissues. No activity of 11β-HSD1 was shown from NZWAR keratocytes and conjunctival fibroblasts which were in stark contrast to the human form of these cells which did exhibit reductase activity. A possible explanation for this is that 11β-HSD1 may play a bigger role in human ocular surfaces compared to in the rabbit.

TLR signalling pathways are important in the elimination of pathogenic bacteria on the ocular surface (Wright et al., 1990, Song et al., 2001). LPS has been shown to bind to CD14 and TLR4 to induce expression of pro-inflammatory cytokines and HBD-2 (Maltseva et al., 2007). The upregulation of 11β-HSD1 activity by LPS in human corneal epithelial cells may represent the functionality of 11β-HSD1 to produce cortisol to modulate immune events. LPS a component of bacterial cell wall has been shown to upregulate immune components that facilitate the inflammatory process such as pro inflammatory cytokines (e.g. IL-6 and TNFα) and activates the innate immune response via CD14 and TLRs on the ocular surface (Liang et al., 2007b, Wu et al., 2005, Sun et al., 2006, Lu et al., 2006). However, LPS is also known to induce immunomodulatory

components to regulate inflammation such as mucins to protect the mucosal barrier (Smirnova et al., 2003). In light of these data, LPS may induce the immunomodulatory properties of 11β-HSD1 in ocular tissue by promoting cortisol generation.

It was shown that the pro-inflammatory cytokine IL-6 and not TNFα was able to upregulate 11β-HSD1 reductase activity in human corneal epithelial and TNFα and not IL-6 upregulates 11β-HSD1 reductase activity in corneal fibroblast and conjunctival fibroblast cells. IL-6 has been shown to upregulate 11β-HSD1 activity in adipocytes (Friedberg et al., 2003) while TNFα has been shown to up-regulate 11β-HSD1 activity in other cell systems such as glomerular mesangial cells of the kidney and in osteoblasts (Escher et al., 1997b). However, these data may suggest that specific cytokines have cell specific roles in modulating 11β-HSD1 activity on the ocular surface.

GR has been localised in ocular tissues which include the cornea, ciliary body, trabecular meshwork, lens, retina and sclera (Rauz et al., 2001, Suzuki et al., 2001, Mirshahi et al., 1997, Stokes et al., 2000). GR interaction with glucocorticoids is important for genomic mediated responses during inflammation (Chinenov and Rogatsky, 2007). Studies have shown that GR agonists such as dexamethasone can activate the GR in-vitro (Takahashi et al., 2000, Wenzel et al., 2001). The immortalized human corneal epithelial cell-line does not express 11β-HSD1 and therefore is not able to reactivate cortisol from cortisone. However, although the immortilized human corneal epithelial cell-line cannot be used to study 11β-HSD1 directly (unless the enzyme can be transfected into the cell-line), it is still useful for the study of other glucocorticoid related components such as GR and H6PD on the ocular surface. GR expression was shown in the immortalized human corneal epithelial cells and although some GR activation was shown in the immortalized human corneal epithelial cells by dexamethasone, this was not as strong as what might have been

expected. This may suggest that the assay used for these particular cells, was not optimally sensitive to detect increases in GR DNA-binding. LPS had no effect on GR activity and therefore does not influence GR mediated transcription.

With respect to intraocular defence, clinical studies have quantified cortisol in aqueous humour where diffusion or ultrafiltration has been postulated to be the main source of intraocular cortisol (Rauz et al., 2003a, Rauz et al., 2001). This study shows conclusively that the ciliary body has the capacity to synthesise cortisol and may have an additional role in the regulation of protective systems such as Fas-L and human beta defensins contributing to intraocular defence.

To conclude, the potential for pre-receptor regulation of glucocorticoids has been uncovered in both NZWAR and human model and the pre-receptor regulation that occurs may regulate ocular surface renewal and may serve to regulate ocular surface inflammation caused by bacterial pathogenic attack.

# 4. Chapter 4

# The Putative Role of 11beta Hydroxysteroid Dehydrogenase 1 in Orbital Adipose Biology

## *4.1. Introduction*

### 4.1.1. Orbital Inflammatory Disease

Different immune-mediated diseases can affect the tissues of the orbit. These can include Wegner's granulomatosis (WG), sarcoidosis and Graves' disease which is the main focus of this chapter. However, a great proportion of orbital inflammatory disease are idiopathic (IOI) or non-specific orbital inflammation (Lutt et al., 2008). WG is a systemic disease which causes necrotic inflammation to small and medium sized vessels, lungs, protrusion of the eyeballs and affects the nervous system, skin and mucous membranes (Kowalewska et al., 2008). Sarcoidosis is an inflammatory disease which causes the appearance of granulomas in organs which include the lungs, lymph nodes, skin, liver and eyes and is of unknown aetiology. In some cases the disease can be self-limiting however in other cases the disease may promote a progression to postgranulomatous fibrosis in affected areas (Nagai et al., 2008).

### 4.1.2. Thyroid Associated Ophthalmopathy

Thyroid associated ophthalmopathy (TAO) is a specific autoimmune disorder characterised by an increase in orbital adipose/connective tissue and extraocular muscles within the orbit (Kumar et al., 2004, Crisp et al., 2000, Bahn, 2003). The condition more frequently occurs with patients that have autoimmune hyperthyroidism which can be a clinical feature of Graves' disease, however, the condition can precede or follow Graves' disease (Garrity and Bahn, 2006, Wiersinga and Bartalena, 2002). The condition can also occur in hypothyroid and euthyroid patients.

TAO can be diagnosed by carrying out assessments conducted by primary care physicians, general practitioners and specialists and the more severe cases are passed on

to specialised thyroid eye clinics for further assessment (Bartalena et al., 2008). The disease severity that they assess for can be split into mild, moderate and severe as dictated by degree of proptosis, extraocular muscle involvement and presence of optic neuropathy (El-Kaissi et al., 2004). Clinical activity-scoring (CAS) systems have been set up to assess disease activity where an example of this involves a point given to each of the following parameters: spontaneous retrobulbar pain, pain on eye movement, eyelid erythema, eyelid oedema, chemosis, conjunctival injection and swelling of the caruncle (Bartalena et al., 2000). A score equal or greater than 4 indicate active ophthalmopathy (Bartalena et al., 2000). Different types of orbital imagery can be used to assess the extent at which the orbit is affect by TAO and help eliminate doubt about the diagnosis. The types of imagery that can be used include, computed tomography (CT), magnetic resonance imaging (MRI) ultrasound (US) and Octreoscan (Kahaly, 2001).

Other tests can be performed if there is suspicion of optic neuropathy and these can include perimetry and visual evoked potentials (El-Kaissi et al., 2004, Dickinson and Perros, 2001).

TAO management, especially for severe cases, can include the use of immunosuppressants such as steroids/glucocorticoids. The time period for which glucocorticoids are used is usually reduced sequentially dependent on the outcome measures (Cawood et al., 2004). Radiotherapy became a controversial treatment in TAO due to doubts in the efficacy of its use as some trials mistakenly correlated that the improvements seen were due to the treatment and not the natural resolution of the disease (Prummel et al., 2004, Mourits et al., 2000, Gorman et al., 2001). When radiotherapy is used in conjunction with a glucocorticoid, there is an improvement in diplopia, however, there is a 12-24 week delay in response to treatment therefore is unsuitable for individuals

suspected of optic neuropathy which needs to be treated within 48 hours to avoid irreversible blindness (Cawood et al., 2004, El-Kaissi et al., 2004).

The option of surgery is only recommended when the condition may become sight threatening, is unresponsive to other interventions or for functional and cosmetic purposes and only occurs after TAO becomes 'inactive' or 'burnt out' (Cawood et al., 2004). The types of surgery performed include orbital decompression, eye muscle surgery and eyelid surgery. Eye muscle surgery is executed when patients are shown to have diplopia in normal gaze or reading position (El-Kaissi et al., 2004). Eyelid surgery improves lid retraction (seen as the characteristic 'stare' in TAO) whilst orbital decompression surgery consists of relieving orbital content by either removing floor and medial orbital bony wall, or roof, lateral and anterior bony wall with some orbital adipose tissue at the same time (El-Kaissi et al., 2004).

Graves' disease is the most common form of hyperthyroidism (Khoo and Bahn, 2007). In the normal thyroid, thyrotropin-releasing hormone (TRH) released from the hypothalamus stimulates thyrotropin (TSH) production from the anterior pituitary. TSH then targets the follicle cells of the thyroid where iodine is combined with the amino acid tyrosine to form thyroxine ($T_4$) and the active thyroid hormone, triiodothyronine ($T_3$). The levels of $T_3$ and $T_4$ can positively or negatively feedback on the hypothalamus-pituitary-thyroid axis. The characteristic of the hyperthyroidism seen in Graves' disease is increased levels of thyrotropin secondary to autoantibodies against thyroid antigens. One of these thyroid antigens is the thyroid-stimulating hormone receptor (TSHR) which mediates active thyroid hormone action. Thyroid-stimulating autoantibodies (TSAB) against the TSHR on thyrocytes of the thyroid mediate the heightened production of thyroid hormone (Akamizu, 2001).

Some patients with Graves' disease do not experience ophthalmopathy, however, some may experience general lid lag, lid retraction and stare, all of which are features of TAO. Features of ocular involvement in Graves' disease can be moderate or severe in nature (Figure 4-1) with experiences of chemosis, conjunctival injection (redness of the conjunctiva), periobital oedema (swelling of the eyelids) and pain (Bahn and Heufelder, 1993). However those with severe ocular disease are at risk of blindness due to compression of the optic nerve (Garrity and Bahn, 2006).

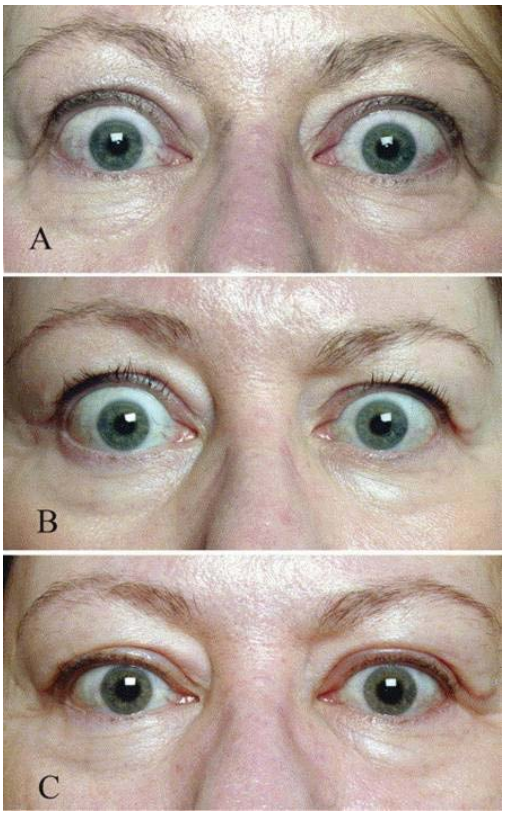



*Figure 4-1: An example of the features in thyroid associated ophthalmopathy.*

*(A) Female patient with moderate to severe proptosis and eyelid retraction. (B) Seven months after orbital decompression and eyelid retraction surgery, there appears to still be some eyelid retraction but a 4mm reduction in proptosis. (C) After additonal eyelid retraction surgery, intervention appears to improve appearance and gives residual eyelid retraction. Taken from(Ben Simon et al., 2005)*

In addition to Graves' disease, TAO can be associated with other forms of thyroid dysfunction which are very much less common and these include, hypothyroidism, a condition characterised by decreased circulating thyroid hormone, Hashimoto's thyroiditis, which is an acquired form of hypothyroidism, most common in adolescence and euthyroid, where individuals have no apparent thyroid dysfunction but still have TSHR antibodies or thyroglobulin or thyroid peroxidase antibodies (Bahn, 2001, Demirbilek et al., 2007, Weetman, 1991, El-Kaissi et al., 2004).

#### 4.1.2.1. Epidemiology of Thyroid Associated Ophthalmopathy

TAO is thought to affect an estimated 400,000 individuals in the United Kingdom (based on a population of 59 million people) with a prevalence of TAO in Graves' disease at 25-50% (Cawood et al., 2004). Women are 5 times more likely to develop the condition when compared to men, however when men develop the condition, the condition is likely to be more severe, particularly with increasing age (Marcocci et al., 1989, Kendler et al., 1993, Manji et al., 2006, Perros et al., 1993).

There have been many susceptibility genes that have been proposed to be involved in TAO including human leukocyte antigen (HLA, 6p21.3) (Kendalltaylor et al., 1988), cytotoxic T-lymphocyte antigen 4 (CTLA-4, 2q33) (Vaidya et al., 1999), tumour necrosis factor (TNF, 6p21.3) (Kamizono et al., 2000), interferon gamma (IFNγ, 12q14) (Kaijzel et al., 1998), intracellular adhesion molecule-1 (ICAM-1, 19p13) (Kretowski et al., 2003) and thyroid-stimulating hormone receptor (TSHR, 14q31) (Bahn et al., 1994). However, the results of these studies are not conclusive and need further replications with larger cohorts. A recently discovered susceptibility gene, IL-23R, has been associated with several autoimmune diseases that include Crohn's disease (Duerr et al., 2006), rheumatoid arthritis (Farago et al., 2008) and Graves' disease (Huber et al., 2008). IL-23R is thought

to be involved promoting cell survival of Th1- derived cells and production of IL-17 produced from Th17 cells that have been implicated in chronic inflammatory responses and tissue damage (Lankford and Frucht, 2003).

Finally, another factor to affect progression of TAO is smoking. It is actually considered to be the most important factor and has an independent association with ophthalmopathy as there is increasing evidence that individuals who smoke while being affected by Graves' disease are more likely to have an increased risk of TAO compared to individuals that do not smoke (Vestergaard, 2002, Manji et al., 2006, Shine et al., 1990, Bartalena et al., 1989, Prummel and Wiersinga, 1993, Cawood et al., 2007).

#### 4.1.2.2. Pathogenesis of Thyroid Associated Ophthalmopathy

Individuals with severe TAO are frequently found to have high levels of circulating thyrotropin receptor antibodies (Gerding et al., 2000). The thyrotropin-stimulating receptor (TSR) has also been found to be expressed on orbital differentiating preadipocytes (Valyasevi et al., 1999, Crisp et al., 2000). TSHR is considered to be a shared antigen in Graves' disease and TAO but this is yet to be proven. The events leading to progression of the disease process in TAO are considered to be quite complex and it may be the case that TSHR may be involved in mediating cytokine role in TAO (Cawood et al., 2004). Figure 4-2 shows an overview of the immune cells/mediators involved in the process of inflammation which can infiltrate orbital fibroblasts, preadipocytes, adipocytes and muscle cells in the pathogenesis of oedema, hyperplasia and hypertrophy that occurs in TAO.

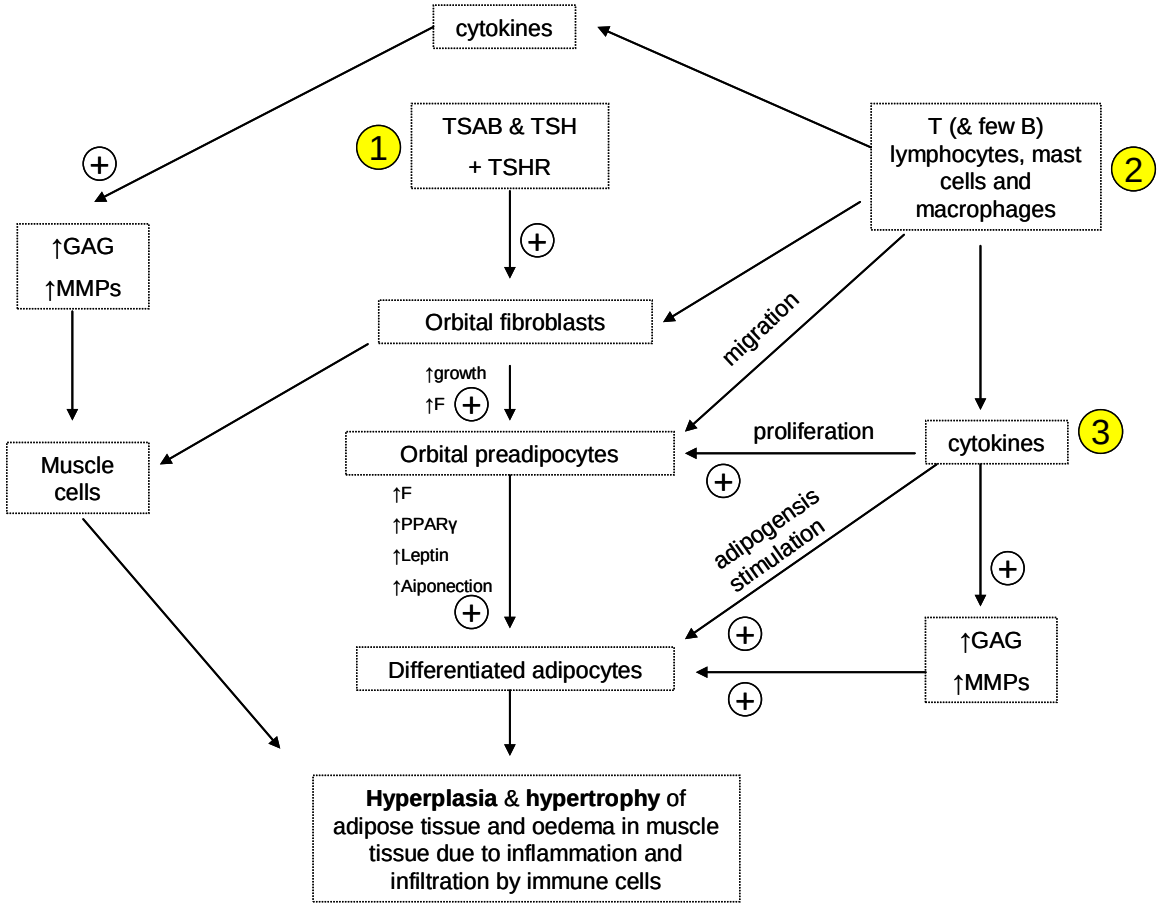



*Figure 4-2: Overview of the mediators of inflammation and adipogenesis which may be involved in expanding of orbital fibroblasts and orbital adipocytes in orbital adipose tissue.*

*1. Thyroid stimulating antibodies (TSAB) and TSH interact with the TSHR to activate orbital fibroblasts. This increases cell growth and cortisol production which causes a change in morphology to orbital preadipocytes. Preadipocytes release adipogenic factors such as PPARγ, leptin and adiponectin and cortisol and induces differentiation to mature-like adipocytes. 2. T and some B lymphocytes release cytokines such as IL-6 to increase glycosaminoglycan (GAG) and matrixmetalloproteinase production which cause muscle oedema. 3. Cytokines released from immune cells such as T lymphocytes increase preadipocyte proliferation and stimulate the differentiation process of preadipocytes to mature-like adipocytes. These actions ultimately result in the hyperplasia and hypertrophy of adipose tissue and oedma in muscle tissue that causes the clinical presentation associated with thyroid associated ophthalmopathy.*

#### *4.1.2.2.1 Thyroid Stimulating Autoantibodies*

Reports have shown that TSHR antibodies are directed against orbital muscle and connective tissue, however, these antibodies are not restricted to these areas (Atkinson et

al., 1984, Kadlubowski et al., 1986). Previous data has suggest that autoantibodies may play a main role in TAO and recently there has been data on a possible involvement of insulin-like growth factor 1 receptor (IGF-R1) activated by immunoglobulins (IgG) from Graves disease patients which mediates stimulation of activated T cell infiltration into inflammatory areas (Garrity and Bahn, 2006, Tandon et al., 1994).

*4.1.2.2.2 The Role of T Lymphocytes*

There is increasing evidence suggesting a role for activated T cells in the pathogenesis of TAO which include T helper/inducer (Th1 $CD4^+$ cells) and T suppressor/cytotoxic (Th2 $CD8^+$ cells) which secrete certain cytokines that are involved in propagating the immune response, such as interleukin 2 (IL-2) from Th1 cells and IL-4 from Th2 cells (Otto et al., 1996, Grubeckloebenstein et al., 1994, Heufelder and Bahn, 1993a, Xia et al., 2006, Bahn, 2003). Additionally, macrophages are increased in TAO orbital adipose tissue compared to those without TAO and this may be mediated by the increase of monocyte chemotactic protein 1 (MCP-1), (Chen et al., 2008).

Adhesion molecules such as ICAM-1 (inter-cellular adhesion molecule-1), ELAM-1 (endothelial cell leukocyte adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and LFA-1 (lymphocyte function-associated antigen-1) stimulated by pro-inflammatory cytokines aid the infiltration of lymphocytes and monocytes into the orbit during the progression of TAO (Heufelder and Bahn, 1993b, Ludgate and Baker, 2002). Furthermore, chemoattractants such as IL-6, IL-16, IL-8, RANTES (regulated on activation, normal T expressed and secreted) and MCP-1 are available to traffic $CD4^+$ T cells into the orbit (Sciaky et al., 2000, Ludgate and Baker, 2002). The CD40/CD154 costimulatory pathway has been found to possibly induce the expression of IL-6 and IL-8 in orbital fibroblasts (Ludgate and Baker, 2004, Sempowski et al., 1998). When T cells

enter the orbit, the antigen presentation to these T cells by antigen presenting cells (APC) such as dendritic cells and macrophages is thought to be mediated through major histocompatibility complex (MHC) class II HLA DR antigen (Hiromatsu et al., 1995). The processing of antigen and presentation to lymphocytes could also involve heat shock proteins that have been shown to be expressed in severe cases of TAO orbital and pretibial fibroblasts (Heufelder et al., 1991, Heufelder et al., 1992b).

*4.1.2.2.3 Activation of Orbital Fibroblasts*

Activation of orbital fibroblasts during the course of TAO may undergo one of two pathways. One involves production of prostaglandins that induce inflammatory cytokines, glycosaminoglycans (GAGs), in which hyaluronan dominates. This causes GAGs to accumulate through the action of inflammatory cytokines such as leukoregulin (Smith et al., 1995b). Matrix metalloproteinases (MMPs) then aid the movement of water into the orbital tissues which is the characteristic swelling seen with orbital fibroblasts and muscles cells implicated in the pathogenesis of TAO (Bahn and Heufelder, 1993, Wang et al., 1996, Smith et al., 1991, Kaback and Smith, 1999). The ability of IFNγ to stimulate GAGs appears to be restricted to orbital fibroblasts, as the same effect is not seen in fibroblasts from the skin (Korducki et al., 1992). Moreover, other inflammatory cytokines in addition to thyrotropin, growth factors and immunoglobulins, do not have an effect on GAGs whereas IL-1α and TGFβ are potent stimulators, suggesting a differential role of inflammatory mediators in TAO (Bahn, 2003).

The other pathway of fibroblast activation during the inflammatory process in TAO could also involve a subset of fibroblasts known as preadipocytes undergoing a state of differentiation leading to adipogenesis (Sorisky et al., 1996, Crisp et al., 2000). Their proliferation can also be stimulated by cytokines which include IL-4, IL-1α, IGF-1, TGFβ

and PDGF-1, but glucocorticoids can oppose this effect (Bahn and Heufelder, 1993). In addition, there is an increase in expression of genes for leptin, adiponectin and peroxisome proliferators-activated receptor gamma (PPARγ) which are mediators and known markers of adipogenesis, within preadipocytes and differentiated adipocytes from TAO individuals, compared to unaffected individuals (Kumar et al., 2004). Other subsets of orbital fibroblasts that exist in the orbit include Thy-1 positive (CD90) and Thy-1 negative. These fibroblasts have an unknown function as yet, however, they may have some role in mediating the inflammation seen in TAO (Smith et al., 2002, Laura Koumas, 2002, Smith et al., 1995a).

#### *4.1.2.2.4 Smoking*

Smoking is associated with a poor prognosis for TAO and affects the incidence, severity and response to treatment of the disease (Thornton et al., 2006, Manji et al., 2006). The dose response of cigarette smoking is found to strongly correlate with TAO where patients who are current heavy smokers develop more severe TAO (Bartalena et al., 1989, Pfeilschifter et al., 1996). It has also been suggested that cigarette smoke may be involved in oxidative stress which has also been shown to correlate with disease activity of TAO (Tsai et al., 2008). Cigarette smoke increases GAG production and adipogenesis in an in-vitro model of TAO (primary cultures of orbital adipose tissue from TAO patients) and a correlation has been found with the increase in volume of connective tissue in certain parts of the orbit of TAO patients. Extraocular muscle volume, however, is not affected by smoking in TAO patients (Cawood et al., 2007, Szucs-Farkas et al., 2005).

#### *4.1.2.2.5 Animal Models*

The development of an animal model to understand pathogenic mechanisms in TAO dates back to Smelser in 1936. He reproduced signs of exophthalmos in guinea-pigs, first with

injection of pituitary extract and then a more severe case after thyroidectomy (Smelser, 1936). Other models include inserting TSHR primed T cells into BALBc (albino, laboratory-bred strain of the house mouse) and NOD (non-obese diabetic) mice, which produced thyroidtis (Many et al., 1999). When their orbits were examined, the BALBc mice showed signs of adipose tissue accumulation and cytokine infiltration into the orbit, where as NOD mice and control BALBc mice appeared to have no eye pathology (Many et al., 1999).

### 4.1.3. Hypothesis and Aims

To date, no studies have evaluated the putative role of 11β-HSD1 in the pathogenesis of TAO. It has already been found that macrophages express 11β-HSD1 and there is now evidence that TNFα can induce 11β-HSD1 activity and expression in subcutaneous and omental adipose tissue depots (Friedberg et al., 2003). The increase in 11β-HSD1 expression has already been linked to visceral obesity and the metabolic syndrome phenotype which consists of hypertension, insulin resistance and dyslipidaemia and involves the circulation of excess glucocorticoids as shown with the phenotype of Cushing's syndrome (Wake & Walker, 2004).

It may be possible that the release of other cytokines and growth factors released from immune cells such as macrophages may be involved in enhancing 11β-HSD1 mediated cortisol action. This could therefore contribute to enhanced differentiation involved in the adipogenesis process which occurs in TAO human orbital adipose tissue (HOAT) This may include hyperplasia (orbital adipose tissue proliferation) and hypertrophy (increase in size of orbital adipose tissue). As a consequence, direct or indirect inhibition (via the manipulation of inflammatory cytokines) of 11β-HSD1 may become a novel target for treatment of local adipose tissue inflammation

The research aims are therefore to:

- Compare SC and OM and HOAT depots morphologically and with PCR of glucocorticoid genes, including 11β-HSD1, GR, H6PD and some cytokines and chemokines.

- Study glucocorticoid activation in HOAT.

- Compare normal control HOAT (cHOAT), (HOAT which has had no known previous inflammation) vs. quiescent HOAT (qHOAT), (HOAT that was previously inflamed but is no longer inflamed) vs. inflamed HOAT (iHOAT), (active inflammation) tissue morphologically and study transcription of glucocorticoid-related genes (11β-HSD1, GRα, H6PD), adipose tissue differentiation-related genes (fatty acid binding protein 4 - FABP4 and glycerol-3-phosphate dehydrogenase - G3PDH), myeloid-related genes (Cluster of Differentiation 200 - CD200, CD123 and CD68), cytokine-related genes (IL-6, TNFα, TNFR, tumour necrosis factor- alpha induced protein - TNFαIP, IL-1β, IL-1R and transforming growth factor-beta - TGFβ), chemokine-related genes (chemokine (C-C motif) receptor 2 - CCR2 and chemokine (C-C motif) ligand 2 - CCL2) and TSHR.

- Compare 11β-HSD1 activity between preadipocytes from cHOAT tissue, qHOAT tissue and iHOAT.

### *4.2. Methodology*

The definitions of the samples used for the study are as follows:

cHOAT = normal control human orbital adipose tissue. These samples are taken from individuals that have no inflammation or TAO.

qHOAT = quiescent human orbital adipose tissue. These samples are taken from individuals that previously experienced TAO but have currently no clinical signs of inflammation.

iHOAT = inflamed human orbital adipose tissue. These samples are taken from individuals that are currently experiencing TAO and have clinical signs of inflammation which include exophthalmos (increased volume in the orbit which causes the eye to protrude from the eye socket), increased palpebral fissure width (separation between the upper and lower eyelids), conjunctival injection (non-uniform redness of the conjunctiva due to inflammation) and chemosis (swelling of the conjunctiva and the membranes lining the eyelids), (Yoon et al., 2009).

Throughout the whole of the study a total of 136 HOAT samples were collected (Table 4-1). Table 4-1 shows the age range and median age of the participants in the study. There were more women in the study compared to men (58 men compared to 87 women).

*Table 4-1: Sample demographics showing total number used for study, their age range and median age*

| | Total | cHOAT | qHOAT | iHOAT |
|---|---|---|---|---|
| **Number of Samples** | 136 | 66 | 51 | 17 |
| **Age range (years)** | 24-93 | 26-93 | 24-80 | 34-81 |
| **Median age (years)** | 61 | 69 | 52 | 56 |

Orbital adipose tissues were processed as described in chapter 2.2.3.2 in preparation of immunohistochemistry, cell culture and RNA extraction. Haematoxylin and eosin staining was performed on HOAT, SC and OM samples (chapter 2.6.2) while immunohistochemistry for CD68, 11β-HSD1 and 2 was performed on HOAT samples (chapter 2.6.3, 2.6.4 and 2.6.5). CD68 counts on HOAT, SC and OM samples were achieved by visually counting the $CD68^+$ cells in triplicate per slide for each adipose depot. Histomorhometric analyses were performed on HOAT, SC and OM samples as described in chapter 2.8. HOAT specimens were homogenised using a rotar-stator blade in a class 3 laminar flow cell culture hood. RNA extraction and subsequent Real-time RT-PCR was performed on whole HOAT, SC tissue and OM tissue samples (chapter 2.4 and 2.5.2). Real-time RT-PCR analysis was carried out on the genes: FABP4, CD163, CD68, G3PDH, GRα, H6PDH, and 11β-HSD1, IL-6, TNFα, TNFR, TNFαIP, CD200, CCR2, CCL2, IL-1β, IL-1R, TGFβ and TSHR.

Preadipocytes were cultured from HOAT samples (chapter 2.3.1.7) and differentiated as detailed in chapter 2.3.1.8. The 11β-HSD activity assay was performed the cultured preadipocytes from HOAT and separate assays were performed where cultures were co-incubated with either the cytokines TNFα (Peprotech, New Jersey), IL-6 (Peprotech, New Jersey), IL-1β (Peprotech, New Jersey) or TGFβ for 24 hours prior to carrying out 11β-

HSD enzyme assay. For assessment of proliferation, HOAT preadipocytes grown in a 96 well plate were assessed on day 1 (untreated). The cells were treated with 1μM cortisol (F) for 4 days and the cells untreated were assessed on day 4 of growth while the cells treated with 1μM cortisol (F) were also assessed on day 4 of growth. The proliferation assay was performed as detailed in chapter 2.12.

## *4.3. Results*

### 4.3.1. Comparative Analysis of Orbital, Subcutaneous and Omental Adipose Tissue Depots

#### 4.3.1.1. Characterisation of Whole Tissue Adipose Morphology

To compare whole cHOAT with subcutaneous (SC) and omental (OM) adipose tissue, a number of parameters were chosen to study differences in cell morphology. These were cell area, minimum and maximum diameter, radius ratio, roundness, minimum and maximum feret (dimensions are taken from a rectangle drawn around the cell) and perimeter.

Figure 4-3 shows an example of photographs of the three depots (SC, OM and HOAT) used for the histomorphometric analysis.

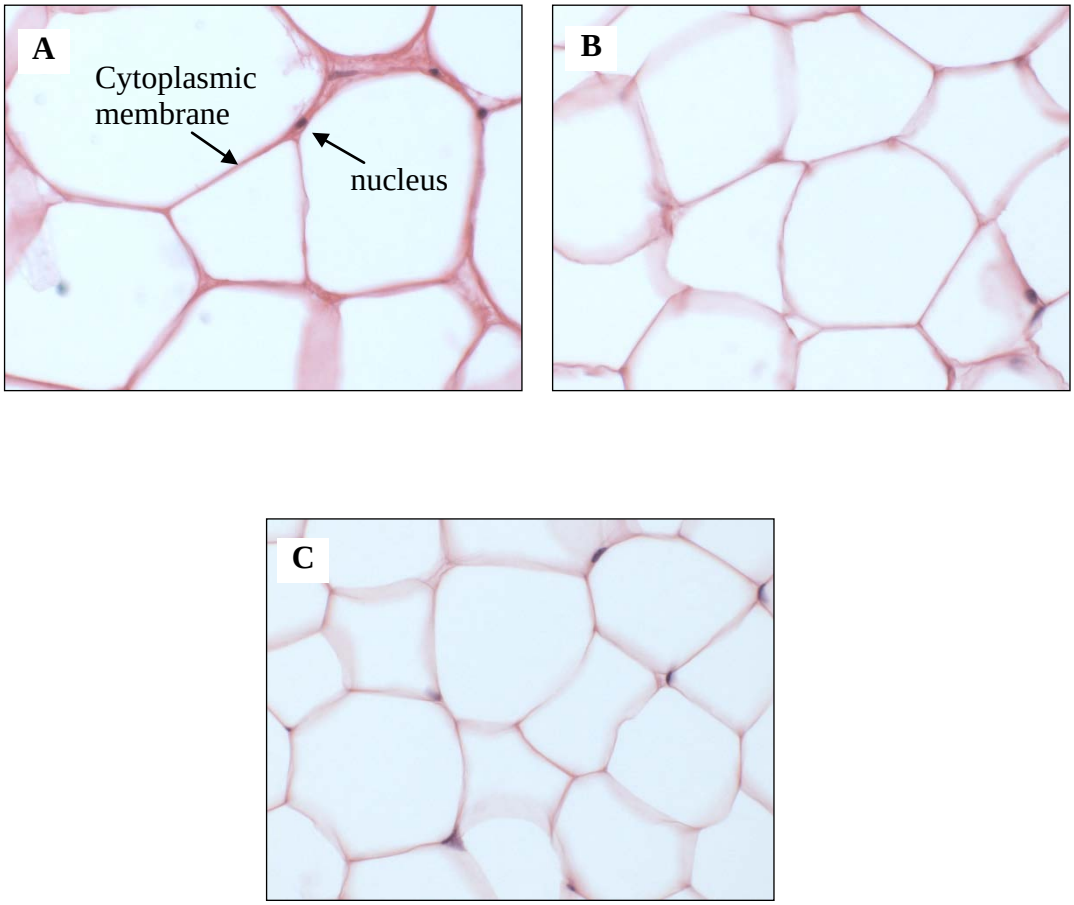
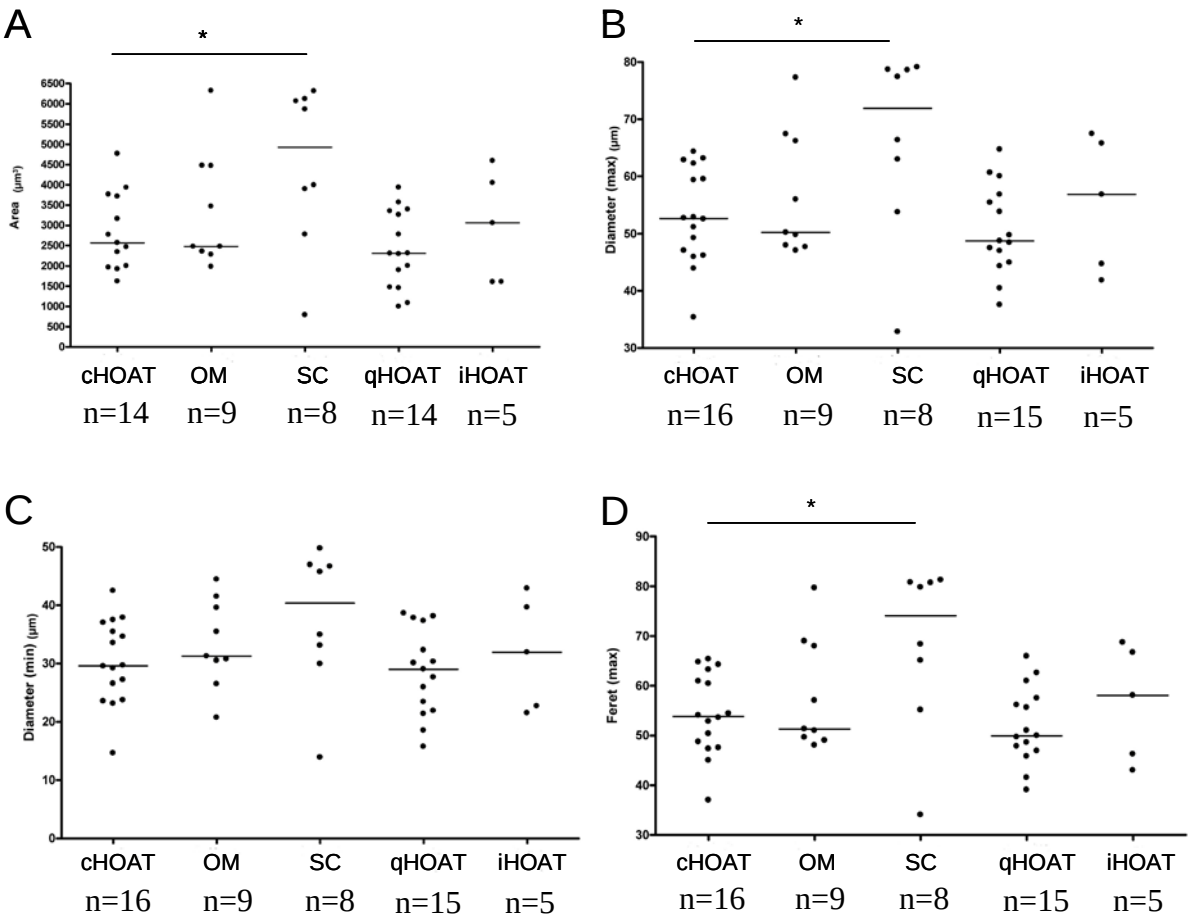



*Figure 4-3: Haematoxylin and Eosin staining in human adipose tissue.*

*(A) Human subcutaneous adipose tissue, (B) omental human adipose tissue, (C) normal control human orbital human adipose tissue. HOAT, human orbital adpose tissue. All at x 40 magnification*

When comparing area amongst the different adipose tissue types, cHOAT area was significantly smaller when compared to subcutaneous (SC) adipose (Figure 4-4). The cHOAT depot has a significantly lower maximum diameter (Figure 4-4), maximum feret (Figure 4-4), minimum feret (Figure 4-5), perimeter (Figure 4-5) and radius ratio (Figure 4-5) when compared to SC adipose. The measure of roundness showed that cHOAT appears to be significantly rounder when compared to OM (Figure 4-5). When the

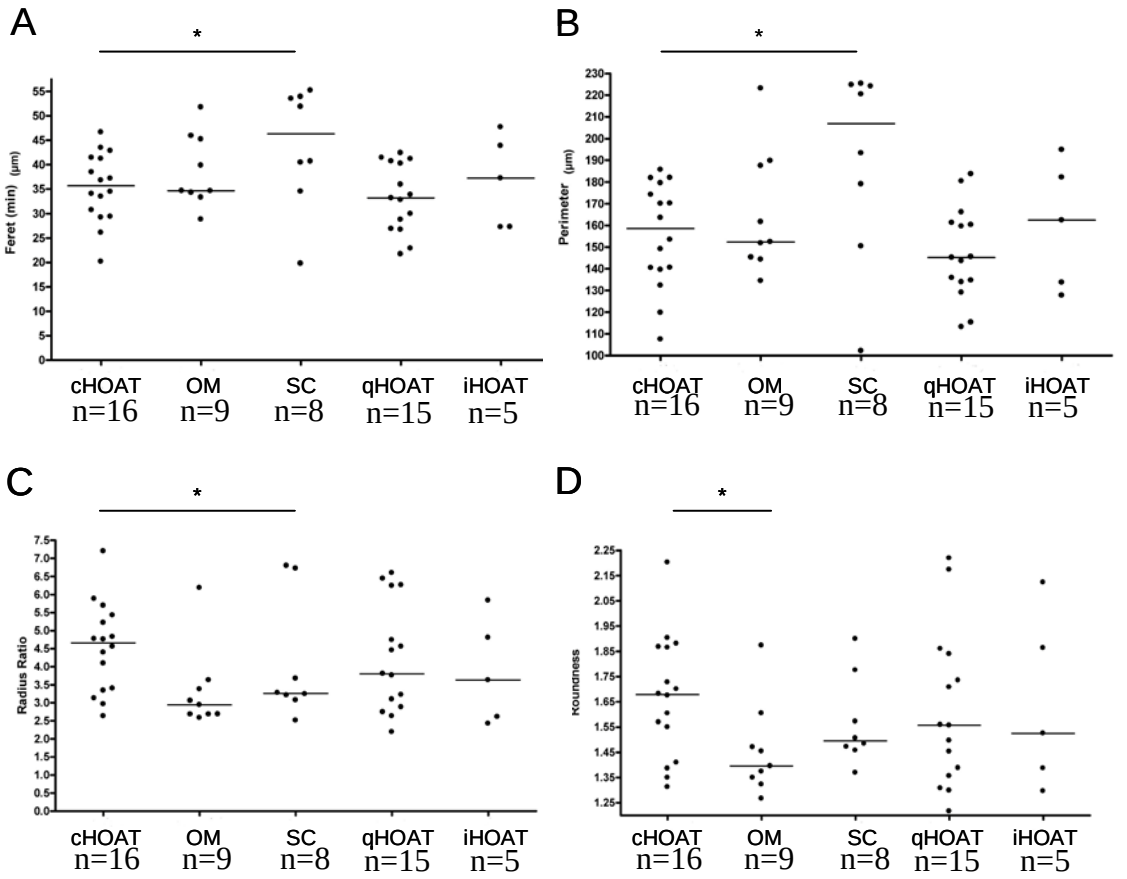
minimum diameter of cHOAT was compared to the other depots (SC, OM, qHOAT and iHOAT), there was no significant difference (Figure 4-4). The cHOAT depot was not found to be significantly different in area, maximum diameter, minimum diameter, maximum feret, minimum feret, perimeter, radius ratio or roundness when compared to qHOAT and iHOAT. These parameters from cHOAT were not significant when compared to OM except for roundness which was found significant. Figure 4-4 A, B and Figure 4-5 B are different from figure 1 published in the paper Bujalska, 2007 due to different presentation of the values in the graph. Figure 1 from the published paper Bujalska, 2007 refers to the means of the values for each of the parameters. Figure 4-4 A, B and Figure 4-5 B was produced from a different data set and the graphs show the median value and the full ranges for the parameters in each HOAT, OM and SC tissue sample.




| | | cHOAT | OM | SC | qHOAT | iHOAT |
|---|---|---|---|---|---|---|
| **Area** | *min* | 1620.99 | 1981.82 | 790.61 | 996.58 | 1604.69 |
| | *max* | 4771.71 | 6325.23 | 6313.72 | 3938.06 | 4593.11 |
| **Max Diameter** | *min* | 35.39 | 47.07 | 32.83 | 37.57 | 41.85 |
| | *max* | 64.36 | 77.31 | 79.13 | 64.74 | 67.48 |
| **Min Diameter** | *min* | 14.65 | 20.72 | 13.93 | 15.74 | 21.52 |
| | *max* | 42.47 | 44.42 | 49.73 | 38.62 | 42.89 |
| **Max Feret** | *min* | 37.01 | 48.03 | 34.06 | 39.08 | 43.03 |
| | *max* | 65.37 | 79.66 | 81.25 | 65.92 | 68.72 |

*Figure 4-4: Histomorphometic analysis of whole adipose tissue depots part 1.*

*The cHOAT depot has significantly ($p<0.05$) smaller area (A), maximum diameter (B) and maximum feret when compared to SC (D). The cHOAT was not found to be significantly different in minimum feret with any of depots (SC, OM, qHOAT and iHOAT), (C). Data in table shows the full ranges. The median line for each parameter is shown on the graph. OM, omental; SC, subcutaneous. cHOAT, control human orbital adipose tissue; qHOAT, queiscent control human orbital adipose tissue; iHOAT, inflamed control human orbital adipose tissue.*




| | | cHOAT | OM | SC | qHOAT | iHOAT |
|---|---|---|---|---|---|---|
| **Min Feret** | *min* | 20.18 | 28.79 | 19.80 | 21.73 | 27.26 |
| | *max* | 46.67 | 51.78 | 55.22 | 42.41 | 47.69 |
| **Perimeter** | *min* | 107.47 | 134.47 | 102.18 | 113.24 | 127.76 |
| | *max* | 185.70 | 223.18 | 225.38 | 183.67 | 194.87 |
| **Radius Ratio** | *min* | 2.63 | 2.59 | 2.52 | 2.19 | 2.43 |
| | *max* | 7.20 | 6.19 | 6.80 | 6.60 | 5.84 |
| **Roundness** | *min* | 1.31 | 1.27 | 1.37 | 1.22 | 1.30 |
| | *max* | 2.20 | 1.87 | 1.90 | 2.22 | 2.12 |

*Figure 4-5: Histomorphometic analysis of whole adipose tissue depots part 2.*

*The cHOAT depot has significantly ($p<0.05$) smaller minimum feret (A), perimeter (B) and radius ratio when compared to SC (C). The cHOAT was also significantly ( $p<0.05$) rounder when compared to OM (D). Data in table shows the full ranges. The median line for each parameter is shown on the graph. OM, omental; SC, subcutaneous. cHOAT, control human orbital adipose tissue; qHOAT, queiscent control human orbital adipose tissue; iHOAT, inflamed control human orbital adipose tissue.*

#### 4.3.1.2. Evaluation of $CD68^+$ protein and mRNA In Human Control Orbital, Subcutaneous and Omental Adipose Tissue

The CD68 antibody was used to identify $CD68^+$ cells that could be representative of resident macrophages within adipose tissue. CD68 positive ($CD68^+$) cells were found in the thin stromal beds of the normal control HOAT samples, SC and OM adipose tissue samples (Figure 4-6). When comparing the number of $CD68^+$ cells in each adipose tissue, there is significantly ($p<0.001$) higher counts per field of view from cHOAT compared SC and OM (Figure 4-7). Real-time RT-PCR analysis found that CD68 mRNA expression was significantly higher in cHOAT compared to SC and OM (Figure 4-8).

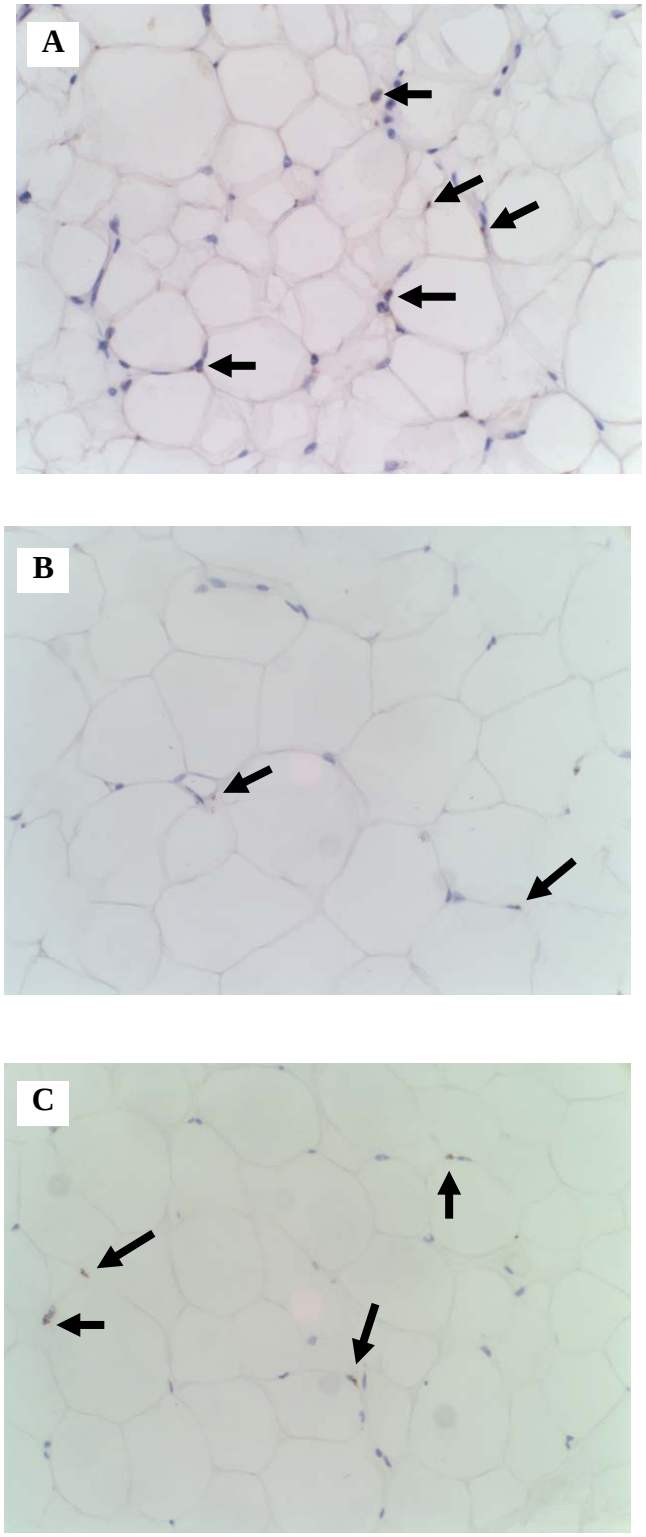



*Figure 4-6: Immunohistochemistry in human adipose tissue with CD68.*

*$CD68^+$ stained cells (arrowed) in cHOAT (A), SC adipose (B) and OM adipose (C). cHOAT, control human orbital adipose tissue; SC, subcutaneous; OM, omental. All at x 20 magnification*

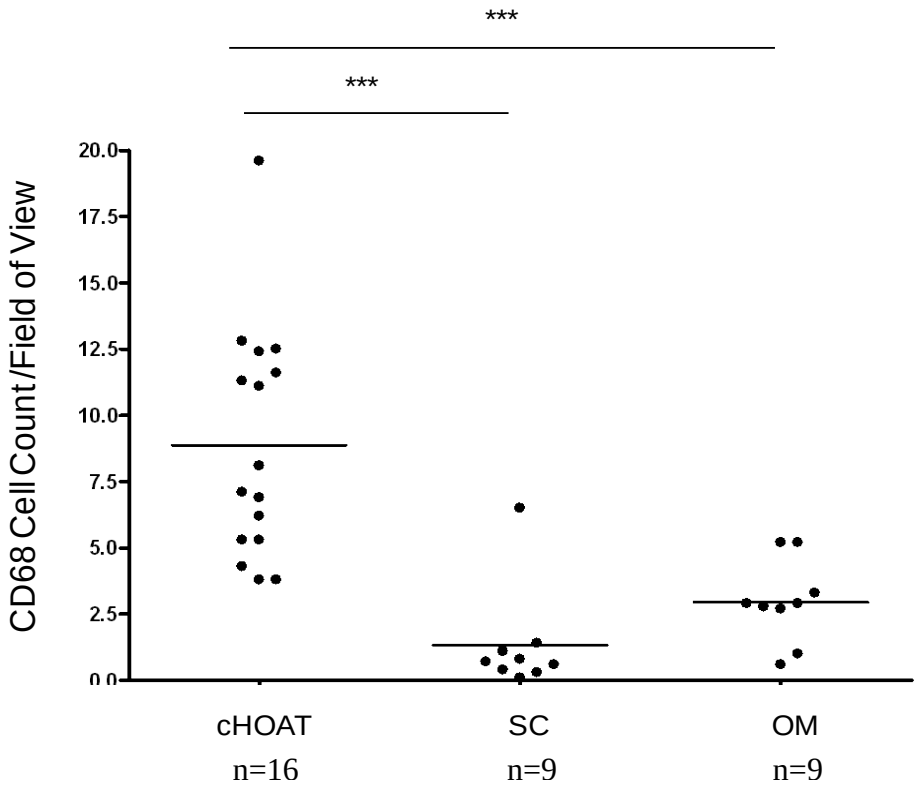
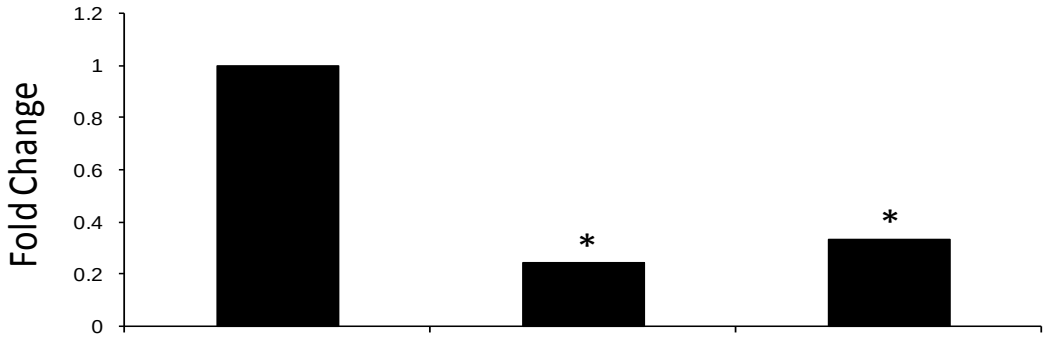



*Figure 4-7: Comparison of CD68+ cell count in human adipose tissue.*

*Cell count of CD68$^+$ stained cells revealed cHOAT as having the significantly ($p<0.001$) larger basal population of macrophages when compared to SC and OM. Results displayed are medians with full ranges. cHOAT, control human orbital adipose tissue; OM, omental; SC, subcutaneous.*




| | cHOAT | SC | OM |
|---|---|---|---|
| dCt | 14.06 | 16.10 | 15.65 |
| *SEM* | 0.26 | 0.24 | 0.63 |

*Figure 4-8: Real-time RT-PCR analysis of CD68+in human adipose tissue depots.*

*There is significantly higher expression ($p<0.05$) from cHOAT compared to SC and OM. Results are expressed as fold change when compared to cHOAT and dCt and standard error of the mean (SEM) values are shown in table.*

#### 4.3.1.3. Localisation of 11β-HSD1 in Control Human Orbital Adipose Tissue

11β-HSD1 immunoreactivity is localised to the cytoplasm of control HOAT adipocytes (Figure 4-9). The preabsorbed 11β-HSD1 antibody with immunising peptide was used as a control for the staining (Figure 4-9). The positive control tissue used for this staining was human liver which was found centripetally around the central vein (Figure 4-9). 11β-HSD2 immunostaining was also performed on cHOAT; however, no staining was found (Figure 4-9). The positive control for this was human kidney where staining was found on many tubules of the kidney cortex (Figure 4-9).

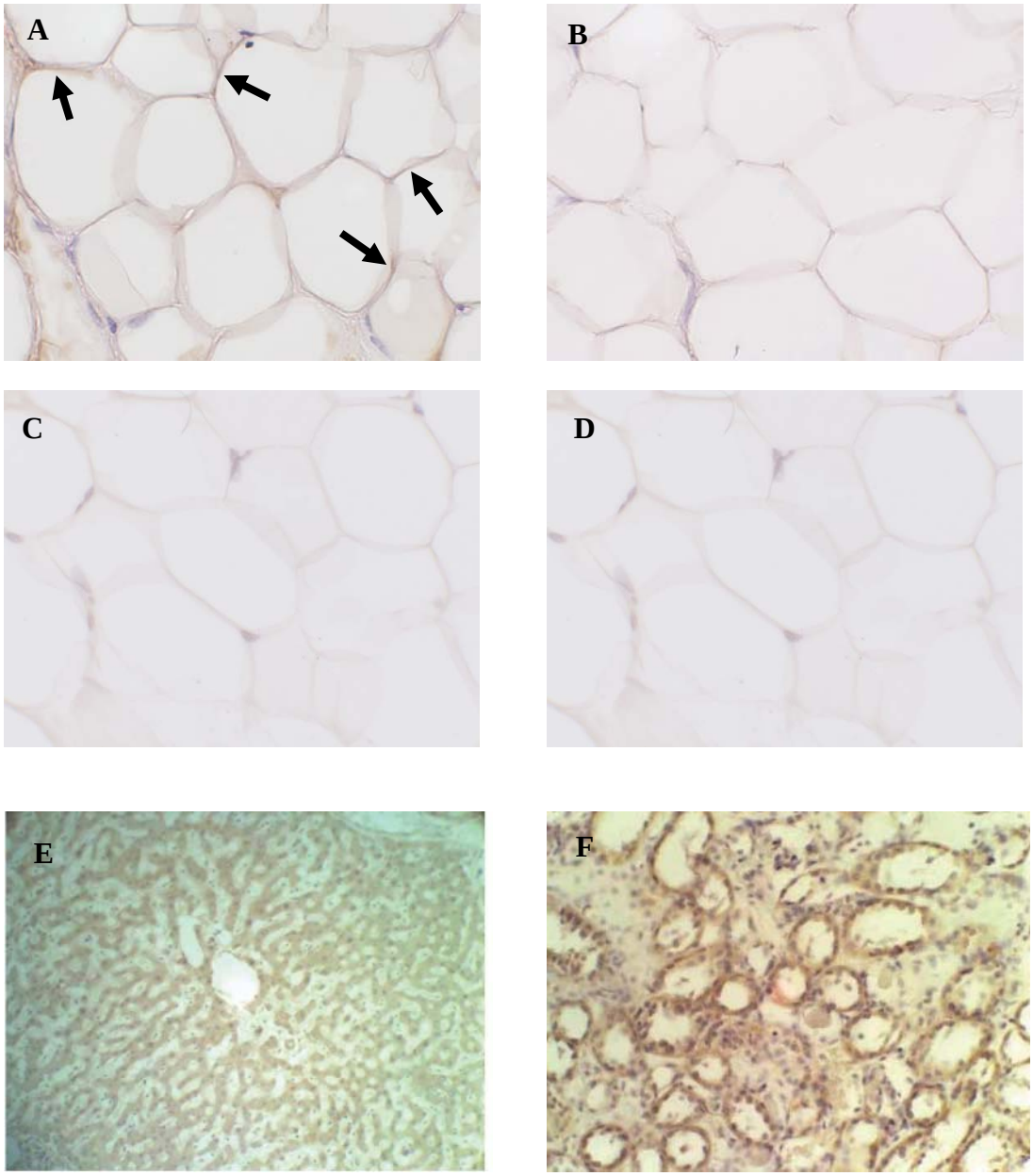
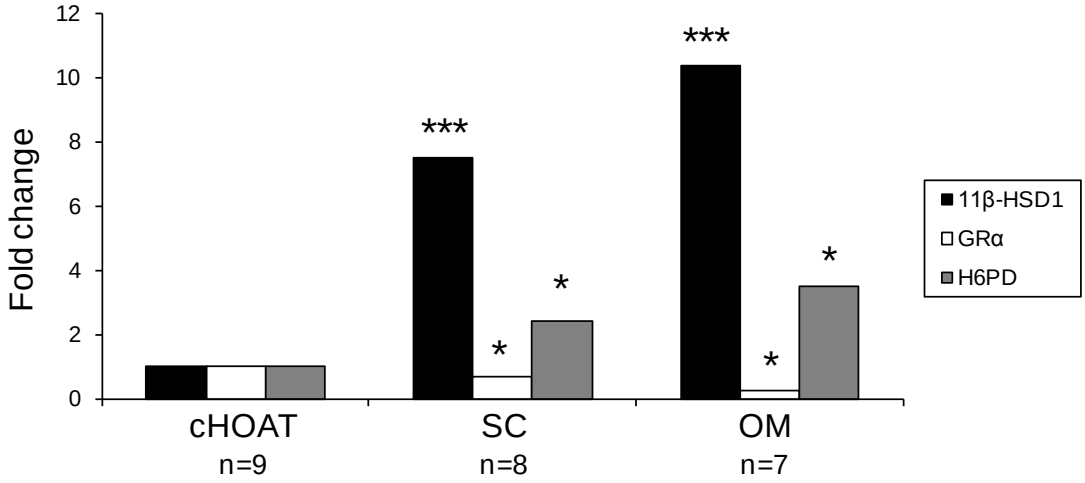



*Figure 4-9: Immunohistochemistry in human control orbital adipose tissue and control tissues with 11β-HSD1 and 11β-HSD2*

*Immunoreactivity for 11β-HSD1 (arrowed) in cHOAT (x 40 magnification), (A), compared to negative control section which is 11β-HSD1 preabsorbed with immunising peptide (x 40 magnification), (B). No 11β-HSD2 was found in cHOAT (x 40 magnification), (C) and negatve control (x 40 magnification), (D). The positive control for 11β-HSD1 was human liver, (x 20 magnification), (E) and for 11β-HSD2 was human kidney cortex, (x 20 magnification), (E). cHOAT, normal control human orbital adipose tissue. Arrows represent examples of 11β-HSD1 staining.*

### 4.3.1.4. Evaluation of Glucocorticoid-Related and Marker of Adipogenesis-Related Gene Expression in Whole Adipose Tissue Depots

Real-time RT-PCR analysis of glucocorticoid-related genes (11β-HSD1, GR and H6PD) showed that basal levels of 11β-HSD1 and H6PD mRNA expression were significantly higher in SC ($p<0.001$ and $p<0.05$ respectively) and OM ($p<0.001$ and $p<0.05$ respectively) compared cHOAT (Figure 4-10). GR mRNA expression was significantly lower in SC ($p<0.05$) and OM ($p<0.05$) compared to cHOAT (Figure 4-10).

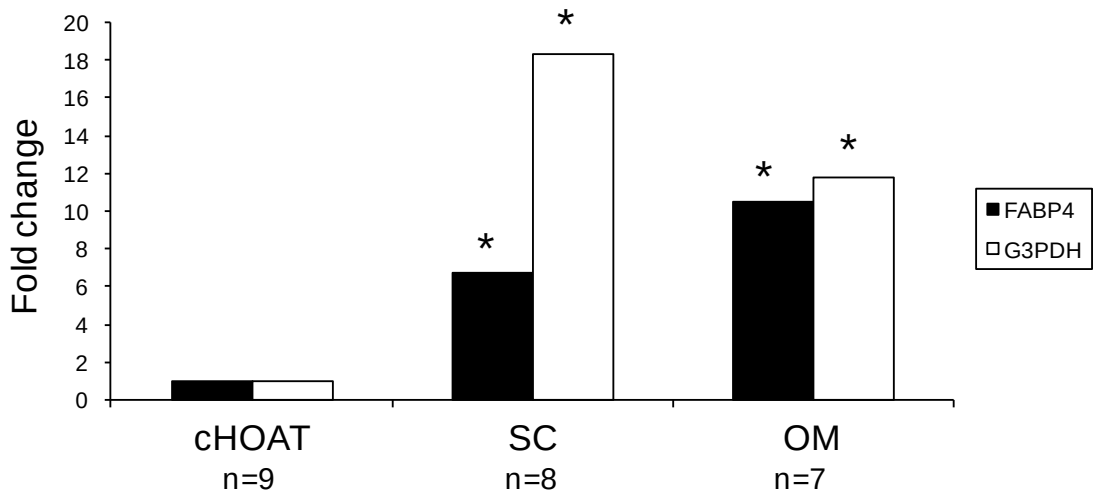



| dct/SEM | cHOAT | SC | OM |
|---|---|---|---|
| 11β-HSD1 | 17.30 | 14.39 | 13.92 |
| *SEM* | 0.33 | 0.24 | 0.45 |
| GRα | 15.57 | 16.09 | 17.59 |
| *SEM* | 0.61 | 0.54 | 0.91 |
| H6PD | 16.89 | 14.32 | 15.07 |
| *SEM* | 0.60 | 0.48 | 0.60 |

*Figure 4-10: Real-time RT-PCR analysis of glucocorticoid-related genes in human adipose tissue depots.*

*11β-HSD1 and H6PD mRNA expression is significantly higher in SC ($p<0.001$ and $p<0.05$ respectively) and OM ($p<0.001$ and $p<0.05$ respectively) compared to cHOAT. GRα mRNA expression is significantly lower in SC and OM compared to cHOAT ($p<0.05$). Results are expressed as fold change when compared to cHOAT and dCt and standard error of the mean (SEM) values are shown in table.*

Real-time RT-PCR analysis of markers of differentiation genes (FABP4 and G3PDH) showed that basal levels of FABP4 and G3PDH were significantly higher in SC ($p<0.05$ for both FABP4 and G3PDH) and OM ($p<0.05$ for both FABP4 and G3PDH) compared cHOAT (Figure 4-11).




| dct/SEM | cHOAT | SC | OM |
|---|---|---|---|
| FABP4 | 11.41 | 7.38 | 8.02 |
| *SEM* | 0.48 | 0.70 | 1.00 |
| G3PDH | 15.13 | 9.64 | 11.57 |
| *SEM* | 0.66 | 0.59 | 0.91 |

*Figure 4-11: Real-time RT-PCR analysis of adipogeneic-related genes in human adipose tissue depots.*

*FABP4 and G3PDH mRNA expression is significantly higher in SC ($p<0.05$ for both FABP4 and G3PDH) and OM ($p<0.05$ for both FABP4 and G3PDH) compared to cHOAT. Results are expressed as fold change when compared to cHOAT and dCt and standard error of the mean values are shown in table.*

### 4.3.2. Comparative Analysis of Control Orbital, Quiescent and Inflamed Orbital Adipose Tissue

#### 4.3.2.1. Characterisation of Whole Tissue Morphology

When comparing cHOAT with qHOAT and iHOAT (Figure 4-12), there were no significant differences in the morphological parameters studied (area, diameter, perimeter, radius ratio, roundness and feret), (Figure 4-4 and Figure 4-5).

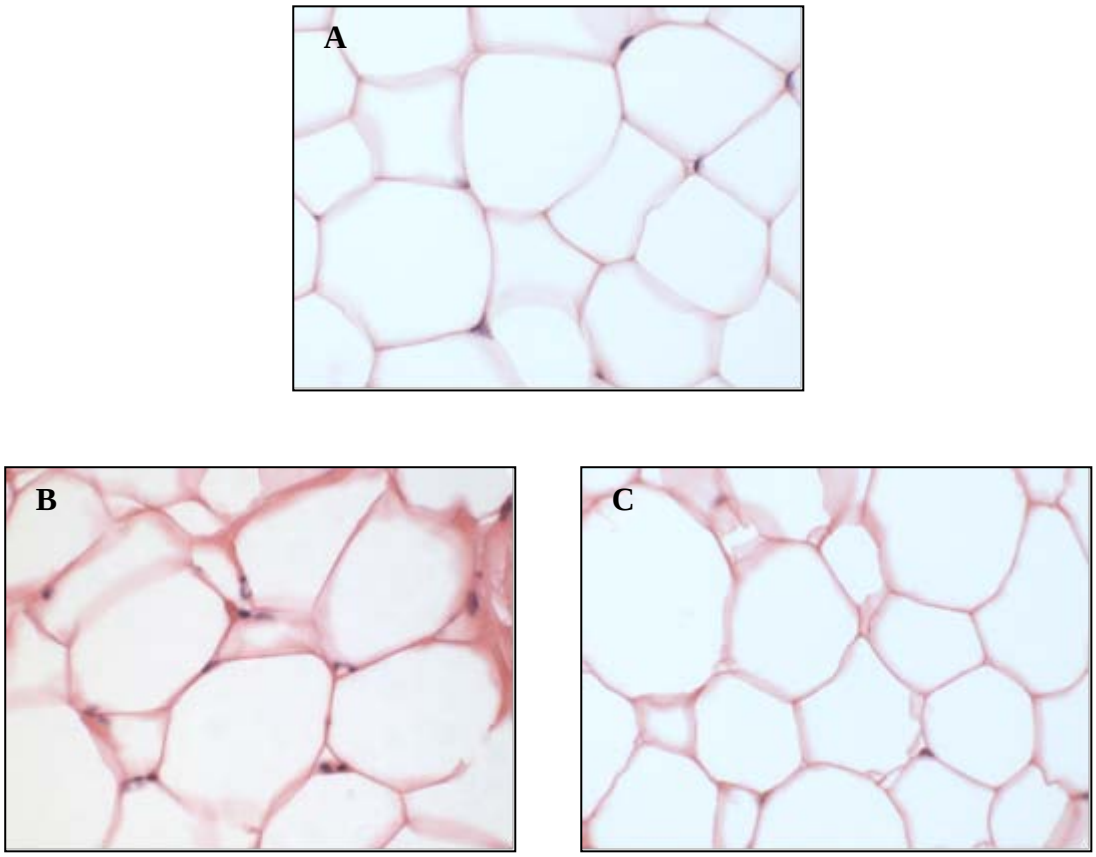



*Figure 4-12: Haematoxylin and Eosin staining in human orbital adipose tissue.*

*(A) control orbital adipose tissue (B) quiescent orbital adipose tissue and (C) inflamed orbital adipose tissue.*

#### 4.3.2.2. Evaluation of CD68 Positive Protein and mRNA in Human Control and Quiescent Orbital Adipose Tissue

Two methodologies were employed to assess the relative levels of CD68 in orbital adipose tissue: cell staining and mRNA expression. Using a specific antibody, $CD68^+$ cells were localised to the stromal bed of qHOAT (Figure 4-13).

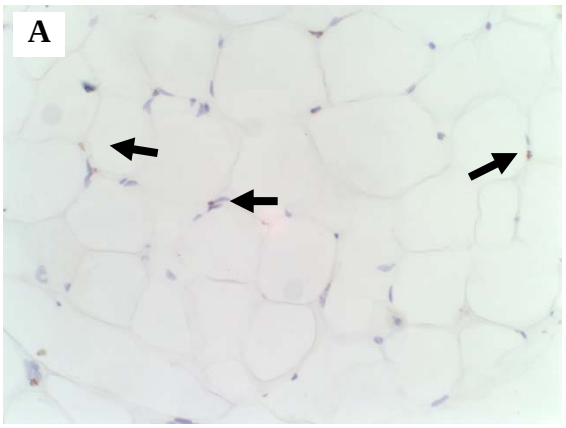



*Figure 4-13: Immunohistochemistry in human orbital adipose tissue with CD68.*

*Localisation of $CD68^+$ (arrowed) in stromal bed of qHOAT. qHOAT, queiscent human orbital adipose tissue at x 20 magnification.*

When control HOAT was compared with inactive TAO HOAT, there was also no significant difference in the number of $CD68^+$ cells per area (Figure 4-14).

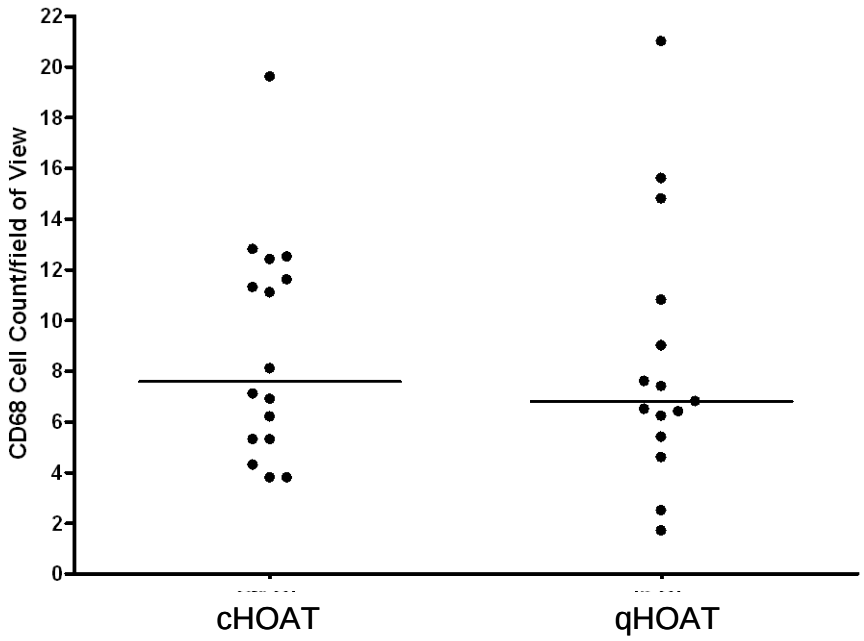
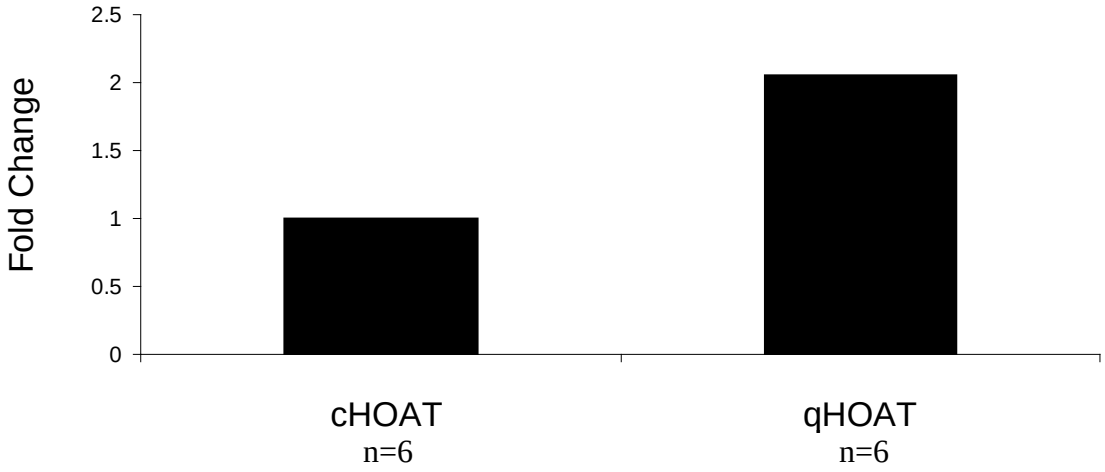



*Figure 4-14: Comparison of CD68$^+$ cell count in human orbital adipose tissue.*

*Cell count of CD68$^+$ staining macrophages revealed no difference in macrophage populations between cHOAT and qHOAT samples. Results displayed are medians with full ranges. cHOAT, control human orbital adipose tissue; qHOAT, queiscent human orbital adipose tissue.*

When comparing CD68$^+$ mRNA expression between cHOAT with qHOAT, expression was higher in inactive TAO HOAT but was found non-significant (Figure 4-15).




| | cHOAT | qHOAT |
|---|---|---|
| dCt | 16.09 | 15.05 |
| SEM | 0.32 | 0.35 |

*Figure 4-15: Real-time RT-PCR analysis of CD68$^+$ in human orbital adipose tissue.*

*CD68$^+$ showed no significant difference in expression between cHOAT and qHOAT. Results are expressed as fold change when compared to cHOAT and dCt and standard error of the mean values are shown in table.*

#### 4.3.2.3. Localisation of 11β-HSD1 in Quiescent and Inflamed Human Orbital Adipose Tissue

11β-HSD1 was localised to the thin film of cytoplasm in qHOAT cells compared to the negative control (preabsorbed 11β-HSD1 with immunising peptide), (Figure 4-16 and Figure 4-16). Similarly to qHOAT, 11β-HSD1 immunoreactivity was also localised to the cytoplasm of the iHOAT cells when compared to its control (Figure 4-16 and Figure 4-16). The positive control tissue used for this staining was human liver (Figure 4-16).

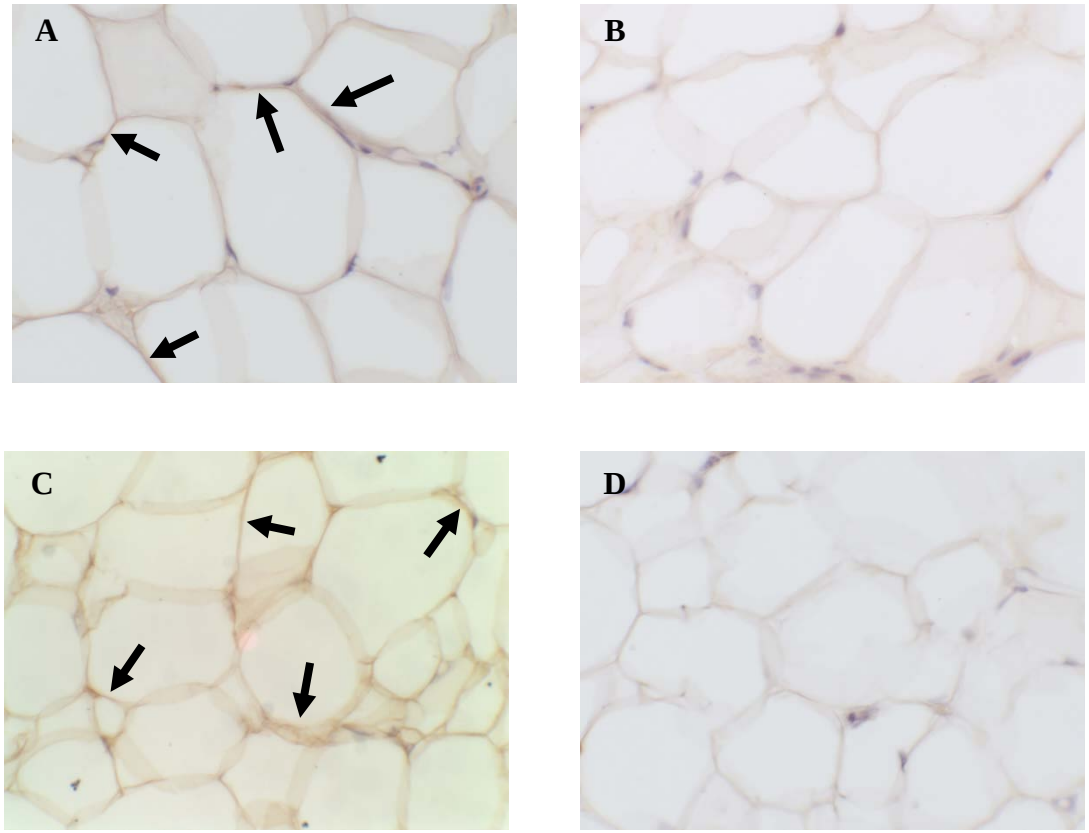



*Figure 4-16: Immunohistochemistry in human orbital adipose tissue with 11β-HSD1.*

*Immunoreactivity for 11β-HSD1 in qHOAT (A), compared to negative control (B) and 11β-HSD1 in iHOAT (C) compared to negative control section (D) which was 11β-HSD1 antibody pre-absorbed with immunising peptide. qHOAT, quiescent human orbital adipose tissue; iHOAT, inflamed human orbital adipose tissue. Arrows correspond to examples of 11β-HSD1 staining. All at x 40 magnification.*

#### 4.3.2.4. Immunohistochemistry with 11β-HSD2 in Quiescent and Inflamed Human Orbital Adipose Tissue

There was no expression of 11β-HSD2 found in qHOAT and iHOAT (Figure 4-17) when compared to their negative control sections (Figure 4-17).

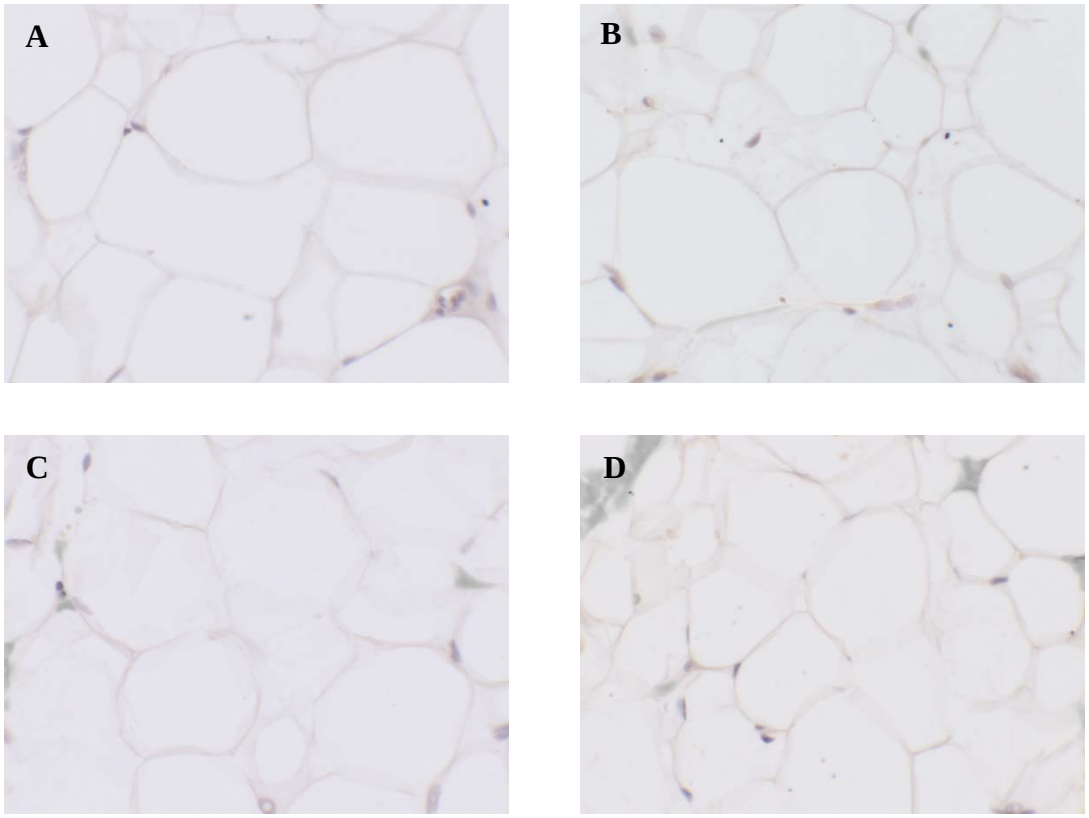
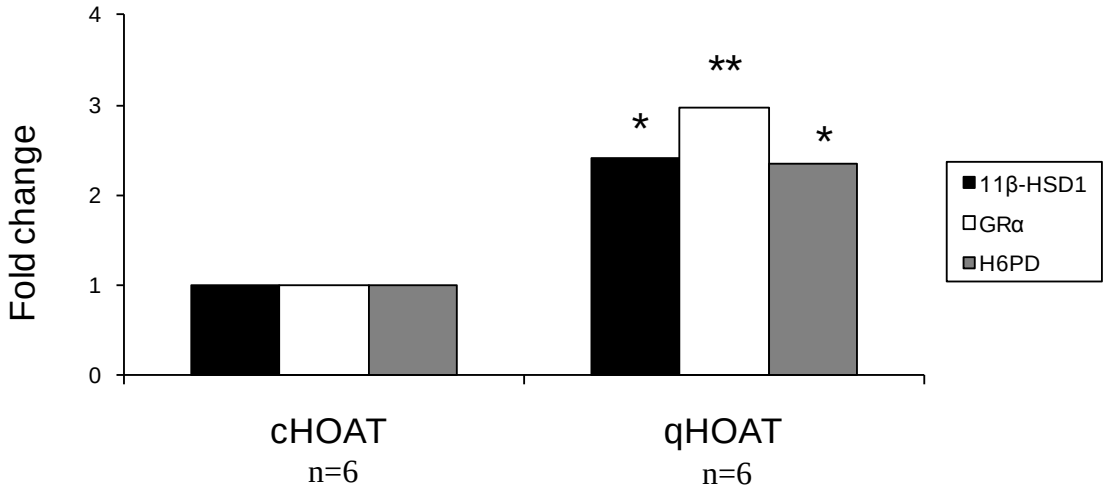



*Figure 4-17: Immunohistochemistry in human orbital adipose tissue with 11β-HSD2.*

*11β-HSD2 immunohistochemistry showed no expression within qHOAT (A) vs negative control (B) and iHOAT (C) vs negative control (D). All at x 40 magnification. Negative control was no primary antibody.*

#### 4.3.2.5. Evaluation of Glucocorticoid-Related Gene Expression and Marker of Adipogenesis-Related Gene Expression in Whole Orbital Adipose Tissue

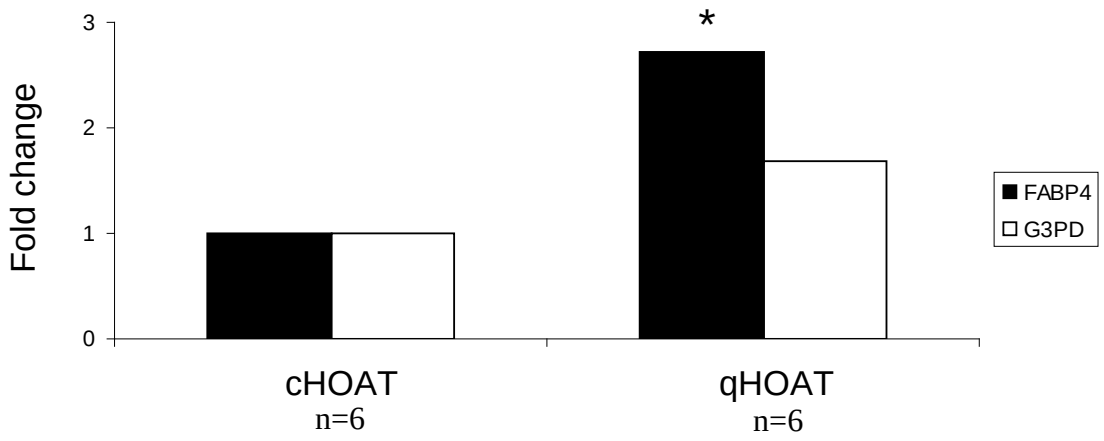
Real-time RT-PCR analyses revealed that qHOAT exhibited significantly higher expression of 11β-HSD1 when compared to cHOAT (Figure 4-18). This was also true for the glucocorticoid related genes, H6PD and GRα (Figure 4-18). There was significantly higher FABP4 expression, a known marker of adipogenesis in qHOAT compared to cHOAT. Although higher expression was shown for the marker of adipogenesis, G3PDH, this was found not to be significant (Figure 4-19).




| dct/SEM | cHOAT | qHOAT |
|---|---|---|
| 11β-HSD1 | 17.86 | 16.59 |
| *SEM* | 0.26 | 0.50 |
| GRα | 15.87 | 14.29 |
| *SEM* | 0.40 | 0.22 |
| H6PD | 15.39 | 14.15 |
| *SEM* | 0.13 | 0.50 |

*Figure 4-18: Real-time RT-PCR analysis of glucocorticoid-related genes in human orbital adipose tissues.*

*qHOAT showed significantly higher 11β-HSD1 ($p<0.05$) , GRα ($p<0.05$) and H6PD ($p<0.01$) mRNA expression in qHOAT compared to cHOAT. Results are expressed as fold change when compared to cHOAT and dCt and standard error of the mean values are shown in table.*



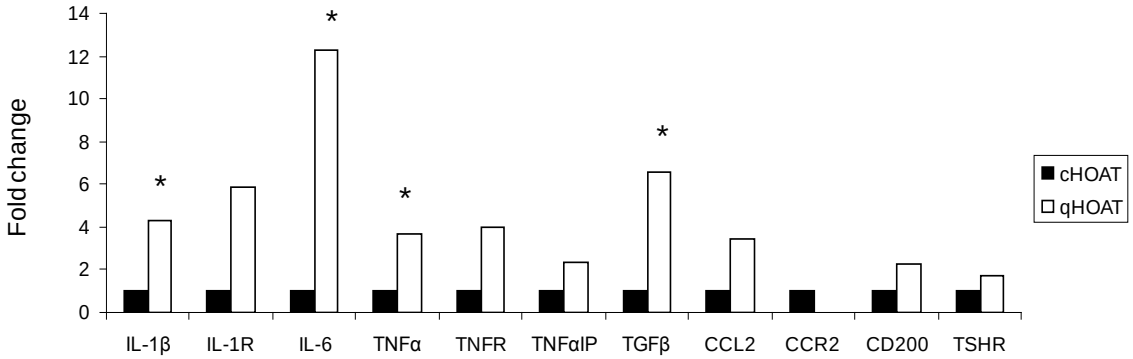

| dct/SEM | cHOAT | qHOAT |
|---|---|---|
| FABP4 | 12.57 | 11.13 |
| *SEM* | 0.34 | 0.29 |
| G3PDH | 13.63 | 12.89 |
| *SEM* | 0.22 | 0.24 |

*Figure 4-19: Real-time RT-PCR analysis of adipogeneic-related genes in human orbital adipose tissue.*

*FABP4 mRNA expression in qHOAT was significantly higher when compared to cHOAT. G3PDH was higher in qHOAT compared to cHOAT but was found not to be significant. Results are expressed as fold change when compared to cHOAT and dCt and standard error of the mean values are shown in table.*

#### 4.3.2.6. Evaluation of Cytokine-Related Gene Expression in Whole Orbital Adipose Tissue

There is higher expression of all of the inflammatory cytokine related gene expression (IL-1β, IL-R, IL-6, TNFα, TNFR, TNFαIP, TGFβ, CCL2, CD200 and TSHR) in qHOAT compared to cHOAT except for CCR2 where there was no expression in cHOAT or qHOAT (Figure 4-20). The significantly expressed genes in qHOAT compared to cHOAT were IL-1β ($p<0.05$), IL-6 ($p<0.05$), TNFα ($p<0.05$) and TGFβ ($p<0.05$).




| dct/SEM | cHOAT n=6 | qHOAT n=6 |
|---|---|---|
| IL-1β | 22.37 | 20.26 |
| *SEM* | 0.19 | 0.84 |
| IL-1R | 24.16 | 18.66 |
| *SEM* | 0.33 | 0.55 |
| IL-6 | 21.52 | 17.90 |
| *SEM* | 0.75 | 0.97 |
| TNFα | 23.10 | 21.21 |
| *SEM* | 0.37 | 0.59 |
| TNFR | 16.06 | 14.08 |
| *SEM* | 0.51 | 0.48 |
| TNFαIP | 20.71 | 19.49 |
| *SEM* | 0.18 | 0.43 |
| TGFβ | 23.12 | 20.41 |
| *SEM* | 0.38 | 0.50 |
| CCL2 | 18.81 | 17.04 |
| *SEM* | 0.54 | 0.77 |
| CCR2 | 0 | 0 |
| *SEM* | 0 | 0 |
| CD200 | 19.83 | 12.89 |
| *SEM* | 0.36 | 0.40 |
| TSHR | 21.31 | 20.51 |
| *SEM* | 0.59 | 1.05 |

*Figure 4-20: Real-time PCR analysis of inflammatory-cytokine related gene in human orbital adipose tissue.*

*There is significantly higher expression of IL-1β, IL-R, IL-6, TNFα, TNFR, TNFαIP, TGFβ, CCL2, CD200 and TSHR in qHOAT compared to cHOAT. However, there was no expression of CCR2 in cHOAT or qHOAT. Results are expressed as fold change when compared to cHOAT and dCt and standard error of the mean values are shown in table.*

### 4.3.3. Comparative Analysis of Cultured Preadipocytes: Control Orbital Preadipocytes vs. Quiescent Orbital Preadipocytes vs. Inflamed Orbital Preadipocytes

#### 4.3.3.1. Analysis of Cultured Preadipocytes

After attempting to culture 28 samples of qHOAT, 18 samples were successful and carried forward for enzyme activity studies compared to 15 out of 30 cHOAT samples and 7 out of 13 iHOAT samples successful for the same enzyme activity studies. Figure 4-21 shows photographs of preadipocytes from HOAT cultured (A) and after 14 day of differentiation – termed ‘day 14’, where cells exhibited lipid droplets in the cell which are representative of mature-like adipocytes (B).

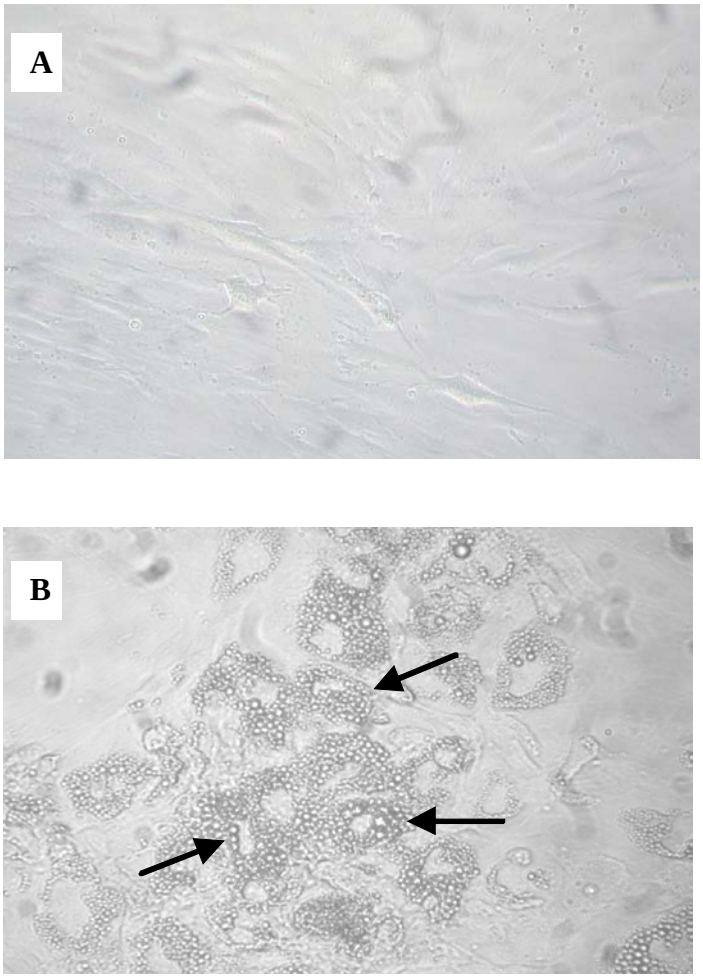



*Figure 4-21: Photographs of human preadipocyes before and after differentiation.*

*Naive human orbital preadipocytes (A). Cell phenotype changes to mature-like adipocytes filled with lipid droplets (arrowed) after differentiating in chemically defined media (B).*

#### 4.3.3.2. Characterisation of 11β-HSD1 Activity in Human Orbital Preadipocytes

Predominant 11β-HSD1 reductase activity (Figure 4-22) is exhibited by untreated preadipocytes from cHOAT when compared to dehydrogenase activity. Preadipocytes from qHOAT and iHOAT also showed predominately reductase activity compared to dehydrogenase activity (Figure 4-22). From the HOAT samples cultured for

differentiation for 14 days, 3 inflamed orbital preadipocytes, 11 quiescent orbital preadipocytes and 6 control orbital preadipocytes were successfully differentiated.

An 11β-HSD enzyme assay performed showed that 11β-HSD1 reductase activity was higher after differentiation of the cells to mature-like adipocytes. This increase was significant in the preadipocytes from cHOAT, qHOAT and iHOAT following differentiation (Control orbital preadipocytes, 1.1±0.13 pmol $mg^{-1}$ $h^{-1}$ day 0 vs. 2.17±0.11 pmol $mg^{-1}$ $h^{-1}$ day 14, $p<0.05$; quiescent orbital preadipocytes, 3.05±0.15 pmol $mg^{-1}$ $h^{-1}$ day 0 vs. 9.42±0.36 pmol $mg^{-1}$ $h^{-1}$ day 14, $p<0.01$; inflamed orbital preadipocytes, 1.83±0.05 pmol $mg^{-1}$ $h^{-1}$ day 0 vs. 5.62±0.11 pmol $mg^{-1}$ $h^{-1}$ day14, $p<0.05$), (Figure 4-22).

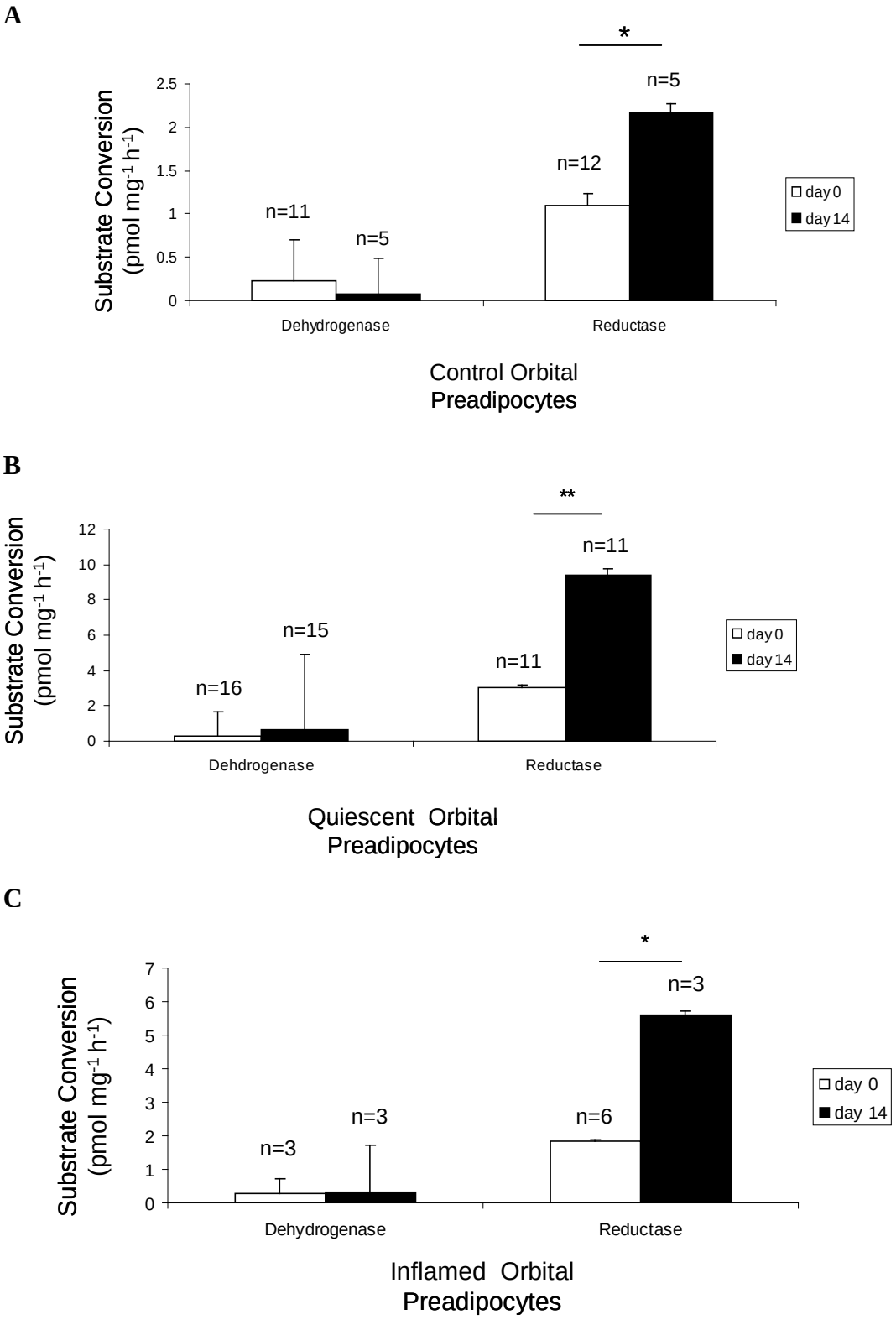



*Figure 4-22: Specific enzyme assay for 11β-HSD1 in primary human orbital preadipocytes before and after differentiation.*

*(A) Predominant reductase activity was exhibited by control orbital preadipocytes, (B) quiescent orbital preadipocytes and (C) inflamed orbital preadipocytes. This activity increased after differentiation of the preadipocytes (termed 'day14') to mature-like adipocytes. (*, p<0.05; **, p<0.01; values represent means with error bars as standard error of the mean)*

The treatment of preadipocytes from qHOAT with IL-6, TNFα and TGFβ (Figure 4-23) showed an increase in 11β-HSD1 reductase activity compared to preadipocytes from cHOAT. TNFα exhibited a significant increase in reductase activity in quiescent orbital preadipocyte, however, IL-1β failed to show an increase in quiescent preadipocyte (quiescent preadipocytes with TNFα, 8.32±2.2 pmol mg-$^{1}$ h$^{-1}$ vs. control orbital preadipocyte with TNFα, 2.16±0.18 pmol mg-$^{1}$ h$^{-1}$, p<0.01).

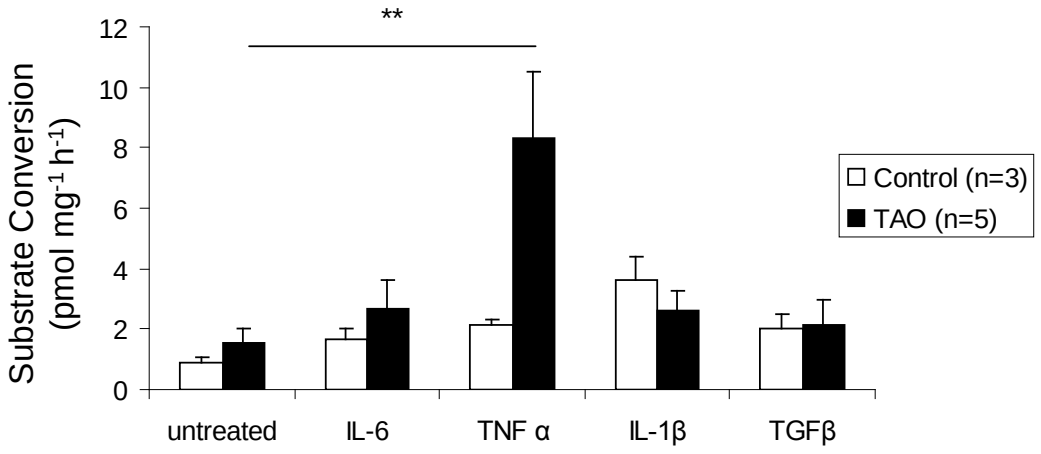



*Figure 4-23: Specific enzyme assay for 11β-HSD1 in primary human orbital preadipocytes before and after cytokine treatment.*

*11β-HSD1 reductase activity after cytokine treatment. 11β-HSD1 reductase activity increased by IL-6, TNFα and TGFβ in preadipocytes from qHOAT when compared to preadipocytes from cHOAT. IL-1β did not increase 11β-HSD1 reductase activity in quiescent preadipocytes, however, TNFα had a significant effect on quiescent preadipocyte 11β-HSD1 reductase activity when compared to preadipocytes from cHOAT (**, p<0.01; values represent means with error bars as standard error of the mean).*

#### 4.3.3.3. Human Orbital Preadipocyte Proliferation

There was a significant increase in the number of control orbital preadipocytes following 4 days in culture (day 1, 0.09±0.02 vs. day 4, 0.19±0.03, $p<0.05$). The proliferation of control orbital preadipocytes did not significantly increase further after the addition of cortisol (Figure 4-24). Quiescent orbital preadipocytes also underwent proliferation at approximately the same rate as the control orbital preadipocytes (day 1, 0.18±0.05 vs. day 4, 0.37±0.07, $p< 0.05$) and this proliferation was significantly reduced upon co-treatment with cortisol (day 4 untreated, 0.36±0.08 vs. day 4 + cortisol, 0.31±0.07, $p<0.05$), (Figure 4-24).

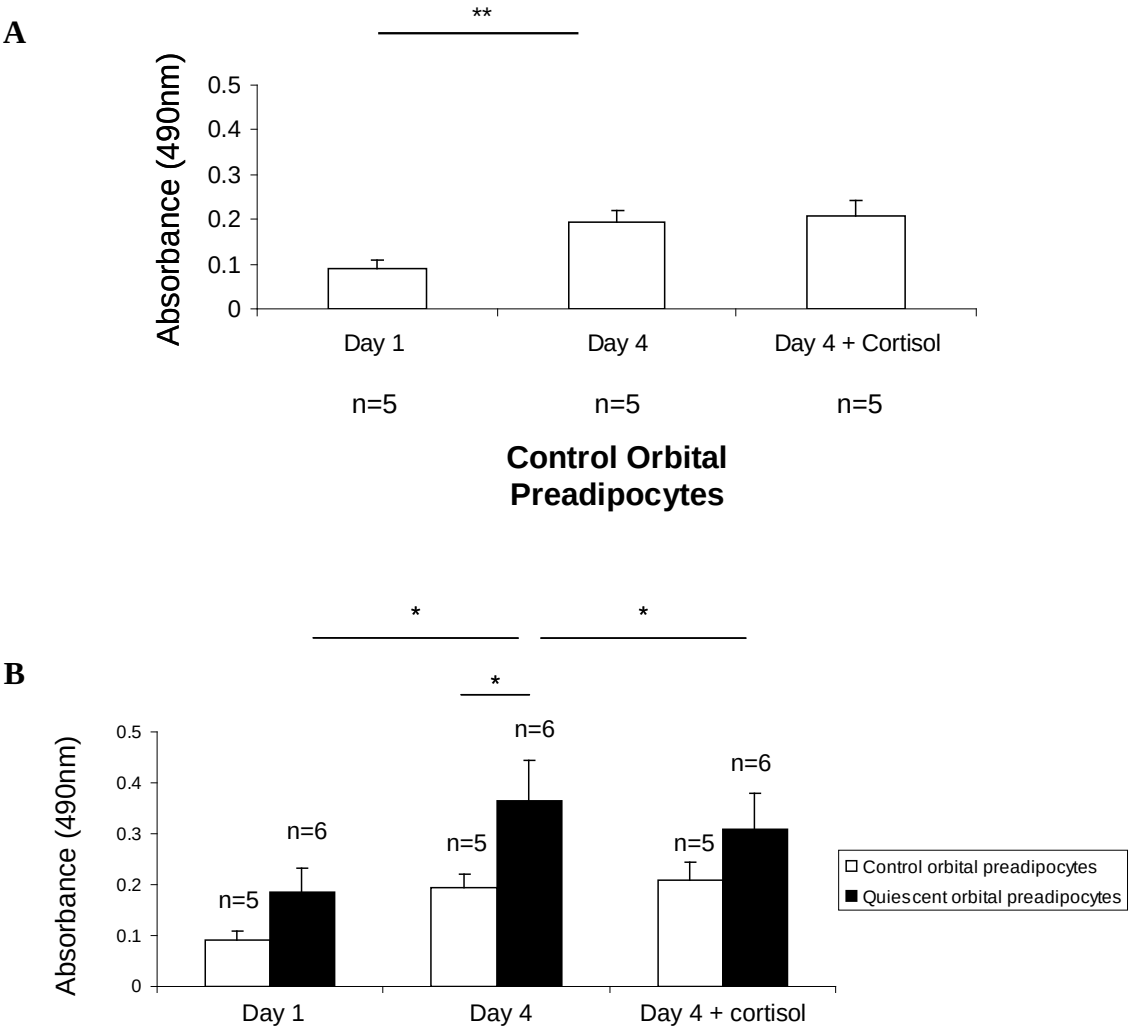



*Figure 4-24: Human orbital preadipocyte proliferation.*

*(A) Control orbital preadipocytes demonstrated a significant increase in cell number following 4 days in culture. This increase was not altered by treatment with 1μM cortisol. (B) Preadipocytes from qHOAT underwent significant proliferation from day 1 to day 4. This increase in cell number by day 4, was significantly reduced by co-incubation with cortisol (*, $p<0.05$; values represent means with error bars as standard error of the mean).*

### *4.4. Discussion*

In light of the knowledge that adipose tissue from different depots possess differing adipocyte biology and glucocorticoid metabolism (Goedecke et al., 2006), this chapter sought to investigate the morphological and biological differences between SC, OM and HOAT depots. The novel reproducible automated method for the histomorphometric analysis of adipose tissue from different depots has allowed us to note the adipose cell characteristic differences in a quantitative manner. The method revealed that cHOAT adipocytes are generally smaller than SC and OM adipocytes and cHOAT adipocytes expressed lower levels of adipocyte differentiation markers (FABP4 and G3PDH). Histomorphometric analysis of qHOAT and iHOAT revealed no significant difference in morphological parameters when compared to cHOAT suggesting that they are of similar size and shape.

The three adipose depots, SC, OM and cHOAT all expressed GRα mRNA as previously found in other studies (Heufelder et al., 1992a, Burnstine et al., 1998, Muhlberg et al., 2000, Boullu-Ciocca et al., 2003), however this was much higher in cHOAT. In addition to this data, I defined 11β-HSD1 activity and mRNA expression along with its regulatory enzyme, H6PDH in cHOAT and qHOAT tissue and preadipocytes. These observations highlight that HOAT is distinct from SC and OM as each depot has differing propensities of adipocyte differentiation, proliferation, apoptosis and lipid metabolism (Niesler et al., 1998, Baglioni et al., 2009, Mito et al., 2009, Lundgren et al., 2008, Tchkonia et al., 2005).

Orbital fibroblasts are known to have defining characteristics such as expression of surface receptors, cellular proteins (such as orbit specific (OS) proteins, cytokine

responsive proteins (CRP) and actin neighbour proteins (ANP)), (Young et al., 1998), response to cytokines and the ability to secrete matrix proteins (Smith and Smith, 2004, Ajjan et al., 2004, Prabhakar et al., 2003).

The higher $CD68^+$ cells and CD68 mRNA in cHOAT compared to SC and OM is important because the cytokines they produce may have effects on adipocyte biology. In addition to this, these data reflect an orbital molecular environment that has an increased potential and plasticity that is vital to combat inflammatory, immune-mediated or infective insult. Recent studies have shown that chronic inflammation activates adipose tissue in simple obesity which is defined by an increase in $CD68^+$ macrophages, increased adipocyte size and increased TNFα in patients with high BMIs (Weisberg et al., 2003, Neels et al., 2006, Pietilainen et al., 2006).

There is a great deal of heterogeneity in monocyte-derived lineages (such as macrophages) and these different subsets from monocytes have specific roles in physiology. For example, when adipose tissue increases during weight gain, this causes a range of signalling pathways to activate the adipose vascular stromal fraction thus allowing monocyte migration into the adipose tissue via the endothelial layer where they differentiate into macrophages (Neels et al., 2006). No significant difference in $CD68^+$ cell number or mRNA expression between cHOAT and qHOAT suggests this is confirmatory of the non-inflamed status of qHOAT.

Crosstalk between adipocytes, macrophages and endothelial cells aggravates the inflammatory state, resulting in increased secretion of pro-inflammatory cytokines/chemokines, adipokines and angiogenic factors (Lolmede et al., 2003, Trayhurn et al., 2006, Neels et al., 2006, Hutley et al., 2001). Furthermore, the interaction of cytokines and glucocorticoid metabolism may be an additional contributing factor. TNFα,

IL-6 and IL-1β are potent inducers of 11β-HSD1 expression and activity in adipose tissue (Friedberg et al., 2003, Tomlinson et al., 2001b). Macrophage-derived cytokine production in vivo may serve to augment cortisol generation through induction of 11β-HSD1.

TNFα specifically, in addition to modulating 11β-HSD1 activity, can potentially inhibit adipocyte differentiation and promotes apoptosis and de-differentiation (Torti et al., 1989, Tejerina et al., 2009, Domingo et al., 2005). This process may be of considerable relevance to the orbital fat microenvironment as the high macrophage content and the increased expression of IL-6, IL-1β and TNFα in HOAT maybe regulating adipocyte biology. For example, qHOAT is the status which represents clinically non-inflamed HOAT and as a consequence, the increased expression of TNFα maybe an indicator of a once inflamed tissue.

TGFβ inhibits TSHR expression and adipogenesis by orbital fibroblasts, which would indicate favourably towards disease remission and its is presumed that these inhibitory and stimulatory cytokine effects occurring simultaneously within the orbit maybe contributing to the severity of TAO (Bahn and Bahn, 2002). Therefore, the only slight increase in TGFβ expression from qHOAT compared to cHOAT may suggestive of disease remission in qHOAT.

The expression of other chemokines such as CD200 and CCL2 maybe of interest, for example, CD200, the myeloid cell regulator, exhibited an expression slightly higher in qHOAT compared to cHOAT. This maybe involved in regulating myeloid immune cell infiltration into qHOAT which may include macrophages.

With respect to MCP-1 (CCL2), this was also increased in qHOAT compared to cHOAT. MCP-1 has a significant part to play in TAO due to its involvement in macrophage infiltration into the tissue. MCP-1 has already been characterised in adipose tissue from obese individuals and is also linked to the Th2 chemokine (Karpus et al., 1997, Chen et al., 2008). One study by Chen et al, 2008, found a correlation between MCP-1 and the degree of infiltration of macrophage into the HOAT of Graves' individuals (Chen et al., 2008). This may indicate that MCP-1 may well be an important mediator of lymphoid cell interactions with HOAT in TAO individuals. The lack of expression of the receptor for MCP-1, CCR2 in orbital adipose tissue may suggest that CCL2 from orbital adipose tiisue may be recruiting macrophages by interacting with CCR2 on macrophage surface.

Studies have shown that there is increased expression of the TSH receptor on both preadipocytes and differentiated adipocytes derived from primary cultures of orbital adipose tissue taken from patients with TAO when compared to controls (Crisp et al., 2000). TSH receptor gene expression in TAO preadipocytes, however, is inhibited by cytokines such as TNFα, IFNγ and TGFβ. Downstream TSH-dependent cAMP production is also decreased. In addition, TNFα and IFNγ attenuate morphological adipocyte maturation, indicating that TSH receptor expression may not be essential for adipogenesis, but may be a characteristic feature of mature differentiated adipocytes (Valyasevi et al., 2001). However, increased positive correlations between expression of TSH receptor mRNA and obesity-related markers (leptin, adiponectin, PPARγ) are also seen in TAO fibroblasts compared with controls (Kumar et al., 2004). We confirmed TSHR is expressed in HOAT and therefore these data suggest de novo adipogenesis within orbital tissues in patients with TAO.

Glucocorticoids are essential for adipocyte differentiation (Hauner et al., 1987), inhibit omental preadipocyte proliferation (Tomlinson et al., 2002), but interestingly, promote proliferation in the SC depot (Bader et al., 2002). Previous data has shown that fibroblasts can be differentiated to mature-like adipocytes using glucocortiocoids in a similar manner as I have done with the orbital preadipocytes and this suggests the unique nature of the orbital molecular environment accounts for its underlying susceptibility to autoimmune diseases (Kaminski et al., 2003).

The identification of 11β-HSD1 in human orbital adipose tissue is an important finding and raises the possibility that the local generation of GC within this depot may be functionally important to these processes. 11β-HSD1 potently regulated by cytokines (up-regulated by TNFα, IL-1, IL-4) which is tissue specific (Tomlinson et al., 2001b, Tomlinson et al., 2004). We have shown that IL-6, TNFα and TGFβ increase local generation of cortisol in preadipocytes from cHOAT and qHOAT which suggests the cytokine effect on 11β-HSD1 to generate cortisol may modulate adipocyte biology. The low response of 11β-HSD1 activity after IL-1β treatment is a surprising one as IL-1β is a potent regulator of 11β-HSD1 in other tissues (Friedberg et al., 2003, Peter et al., 2002).

Cell-types that play a role in the adipogenesis process that occurs in qHOAT include activated fibroblasts, preadipocytes and immune cells such as T cells and macrophages (Bahn, 2003). These cells release pro-inflammatory cytokines which can induce 11β-HSD1 to generate cortisol locally in orbital adipose tissue and in doing so; cortisol may enhance adipogenesis (Figure 4-25). Therefore, we postulate that the intracrine regulation is a key determinant of adipogenic and inflammatory pathways in the pathogenesis of TAO and modulation of 11β-HSD1 may provide a novel therapeutic target for this sight-threatening condition.

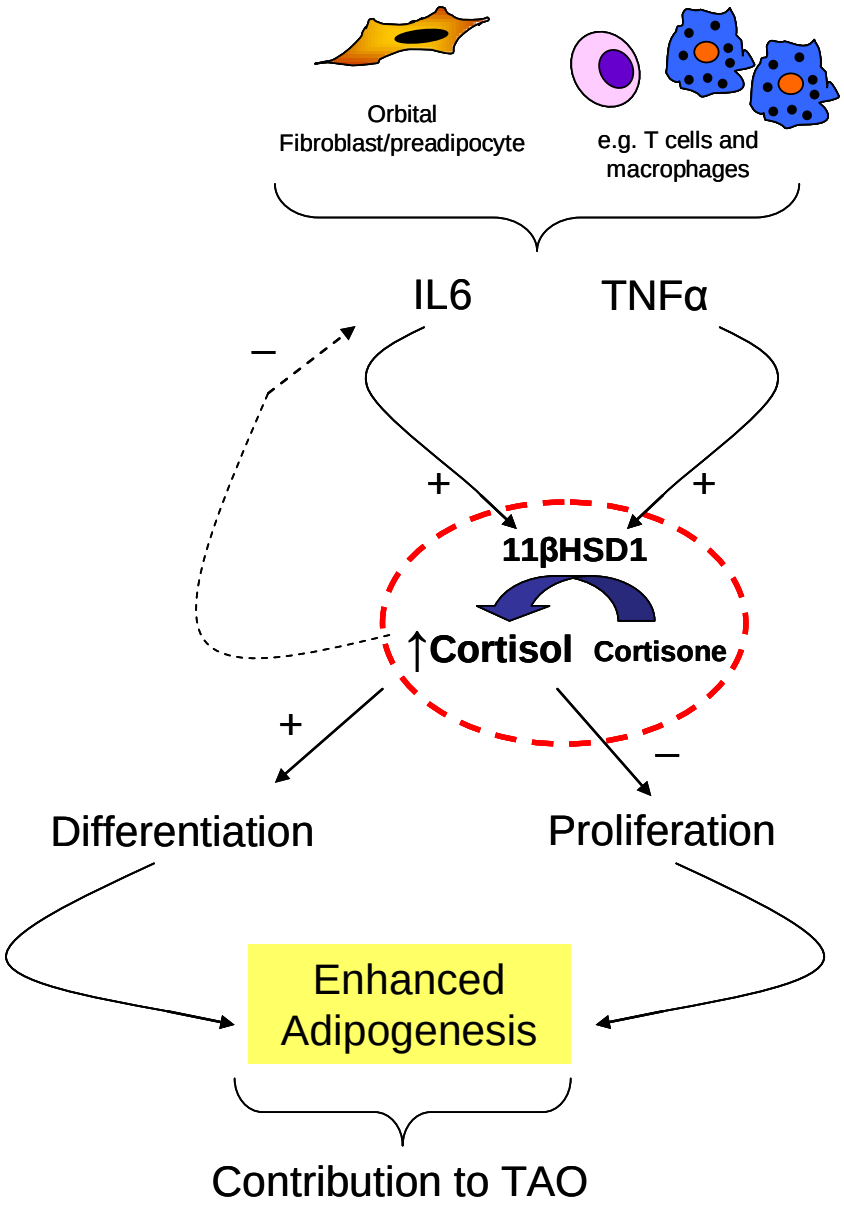



*Figure 4-25: The role of pre-receptor cortisol metabolism in the pathogenesis of TAO.*

*Pro-inflammatory cytokines increase 11β-HSD1 activity and expression enhancing the local generation of cortisol thus promoting preadipocyte/fibroblast differentiation and potentially limiting proliferation. In addition, increased cortisol generation may have a local anti-inflammatory action to suppress cytokine production. Therapeutic use of glucocorticoids suppresses pro-inflammatory cytokine production and therefore relieves the induction of 11β-HSD1. However, cortisol generation by 11β-HSD1 involved in differentiation and increased cell proliferation may contribute to the enhanced adipogenesis in TAO.*

# 5. Final Conclusions and Future Perspectives

Glucocorticoids affect physiological process which include carbohydrate metabolism, adipocyte biology and they have an important role in immunomodulation of inflammatory processes due their anti-inflammatory and immunosuppressive properties. As a consequence of these properties, glucocorticoids are used abundantly in ophthalmic practice for the treatment of ocular ailments such as orbital diseases for example in thyroid associated ophthalmopathy and idiopathic orbital and ocular inflammatory diseases such as uveitis, diabetic macular oedema, and ocular surface inflammation such as allergic conjunctivitis and peri-operatively and post-operatively in peripheral ulcerative keratitis (Leibovitch et al., 2007, Toker et al., 2002, Grenga et al., 2008, Arcangelo and Peterson, 2005, Wahid et al., 2008).

However, the therapeutic use of glucocorticoids can have adverse effects on cellular processes. For example, glucocorticoids have been shown to induce osteoporosis and osteonecrosis; induce diabetes mellitus; are associated with obesity; delay wound healing and promote dyslipidaemia and atherosclerosis (Adam and Epel, 2007, Chedid et al., 1996, Da Silva et al., 2006, Dalle Carbonare et al., 2001, McCurdy and Friedman, 2006, Weinstein et al., 2000). As a consequence of this, there is a need for a tissue specific regulator of glucocorticoids and one way to achieve this is to through the pre-receptor regulation of glucocorticoids via 11β-HSD.

The enzyme, 11β-HSD, interconverts cortisone and cortisol to regulate their levels locally in tissues such as the liver, kidney and adipose tissue. The two isozymes which are responsible for these reactions are 11β-HSD1 and 11β-HSD2. 11β-HSD1 is typically located in GR rich tissues while 11β-HSD2 is found in MR rich tissues.

11β-HSD1 has numerous roles in physiology, which include, inflammation (Chapman and Seckl, 2008, Gilmour et al., 2006), adipogenesis (Bujalska et al., 1997), and bone

regulation (Hong et al., 2008, Delany et al., 1995). Its role in vivo is to act primarily as an oxo-reductase converting biologically inactive cortisone to active cortisol.

There are a handful of studies into specific roles of 11β-HSD1 in the eye. These include expression of 11β-HSD1 in ocular tissues (Rauz et al., 2001, Suzuki et al., 2001, Stokes et al., 2000, Mirshahi et al., 1997) and studies concerning the manipulation of 11β-HSD1 with non-specific inhibitor carbenoxolone to reduce IOP (Rauz et al., 2003a).

The aim of this thesis was to study putative roles in the eye and orbit in normal and diseased states. This was achieved by using animal and human tissue to study generation of glucocorticoids in intraocular environment, in the anterior ocular surface and in orbital adipose tissue.

The limitations of the study involve the restricted availability of human ocular tissues and the quantity required for each specific experiment. However, we have confirmed active 11β-HSD1 in reactivating cortisol from cortisone in corneal epithelial cells. The local production of glucocorticoids could be up-regulated by inflammatory cytokines such as IL-6 and could lead to the up-regulation of glucocorticoid receptor genes that maybe involved in corneal wound repair and anti-inflammatory action against pathogens; however, this still needs to be determined.

The use of synthetic glucocorticoids at high concentrations are known to inhibit the corneal wound healing process (Bourcier et al., 2000) and our data suggests that reactivated glucocorticoid (cortisone to cortisol) reduces corneal epithelial cell proliferation.

As no expression of 11β-HSD2 was found in the corneal epithelium and corneal epithelial cells of the NZWAR which is comparable to the human (Rauz et al., 2001), it is deduced that this enzyme has no major role in these cell-types.

The New Zealand White Albino Rabbit (NZWAR) was used as a model in these studies because the model has previously been used in different studies concerning the ocular surface and the intraocular environment. There are still many tissue types to be investigated for possible 11β-HSD1 action, however, tools such as the use of an animal model just like the NZWAR can accelerate research and provide good comparisons with the limited human data to eventually lead to novel therapeutic treatments.

To help establish 11β-HSD1's role in human ocular tissues, I have characterised the pre-receptor regulation of glucocorticoids and the components involved in the process in human ocular surface (corneal epithelial cells, corneal fibroblasts and conjunctival fibroblasts) and rabbit ocular surface (corneal epithelial cells, corneal fibroblasts, conjunctival fibroblasts) and intraocular (non-pigmented and pigmented epithelium) tissues.

11β-HSD1 staining in cultured human corneal epithelial cells by immunocytochemistry confirmed that 11β-HSD1 protein is present in the corneal epithelium. 11β-HSD1 was also confirmed in NZWAR corneal and conjunctival epithelium using a human anti-11β-HSD1 antibody. This proved that cross-reactivity of the 11β-HSD1 antibody exists between the rabbit and human models and highlighted the potential for these cell types to locally generate cortisol.

We checked whether this enzyme was active by using a specific 11β-HSD enzyme assay on cultured NZWAR and human corneal epithelial cells, corneal fibroblasts, conjunctival

fibroblasts and NZWAR ciliary epithelium (non-pigmented and pigmented) of which predominant activity was present in NZWAR NPE compared to PE.

With respect to intraocular defence, clinical studies have quantified cortisol in aqueous humour where diffusion or ultrafiltration has been postulated to be the main source of intraocular cortisol (Rauz et al., 2003a, Rauz et al., 2001). This study shows conclusively that the non-pigmented epithelium has the capacity to generate cortisol locally in tissue cortisol which can have intracrine and paracrine effects to regulate aqueous humour production. This is important because aqueous humour provides an additional role in the regulation of protective systems which may include Fas-L and human beta defensins contributing to intraocular defence.

One way to test the effect of cortisol on Fas-L on the cornea and in the ciliary epithelium could be carried out by showing Fas-L mRNA expression (real-time RT-PCR) or protein expression (western blot or ELISA) in these cell-lines. The expression of antimicrobial products such as human beta defensin 2 and LL37 after cortisol and LPS treatment in primary corneal epithelial cells can be evaluated using ELISA and western blotting techniques.

There was predominant reductase activity from NZWAR and human corneal epithelial cells which highlights the ability of these cells to take part in the pre-receptor regulation of glucocorticoids in intact cells. This is in addition to cells from other systems having predominantly reductase activity in intact cells, for example, from liver, lung, and adipose tissue (Jamieson et al., 1995, Hundertmark et al., 1995, Bujalska et al., 1997) and therefore highlights the role 11β-HSD1 plays in regulating cortisol levels at a tissue specific level. No activity of 11β-HSD1 was shown from NZWAR keratocytes and

conjunctival fibroblasts; however, the human form of these cells did exhibit this reductase activity suggesting a possible role in these human cells also.

The use of the NZWAR and human eye as an in-vitro means of studying the effects of many other cytokines (such as IL-1) involved in ocular diseases can be performed on other primary cells which could include retinal epithelial cells and decipher what consequences this may have for autoimmune condition of the eye and how this affects 11β-HSD1 activity. This may involve specific 11β-HSD enzyme assays, protein analysis such as western blotting or and quantitative mRNA expression with real-time RT-PCR.

TLR signalling pathways are important in the elimination of pathogenic bacteria on the ocular surface such as that shown with TLR4 (Wright et al., 1990, Song et al., 2001). LPS has been shown to bind to CD14 and TLR4 to induce expression of pro-inflammatory cytokines and human beta defensin 2 (Maltseva et al., 2007). There was confirmed expression of glucocorticoid related and certain pro-inflammatory cytokine genes (IL-1β and IL-6) in the human corneal epithelial cells which may be the necessary apparatus in responding to a potential inflammatory stimulus such as LPS. The confirmation of CD14, HBD-2 and TLR4 gene expression in primary human corneal epithelial cells was also keeping in-line with other literature (Song et al., 2001, Terai et al., 2004, Li et al., 2007).

The upregulation of 11β-HSD1 activity by LPS in human corneal epithelial cells may represent the functionality of 11β-HSD1 to produce cortisol to modulate immune events. LPS a component of bacterial cell wall has been shown to upregulate immune components that facilitate the inflammatory process such as pro-inflammatory cytokines (e.g. IL-6 and TNFα) and activates the innate immune response via CD14 and TLRs on the ocular surface (Liang et al., 2007b, Wu et al., 2005, Sun et al., 2006, Lu et al., 2006).

However, LPS is also known to induce immunomodulatory components to regulate inflammation such as mucins to protect the mucosal barrier (Smirnova et al., 2003). In light of these data, LPS could induce the immunomodulatory properties of 11β-HSD1 in ocular tissue by promoting cortisol generation.

The pro-inflammatory cytokine IL-6 and not TNFα was able to upregulate 11β-HSD1 reductase activity in human corneal epithelial while TNFα and not IL-6 upregulates 11β-HSD1 reductase activity in corneal fibroblast and conjunctival fibroblast cells. IL-6 has been shown to upregulate 11β-HSD1 activity in adipocytes (Friedberg et al., 2003) while TNFα has been shown to up-regulate 11β-HSD1 activity in other cell systems such as glomerular mesangial cells of the kidney and in osteoblasts (Escher et al., 1997b). However, these data may suggest that specific cytokines have cell specific roles in modulating 11β-HSD1 activity on the ocular surface.

GR has been localised in ocular tissues which include the cornea, ciliary body, trabecular meshwork, lens, retina and sclera (Rauz et al., 2001). GR interaction with glucocorticoids is important for genomic mediated responses during inflammation (Chinenov and Rogatsky, 2007). Studies have shown that GR agonists such as dexamethasone can activate the GR in vitro (Takahashi et al., 2000, Wenzel et al., 2001).

GR expression was shown in the immortalized human corneal epithelial cells and although some GR activation was shown in the immortalized human corneal epithelial cells by dexamethasone, this was not as strong as what might have been expected. This may suggest that the assay used for these particular cells, was not optimally sensitive to detect large increases in GR activity. LPS had no effect on GR activity and therefore does not influence GR mediated transcription.

Further studies on 11β-HSD1 in corneal epithelial cells and its role in inflammation can include studying the effects locally generated cortisol by 11β-HSD1 reductase has on the immune response that is mediating TLR regulated immunoprotective mechanisms. We hypothesize that the induction of TLR signalling pathway by infectious agents can promote 11β-HSD1 reductase to generate cortisol. In doing so, the cortisol generated could trigger anti-inflammatory or anti-microbial GR responses which could contribute to the immune privilege state of the cornea and provide ocular surface immunoprotection. The experiments that can be carried out will include, immunohistochemistry analysis of TLRs in formalin-fixed paraffin embedded corneas to ascertain which resident and recruited cells express specific TLRs. Sections could also be co-incubated with TLR antibodies and markers of immune cells (such as CD4 and MHC I) to visualise antigen-antibody interactions.

Further experiments will focus on obtaining purified corneal epithelial cells and using the immortalized corneal epithelial cells to study TLR expression after co-incubation with specific ligands. Specific inhibitors will also be used to ascertain whether the downstream signalling is mediated through induction of 11β-HSD1 and GR.

Co-cultures of macrophages and dendritic cells with corneal epithelial cells could also be established to study their crosstalk through direct cell-cell contact and establish whether corneal epithelial cells influence the function of macrophages and dendritic cells.

Adipose tissues from different depots possess differing adipocyte biology and perform glucocorticoid metabolism. In this thesis, we sought to investigate the morphological and biological differences between SC, OM and HOAT depots. The novel reproducible automated method for the histomorphometric analysis of adipose tissue from different depots has allowed us to note the adipose cell characteristic differences in a quantitative

manner. The method revealed that cHOAT adipocytes are generally smaller than SC and OM adipocytes and cHOAT adipocytes expressed lower levels of adipocyte differentiation markers (FABP4 and G3PDH. Histomorphometric analysis of qHOAT and iHOAT revealed no significant difference in morphological parameters when compared to cHOAT suggesting that that are of similar size and shape.

SC, OM and cHOAT all expressed GRα mRNA as previously found in other studies (Heufelder et al., 1992a, Burnstine et al., 1998, Muhlberg et al., 2000, Boullu-Ciocca et al., 2003), however this was much higher in cHOAT. In addition to this, the data defined 11β-HSD1 activity and mRNA expression along with its regulatory enzyme, H6PDH in cHOAT and qHOAT tissue and preadipocytes. These observations highlight that HOAT is distinct from SC and OM as each depot has differing propensities of adipocyte differentiation, proliferation, apoptosis and lipid metabolism (Niesler et al., 1998, Baglioni et al., 2009, Mito et al., 2009, Lundgren et al., 2008, Tchkonia et al., 2005). Further work related to this will include glucocorticoid-related gene expression in iHOAT to establish whether this is different to qHOAT.

The different properties between SC, OM and HOAT, maybe indicative of the difference in cell lineage as SC and OM ‘peripheral' fibroblasts originate from the mesoderm and the cHOAT fibroblasts originally come from the neuroectoderm (Smith and Smith, 2004). It has already been shown that fibroblasts can be differentiated to mature-like adipocytes in the same manner as we have done with the orbital preadipocytes and this suggests the unique nature of the orbital molecular environment accounts for its underlying susceptibility to autoimmune diseases (Kaminski et al., 2003).

$CD68^+$ cells and CD68 mRNA was higher in cHOAT compared to SC and OM which is important because the cytokines they produce may have effects on adipocyte biology. In

addition to this, these data reflect an orbital molecular environment that has an increased potential and plasticity that is vital to combat inflammatory, immune-mediated or infective insult. Recent studies have shown that chronic inflammation activates adipose tissue in simple obesity which is defined by an increase in $CD68^+$ macrophages, increased adipocyte size and increased TNFα in patients with high BMIs (Weisberg et al., 2003, Neels et al., 2006, Pietilainen et al., 2006).

There is a great deal of heterogeneity in monocyte-derived lineages (such as macrophages) and these different subsets from monocytes have specific roles in physiology. No significant difference in $CD68^+$ cell number or mRNA expression between cHOAT and qHOAT suggests this is confirmatory of the non-inflamed status of qHOAT. Obtaining more samples of iHOAT and other inflamed responses such as dermatitis will help establish if there is a difference in expression of $CD68^+$ when compared to cHOAT and qHOAT. This could confirm the use of this technique a an indicator of active inflammation.

The crosstalk that occurs between adipocytes, macrophages and endothelial cells aggravates the inflammatory state, resulting in increased secretion of pro-inflammatory cytokines/chemokines, adipokines and angiogenic factors. Furthermore, the interaction of cytokines and glucocorticoid metabolism may be an additional contributing factor. TNFα, IL-6 and IL-1β are potent inducers of 11β-HSD1 expression and activity in adipose tissue. Macrophage-derived cytokine production in-vivo may serve to augment cortisol generation through induction of 11β-HSD1. TNFα specifically, in addition to modulating 11β-HSD1 activity, inhibits adipocyte differentiation, proliferation and promotes apoptosis and de-differentiation. This process may be of considerable relevance to the orbital fat microenvironment as the high macrophage content and the increased expression

of IL-6, IL-1β and TNFα in HOAT maybe regulating adipocyte biology. For example, qHOAT is a clinically non-inflamed state and as a consequence, the increased expression of TNFα is an indicator of a once inflamed tissue, however the expression of TNFα in qHOAT maybe lower that that in iHOAT and needs to be defined.

TGFβ inhibits TSHR expression and adipogenesis by orbital fibroblasts, which would indicate favourably towards disease remission and its is presumed that these inhibitory and stimulatory cytokine effects occurring simultaneously within the orbit maybe contributing to the severity of TAO (Bahn and Bahn, 2002). Therefore, the only slight increase in TGFβ expression from qHOAT compared to cHOAT may suggestive of disease remission in qHOAT. CD200, the myeloid cell regulator, exhibited an expression slightly higher in qHOAT compared to cHOAT. This maybe involved in regulating myeloid immune cell infiltration into qHOAT which may include macrophages.

With respect to CCL2 (also known as MCP-1) this was also increased in qHOAT compared to cHOAT. CCL2 has a significant part to play in TAO due to its involvement in macrophage infiltration into the tissue. CCL2 has already been characterised in adipose tissue from obese individuals and is also linked to the Th2 chemokine (Karpus et al., 1997, Chen et al., 2008). One study by Chen et al, 2008, found a correlation between CCL2 and the degree of infiltration of macrophage into the HOAT of Graves' individuals (Chen et al., 2008). This may indicate that CCL2 may well be an important mediator of lymphoid cell interactions with HOAT in TAO individuals. These studies looking at cytokine expression in HOAT can be extended to iHOAT to establish whether there are any differences. Other cytokines can be studied for effects on 11β-HSD1 expression and activity in HOAT which can include IFNγ and TGFβ.

Studies have shown that there is increased expression of the TSH receptor on both preadipocytes and differentiated adipocytes derived from primary cultures of orbital adipose tissue taken from patients with TAO when compared to controls (Crisp et al., 2000). TSH receptor gene expression in TAO preadipocytes, however, is inhibited by cytokines such as TNFα, IFNγ and TGFβ. Downstream TSH-dependent cAMP production is also decreased. In addition, TNFα and IFNγ attenuate morphological adipocyte maturation, indicating that TSH receptor expression may not be essential for adipogenesis, but may be a characteristic feature of mature differentiated adipocytes (Valyasevi et al., 2001). However, increased positive correlations between expression of TSH receptor mRNA and obesity-related markers (leptin, adiponectin, PPARγ) are also seen in TAO fibroblasts compared with controls (Kumar et al., 2004). We confirmed TSHR is expressed in HOAT and therefore these data suggest de novo adipogenesis within orbital tissues in patients with TAO.

Glucocorticoids are essential for adipocyte differentiation (Hauner et al., 1987), inhibit omental preadipocyte proliferation (Tomlinson et al., 2002), but interestingly, promote proliferation in the SC depot (Bader et al., 2002). The identification of 11β-HSD1 in human orbital adipose tissue is an important finding and raises the possibility that the local generation of GC within this depot may be functionally important to these processes. With respect to TAO, this may see some of the beneficial effects of regulating 11β-HSD1 activity at least in the active stages of the disease.

11β-HSD1 is potently regulated by cytokines (up-regulated by TNFα, IL-1, IL-4) which is tissue specific (Tomlinson et al., 2001b, Tomlinson et al., 2004). I have shown that IL-6, TNFα and TGFβ increase local generation of cortisol in preadipocytes from cHOAT and qHOAT which suggests the cytokine effect on 11β-HSD1 to generate cortisol may

modulate adipocyte biology. The low response of 11β-HSD1 activity after IL-1β treatment is a surprising one as IL-1 is a potent regulator of 11β-HSD1 in other tissues (Friedberg et al., 2003, Peter et al., 2002).

Studies into 11β-HSD1 activity and expression with active TAO samples could be performed to show whether down-regulation of 11β-HSD1 in the early stages of TAO will be beneficial or detrimental. This work may include a mouse animal model of over expression of 11β-HSD1 specifically in orbital adipose tissue to see if adipose biology mimics that seen in TAO. Other avenues for mouse models to study ocular disease could include a knockout model of 11β-HSD1 to establish effects on eye pathology and inflammation following treatment with bacterial endotoxins.

To conclude, data presented here emphasise the putative role of 11β-HSDs in the ocular microenvironment. An important facet of this study is that it highlights the possible therapeutic application of 11β-HSD modulators in the treatment of a variety of ocular and orbital diseases. Specific 11β-HSD1 inhibitors are currently being tested in a variety of model systems and it will be interesting to see if there are any benefits of these inhibitors in ocular orientated diseases.

# 6. Publications and Presentations Relating to this Study

**Peer Reviewed Original Articles**

Bujalska IJ. Durrani OM. Abbott J. **Onyimba CU**. Khosla P. Moosavi AH. Reuser TT. Stewart PM. Tomlinson JW. Walker EA. Rauz S. Characterisation of 11beta-hydroxysteroid dehydrogenase 1 in human orbital adipose tissue: a comparison with subcutaneous and omental fat. **Journal of Endocrinology. 192(2):279-88, 2007 Feb.**

**Onyimba CU**. Vijapurapu N. Curnow SJ. Khosla P. Stewart PM. Murray PI. Walker EA. Rauz S. Characterisation of the prereceptor regulation of glucocorticoids in the anterior segment of the rabbit eye. **Journal of Endocrinology. 190(2):483-93, 2006 Aug**

**Oral Presentations**

**Onyimba CU**, Vijapurapu N. Curnow J. Bujalska IJ. Hughes SV. Khosla P. Stewart PM. Murray PI. Walker EA. Rauz S. Characterisation of Pre–Receptor Glucocorticoid Regulation in the Rabbit Eye. **Association for Research in Vision and Ophthalmology (ARVO) 2006 meeting, Fort Lauderdale, Florida. Investigative ophthalmology & visual science, 47: E-Abstract 5431**

**Poster Presentations**

**Onyimba CU**. Bujalska IJ. Durrani OM. Abbott J. Khosla P. Moosavi AH. Reuser TTQ. Tomlinson JW. Walker EA. Rauz S. Glucocorticoid Metabolism in Human Orbital Adipose Tissue. **Association for Research in Vision and Ophthalmology (ARVO) 2007 meeting, Fort Lauderdale, Florida. Investigative ophthalmology & visual science 2007 48: E-Abstract 3157.**

**Onyimba CU**. Bujalska IJ. Durrani OM. Abbott J. Khosla P. Moosavi AH. Reuser TTQ. Stewart PM. Tomlinson JW. Walker EA. Rauz S. Glucocorticoid metabolic pathways in human orbital adipose tissue: a comparison with subcutaneous and omental depots. **British Endocrine Society meeting 2007, Birmingham, UK. Endocrine Abstracts 13 P115**

**Onyimba CU**. Khosla P. Hughes SV. Murray PI. Stewart PM. EA. Rauz S. The New Zealand White Albino Rabbit is a suitable model for the evaluation of 11β-hydroxysteroid dehydrogenase type 1 activity in ocular tissues. **British Endocrine Society meeting 2006, Glasgow, UK. Endocrine Abstracts 11 P736**

## 7. References

ABRAMOVITZ, M., BRANCHAUD, C. L. & MURPHY, B. E. P. (1982) Cortisol-cortisone interconversion in human-fetal lung - contrasting results using explant and monolayer-cultures suggest that 11 beta-hydroxysteroid dehydrogenase (EC 1.1.1.146) comprises 2 enzymes. *Journal of Clinical Endocrinology and Metabolism,* 54**,** 563-568.

ADAM, T. C. & EPEL, E. S. (2007) Stress, eating and the reward system. *Physiology and Behavior,* 91**,** 449-458.

AGARWAL, A. (2006) *Handbook of Ophthalmology*, SLACK Incorporated.

AGARWAL, A. K., MONDER, C., ECKSTEIN, B. & WHITE, P. C. (1989) Cloning and expression of rat cDNA encoding corticosteroid 11 beta- dehydrogenase. *Journal of Biological Chemistry,* 264**,** 18939-18943.

AGRAWAL, V. B. & TSAI, R. J. F. (2003) Corneal epithelial wound healing. *Indian J Ophthalmol,* 51**,** 5-15.

AGUILERA, G., HARWOOD, J. P. & CATT, K. J. (1981) Somatostatin modulates effects of angiotensin II in adrenal glomerulosa zone. *Nature,* 292**,** 262-263.

AHIMA, R. S. & FLIER, J. S. (2000a) Leptin. *Annual Review of Physiology,* 62**,** 413-437.

AHIMA, R. S. & FLIER, J. S. (2000b) Adipose tissue as an endocrine organ. *Trends in Endocrinology and Metabolism,* 11**,** 327-332.

AHO, H. J., SAARI, K. M., KALLAJOKI, M. & NEVALAINEN, T. J. (1996) Synthesis of group II phospholipase A2 and lysozyme in lacrimal glands. *Investigative Ophthalmology & Visual Science,* 37**,** 1826-1832.

AI-QUN, Q., JIAN, Q., LIN, X. & YI-ZHANG, C. (2005) Rapid activation of JNK and p38 by glucocorticoids in primary cultured hippocampal cells. *Journal of Neuroscience Research,* 80**,** 510-517.

AJJAN, R. A., WEETMAN, A. P., AJJAN, R. A. & WEETMAN, A. P. (2004) New understanding of the role of cytokines in the pathogenesis of Graves' ophthalmopathy. *Journal of Endocrinological Investigation,* 27**,** 237-45.

AKAMIZU, T. (2001) Antithyrotropin Receptor Antibody: An Update. *Thyroid,* 11**,** 1123-1134.

AL-SHAKARCHI, F. (2007) Initial therapy for suppurative microbial keratitis in Iraq. *British Journal of Ophthalmology,* 91**,** 1583-1587.

ALBERTIN, G., TORTORELLA, C., MALENDOWICZ, L. K., ARAGONA, F., NERI, G. & NUSSDORFER, G. G. (2002) Human adrenal cortex and aldosterone secreting adenomas express both 11 beta-hydroxysteroid dehydrogenase type 1 and type 2 genes. *International Journal of Molecular Medicine,* 9**,** 495-498.

ALBISTON, A. L., OBEYESEKERE, V. R., SMITH, R. E. & KROZOWSKI, Z. S. (1994) Cloning and tissue distribution of the human 11beta-hydroxysteroid dehydrogenase type 2 enzyme. *Molecular and Cellular Endocrinology,* 105**,** R11-17.

AMELUNG, D., HUBENER, H. J., ROKA, L. & MEYERHEIM, G. (1953) Conversion of cortisone to compound F. *Journal of Clinical Endocrinology and Metabolism,* 13**,** 1125-1126.

AMY LI, S. L. (2000) *Corynebacterium pseudodiphtheriticum* keratitis and conjunctivitis: a case report.

ANDERSSON, A. K., ATKINSON, S. E., KHANOLKAR-YOUNG, S., CHADUVULA, M., JAIN, S., SUNEETHA, L., SUNEETHA, S. & LOCKWOOD, D. N. J. (2007) Alteration of the cortisol-cortisone shuttle in leprosy type 1 reactions in leprosy patients in Hyderabad, India. *Immunology Letters,* 109**,** 72-75.

ANDERSSON, J., TRAN, D. Q., PESU, M., DAVIDSON, T. S., RAMSEY, H., O'SHEA, J. J. & SHEVACH, E. M. (2008) $CD4^+$ $FoxP3^+$ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *Journal of Experimental Medicine,* 205**,** 1975-81.

ANDREW J. MACPHERSON, M. B. G. K. D. M. (2005) Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria.

APPEL, B. & FRIED, S. K. (1992) Effects of insulin and dexamethasone on lipoprotein lipase in human adipose tissue. *American Journal of Physiology,* 262**,** E695-699.

ARANDA, A. & PASCUAL, A. (2001) Nuclear hormone receptors and gene expression. *Physiol. Rev.,* 81**,** 1269-1304.

ARCANGELO, V. P. & PETERSON, A. M. (2005) *Pharmacotherapeutics for Advanced Practice: A Practical Approach*, Lippincott Williams & Wilkins.

ARCURI, F., BATTISTINI, S., HAUSKNECHT, V., CINTORINO, M., LOCKWOOD, C. J. & SCHATZ, F. (1997) Human endometrial decidual cell-associated 11 beta-hydroxysteroid dehydrogenase expression: its potential role in implantation. *Early Pregnancy,* 3**,** 259-64.

ARCURI, F., MONDER, C., LOCKWOOD, C. J. & SCHATZ, F. (1996) Expression of 11beta-hydroxysteroid dehydrogenase during decidualization of human endometrial stromal cells. *Endocrinology,* 137**,** 595-600.

ASHTON, N. & COOK, C. (1951) Effect of cortisone on healing of corneal wounds. *British Journal of Ophthalmology,* 35**,** 708-717.

ATKINSON, S., HOLCOMBE, M. & KENDALLTAYLOR, P. (1984) Ophthalmopathic immunoglobulin in patients with Graves ophthalmopathy. *Lancet,* 2**,** 374-376.

AUPHAN, N., DIDONATO, J. A., ROSETTE, C., HELMBERG, A. & KARIN, M. (1995) Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science,* 270**,** 286-90.

BADER, T., ZOUMAKIS, E., FRIEDBERG, M., HIROI, N., CHROUSOS, G. P., HOCHBERG, Z., BADER, T., ZOUMAKIS, E., FRIEDBERG, M., HIROI, N., CHROUSOS, G. P. & HOCHBERG, Z. (2002) Human adipose tissue under in vitro inhibition of 11beta-hydroxysteroid dehydrogenase type 1: differentiation and metabolism changes.[erratum appears in Horm Metab Res. 2003 Jun;35(6):390]. *Hormone & Metabolic Research,* 34**,** 752-7.

BAGLIONI, S., FRANCALANCI, M., SQUECCO, R., LOMBARDI, A., CANTINI, G., ANGELI, R., GELMINI, S., GUASTI, D., BENVENUTI, S., ANNUNZIATO, F., BANI, D., LIOTTA, F., FRANCINI, F., PERIGLI, G., SERIO, M. & LUCONI, M. (2009) Characterization of human adult stem-cell populations isolated from visceral and subcutaneous adipose tissue. *FASEB J.,* 23**,** 3494-3505.

BAHN, R. S. (2001) *Thyroid Eye Disease,* Kluwer Academic Publishers.

BAHN, R. S. (2003) Clinical review 157: Pathophysiology of Graves' ophthalmopathy: the cycle of disease. *Journal of Clinical Endocrinology & Metabolism,* 88**,** 1939-46.

BAHN, R. S. & BAHN, R. S. (2002) Thyrotropin receptor expression in orbital adipose/connective tissues from patients with thyroid-associated ophthalmopathy. *Thyroid,* 12**,** 193-5.

BAHN, R. S., DUTTON, C. M., HEUFELDER, A. E. & SARKAR, G. (1994) A Genomic Point Mutation in the Extracellular Domain of the Thyrotropin Receptor in Patients with Graves Ophthalmopathy. *Journal of Clinical Endocrinology and Metabolism,* 78**,** 256-260.

BAHN, R. S. & HEUFELDER, A. E. (1993) Pathogenesis of Graves' ophthalmopathy. *New England Journal of Medicine,* 329**,** 1468-1475.

BALACHANDRAN, A., GUAN, H., SELLAN, M., VAN UUM, S. & YANG, K. (2008) Insulin and dexamethasone dynamically regulate adipocyte 11 beta-hydroxysteroid dehydrogenase type 1. *Endocrinology***,** en.2008-0088.

BARTALENA, L., BALDESCHI, L., DICKINSON, A., ECKSTEIN, A., KENDALL-TAYLOR, P., MARCOCCI, C., MOURITS, M., PERROS, P., BOBORIDIS, K., BOSCHI, A., CURRO, N., DAUMERIE, C., KAHALY, G. J., KRASSAS, G. E., LANE, C. M., LAZARUS, J. H., MARINO, M., NARDI, M., NEOH, C., ORGIAZZI, J., PEARCE, S., PINCHERA, A., PITZ, S., SALVI, M., SIVELLI, P., STAHL, M., VON ARX, G. & WIERSINGA, W. M. (2008) Consensus statement of the European Group on Graves' orbitopathy (EUGOGO) on management of GO. *European Journal of Endocrinology,* 158**,** 273-285.

BARTALENA, L., MARTINO, E., MARCOCCI, C., BOGAZZI, F., PANICUCCI, M., VELLUZZI, F., LOVISELLI, A. & PINCHERA, A. (1989) More on Smoking-Habits and Graves Ophthalmopathy. *Journal of Endocrinological Investigation,* 12**,** 733-737.

BARTALENA, L., PINCHERA, A. & MARCOCCI, C. (2000) Management of Graves' ophthalmopathy: reality and perspectives. *Endocrine Reviews,* 21**,** 168-199.

BARTNESS, T. J. & BAMSHAD, M. (1998) Innervation of mammalian white adipose tissue: implications for the regulation of total body fat. *American Journal of Physiology,* 275**,** R1399-1411.

BASAK, S. K., BASAK, S., MOHANTA, A., BHOWMICK, A., BASAK, S. K., BASAK, S., MOHANTA, A. & BHOWMICK, A. (2005) Epidemiological and microbiological diagnosis of suppurative keratitis in Gangetic West Bengal, eastern India.[see comment]. *Indian Journal of Ophthalmology,* 53**,** 17-22.

BASKIN, D. G., FIGLEWICZ LATTEMANN, D., SEELEY, R. J., WOODS, S. C., PORTE, D. & SCHWARTZ, M. W. (1999) Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Research,* 848**,** 114-123.

BASTARD, J.-P., JARDEL, C., BRUCKERT, E., BLONDY, P., CAPEAU, J., LAVILLE, M., VIDAL, H. & HAINQUE, B. (2000) Elevated levels of interleukin 6 are reduced in serum and subcutaneous

adipose tissue of obese women after weight loss. *Journal of Clinical Endocrinology & Metabolism,* 85**,** 3338-3342.

BASTARD, J.-P., MAACHI, M., VAN NHIEU, J. T., JARDEL, C., BRUCKERT, E., GRIMALDI, A., ROBERT, J.-J., CAPEAU, J. & HAINQUE, B. (2002) Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. *Journal of Clinical Endocrinology & Metabolism,* 87**,** 2084-2089.

BEER, H. D., FASSLER, R., WERNER, S., BEER, H. D., FASSLER, R. & WERNER, S. (2000) Glucocorticoid-regulated gene expression during cutaneous wound repair. *Vitamins & Hormones,* 59**,** 217-39.

BELL, M. P., SVINGEN, P. A., RAHMAN, M. K., XIONG, Y. & FAUBION, W. A., JR. (2007) FOXP3 regulates TLR10 expression in human T regulatory cells. *The Journal of Immunology,* 179**,** 1893-900.

BEN SIMON, G. J., MANSURY, A. M., SCHWARCZ, R. M., LEE, S., MCCANN, J. D. & GOLDBERG, R. A. (2005) Simultaneous orbital decompression and correction of upper eyelid retraction versus staged procedures in thyroid-related orbitopathy. *Ophthalmology,* 112**,** 923-932.

BENNETT, E. S. & WEISSMAN, B. A. (2004) *Clinical Contact Lens Practice,* Lippincott Williams & Wilkins.

BENSCH, K. W., RAIDA, M., MAGERT, H.-J., SCHULZ-KNAPPE, P. & FORSSMANN, W.-G. (1995) hBD-1: a novel beta-defensin from human plasma. *FEBS Letters,* 368**,** 331-335.

BERGER, A. (2001) Resistin: a new hormone that links obesity with type 2 diabetes. *BMJ,* 322**,** 193c-.

BERMAN, E. R. (1991) *Biochemistry of the Eye,* Springer.

BERNAL, A. L., ANDERSON, A. B. & TURNBULL, A. C. (1982) Cortisol:cortisone interconversion by human decidua in relation to parturition: effect of tissue manipulation on 11 beta-hydroxysteroid dehydrogenase activity. *Journal of Endocrinology,* 93**,** 141-149.

BERNAL, A. L., FLINT, A. P. F., ANDERSON, A. B. M. & TURNBULL, A. C. (1980) 11beta-hydroxysteroid dehydrogenase activity (E.C. 1.1.1.146) in human placenta and decidua. *Journal of Steroid Biochemistry,* 13**,** 1081-1087.

BERNAUER, W., WRIGHT, P., DART, J. K., LEONARD, J. N. & LIGHTMAN, S. (1993) Cytokines in the Conjunctiva of Acute and Chronic Mucous-Membrane Pemphigoid - an Immunohistochemical Analysis. *Graefes Archive for Clinical and Experimental Ophthalmology,* 231**,** 563-570.

BLAND, R. (2000) Steroid hormone receptor expression and action in bone. *Clinical Science,* 98**,** 217-240.

BLAND, R., WORKER, C. A., NOBLE, B. S., EYRE, L. J., BUJALSKA, I. J., SHEPPARD, M. C., STEWART, P. M. & HEWISON, M. (1999) Characterization of 11beta-hydroxysteroid dehydrogenase activity and corticosteroid receptor expression in human osteosarcoma cell lines. *Journal of Endocrinology,* 161**,** 455-464.

BOCKMAN, R. S. & WEINERMAN, S. A. (1990) Steroid-induced osteoporosis. *Orthopedic Clinics of North America,* 21**,** 97-107.

BOEHLKE, C. S., YUAN, C., KAO, W. W., HUANG, A. J., BOEHLKE, C. S., YUAN, C., KAO, W. W. Y. & HUANG, A. J. W. (2004) Cytokeratin 12 in human ocular surface epithelia is the antigen reactive with a commercial anti-Galpha q antibody. *Molecular Vision,* 10**,** 867-73.

BOES, M., CERNY, J., MASSOL, R., OP DEN BROUW, M., KIRCHHAUSEN, T., CHEN, J. & PLOEGH, H. L. (2002) T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature,* 418**,** 983-988.

BOUCHARD, C., DESPRES, J. P. & MAURIEGE, P. (1993) Genetic and nongenetic determinants of regional fat distribution. *Endocrine Reviews,* 14**,** 72-93.

BOULLU-CIOCCA, S., PAULMYER-LACROIX, O., FINA, F., OUAFIK, L., ALESSI, M. C., OLIVER, C., GRINO, M., BOULLU-CIOCCA, S., PAULMYER-LACROIX, O., FINA, F., OUAFIK, L. H., ALESSI, M.-C., OLIVER, C. & GRINO, M. (2003) Expression of the mRNAs coding for the glucocorticoid receptor isoforms in obesity. *Obesity Research,* 11**,** 925-9.

BOURCIER, T., FORGEZ, P., BORDERIE, V., SCHEER, S., ROSTENE, W. & LAROCHE, L. (2000) Regulation of human corneal epithelial cell proliferation and apoptosis by dexamethasone. *Investigative Ophthalmology & Visual Science,* 41**,** 4133-4141.

BOWERS, R. R., FESTUCCIA, W. T. L., SONG, C. K., SHI, H., MIGLIORINI, R. H. & BARTNESS, T. J. (2004) Sympathetic innervation of white adipose tissue and its regulation of fat cell number. *American Journal of Physiology - Regulatory Integrative & Comparative Physiology,* 286**,** R1167-1175.

BREM, A. S., BINA, R. B., KING, T., CHOBANIAN, M. C. & MORRIS, D. J. (1997) Influence of dietary sodium on the renal isoforms of 11 beta-hydroxysteroid dehydrogenase. *Proceedings of the Society for Experimental Biology and Medicine,* 214**,** 340-345.

BREM, A. S., BINA, R. B., KING, T. & MORRIS, D. J. (1995) Bidirectional activity of 11beta-hydroxysteroid dehydrogenase in vascular smooth muscle cells. *Steroids,* 60**,** 406-410.

BREMOND-GIGNAC, D., COPIN, H., CUSSENOT, O., LASSAU, J. P. & HENIN, D. (2004) Anatomical histological and mesoscopic study of the adipose tissue of the orbit. *Surgical and Radiologic Anatomy,* 26**,** 297-302.

BROOK, C. G. D. & MARSHALL, N. J. (2001) *Essential endocrinology,* Oxford, Blackwell Science.

BROOKS, G. F., BUTEL,J ANET S., MORSE, STEPHEN A., JAWETZ, ERNEST, MELNICK, JOSEPH L., ADELBERG, EDWARD A. (2004) *Jawetz, Melnick, & Adelberg's Medical Microbiology,* McGraw-Hill Professional.

BROWN, R. W., CHAPMAN, K. E., EDWARDS, C. R. & SECKL, J. R. (1993) Human placental 11beta-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology,* 132**,** 2614-2621.

BRUBAKER, R. F. (1991) Flow of aqueous humor in humans [The Friedenwald Lecture]. *Investigative Ophthalmology & Visual Science,* 32**,** 3145-3166.

BUJALSKA, I. J., DRAPER, N., MICHAILIDOU, Z., TOMLINSON, J. W., WHITE, P. C., CHAPMAN, K. E., WALKER, E. A. & STEWART, P. M. (2005) Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11 beta-hydroxysteroid dehydrogenase type 1. *Journal of Molecular Endocrinology,* 34**,** 675-684.

BUJALSKA, I. J., DURRANI, O. M., ABBOTT, J., ONYIMBA, C. U., KHOSLA, P., MOOSAVI, A. H., REUSER, T. T., STEWART, P. M., TOMLINSON, J. W., WALKER, E. A., RAUZ, S., BUJALSKA, I. J., DURRANI, O. M., ABBOTT, J., ONYIMBA, C. U., KHOSLA, P., MOOSAVI, A. H., REUSER, T. T. Q., STEWART, P. M., TOMLINSON, J. W., WALKER, E. A. & RAUZ, S. (2007) Characterisation of 11beta-hydroxysteroid dehydrogenase 1 in human orbital adipose tissue: a comparison with subcutaneous and omental fat. *Journal of Endocrinology,* 192**,** 279-88.

BUJALSKA, I. J., GATHERCOLE, L. L., TOMLINSON, J. W., DARIMONT, C., ERMOLIEFF, J., FANJUL, A. N., REJTO, P. A. & STEWART, P. M. (2008) A novel selective 11beta-hydroxysteroid dehydrogenase type 1 inhibitor prevents human adipogenesis. *Journal of Endocrinology,* 197**,** 297-307.

BUJALSKA, I. J., KUMAR, S. & STEWART, P. M. (1997) Does central obesity reflect "Cushing's disease of the omentum"? *The Lancet,* 349**,** 1210-1213.

BUJALSKA, I. J., QUINKLER, M., TOMLINSON, J. W., MONTAGUE, C. T., SMITH, D. M. & STEWART, P. M. (2006) Expression profiling of 11beta-hydroxysteroid dehydrogenase type-1 and glucocorticoid-target genes in subcutaneous and omental human preadipocytes. *Journal of Molecular Endocrinology,* 37**,** 327-340.

BUJALSKA, I. J., WALKER, E. A., TOMLINSON, J. W., HEWISON, M. & STEWART, P. M. (2002b) 11 beta-hydroxysteroid dehydrogenase type 1 in differentiating omental human preadipocytes: From de-activation to generation of cortisol. *Endocrine Research,* 28**,** 449-461.

BURBACH, G. J., NAIK, S. M., HARTEN, J. B., LIU, L., DITHMAR, S., GROSSNIKLAUS, H., WARD, S. L., ARMSTRONG, C. A., CAUGHMAN, S. W. & ANSEL, J. C. (2001) Interleukin-18 expression and modulation in human corneal epithelial cells. *Current Eye Research,* 23**,** 64-8.

BUREN, J., LIU, H. X., JENSEN, J. & ERIKSSON, J. W. (2002) Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. *European Journal of Endocrinology,* 146**,** 419-429.

BURNSTINE, M. A., ELNER, S. G., ELNER, V. M., BURNSTINE, M. A., ELNER, S. G. & ELNER, V. M. (1998) Orbital fibroblast chemokine modulation: effects of dexamethasone and cyclosporin A. *British Journal of Ophthalmology,* 82**,** 318-22.

BURNSTOCK, G. & SILLITO, A. M. (2000) *Nervous Control of the Eye,* Informa Health Care.

BUSH, I. E., HUNTER, S. A. & MEIGS, R. A. (1968) Metabolism of 11-oxygenated steroids. Metabolism in vitro by preparations of liver. *Biochemical Journal,* 107**,** 239-58.

BÖHMER, S., CARAPITO, C., WILZEWSKI, B., LEIZE, E., DORSSELAER, A. V. & BERNHARDT, R. (2006) Analysis of aldosterone-induced differential receptor-independent protein patterns using 2D-electrophoresis and mass spectrometry. *Biological Chemistry,* 387**,** 917.

C.W. NOORLANDER, P. N. E. D. G. J. M. J. J. B. C. V. B. G. H. A. V. (2006) Ontogeny of hippocampal corticosteroid receptors: Effects of antenatal glucocorticoids in human and mouse.

CAI, T.-Q., WONG, B., MUNDT, S. S., THIERINGER, R., WRIGHT, S. D. & HERMANOWSKI-VOSATKA, A. (2001) Induction of 11[beta]-hydroxysteroid dehydrogenase type 1 but not -2 in human aortic smooth muscle cells by inflammatory stimuli. *The Journal of Steroid Biochemistry and Molecular Biology,* 77**,** 117-122.

CALONGE, M. & STERN, M. E. (2004) The conjunctiva and tear film maintenance. *Dry Eye and Ocular Surface Disorders.* Informa Health Care.

CANTU, R. C. & GOODMAN, H. M. (1967) Effects of denervation and fasting on white adipose tissue. *American Journal of Physiology,* 212**,** 207-212.

CARDILLO, J. A., MELO JR, L. A. S., COSTA, R. A., SKAF, M., BELFORT JR, R., SOUZA-FILHO, A. A., FARAH, M. E. & KUPPERMANN, B. D. (2005) Comparison of intravitreal versus posterior sub-tenon's capsule injection of triamcinolone acetonide for diffuse diabetic macular edema. *Ophthalmology,* 112**,** 1557-1563.

CATERINA ROMANO, C., NELLO, M., ROSSELLA, P., SABATO, S. & ANTONIETTA, R. (2009) Toll-like receptor-4 (TLR4) mediates human beta-defensin-2 (HBD-2) induction in response to Chlamydia pneumoniae in mononuclear cells. *FEMS Immunology & Medical Microbiology,* 57**,** 116-124.

CAUX, C. & DUBOIS, B. (2001) Antigen Uptake by Dendritic Cells. *Dendritic Cell Protocols.*

CAWOOD, T., MORIARTY, P. & O'SHEA, D. (2004) Recent developments in thyroid eye disease. *BMJ,* 329**,** 385-390.

CAWOOD, T. J., MORIARTY, P., O'FARRELLY, C. & O'SHEA, D. (2007) Smoking and thyroid-associated ophthalmopathy: A novel explanation of the biological link. *Journal of Clinical Endocrinology & Metabolism,* 92**,** 59-64.

CEPEK, K. L., SHAW, S. K., PARKER, C. M., RUSSELL, G. J., MORROW, J. S., RIMM, D. L. & BRENNER, M. B. (1994) Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the [alpha]E[beta]7 integrin. *Nature,* 372**,** 190-193.

CHAN, J. H., AMANKWAH, R., ROBINS, R. A., GRAY, T. & DUA, H. S. (2008) Kinetics of immune cell migration at the human ocular surface. *British Journal of Ophthalmology,* 92**,** 970-975.

CHANG, J. H., MCCLUSKEY, P. J. & WAKEFIELD, D. (2006) Toll-like receptors in ocular immunity and the immunopathogenesis of inflammatory eye disease. *British Journal of Ophthalmology,* 90**,** 103-108.

CHAPMAN, K. & SECKL, J. (2008) 11beta-HSD1, inflammation, metabolic disease and age-related cognitive (dys)function. *Neurochemical Research,* 33**,** 624-636.

CHEDID, M., HOYLE, J. R., CSAKY, K. G. & RUBIN, J. S. (1996) Glucocorticoids inhibit keratinocyte growth factor production in primary dermal fibroblasts. *Endocrinology,* 137**,** 2232-2237.

CHEN, H., XU, Z., PENG, L., FANG, X., YIN, X., XU, N. & CEN, P. (2006a) Recent advances in the research and development of human defensins. *Peptides,* 27**,** 931-940.

CHEN, J.-J., SUN, Y. & NABEL, G. J. (1998) Regulation of the Proinflammatory Effects of Fas Ligand (CD95L). *Science,* 282**,** 1714-1717.

CHEN, J. J., TSENG, S. C., CHEN, J. J. & TSENG, S. C. (1990) Corneal epithelial wound healing in partial limbal deficiency. *Investigative Ophthalmology & Visual Science,* 31**,** 1301-14.

CHEN, J. J., TSENG, S. C., CHEN, J. J. & TSENG, S. C. (1991) Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. *Investigative Ophthalmology & Visual Science,* 32**,** 2219-33.

CHEN, K.-H., HARRIS, D. L. & JOYCE, N. C. (1999) TGF-beta2 in Aqueous Humor Suppresses S-Phase Entry in Cultured Corneal Endothelial Cells. *Investigative Ophthalmology & Visual Science,* 40**,** 2513-2519.

CHEN, M.-H., CHEN, M.-H., LIAO, S.-L., CHANG, T.-C. & CHUANG, L.-M. (2008) Role of macrophage infiltration in the orbital fat of patients with Graves' ophthalmopathy. *Clinical Endocrinology,* 69**,** 332-7.

CHEN, Y. T., HUANG, C. W., HUANG, F. C., TSENG, S. Y., TSENG, S. H., CHEN, Y.-T., HUANG, C.-W., HUANG, F.-C., TSENG, S.-Y. & TSENG, S.-H. (2006b) The cleavage plane of corneal epithelial adhesion complex in traumatic recurrent corneal erosion. *Molecular Vision,* 12**,** 196-204.

CHINENOV, Y. & ROGATSKY, I. (2007) Glucocorticoids and the innate immune system: Crosstalk with the Toll-like receptor signaling network. *Molecular and Cellular Endocrinology,* 275**,** 30-42.

CIANFLONE, K., MASLOWSKA, M. & SNIDERMAN, A. D. (1999) Acylation stimulating protein (ASP), an adipocyte autocrine: new directions. *Seminars in Cell & Developmental Biology,* 10**,** 31-41.

CIVAN, M. M., COCA-PRADOS, M. & PETERSON-YANTORNO, K. I. M. (1996) Regulatory volume increase of human non-pigmented ciliary epithelial cells. *Experimental Eye Research,* 62**,** 627-640.

COLLINGWOOD, T. N., URNOV, F. D. & WOLFFE, A. P. (1999) Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *Journal of Molecular Endocrinology,* 23**,** 255-275.

COOPER, M. S., HEWISON, M. & STEWART, P. M. (1999) Journal of Endocrinology. 163**,** 159-164.

COOPER, M. S., WALKER, E. A., BLAND, R., FRASER, W. D., HEWISON, M. & STEWART, P. M. (2000) Expression and functional consequences of 11beta-hydroxysteroid dehydrogenase activity in human bone. *Bone,* 27**,** 375-381.

COPE, C. (1972) Metabolic breakdown. *Adrenal steroids and disease.* London, Pitman Medical.

COPE, C. L. & BLACK, E. (1958) The Production Rate of Cortisol in Man. *British Medical Journal,* 1**,** 1020-1024.

CORIC, T., HERNANDEZ, N., DE LA ROSA, D. A., SHAO, D., WANG, T. & CANESSA, C. M. (2004) Expression of ENaC and serum- and glucocorticoid-induced kinase 1 in the rat intestinal epithelium. *American Journal of Physiology - Gastrointestinal & Liver Physiology,* 286**,** G663-670.

CORN, A. L. & KOENIG, A. J. (1996) *Foundations of Low Vision: Clinical and Functional Perspectives,* American Foundation for the Blind.

COTSARELIS, G., CHENG, S.-Z., DONG, G., SUN, T.-T. & LAVKER, R. M. (1989) Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: Implications on epithelial stem cells. *Cell,* 57**,** 201-209.

CREAGH, E. M. & O'NEILL, L. A. J. (2006) TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends in Immunology,* 27**,** 352-357.

CRISP, M., STARKEY, K. J., LANE, C., HAM, J. & LUDGATE, M. (2000) Adipogenesis in Thyroid Eye Disease. *Investigative Ophthalmology & Visual Science,* 41**,** 3249-3255.

CURNOW, S. J., FALCIANI, F., DURRANI, O. M., CHEUNG, C. M. G., ROSS, E. J., WLOKA, K., RAUZ, S., WALLACE, G. R., SALMON, M. & MURRAY, P. I. (2005) Multiplex bead immunoassay analysis of aqueous humor reveals distinct cytokine profiles in uveitis. *Investigative Ophthalmology & Visual Science,* 46**,** 4251-4259.

D'ORAZIO, T. J. & NIEDERKORN, J. Y. (1998) A novel role for TGF-beta and IL-10 in the induction of immune privilege. *Journal of Immunology,* 160**,** 2089-2098.

DA SILVA, J. A. P., JACOBS, J. W. G., KIRWAN, J. R., BOERS, M., SAAG, K. G., INES, L. B. S., DE KONING, E. J. P., BUTTGEREIT, F., CUTOLO, M., CAPELL, H., RAU, R. & BIJLSMA, J. W. J. (2006) Safety of low dose glucocorticoid treatment in rheumatoid arthritis: published evidence and prospective trial data. *Annals of the Rheumatic Diseases,* 65**,** 285-293.

DALLE CARBONARE, L., ARLOT, M. E., CHAVASSIEUX, P. M., ROUX, J. P., PORTERO, N. R., MEUNIER, P. J., DALLE CARBONARE, L., ARLOT, M. E., CHAVASSIEUX, P. M., ROUX, J. P., PORTERO, N. R. & MEUNIER, P. J. (2001) Comparison of trabecular bone microarchitecture and remodeling in glucocorticoid-induced and postmenopausal osteoporosis. *Journal of Bone & Mineral Research,* 16**,** 97-103.

DARDICK, I., POZNANSKI, W. J., WAHEED, I. & SETTERFIELD, G. (1976) Ultrastructural observations on differentiating human preadipocytes cultured in vitro. *Tissue and Cell,* 8**,** 561-571.

DARNEL, A. D., ARCHER, T. K. & YANG, K. (1999) Regulation of 11 beta-hydroxysteroid dehydrogenase type 2 by steroid hormones and epidermal growth factor in the Ishikawa human endometrial cell line. *Journal of Steroid Biochemistry and Molecular Biology,* 70**,** 203-210.

DAVANGER, M. & EVENSEN, A. (1971) Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature,* 229**,** 560-561.

DAVANI, B., KHAN, A., HULT, M., MARTENSSON, E., OKRET, S., EFENDIC, S., JORNVALL, H. & OPPERMANN, U. C. T. (2000) Type 1 11beta -hydroxysteroid dehydrogenase mediates glucocorticoid activation and insulin release in pancreatic islets. *The Journal of Biological Chemistry,* 275**,** 34841-34844.

DAVSON, H. (1990) *Davson's physiology of the eye,* London, Macmillan Press.

DE PAIVA, C. S., CHEN, Z., CORRALES, R. M., PFLUGFELDER, S. C. & LI, D.-Q. (2005) ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells,* 23**,** 63-73.

DE SOUSA PEIXOTO, R. A., TURBAN, S., BATTLE, J. H., CHAPMAN, K. E., SECKL, J. R. & MORTON, N. M. (2008) Preadipocyte 11beta-hydroxysteroid dehydrogenase type 1 is a keto-reductase and contributes to diet-induced visceral obesity in vivo. *Endocrinology,* 149**,** 1861-8.

DELANY, A. M., JEFFREY, J. J., RYDZIEL, S. & CANALIS, E. (1995) Cortisol increases interstitial collagenase expression in osteoblasts by post-transcriptional mechanisms. *Journal of Biological Chemistry,* 270**,** 26607-26612.

DEMIRBILEK, H., KANDEMIR, N., GONC, E. N., OZON, A., ALIKASIFOGLU, A. & YORDAM, N. (2007) Hashimoto's thyroiditis in children and adolescents: A retrospective study on clinical, epidemiological and laboratory properties of the disease. *Journal of Pediatric Endocrinology & Metabolism,* 20**,** 1199-1205.

DI IORIO, E., BARBARO, V., RUZZA, A., PONZIN, D., PELLEGRINI, G. & DE LUCA, M. (2005) Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proceedings of the National Academy of Sciences of the United States of America.*

DICKINSON, A. J. & PERROS, P. (2001) Controversies in the clinical evaluation of active thyroid-associated orbitopathy: use of a detailed protocol with comparative photographs for objective assessment. *Clinical Endocrinology,* 55**,** 283-303.

DING, X. F., ANDERSON, C. M., MA, H., HONG, H., UHT, R. M., KUSHNER, P. J. & STALLCUP, M. R. (1998) Nuclear receptor-binding sites of coactivators glucocorticoid receptor interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. *Molecular Endocrinology,* 12**,** 302-313.

DOMINGO, P., VIDAL, F., DOMINGO, J. C., VELOSO, S., SAMBEAT, M. A., TORRES, F., SIRVENT, J. J., VENDRELL, J., MATIAS-GUIU, X. & RICHART, C. (2005) Tumour necrosis factor alpha in fat redistribution syndromes associated with combination antiretroviral therapy in HIV-1-infected patients: potential role in subcutaneous adipocyte apoptosis. *European Journal of Clinical Investigation,* 35**,** 771-780.

DRAVIDA, S., PAL, R., KHANNA, A., TIPNIS, S. P., RAVINDRAN, G. & KHAN, F. (2005) The transdifferentiation potential of limbal fibroblast-like cells. *Developmental Brain Research,* 160**,** 239-251.

DRIVER, P. M., RAUZ, S., WALKER, E. A., HEWISON, M., KILBY, M. D. & STEWART, P. M. (2003) Characterization of human trophoblast as a mineralocorticoid target tissue. *Molecular Human Reproduction,* 9**,** 793-798.

DUA, H. S., AZUARA-BLANCO, A., DUA, H. S. & AZUARA-BLANCO, A. (2000) Limbal stem cells of the corneal epithelium. *Survey of Ophthalmology,* 44**,** 415-25.

DUA, H. S., DONOSO, L. A. & LAIBSON, P. R. (1994a) Conjunctival instillation of retinal antigens induces tolerance. Does it invoke mucosal tolerance mediated via conjunctiva associated lymphoid tissues (CALT)? *Ocular Immunology and Inflammation,* 2**,** 29 - 36.

DUA, H. S., GOMES, J. A. P., JINDAL, V. K., APPA, S. N., SCHWARTING, R., EAGLE, R. C., DONOSO, L. A. & LAIBSON, P. R. (1994b) Mucosa specific lymphocytes in the human conjunctiva, corneoscleral limbus and lacrimal gland. *Current Eye Research.*

DUA, H. S., JOSEPH, A., SHANMUGANATHAN, V. A. & JONES, R. E. (2003) Stem cell differentiation and the effects of deficiency. *Eye,* 17**,** 877-885.

DUAN, X. & SHEARDOWN, H. (2007) Incorporation of cell-adhesion peptides into collagen scaffolds promotes corneal epithelial stratification. *Journal of Biomaterials Science, Polymer Edition,* 18**,** 701-711.

DUANE, T. D. & JAEGER, E. A. (Eds.) (1985) *Biomedical Foundations of Ophthalmology,* Philadelphia; London, Harper & Row.

DUERR, R. H., TAYLOR, K. D., BRANT, S. R., RIOUX, J. D., SILVERBERG, M. S., DALY, M. J., STEINHART, A. H., ABRAHAM, C., REGUEIRO, M., GRIFFITHS, A., DASSOPOULOS, T., BITTON, A., YANG, H., TARGAN, S., DATTA, L. W., KISTNER, E. O., SCHUMM, L. P., LEE, A. T., GREGERSEN, P. K., BARMADA, M. M., ROTTER, J. I., NICOLAE, D. L. & CHO, J. H. (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science,* 314**,** 1461-1463.

EICHHORN, M., HORNEBER, M., STREILEIN, J. W. & LUTJEN-DRECOLL, E. (1993) Anterior chamber-associated immune deviation elicited via primate eyes. *Investigative Ophthalmology & Visual Science,* 34**,** 2926-2930.

EL-KAISSI, S., FRAUMAN, A. G. & WALL, J. R. (2004) Thyroid-associated ophthalmopathy: a practical guide to classification, natural history and management. *Internal Medicine Journal,* 34**,** 482-491.

ELDER, M. J., HISCOTT, P., DART, J. K., ELDER, M. J., HISCOTT, P. & DART, J. K. (1997) Intermediate filament expression by normal and diseased human corneal epithelium. *Human Pathology,* 28**,** 1348-54.

ERICH KNOP, N. K. (2005) The role of eye-associated lymphoid tissue in corneal immune protection. *Journal of Anatomy,* 206**,** 271-285.

ERIE, J. C., NEVITT, M. P., HODGE, D. O., BALLARD, D. J., ERIE, J. C., NEVITT, M. P., HODGE, D. O. & BALLARD, D. J. (1993) Incidence of ulcerative keratitis in a defined population from 1950 through 1988. *Archives of Ophthalmology,* 111**,** 1665-71.

ESCHER, G., GALLI, I., VISHWANATH, B. S., FREY, B. M. & FREY, F. J. (1997a) Tumor Necrosis Factor alpha and Interleukin 1beta Enhance the Cortisone/Cortisol Shuttle.

ESCHER, G., GALLI, I., VISHWANATH, B. S., FREY, B. M. & FREY, F. J. (1997b) Tumor necrosis factor alpha and interleukin 1beta enhance the cortisone/cortisol shuttle. *Journal of Experimental Medicine.*

ESTEBAN, N. V., LOUGHLIN, T., YERGEY, A. L., ZAWADZKI, J. K., BOOTH, J. D., WINTERER, J. C. & LORIAUX, D. L. (1991) Daily cortisol production-rate in man determined by stable isotope-dilution mass-spectrometry. *Journal of Clinical Endocrinology and Metabolism,* 72**,** 39-45.

EVAGELATOU, M., PETERSON, S. L. & COOKE, B. A. (1997) Leukocytes modulate 11[beta]-hydroxysteroid dehydrogenase (11[beta]-HSD) activity in human granulosa-lutein cell cultures. *Molecular and Cellular Endocrinology,* 133**,** 81-88.

FARAGO, B., MAGYARI, L., SAFRANY, E., CSONGEI, V., JAROMI, L., HORVATOVICH, K., SIPEKY, C., MAASZ, A., RADICS, J., GYETVAI, A., SZEKANECZ, Z., CZIRJAK, L. & MELEGH, B. (2008) Functional variants of interleukin-23 receptor gene confer risk for rheumatoid arthritis but not for systemic sclerosis. *Annals of the Rheumatic Diseases,* 67**,** 248-250.

FEGAN, K. S., RAE, M. T., CRITCHLEY, H. O. D. & HILLIER, S. G. (2008) Anti-inflammatory steroid signalling in the human peritoneum. *Journal of Endocrinology,* 196**,** 369-376.

FEJES-TOTH, G., FRINDT, G., NARAY-FEJES-TOTH, A. & PALMER, L. G. (2008) Epithelial $Na^+$ channel activation and processing in mice lacking SGK1. *American Journal of Physiology - Renal Physiology,* 294**,** F1298-1305.

FERNANDO, A. I. & PANDIT, J. C. (2005) Intravitreal triamcinolone acetonide for refractory diabetic macular oedema. *Journal of the Royal Society of Medicine,* 98**,** 421-422.

FISCHBARG, J. (1997) Mechanism of fluid transport across corneal endothelium and other epithelial layers: a possible explanation based on cyclic cell volume regulatory changes. *British Journal of Ophthalmology,* 81**,** 85-89.

FISCHBARG, J. & DIECKE, F. P. J. (2005) A mathematical model of electrolyte and fluid transport across corneal endothelium. *Journal of Membrane Biology,* 203**,** 41-56.

FITZGERALD, K. A. & GOLENBOCK, D. T. (2007) Immunology: The Shape of Things to Come. *Science,* 316**,** 1574-1576.

FITZGERALD, K. A., MCWHIRTER, S. M., FAIA, K. L., ROWE, D. C., LATZ, E., GOLENBOCK, D. T., COYLE, A. J., LIAO, S.-M. & MANIATIS, T. (2003) IKK[epsi] and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol,* 4**,** 491-496.

FLEISHER, L. N., MCGAHAN, M. C. & FERRELL, J. B. (2000) Rabbit pigmented ciliary epithelium produces interleukin-6 in response to inflammatory cytokines. *Experimental Eye Research,* 70**,** 271-279.

FLEISZIG, S. M. J., KWONG, M. S. F. & EVANS, D. J. (2003) Modification of Pseudomonas aeruginosa interactions with corneal epithelial cells by human tear fluid. *Infection & Immunity,* 71**,** 3866-3874.

FORRESTER, J. V., DICK, A. D., MCMENAMIN, P. G. & LEE, W. R. (2001) *The Eye: Basic Sciences in Practice,* Elsevier Health Sciences.

FOX, C. S., MASSARO, J. M., HOFFMANN, U., POU, K. M., MAUROVICH-HORVAT, P., LIU, C.-Y., VASAN, R. S., MURABITO, J. M., MEIGS, J. B., CUPPLES, L. A., D'AGOSTINO, R. B., SR. & O'DONNELL, C. J. (2007) Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation,* 116**,** 39-48.

FRATTI, R. A., BACKER, J. M., GRUENBERG, J., CORVERA, S. & DERETIC, V. (2001) Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *Journal of Cell Biology,* 154**,** 631-644.

FREDERICH, R. C., KAHN, B. B., PEACH, M. J. & FLIER, J. S. (1992) Tissue-specific nutritional regulation of angiotensinogen in adipose-tissue. *Hypertension,* 19**,** 339-344.

FRIEDBERG, M., ZOUMAKIS, E., HIROI, N., BADER, T., CHROUSOS, G. P. & HOCHBERG, Z. E. (2003) Modulation of 11beta-Hydroxysteroid Dehydrogenase Type 1 in Mature Human Subcutaneous Adipocytes by Hypothalamic Messengers. *Journal of Clinical Endocrinology & Metabolism.*

FRIEDMAN, J. M. & HALAAS, J. L. (1998) Leptin and the regulation of body weight in mammals. *Nature,* 395**,** 763-770.

FUENTES, G. C. & NEWGREN, J. (2008) Physiology and clinical pathology of laboratory new zealand white rabbits housed individually and in groups. *Journal of the American Association for Laboratory Animal Science: JAALAS,* 47**,** 35-8.

FUNDER, J. W. (2004) Aldosterone, mineralocorticoid receptors and vascular inflammation. *Molecular and Cellular Endocrinology,* 217**,** 263-269.

FUSE, H., KITAGAWA, H. & KATO, S. (2000) Characterization of transactivational property and coactivator mediation of rat mineralocorticoid receptor activation function-1 (AF-1). *Molecular Endocrinology,* 14**,** 889-899.

GALOR, A. & THORNE, J. E. (2007) Scleritis and peripheral ulcerative keratitis. *Rheumatic Disease Clinics of North America,* 33**,** 835-854.

GANZ, T. & LEHRER, R. I. (1994) Defensins. *Current Opinion in Immunology,* 6**,** 584-589.

GARBRECHT, M. R., SCHMIDT, T. J., KROZOWSKI, Z. S. & SNYDER, J. M. (2006) 11beta-Hydroxysteroid dehydrogenase type 2 and the regulation of surfactant protein A by dexamethasone metabolites.

GARCIA-VALENZUELA, E. & SONG, C. D. (2005) Intracorneal injection of amphothericin B for recurrent fungal keratitis and endophthalmitis. *Archives of Ophthalmology,* 123**,** 1721-1723.

GARRETT, Q., XU, S., SIMMONS, P. A., VEHIGE, J., XIE, R. Z., KUMAR, A., FLANAGAN, J. L., ZHAO, Z. & WILLCOX, M. D. P. (2008) Carboxymethyl cellulose stimulates rabbit corneal epithelial wound healing. *Current Eye Research,* 33**,** 567 - 573.

GARRITY, J. A. & BAHN, R. S. (2006) Pathogenesis of graves ophthalmopathy: implications for prediction, prevention, and treatment. *American Journal of Ophthalmology,* 142**,** 147-147.

GELDING, TAYLOR, WOOD, NOONAN, WEAVER, WOOD & MONSON (1998) The effect of growth hormone replacement therapy on cortisol-cortisone interconversion in hypopituitary adults: evidence for growth hormone modulation of extrarenal 11 beta-hydroxysteroid dehydrogenase activity. *Clinical Endocrinology,* 48**,** 153-162.

GERDING, M. N., VAN DER MEER, J. W. C., BROENINK, M., BAKKER, O., WIERSINGA, W. M. & PRUMMEL, M. F. (2000) Association of thyrotrophin receptor antibodies with the clinical features of Graves' ophthalmopathy. *Clinical Endocrinology,* 52**,** 267-271.

GERHARDT, C. C., ROMERO, I. A., CANCELLO, R., CAMOIN, L. & STROSBERG, A. D. (2001) Chemokines control fat accumulation and leptin secretion by cultured human adipocytes. *Molecular and Cellular Endocrinology,* 175**,** 81-92.

GILMOUR, J. S., COUTINHO, A. E., CAILHIER, J.-F., MAN, T. Y., CLAY, M., THOMAS, G., HARRIS, H. J., MULLINS, J. J., SECKL, J. R., SAVILL, J. S. & CHAPMAN, K. E. (2006) Local amplification of glucocorticoids by 11beta-Hydroxysteroid dehydrogenase Type 1 promotes macrophage phagocytosis of apoptotic leukocytes. *Journal of Immunology.*

GIPSON, I. K. & GIPSON, I. K. (2004) Distribution of mucins at the ocular surface. *Experimental Eye Research,* 78**,** 379-88.

GIPSON, I. K., SPURR-MICHAUD, S. J. & TISDALE, A. S. (1987) Anchoring fibrils form a complex network in human and rabbit cornea. *Investigative Ophthalmology & Visual Science.*

GOEDECKE, J. H., WAKE, D. J., LEVITT, N. S., LAMBERT, E. V., COLLINS, M. R., MORTON, N. M., ANDREW, R., SECKL, J. R. & WALKER, B. R. (2006) Glucocorticoid metabolism within superficial subcutaneous rather than visceral adipose tissue is associated with features of the metabolic syndrome in South African women. *Clinical Endocrinology,* 65**,** 81-87.

GOLDBLUM, D., BACHMANN, C., TAPPEINER, C., GARWEG, J. & FRUEH, B. E. (2008) Comparison of oral antiviral therapy with valacyclovir or acyclovir after penetrating keratoplasty for herpetic keratitis. *British Journal of Ophthalmology,* 92**,** 1201-1205.

GONG, R., MORRIS, D. J. & BREM, A. S. (2008) Human renal 11beta-hydroxysteroid dehydrogenase 1 functions and co-localizes with COX-2. *Life Sciences,* 82**,** 631-637.

GORDON, M. K., FOLEY, J. W., BIRK, D. E., FITCH, J. M. & LINSENMAYER, T. F. (1994) Type V collagen and Bowman's membrane. Quantitation of mRNA in corneal epithelium and stroma. *Journal of Biological Chemistry,* 269**,** 24959-24966.

GORDON, Y. J., HUANG, L. C., ROMANOWSKI, E. G., YATES, K. A., PROSKE, R. J. & MCDERMOTT, A. M. (2005) Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Current Eye Research,* 30**,** 385 - 394.

GORMAN, C. A., GARRITY, J. A., FATOURECHI, V., BAHN, R. S., PETERSEN, I. A., STAFFORD, S. L., EARLE, J. D., FORBES, G. S., KLINE, R. W., BERGSTRALH, E. J., OFFORD, K. P., RADEMACHER, D. M., STANLEY, N. M. & BARTLEY, G. B. (2001) A prospective, randomized, double-blind, placebo-controlled study of orbital radiotherapy for Graves' ophthalmopathy. *Ophthalmology,* 108**,** 1523-1534.

GRANSTEIN, R. D., STASZEWSKI, R., KNISELY, T. L., ZEIRA, E., NAZARENO, R., LATINA, M. & ALBERT, D. M. (1990) Aqueous humor contains transforming growth factor-beta and a small (less than 3500 daltons) inhibitor of thymocyte proliferation. *Journal of Immunology,* 144**,** 3021-3027.

GRENGA, P. M., LUPO, S. M., DOMANICO, D. M. & VINGOLO, E. M. M. (2008) Efficacy of intravitreal triamcinolone acetonide in long standing diabetic macular edema: A microperimetry and optical Coherence tomography study. *Retina,* 28**,** 1270-1275.

GRIFFITH, T. S., BRUNNER, T., FLETCHER, S. M., GREEN, D. R. & FERGUSON, T. A. (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science,* 270**,** 1189-1192.

GROSE, R., WERNER, S., KESSLER, D., TUCKERMANN, J., HUGGEL, K., DURKA, S., REICHARDT, H. M., WERNER, S., GROSE, R., WERNER, S., KESSLER, D., TUCKERMANN, J., HUGGEL, K., DURKA, S., REICHARDT, H. M. & WERNER, S. (2002) A role for endogenous glucocorticoids in wound repair. *EMBO Reports,* 3**,** 575-82.

GRUBECKLOEBENSTEIN, B., TRIEB, K., SZTANKAY, A., HOLTER, W., ANDERL, H. & WICK, G. (1994) Retrobulbar T-Cells from patients with graves ophthalmopathy are $CD8^+$ and specifically recognize autologous fibroblasts. *Journal of Clinical Investigation,* 93**,** 2738-2743.

GUERRE-MILLO, M. (2004) Adipose tissue and adipokines: for better or worse. *Diabetes & Metabolism,* 30**,** 13-19.

GUPTA, V., GALANTE, A., SOTEROPOULOS, P., GUO, S. Q. & WAGNER, B. J. (2005) Global gene profiling reveals novel glucocorticoid induced changes in gene expression of human lens epithelial cells. *Molecular Vision,* 11**,** 1018-1040.

GUZEK, J. P., CLINE, D. J., ROW, P. K., WESSELS, I. F., BEEVE, S., ISPIRESCU, S., APRECIO, R. M., KETTERING, J. D., GANO, D. L. & NELSON, G. M. (1998) Rabbit Streptococcus pneumoniae keratitis model and topical therapy. *Investigative Ophthalmology and Visual Science.*

HALL, L. R., LASS, J. H., DIACONU, E., STRINE, E. R. & PEARLMAN, E. (1999) An essential role for antibody in neutrophil and eosinophil recruitment to the cornea: B cell-deficient (microMT) mice fail to develop Th2-dependent, helminth-mediated keratitis. *Journal of Immunology,* 163**,** 4970-4975.

HALLER, J., MIKICS, É. & MAKARA, G. B. (2008) The effects of non-genomic glucocorticoid mechanisms on bodily functions and the central neural system. A critical evaluation of findings. *Frontiers in Neuroendocrinology,* 29**,** 273.

HAMMAMI, M. M. & SIITERI, P. K. (1991) Regulation of 11 beta-hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action. *Journal of Clinical Endocrinology and Metabolism,* 73**,** 326-334.

HAMRAH, P., HUQ, S. O., LIU, Y., ZHANG, Q. & DANA, M. R. (2003a) Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. *Journal of Leukocyte Biology,* 74**,** 172-178.

HAMRAH, P., LIU, Y., ZHANG, Q. & DANA, M. R. (2003b) The corneal stroma is endowed with a significant number of resident dendritic cells. *Investigative Ophthalmology & Visual Science,* 44**,** 581-589.

HAMRAH, P., ZHANG, Q., LIU, Y. & DANA, M. R. (2002) Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Investigative Ophthalmology & Visual Science,* 43**,** 639-646.

HAMSTEN, A., WIMAN, B., DE FAIRE, U. & BLOMBACK, M. (1985) Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. *New England Journal of Medicine,* 313**,** 1557-1563.

HANDOKO, K., YANG, K., STRUTT, B., KHALIL, W. & KILLINGER, D. (2000) Insulin attenuates the stimulatory effects of tumor necrosis factor [alpha] on 11[beta]-hydroxysteroid dehydrogenase 1 in human adipose stromal cells. *The Journal of Steroid Biochemistry and Molecular Biology,* 72**,** 163-168.

HANSSEN, E., FRANC, S. & GARRONE, R. (2001) Synthesis and structural organization of zonular fibers during development and aging. *Matrix Biology,* 20**,** 77-85.

HARDER, J., BARTELS, J., CHRISTOPHERS, E. & SCHRODER, J. M. (1997) A peptide antibiotic from human skin. *Nature,* 387**,** 861-861.

HARDY, R., FILER, A., COOPER, M., PARSONAGE, G., RAZA, K., HARDIE, D., RABBITT, E., STEWART, P., BUCKLEY, C. & HEWISON, M. (2006) Differential expression, function and response to inflammatory stimuli of 11beta-hydroxysteroid dehydrogenase type 1 in human fibroblasts: a mechanism for tissue-specific regulation of inflammation. *Arthritis Research & Therapy,* 8**,** R108.

HATAKEYAMA, H., INABA, S. & MIYAMORI, I. (2001) 11 beta-hydroxysteroid dehydrogenase activity in human aortic smooth muscle cells. *Hypertension Research,* 24**,** 33-37.

HAUNER, H., SCHMID, P. & PFEIFFER, E. F. (1987) Glucocorticoids and insulin promote the differentiation of human adipocyte precursor cells into fat cells. *Journal of Clinical Endocrinology & Metabolism,* 64**,** 832-835.

HAYASHI, T., BOYKO, E. J., LEONETTI, D. L., MCNEELY, M. J., NEWELL-MORRIS, L., KAHN, S. E. & FUJIMOTO, W. Y. (2003) Visceral adiposity and the risk of impaired glucose tolerance: a prospective study among Japanese Americans. *Diabetes Care,* 26**,** 650-655.

HAYASHI, T., BOYKO, E. J., LEONETTI, D. L., MCNEELY, M. J., NEWELL-MORRIS, L., KAHN, S. E. & FUJIMOTO, W. Y. (2004) Visceral adiposity is an independent predictor of incident hypertension in Japanese Americans. *Annals of Internal Medicine,* 140**,** 992-1000.

HAYASHIDA, Y., NISHIDA, K., YAMATO, M., WATANABE, K., MAEDA, N., WATANABE, H., KIKUCHI, A., OKANO, T. & TANO, Y. (2005) Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature-responsive culture surface. *Investigative Ophthalmology and Visual Science.*

HAYNES, R. J., TIGHE, P. J. & DUA, H. S. (1999) Antimicrobial defensin peptides of the human ocular surface. *Br J Ophthalmol,* 83**,** 737-741.

HEIL, F., HEMMI, H., HOCHREIN, H., AMPENBERGER, F., KIRSCHNING, C., AKIRA, S., LIPFORD, G., WAGNER, H. & BAUER, S. (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science.*

HERMANOWSKI-VOSATKA, A., BALKOVEC, J. M., CHENG, K., CHEN, H. Y., HERNANDEZ, M., KOO, G. C., LE GRAND, C. B., LI, Z., METZGER, J. M., MUNDT, S. S., NOONAN, H., NUNES, C. N., OLSON, S. H., PIKOUNIS, B., REN, N., ROBERTSON, N., SCHAEFFER, J. M., SHAH, K., SPRINGER, M. S., STRACK, A. M., STROWSKI, M., WU, K., WU, T., XIAO, J., ZHANG, B. B., WRIGHT, S. D. & THIERINGER, R. (2005) 11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. *J. Exp. Med.,* 202**,** 517-527.

HEUFELDER, A. E. & BAHN, R. S. (1993a) Detection and localization of cytokine immunoreactivity in retroocular connective-tissue in graves ophthalmopathy. *European Journal of Clinical Investigation,* 23**,** 10-17.

HEUFELDER, A. E. & BAHN, R. S. (1993b) Elevated expression in situ of selectin and immunoglobulin superfamily type adhesion molecules in retroocular connective tissues from patients with Graves' ophthalmopathy. *Clinical and Experimental Immunology,* 91**,** 381-389.

HEUFELDER, A. E. & BAHN, R. S. (1994) Modulation of Graves' orbital fibroblast proliferation by cytokines and glucocorticoid receptor agonists. *Investigative Ophthalmopathy & Visual Science,* 35**,** 120-127.

HEUFELDER, A. E., BAHN, R. S., SMITH, T. J., HEUFELDER, A. E., BAHN, R. S. & SMITH, T. J. (1992a) Regulation by glucocorticoids of interferon gamma-induced HLA-DR antigen expression in cultured human orbital fibroblasts. *Clinical Endocrinology,* 37**,** 59-63.

HEUFELDER, A. E., WENZEL, B. E. & BAHN, R. S. (1992b) Cell surface localization of a 72 kilodalton heat shock protein in retroocular fibroblasts from patients with Graves' ophthalmopathy. *Journal of Clinical Endocrinology & Metabolism,* 74**,** 732-736.

HEUFELDER, A. E., WENZEL, B. E., GORMAN, C. A. & BAHN, R. S. (1991) Detection, cellular localization, and modulation of heat shock proteins in cultured fibroblasts from patients with extrathyroidal manifestations of Graves' disease. *Journal of Clinical Endocrinology & Metabolism,* 73**,** 739-745.

HINGORANI, M., METZ, D. & LIGHTMAN, S. L. (1997) Characterisation of the Normal Conjunctival Leukocyte Population. *Experimental Eye Research,* 64**,** 905-912.

HIRASAWA, G., SASANO, H., TAKAHASHI, K.-I., FUKUSHIMA, K., SUZUKI, T., HIWATASHI, N., TOYOTA, T., KROZOWSKI, Z. S. & NAGURA, H. (1997) Colocalization of 11 beta-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human epithelia. *Journal of Clinical Endocrinology & Metabolism,* 82**,** 3859-3863.

HIROMATSU, Y., TANAKA, K., ISHISAKA, N., KAMACHI, J., KUROKI, T., HOSHINO, T., INOUE, Y., WALL, J. R. & NONAKA, K. (1995) Human histocompatibility leukocyte antigen-DR and heat shock protein-70 expression in eye muscle tissue in thyroid-associated ophthalmopathy. *Journal of Clinical Endocrinology & Metabolism,* 80**,** 685-691.

HOANG-XUAN, T., BAUDOUIN, CHRISTOPHE, CREUZOT-GARCHER, CATHERINE (2001) *Inflammatory Diseases of the Conjunctiva*, Thieme.

HOLTHÖFER, H., SIEGEL, G., TARKKANEN, A. & TERVO, T. (1991) Immunocytochemical localization of carbonic anhydrase, NaK-ATPase and the bicarbonate chloride exchanger in the anterior segment of the human eye. *Acta Ophthalmologica,* 69**,** 149-54.

HONG, D., CHEN, H.-X., GE, R.-S. & LI, J.-C. (2008) The Biological Roles of Extracellular and Intracytoplasmic Glucocorticoids in Skeletal Cells. *The Journal of Steroid Biochemistry and Molecular Biology,* 111**,** 164-170.

HONG, H., DARIMONT, B. D., MA, H., YANG, L., YAMAMOTO, K. R. & STALLCUP, M. R. (1999) An additional region of coactivator GRIP1 required for interaction with the hormone-binding domains of a subset of nuclear receptors. *Journal of Biological Chemistry,* 274**,** 3496-3502.

HONGXIU, N., GANG, L., GUITING, L., RONG, Y., TOM, F. L. & CHING-SHWUN, L. (2009) Fibroblast growth factor 2 promotes endothelial differentiation of adipose tissue-derived stem cells. *Journal of Sexual Medicine,* 6**,** 967-979.

HORWITZ, D. A., GRAY, J. D. & OHTSUKA, K. (1999) Role of NK cells and TGF-beta in the regulation of T-cell-dependent antibody production in health and autoimmune disease. *Microbes and Infection,* 1**,** 1305-1311.

HSUEH, A. J. W. & ERICKSON, G. F. (1978) Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. *Steroids,* 32**,** 639-648.

HUANG, A. J., TSENG, S. C., HUANG, A. J. & TSENG, S. C. (1991) Corneal epithelial wound healing in the absence of limbal epithelium. *Investigative Ophthalmology & Visual Science,* 32**,** 96-105.

HUANG, T., HE, D., KLEINER, G. & KULUZ, J. (2007) Neuron-like differentiation of adipose-derived stem cells from infant piglets in vitro. *Journal of Spinal Cord Medicine,* 30 Suppl 1**,** S35-40.

HUBER, A. K., JACOBSON, E. M., JAZDZEWSKI, K., CONCEPCION, E. S. & TOMER, Y. (2008) Interleukin (IL)-23 receptor is a major susceptibility gene for Graves' ophthalmopathy: the IL-23/T-helper 17 axis extends to thyroid autoimmunity. *Journal of Clinical Endocrinology & Metabolism,* 93**,** 1077-1081.

HUNDERTMARK, S., BUHLER, H., RAGOSCH, V., DINKELBORG, L., ARABIN, B. & WEITZEL, H. K. (1995) Correlation of surfactant phosphatidylcholine synthesis and 11 beta- hydroxysteroid dehydrogenase in the fetal lung. *Endocrinology.*

HUTLEY, L. J., HERINGTON, A. C., SHURETY, W., CHEUNG, C., VESEY, D. A., CAMERON, D. P. & PRINS, J. B. (2001) Human adipose tissue endothelial cells promote preadipocyte proliferation. *Am J Physiol Endocrinol Metab,* 281**,** E1037-1044.

IN-KYUNG, J., SEUNG-HOON, O., BYUNG-JOON, K., JAE-HOON, C., YONG-KI, M., MYUNG-SHIK, L., MOON-KYU, L. & KWANG-WON, K. (2001) The effects of dexamethasone on insulin release and biosynthesis are dependent on the dose and duration of treatment. *Diabetes research and clinical practice,* 51**,** 163-171.

ISHIKAWA, T. (1962) Fine structure of the human cillary muscle. *Investigative Ophthalmology,* 1**,** 587-608.

ITOH, Y., KAMATA-SAKURAI, M., DENDA-NAGAI, K., NAGAI, S., TSUIJI, M., ISHII-SCHRADE, K., OKADA, K., GOTO, A., FUKAYAMA, M. & IRIMURA, T. (2008) Identification and expression of human epiglycanin/MUC21: a novel transmembrane mucin. *Glycobiology,* 18**,** 74-83.

JAMIESON, A., WALLACE, A. M., ANDREW, R., NUNEZ, B. S., WALKER, B. R., FRASER, R., WHITE, P. C. & CONNELL, J. M. C. (1999) Apparent cortisone reductase deficiency: a functional defect in 11beta-hydroxysteroid dehydrogenase type 1. *Journal of Clinical Endocrinology & Metabolism,* 84**,** 3570-3574.

JAMIESON, P. M., CHAPMAN, K. E., EDWARDS, C. R. & SECKL, J. R. (1995) 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta- reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. *Endocrinology,* 136**,** 4754-4761.

JIA, H. P., SCHUTTE, B. C., SCHUDY, A., LINZMEIER, R., GUTHMILLER, J. M., JOHNSON, G. K., TACK, B. F., MITROS, J. P., ROSENTHAL, A., GANZ, T., MCCRAY, P. B., JR., JIA, H. P., SCHUTTE, B. C., SCHUDY, A., LINZMEIER, R., GUTHMILLER, J. M., JOHNSON, G. K., TACK, B. F., MITROS, J. P., ROSENTHAL, A., GANZ, T. & MCCRAY, P. B., JR. (2001) Discovery of new human beta-defensins using a genomics-based approach. *Gene,* 263**,** 211-8.

JIANG, L. Q. & STREILEIN, J. W. (1991) Immune privilege extended to allogeneic tumor cells in the vitreous cavity. *Investigative Ophthalmology & Visual Science,* 32**,** 224-228.

JIN, X., QIN, Q., CHEN, W., QU, J., JIN, X., QIN, Q., CHEN, W. & QU, J. (2007) Expression of toll-like receptors in the healthy and herpes simplex virus-infected cornea. *Cornea,* 26**,** 847-52.

JIN, X., QIN, Q., LIN, Z., CHEN, W. & QU, J. (2008) Expression of Toll-Like Receptors in the *Fusarium solani* Infected Cornea. *Current Eye Research,* 33**,** 319 - 324.

JOHANSSON, A., ANDREW, R., FORSBERG, H., CEDERQUIST, K., WALKER, B. R. & OLSSON, T. (2001) Glucocorticoid metabolism and adrenocortical reactivity to ACTH in myotonic dystrophy. *Journal of Clinical Endocrinology & Metabolism,* 86**,** 4276-4283.

JOHNSON, M. (2006) 'What controls aqueous humour outflow resistance?' *Experimental Eye Research,* 82**,** 545-557.

JOHNSTONE, C. & DEL VAL, M. (2007) Traffic of proteins and peptides across membranes for immunosurveillance by CD8(+) T lymphocytes: a topological challenge. *Traffic,* 8**,** 1486-1494.

JONAS, J. B., AKKOYUN, I., KREISSIG, I. & DEGENRING, R. F. (2005) Diffuse diabetic macular oedema treated by intravitreal triamcinolone acetonide: a comparative, non-randomised study. *British Journal of Ophthalmology,* 89**,** 321-326.

JONES, K. M., LLOYDJONES, R., RIONDEL, A., TAIT, J. F., TAIT, S. A. S., BULBROOK, R. D. & GREENWOOD, F. C. (1959) Aldosterone secretion and metabolism in normal men and women and in pregnancy. *Acta Endocrinologica,* 30**,** 321-342.

JONES, R. F. & MAURICE, D. M. (1966) New Methods of Measuring Rate of Aqueous Flow in Man with Fluorescein. *Experimental Eye Research,* 5**,** 208-&.

JOSEPH, A., POWELL-RICHARDS, A. O. R., SHANMUGANATHAN, V. A. & DUA, H. S. (2004) Epithelial cell characteristics of cultured human limbal explants. *British Journal of Ophthalmology,* 88**,** 393-398.

JUNQUEIRA, L. C. U. & CARNEIRO, J. (2005) *Basic Histology: Text & Atlas*, McGraw-Hill Professional.

KABACK, L. A. & SMITH, T. J. (1999) Expression of hyaluronan synthase messenger ribonucleic acids and their induction by interleukin-1beta in human orbital fibroblasts: potential insight into the molecular pathogenesis of thyroid-associated ophthalmopathy. *Journal of Clinical Endocrinology & Metabolism,* 84**,** 4079-4084.

KADLUBOWSKI, M., IRVINE, W. J. & ROWLAND, A. C. (1986) The lack of specificity of ophthalmic immunoglobulins in Graves' disease. *Journal of Clinical Endocrinology & Metabolism,* 63**,** 990-995.

KAHALY, G. J. (2001) Imaging in thyroid-associated orbitopathy. *European Journal of Endocrinology,* 145**,** 107-118.

KAIJZEL, E. L., VAN KRUGTEN, M. V., BRINKMAN, B. M. N., HUIZINGA, T. W. J., VAN DER STRAATEN, T., HAZES, J. M. W., ZIEGLER-HEITBROCK, H. W. L., NEDOSPASOV, S. A., BREEDVELD, F. C. & VERWEIJ, C. L. (1998) Functional analysis of a human tumor necrosis factor alpha (TNF-alpha) promoter polymorphism related to joint damage in rheumatoid arthritis. *Molecular Medicine,* 4**,** 724-733.

KAMEI, Y., XU, L., HEINZEL, T., TORCHIA, J., KUROKAWA, R., GLOSS, B., LIN, S.-C., HEYMAN, R. A., ROSE, D. W., GLASS, C. K. & ROSENFELD, M. G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell,* 85**,** 403-414.

KAMINSKI, H. J., LI, Z., RICHMONDS, C., RUFF, R. L., KUSNER, L., KAMINSKI, H. J., LI, Z., RICHMONDS, C., RUFF, R. L. & KUSNER, L. (2003) Susceptibility of ocular tissues to autoimmune diseases. *Annals of the New York Academy of Sciences,* 998**,** 362-74.

KAMIZONO, S., HIROMATSU, Y., SEKI, N., BEDNARCZUK, T., MATSUMOTO, H., KIMURA, K. & ITOH, I. (2000) A polymorphism of the 5 ' flanking region of tumour necrosis factor alpha gene is associated with thyroid-associated ophthalmopathy in Japanese. *Clinical Endocrinology,* 52**,** 759-764.

KARPUS, W. J., LUKACS, N. W., KENNEDY, K. J., SMITH, W. S., HURST, S. D., BARRETT, T. A., KARPUS, W. J., LUKACS, N. W., KENNEDY, K. J., SMITH, W. S., HURST, S. D. & BARRETT, T. A. (1997) Differential CC chemokine-induced enhancement of T helper cell cytokine production. *Journal of Immunology,* 158**,** 4129-36.

KATZMARZYK, P. T., PÉRUSSE, L. & BOUCHARD, C. (1999) Genetics of abdominal visceral fat levels. *American Journal of Human Biology,* 11**,** 225-235.

KENDALLTAYLOR, P., STEPHENSON, A., STRATTON, A., PAPIHA, S. S., PERROS, P. & ROBERTS, D. F. (1988) Differentiation of autoimmune ophthalmopathy from Graves' hyperthyroidism by analysis of genetic markers. *Clinical Endocrinology,* 28**,** 601-610.

KENDLER, D. L., LIPPA, J. & ROOTMAN, J. (1993) The initial clinical characteristics of Graves' orbitopathy vary with age and sex. *Archives of Ophthalmology,* 111**,** 197-201.

KERSHAW, E. E. & FLIER, J. S. (2004) Adipose tissue as an endocrine organ. *Journal of Clinical Endocrinology & Metabolism,* 89**,** 2548-2556.

KESSLER, E., MONDINO, B. J. & BROWN, S. I. (1977) The corneal response to Pseudomonas aeruginosa: histopathological and enzymatic characterization. *Investigative Ophthalmology & Visual Science,* 16**,** 116-125.

KHOO, T. K. & BAHN, R. S. (2007) Pathogenesis of Graves' ophthalmopathy: the role of autoantibodies. *Thyroid,* 17**,** 1013-1018.

KIM, H. J., ZHAO, H., KITAURA, H., BHATTACHARYYA, S., BREWER, J. A., MUGLIA, L. J., ROSS, F. P. & TEITELBAUM, S. L. (2007) Dexamethsone suppresses bone formation via the osteoclast. *Osteoimmunology,* 602**,** 43-46.

KIMURA, K., ENDOU, H., SUDO, J. & SAKAI, F. (1979) Glucose dehydrogenase (hexose 6-phosphate dehydrogenase) and the microsomal electron transport system. Evidence supporting their possible functional relationship. *Journal of Biochemistry,* 85**,** 319-326.

KINO, T., NORDEEN, S. K. & CHROUSOS, G. P. (1999) Conditional modulation of glucocorticoid receptor activities by CREB-binding protein (CBP) and p300. *Journal of Steroid Biochemistry and Molecular Biology,* 70**,** 15-25.

KLIPPEL, J. H. (2007) *Primer on the Rheumatic Diseases,* Springer.

KNISELY, T. L., HOSOI, J., NAZARENO, R. & GRANSTEIN, R. D. (1994) The presence of biologically significant concentrations of glucocorticoids but little or no cortisol binding globulin within aqueous humor: relevance to immune privilege in the anterior chamber of the eye. *Investigative Ophthalmology & Visual Science,* 35**,** 3711-3723.

KNOP, N. & KNOP, E. (2000) Conjunctiva-associated lymphoid tissue in the human eye. *Investigative Ophthalmology & Visual Science,* 41**,** 1270-1279.

KNUDSEN, L. L. (2004) Retrobulbar injection of methylprednisolone in diffuse diabetic macular edema. *Retina,* 24**,** 905-909.

KOERNER, D. R. (1969) Assay and substrate specificity of liver 11[beta]-hydroxysteroid dehydrogenase. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 176**,** 377-382.

KOEVARY, S. B. (2000) Ocular immune privilege: a review. *Clinical Eye and Vision Care,* 12**,** 97-106.

KOLEHMAINEN, M., VIDAL, H., OHISALO, J. J., PIRINEN, E., ALHAVA, E. & UUSITUPA, M. I. J. (2002) Hormone sensitive lipase expression and adipose tissue metabolism show gender difference in obese subjects after weight loss. *International Journal of Obesity,* 26**,** 6-16.

KOLLS, J. K., MCCRAY, P. B. & CHAN, Y. R. (2008) Cytokine-mediated regulation of antimicrobial proteins. *Nat Rev Immunol,* 8**,** 829-835.

KONSTAS, A. G. P., KOLIAKOS, G. G., KARABATSAS, C. H., LIAKOS, P., SCHLÖTZER-SCHREHARDT, U., GEORGIADIS, N. & RITCH, R. (2006) Latanoprost therapy reduces the

levels of TGF beta 1 and gelatinases in the aqueous humour of patients with exfoliative glaucoma. *Experimental Eye Research,* 82**,** 319-322.

KORBONITS, M., BUJALSKA, I., SHIMOJO, M., NOBES, J., JORDAN, S., GROSSMAN, A. B. & STEWART, P. M. (2001) Expression of 11 beta-hydroxysteroid dehydrogenase isoenzymes in the human pituitary: induction of the type 2 enzyme in corticotropinomas and other pituitary tumors. *Journal of Clinical Endocrinology & Metabolism,* 86**,** 2728-2733.

KORDUCKI, J. M., LOFTUS, S. J. & BAHN, R. S. (1992) Stimulation of glycosaminoglycan production in cultured human retroocular fibroblasts. *Investigative Ophthalmology & Visual Science,* 33**,** 2037-2042.

KOTELEVTSEV, Y., HOLMES, M. C., BURCHELL, A., HOUSTON, P. M., SCHMOLL, D., JAMIESON, P., BEST, R., BROWN, R., EDWARDS, C. R. W., SECKL, J. R. & MULLINS, J. J. (1997) 11beta-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proceedings of the National Academy of Sciences of the United States of America.*

KOWALEWSKA, B., SZECHINSKI, J., ROSZKOWSKA, E., KOWALEWSKA, B., SZECHINSKI, J. & ROSZKOWSKA, E. (2008) Wegener's granulomatosis effectively treated with rituximab: a case study. *Polskie Archiwum Medycyny Wewnetrznej,* 118**,** 381-5.

KRETOWSKI, A., WAWRUSIEWICZ, N., MIRONCZUK, K., MYSLIWIEC, J., KRETOWSKA, M. & KINALSKA, I. (2003) Intercellular adhesion molecule 1 gene polymorphisms in Graves' disease. *Journal of Clinical Endocrinology and Metabolism,* 88**,** 4945-4949.

KROZOWSKI, Z. (1999) The 11[beta]-hydroxysteroid dehydrogenases: functions and physiological effects. *Molecular and Cellular Endocrinology,* 151**,** 121-127.

KROZOWSKI, Z., MAGUIRE, J. A., STEIN-OAKLEY, A. N., DOWLING, J., SMITH, R. E. & ANDREWS, R. K. (1995) Immunohistochemical localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. *Journal of Clinical Endocrinology & Metabolism,* 80**,** 2203-2209.

KRUSE, F. E., CHEN, J. J., TSAI, R. J., TSENG, S. C., KRUSE, F. E., CHEN, J. J., TSAI, R. J. & TSENG, S. C. (1990) Conjunctival transdifferentiation is due to the incomplete removal of limbal basal epithelium. *Investigative Ophthalmology & Visual Science,* 31**,** 1903-13.

KUMAR, S., COENEN, M. J., SCHERER, P. E. & BAHN, R. S. (2004) Evidence for enhanced adipogenesis in the orbits of patients with Graves' ophthalmopathy. *Journal of Clinical Endocrinology & Metabolism,* 89**,** 930-935.

KYOSSEV, Z., WALKER, P. D., REEVES, W. B., KYOSSEV, Z., WALKER, P. D. & REEVES, W. B. (1996) Immunolocalization of NAD-dependent 11 beta-hydroxysteroid dehydrogenase in human kidney and colon. *Kidney International,* 49**,** 271-81.

LAKSHMI, V. & MONDER, C. (1988) Purification and Characterization of the Corticosteroid 11 Beta-Dehydrogenase Component of the Rat-Liver 11 Beta-Hydroxysteroid Dehydrogenase Complex. *Endocrinology,* 123**,** 2390-2398.

LAM, D. S., HOUANG, E., FAN, D. S., LYON, D., SEAL, D., WONG, E., HONG KONG MICROBIAL KERATITIS STUDY, G., LAM, D. S. C., HOUANG, E., FAN, D. S. P., LYON, D., SEAL, D., WONG, E. & HONG KONG MICROBIAL KERATITIS STUDY, G. (2002) Incidence and risk factors for microbial keratitis in Hong Kong: comparison with Europe and North America. *Eye,* 16**,** 608-18.

LANKFORD, C. S. R. & FRUCHT, D. M. (2003) A unique role for IL-23 in promoting cellular immunity. *Journal of Leukocyte Biology,* 73**,** 49-56.

LAURA KOUMAS, T. J. S. R. P. P. (2002) Fibroblast subsets in the human orbit: Thy-1 subpopulations exhibit distinct phenotypes. *European Journal of Immunology,* 32**,** 477-485.

LECLERC, M., BRUNETTE, M. G. & COUCHOUREL, D. (2004) Aldosterone enhances renal calcium reabsorption by two types of channels. *Kidney Int,* 66**,** 242-250.

LEE, S. K., CHOI, B. K., KANG, W. J., KIM, Y. H., PARK, H. Y., KIM, K. H., KWON, B. S., LEE, S. K., CHOI, B. K., KANG, W. J., KIM, Y. H., PARK, H. Y., KIM, K. H. & KWON, B. S. (2008) MCP-1 derived from stromal keratocyte induces corneal infiltration of $CD4^+$ T cells in herpetic stromal keratitis. *Molecules & Cells,* 26**,** 67-73.

LEIBOVITCH, I., PRABHAKARAN, V. C., DAVIS, G. & SELVA, D. (2007) Intraorbital injection of triamcinolone acetonide in patients with idiopathic orbital inflammation. *Archives of Ophthalmology,* 125**,** 1647-1651.

LEVETAN, C. S. & MAGEE, M. F. (2000) Hospital management of diabetes. *Endocrinology & Metabolism Clinics of North America,* 29**,** 745-770.
LI, D.-Q., LOKESHWAR, B. L., SOLOMON, A., MONROY, D., JI, Z. & PFLUGFELDER, S. C. (2001) Regulation of MMP-9 Production by Human Corneal Epithelial Cells. *Experimental Eye Research,* 73**,** 449.
LI, J., SHEN, J., BEUERMAN, R. W., LI, J., SHEN, J. & BEUERMAN, R. W. (2007) Expression of toll-like receptors in human limbal and conjunctival epithelial cells. *Molecular Vision,* 13**,** 813-22.
LI, W., GAO, L., WANG, Y., DUAN, T., MYATT, L. & SUN, K. (2006) Enhancement of cortisol-induced 11beta-hydroxysteroid dehydrogenase type 1 expression by interleukin 1beta in cultured human chorionic trophoblast cells. *Endocrinology,* 147**,** 2490-2495.
LI, X., WONG, J., TSAI, S. Y., TSAI, M.-J. & O'MALLEY, B. W. (2003) Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. *Molecular and Cellular Biology,* 23**,** 3763-3773.
LI, Y., SUINO, K., DAUGHERTY, J. & XU, H. E. (2005) Structural and biochemical mechanisms for the specificity of hormone binding and coactivator assembly by mineralocorticoid receptor. *Molecular Cell,* 19**,** 367-380.
LIANG, F., KAPOUN, A. M., LAM, A., DAMM, D. L., QUAN, D., O'CONNELL, M. & PROTTER, A. A. (2007a) B-Type natriuretic peptide inhibited angiotensin II-stimulated cholesterol biosynthesis, cholesterol transfer, and steroidogenesis in primary human adrenocortical cells. *Endocrinology,* 148**,** 3722-3729.
LIANG, H., BRIGNOLE-BAUDOUIN, F., LABBE, A., PAULY, A., WARNET, J. M., BAUDOUIN, C., LIANG, H., BRIGNOLE-BAUDOUIN, F., LABBE, A., PAULY, A., WARNET, J.-M. & BAUDOUIN, C. (2007b) LPS-stimulated inflammation and apoptosis in corneal injury models. *Molecular Vision,* 13**,** 1169-80.
LIESEGANG, T. J. (1997) Contact lens-related microbial keratitis: Part I: Epidemiology. *Cornea,* 16**,** 125-31.
LOLMEDE, K., DUNAND DE SAINT FRONT, V., GALITZHY, J., LAFONTAN, M. & BOULOUMIE, A. (2003) Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int J Obes Relat Metab Disord,* 27**,** 1187.
LU, H., LU, Q. J., FU, T., PAN, Z. Q. & WANG, N. L. (2008) The effects of glaucoma filtering surgery on anterior chamber-associated immune deviation and contribution of lymphatic drainage in rats. *Eye,* 23**,** 215-21.
LU, Y., LIU, Y., FUKUDA, K., NAKAMURA, Y., KUMAGAI, N., NISHIDA, T., LU, Y., LIU, Y., FUKUDA, K., NAKAMURA, Y., KUMAGAI, N. & NISHIDA, T. (2006) Inhibition by triptolide of chemokine, proinflammatory cytokine, and adhesion molecule expression induced by lipopolysaccharide in corneal fibroblasts. *Investigative Ophthalmology & Visual Science,* 47**,** 3796-800.
LUDGATE, M. & BAKER, G. (2002) Unlocking the immunological mechanisms of orbital inflammation in thyroid eye disease. *Clinical and Experimental Immunology,* 127**,** 193-198.
LUDGATE, M. & BAKER, G. (2004) Inducing Graves' ophthalmopathy. *Journal of Endocrinological Investigation,* 27**,** 211-215.
LUETSCHE.JA, HANCOCK, E. W., CAMARGO, C. A., DOWDY, A. J. & NOKES, G. W. (1965) Conjugation of 1,2-3H-aldosterone in human liver and kidneys and renal extraction of aldosterone and labeled conjugates from blood plasma. *Journal of Clinical Endocrinology and Metabolism,* 25**,** 628-38.
LUNDGREN, M., BUREN, J., LINDGREN, P., MYRNAS, T., RUGE, T. & ERIKSSON, J. W. (2008) Sex- and depot-specific lipolysis regulation in human adipocytes: interplay between adrenergic stimulation and glucocorticoids. *Hormone & Metabolic Research,* 40**,** 854-60.
LUTT, J. R., LIM, L. L., PHAL, P. M. & ROSENBAUM, J. T. (2008) Orbital inflammatory disease. *Seminars in Arthritis and Rheumatism,* 37**,** 207-222.
LYNGHOLM, M., HØYER, P. E., VORUM, H., NIELSEN, K., EHLERS, N. & MØLLGÅRD, K. (2008) Immunohistochemical markers for corneal stem cells in the early developing human eye. *Experimental Eye Research,* 87**,** 115-121.
MA, A., BOULTON, M., ZHAO, B., CONNON, C., CAI, J. & ALBON, J. (2007) A role for notch signaling in human corneal epithelial cell differentiation and proliferation. *Investigative Ophthalmology & Visual Science,* 48**,** 3576-3585.

MACFARLANE, D. P., FORBES, S. & WALKER, B. R. (2008) Glucocorticoids and fatty acid metabolism in humans: fuelling fat redistribution in the metabolic syndrome. *Journal of Endocrinology,* 197**,** 189-204.

MACREDMOND, R., GREENE, C., TAGGART, C., MCELVANEY, N. & O'NEILL, S. (2005) Respiratory epithelial cells require Toll-like receptor 4 for induction of Human beta-defensin 2 by Lipopolysaccharide. *Respiratory Research,* 6**,** 116.

MAEDA, K., OKUBO, K., SHIMOMURA, I., FUNAHASHI, T., MATSUZAWA, Y. & MATSUBARA, K. (1996) cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). *Biochemical and Biophysical Research Communications,* 221**,** 286-289.

MAGONE, M. T., NASSER, R. E., CEVALLOS, A. V. & MARGOLIS, T. P. (2005) Chronic recurrent varicella-zoster virus keratitis confirmed by polymerase chain reaction testing. *American Journal of Ophthalmology,* 139**,** 1135-1136.

MAKKONEN, J., WESTERBACKA, J., KOLAK, M., SUTINEN, J., CORNER, A., HAMSTEN, A., FISHER, R. M. & YKI-JARVINEN, H. (2007) Increased expression of the macrophage markers and of 11beta-HSD-1 in subcutaneous adipose tissue, but not in cultured monocyte-derived macrophages, is associated with liver fat in human obesity. *Int J Obes,* 31**,** 1617-1625.

MALTSEVA, I. A., FLEISZIG, S. M. J., EVANS, D. J., KERR, S., SIDHU, S. S., MCNAMARA, N. A. & BASBAUM, C. (2007) Exposure of human corneal epithelial cells to contact lenses in vitro suppresses the upregulation of human beta-defensin-2 in response to antigens of Pseudomonas aeruginosa. *Experimental Eye Research,* 85**,** 142-153.

MANJI, N., CARR-SMITH, J. D., BOELAERT, K., ALLAHABADIA, A., ARMITAGE, M., CHATTERJEE, V. K., LAZARUS, J. H., PEARCE, S. H. S., VAIDYA, B., GOUGH, S. C. & FRANKLYN, J. A. (2006) Influences of age, gender, smoking, and family history on autoimmune thyroid disease phenotype. *Journal of Clinical Endocrinology & Metabolism,* 91**,** 4873-4880.

MANOLAGAS, S. C., WEINSTEIN, R. S., MANOLAGAS, S. C. & WEINSTEIN, R. S. (1999) New developments in the pathogenesis and treatment of steroid-induced osteoporosis. *Journal of Bone & Mineral Research,* 14**,** 1061-6.

MANY, M. C., COSTAGLIOLA, S., DETRAIT, M., DENEF, J. F., VASSART, G. & LUDGATE, M. (1999) Development of an animal model of autoimmune thyroid eye disease. *Journal of Immunology,* 162**,** 4966-4974.

MARCOCCI, C., BARTALENA, L., BOGAZZI, F., PANICUCCI, M. & PINCHERA, A. (1989) Studies on the occurrence of ophthalmopathy in graves Disease. *Acta Endocrinologica,* 120**,** 473-478.

MARCOCCI, C., BARTALENA, L., TANDA, M. L., MANETTI, L., DELL'UNTO, E., ROCCHI, R., BARBESINO, G., MAZZI, B., BARTOLOMEI, M. P., LEPRI, P., CARTEI, F., NARDI, M. & PINCHERA, A. (2001) Comparison of the effectiveness and tolerability of intravenous or oral glucocorticoids associated with orbital radiotherapy in the management of severe Graves' ophthalmopathy: results of a prospective, single-blind, randomized study. *Journal of Clinical Endocrinology & Metabolism,* 86**,** 3562-3567.

MARTTILA-ICHIHARA, F., TURJA, R., MIILUNIEMI, M., KARIKOSKI, M., MAKSIMOW, M., NIEMELA, J., MARTINEZ-POMARES, L., SALMI, M. & JALKANEN, S. (2008) Macrophage mannose receptor on lymphatics controls cell trafficking. *Blood,* 112**,** 64-72.

MASCARETTI, O. A. (2003) *Bacteria Versus Antibacterial Agents: An Integrated Approach*, ASM Press.

MASLOWSKA, M., LEGAKIS, H., ASSADI, F. & CIANFLONE, K. (2006) Targeting the signaling pathway of acylation stimulating protein. *Journal of Lipid Research,* 47**,** 643-652.

MATOS, J., SORENSEN, M., GEYTI, C., ROBAYE, B., BOEYNAEMS, J. & LEIPZIGER, J. (2007) Distal colonic Na+ absorption inhibited by luminal P2Y2 receptors. *Pflügers Archiv European Journal of Physiology,* 454**,** 977-987.

MATTSSON, C. & OLSSON, T. (2007) Estrogens and glucocorticoid hormones in adipose tissue metabolism. *Current Medicinal Chemistry,* 14**,** 2918-2924.

MCCORMICK, J. A., BHALLA, V., PAO, A. C. & PEARCE, D. (2005) SGK1: a rapid aldosterone-induced regulator of renal sodium reabsorption. *Physiology,* 20**,** 134-139.

MCCURDY, C. & FRIEDMAN, J. (2006) Early foetal programming of hepatic gluconeogenesis: glucocorticoids strike back. *Diabetologia,* 49**,** 1138-1141.

MCDERMOTT, A. M., REDFERN, R. L. & ZHANG, B. (2001) Human beta-defensin 2 is up-regulated during re-epithelialization of the cornea. *Current Eye Research,* 22**,** 64-67.

MCDONALD, T. O. (1970) Corneal wound healing: 1. Inhibition of stromal healing by 3 dexamethasone derivatives. *Investigative Ophthalmology,* 9**,** 703-&.

MCNAMARA, N. A., VAN, R., TUCHIN, O. S. & FLEISZIG, S. M. (1999) Ocular surface epithelia express mRNA for human beta defensin-2. *Experimental Eye Research,* 69**,** 483-90.

MEDZHITOV, R., PRESTON-HURLBURT, P., KOPP, E., STADLEN, A., CHEN, C., GHOSH, S. & JANEWAY, C. A. (1998) MyD88 Is an Adaptor Protein in the hToll/IL-1 Receptor Family Signaling Pathways. *Molecular Cell,* 2**,** 253-258.

MIRSHAHI, M., MIRSHAHI, A., SEDIGHIAN, R., HECQUET, C., FAURE, J. P. & AGARWAL, M. K. (1997) Immunochemical demonstration of the mineralocorticoid receptor in ocular tissues. *Neuroendocrinology,* 65**,** 70-78.

MITO, T., YOSHINORI, M., KONGHUA, L., KEIZOU, S. & MASUTAKA, F. (2009) Characterization and comparison of adipose tissue-derived cells from human subcutaneous and omental adipose tissues. *Cell Biochemistry and Function,* 27**,** 440-447.

MOHAN, R. & MURALIDHARAN, A. R. (1989) Steroid induced glaucoma and cataract. *Indian Journal of Ophthalmology,* 37**,** 13-16.

MOISAN, M. P., SECKL, J. R. & EDWARDS, C. R. W. (1990) 11 beta-hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus, and cortex. *Endocrinology,* 127**,** 1450-1455.

MOLLER, N., JORGENSEN, J. O., SCHMITZ, O., MOLLER, J., CHRISTIANSEN, J., ALBERTI, K. G. & ORSKOV, H. (1990) Effects of a growth hormone pulse on total and forearm substrate fluxes in humans. *American Journal of Physiology,* 258**,** E86-91.

MONDER, C. & BRADLOW, H. (1980) Cortoic acids: explorations at the frontier of corticosteroid metabolism. *Recent Progress in Hormone Research,* 36**,** 345–400.

MONDER, C. & LAKSHMI, V. (1989) Evidence for kinetically distinct forms of corticosteroid 11[beta]-dehydrogenase in rat liver microsomes. *Journal of Steroid Biochemistry,* 32**,** 77-83.

MONIAUX, N., ESCANDE, F., PORCHET, N., AUBERT, J. P., BATRA, S. K., MONIAUX, N., ESCANDE, F., PORCHET, N., AUBERT, J. P. & BATRA, S. K. (2001) Structural organization and classification of the human mucin genes. *Frontiers in Bioscience,* 6**,** D1192-206.

MOORE, J. S., MONSON, J. P., KALTSAS, G., PUTIGNANO, P., WOOD, P. J., SHEPPARD, M. C., BESSER, G. M., TAYLOR, N. F. & STEWART, P. M. (1999) Modulation of 11beta-hydroxysteroid dehydrogenase isozymes by growth hormone and insulin-like growth factor: in vivo and in vitro studies. *Journal of Clinical Endocrinology & Metabolism,* 84**,** 4172-4177.

MORRIS, D. J. (1981) The metabolism and mechanism of action of aldosterone. *Endocrine Reviews,* 2**,** 234-247.

MOURITS, M. P., VAN KEMPEN-HARTEVELD, M. L., GARCÍA, M. B. G., KOPPESCHAAR, H. P. F., TICK, L. & TERWEE, C. B. (2000) Radiotherapy for Graves' orbitopathy: randomised placebo-controlled study. *The Lancet,* 355**,** 1505-1509.

MÜLLER, M. J., ACHESON, K. J., JEQUIER, E. & BURGER, A. G. (1990) Thyroid hormone action on lipid metabolism in humans: A role for endogenous insulin. *Metabolism,* 39**,** 480-485.

MUHLBERG, T., JOBA, W., SPITZWEG, C., SCHWORM, H. D., HEBERLING, H. J., HEUFELDER, A. E., MUHLBERG, T., JOBA, W., SPITZWEG, C., SCHWORM, H. D., HEBERLING, H. J. & HEUFELDER, A. E. (2000) Interleukin-1 receptor antagonist ribonucleic acid and protein expression by cultured Graves' and normal orbital fibroblasts is differentially modulated by dexamethasone and irradiation. *Journal of Clinical Endocrinology & Metabolism,* 85**,** 734-42.

MUKAIDA, N., MORITA, M., ISHIKAWA, Y., RICE, N., OKAMOTO, S., KASAHARA, T. & MATSUSHIMA, K. (1994) Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *Journal of Biological Chemistry,* 269**,** 13289-13295.

NAGAI, S., HANDA, T., ITO, Y., OHTA, K., TAMAYA, M. & IZUMI, T. (2008) Outcome of sarcoidosis. *Clinics in Chest Medicine,* 29**,** 565-574.

NAGAI, Y., AKASHI, S., NAGAFUKU, M., OGATA, M., IWAKURA, Y., AKIRA, S., KITAMURA, T., KOSUGI, A., KIMOTO, M. & MIYAKE, K. (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol,* 3**,** 667-672.

NAGATA, S. & GOLSTEIN, P. (1995) The Fas death factor. *Science,* 267**,** 1449-1456.

NÁRAY-FEJES-TÓTH, A., FEJES-TÓTH, G., VOLK, K. A. & STOKES, J. B. (2000) SGK is a primary glucocorticoid-induced gene in the human. *The Journal of Steroid Biochemistry and Molecular Biology,* 75**,** 51-56.

NEELS, J. G., OLEFSKY, J. M., NEELS, J. G. & OLEFSKY, J. M. (2006) Inflamed fat: what starts the fire? *Journal of Clinical Investigation,* 116**,** 33-5.
NGUYEN, T. X., COLE, A. M. & LEHRER, R. I. (2003) Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides,* 24**,** 1647-1654.
NIESLER, C. U., SIDDLE, K., PRINS, J. B., NIESLER, C. U., SIDDLE, K. & PRINS, J. B. (1998) Human preadipocytes display a depot-specific susceptibility to apoptosis. *Diabetes,* 47**,** 1365-8.
NIIJIMA, A. (1989) Nervous regulation of metabolism. *Progress in Neurobiology,* 33**,** 135-147.
NIKKILA, H., TANNIN, G. M., NEW, M. I., TAYLOR, N. F., KALAITZOGLOU, G., MONDER, C. & WHITE, P. C. (1993) Defects in the HSD11 gene encoding 11 beta-hydroxysteroid dehydrogenase are not found in patients with apparent mineralocorticoid excess or 11- oxoreductase deficiency. *Journal of Clinical Endocrinology & Metabolism,* 77**,** 687-691.
NISHIMURA, J. & IKUYAMA, S. (2000) Glucocorticoid-induced osteoporosis: pathogenesis and management. *Journal of Bone and Mineral Metabolism,* 18**,** 350-352.
NOËL, D., CATON, D., ROCHE, S., BONY, C., LEHMANN, S., CASTEILLA, L., JORGENSEN, C. & COUSIN, B. (2008) Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. *Experimental Cell Research,* 314**,** 1575.
NOVAK, N., SIEPMANN, K., ZIERHUT, M. & BIEBER, T. (2003) The good, the bad and the ugly - APCs of the eye. *Trends in Immunology,* 24**,** 570-574.
NUSSEY, S. & WHITEHEAD, S. A. (2001) *Endocrinology: an integrated approach,* Taylor & Francis.
OBENBERGER, J., STÁRKA, L. & HAMPL, R. (1971) Quantitative determination of endogenous corticosteroids in the rabbit plasma and aqueous humor. *Graefe's Archive for Clinical and Experimental Ophthalmology,* 183**,** 203.
OH, J. Y., IN, Y. S., KIM, M. K., KO, J. H., LEE, H. J., SHIN, K. C., LEE, S. M., WEE, W. R., LEE, J. H. & PARK, M. (2007) Protective effect of uridine on cornea in a rabbit dry eye model. *Investigative Ophthalmology and Visual Science*.
OHNISHI, T., MUROI, M. & TANAMOTO, K. I. (2007) The lipopolysaccharide-recognition mechanism in cells expressing TLR4 and CD14 but lacking MD-2. *Fems Immunology and Medical Microbiology,* 51**,** 84-91.
OKADA, Y., IKEDA, K., YAMANAKA, O., MIYAMOTO, T., KITANO, A., KAO, W. W. Y. & SAIKA, S. (2007) TNF alpha suppression of corneal epithelium migration. *Molecular Vision,* 13**,** 1428-1435.
ONO, S. J. & ABELSON, M. B. (2005) Allergic conjunctivitis: Update on pathophysiology and prospects for future treatment. *Journal of Allergy and Clinical Immunology,* 115**,** 118-122.
OOI, K. G. J., GALATOWICZ, G., TOWLER, H. M. A., LIGHTMAN, S. L. & CALDER, V. L. (2006) Multiplex cytokine detection versus ELISA for aqueous humor: IL-5, IL-10, and IFNgamma profiles in uveitis. *Investigative Ophthalmology & Visual Science,* 47**,** 272-277.
OTTO, E. A., OCHS, K., HANSEN, C., WALL, J. R. & KAHALY, G. J. (1996) Orbital tissue-derived T lymphocytes from patients with Graves' ophthalmopathy recognize autologous orbital antigens. *Journal of Clinical Endocrinology & Metabolism,* 81**,** 3045-3050.
OZOLS, J. (1993) Isolation and the complete amino acid sequence of lumenal endoplasmic reticulum glucose-6-phosphate dehydrogenase. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 5302-5306.
OZOLS, J. (1995) Lumenal orientation and post-translational modifications of the liver microsomal 11 beta-hydroxysteroid dehydrogenase. *Journal of Biological Chemistry,* 270**,** 2305-2312.
PALSSON-MCDERMOTT, E. M. & O'NEILL, L. A. J. (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4.
PANDIT, J. C., NAGYOVÁ, B., BRON, A. J. & TIFFANY, J. M. (1999) Physical properties of stimulated and unstimulated tears. *Experimental Eye Research,* 68**,** 247-253.
PAPINI, S., ROSELLINI, A., NARDI, M., GIANNARINI, C. & REVOLTELLA, R. P. (2005) Selective growth and expansion of human corneal epithelial basal stem cells in a three-dimensional-organ culture. *Differentiation,* 73**,** 61-68.
PATERSON, J. M., MORTON, N. M., FIEVET, C., KENYON, C. J., HOLMES, M. C., STAELS, B., SECKL, J. R. & MULLINS, J. J. (2004) Metabolic syndrome without obesity: Hepatic overexpression of 11 beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 7088-7093.
PAULSEN, S. K., PEDERSEN, S. B., JORGENSEN, J. O. L., FISKER, S., CHRISTIANSEN, J. S., FLYVBJERG, A. & RICHELSEN, B. (2006) Growth hormone (GH) substitution in GH-deficient

patients inhibits 11beta-hydroxysteroid dehydrogenase type 1 messenger ribonucleic acid expression in adipose tissue. *Journal of Clinical Endocrinology & Metabolism,* 91**,** 1093-1098.

PAYNE, A. H. & SHA, L. (1991) Multiple mechanisms for regulation of 3 beta-hydroxysteroid dehydrogenase/delta5-delta 4-isomerase, 17 alpha-hydroxylase/C17-20 lyase cytochrome P450, and cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid levels in primary cultures of mouse Leydig cells. *Endocrinology,* 129**,** 1429-1435.

PELLEGRINI, G., GOLISANO, O., PATERNA, P., LAMBIASE, A., BONINI, S., RAMA, P. & DE LUCA, M. (1999) Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *Journal of Cell Biology,* 145**,** 769-782.

PERROS, P., CROMBIE, A. L., MATTHEWS, J. N. S. & KENDALLTAYLOR, P. (1993) Age and gender influence the severity of thyroid-associated ophthalmopathy: a study of 101 patients attending a combined thyroid-eye clinic. *Clinical Endocrinology,* 38**,** 367-372.

PETER, Y. K. Y., CHRISTOPHER, H., THONG, K. J. & STEPHEN, G. H. (2002) Regulation of 11beta-hydroxysteroid dehydrogenase type 1 gene expression in human ovarian surface epithelial cells by interleukin-1. *Human Reproduction,* 17**,** 2300-2306.

PFEILSCHIFTER, J., ZIEGLER, R., PFEILSCHIFTER, J. & ZIEGLER, R. (1996) Smoking and endocrine ophthalmopathy: impact of smoking severity and current vs lifetime cigarette consumption. *Clinical Endocrinology,* 45**,** 477-81.

PFLUGFELDER, S., BEUERMAN, R. W. & STERN, M. E. (2004) *Dry Eye and Ocular Surface Disorders,* Informa Health Care.

PFLUGFELDER, S. C., JONES, D., JI, Z., AFONSO, A. & MONROY, D. (1999) Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjögren's syndrome keratoconjunctivitis sicca. *Current Eye Research,* 19**,** 201 - 211.

PIETILAINEN, K. H., KANNISTO, K., KORSHENINNIKOVA, E., RISSANEN, A., KAPRIO, J., EHRENBORG, E., HAMSTEN, A., YKI-JARVINEN, H., PIETILAINEN, K. H., KANNISTO, K., KORSHENINNIKOVA, E., RISSANEN, A., KAPRIO, J., EHRENBORG, E., HAMSTEN, A. & YKI-JARVINEN, H. (2006) Acquired obesity increases CD68 and tumor necrosis factor-alpha and decreases adiponectin gene expression in adipose tissue: a study in monozygotic twins. *Journal of Clinical Endocrinology & Metabolism,* 91**,** 2776-81.

PITT, B., REMME, W., ZANNAD, F., NEATON, J., MARTINEZ, F., RONIKER, B., BITTMAN, R., HURLEY, S., KLEIMAN, J., GATLIN, M. & INVESTIGATORS, E. P.-A. M. I. H. F. E. A. S. S. (2003) Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *New England Journal of Medicine,* 348**,** 1309-1321.

POLISETTY, N., FATIMA, A., MADHIRA, S. L., SANGWAN, V. S. & VEMUGANTI, G. K. (2008) Mesenchymal cells from limbal stroma of human eye. *Mol Vis,* 14**,** 431-42.

PRABHAKAR, B. S., BAHN, R. S., SMITH, T. J., PRABHAKAR, B. S., BAHN, R. S. & SMITH, T. J. (2003) Current perspective on the pathogenesis of Graves' disease and ophthalmopathy. *Endocrine Reviews,* 24**,** 802-35.

PRATT, W. B. (1987) Transformation of glucocorticoid and progesterone receptors to the DNA-binding state. *Journal of Cellular Biochemistry,* 35**,** 51-68.

PRUMMEL, M. F., TERWEE, C. B., GERDING, M. N., BALDESCHI, L., MOURITS, M. P., BLANK, L., DEKKER, F. W. & WIERSINGA, W. M. (2004) A randomized controlled trial of orbital radiotherapy versus sham irradiation in patients with mild Graves' ophthalmopathy. *Journal of Clinical Endocrinology & Metabolism,* 89**,** 15-20.

PRUMMEL, M. F. & WIERSINGA, W. M. (1993) Smoking and risk of Graves' disease. *Jama-Journal of the American Medical Association,* 269**,** 479-482.

QUAYLE, A. J., PORTER, E. M., NUSSBAUM, A. A., WANG, Y. M., BRABEC, C., YIP, K. P., MOK, S. C., QUAYLE, A. J., PORTER, E. M., NUSSBAUM, A. A., WANG, Y. M., BRABEC, C., YIP, K. P. & MOK, S. C. (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *American Journal of Pathology,* 152**,** 1247-58.

R. WOLFRAM-GABEL, J. L. K. (2002) Adipose body of the orbit. *Clinical Anatomy,* 15**,** 186-192.

RAE, M. T., NIVEN, D., CRITCHLEY, H. O. D., HARLOW, C. R. & HILLIER, S. G. (2004) Antiinflammatory steroid action in human ovarian surface epithelial cells. *Journal of Clinical Endocrinology & Metabolism,* 89**,** 4538-4544.

RAJAN, V., EDWARDS, C. R. & SECKL, J. R. (1996) 11 beta-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. *Journal of Neuroscience,* 16**,** 65-70.

RAUH, R., DINUDOM, A., FOTIA, A. B., PAULIDES, M., KUMAR, S., KORBMACHER, C. & COOK, D. I. (2006) Stimulation of the epithelial sodium channel (ENaC) by the serum- and glucocorticoid-inducible kinase (Sgk) involves the PY motifs of the channel but is independent of sodium feedback inhibition. *Pflugers Archiv-European Journal of Physiology,* 452**,** 290-299.

RAUZ, S., CHEUNG, C. M. G., WOOD, P. J., COCA-PRADOS, M., WALKER, E. A., MURRAY, P. I. & STEWART, P. M. (2003) Inhibition of 11{beta}-hydroxysteroid dehydrogenase type 1 lowers intraocular pressure in patients with ocular hypertension.

RAUZ, S., CHEUNG, C. M. G., WOOD, P. J., COCA-PRADOS, M., WALKER, E. A., MURRAY, P. I. & STEWART, P. M. (2003a) Inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 lowers intraocular pressure in patients with ocular hypertension. *QJM,* 96**,** 481-490.

RAUZ, S., WALKER, E. A., HUGHES, S. V., COCA-PRADOS, M., HEWISON, M., MURRAY, P. I. & STEWART, P. M. (2003b) Serum- and glucocorticoid-regulated kinase isoform-1 and epithelial sodium channel subunits in human ocular ciliary epithelium. *Investigative Ophthalmology & Visual Science,* 44**,** 1643-1651.

RAUZ, S., WALKER, E. A., MURRAY, P. I. & STEWART, P. M. (2003c) Expression and distribution of the serum and glucocorticoid regulated kinase and the epithelial sodium channel subunits in the human cornea. *Experimental Eye Research,* 77**,** 101-108.

RAUZ, S., WALKER, E. A., SHACKLETON, C. H. L., HEWISON, M., MURRAY, P. I. & STEWART, P. M. (2001) Expression and putative role of 11beta-hydroxysteroid dehydrogenase isozymes within the human eye. *Investigative Ophthalmology and Visual Science,* 42**,** 2037-2042.

RAVIOLA, G. & RAVIOLA, E. (1978) Intercellular junctions in the ciliary epithelium. *Investigative Ophthalmology & Visual Science,* 17**,** 958-981.

RAY, A. & PREFONTAINE, K. E. (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 752-6.

RAZZAQUE, M. S., FOSTER, C. S. & AHMED, A. R. (2004) Role of macrophage migration inhibitory factor in conjunctival pathology in ocular cicatricial pemphigoid. *Investigative Ophthalmology & Visual Science,* 45**,** 1174-1181.

REIDY, J. J., HERSHBERGER, D. H., CAMPBELL, L. M. & SEIGEL, G. M. (2004) p63 and ABCG2 expression in the corneoscleral limbus: Implications for stem cell purification. *Investigative Ophthalmology & Visual Science.*

REYNISDOTTIR, S., ANGELIN, B., LANGIN, D., LITHELL, H., ERIKSSON, M., HOLM, C. & ARNER, P. (1997) Adipose tissue lipoprotein lipase and hormone-sensitive lipase. Contrasting findings in familial combined hyperlipidemia and insulin resistance syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 17**,** 2287-2292.

RIBOT, J., RODRIGUEZ, A. M., RODRIGUEZ, E. & PALOU, A. (2008) Adiponectin and resistin response in the onset of obesity in male and female rats. *Obesity,* 16**,** 723-730.

RICKETTS, M. L., SHOESMITH, K. J., HEWISON, M., STRAIN, A., EGGO, M. C. & STEWART, P. M. (1998b) Regulation of 11 beta-hydroxysteroid dehydrogenase type 1 in primary cultures of rat and human hepatocytes. *Journal of Endocrinology,* 156**,** 159-168.

RICKETTS, M. L., VERHAEG, J. M., BUJALSKA, I., HOWIE, A. J., RAINEY, W. E. & STEWART, P. M. (1998a) Immunohistochemical localization of type 1 11beta-hydroxysteroid dehydrogenase in human tissues. *Journal of Clinical Endocrinology & Metabolism,* 83**,** 1325-1335.

ROSATO, E. F., VEMULAPALLI, P., LANG, C. H. & LANZA-JACOBY, S. (1997) Insulin stimulates lipoprotein lipase activity and synthesis in adipocytes from septic rats. *Journal of Surgical Research,* 73**,** 73-79.

ROSELLINI, A., PAPINI, S., GIANNARINI, C., NARDI, M., REVOLTELLA, R. P., ROSELLINI, A., PAPINI, S., GIANNARINI, C., NARDI, M. & REVOLTELLA, R. P. (2007) Human conjunctival epithelial precursor cells and their progeny in 3D organotypic culture. *International Journal of Developmental Biology,* 51**,** 739-43.

ROSMOND, R. & BJORNTORP, P. (2000) Occupational status, cortisol secretory pattern, and visceral obesity in middle-aged men. *Obesity,* 8**,** 445-450.

ROUND, A. N., BERRY, M., MCMASTER, T. J., STOLL, S., GOWERS, D., CORFIELD, A. P. & MILES, M. J. (2002) Heterogeneity and persistence length in human ocular mucins. *Biophysical Journal,* 83**,** 1661-1670.

RUAN, H. & LODISH, H. F. (2003) Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-[alpha]. *Cytokine & Growth Factor Reviews,* 14**,** 447-455.

RUSSO, S., PAPA, V., DI BELLA, A., FAVERO, A., RADULESCU, C., GAFENCU, O., CARSTOCEA, B. & MILAZZO, G. (2007) Dexamethasone-netilmicin: a new ophthalmic steroid-antibiotic combination. Efficacy and safety after cataract surgery. *Eye,* 21**,** 58-64.

SABAN, D. R., ELDER, I. A., NGUYEN, C. Q., SMITH, W. C., TIMMERS, A. M., GRANT, M. B., PECK, A. B., SABAN, D. R., ELDER, I. A., NGUYEN, C. Q., SMITH, W. C., TIMMERS, A. M., GRANT, M. B. & PECK, A. B. (2008) Characterization of intraocular immunopathology following intracameral inoculation with alloantigen. *Molecular Vision,* 14**,** 615-24.

SAI, S., ESTEVES, C. L., KELLY, V., MICHAILIDOU, Z., ANDERSON, K., COLL, A. P., NAKAGAWA, Y., OHZEKI, T., SECKL, J. R. & CHAPMAN, K. E. (2008) Glucocorticoid regulation of the promoter of 11beta-hydroxysteroid dehydrogenase type 1 is indirect and requires CCAAT/enhancer-binding protein-beta. *Molecular Endocrinology***,** me.2007-0489.

SAIKA, S. (2007) Yin and Yang in cytokine regulation of corneal wound healing - Roles of TNF-alpha. *Cornea,* 26**,** S70-S74.

SAINT-MARC, P., KOZAK, L. P., AILHAUD, G., DARIMONT, C. & NEGREL, R. (2001) Angiotensin II as a trophic factor of white adipose tissue: stimulation of adipose cell formation. *Endocrinology,* 142**,** 487-492.

SANDER, B., LARSEN, M., ENGLER, C., STROM, C., MOLDOW, B., LARSEN, N. & LUND-ANDERSEN, H. (2002) Diabetic macular oedema: a comparison of vitreous fluorometry, angiography, and retinopathy. *British Journal of Ophthalmology,* 86**,** 316-320.

SARCHAHI, A. A., MAIMANDI, A., TAFTI, A. K. & AMANI, M. (2008) Effects of acetylcysteine and dexamethasone on experimental corneal wounds in rabbits. *Ophthalmic Research,* 40**,** 41-48.

SATO, K., CHISAKA, H., OKAMURA, K. & CHALLIS, J. R. G. (2008a) Effect of the Interaction Between Lipoxygenase Pathway and Progesterone on the Regulation of Hydroxysteroid 11-Beta Dehydrogenase 2 in Cultured Human Term Placental Trophoblasts.

SATO, K., CHISAKA, H., OKAMURA, K. & CHALLIS, J. R. G. (2008b) Effect of the interaction between lipoxygenase pathway and progesterone on the regulation of hydroxysteroid 11-Beta dehydrogenase 2 in cultured human term placental trophoblasts. *Biology of Reproduction,* 78**,** 514-520.

SCHAFFLER, A. & BUCHLER, C. (2007) Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem Cells,* 25**,** 818-827.

SCHEINMAN, R. I., COGSWELL, P. C., LOFQUIST, A. K. & BALDWIN, A. S., JR. (1995a) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science,* 270**,** 283-286.

SCHEINMAN, R. I., GUALBERTO, A., JEWELL, C. M., CIDLOWSKI, J. A. & BALDWIN, A. S., JR. (1995b) Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Molecular and Cellular Biology,* 15**,** 943-953.

SCHERER, P. E. (2006) Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes,* 55**,** 1537-1545.

SCHERMER, A., GALVIN, S. & SUN, T. T. (1986a) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *Journal of Cell Biology,* 103**,** 49-62.

SCHERMER, A., GALVIN, S. & SUN, T. T. (1986b) Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells.

SCHLÖTZER-SCHREHARDT, U. & KRUSE, F. E. (2005) Identification and characterization of limbal stem cells. *Experimental Eye Research,* 81**,** 247-264.

SCHULZ, M., EGGERT, M., BANIAHMAD, A., DOSTERT, A., HEINZEL, T. & RENKAWITZ, R. (2002) RU486-induced glucocorticoid receptor agonism is controlled by the receptor N terminus and by corepressor binding. *Journal of Biological Chemistry,* 277**,** 26238-26243.

SCHUTTE, B. C., MITROS, J. P., BARTLETT, J. A., WALTERS, J. D., JIA, H. P., WELSH, M. J., CASAVANT, T. L., MCCRAY, P. B., JR., SCHUTTE, B. C., MITROS, J. P., BARTLETT, J. A., WALTERS, J. D., JIA, H. P., WELSH, M. J., CASAVANT, T. L. & MCCRAY, P. B., JR. (2002) Discovery of five conserved beta -defensin gene clusters using a computational search strategy. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 2129-33.

SCIAKY, D., BRAZER, W., CENTER, D. M., CRUIKSHANK, W. W. & SMITH, T. J. (2000) Cultured human fibroblasts express constitutive IL-16 mRNA: cytokine induction of active IL-16 protein synthesis through a caspase-3-dependent mechanism. *Journal of Immunology,* 164**,** 3806-3814.

SEIDEL, E. (1920) Weitere experimentelle Untersuchungen über die Quelle und den Verlauf der intraokularen Saftströmung. *Graefe's Archive for Clinical and Experimental Ophthalmology,* 102**,** 366-382.

SELSTED, M. E., HARWIG, S. S., GANZ, T., SCHILLING, J. W., LEHRER, R. I., SELSTED, M. E., HARWIG, S. S., GANZ, T., SCHILLING, J. W. & LEHRER, R. I. (1985) Primary structures of three human neutrophil defensins. *Journal of Clinical Investigation,* 76**,** 1436-9.

SEMPOWSKI, G. D., ROZENBLIT, J., SMITH, T. J. & PHIPPS, R. P. (1998) Human orbital fibroblasts are activated through CD40 to induce proinflammatory cytokine production. *American Journal of Physiology,* 274**,** C707-714.

SHACKLETON, C. H. L. (1993) Mass-spectrometry in the diagnosis of steroid-related disorders and in hypertension research. *Journal of Steroid Biochemistry and Molecular Biology,* 45**,** 127-140.

SHEPPARD, K.-A., PHELPS, K. M., WILLIAMS, A. J., THANOS, D., GLASS, C. K., ROSENFELD, M. G., GERRITSEN, M. E. & COLLINS, T. (1998) Nuclear integration of glucocorticoid receptor and nuclear factor-kappaB signaling by CREB-binding protein and steroid receptor coactivator-1. *Journal of Biological Chemistry,* 273**,** 29291-29294.

SHERBET, D. P., PAPARI-ZAREEI, M., KHAN, N., SHARMA, K. K., BRANDMAIER, A., RAMBALLY, S., CHATTOPADHYAY, A., ANDERSSON, S., AGARWAL, A. K. & AUCHUS, R. J. (2007) Cofactors, redox state, and directional preferences of hydroxysteroid dehydrogenases. *Molecular and Cellular Endocrinology,* 265-266**,** 83-88.

SHIMAZU, R., AKASHI, S., OGATA, H., NAGAI, Y., FUKUDOME, K., MIYAKE, K. & KIMOTO, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4. *J. Exp. Med.,* 189**,** 1777-1782.

SHIMOMURA, I., FUNAHASM, T., TAKAHASHI, M., MAEDA, K., KOTANI, K., NAKAMURA, T., YAMASHITA, S., MIURA, M., FUKUDA, Y., TAKEMURA, K., TOKUNAGA, K. & MATSUZAWA, Y. (1996) Enhanced expression of PAI-1 in visceral fat: Possible contributor to vascular disease in obeisty. *Nat Med,* 2**,** 800-803.

SHINE, B., FELLS, P., EDWARDS, O. M. & WEETMAN, A. P. (1990) Association between Graves' ophthalmopathy and smoking. *The Lancet,* 335**,** 1261-1263.

SHOELSON, S. E., LEE, J. & GOLDFINE, A. B. (2006) Inflammation and insulin resistance. *Journal of Clinical Investigation,* 116**,** 1793-1801.

SHORTT, A. J., SECKER, G. A., MUNRO, P. M., KHAW, P. T., TUFT, S. J. & DANIELS, J. T. (2007) Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. *Stem Cells,* 25**,** 1402-1409.

SIGURJONSDOTTIR, H. A., KORANYI, J., AXELSON, M., BENGTSSON, B.-A. & JOHANNSSON, G. (2006) GH effect on enzyme activity of 11beta HSD in abdominal obesity is dependent on treatment duration. *European Journal of Endocrinology,* 154**,** 69-74.

SINCLAIR, A. J., ONYIMBA, C. U., KHOSLA, P., VIJAPURAPU, N., TOMLINSON, J. W., BURDON, M. A., STEWART, P. M., MURRAY, P. I., WALKER, E. A. & RAUZ, S. (2007) Corticosteroids, 11 beta-hydroxysteroid dehydrogenase isozymes and the rabbit choroid plexus. *Journal of Neuroendocrinology,* 19**,** 614-620.

SIRARD, J.-C., VIGNAL, C. C., DESSEIN, R. & CHAMAILLARD, M. (2007) Nod-Like Receptors: Cytosolic Watchdogs for Immunity against Pathogens. *PLoS Pathogens,* 3**,** e152.

SMELSER, G. K. (1936) Experimental production of exophthalmos resembling that found in Graves disease. *Proceedings of the Society for Experimental Biology and Medicine,* 35**,** 128-130.

SMIRNOVA, M. G., GUO, L., BIRCHALL, J. P., PEARSON, J. P., SMIRNOVA, M. G., GUO, L., BIRCHALL, J. P. & PEARSON, J. P. (2003) LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells. *Cellular Immunology,* 221**,** 42-9.

SMITH, R. E., MAGUIRE, J. A., STEIN-OAKLEY, A. N., SASANO, H., TAKAHASHI, K., FUKUSHIMA, K. & KROZOWSKI, Z. S. (1996) Localization of 11 beta-hydroxysteroid dehydrogenase type II in human epithelial tissues. *Journal of Clinical Endocrinology & Metabolism,* 81**,** 3244-3248.

SMITH, T. J., BAHN, R. S., GORMAN, C. A. & CHEAVENS, M. (1991) Stimulation of Glycosaminoglycan Accumulation by Interferon-Gamma in Cultured Human Retroocular Fibroblasts. *Journal of Clinical Endocrinology and Metabolism,* 72**,** 1169-1171.

SMITH, T. J., KOUMAS, L., GAGNON, A., BELL, A., SEMPOWSKI, G. D., PHIPPS, R. P. & SORISKY, A. (2002) Orbital fibroblast heterogeneity may determine the clinical presentation of

thyroid-associated ophthalmopathy. *Journal of Clinical Endocrinology & Metabolism,* 87**,** 385-392.

SMITH, T. J., SEMPOWSKI, G. D., WANG, H. S., DEL VECCHIO, P. J., LIPPE, S. D. & PHIPPS, R. P. (1995a) Evidence for cellular heterogeneity in primary cultures of human orbital fibroblasts. *Journal of Clinical Endocrinology & Metabolism,* 80**,** 2620-2625.

SMITH, T. J. & SMITH, T. J. (2004) Novel aspects of orbital fibroblast pathology. *Journal of Endocrinological Investigation,* 27**,** 246-53.

SMITH, T. J., WANG, H. S. & EVANS, C. H. (1995b) Leukoregulin is a potent inducer of hyaluronan synthesis in cultured human orbital fibroblasts. *American Journal of Physiology,* 268**,** C382-388.

SNYDER, P. M., OLSON, D. R. & THOMAS, B. C. (2002) Serum and glucocorticoid-regulated kinase modulates Nedd4-2-mediated inhibition of the epithelial $Na^+$ channel. *Journal of Biological Chemistry,* 277**,** 5-8.

SO-JUNG, G., SUK, H. B., HEE, S. Y., SANG-SOO, K., DAE-HEE, L., SOO-HONG, L. & BYUNG-SOO, K. (2009) In vitro cardiomyogenic differentiation of adipose-derived stromal cells using transforming growth factor-beta 1. *Cell Biochemistry and Function,* 27**,** 148-154.

SONG, P. I., ABRAHAM, T. A., PARK, Y., ZIVONY, A. S., HARTEN, B., EDELHAUSER, H. F., WARD, S. L., ARMSTRONG, C. A. & ANSEL, J. C. (2001) The expression of functional LPS receptor proteins CD14 and toll-like receptor 4 in human corneal cells. *Investigative Ophthalmology & Visual Science,* 42**,** 2867-77.

SORISKY, A., PARDASANI, D., GAGNON, A. & SMITH, T. J. (1996) Evidence of adipocyte differentiation in human orbital fibroblasts in primary culture. *Journal of Clinical Endocrinology & Metabolism,* 81**,** 3428-3431.

SOTOZONO, C., HE, J. C., MATSUMOTO, Y., KITA, M., IMANISHI, J. & KINOSHITA, S. (1997) Cytokine expression in the alkali-burned cornea. *Current Eye Research,* 16**,** 670-676.

SQUIRRELL, D. M., WINFIELD, J. & AMOS, R. S. (1999) Peripheral ulcerative keratitis `corneal melt' and rheumatoid arthritis: a case series. *Rheumatology,* 38**,** 1245-1248.

STÁRKA, L., KOLÍN, J. & OBENBERGER, J. (1975) Cortisol Levels in Human Aqueous Humour and Blood Plasma. *Ophthalmic Research,* 7**,** 303.

STEGEMAN, J. J. & KLOTZ, A. V. (1979) A possible role for microsomal hexose-6-phosphate dehydrogenase in microsomal electron transport and mixed-function oxygenase activity. *Biochemical and Biophysical Research Communications,* 87**,** 410-415.

STEIN-STREILEIN, J. & TAYLOR, A. W. (2007) An eye's view of T regulatory cells. *Journal of Leukocyte Biology,* 81**,** 593-598.

STEWART, P. M., MURRY, B. A. & MASON, J. I. (1994) Human kidney 11 beta-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform.

STEWART, P. M., WHORWOOD, C. B. & MASON, J. I. (1995) Type 2 11beta-hydroxysteroid dehydrogenase in foetal and adult life. *The Journal of Steroid Biochemistry and Molecular Biology,* 55**,** 465-471.

STIER, C. T., CHANDER, P. N. & ROCHA, R. (2002) Aldosterone as a Mediator in Cardiovascular Injury. *Cardiology in Review,* 10**,** 97-107.

STOCUM, D. L. (2006) *Regenerative Biology and Medicine,* Academic Press.

STOKES, J., NOBLE, J., BRETT, L., PHILLIPS, C., SECKL, J. R., O'BRIEN, C. & ANDREW, R. (2000) Distribution of glucocorticoid and mineralocorticoid receptors and 11beta-hydroxysteroid dehydrogenases in human and rat ocular tissues. *Investigative Ophthalmology & Visual Science,* 41**,** 1629-1638.

STOKES, J., WALKER, B. R., CAMPBELL, J. C., SECKL, J. R., O'BRIEN, C. & ANDREW, R. (2003) Altered peripheral sensitivity to glucocorticoids in primary open-angle glaucoma. *Investigative Ophthalmology & Visual Science,* 44**,** 5163-5167.

STREILEIN, J. W. (2003a) Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol,* 3**,** 879-889.

STREILEIN, J. W. (2003b) Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation. *Journal of Leukocyte Biology***,** jlb.1102574.

SUDHA, B., MADHAVAN, H. N., SITALAKSHMI, G., MALATHI, J., KRISHNAKUMAR, S., MORI, Y., YOSHIOKA, H., ABRAHAM, S., SUDHA, B., MADHAVAN, H. N., SITALAKSHMI, G., MALATHI, J., KRISHNAKUMAR, S., MORI, Y., YOSHIOKA, H. & ABRAHAM, S. (2006)

Cultivation of human corneal limbal stem cells in Mebiol gel--A thermo-reversible gelation polymer. *Indian Journal of Medical Research,* 124**,** 655-64.

SUN, K. & MYATT, L. (2003) Enhancement of glucocorticoid-induced 11beta-hydroxysteroid dehydrogenase type 1 expression by proinflammatory cytokines in cultured human amnion fibroblasts. *Endocrinology,* 144**,** 5568-5577.

SUN, K., YANG, K. & CHALLIS, J. R. (1998) Regulation of 11beta-hydroxysteroid dehydrogenase type 2 by progesterone, estrogen, and the cyclic adenosine 5'-monophosphate pathway in cultured human placental and chorionic trophoblasts. *Biology of Reproduction,* 58**,** 1379-1384.

SUN, Y., HISE, A. G., KALSOW, C. M. & PEARLMAN, E. (2006) Staphylococcus aureus-induced corneal inflammation is dependent on Toll-like receptor 2 and myeloid differentiation factor 88. *Infection and Immunity,* 74**,** 5325-5332.

SUZUKI, T., SASANO, H., KANEKO, C., OGAWA, S., DARNEL, A. D. & KROZOWSKI, Z. S. (2001) Immunohistochemical distribution of 11[beta]-hydroxysteroid dehydrogenase in human eye. *Molecular and Cellular Endocrinology,* 173**,** 121-125.

SZE, P. Y. & IQBAL, Z. (1994) Regulation of calmodulin content in synaptic plasma membranes by glucocorticoids. *Neurochemical Research,* 19**,** 1455.

SZETO, A., GONZALES, J. A., SPITZER, S. B., LEVINE, J. E., ZAIAS, J., SAAB, P. G., SCHNEIDERMAN, N. & MCCABE, P. M. (2004) Circulating levels of glucocorticoid hormones in WHHL and NZW rabbits: circadian cycle and response to repeated social encounter. *Psychoneuroendocrinology,* 29**,** 861.

SZUCS-FARKAS, Z., TOTH, J., KOLLAR, J., GALUSKA, L., BURMAN, K. D., BODA, J., LEOVEY, A., VARGA, J., UJHELYI, B., SZABO, J., BERTA, A., NAGY, E. V., SZUCS-FARKAS, Z., TOTH, J., KOLLAR, J., GALUSKA, L., BURMAN, K. D., BODA, J., LEOVEY, A., VARGA, J., UJHELYI, B., SZABO, J., BERTA, A. & NAGY, E. V. (2005) Volume changes in intra- and extraorbital compartments in patients with Graves' ophthalmopathy: effect of smoking. *Thyroid,* 15**,** 146-51.

SZYMANSKI, E. S. & FURFINE, C. S. (1977) 20beta-Hydroxysteroid oxidoreductase. Kinetics and binding of corticosteroids and corticosteroid-21-aldehydes. *Journal of Biological Chemistry,* 252**,** 205-211.

TABBARA, K. F., EL-SHEIKH, H. F. & AABED, B. (2000) Extended wear contact lens related bacterial keratitis. *British Journal of Ophthalmology,* 84**,** 327-328.

TAKAHASHI, S., WAKUI, H., GUSTAFSSON, J.-Å., ZILLIACUS, J. & ITOH, H. (2000) Functional Interaction of the Immunosuppressant Mizoribine with the 14-3-3 Protein. *Biochemical and Biophysical Research Communications,* 274**,** 87-92.

TAKEDA, K., KAISHO, T. & AKIRA, S. (2003) Toll-like receptors. *Annual Review of Immunology,* 21**,** 335-376.

TAKEUCHI, M., KEINO, H., SUZUKI, J., USUI, Y., HATTORI, T., TAKEUCHI, A., OH-I, K., OKUNUKI, Y., KEZUKA, T. & USUI, M. (2006) Possibility of inducing anterior chamber-associated immune deviation by TGF-[beta]2 treatment of monocytes isolated from Behcet's patients. *Experimental Eye Research,* 83**,** 981-988.

TAKEUCHI, O., SATO, S., HORIUCHI, T., HOSHINO, K., TAKEDA, K., DONG, Z., MODLIN, R. L. & AKIRA, S. (2002) Cutting edge: Role of toll-like receptor 1 in mediating immune response to microbial lipoproteins. *The Journal of Immunology.*

TANDON, N., YAN, S. L., ARNOLD, K., METCALFE, R. A. & WEETMAN, A. P. (1994) Immunoglobulin class and subclass distribution of eye muscle and fibroblast antibodies in patients with thyroid-associated ophthalmopathy. *Clinical Endocrinology,* 40**,** 629-639.

TANG, Y. Q., YUAN, J., OSAPAY, G., OSAPAY, K., TRAN, D., MILLER, C. J., OUELLETTE, A. J., SELSTED, M. E., TANG, Y. Q., YUAN, J., OSAPAY, G., OSAPAY, K., TRAN, D., MILLER, C. J., OUELLETTE, A. J. & SELSTED, M. E. (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science,* 286**,** 498-502.

TANIGUCHI, A., KATAOKA, K., KONO, T., OSEKO, F., OKUDA, H., NAGATA, I. & IMURA, H. (1987) Parathyroid hormone-induced lipolysis in human adipose tissue. *Journal of Lipid Research,* 28**,** 490-494.

TANNIN, G. M., AGARWAL, A. K., MONDER, C., NEW, M. I. & WHITE, P. C. (1991) The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. *Journal of Biological Chemistry,* 266**,** 16653-16658.

TASKER, J. G., DI, S. & MALCHER-LOPES, R. (2006) Minireview: rapid glucocorticoid signaling via membrane-associated receptors. *Endocrinology,* 147**,** 5549-5556.

TAYEK, J. A. & KATZ, J. (1997) Glucose production, recycling, Cori cycle, and gluconeogenesis in humans: Relationship to serum cortisol. *American Journal of Physiology-Endocrinology and Metabolism,* 272**,** E476-E484.

TCHKONIA, T., TCHOUKALOVA, Y. D., GIORGADZE, N., PIRTSKHALAVA, T., KARAGIANNIDES, I., FORSE, R. A., KOO, A., STEVENSON, M., CHINNAPPAN, D., CARTWRIGHT, A., JENSEN, M. D. & KIRKLAND, J. L. (2005) Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots. *Am J Physiol Endocrinol Metab,* 288**,** E267-277.

TEELUCKSINGH, S., MACKIE, A. D. R., BURT, D., EDWARDS, C. R. W., MCLNTYRE, M. A. & BRETT, L. (1990) Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *The Lancet,* 335**,** 1060-1063.

TEJERINA, S., DE PAUW, A., VANKONINGSLOO, S., HOUBION, A., RENARD, P., DE LONGUEVILLE, F., RAES, M. & ARNOULD, T. (2009) Mild mitochondrial uncoupling induces 3T3-L1 adipocyte de-differentiation by a PPARgamma-independent mechanism, whereas TNFalpha-induced de-differentiation is PPARgamma dependent. *J Cell Sci,* 122**,** 145-155.

TERAI, K., SANO, Y., KAWASAKI, S., ENDO, K.-I., ADACHI, W., HIRATSUKA, T., IHIBOSHI, H., NAKAZATO, M. & KINOSHITA, S. (2004) Effects of dexamethasone and cyclosporin A on human beta-defensin in corneal epithelial cells. *Experimental Eye Research,* 79**,** 175-180.

THIERINGER, R., LE GRAND, C. B., CARBIN, L., CAI, T.-Q., WONG, B., WRIGHT, S. D. & HERMANOWSKI-VOSATKA, A. (2001a) 11{{beta}}-Hydroxysteroid Dehydrogenase Type 1 Is Induced in Human Monocytes upon Differentiation to Macrophages. *Journal of Immunology.*

THIERINGER, R., LE GRAND, C. B., CARBIN, L., CAI, T.-Q., WONG, B., WRIGHT, S. D. & HERMANOWSKI-VOSATKA, A. (2001b) 11beta-Hydroxysteroid Dehydrogenase Type 1 Is Induced in Human Monocytes upon Differentiation to Macrophages. *Journal of Immunology.*

THOMAS, P. A., GERALDINE, P., THOMAS, P. A. & GERALDINE, P. (2007) Infectious keratitis. *Current Opinion in Infectious Diseases,* 20**,** 129-41.

THOMAS, P. B., ZHU, Z., SELVAM, S., SAMANT, D. M., STEVENSON, D., MIRCHEFF, A. K., SCHECHTER, J. E., SONG, S. W. & TROUSDALE, M. D. (2008) Autoimmune dacryoadenitis and keratoconjunctivitis induced in rabbits by subcutaneous injection of autologous lymphocytes activated ex vivo against lacrimal antigens. *Journal of Autoimmunity,* 31**,** 116-122.

THOMPSON, P. P., KOWALSKI, R. P., SHANKS, R. M. Q. & GORDON, Y. J. (2008) Validation of real-time PCR for laboratory diagnosis of Acanthamoeba keratitis. *Journal of Clinical Microbiology***,** JCM.00908-08.

THORNTON, J., KELLY, S. P., HARRISON, R. A. & EDWARDS, R. (2006) Cigarette smoking and thyroid eye disease: a systematic review. *Eye,* 21**,** 1135-1145.

TOKER, E., KAZOKOGLU, H. & ACAR, N. (2002) High dose intravenous steroid therapy for severe posterior segment uveitis in Behcet's disease. *British Journal of Ophthalmology,* 86**,** 521-523.

TOMLINSON, J. J., BOUDREAU, A., WU, D., ATLAS, E. & HACHE, R. J. G. (2006) Modulation of early human preadipocyte differentiation by glucocorticoids. *Endocrinology,* 147**,** 5284-5293.

TOMLINSON, J. W., MOORE, J., COOPER, M. S., BUJALSKA, I., SHAHMANESH, M., BURT, C., STRAIN, A., HEWISON, M. & STEWART, P. M. (2001b) Regulation of expression of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue: tissue-specific induction by cytokines. *Endocrinology.*

TOMLINSON, J. W., SINHA, B., BUJALSKA, I., HEWISON, M. & STEWART, P. M. (2002) Expression of 11beta-hydroxysteroid dehydrogenase type 1 in adipose tissue is not increased in human obesity. *Journal of Clinical Endocrinology & Metabolism.*

TOMLINSON, J. W., WALKER, E. A., BUJALSKA, I. J., DRAPER, N., LAVERY, G. G., COOPER, M. S., HEWISON, M. & STEWART, P. M. (2004) 11beta-hydroxysteroid dehydrogenase type 1: A tissue-specific regulator of glucocorticoid response. *Endocrine Reviews,* 25**,** 831-866.

TORTI, F. M., TORTI, S. V., LARRICK, J. W. & RINGOLD, G. M. (1989) Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor beta. *J. Cell Biol.,* 108**,** 1105-1113.

TRAN, M. T., TELLAETXE-ISUSI, M., ELNER, V., STRIETER, R. M., LAUSCH, R. N. & OAKES, J. E. (1996) Proinflammatory cytokines induce RANTES and MCP-1 synthesis in human corneal

keratocytes but not in corneal epithelial cells. Beta-chemokine synthesis in corneal cells. *Investigative Ophthalmology & Visual Science,* 37**,** 987-996.

TRAYHURN, P., BING, C. & WOOD, I. S. (2006) Adipose tissue and adipokines--Energy Regulation from the human perspective. *J. Nutr.,* 136**,** 1935S-1939.

TSAI, C. C., CHENG, C. Y., LIU, C. Y., KAO, S. C., KAU, H. C., HSU, W. M. & WEI, Y. H. (2008) Oxidative stress in patients with Graves' ophthalmopathy: relationship between oxidative DNA damage and clinical evolution. *Eye,* 23**,** 1725-30.

TUCKERMANN, J. P., KLEIMAN, A., MCPHERSON, K. G. & REICHARDT, H. M. (2005) Molecular mechanisms of glucocorticoids in the control of inflammation and lymphocyte apoptosis. *Critical Reviews in Clinical Laboratory Sciences,* 42**,** 71-104.

TULLOCH-REID, M. K., HANSON, R. L., SEBRING, N. G., REYNOLDS, J. C., PREMKUMAR, A., GENOVESE, D. J. & SUMNER, A. E. (2004) Both subcutaneous and visceral adipose tissue correlate highly with insulin resistance in African Americans. *Obesity Research,* 12**,** 1352-1359.

TURGEON, J. L., CARR, M. C., MAKI, P. M., MENDELSOHN, M. E. & WISE, P. M. (2006) Complex actions of sex steroids in adipose tissue, the cardiovascular system, and brain: Insights from basic science and clinical studies. *Endocrine Reviews,* 27**,** 575-605.

UETA, M., IIDA, T., SAKAMOTO, M., SOTOZONO, C., TAKAHASHI, J., KOJIMA, K., OKADA, K., CHEN, X., KINOSHITA, S. & HONDA, T. (2007a) Polyclonality of Staphylococcus epidermidis residing on the healthy ocular surface.

UETA, M., IIDA, T., SAKAMOTO, M., SOTOZONO, C., TAKAHASHI, J., KOJIMA, K., OKADA, K., CHEN, X., KINOSHITA, S. & HONDA, T. (2007b) Polyclonality of Staphylococcus epidermidis residing on the healthy ocular surface. *Journal of Medical Microbiology,* 56**,** 77-82.

VAIDYA, B., IMRIE, H., PERROS, P., DICKINSON, J., MCCARTHY, M. I., KENDALL-TAYLOR, P. & PEARCE, S. H. S. (1999) Cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphism confers susceptibility to thyroid associated orbitopathy. *Lancet,* 354**,** 743-744.

VALYASEVI, R. W., ERICKSON, D. Z., HARTENECK, D. A., DUTTON, C. M., HEUFELDER, A. E., JYONOUCHI, S. C. & BAHN, R. S. (1999) Differentiation of human orbital preadipocyte fibroblasts induces expression of functional thyrotropin receptor. *Journal of Clinical Endocrinology & Metabolism,* 84**,** 2557-2562.

VALYASEVI, R. W., JYONOUCHI, S. C., DUTTON, C. M., MUNSAKUL, N. & BAHN, R. S. (2001) Effect of Tumor Necrosis Factor-alpha, Interferon-gamma, and Transforming Growth Factor-beta on Adipogenesis and Expression of Thyrotropin Receptor in Human Orbital Preadipocyte Fibroblasts. *Journal of Clinical Endocrinology & Metabolism,* 86**,** 903-908.

VAN GELDER, R. & KAPLAN, H. (1999) Immunosuppression in uveitis therapy. *Springer Seminars in Immunopathology,* 21**,** 179-190.

VAN HARMELEN, V., REYNISDOTTIR, S., CIANFLONE, K., DEGERMAN, E., HOFFSTEDT, J., NILSELL, K., SNIDERMAN, A. & ARNER, P. (1999) Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin. *Journal of Biological Chemistry,* 274**,** 18243-18251.

VANDER KOOI, B. T., ONUMA, H., OESER, J. K., SVITEK, C. A., ALLEN, S. R., VANDER KOOI, C. W., CHAZIN, W. J. & O'BRIEN, R. M. (2005) The glucose-6-phosphatase catalytic subunit gene promoter contains both positive and negative glucocorticoid response elements. *Molecular Endocrinology,* 19**,** 3001-3022.

VERHOEF, J. & MATTSSON, E. (1995) The role of cytokines in Gram-positive bacterial shock. *Trends in Microbiology,* 3**,** 136-140.

VESTERGAARD, P. (2002) Smoking and thyroid disorders--a meta-analysis. *European Journal of Endocrinology,* 146**,** 153-161.

VISINTIN, A., HALMEN, K. A., KHAN, N., MONKS, B. G., GOLENBOCK, D. T. & LIEN, E. (2006) MD-2 expression is not required for cell surface targeting of Toll-like receptor 4 (TLR4). *Journal of Leukocyte Biology,* 80**,** 1584-1592.

VOEGEL, J. J., HEINE, M. J. S., ZECHEL, C., CHAMBON, P. & GRONEMEYER, H. (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *Embo Journal,* 15**,** 3667-3675.

WAHID, W. A. S. M., SELVARAJA, V. M., BAKIAH, S. M. & IBRAHIM, M. M. (2008) Recurrence of peripheral ulcerative keratitis on the corneoscleral button in a young man treated successfully with oral corticosteroids. *Cornea,* 27**,** 837-839.

WAJCHENBERG, B. L. (2000) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocrine Reviews,* 21**,** 697-738.

WAKE, D. J., HOMER, N. Z. M., ANDREW, R. & WALKER, B. R. (2006) Acute in vivo regulation of 11beta-hydroxysteroid dehydrogenase type 1 activity by insulin and intralipid infusions in humans. *Journal of Clinical Endocrinology & Metabolism,* 91**,** 4682-4688.

WALKER, B. R., STEWART, P. M., SHACKLETON, C. H. L., PADFIELD, P. L. & EDWARDS, C. R. W. (1993) Deficient inactivation of cortisol by 11beta-hydroxysteroid dehydrogenase in essential-hypertension. *Clinical Endocrinology,* 39**,** 221-227.

WALKER, E. A., AHMED, A., LAVERY, G. G., TOMLINSON, J. W., KIM, S. Y., COOPER, M. S., RIDE, J. P., HUGHES, B. A., SHACKLETON, C. H. L., MCKIERNAN, P., ELIAS, E., CHOU, J. Y. & STEWART, P. M. (2007) 11beta-Hydroxysteroid Dehydrogenase Type 1 Regulation by Intracellular Glucose 6-Phosphate Provides Evidence for a Novel Link between Glucose Metabolism and Hypothalamo-Pituitary-Adrenal Axis Function. *Journal of Biological Chemistry,* 282**,** 27030-27036.

WANG, H.-S., CAO, H. J., WINN, V. D., REZANKA, L. J., FROBERT, Y., EVANS, C. H., SCIAKY, D., YOUNG, D. A. & SMITH, T. J. (1996) Leukoregulin induction of prostaglandin-endoperoxide H synthase-2 in human orbital fibroblasts. An in vitro model for connective tissue inflammation. *Journal of Biological Chemistry,* 271**,** 22718-22728.

WANG, Q., ANZICK, S., RICHTER, W. F., MELTZER, P. & SIMONS, J. S. S. (2004) Modulation of transcriptional sensitivity of mineralocorticoid and estrogen receptors. *The Journal of Steroid Biochemistry and Molecular Biology,* 91**,** 197-210.

WATSKY, M. A., COOPER, K. & RAE, J. L. (1991) Sodium-channels in ocular epithelia. *Pflugers Archiv-European Journal of Physiology,* 419**,** 454-459.

WEETMAN, A. P. (1991) Thyroid-associated eye disease: pathophysiology. *The Lancet,* 338**,** 25-28.

WEINSTEIN, R. S., NICHOLAS, R. W. & MANOLAGAS, S. C. (2000) Apoptosis of ssteocytes in glucocorticoid-induced osteonecrosis of the hip. *Journal of Clinical Endocrinology & Metabolism,* 85**,** 2907-2912.

WEINSTEIN, S. P., WILSON, C. M., PRITSKER, A. & CUSHMAN, S. W. (1998) Dexamethasone inhibits insulin-stimulated recruitment of GLUt4 to the cell surface in rat skeletal muscle. *Metabolism,* 47**,** 3-6.

WEISBERG, S. P., MCCANN, D., DESAI, M., ROSENBAUM, M., LEIBEL, R. L. & FERRANTE, A. W. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation,* 112**,** 1796-1808.

WENKEL, H., CHEN, P. W., KSANDER, B. R. & STREILEIN, J. W. (1999) Immune privilege is extended, then withdrawn, from allogeneic tumor cell grafts placed in the subretinal space. *Investigative Ophthalmology & Visual Science,* 40**,** 3202-3208.

WENZEL, A., GRIMM, C., SEELIGER, M. W., JAISSLE, G., HAFEZI, F., KRETSCHMER, R., ZRENNER, E. & REME, C. E. (2001) Prevention of photoreceptor apoptosis by activation of the glucocorticoid receptor. *Investigative Ophthalmology and Visual Science.*

WERNER, S. & GROSE, R. (2003) Regulation of wound healing by growth factors and cytokines. *Physiological Reviews.,* 83**,** 835-870.

WESCHE, H., HENZEL, W. J., SHILLINGLAW, W., LI, S. & CAO, Z. (1997) MyD88: An Adapter That Recruits IRAK to the IL-1 Receptor Complex. *Immunity,* 7**,** 837-847.

WEYER, C., FUNAHASHI, T., TANAKA, S., HOTTA, K., MATSUZAWA, Y., PRATLEY, R. E. & TATARANNI, P. A. (2001) Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *Journal of Clinical Endocrinology & Metabolism,* 86**,** 1930-1935.

WHORWOOD, C. B., MASON, J. I., RICKETTS, M. L., HOWIE, A. J. & STEWART, P. M. (1995) Detection of human 11[beta]-hydroxysteroid dehydrogenase isoforms using reverse-transcriptase-polymerase chain reaction and localization of the type 2 isoform to renal collecting ducts. *Molecular and Cellular Endocrinology,* 110**,** R7-R12.

WHORWOOD, C. B., RICKETTS, M. L. & STEWART, P. M. (1994) Epithelial cell localization of type 2 11 beta-hydroxysteroid dehydrogenase in rat and human colon. *Endocrinology,* 135**,** 2533-2541.

WIERSINGA, W. M. & BARTALENA, L. (2002) Epidemiology and prevention of graves' ophthalmopathy. *Thyroid,* 12**,** 855-860.

WILSON, J. W., SCHURR, M. J., LEBLANC, C. L., RAMAMURTHY, R., BUCHANAN, K. L. & NICKERSON, C. A. (2002) Mechanisms of bacterial pathogenicity. *Postgraduate Medical Journal,* 78**,** 216-224.

WILSON, S. E., MOHAN, R. R., MOHAN, R. R., AMBRÓSIO, R., HONG, J. & LEE, J. (2001b) The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Progress in Retinal and Eye Research,* 20**,** 625-637.

WONG, T., ORMONDE, S., GAMBLE, G. & MCGHEE, C. N. J. (2003) Severe infective keratitis leading to hospital admission in New Zealand. *British Journal of Ophthalmology,* 87**,** 1103-1108.

WRIGHT, S. D., RAMOS, R. A., TOBIAS, P. S., ULEVITCH, R. J. & MATHISON, J. C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science,* 249**,** 1431-1433.

WU, H., WANG, H., XIONG, W., CHEN, S., TANG, H. & HAN, D. (2008) Expression patterns and functions of Toll-like receptors in mouse Sertoli cells. *Endocrinology***,** en.2007-1776.

WU, K.-Y., WANG, H.-Z. & HONG, S.-J. (2006) Effects of antibiotics and corticosteroid eyedrops on cellular proliferation in cultured human corneal keratocytes. *Kaohsiung Journal of Medical Sciences,* 22**,** 385-389.

WU, X. Y., GAO, J. L., REN, M. Y., WU, X.-Y., GAO, J.-L. & REN, M.-Y. (2007) Expression profiles and function of Toll-like receptors in human corneal epithelia. *Chinese Medical Journal,* 120**,** 893-7.

WU, X. Y., HAN, S. P., REN, M. Y., CHANG, Y., YU, F. X., WU, X.-Y., HAN, S.-P., REN, M.-Y., CHANG, Y. & YU, F.-X. (2005) The role of NF-kappaB activation in lipopolysaccharide induced keratitis in rats. *Chinese Medical Journal,* 118**,** 1893-9.

XIA, N., ZHOU, S. X., LIANG, Y. Z., XIAO, C. Q., SHEN, H. L., PAN, H. L., DENG, H. M., WANG, N. Z. & LI, Q. D. Q. (2006) CD4(+) T cells and the Th1/Th2 imbalance are implicated in the pathogenesis of Graves' ophthalmopathy. *International Journal of Molecular Medicine,* 17**,** 911-916.

YAMAGAMI, S., EBIHARA, N., USUI, T., YOKOO, S. & AMANO, S. (2006) Bone Marrow-Derived Cells in Normal Human Corneal Stroma. *Archives of Ophthalmology,* 124**,** 62-69.

YAMAGAMI, S., YOKOO, S., USUI, T., YAMAGAMI, H., AMANO, S. & EBIHARA, N. (2005) Distinct Populations of Dendritic Cells in the Normal Human Donor Corneal Epithelium. *Investigative Ophthalmology & Visual Science,* 46**,** 4489-4494.

YANG, K., KHALIL, M. W., STRUTT, B. J. & KILLINGER, D. W. (1997) 11[beta]-Hydroxysteroid dehydrogenase 1 activity and gene expression in human adipose stromal cells: Effect on aromatase activity. *The Journal of Steroid Biochemistry and Molecular Biology,* 60**,** 247-253.

YAO, S., ZHANG, X., XU, Y., ZHANG, X. & ZHU, S. (1999) Insulin and insulin mutants stimulate glucose uptake in rat adipocytes. *Science in China Series C: Life Sciences,* 42**,** 63-67.

YAO, T. P., KU, G., ZHOU, N. D., SCULLY, R. & LIVINGSTON, D. M. (1996) The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. *Proceedings of the National Academy of Sciences of the United States of America,* 93**,** 10626-10631.

YASIN, B., WANG, W., PANG, M., CHESHENKO, N., HONG, T., WARING, A. J., HEROLD, B. C., WAGAR, E. A. & LEHRER, R. I. (2004) Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. *Journal of Virology,* 78**,** 5147-5156.

YONEDA, K., NAKANO, M., MORI, K., KINOSHITA, S. & TASHIRO, K. (2007) Disease-related quantitation of TGF-beta3 in human aqueous humor. *Growth Factors,* 25**,** 160-167.

YONG, P. Y. K., HARLOW, C., THONG, K. J. & HILLIER, S. G. (2002) Regulation of 11beta-hydroxysteroid dehydrogenase type 1 gene expression in human ovarian surface epithelial cells by interleukin-1.

YOON, J., CHOI, S., LEE, J., LEE, S. & LEE, S. (2009) Ocular surface inflammation, and nerve growth factor level in tears in active thyroid-associated ophthalmopathy. *Graefe's Archive for Clinical and Experimental Ophthalmology,* 248**,** 271.

YOUNG, D. A., EVANS, C. H. & SMITH, T. J. (1998) Leukoregulin induction of protein expression in human orbital fibroblasts: evidence for anatomical site-restricted cytokine-target cell interactions. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 8904-9.

YULEK, F., OZDEK, S., GURELIK, G. & HASANREISOGLU, B. (2006) Effect of topical steroids on corneal epithelial healing after vitreoretinal surgery. *Acta Ophthalmologica Scandinavica,* 84**,** 319-322.

ZBANKOVA, S., BRYNDOVA, J., LEDEN, P., KMENT, M., SVEC, A. & PACHA, J. (2007) 11 beta-hydroxysteroid dehydrogenase 1 and 2 expression in colon from patients with ulcerative colitis. *Journal of Gastroenterology and Hepatology,* 22**,** 1019-1023.

ZBOROWSKA, B., GHABRIAL, R., SELVA, D. & MCCLUSKEY, P. (2005) Idiopathic orbital inflammation with extraorbital extension: case series and review. *Eye,* 20**,** 107-113.

ZELENKA, P. S. & ARPITHA, P. (2008) Coordinating cell proliferation and migration in the lens and cornea. *Seminars in Cell & Developmental Biology,* 19**,** 113-124.

ZHANG, S.-X. (1999) *An Atlas of Histology,* Springer.

ZHANG, Z., CHERRYHOLMES, G. & SHIVELY, J. E. (2008) Neutrophil secondary necrosis is induced by LL-37 derived from cathelicidin. *Journal of Leukocyte Biology,* jlb.0208086.

ZHAO, B., ALLINSON, S. L., MA, A., BENTLEY, A. J., MARTIN, F. L. & FULLWOOD, N. J. (2008) Targeted cornea limbal stem/progenitor cell transfection in an organ culture model. *Investigative Ophthalmology & Visual Science,* 49**,** 3395-3401.

ZHENG, X., YAMAGUCHI, M., GOTO, T., OKAMOTO, S. & OHASHI, Y. (2000) Experimental corneal endotheliitis in rabbit. *Investigative Ophthalmology & Visual Science,* 41**,** 377-385.

ZHONG, X., LIU, H., PU, A., XIA, X. & ZHOU, X. (2007) M cells are involved in pathogenesis of human contact lens-associated giant papillary conjunctivitis. *Archivum Immunologiae et Therapiae Experimentalis,* 55**,** 173-177.

ZHU, X., YANG, P., ZHOU, H., LI, B., HUANG, X., MENG, Q., WANG, L. & KIJLSTRA, A. (2007) CD4+CD25+Tregs express an increased LAG-3 and CTLA-4 in anterior chamber-associated immune deviation. *Graefe's Archive for Clinical and Experimental Ophthalmology,* 245**,** 1549-1557.

ZIESKE, J. D., HUTCHEON, A. E. K., GUO, X., CHUNG, E.-H. & JOYCE, N. C. (2001) TGF-beta receptor types I and II are differentially expressed during corneal epithelial wound repair. *Investigative Ophthalmology & Visual Science,* 42**,** 1465-1471.

ZÖLLNER, S., HWANG, K. H., WILZEWSKI, B., CARAPITO, C., LEIZE-WAGNER, E., VAN DORSSELAER, A. & BERNHARDT, R. (2008) Aldosterone: From biosynthesis to non-genomic action onto the proteome. *Steroids,* 73**,** 966-972.

ZUK, P. A., ZHU, M., ASHJIAN, P., DE UGARTE, D. A., HUANG, J. I., MIZUNO, H., ALFONSO, Z. C., FRASER, J. K., BENHAIM, P. & HEDRICK, M. H. (2002) Human adipose tissue is a source of multipotent stem cells. *Molecular Biology of the Cell,* 13**,** 4279-4295.