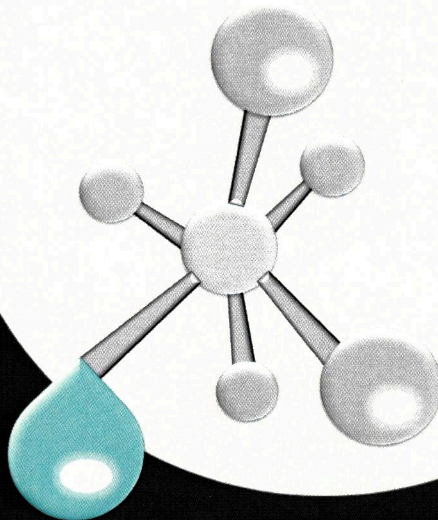
ENVIRONMENTAL STRESS AND THE CORNEAL EPITHELIUM

Alexandra Robciuc



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ENVIRONMENTAL STRESS AND THE CORNEAL EPITHELIUM

Alexandra Robciuc

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Medicine, University of Helsinki, in Lecture Hall 2, Haartman Institute, Haartmaninkatu 3, Helsinki, on September 22nd 2017, at noon.

Helsinki 2017

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ISBN 978-951-51-3519-3 (paperback)
ISBN 978-951-51-3520-9 (PDF)

Unigrafia 2017

To my family

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List of publications not included in the dissertation project:

1. Arvola RP, Robciuc A, Holopainen JM. Matrix Regeneration Therapy: A Case Series of Corneal Neurotrophic Ulcers. Cornea. 2016 Apr;35(4):451-5.
2. Mikkola SK, Robciuc A, Lokajová J, Holding AJ, Lämmerhofer M, Kilpeläinen I, Holopainen JM, King AW, Wiedmer SK. Impact of amphiphilic biomass-dissolving ionic liquids on biological cells and liposomes. Environ Sci Technol. 2015 Feb 3;49(3):1870-8.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based the following original publications, which will be referred to in the text by their Roman numerals. The thesis also includes unpublished data.

I. Holopainen JM, Robciuc A, Cafaro TA, Suarez MF, Konttinen YT, Alkatan HM, Tabbara KF, Tervahartiala T, Sorsa T, Urrets-Zavalia JA, Serra HM. Pro-inflammatory cytokines and gelatinases in climatic droplet keratopathy. Invest Ophthalmol Vis Sci. 2012;53(7):3527-35.

II. Robciuc A, Rantamäki AH, Jauhiainen M, Holopainen JM. Lipid-modifying enzymes in human tear fluid and corneal epithelial stress response. Invest Ophthalmol Vis Sci. 2014;55(1):16-24.

III. Robciuc A, Hyötyläinen T, Jauhiainen M, Holopainen JM. Hyper-osmolarity-induced lipid droplet formation depends on ceramide production by neutral sphingomyelinase 2. J Lipid Res. 2012;53(11):2286-95.

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ABBREVIATIONS

ASM – acid sphingomyelinase
ASAH1 – acid ceramidase
ASAH2- neutral ceramidase
BEL – bromoenol lactone
Cer – ceramide
C1P – ceramide-1-phosphate
CDK – climatic droplet keratopathy
CERK – ceramide kinase
DED – dry eye disease
DEWS – Dry Eye Workshop
ERK – extracellular-signal regulated kinase
HCE – human corneal epithelial cell line
HO – hyperosmolarity
Ig – immunoglobulin
iPLA2b – calcium-independent phospholipase A2beta
JNK – Jun N-terminal kinase
LPS – lipopolysaccharide
MAP kinase – mitogen activated protein kinase
MGD – meibomian gland dysfunction
MHC – major histocompatibility complex
MMP – matrix metalloproteinase
NSMs – neutral sphingomyelinases
PC – phosphatidylcholine
PE – phosphatidylethanolamine
PLs – phospholipids
PS – phosphatidylserine
S1P – sphingosine-1-phosphate
SK1 – sphingosine kinase
SL – sphingolipids
SM – sphingomyelin
Sph – sphingosine
SPL – sphingosine-1 phosphate lyase
PMN – polymorphonuclear
PG – proteoglycan
TBUT – tear break-up time
TLR – Toll-like receptor
UVB – ultraviolet radiation B

ABSTRACT

The cornea is the first optical element of the eye and, together with the eyelids, eye socket, tears, and sclera, shares an important part in ocular protection. It is a thin, transparent, avascular tissue with a rigorous layered structure. The continuous contact with the outside environment exposes the ocular surface tissues, such as the corneal epithelium, to pathogens, mechanical traumas, irritants, toxins, allergens, or radiation from the sun. The cellular stress response represents an adaptive reaction to environmental stimuli and defines the health-state of the tissue, the absence/presence of clinical manifestations. We have aimed in this thesis project to study the stress response of the corneal epithelium to environmental stimuli and to determine its contribution to ocular surface diseases such as climatic droplet keratopathy (CDK), infection, or dry eye disease. A cell culture model of the corneal epithelium was exposed to environmental stress – UV radiation, LPS, or hyperosmolarity (HO) – to identify macromolecular alterations: mRNA expression, protein localization, enzyme activation, lipid conversions.

CDK is a degenerative disease of the cornea with increased prevalence in warm, dry climate. Examination of corneal tissue and tears from patients with CDK suggested an involvement of metalloproteinases (MMPs) in the disease-associated tissue degradation. Our cellular model helped reveal the connexion between UV radiation and the unbalanced secretion of gelatinases (MMP-2 and MMP-9) and thus explain in part the pathogenesis of this rare disease. The evaluation of the inflammatory response to UV, initially, and then to LPS, or HO, highlighted IL-8 secretion as an acute stress marker and followed throughout the studies. Human corneal epithelial cells were found also to release lipid-modifying enzymes into the cell culture medium as a response to stress. Of particular importance to us were the enzymes of the sphingolipid metabolism, a lipid signalling pathway of great importance in the stress response. These enzymes were released as part of cell-derived extracellular vesicles, the vesicle-lipids, however, were the mediators of a significant decrease in IL-8 levels. The same sphingolipid enzymes appeared responsible for the intracellular response to HO, controlling the IL-8 production but also the stress-induced neutral lipid loading.

We have therefore succeeded to establish a causative link between UV radiation and tissue degeneration in CDK, to determine the role of the sphingolipid signalling pathway in ocular surface stress and to discover more about HO consequences in the corneal epithelium. The stress response at the ocular surface is a thin balance between tissue protection and maintenance of function. Inflammation represents one of the most relevant clinical signs of distress and we aimed to identify targets for therapies that seek to restore tissue homeostasis.

1. INTRODUCTION

The most important visual component of the eye is the cornea. Its structure comprises three easily distinguished cellular layers – the epithelium, stroma, and endothelium – separated by two lamellae: Bowman's layer and Descemet's membrane. The superficial cells in the corneal epithelium bathe in the tear fluid and are in contact with the exterior, while the endothelium lines the anterior chamber, in contact with the aqueous humour. The cornea is responsible for more than two-thirds of the eyes' optical power. This function is made possible by two very important characteristics: transparency and curvature. Transparency is the result of complex interactions between physiology and physical structure and permits light to enter the eye and stimulate the retina. Maintenance of corneal shape depends on a special arrangement of stromal collagen fibrils (1). This collagen structure is sufficient to maintain the proper curvature even under extreme hydration stress. Corneal curvature allows correct refraction of the light on the visual axis and is the first element in ocular optics.

Besides its visual function the cornea also serves as a barrier tissue, separating the eye from the external environment. Achievement of the barrier function occurs via the strong junctions between cells in the epithelial layer (2). As a limit tissue the cornea, and more precisely the corneal epithelium, is under constant exposure to external stressors such as pathogens, foreign particles, debris, UV radiation, or osmotic changes. These environmental stressors affect the barrier function and with it corneas' functional integrity. The physiological response to such stressors is inflammation; one of the consequences of an inflammatory reaction in the affected tissue is *loss of function*. Because of the dramatic consequences of inflammation for vision, in the cornea, immune responses are diminished – the *cornea immune privilege* (3). This unique characteristic identifies our study of environmental stress response in such a tissue as being equally challenging and motivating.

Cellular stress response is a universal and complex mechanism that decides the fate of cells, tissues, and even organisms. Environmental stress represents the driving force of adaptation. Many aspects of this mechanism are not stressor-specific, there exist, however, tissue-specific differences. The aim of the present thesis was to study the particularities of the corneal epithelium response to relevant stressors. The cellular stress response of the corneal epithelium to environmental stimuli such as UV-radiation, lipopolysaccharides (LPS), or hyperosmolar stress, manifests clinically as UV burns, infection, or dry eye syndrome (DES). The unique features of the cornea make the extrapolation of information from other tissues impractical. New insights into the corneal epithelium's interaction with tear

film and into each component's contribution to inflammation will provide better understanding of adaptive response pathways and lead to better and more specific treatment for ocular surface disease. We directed special attention to the sphingolipid metabolic pathway and its involvement in the epithelial inflammatory response. Sphingolipids are indispensable molecules that influence cellular responses through their effects on membrane biophysical properties or direct interaction with target proteins (4,5). Their role in inflammation has long been established (6), their involvement in ocular physiology has been of only moderate interest, however. We have investigated the role of UV-B radiation in the aetiology of a climate-related corneal dystrophy. Subsequently, we have focused on the participation of the sphingolipid signalling pathway in the corneal epithelium response to these stressors as well as in tear fluid homeostasis.

2. REVIEW OF LITERATURE

2.1. Cornea

"The complexity of structure and function necessary to maintain such elegant simplicity is the wonder that draws us to one of the most important components of our visual system." (7)

Cornea forms the transparent slightly protruding part of the anterior of the eye (**Figure 1A**). The cornea is the primary barrier of the eye, structurally and functionally, protecting the interior of the eye from infection. Together with the tear fluid it provides a smooth surface for refraction, contributing with almost two thirds of the total refractive power of the eye. The thickness of the cornea varies throughout its length from only 0.5 mm in the centre to almost 1 mm at the periphery (8). In adults, the average horizontal diameter of the cornea is 11.5 to 12 mm (9), while the vertical diameter is about 1 mm shorter (7). Shape is the main determinant of its optical function, allowing for an average power of 43 dioptres. Transparency is the result of both structural details as well as tissue physiology.

2.1.1 Corneal structure

There are three very well distinguished cell layers in the cornea that are separated by two important lamellae. Thus, the five layers from the exterior of the eye to the

anterior chamber are: corneal epithelium, Bowman layer, stroma, Descemet's membrane and the corneal endothelium (**Figure 1B**).

Corneal epithelium

Epithelium is defined as an aggregation of cells in close contact with each other and with a free surface (8). Therefore, the epithelia can be found on the exterior of the body as well as lining the internal cavities that communicate with the exterior (respiratory, gastro-intestinal and genitourinary tracts).

Corneal epithelium, represents the first barrier of the eye to the external environment. It is of non-keratinized stratified squamous type and derives from the embryological ectoderm. Therefore, the epithelium is organised in layers (5 to 7 layers) of cells that become flattened towards the surface but do not keratinize like the epithelium from the skin for example. The epithelium measures about 50 µm in thickness (10) and continues with the conjunctival epithelium that overlays the sclera. The coating film of tear fluid completes the anatomical and functional unit.

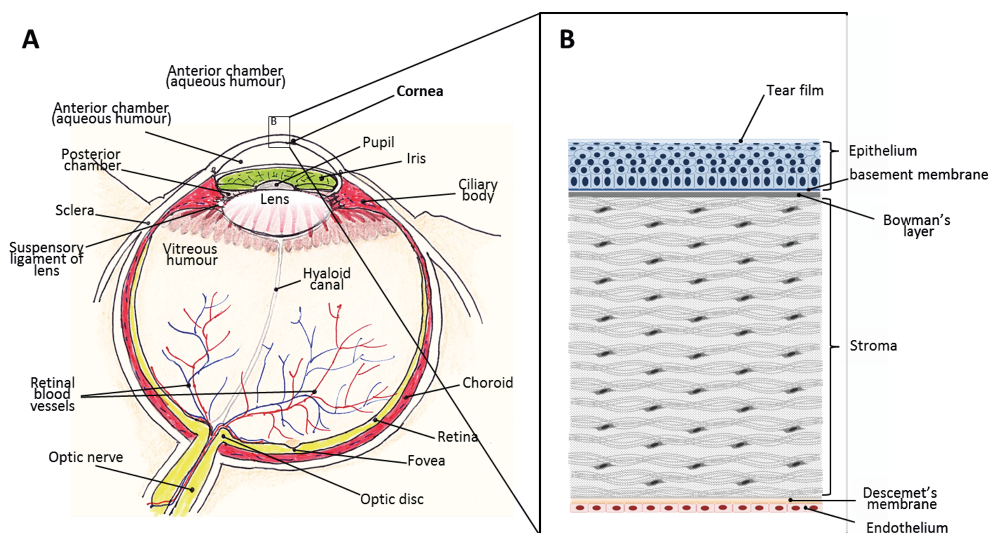


Figure 1: Structure of the eye (A) and cornea (B).

Like other epithelia, the corneal epithelium is maintained through continuous renewal (11). Proliferation of the basal cells is synchronized with the differentiation of the daughter cells as they move towards the surface. The average life span of the epithelial cell is of 7 to 10 days (12). After complete differentiation cells undergo involution, apoptosis and desquamation. The tight junctional complexes between the uppermost cells in the epithelial layer prevent the diffusion of tear fluid into the cornea. This physical barrier is effective against usual dyes used in clinical

practice (fluorescein or rose Bengal), but also against toxins and microbes. This is realized by tight junctions, zonulae occludens, which serve as a semipermeable, highly resistive (12–16 kohms/cm2) membrane (2). Beneath the superficial layers of squamous cells lie 2-3 layers of wing cells. These cells are less flat but possess the same tight lateral junctions. The basal cells of the epithelium are one layer of columnar cells which are the only ones (apart from the limbal cells) able to divide (13). They are the source of wing cells and are connected to each other through zonula adherens as well as gap junctions. The basal cells produce their underlying basement membrane that offers support for the cells atop and attaches the epithelium to the neighbouring layers. The basement membrane is approximately 0.05 μm thick and mostly comprised of collagen type IV and laminin.

The source of basal cells, and therefore all cells in the epithelium, has been localized to the periphery of the cornea in the stem cell reserve of the limbus. The cells from this area migrate towards the central cornea and gradually lose their mitotic capacity and differentiate into transient amplifying cells and basal cells. The limbal epithelium is a vascular tissue of about 10 to 12 cell layers thick. The limbal stroma and epithelium are arranged in radial fibro-vascular ridges called the Palisades of Vogt (14). These palisades are most defined in the upper and inferior cornea but are present on the entire circumference of the cornea.

Bowman layer

Bowman layer is approximately 15 μm thick and is comprised of collagen type I, III and V fibres and other acellular components mediating the epithelium-stroma interaction (15). During development, it is populated consecutively by both epithelial cells and keratocytes (16). Its acellular quality could serve as biological barrier to viral expansion to the stroma but also to prevent the invasion of keratocytes from the stroma (17). When injured it does not regenerate and can form a scar, yet no study has proven a vital role for the Bowman layer in the corneal structure or function.

Stroma

Corneal stroma or *substantia propria* represents the clear majority of the corneal thickness (80 – 85 % of the corneal thickness). The primary corneal stroma is secreted by the epithelium while the secondary stroma is the result of keratinocyte activity. The structure of the stroma is a unique example of natural engineering. The fibre-forming collagen in the stroma (mainly collagen type I, III and V) is arranged in parallel bundles called fibrils; parallel fibrils form lamellae. More than 200 of these lamellae form the bulk of the stroma and they must: i) maintain the corneal curvature and resist the intraocular pressure; and ii) minimize light

scattering. The lamellae run parallel to the surface of the tissue, while adjacent lamellae traverse the cornea at varying angles to each-other (with a preference towards the superior-inferior and nasal-temporal directions). At the periphery of the cornea the stroma is thicker and collagen fibrils may adopt a circumferential orientation close to the limbus (18) (**Figure 2**). This special arrangement of the collagen lamellae has no effect on transparency but is crucial for the strength of the tissue and its anchoring in the neighbouring structures.

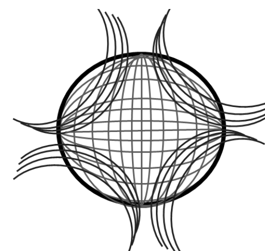


Figure. 2: Arrangement of collagen lamellae in the cornea, based on X-ray diffraction studies. (Modified from Meek and Boote, (18).

The collagen fibrils are embedded in a hydrated matrix of non-fibrilar collagens, proteoglycans (PGs), other soluble proteins, salts and cells. The cells that populate the stroma, keratinocytes, have their embryological origin in the neural crest mesenchymal cells (19). They produce and maintain the highly organized stromal structure by synthesizing the collagen molecules, PGs (mostly keratan or chondroitin sulphate) and the matrix metalloproteinases (MMPs) responsible for remodelling and renewal. Most of the cells reside in the anterior stroma and contain intracellular proteins called "crystallins" that help lower light scattering. A concession from this high degree of organization seems to be made for the anterior part of the stroma where the packing of the collagen layers is slightly more rigid and interwoven. This aspect plus the importance of PGs is discussed further in a following chapter.

Descemet membrane

Descemet membrane is continually secreted by the cells in the endothelial layer. The anterior part is secreted before birth and seems to have a precise organization, while the part secreted after birth is more amorphous (20,21). The layer can reach a 10 μm thickness with age.

Corneal endothelium

The endothelial cells form a monolayer on the posterior side of the cornea. They are of mesenchymal origin and with a crucial role in maintaining the proper hydration level of the stroma. Endothelial cells have a specific polygonal shape, are flattened and tightly bound to one-another. Adjacent cells possess tight-junctions but also gap junctions on their lateral sides and hemi-desmosomes maintain the

cells bound to the Descemet membrane (7). Their main cellular function is to produce an osmotic gradient from the stroma to the aqueous humour in the anterior chamber. This gradient facilitates the removal of water from the stroma to preserve transparency (22,23). At the corneal periphery, the endothelium fuses with the cells of the trabecular meshwork.

Because endothelial cells are non-dividing their number decreases throughout life with 20 cells/year, on average, from the initial density of circa 3500 cells/mm^2 (24,25). The minimum number of cells necessary to maintain function was suggested to be around 500 cells/mm^2 (26). This progressive loss of cells is exacerbated by trauma, inflammation and other cellular stressors. To cover the surface and conserve the cellular barrier the endothelial cells migrate and/or grow in size.

2.1.2 Tear fluid

The ocular surface, and therefore corneal epithelium, is covered with a protective film of tear fluid of approximately 4 – 11µm. The functions of this tear film include: lubrication for lids and ocular surface, antibacterial protection, flushing of contaminants from the ocular surface, provide nutrition to the corneal epithelium as well as participation in the visual function by providing a smooth surface for light refraction (27,28). The tear fluid is structured in three layers in close interaction with each other: i) a mucinous matrix close to the ocular surface cells and in tight relation with the cellular glycocalyx; ii) the aqueous of tears that dissolves the mucins and other components of the tears; iii) the tear fluid lipid layer at the air-aqueous interface (**Figure 3**).

The tear fluid layers have different chemical, physical, and biological properties as well as distinct origins. The mucinous layer is comprised of mucins, water-retaining, high-molecular-weight glycoproteins that originate from the ocular surface epithelia glycocalix (MUC1, 4 and 16 – transmembrane mucins) or secreted from conjunctival goblet cells (MUC5AC – gel-forming mucin) (29,30). Soluble forms of the transmembrane mucins together with gel-forming mucins generate a loose network-like matrix that dilutes in the aqueous tears towards the air.

The aqueous of the tear fluid contains the mucous gradient, electrolytes, proteins, metabolites and it is produced by the lacrimal gland and accessory lacrimal glands. The composition of the tears aqueous is conditioned also by the surface epithelia. There is a high concentration of proteins (7 – 10 mg/mL) (27,28) with almost 500 proteins identified (31). Main functions of the proteins are: wound healing, inflammation or microbial protection. Most abundant proteins are

lysozyme, lactoferrin, tear lipocalin, secretory immunoglobulin (Ig) A, lipophilin, IgG, and serum albumin (28).

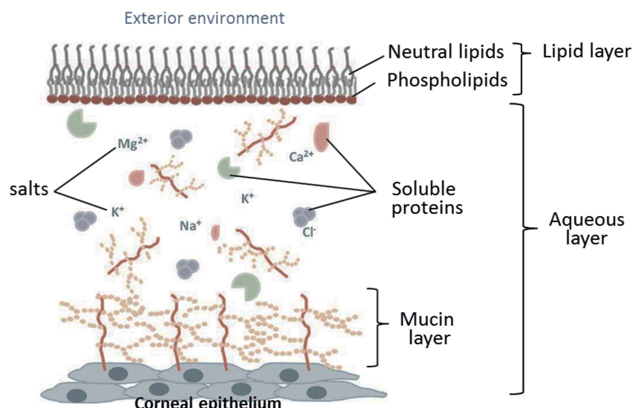


Figure 3: Schematic structure of the tear fluid.

The tear fluid lipid layer is a thin oily layer on top of the aqueous tears. The lipids forming this layer are partly secreted by specialized glands in the eyelids called meibomian glands (32). There is an intensely discussed non-synonymy between meibum and tear fluid lipid layer. Meibum is mainly composed of sterol esters (30%, (33)) and wax esters (30-50%, (34)). Other non-polar lipids in its composition are triacylglycerols, fatty acids, cholesterol (35,36). Besides the non-polar lipids from the meibum the tear fluid lipid layer also includes polar lipids (37-39) of yet unknown origin. The presence of such a lipid layer is justified by the stabilizing activity of the lipids, by lowering the aqueous surface tension they help tears spread evenly and remain on the surface of the eye. The lipids are also thought to retard water evaporation from the tears, however, this issue has been argued intensely in the literature (40-43).

2.1.3 Corneal physiology

Shape and transparency

The main visual function is accomplished by the cornea's transparency and shape. The transparency is acquired and maintained through the cooperation between the epithelium's barrier function, the special structure of the corneal stroma and the continuous removal of fluid from the stroma through the activity of the endothelial pumps. Because of their reduced thickness Bowman layer and Descemet membrane do not contribute to light scattering.

The epithelium participates in maintaining transparency by keeping a relative water-tight barrier to the tears. This barrier function is dependent on the

physiological integrity of the layers and is sustained by the epithelial tight junctions and the continual renewal of the epithelial cells. Its own transparency is explained by the high homogeneity of refractive index throughout the cellular layers (i.e. the virtual absence of intercellular spaces) (44). In the stroma, transparency is the result of the perfect correlation between the diameter of the collagen fibrils and the spacing between the fibrils in the lamellae (45). The diameter of the fibrils is controlled by the proportion of collagen V (46), while the spacing between the fibrils is maintained by the PGs, and more accurately by their hydration level (**Figure 4**).

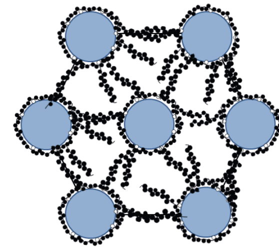


Figure 4: Fibril arrangement in the collagen lamellae that minimizes light scattering. Fibrils are represented by the circles in the PG matrix.

The last cellular layer of the cornea facilitates, through interdepended passive and active ion transports, a net outflow of water from the stroma towards the aqueous conserving the relative dehydrated state (i.e. deturgescence) of the stroma. By controlling the hydration level of the PGs, the endothelium controls the spacing between the collagen fibres and therefore the transparency of the stroma. This process relies most relevantly on the activity of the Na^+/K^+ ATP-ase and the carbonic anhydrase.

Muller *et al.* suggested that corneal curvature is maintained by the specific architecture of the anterior cornea, more precisely the first 120 μm of the corneal stroma (1). The particular arrangement of collagen fibrils in this part of the stroma, together with the difference in PG composition (dermatan sulphate as opposed to keratan sulphate in posterior stroma), are thought to protect the anterior stroma from swelling and therefore preserve the corneal curvature even under extreme hydration. Here the collagen is organized in few straight lamellae with random directions, many undulating collagen bundles, and a high degree of interlacing. This specific arrangement almost doubles the light scattering in the anterior stroma (47) but is essential in maintaining the functional curvature.

Barrier function

As the first layer in ocular structure, the cornea is a shape defining and protective tissue. It must withstand and maintain intraocular pressure and block external insults. As mentioned before, the strength of the cornea is provided by the stromal collagen, the rest of the protective functions are supported by the corneal epithelium. The very tight junction between the epithelial cells are a very efficient barrier to microorganisms, electrolytes, and most importantly water (48). The small amount of water that does make it across the epithelium is removed by the activity of the endothelium (explained in more detail in Corneal transparency section).

The epithelium is responsible for protection against exterior stress stimuli. One such example is offered by UV radiation. Epithelial cells absorb UV wavelengths shorter than 310nm (49), while longer wavelengths are absorbed by the lens thus avoiding UV-induced damage to the retina (50). Additionally, a healthy epithelium, aided greatly by the flushing activity of the tears, is an almost impenetrable barrier for microorganisms. Most of corneal infections are thus made possible by the events that weaken this barrier, such as: inflammatory reactions or variable traumas of the epithelium. Notable exceptions are viral infections.

Blood supply to the cornea

The cornea has no direct blood supply since that would compromise transparency, it is, however, reliant on nutrients from the blood stream. All nutrients but oxygen are delivered to corneal tissues through the aqueous humour (44) and to a smaller degree from the conjunctival blood vessels. Oxygen is taken up directly from the air, or from the surrounding vascularized tissues during sleep (51).

Cornea innervation

The cornea is one of the most innervated tissues in the body. Most of the nerve fibres are sensorial and derive from the ophthalmic branch of the trigeminal nerve (52). A lesser number may originate also from the trigeminal maxillary branch. The cornea also contains autonomic sympathetic and parasympathetic innervation (53,54). The unmyelinated nerve bundles enter the stroma in radial manner, parallel with the collagen lamellae, and then move towards the epithelium, perforate Bowman's and from the sub-basal nerve plexus beneath the basal epithelial layer (55,56). From here, individual fibres separate and penetrate the epithelium to stop in the superficial layers.

Corneal immune defence

Good vision outweighed the necessity of a complex immune response; corneal

localization, however, prompted the existence of essential protection mechanisms. Physical protection is provided by the orbital skeleton, eye lids, and tears. Immune protection is supplied by components of both innate and adaptive immunity.

Innate immunity is represented by soluble components in the tear fluid, corneal nerves, the epithelium, keratocytes, polymorphonuclear cells (PMNs), and cytokines and is responsible for the non-specific, timely response to aggression. Tear fluid is abundant in anti-microbial proteins (lactoferrin, lysozyme, beta-lysin) and Igs that help prevent infections (57). Secretory IgA is found in much higher quantities in tears than serum (58). Apart from its passive role in immune protection (i.e. the barrier function), the epithelium secretes a series of cytokines (TGF-β, IL-1a, IL-1b, IL-6, IL-10) and chemokines (IL-8, MCP-1 and CCL20) that modulate the ocular immune reaction (59). Stromal keratocytes also secrete IL-1a, IL-6 and defensins (peptides with a broad anti-microbial role). As a response to pain, sensory termination release neuropeptides (calcitonin gene-related peptide, substance P) that induce secretion of IL-8 from epithelial cells (60). Components of the complement diffuse from limbal blood vessels to participate in the corneal immune defence (61).

The cellular components of the innate immunity are: neutrophils, eozinophils, macrophages and natural killer (NK) cells. Neutrophils are the most abundant PMNs at the ocular surface and play vital anti-microbial roles (62). They are recruited from the limbal vasculature, IL-8 being a very potent chemoattractant. Macrophages recognize and phagocytose microbes and activate and modulates the adaptive immunity (63). NK cells recognize MHC class I molecules and lyse the cells that express insufficient such molecules on their surface: tumour cells, virus-infected cells, antibody-coated cells or cells that are undifferentiated. Similar to macrophages, the NK cells secrete TNFα and IFNα.

When the immune threat persists, the adaptive immune response is activated. Unlike the innate immunity, this delayed response is difficult to contain and most often leads to irreversible tissue destruction. Langerhans cells are the essential component of this surveillance system. These cells express MHC class II and are antigen-presenting cells. They activate the summoned T cells that will differentiate into effector cells T_H (CD4/helper) or T_C (CD8/cytotoxic). The activated T cells will then take over the immune response until the pathogens/antigens are eliminated. The selection of cytokines secreted by the different T_H cells dictate the intensity, chronicity, and type of immune/inflammatory response that is produced.

The *immune privilege* of the cornea was proposed first time in the 1940s by Medawar (64), prompted by the very low graft rejection rate. This process has evolved to protect the vital functions of organs from immuno-pathogenic damage

(3). The immune privilege is achieved in the cornea through: i) anatomical and molecular barriers; ii) immuno-tolerance; iii) an immunosuppressive environment. Corneal avascularity (*angiogenic privilege*) contributes to transparency but also physically disconnects the cornea from the immune components circulating in blood and lymph. Anti-angiogenic factors (pigmented epithelium-derived factor or angiostatin) outweigh the pro-angiogenic ones (vascular endothelial growth factor) at the corneal limbus preventing blood vessels (presumably also lymphatic vessels) from entering the cornea (65). This anatomical detail aims to delay the involvement of the cellular immune response and allow innate immunity to resolve antigen threats. Additionally, the cornea is almost devoid of MHC class II cells (except for rare macrophages and Langerhans cells) and shows high immunotolerance for antigens placed within or arising from the eye (66). Another level in the immune privilege is exemplified by the presence of immunosuppressing factors in the aqueous humour. Soluble factors, such as TGF-β2, α -melanocyte stimulating hormone, vasoactive intestinal peptide, calcitonin gene-related peptide or macrophage migration inhibitory factor, suppress effector functions of both adaptive and innate immunity (3). The eye offers for analysis a naturally evolved self-regulatory mechanisms for inflammation.

2.2. Environmental stress response

2.2.1. Environmental stress

Except when sleeping, the cornea is exposed to the external environment, either natural or man-made. Environmental stress can be defined as an action, agent, or condition that affects the function or structure of a biological system (67). This definition implies the existence of a receptor – the biological system – and the adverse response – the change in the physiology of the receptor. To be qualified as environmental stress the stimuli must be a threat to the survival of that biological system. The word "stress", however, is associated with a high degree of ambiguity that originates from the great interest across many biology fields: biomedical, populational, whole-organism or cellular level only (68). In our studies, we have concentrated on the cellular stress imposed by the external medium.

For the cornea, there are biotic stress factors such as metabolic stress or pathogens, as well as abiotic factors such as temperature, humidity, atmospheric pressure, toxins, allergens, radiation, chemicals, mechanical or oxidative stress. The longer the environment diverges from optimum, the greater the stress and the

greater the effect on cellular survival. Each stressor activates its specific response pathways in the cells all paths, however, converge downstream to inflammation.

Pathogen aggression

The cornea is exposed to bacteria, fungi, parasites and viruses. The herpes viruses (herpes simplex and herpes zoster) are DNA viruses and are the most common viral infections of the cornea, associated often with increased morbidity and disability (69). Infection occurs by direct contact with active viral lesions or secretions. Although both types of herpes simplex viruses can affect the cornea, herpes simplex type 1 is primarily responsible for orofacial and ocular infections. The virus travels the trigeminal nerve sensory endings and affects the nearby epithelial cells. Parasitic invasion of the cornea is rare in the developed world and are almost always associated with increased outdoor activities.

Contamination with bacteria and fungi can occur from air, waters (oceans, rivers, lakes and hot tubs), hands, various objects. Fungi involved in ocular surface disease belong to the genera such as *Fusarium*, *Alternaria*, *Aspergillus* or *Candida* spp (70). Both Gram-negative and Gram-positive bacteria infect the ocular surface. S*taphylococcus* and *Streptococcus* species (Gram-positive), Entero-bacteriaceae and *Pseudomonas aeruginosa* (Gram-negative) are most common causes for bacterial keratitis (71,72). Since a healthy cornea is unlikely to become infected, a previous lesion of the epithelium represents a necessary condition for the above-mentioned pathogens (bacteria and fungi). This pre-conditioning occurs through traumas with contaminated objects or plant material, contact lens wear, inflammation.

UV radiation

Approximately 10% of the total light from the Sun consists of ultraviolet (UV) radiation. UV radiation wavelengths are from 10nm to 400nm where the visible spectrum of the light begins. Artificial UV radiation is produced by tanning lamps, black lights or mercury-vapour lamps. Although a non-ionizing radiation, UV can cause chemical reactions, affecting the physiology of the exposed tissues. Based on the wavelength, three types of UV reach the surface of the Earth: mostly UV-A (315 – 400 nm), and in a smaller proportion UV-B (280 – 315 nm) and UV-C (100 – 280 nm); shorter UV rays being absorbed by the atmosphere (73). The energy of the radiation is inversely correlated with the wavelength, UV-B and UV-C being 100- to 100 000-fold more damaging than UV-A (50). UV-C, although extremely damaging, represents an extremely small proportion of the Sun light reaching the eye, but it is the main UV radiation produced by the germicidal irradiation lamps. This method is used in medical sanitation, as well as for purification of food, water, air, and relies on the capacity of the short-wave UV to damage deoxyribonucleic

acid (DNA), affecting cell survival (74). UV-B can also cause direct DNA damage by inducing covalent bonds between consecutive thymine base pairs (75). UV-A and UV-B are two types of solar radiation most responsible for sun burns. UV-A cannot cause direct DNA damage, but it can generate free radicals and reactive oxygen species (76). Apart from the negative effects, UV radiation has a positive impact on human health through vitamin D production.

Osmolar stress

Not all stressors originate from the external environment. Qualitative changes in the tear fluid may also induce adaptive responses from the corneal epithelium. One such example is tear osmolarity, the measure of solute concentration. This defines the osmotic pressure across the plasma membrane of the cells in contact with the tear fluid and therefore direct the movement of water molecules across those membranes: in hypo-osmolar environment cells swell, while in hyper-osmolarity (HO) cell volume decreases. These changes affect intracellular ion homeostasis (in particular Na^+ and K^+) and macromolecule density and are generally detrimental (77). Regaining intracellular ion-balance straggles compared to the almost instantaneous volume change, therefore, until compensatory mechanisms come in place, osmotic stress may damage cellular macromolecules and impair cell function. Failure to regulate ionic balance can trigger cell death (78,79).

The cellular response to HO was first discovered in yeast (80) where the signalling cascade involves the high-osmolarity glycerol (HOG) pathway with events closely linked to the plasma membrane (81). The HOG proteins are kinases from the MAPK (mitogen-activated protein kinase) family. Yeast cells have two classes of sensor molecules for osmolarity: Sho1 and Msb2 (where Msb2 is a mucin-like protein) for high osmolar stress and the Sln1 protein for milder osmolar conditions (80). Nevertheless, both pathways activate the effector kinase (Hog kinases) that in turn affects the production of osmoregulators (small molecules that increase the intracellular solute concentration, thereby providing osmotic stabilization). The established homologue of the Hog kinase in mammalian cells is the p38 MAPK (82) although HO may activate all three main MAPK pathways. Proposed mammalian sensors for osmolarity are: i) the integrins that sense the mechanical stress that the plasma membrane is subjected to under osmotic stress (83); ii) the transient receptor potential vanilloid family (TRPV1-7) (84); iii) transmembrane mucins, due to their structural homology with the Msb2 proteins in yeast and the capability to activate receptor tyrosine kinases of the ErbB family (85). In mice, HO upregulated the expression of kinases, heat-shock response, or homeostatic genes whereas macromolecule synthesis, cell cycle, telomere maintenance, as well as the response to DNA-damage stimulus were reduced (86).

2.2.2. Cellular stress response

At the organism level the response to stress is controlled by hormones and gives the "fight-or-flight response" (87) or the "general adaptation syndrome" (88) to an aggression/aggressor. At cellular level the stress response is activated by the detection of damaged macromolecules (i.e. lipids, proteins and/or DNA).

The evolutionary conserved principles of the cellular stress response include cell cycle control proteins, chaperones, DNA stabilization and repair machinery, removal of damaged macromolecules and specific aspects of metabolism (89). The proteomic analysis of human, yeast, bacterial, and archaeal bacteria provided approximately 300 proteins that are highly conserved between these species (90). From these, 44 proteins have been implicated in the cellular stress response, highlighting the importance and universality of this process. Many more proteins are involved in the stress response but are not as conserved on the evolutionary axis. The detailed structure and the magnitude of the cellular stress response is cell type- and species-specific, because it depends on what effectors the cells are expressing at the time of the damage. The severity and duration of stress determines whether the cells: i) induce cell repair mechanisms to re-establish homeostasis; ii) use coping mechanisms for temporary adaptation; iii) induce autophagy; iv) trigger cell death. All these paths may be involved, however, in the cellular response to one stressor.

Cellular stress response pathways

Conserved cell stress response pathways are the **heath-shock response**, the **unfolded protein response** (UPR), the response to **oxidative stress** or to **DNA damage**. The latter is activated by stressors that directly target DNA, such as chemotherapeutic agents, irradiation (including UV light) and other genotoxic agents (91). Single or double-strand breaks in the DNA structure signal the intervention of the DNA-repair machinery controlled by p53 and other checkpoint proteins (92). Oxidative stress response is prompted by one of the most potent threats to cellular viability – reactive oxygen species such as superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl or peroxide radicals (93). These molecules can damage all major classes of macromolecules therefore many other response pathways cross-talk with the oxidative stress response. The heat-shock response was initially described as the response to temperature increase (94), currently more stressors have been shown to induce this response (heavy metals or even oxidative stress). Heat-shock response is controlled by specific transcription factors (heat shock factors - HSFs) that induce the expression of the heat shock proteins (Hsps). These proteins grouped by their molecular weight (Hsp90, Hsp70,

Hsp40) act as molecular chaperones that help refold misfolded or aggregated proteins, thus conferring transient thermotolerance (increased resistance to various noxious stimuli). Another pro-survival mechanism is the UPR (95), a stress response initiated in the endoplasmic reticulum (ER) by three main proteins: the inositol-requiring protein-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Each of the three initiator proteins has specific downstream targets that cover a large spectrum of cellular effects (apoptosis, metabolic stress, inflammation, alterations in lipid metabolism).

Sphingolipid signalling pathway

An evolutionary advantageous alternative to conventional signalling pathways are lipid signalling pathways. Lipids mediate their membrane-associated functions majorly through two mechanisms: by direct interaction with an effector (lipid-protein interaction) or by modulation of the biophysical environment of the membrane (lipid-lipid and lipid-protein interactions). The details of either pathway are still largely unknown but enormous progress has been achieved in the field of the bioactive lipids. Bioactive lipids are intermediates or end-products in the metabolic pathways and are under tight homeostatic control. First molecules recognized to have profound physiological effects were the members of the eicosanoid signalling (96), followed by the complicated network of the phosphoinositide metabolites (97,98). Last to be recognized were sphingolipids, a complex class of lipids involved in almost all aspects of survival - proliferation, differentiation, migration, cell death and inflammation (4,6,99-101). The **sphingolipid signalling pathway** is of special interest to us and of major importance in the cellular stress response.

The pathway is centred on ceramides (Cers), the classic sphingolipid (SL) that constitutes the hydrophobic core of sphingomyelins (SMs) and glycosphingolipids. SM is a common component of cellular membranes (plasma membrane in particular), representing almost 20% of all phospholipids. Over 200 different Cers are used or produced in cells by an estimated number of 28 enzymes (102). The considerable number of Cer species and their relatively low intracellular levels suggest a specificity of interaction between the lipids and their protein partners, supporting the second messenger quality of Cers (102). Their unique biophysical properties represent the great advantage that made SLs indispensable for eukaryote survival. The increased hydrophobicity, resulting from the relative high degree of saturation in the lipid chains and the small hydrophilic head-group (the hydroxyl moiety), restricts Cers to membranes and affects significantly the lateral organization of lipids. In other words, due to their increased molecular rigidity Cers segregate from membrane phospholipids (PLs) into transitory domains called

rafts (103). This local separation selects specific residing proteins that complete the functional unit. The spatial confinement or the increase hydrophobicity of their milieu act as activating signals for residing proteins transforming the lipid rafts into membrane signalling hubs (104). These biophysical properties are modulated in cells by the enzymes of the SL metabolic pathway. Cers offer an illustrating example of the SLs structure/function specificity: the double bond at C4,5 of the sphingoid base in Cer can be removed producing dehydroCers and while both lipids (Cers and dehydroCers) can induce autophagy (105,106), only Cers are able to initiate apoptosis (99).

In the SL pathway Cers originate from *de novo* synthesis in the ER or SM hydrolysis and can be phosphorylated to ceramide-1-phosphate (C1P) or glucosylated to glucosyl-Cer (precursor of complex glycosphingolipids). By receiving a phospho-choline group Cer turns into SM, while if hydrolysed Cer converts to sphingosine, the SL backbone (5). Sphingosine can be reused for Cer production or it can be phosphorylated to sphingosine-1-phosphate (S1P). Apart from the non-reversible *de novo* input of Cer and the breakdown of S1P into non-SL molecules, all other conversions are reversible (5) (**Figure 5**). Each SL possesses specific biophysical activity and interaction partners, therefore the outcome of the SL signalling engagement is determined by the lipid species that transiently accumulates in a membrane at one time. Cers build-up is associated with apoptosis and senescence, while C1P promotes inflammation. Sphingosine (Sph) is toxic for cells and rarely accumulates, it is, however, the source of S1P. S1P is a particularly versatile molecule with intracellular and extracellular targets that controls proliferation, migration, differentiation, inflammation. The effects of extracellular S1P are mediated by the five G-protein coupled receptors ($S1PR_{1-5}$). And because S1P is the measure of Cer hydrolysis, these two lipids maintain the balance between cell death and survival of cells under stress (107).

In stress, the SL pathway is engaged through Cer production from membrane resident SM (108). This key step is mediated by sphingomyelinases (SMases). According to the optimum pH SMases are classified as acidic (ASMases), alkaline, and neutral (NSMases) (109). Alkaline SMase is present mainly in the intestine and is thought to play a role in SM digestion (110). Cells produce two isoforms of the ASMase, one residing in lysosomes (ASM) and a secreted form of ASM (111,112). Three neutral SMases (NSMase1, 2 and 3) have been identified, with little to none structural homology, apart from the Mg^{2+} requirement. NSM2 is suggested to have essential regulatory role in SL metabolism (113-115). NSM2 together with the two acidic SMases constitute great candidates for the stress response studies. Stimuli reported to activate this signaling pathway range from UV radiation to hypoxia or bacterial infection (108,116-118).

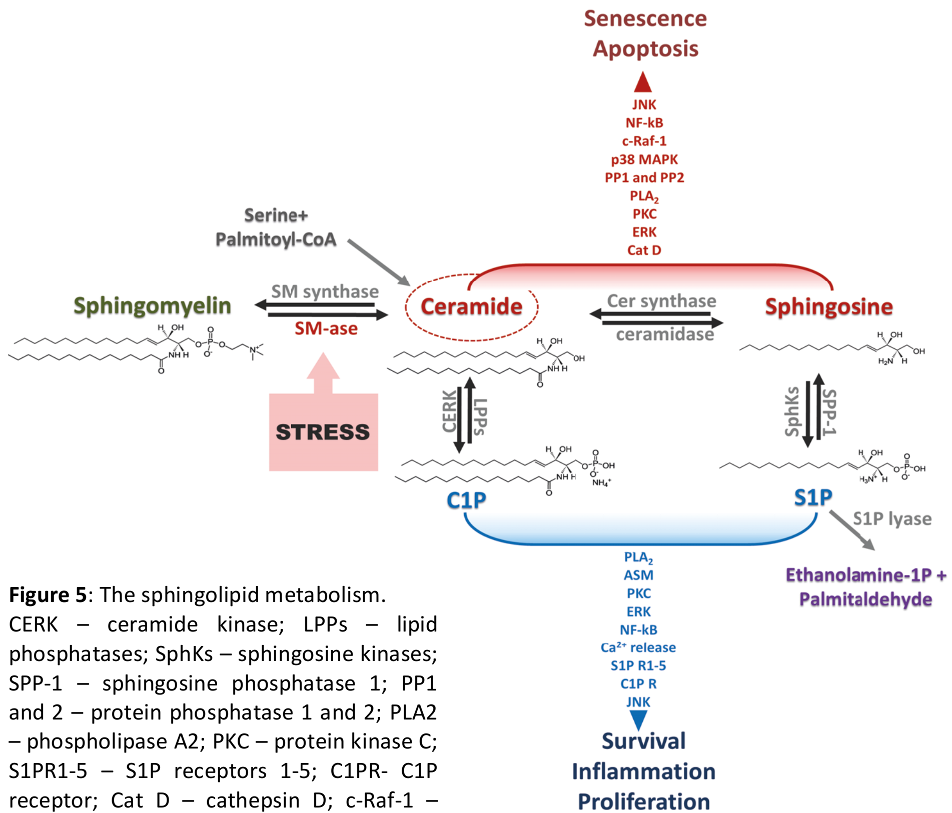


Figure 5: The sphingolipid metabolism. CERK – ceramide kinase; LPPs – lipid phosphatases; SphKs – sphingosine kinases; SPP-1 – sphingosine phosphatase 1; PP1 and 2 – protein phosphatase 1 and 2; PLA2 – phospholipase A2; PKC – protein kinase C; S1PR1-5 – S1P receptors 1-5; C1PR- C1P receptor; Cat D – cathepsin D; c-Raf-1 – MAP3K.

2.2.3 Consequences of stress

2.2.3.1 Stress-induced cell death

Although designed to promote survival and adaptation in stress-affected cells, all stress pathways can activate effectors of cell death when the damage is too severe (119). Depending on the cells' ability to cope with the environmental condition, cell death can be apoptotic, necrotic, pyroptotic, or autophagic.

Apoptosis is a highly conserved morphologically distinct cell death (120) that can be triggered by many stress stimuli (irradiation, oxidative stress, ER stress). Most prominent effectors of this programmed cell death are the caspases (cysteine-dependent aspartate-specific protease) family (121). Caspases are normally inactive in cells until the apoptotic stimuli induces proteolytic cleavage of one of the initiating caspases -2, -8, -9 or -10. This event starts a cascade of proteolytic events

that culminates with the activation of the executioner caspases, -3, -6 and -7, and initiation of the apoptosis morphology (blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, DNA and mRNA degradation). In the extrinsic pathway apoptosis is triggered by signals from the cell surface (cytokine receptors, CD95 clustering) and activate caspase 8; while in the intrinsic pathway, controlled by the mitochondria, caspase-9 is activated (122). Common components are the activation of caspase-3 and the Bcl-2 family proteins. Cers were shown to be involved in both pathways of apoptosis, either by favouring CD95 receptor clustering in Cer-rich domains or by assisting in cytochrome c release from mitochondria (123,124). If over-stimulated all three branches of the UPR can lead to apoptosis through caspase-4 (125), CHOP (C/EBP homologous protein) (126) or Jun kinase pathways (127).

Autophagy is an adaptational response to a variety of metabolic and therapeutic stresses such as: nutrient or growth-factor deprivation, ER stress, oxidative stress. This progressive mechanism is characterized by vesicular sequestration and digestion of cytoplasmic proteins and organelles (128). Autophagic cell death is rare in mature organisms and acts as a replacement for inhibited apoptosis (129).

Necrosis is characterized by cellular and organelle swelling, plasma membrane accidental cell death, recent evidence suggests that necrosis is a regulated process. Most notable examples are death-receptor or Toll-like receptor induced necrosis (130).

2.2.3.2 Pro-inflammatory signalling

Inflammation is a protective response to harmful stimuli that involves the immune system components. At tissue level the inflammatory reaction is designed to eliminate the cause of injury, clear out the damaged tissue and initiate tissue repair. Inflammation is always associated with loss of function of the affected tissue and is therefore closely regulated. The initial phase is the activation of inflammatory pathways in the affected cells. A multitude of signalling pathways have evolved to mediate the cells' communication with the extracellular environment.

The MAPKs are ubiquitous enzymes that specialize in extracellular signal transduction. These enzymes regulate protein activity by tyrosine-, serine-, or threonine-phosphorylation. They respond to a wide variety extracellular receptors (receptor tyrosine kinases, cytokine receptors, G-protein coupled receptors), and environmental stresses such as osmotic shock, ionizing radiation and ischemic injury (131). MAPKs operate in three-step activation cascade: MAP3K (MAP Kinase Kinase Kinase) activates MAP2K (MAP Kinase Kinase), which then

activates a MAP Kinase. In mammalian cells, 19 genes code for MAP3Ks, 7 for MAP2Ks, and 12 for MAPKs. Best characterised MAPKs are ERK (extracellular signal-regulated kinase), JNK/SAPK (C-Jun N-terminal kinase/ stress-activated protein kinase) and p38 Kinase (132). The JNK/SAPK usually leads to apoptosis induction but also plays a role in pro-inflammatory signalling. Activation of ERK pathway regulates cell division, migration and survival. The p38 MAPKs responds to UV irradiation, heat shock, high osmotic stress, lipopolysaccharide (LPS), cytokines (IL-1 or TNFα) by modulating response gene transcription. p38 pathways cross-talk with CHOP-mediated gene transcription or with the NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells).

NF-kB is a transcription factor complex present in almost all cells and controls the expression of cytokines, chemokines, or growth factors. It can be activated by stress or indirectly by cross-talk with other stress response pathways. NF-kB is a cytosolic heterodimer of a Rel-family protein with a p50/p52 proteins (133) associated with the IkB inhibitor. Upon activation IkB is phosphorylated and targeted for proteasomal degradation. The released NF-kB translocate to the nucleus where it activates genes involved in cellular replication, inflammatory responses (**Figure 6**).

These two pivotal signalling pathways (MAPK and NF-kB) control the expression of most pro-inflammatory genes: cytokines, chemokines and other inflammatory mediators.

IL-8 secretion

Chemokines are small cytokines with the ability to induce chemotaxis in nearby cells. The first discovered CXC (cysteine, X, cysteine) chemokine was termed IL-8 (134) and it is an acute phase, potent pro-inflammatory chemokine with strong chemotactic properties for neutrophils. IL-8 is produced upon stimulation by macrophages and different epithelial and endothelial cells (**Figure 6**). Its expression is controlled by numerous stress pathways with the MAP kinase and NF-kB as most important ones. Once secreted from the cells, IL-8 is a specific ligand for two receptors CXCR1 and CXCR2, with more binding affinity for CXCR1 (135). These receptors are G-protein coupled receptors that are upstream activators of numerous signalling pathways such as AKT (protein kinase B), JAK/STAT, or MAPK/ERK. The two IL-8 receptors are present primarily on neutrophils, monocytes/macrophages, and vascular endothelial cells. Thus, IL-8 effects in these cells range from chemotaxis (neutrophils), phagocytosis (macrophages), and angiogenesis (by stimulating the division and migration of endothelial cells). In neutrophils IL-8 also inhibits apoptosis, prolonging their life

and involvement in the inflammatory reaction. Because of its very strong pro-inflammatory profile and angiogenic effects, IL-8 has been associated with many inflammatory disorders and even with tumorigenesis and tumour progression (136). The wide-spread clinical relevance and effectiveness make IL-8 an ideal marker for the evaluation of the inflammatory response. This is of particular significance in the cornea since corneal epithelial cells (CECs) secrete IL-8 (137), among other cytokines, as a response to stress and neutrophils represent the first line of immune defence.

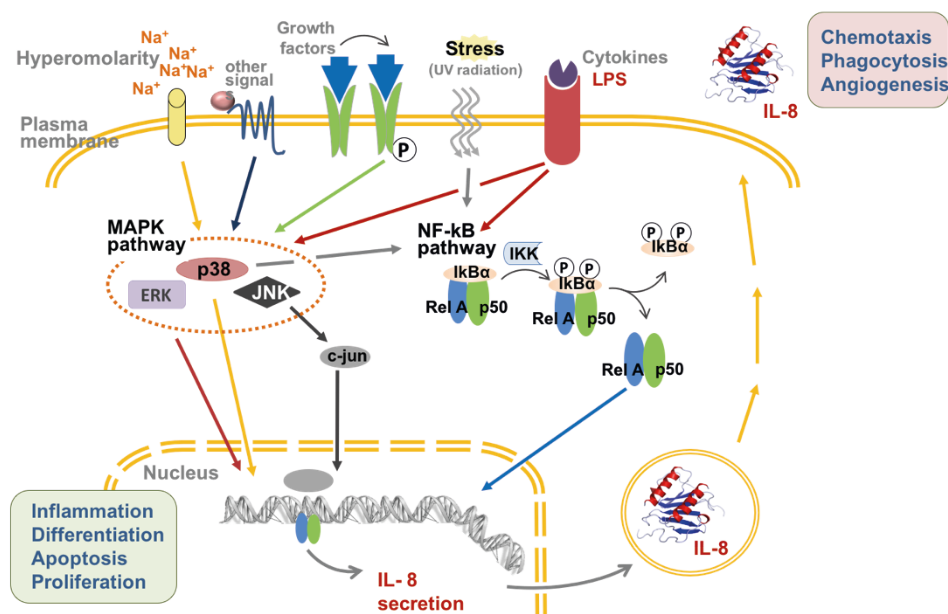


Figure 6: IL-8 secretion inducers and consequences.

Matrix metalloproteinase activation

Matrix metalloproteinases (MMPs) are a family of neutral zinc proteolytic enzymes that remodel and maintain tissue architecture. Their large variety of substrates (extracellular matrix, cytokines, cell surface molecules) has implicated them in a wide range of physiological and pathological processes including wound healing, angiogenesis, inflammation, or tumour metastasis (138-140). MMPs are not synthesized unless needed and are usually secreted as pro-enzymes that are activated in the extracellular environment. Identified with consecutive numbers (MMP1, MMP2, etc.), the MMP family numbers more than 20 members divided into four general categories: collagenases (MMP8 – for native fibrillar collagens), gelatinases (MMP2, MMP9 – for denaturated collagens and basement membrane

components), stromelysins (for collagens, PGs, and MMP activation), and membrane-type MMPs involved in various activities in the cells' surroundings (139-141).

The activity of the MMPs is modulated by the tissue inhibitors of MMPs (TIMPs). These protease inhibitors have broad specificity and block enzymatic activity by binding the MMP active site (142). Cytokines and growth factors (ILs, TNFα, TGF-β) may participate in MMP regulation by either inducing or inhibiting them (143). An additional level of control is provided by inflammatory and stress-associated factors such as the NF-kB pathway (144). In the cornea, the MMPs are responsible for wound healing and maintenance of stromal collagen structure; thus, uncontrolled activation is responsible for corneal ulceration, persisting inflammation, neovascularization.

2.2.3.3 Extracellular vesicles release

Extracellular vesicles (EVs) are membrane enclosed structures loosely categorised based on their size and origin in exosomes, microparticles, and apoptotic bodies. Although their size ranges are overlapping, the exosome fraction contains vesicles of 50 to 100 nm that originate in the endosomal compartment (i.e. the exocytosis of multivesicular bodies), microparticles (100 – 1000 nm) are produces by plasma membrane budding, while apoptotic bodies can be larger than 1000 nm in diameter and emerge from the apoptotic cell membrane-blebbing (145). EVs were found to carry, and thus protect from degradation, genetic information such as mRNAs, micro RNAs, and even DNA as well as signalling molecules – membrane proteins, carbohydrates, lipids, or metabolites (146,147). Their roles range from inter-cellular communication to signalling regulation, modulation of the immune function, and tumour development (148).

All cell types, including bacteria (149), were found to release EVs in response to varied stimuli but also in basal conditions (150). Stress stimuli particularly, are known to substantially increase their release (151). Their composition changes depending on the site of release and stimulus and the messages conveyed by these vehicles may be protective but also pro-inflammatory (152,153). The EV's immunosuppressive function (148), may be of particular importance in the resolution of the inflammatory reaction.

The study of EVs is very rapidly progressing and their wide-ranging cellular and biological functions confer them an undeniable diagnostic and therapeutic value (145,150).

2.3 Clinical manifestation of stress at the ocular surface

The cellular stress response manifests clinically as corneal diseases. The most frequent sign of ocular distress is inflammation (keratitis) and the priming event for the immune feedback activation. Its consequences range from mild discomfort to vision loss. The stress-induced inflammation challenges the barrier function by promoting cell permeability and affecting cell viability. This translates clinically into loss of transparency. More permanent damage is associated with the involvement of the adaptive immunity, culminating with the activation of T lymphocytes. The integrity of the tissue is compromised by cell death, necrosis, enzymatic digestion of cellular and extracellular matter.

2.3.1 Infections

Any bacteria can virtually cause a corneal infection, in practice, however, only a compromised corneal barrier allows for pathogen aggression. Thus, bacterial keratitis remains one of the most important potential complications of contact lens use and refractive surgery. Each pathogen prompts a specific defensive response, but all involve the innate and adaptive immunity. In the first stage (from minutes to 8 – 10 hours) the stressed-induced inflammation attempts to contain the infection and induces neutrophil migration towards the affected site (154). The adaptive response would follow 24 to 48 hours later (154). While there is an active infection, bacterial toxins and secreted enzymes (bacterial or from resident cells) damage the tissular structure causing ulceration, edema, necrosis. Most frequent complications caused by severe inflammation are: i) scar tissue formation; ii) neo-vascularization; iii) uneven healing of the stroma resulting in irregular astigmatism; iv) corneal perforation.

Pseudomonas aeruginosa infection is one of the most common complications of contact lens wear. An important component of the surface antigens of all gram-negative bacteria is the outer membrane LPS (endotoxin). This conserved molecular signature is recognized as a sign of microbial invasion and prompts immediate defensive response. The membrane structures specialized to recognize these molecular signatures are the toll-like receptors (TLR) (155). The TLRs are pattern recognition receptors with rigorous specificity: TLR2 identifies the proteoglycans in gram-positive bacterial wall, TLR3 is specific for double-stranded RNA, TLR5 for flagellin, while TLR4 recognizes LPS. TLRs are usually expressed by immune cells, however, many TLRs, including TLR4, were identified also on the surface of corneal epithelial cells (156-158). Activated TLRs induce a kinase

cascade that results in the engagement of both MAPK and NF-kB pathways and the associated secretion of pro-inflammatory cytokines (IL-1, IL-6, IL-8, macrophage inflammatory protein (MIP)-2 or interferon-gamma) (159). Therefore, the interaction with its receptor allows LPS to induce an immune reaction in the absence of live bacteria (160). Huang *et al.* showed in mice, however, that TLR4 expression is upregulated in the cornea after infection and that TLR4 deficiency sensitized mice to *P. aeruginosa* because of impaired bacterial removal, increased neutrophil infiltration, enhanced cytokine expression, and inhibition of protective factors such as beta-defensin-2 (161). These suggest complex immunomodulatory roles for LPS recognition at the cellular surface that might influence early-stage response as well as late adaptive events.

2.3.2 Photo-keratitis

The cornea absorbs all UV radiation bellow 310 nm (UV-B and UV-C) (49) and partially UV-A (162). Most of this is due to the high levels of proteins and nucleic acids in the corneal epithelium. Residual UV radiation is eliminated by the aqueous humour and the lens, with only visible light reaching the retina. UV energy in the range of 10 – 100 J/cm2 is sufficient to induce cell death in corneal epithelial cells (50). Corneal disease associated with pathological exposure to UV radiation include pterygia, UV keratitis, and climatic droplet keratopathy (CDK) (162).

CDK is a degenerative disease characterised by progressive opacity of the cornea, affecting predominantly males over 40 years old (163). Initially, at the periphery of the cornea, multiple translucent droplet-like deposits appear in the Bowman's layer and anterior stroma. In its progression, the hazy droplets coalesce and extend towards the central cornea affecting vision and corneal sensitivity (164,165). The decreased corneal sensitivity predisposes to ulceration, spontaneous perforation and eventual blindness. The origin of the droplets was suggested to be from UV-induced protein degradation (166,167). A study of canine chronic superficial keratitis proposed MMPs as possible effectors in the CDK pathogenesis (168). In a later study, Holopainen *et al.* found MMPs (MMP-2 and MMP-9) and pro-inflammatory cytokines to be increased in tears from CDK patients compared to controls (169). The droplet-like deposits could therefore be the consequence of UV-stimulated inflammatory signalling and unbalanced activation of MMPs.

2.3.3 Dry eye disease

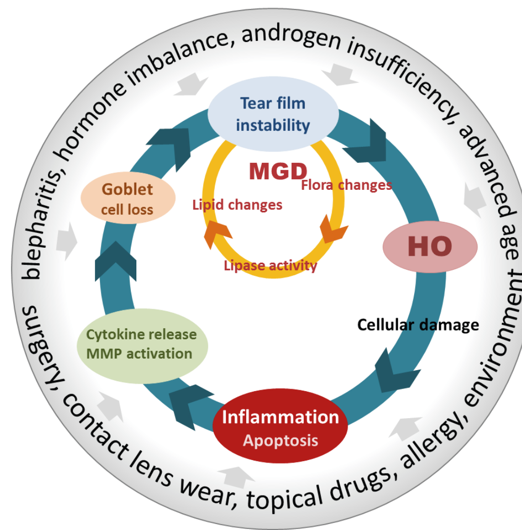
The 2007 DEWS report defined dry eye disease (DED) as "*a multifactorial disease of tears and ocular surface that results in symptoms of discomfort, visual disturbance and tear*

instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and ocular surface inflammation" (170). If counting also episodic manifestation of the disease, almost 20% of the population suffer from DED. Most prevalent risk factors are advancing age, female gender, and hormonal changes. The hallmark of DED is tear film instability that greatly impacts the visual function (171). Tear film instability is characterised by the shortened tear break-up time (TBUT) that results in local dry spots, increased osmolarity, epithelial damage and disturbance of mucous production (170). These translate as DED symptoms such as burning sensation, itching, redness or foreign body sensation. Thus, DED burden has been compared to that of angina (172).

The core mechanism of DED is tear film hyperosmolarity (HO) leading to ocular surface inflammation and consequent tissular damage (170). Normal tears osmolarity is 295 mOsm/L (173) HO arising either from decreased production of aqueous tears by the lacrimal glands (aqueous-deficient dry eye) or from increased evaporation (evaporative dry eye). The faulty production of tears may have several causes: lacrimal gland dysfunction (174-176); Sjögren Syndrome (177); aging (178); tearing reflex block (179). Evaporative dry eye is commonly due to meibomian gland dysfunction (180,181), other causes being: large lid aperture (177), low blink rate (182), allergic reactions, cytotoxic eye drops (183) or contact lens wear (184). Whatever the causative implication, HO affects the viability of the ocular surface cells through inflammation and immune cell-infiltration. This events compromise mucin production and promote meibomian and lacrimal gland dysfunction, propelling the ocular surface into a vicious cycle of destruction (**Figure 7**) (173).

The corneal epithelium abides the general rule that places MAPK signalling at the centre of the HO response pathway (185). MMPs or tyrosine kinase receptors were proposed as signal transducers down-stream from sensors, while the activation of pro-inflammatory signalling falls centrally on the p38 MAPK but involves also ERK, JNK and NF-kB signalling (137,185,186). Consequently, the tissue damage is mediated by the prominent secretion of cytokines and chemokines (IL-1, IL-8, IL-17, TNFα, IFN-γ) and activation of tissue remodelling enzymes (MMP-9).

Figure 7: DED mechanism and reinforcing cycle of dysfunction (adapted from Baudouin et al. (173)). MGD – Meibomian gland dysfunction



3. AIMS OF THE STUDY

The aim of the present thesis was to investigate the response of the corneal epithelium to environmental stress and the connection with the inflammatory process to find better clinical approaches for dry eye syndrome, UV-burns or bacterial infections. A cell line of human corneal epithelial cells was used to provide insights on the interaction between epithelium and the tear film and how each component contributes to the inflammation process.

The major objectives of the thesis were:

- To assess the effect of UVB radiation on the proteolytic capacity of corneal epithelium
- To determine the ability of the corneal epithelium to model the tear fluid lipid layer and to alter the tear fluid composition
- To reveal the role of the sphingolipids in ocular surface inflammation
- To detail the corneal epithelial cell response to hyperosmolarity

4. MATERIALS AND METHODS

4.1 Patient samples

The CDK study included 10 patients (9 males, age 67.4±10.2) and 10 matching controls from northwest Patagonia, Argentina. After a complete eye examination 15-20 μl of unstimulated tear fluid were collected using disposable micro-capillaries and stored frozen until analysis. Corneal tissues from 4 CDK patients and 3 controls were used for immunohistochemistry. Tears from 5 volunteers without external eye disease symptoms (3 males; age 30±5) were collected for enzyme secretion studies. Informed consent was obtained from all participants, and all studies were approved by the Ethical Committee of the Helsinki-Uusimaa Hospital District and, where appropriate, by the Institutional Review Board of the Clínica Universitaria Reina Fabiola. All studies respected the principles of the Declaration of Helsinki.

4.2 Cell culture

The SV-40 immortalized line of human corneal epithelial cells (HCE-2) were cultured in DMEM/F12 with 15% FBS, 10 ng/mL epithelial growth factor, 5 μg/mL insulin, 1μg/mL L-glutamine, 40 μg/mL gentamicin (all from Thermo-Fisher Scientific, Waltham, MA), and 0.1 μg/mL cholera toxin (Sigma, St. Louis, MO), at 37°C and 5% CO_2 in a humidified incubator (187). Cells were sub-cultured every 3 days for no more than 10-15 passages and serum-starved for 6 to 18 hours before experiments. The osmolarity of the growth medium (317 mOsm/kg) was increased by adding 5 M NaCl solution. In the UVB experiments, the PBS washed cells were exposed to the UV radiation in PBS in a microprocessor controlled UV cross-linker until the desired UV energy was reached (maximum 10mJ/cm^2). LPS, as well as other active molecules (inhibitors and/or agonists), were added to the serum-deprived medium. After stimulation cells were incubated for different amounts of time (specified for each experiment) and medium and/or cells was collected for analysis. Medium centrifugation at 400 g for 10 min eliminated floating cells and debris. For cells, the monolayer was washed with cold PBS and lysed on ice in the presence of protease and phosphatase inhibitors.

4.3 Cell and tissue staining

4.3.1 Haematoxylin-eosin staining of corneal samples

CDK and control corneas were fixed in 10% buffered formaldehyde (pH 6.8). The paraffin embedded tissue was sectioned perpendicular to the epithelial surface in 5μm thick slices. The 3-min haematoxylin (Sigma, St. Louis, MO) stain was improved by three dips in 0.5% hydrochloric acid in 70% ethanol and blued with 2% $NaHCO_3$ for 1 min and counterstained with 1% aqueous eosin (BHD Chemical, Poole, UK). Sections were mounted on microscopy slides using Mountex medium (Histolab Products, Gothenburg, Sweden).

4.3.2 MMP-2 and MMP-9 immunohistochemistry

The 5μm thick corneal sections were additionally used for immunohistochemical localization of MMP-2 and 9. Affinity purified goat anti-human MMP-2 and MMP-9 IgG (R&D Systems, Minneapolis, MN) were used in tandem with the avidin-biotin peroxidase complex (ABC) staining (VectaStain, Burlingame, CA) following the instructions provided by the manufacturer (188). Non-specific goat, rabbit or mouse IgG, and secondary antibody only samples were used as controls.

4.3.3 OilRed O staining for fluorescence microscopy

Cells grown on glass coverslips were carefully washed with PBS and fixed with 10% buffered formalin. Fresh OilRed O staining solution (3 % OilRed O in isopropanol mixed with water 3:2, vol/vol) was added to the cells for 5 min and then thoroughly rinsed in water. VectaShield mounting medium (Vector Laboratories, Burlingame, CA) stained nuclei with DAPI and mounted the coverslips on the slides.

4.4 Gene expression and silencing

4.4.1 Real time RT-PCR

The total RNA extracted with the RNeasy Mini Kit (QIAGEN, Hilden, Germany) was converted to cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, Waltham, MA). Subsequently, 50 ng cDNA were amplified by Q-PCR (SYBR Green/ROX FastStart Universal master mix - Roche, Basel, Switzerland) in an ABI Prism7000 thermo-cycler (Thermo-Fisher Scientific). The reaction volume was 25 μL and included 0.2 μM of each primer. The primer pairs used for the various genes of interest are presented in **Table 1**. Relative gene expression was calculated using the Ct (Cycle threshold) values of control and

experimental samples versus the expression levels of human actin (ddCt) and 2^{-ddCt} to represent fold change.

Table 1: Q-PCR primer sequence

gene	Forward primer	Reverse primer
h-actin	GATGTGGATCAGCAAGCAGGA	AGCATTTGCGGTGGACGAT
ASM	CTCGGGCTGAAGAAGGAACC	TGGACAATGGATTGGCACAC
ASAH1	GCCGGAGTTGCGTCGCCTTA	GTCCTCTGTCCACGGCGGC
ASAH2	GGACCACTGGCTTCAGCACACC	GCCAGCTGCAAAACTGTAGCCC
CERK	ATCGTTGTTGGGGACTCGCT	GGGACACGGAGTAGCGAAGG
IL-8	CACACTGCGCCAACACAGAAA	AGCCCTCTTCAAAAACTTCTCCACA
NSM1	GCTTCGACCTGGCTTTGCTGGA	AGTGGTGTGCAGCTGGGTAGGT
NSM2	AGCCGTGGGCCATCGGTACT	GCGGCCCTCCTCACTCTCCA
S1P1	TGCTTGAGCGAGGCTGCGGT	AGGGCGAGGCGAGAGAGCCTT
S1P2	CGGGCCGGCCTAGCCAGTTCT	CGAGTACAAGCTGCCCATGGTGGGG
S1P3	CTCAGCCGACGGAGGAGCCC	CGGGGCTGAGGTTCCGGCTT
SK1	GGTCAGGAGCCCAGCTGGC	TTTGGTTCGTGGGGTGGGGG
SPL	GCTTTTGGCCATGGTGCCGC	TCTGCTCATCACCAGCCCAGGA

ASAH1 – acid ceramidase; ASAH2 – neutral ceramidase; NSM1 – neutral SMase 1; SK1-sphingosine kinase 1; SPL – S1P lyase; CERK – ceramide kinase.

4.4.2 siRNA transfection

Cells in antibiotic-free medium at 70% density were transfected with 25 pmols of DharmaFECT SMARTpool (Dharmacon, Lafayette, CO), a mix of 4 different short interfering RNAs (siRNAs) specific for the genes of interest. Cells were transfected in 12-wells plates using 3 μL of DharmaFECT Transfection Reagent 1 and the manufacturer's protocol. Non-targeting siRNA provided the transfection control. Silencing efficacy was tested 36 hours post-transfection.

4.5 Protein analysis

4.5.1 Western blotting

Tear fluid, cell lysate or cell culture medium samples were run on 4-12 % Bis-Tris NuPAGE gels (Thermo-Fisher Scientific, Waltham, MA). Proteins were then electro-transferred on nitrocellulose membrane and detected using specific antibodies and enhanced chemiluminescence detection (GE Healthcare, Buckinghamshire, UK). Equal quantities of total protein were loaded on each well and actin detection as well as Ponceau staining were used as loading reference. The cell-conditioned medium was concentrated by filtration (Nanosep 10K, Pall Life Sciences, Port Washington, NY) before electrophoresis. The specific antibodies used were purchased from R&D Systems (Minneapolis, MN) or Prestige Antibodies (Sigma, St. Louis, MO).

4.5.2 Zymography

Tear fluid or cell culture medium samples (30μg total protein) were electrophoresed in non-reducing 10% polyacrylamide gels containing 1 mg/mL gelatine with 2-methoxy-2,4-dephenyl-3(2H) furanone labelling. Gels were then washed in 2.5% Triton X-100 and incubated in activation solution (50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.5) at 37°C for 1 to 5 days. Gelatine degradation was monitored and photographed using UV light. Blood gelatinases were used as controls and MMP-2 and -9 were identified by their molecular weight using standard molecular ladders (Bio-Rad, Hercules, CA).

4.5.3 Enzymatic activity assays

Pooled tear fluid samples as well as cell lysates were used to measure specific activity of different lipases: phosphatidylcholine-specific phospholipase C (PC-PLC), neutral and acid sphingomyelinase (SMase). Cell lysates were prepared by washing away medium traces and scrapping the cells in PBS. The cells pelleted at 400g for 10 min at 4°C were then re-suspended in distilled water and lysed by sequential freeze/thawing cycles and light sonication using Soniprep 150 (Sanyo Electric, Tokyo, Japan). Enzymatic activity was measured using the Thermo Fisher Scientific Amplex Red kit (PC-PLC, NSMases and ASM) and the EnzChek direct phospholipase C assay kit (PC-PLC) following the manufacturer's suggestions. To fulfil the SMases pH requirements the cell lysate was diluted in either sodium acetate buffer (pH 5.0) or in 0.1 M Tris-HCl, 10 mM $MgCl_2$ (pH 7.4). Reaction mixtures were incubated at 37°C in the dark for 30 to 60 min. The Amplex Red fluorescence was measured at 535/585 nm excitation/emission using Victor 2 Multilabell Counter (Wallac, Turku, Finland). Enzymatic activity was calculated

using the activity curves of provided control enzymes and reported as milliunits (mU). Protein concentration was determined using a commercial kit (Promega, Madison, WI).

4.6 Cytokine/chemokine measurements

4.6.2 Enzyme-Linked ImmunoSorbent Assay (ELISA)

HCE IL-8 secretion was measured using the DuoSet ELISA kit for human CXCL8/IL-8 (R&D Systems, Minneapolis, MN). Medium samples were diluted 1:1 (vol:vol) in Reagent Diluent and pipetted onto the plate-bound anti-IL-8 antibodies. After washing away the unbound material a second biotinylated anti-IL-8 antibody is added, followed by the streptavidin-horseradish peroxidase complex. Finally, the peroxidase-generated end-products reported the amount of plate-bound IL-8 by increasing 450 nm absorbance.

4.6.1 Multiplex Bead-array

The human cytokine/chemokine Multiplex kit (Bio-Rad Laboratories, Richmond, CA) used 5μl of tear fluid to measure simultaneously IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL- 13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, TNFα. This method combines ELISA and flow cytometry by using differently coloured beads and specific antibodies for the molecules of interest. The working protocol followed the instructions provided by the producer.

4.6.3 Cytokine array

Cytokine/chemokine secretion in HCE medium was quantified using the Proteome Profiler Array – Human Cytokine Array Panel A (R&D Systems Inc., Minneapolis, MN). The antibodies to 36 different chemokines and cytokines are spotted on a nitrocellulose paper. The conditioned medium (700 μL) cleared of cell remnants and debris was mixed with the kit reagents and incubated with the arrays as instructed in the assay protocol. Bound cytokines were identified through chemiluminescent detection reagents.

4.7 Lipid analysis

4.7.1 Lipid extraction

For lipid extraction, the cells were washed with cold PBS and scraped in 0.34 M NaCl. A small aliquot of the cell suspension was reserved for total protein

measurement, while the rest was used for lipid extraction with organic solvent mixture (chloroform-methanol) according to the Bligh-Dyer method (189). The organic phase was removed to a new glass tube and solvents evaporated under nitrogen gas. Extracted lipids were stored in methanol at -20ºC.

4.7.2 Non-targeted lipidomic analysis

The extracted lipids were first fractioned using ultra-performance liquid chromatography (Acquity UPLC, Waters, Herts, UK) and analysed with an in-line quadrupole-time of flight mass spectrometer (Waters, Herts, UK) (190). The UPLC separation was performed at 50ºC on an Acquity UPLCTM BEH C18 column of 2.1mm internal diameter and 100mm in length, with 1.7µm particles. The injected sample volume was 2µl and the flow rate 0.4 ml/min. The gradient eluent system consisted of component (A) ultrapure water (1% 1 M NH_4Ac, 0.1% HCOOH) and (B) LC/MS grade acetonitrile/isopropanol (1:1, 1% 1M NH_4Ac, 0.1% HCOOH). The eluent composition changed from 35% B to 80% B in 2 min and to 100% B in 7 min, and persisted at this composition for 7 min. For lipid mass profiles the positive electrospray ionization was used and the mass/charge range was of 300 to 1200. Lipids were identified using the internal spectral library and analysed with MZmine2 software (190).

4.7.3 Lipid assays

We used an enzymatic/colorimetric assay to measure total cholesterol, free cholesterol and choline-containing phospholipids (DiaSys Gmbh, Holzheim, Germany) from buffered cellular lysates or extracellular vesicle sediments. The recommended protocol was adapted to fit the different working volumes. For triglyceride measurements, equal amounts of extracted lipids were dried under nitrogen and then re-suspended in the assay buffer (TG assay from Roche/Hitachi, Rotkreuz, Switzerland). Appropriate standard curves were generated for correct quantification and standardized plasma was used as a positive control.

4.7.4 OilRed O staining quantification

Cells grown on 12-well plates were fixed with 10% formalin and stained with fresh OilRed O 3% and water 2:3 (vol/vol). The plates were rinsed carefully with water and the retained OilRed O dye was extracted with 100% isopropanol. The 490nm absorbance of the extracted solutions was measured with the EnSpire multimodal plate reader (PerkinElmer, Waltham, MA). As blank we used similarly processed cell-free wells.

4.7.5 Lipolysis assay

Triglyceride hydrolysis was measured using a radiometric method (191). Cells in assay buffer (0.2 M Tris-HCl buffer, pH 8.4 with 0.1 M NaCl) were briefly sonicated and mixed with 30 nmol [^{3}H]-triolein. After 60 min incubation at 37ºC enzymatic activity was stopped by adding a 10:9:7 (vol/vol) mixture of methanol/chloroform/heptane and 0.14 M K_2CO_3 - 0.14 M boric acid (pH 10.5) and centrifuged (1600 g for 15 min). The lipolytic activity of the cellular lysate was proportional with the radioactivity measured (WinSpectral liquid scintillation counter, Wallac, Turku, Finland) in the hydrophilic phase (i.e. labelled free fatty acids).

4.7.6 S1P secretion assay

To quantify S1P production, cells were incubated with [^{3}H]sphingosine (Perkin Elmer, Waltham, MA, USA) and after the appropriate stimulation the medium as well as cell lysate was tested for the presence of [^{3}H]S1P (192). This is achieved due to the differential partition of the two sphingolipids between the extraction phases under alkaline conditions. Thus, the cell lysate or medium was mixed with equal volumes of methanol and chloroform and 0.1 N NaOH. After the phase separation, S1P is negatively charged and partitions into the aqueous phase, whereas sphingosine remains in the organic phase. The radioactivity ratio between the two phases reveals the amount of intracellular (cell lysate) or secreted (medium) S1P.

4.7.7 Liposome construction

Pure lipids were mixed in 60:15:15:x:y (mol %) PC:PE:PS:SM:Cer (where the x would decrease from 10, while the y proportionally increase from 0), dried under nitrogen, and hydrated in 20 mM Hepes, 100 mM NaCl, pH 7.5, to yield 2 mM multi-lamellar vesicles. These were extruded through 100 nm polycarbonate filters (Millipore, Bedford, MA) to produce small unilamellar vesicles/liposomes using an Avanti Mini Extruder system (Avanti Polar Lipids, Alabaster AL). Same protocol was followed for the lipids extracted from extracellular vesicles.

4.7.8 Thin layer chromatography (TLC)

TLC separation for non-polar lipids was performed using hexane/diethyl ether/acetic acid organic solvent mixture (80:20:1, vol/vol). After chromatography, the TLC plate was dipped in a 3% copper sulphate and 8% phosphoric acid solution and developed at 180°C for 45 min. Charred lipids were visually identified using appropriate lipid standards.

4.8 Extracellular vesicle isolation from cell culture medium

Extracellular vesicles from HCE-conditioned media were isolated through ultracentrifugation (TLA-100.3 rotor – Optima TL Ultracentrifuge, Beckman Coulter, CA) at 100000 g, 90 min, 4ºC. Alternatively, the medium was first filtered through a 22 μm pore PVDF filter (Millipore, Billerica, MA) to eliminate apoptotic bodies and then centrifuged as above. The resulted sediment included all types of extracellular vesicles (small apoptotic bodies, microparticles and exosomes); the supernatant was denoted vesicle-poor media.

4.9 Microscopy

For microscopy, the following instruments were used: Nikon Eclipse TE2000-E (Nikon Corp., Tokyo, Japan) microscope equipped with a Nikon Digital Sight DS-5MC camera; Axioplan 2 fluorescence microscope (Carl Zeiss, AG, Germany) with a Plan Neofluar 40x/0.75 (DIC II) air objective and AxioCamHR camera.

4.10 Data analysis

Densitometry data (zymographs, western blots, cytokine arrays) was collected using the ImageJ software (NIH free software v1.42q) on non-altered TIFF images. All experiments were performed three times on average and the data is presented as mean values ± standard deviation, unless otherwise specified. Statistical significance was calculated by the Student t-test or ANOVA and P values < 0.05 were considered significant. All data was normalized to protein concentration.

5. RESULTS AND DISCUSSION

5.1. Role of inflammation and gelatinases in CDK pathogenesis

In CDK the highly-organised structure of the cornea is disrupted and our group's previous findings highlighted MMPs as possible effectors of tissular degradation (169). The known link between inflammation and MMP activation, plus the correlation between UV radiation and the degradative effects of the gelatinases (168) encouraged us to define the role played by the UV radiation in the pathogenesis of CDK.

5.1.1. Gelatinases in CDK corneas and tears

Motivated by our previous results we first measured gelatinases (MMP-2 and MMP-9) levels in tear fluid from CDK patients vs. controls (**I**, Fig. 2A). Zymography analysis identified significantly increased expression of the pro-forms of the two MMPs (72 and 92kDa, respectively) in CDK affected eyes (**Figure 8**).

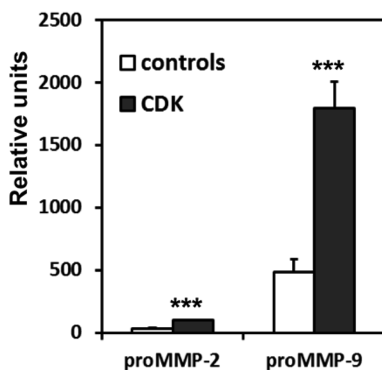


Figure 8: Tear fluid gelatinase levels in CDK patients and controls. *** indicate $P < 0.001$ when compared to control. Adapted from **I**, Fig. 2A

Numerous disorders that affect corneal epithelium integrity are associated with increased presence of gelatinases in the tear fluid (182-185). The MMP-2 and MMP-9 active forms could not be identified in zymographies (**I**, Fig. 2A). This, however, does not exclude their involvement in the CDK pathogenesis and can be justified by substrate binding at the place of secretion or interaction with their inhibitors (TIMPs) in tears or tissues.

Structural damage in CDK corneas was confirmed by hematoxylin-eosin staining. In early stages of the disease the epithelium appeared thinned with granulated material accumulating in the anterior stroma (**I**, Fig. 1A). With the disease progression, the structure of the epithelium was progressively altered

together with the Bowman's layer; in addition, the sub-epithelial deposits increased consistently in size and disturbed stromal architecture (**I**, Fig. 1B). MMP-2 and MMP-9 were identified in the epithelial basement membrane in all samples, with an accentuation in CDK corneas (**I**, Fig. 1D and F). The CDK granules showed strong MMP-2 immunoreactivity at the edges with no staining in the core (**I**, Fig. 1C and D). No traces of MMP-9 were detectable around or inside the granules (**I**, Fig. 1E and F).

The histological localization of the gelatinases reinforces their role in the tissue degeneration, allows for an explanation for the lack of active forms in the tear fluid, and highlights the epithelium as a likely source of secretion. The basement membrane maintains the structural integrity of the corneal epithelium and comprises mostly of collagen type IV and laminin sensitive to MMP activity. Fini *et al*. showed in an animal model of corneal injury that both gelatinases participate in basement membrane remodelling, MMP-2 acting also on extracellular matrix substrates (197,198). This, together with the immuno-detection of MMP-2 surrounding the droplet-like deposits, suggests that proteolytic activity as source of material in the sub-epithelial deposits.

5.1.2. Tear fluid pro-inflammatory profile in patients with CDK

Because the dysregulated activation of the gelatinases could be a consequence of inflammation, a multiplex ELISA was used to characterise the cytokine profile in healthy and CDK tears (**Table 2**). IL-1b and IL-8 appeared in all samples analysed, with a significant increase in CDK. Additionally, IL-5, -6, and -7, MCP-1, and MIP-1b could be detected in all CDK samples and few controls. TNFα was present in 6/10 CDK samples but in only 1/10 control samples. All control samples were negative for IL-12 and GM-CSF, although more abundant in IL-4 and IL-10. This net pro-inflammatory profile supports the presumed involvement of environmental stress in CDK pathogenesis with UV radiation as main candidate.

5.1.3. UVB-induced secretion of cytokine/chemokines in HCE cells

To assess the role played by UV-stress on the development of CDK hallmarks, we used a cell culture model of the corneal epithelium and measured UV-induced cytokine and gelatinase secretion. UVB radiation proved to be the optimum stimulus due to its efficacy and relevance: cornea absorbs most of the UVB wavelengths and its high energy triggers a significant cellular response. Therefore, HCE cells were exposed to 10 mJ/cm^2 UVB and secretion of cytokines in the cell

culture medium was compared to the experimental control (**Table 2**). Five cytokines appeared to be notably modulated by UVB: IL-6, -8 and -16, as well as MIF-1 and PAI-1. These results suggest that the UV-stressed corneal epithelium is an important modulator of the pro-inflammatory response and one of the sources of IL-8 and IL-6 in the tears of CDK affected eyes.

cytokine	CDK tears	UV-stimulated HCEs
IL-1b	5.43 ± 3.94*	–
IL-1a	–	1.39 ± 0.57
IL-8	2.94 ± 1.23*	2.81 ± 0.15
IL-6	1.96 ± 1.63	1.48 ± 0.02
IL-7	2.15 ± 1.12	–
TNFa	4.43 ± 3.84	–
MCP-1	1.89 ± 1.35	–
MIP-1b	1.90 ± 2.61	–
G-CSF	1.81 ± 2.32	–
IL-12	6.53 ± 11.10	–
GM-CSF	89.46 ± 151.9[a]	–
IL-10	0.44 ± 1.11	–
INFg	2.91 ± 1.29	1.15 ± 0.45
IL-16	–	5.10 ± 0.06
MIF	–	1.36 ± 0.02
PAI-1	–	0.53 ± 0.05

Table 2: Cytokines/chemokines in CDK tears and medium from UV-stimulated HCE cells. Results from **I,** Table are presented here as fold-change compared with controls. * indicate $P < 0.05$; [a] – GM-CSF was present only in 3 CDK samples. MCP-1 – monocyte chemoattractant protein 1; MIP-1b – macrophage inflammatory protein 1beta; G-CSF and GM-CSF – granulocyte and macrophage colony stimulating factors; MIF – macrophage migration inhibition factor; PAI-1/Serpin E1 – plasminogen-activator inhibitor 1.

5.1.4. UVB effect on HCEs metalloproteinase secretion

MMP secretion could be detected in the HCE cell media after 24 hours from the UVB exposure. Cells were exposed to UVB intensities in the range 0.5 to 10 mJ/cm^2. Cell medium zymographies suggested the presence of several metalloproteases and protease-inhibitor complexes such as: MMP-2, MMP-9, and their complexes with TIMPs, MMP-8, MMP-13, and we have proceeded to identify individually the most relevant ones. ELISA showed a time and UVB intensity-dependent increase in MMP-2 (pro-form and in complex) secretion from HCE cells (**I**, Fig. 4). On the other hand, MMP-9 proved to be the main gelatinase in the HCE medium, showing significant increase with the UVB intensity and enhanced by time (**Figure 9**).

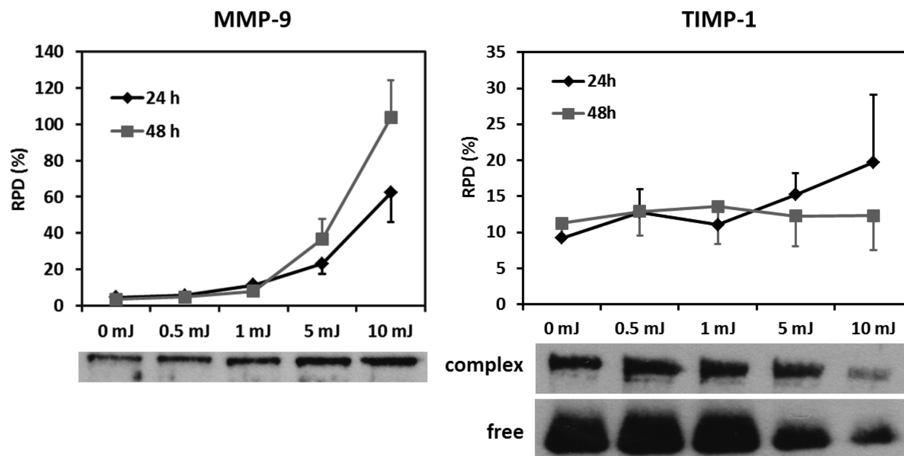


Figure 9: MMP-9 and TIMP-1 levels in media from UV-stimulated HCE cells. The samples in the Western blots are following 48 hours of incubation post-UVB. TIMP-1 could be identified both in complexes with the gelatinases and in free form.

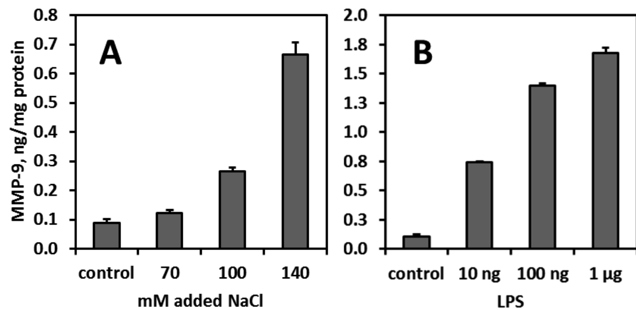
Moderate to high UVB intensities affected also MMP-8 secretion from HCE cells, both PMN-type and mesenchymal MMP-8 appearing in the Western blot analysis. TIMP-1 secretion showed a different response to UVB. After 24 hours, the MMP-inhibitor's secretion was increasing with the UV intensity, at 48 hours, however, the free form of the TIMP-1 was uniform in all samples (**Figure 9**) suggesting an unbalanced activation of the MMPs.

The *in vitro* results indicate a pivotal role for the environmental stress in the CDK pathogenesis. The epithelium contributes to both pro-inflammatory reaction induced by the UV-stress and the structural breakdown associated with the MMP secretion even without the involvement of the immune system. The down-regulation of PAI-1 may contribute to a sustained urokinase-plasmin activation of the MMPs (199) cooperating with the unbalanced secretion of TIMPs. Along other cytokines, IL-8 anticipates immune cell involvement and modulates the secretion of neutrophil MMP-9 (200) reinforcing the initiated tissue damage. The combination between these effects and prolonged daily exposure to sunlight over a period of many years can offer a plausible explanation for the progressive accumulation of molecular debris in the anterior cornea of CDK patients.

5.1.5. Gelatinases in media from LPS or HO stimulated cells

Stimulation with LPS or HO stress had no effect on MMP-2 secretion from the HCE cells, they induced, however, stimulus-proportional secretion of MMP-9 (**Figure 10** – unpublished results).

Figure 10: HO (A) and LPS (B) stimulated secretion of MMP-9. The presence of the proteinase was tested after 48h of incubation using an ELISA method.



Increased secretion of gelatinases does not represent a specific response to UVB, other stress agents having similar effects. LPS proved a very efficient inducer of MMP-9 and this is most likely related to the powerful TLR-4 mediated inflammatory signalling.

5.2. Stress-dependent secretion of IL-8

Continuing with our cell culture model of the corneal epithelium, we subsequently measured the time-line of the stress-induced inflammatory response. Along UVB, we investigated LPS stimulation as well as HO response (**Figure 11**). The cytokine profiling highlighted IL-8 as a consistent stress marker. IL-8, as an acute phase cytokine and a major mediator of the inflammatory response, is a sensitive indicator of the stress response (**Figure 11D** and **III**, Fig. 1A).

LPS and HO proved to be better inducers of IL-8 secretion than UVB, triggering significant secretion as early as 4 hours post-stimulation. In all cases, the chemokine secretion increase with time and with the strength of the stimulus until a viability threshold was reached and the IL-8 secretion decreased (**Figure 11A** and **B**). Stimuli that significantly affect cell viability (25 mJ/cm² UVB or 140 mM NaCl – **II**, fig. 2) failed to induce a proportional inflammatory response, suggesting a divergence towards cell death. LPS stimulation has no effect on cellular viability (**II**, fig. 2), IL-8 secretion, however, seems to be continuously stimulated until a plateau is reached (**Figure 11B**). All stimuli tested induced an acute inflammatory response in HCE cells, IL-8 being a reliable acute phase marker. UVB and HO have direct cytotoxic effects on the cells, while the deleterious effects of LPS stimulation seem to be mediated by the immune cell recruitment. The amount of secreted IL-8 was 20-fold higher for LPS compared to HO at 4 or 6h suggesting a considerably higher inflammatory response to pathogens than to physical stressors.

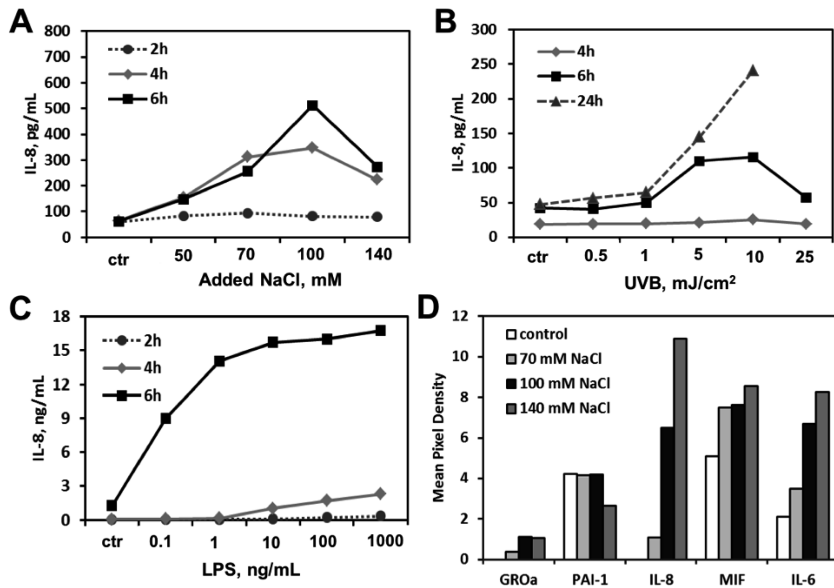


Figure 11: Stress-induced pro-inflammatory signalling. IL-8 secretion in HO (A), UVB (B) and LPS (C). (D) Cytokine array in media from HO-stressed HCE cells;

5.3. Environmental stress modulates lipase secretion from HCE cells

5.3.1. Lipid-modifying enzymes in normal tear fluid

Lipids are of great importance for tear fluid stability and the corneal epithelial cells may modulate their functions in numerous ways. Lipid-interacting proteins such as lipocalin (201,202), lipophilin (203) phospholipid transfer protein (204) or group II phospholipases A2 (205) have already been detected in tear fluid and they either bind, transport or hydrolyse local lipids from tear fluid lipid layer, plasma membranes, bacterial wall, extracellular vesicles or other liposomal aggregates.

The discovery of active ASM (206) together with its substrate, sphingomyelin (SM), and Cer (39) in the tear fluid suggested an important role for the sphingolipids in the ocular surface homeostasis. Since SL metabolism enzymes are ubiquitously expressed we have assessed their presence also in the tear fluid. Thus, using pooled tears from healthy volunteers and specific antibodies we identified four important SL enzymes: ASM, NSM2, acid ceramidase (ASAH1) and neutral ceramidase (ASAH2) (**Figure 12** and **II**, Fig. 1B). Tear fluid enzymes were blotted

next to corneal epithelial cell lysates (CECs) used as control. ASM was present in tears (TF) as both secreted and lysosomal forms, while the lysosomal form (70 kDa) prevailed in cells. The most surprising tear fluid component was NSM2, a palmitoylated membrane protein never identified before in the extracellular space. The two ceramidases (ASAH1 and 2) were more difficult to detected at much than their SM counterparts (either due to less efficient antibodies or to their lower levels in tears). The absence of cellular contamination in the tear fluid was confirmed by actin immunodetection. SMases activity assays showed that the acidic hydrolysis of SM was 2-fold higher than neutral SMase activity and comparable to PC-specific phospholipase activity (**II**, Fig 1C). Ceramidase activity could not be tested due to the limited amount of tear fluid.

Our study confirmed the presence of ASAH1 and ASM (31,206) in normal tears and added NSM2 and ASAH2 as components of the tear proteome. The secretion of the two acidic enzymes was expected because most lysosomal enzymes (such as ASM and ASAH1) are known to be secreted as pro-enzymes. The secreted SMase is also a product of the *smpd1* gene, similar to ASM, but follows a different secretory route from the Golgi apparatus and therefore presents different glycosylation pattern and a higher apparent molecular weight (~80 kDa) in our immunoblots. Its optimum pH lies in the acidic range but it was shown to hydrolyse SM quite efficiently even at neutral pH (207). The extracellular activity of both acidic SMases has been correlated with the progression of numerous diseases and conditions (111). The function of the two *smpd1* gene products is difficult to segregate; ASM activity, however, has been associated predominantly with the stress-induced apoptosis, while serum secreted-SMase has been linked with inflammatory diseases, such as infections and atherosclerosis (208,209), insulin resistance and type 2 diabetes (210) or chronic heart failure (211). We can only speculate about the role of the secreted SMase in tear fluid. Stress-induced production of Cer by a plasma membrane ASM, however, is a key event in the cellular response to many stimuli including UV radiation, LPS, Fas ligand, ischemia/reperfusion or TNFα (212-216). Moreover, ASM was shown to be indispensable for an efficient clearance of *P. aeruginosa* infection (217).

NSM2, on the other hand, is not a secreted enzyme but has a preferred localization to the inner-leaflet of the plasma membrane (218). Palmitoylation discourages the concept of any soluble forms of the protein and suggests a membrane packing for the enzyme in the outer-cell environment. The neutral ceramidase, ASAH2, was also detected in the extracellular space together with caveolin (219). This suggests a vesicular transport initiated at the plasma membrane that could accommodate both enzymes ASAH2 as well as NSM2. There is no data to support a role for these enzymes outside the cell. Their function in the SL

metabolism implies an involvement for the bioactive SLs in the stress response at the ocular surface, however.

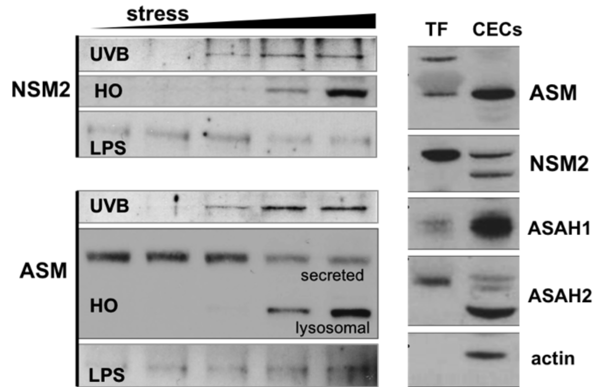


Figure 12: SL metabolism enzymes in HCE cell culture media (left) and tears (right panel). TF – tear fluid; CECs – human corneal epithelial cells. The stimulus intensity is defined by the black triangle.

5.3.2. Stress-induced secretion/leakage of sphingolipid enzymes

To investigate the hypothesis above, we employed the HCE cell model to determine the connection between the secreted enzymes and environmental stress. Considering the high dilution of the cell-originated molecules in the culture medium and the low abundance of the ceramidases, only SMases were targeted in these experiments. The SMases seemed to be constitutive components of the cell-conditioned medium but stress proportionally induced their secretion (**Figure 12**). The constitutive secretion was apparent in the LPS stimulation where the stimulus had minor effects on both enzymes, NSM2 and ASM. Cell-damaging stimuli such as UVB and HO significantly increased the presence of the SL enzymes in the extracellular environment in a dose-dependent manner. Secreted SMase offered contrary results, since here UV-B or HO were neutral stimuli, while LPS seemed to induce its secretion (**Figure 13** – unpublished results).

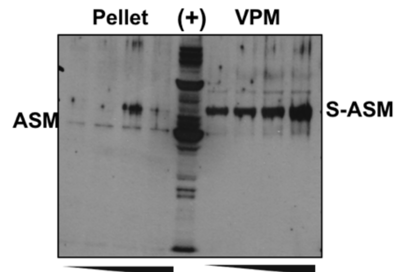


Figure 13: LPS-induced release of ASM and secreted SMase (S-ASM). (+) – HCE cell lysate as positive control; black triangles represent the degree of stress.

These results reinforce the connection between stress and the SL enzymes and urged us to determine a route of secretion and to define their possible roles.

5.4. Extracellular vesicles and HCE cells stress response

5.4.1. Environmental stress and EV release

EVs could represent ideal transporters for the membrane bound SL enzymes and their substrates. Consequently, we have attempted to isolate such vesicles and characterise them using specific markers: CD63 for exosomes, integrin beta1 for microparticles, and histone H3 for apoptotic bodies (220). The EVs were isolated through ultra-centrifugation and the supernatant was labelled vesicle-poor medium (VPM). UVB and HO induced a direct proportional release of all EV populations, while LPS-stimulation was, yet again, an exception (**II**, Fig. 5A). The only marker clearly visible in the LPS pellet was CD63 suggesting exosome enrichment in the EV pellet. Because there was no correlation between LPS levels and CD63 (**II**, Fig. 5A) we concluded that exosomes are most likely released by cells even in the absence of stimulation.

Immunoblotting results were complemented by protein/lipid ratios in the isolated pellets. The HO and LPS EVs seemed to have similar protein/lipid ratios with each other and with plasma membrane (cholesterol levels could not be measured for the LPS-pellets due to insufficient amount of EVs). UVB pellets were richer in protein, most likely due to increased incidence of apoptotic bodies (**II**, Fig. 5B).

5.4.2. Sphingomyelinases are associated with extracellular vesicles

ASM, NSM2, and EVs are detected in the cell culture medium under similar circumstances, therefore, we attempted to determine whether the lipid-modifying enzymes associate with the EV pellets or remain in the medium. The specific antibodies showed that ASM and NSM2 appear associated with the vesicles, while secreted SMase is found only in the VPM (**II**, Fig. 5C and **Figure 13**). The traces of NSM2 in the VPM (**II**, Fig. 5C) suggest a lack of complete sedimentation of the vesicles.

The EVs may potentially accommodate most of the components of the SL metabolism, becoming signalling carriers that cover long distances in the tissues, with the lipid layers as containers for the message: receptors, ligands, enzymes or bioactive lipids (221-223). In this manner, the components of the SL pathway can exert their conserved regulatory roles at a distance from the site of production and from intracellular modulators. One such example is represented by the Cer-

mediated immunosuppression, where apoptosis of recruited immune cells is mediated by local Cer-rich vesicles (224,225). Additionally, apoptotic bodies from differentiated cells are able to induce differentiation of tissue-resident stem cells and thus replace lost cells (226).

5.4.3. Anti-inflammatory properties of extracellular vesicles

Numerous regulatory functions associated with the presence of the SL signalling and EVs are relevant to the ocular surface. The hypothesis selected was that stress-induced vesicles may propagate inflammatory signals throughout the tissue. Naïve-cells were incubated with stress-induced EVs and IL-8 secretion was measured. Contrary to our expectations, the UVB- or HO-induced vesicles failed to induce pro-inflammatory signalling in non-stressed cells and in fact downregulated IL-8 secretion (**II**, Fig. 6B). LPS-induced EVs, however, triggered a massive pro-inflammatory response from the naïve HCE cells (**II**, Fig. 6A). This result, is most likely owing to LPS contamination of the isolated EVs. By incubating HCE cells for 18 hours with EVs released from cells exposed to 5 mJ/cm^2 UVB or an added 100 mM of NaCl we could reduce their inflammatory response to stress with almost 40% (**II**, Fig. 6B). The significant decrease in IL-8 secretion was mirrored by gene expression levels (**II**, Fig. 6C).

Stress-released vesicles diminished the response of naïve cells to environmental stress suggesting that their cargo has anti-inflammatory activity. Considering the relevance of these results for ocular surface inflammation, we have sought to determine whether their effects are mediated by proteins, lipids or other molecules. For this purpose, the lipids extracted from the EVs pellet were reassembled in 100 nm liposomes. Their anti-inflammatory potential was tested on HCE cells and compared to complete EVs. As **Figure 14A** shows, the EVs liposomes restored almost entirely the results obtained with intact vesicles (unpublished results). This was not entirely surprising since liposomes were shown to reduce inflammation (227,228). In the study conducted by Sun *et al.* the anti-inflammatory effects were attributed to short-chain Cer (229). Hence, to emphasise a possible function for the SLs in this process we constructed liposomes with constant PC/PE/PS ratios and variable SM/Cer to mimic SMase activity. Mix 1 in **Figure 14B** is made up only of PLs (60:20:20 mol% PC/PE/PS), while in the following mixes the PLs change to 60:15:15 and the remainder 10% is divided between SM and Cer as follows: SM/Cer 10:0 (mix 2), 8:2 (mix 3), 5:5 (mix 4), and 2:8 in mix 5.

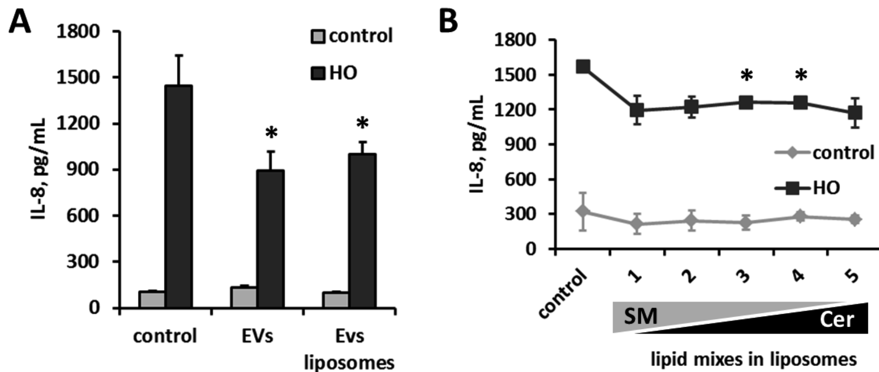


Figure 14: Effect of EVs lipids on IL-8 secretion. (A) Stress-induced IL-8 after incubation with EVs or liposomes reconstituted from EVs-lipids; (B) Effects of synthetic liposomes on inflammation. The triangles bellow the graph signify the SM/Cer ratios. *- P < 0.05 (compared to control).

The artificial liposomes reduced stress-induced IL-8 secretion in HCE cells, however, the cells seemed to be unaffected by the amount of Cer in the vesicles. These results suggest PLs could be responsible for the IL-8 modulation.

5.5. Sphingolipid signalling in hyperosmolarity

5.5.1. The sphingolipid pathway is activated by hyperosmolarity

UVB and LPS are well known inducers of the SL signalling pathway, leading to Cer production within less than 5 min (230,231), the effects of HO were unknown. We have measured SMase activity in HO-stressed HCE cells at various time points and concluded that first significant signs of activation appear after one hour from stimulation. At this early stage in the HO-response, neutral SMases predominate with an up to 2-fold increase, while activity under acidic conditions becomes significant only after 4 hours (**III**, Fig. 3A and B). A subsequent dose-dependent Cers build-up could be measured using MS analysis 2 hours after stress (**III**, Fig. 2C). Because of the very low levels of Cer in membranes, the relative small boost in SMases activity induced a more than 5-fold increase in Cer species.

There are three known intracellular neutral SMases and gene expression analysis helped identify NSM2 as the enzyme responsible for the early production of Cer (**III**, Fig. 3C). The relative mRNA levels of the SL enzymes, after 4 hours of stress, also highlighted ASAH2 and two S1P receptors as HO-responsive genes. Similar to enzymatic activity, NSM2 expression was progressively induced by the added

NaCl up to 90 mM when it started decreasing (**III**, Fig. 3D). This decrease appears to coincide with the boost in ASM enzymatic activity (**III**, Fig. 3B).

Cer production is only a transient point of the SL pathway and the outcome is decided by the lipid that accumulates in the membranes. The activation of the ASAH2 gene expression suggests Cer break-down to smaller SLs. The next crucial step in the SL metabolism is S1P production to promote cell survival. Upon HO intracellular levels of S1P remained unchanged while extracellular S1P slightly decreased (**III**, Fig. 3E). This decrease in S1P secretion is confirmed by the upregulation of the S1P receptors. The activation of the SL pathway could therefore end in accumulation of Cer and apoptosis in some cells, or it might be that the Cer production is balanced partially by phosphorylation to C1P or elimination of S1P by the S1P lyase to non-SL molecules.

5.5.2. Hyperosmolarity induces intracellular neutral lipid accumulation

Besides Cers, the lipidomic analysis emphasised triglycerides (TGs) as another lipid species dramatically modulated by media HO. The rise in media osmolarity caused formation of intracellular neutral lipid droplets visible with OilRed O staining in fluorescence microscopy (**III**, Fig. 4A, B and C). The quantification of the stimulus-sensitive increase in dye uptake matched well the MS results, as shown in **Figure 15**.

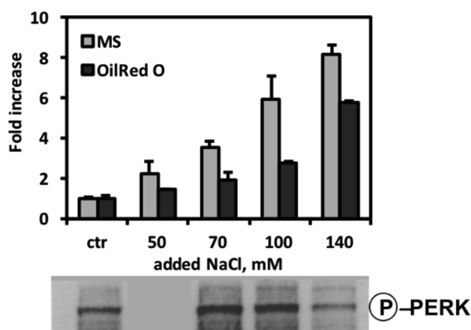


Figure 15: Mass spectrometric (MS) assessment of TGs compared with OilRed O dye uptake quantification. Immunoblotting for phospho-PERK as an indicator of ER stress.

Lipid analysis assays confirmed that the neutral lipid accumulation is represented by TGs with no contribution from cholesterol-esters (**III**, supplemental material). TGs synthesis is controlled by two di-glycerol acyltransferases – DGAT1 and 2 and siRNA knockdown identified DGAT1 as the enzyme responsible for the HO-induced TG accumulation (**III**, Fig. 4G). DGAT1 silencing significantly reduced triglyceride accumulation but promoted the HO-induced IL-8 secretion (**III**, Fig 4H) suggesting a possible protective role for the lipid droplets. This hypothesis was reinforced by the decrease in phospho-PERK

in high osmolar stress (**Figure 15**). Work by Hölttä-Vuori *et al.* showed that intracellular lipid droplets are associated with a decrease in ER stress and positive effects on survival (232). Moreover, numerous other stimuli have been associated with survival-promoting lipid droplet formation (233-235).

5.5.3. Stress droplet formation is dependent on phospholipase and NSM2 activity

If the lipid droplets are produced to alleviate stress, what is the fatty acid source needed for their synthesis? The HO-induced TG synthesis occurs in serum-starved cells and therefore an outside source is excluded. Fatty acid synthase inhibitors (C75) and acetate incorporation assays ruled out *de novo* fatty acids synthesis (**Figure 16A** – unpublished results).

The work of Gubern *et al.* suggested phospholipids as fatty acid source through the action of group VIA phospholipase A2 (235,236). Similarly, we could show that bromoenol lactone (BEL), a calcium-independent phospholipases A2b (iPLA2b) inhibitor could prevent almost entirely the intracellular TG build up (**III**, Fig. 5A). However, iPLA2b is one of the upstream activators of NSMase (237), and in our experiments BEL abolished the HO-stimulated up-regulation of NSM2 (**III**, Fig. 5E). NSM2 knockdown revealed that lipid droplet formation depends also on SMase activity (**III**, Fig. 5C).

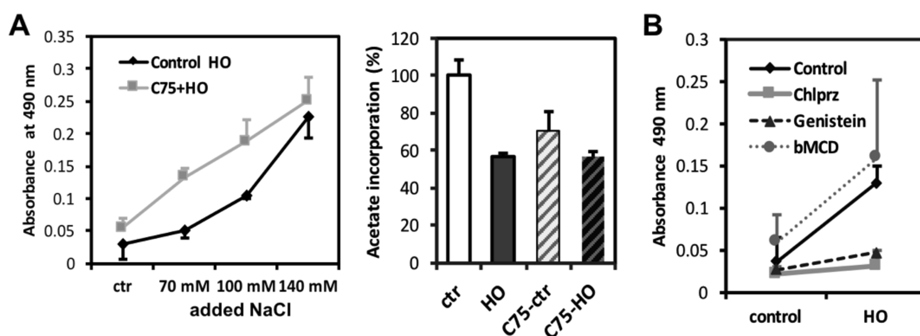


Figure 16: (A) Lipid droplet quantification using the free fatty acid synthase inhibitor C75 (left panel); Acetate incorporation in control and HO with or without C75 (central panel). Acetate incorporation assay measured the *de novo* lipid synthesis. (B) Endocytosis blockers affect TG synthesis. Chlprz – chlorpromazine; bMCD – beta-methyl-cyclodextrine.

Phospholipids are the major building block of cellular membranes and their conversion to TG synthesis might be a consequence of membrane recycling. HO stress prompts intracellular water loss and cell shrinkage. This decrease in volume poses a packing stress on membrane lipids and plasma membrane recycling could

be one of the mechanisms employed to relieve this stress. We have used several endocytosis inhibitors in HO conditions and concluded that both clathrin-dependent (inhibited by chlorpromazine and sometimes beta-methyl-cyclodextrine) and caveolae-mediated endocytosis (inhibited by genistein, beta-methyl-cyclodextrine and sometimes chlorpromazine) could be involved in plasma membrane recycling to lipid droplets (**Figure 16B** – unpublished results). Endocytosis would target the vesicles to the ER where DGAT1 resides. The PL traffic and the activity of the cytosolic iPLA2b promote ER stress which is alleviated by the elimination of excess PLs in TG lipid droplets.

Additional supporting evidence came from a different experimental set up: we have mixed the EVs lipids with [^{14}C]-Dipalmitoylphosphatidylcholine and followed the label inside the cells. We could observe a successful intracellular uptake of the radioactive label that was improved by HO (**Figure 17A** –unpublished results).

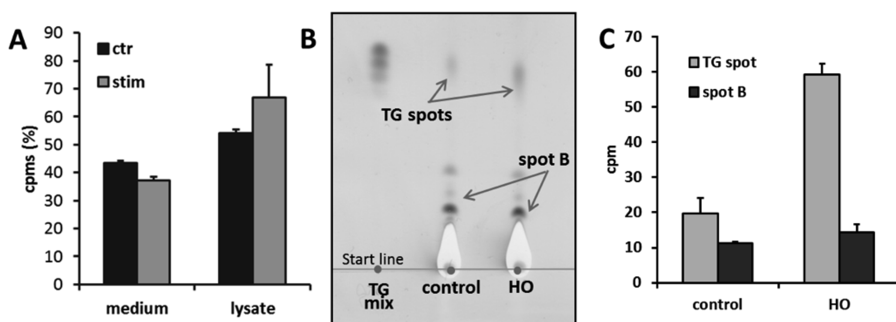


Figure 17: Liposomal lipids are taken up by the cells and excess lipids are transformed into TGs. (A) Radioactivity measurement in media and cellular lysate with/without HO stimulation; (B) TLC for neutral lipids to identify intracellular TGs; (C) Radioactivity assay in TG spots vs. background.

A TLC analysis for neutral lipids allowed us to identify the stress-induced TGs (**Figure 17B** - unpublished) and to measure the transfer of the radioactive label to other lipid classes. Four hours of HO stimulation has permitted a minute transfer of radioactivity from the PL fraction to the TGs; unspecific transfer was controlled by measuring radioactivity of a strong lipid spot on the TLC plate with unknown identity (spot B – **Figure 17C**). Much of the [^{14}C]-label remained, however, at the origin with the polar lipids.

Therefore, the inhibition of plasma membrane recycling through endocytosis blockage impeded the HO-induced lipid droplet formation. Moreover, we could show that the liposomal phospholipids could be used by the HCE cells for TG synthesis, further validating our hypothesis stating membrane recycling as stress-droplets lipid source.

5.5.4 Inhibition of specific phospholipases minimizes stress-induced IL-8 secretion

IL-8 secretion represented the stress marker in all our experiments and the stress-effect we were aiming to decrease or eliminate. Both iPLA2b and NSM2 inhibition significantly reduced HO-induced IL-8 in the cell culture media, confirming the important role of the two lipases in HO-stress response (**III**, Fig. 5B and D). The SB239063 effect on IL-8 (**III**, Fig. 5B) confirmed the involvement of the p38 MAPK in HO response as commonly accepted (82,238). MAPK p38 inhibition, however, had no effect on lipid droplet formation suggesting separate pathways for the two events with the iPLA2b and NSM2 as shared components. Although other studies placed p38 as an NSM2 activator (82), our results propose a downstream position for the MAPK, a concept supported also by others (239). These anti-inflammatory effects could minimize the undesirable consequences of stress by reducing or delaying immune cell recruitment at the ocular surface.

6. SUMMARY AND CONCLUSIONS

Throughout the studies included in this thesis we have succeeded: i) to establish a causative link between UVB and tissue degeneration in CDK; ii) to determine the role of the sphingolipid signalling pathway in ocular surface stress and iii) to learn more about hyperosmolarity effects on the corneal epithelium (**Figure 18**).

The degenerative nature of the CDK was previously associated with multiple environmental factors such as corneal micro-traumas, low humidity or inadequate UV protection, but no causative link was established, nor the nature of the droplet-like deposits was known. The composition analysis of the CDK deposits and evaluation of the proteolytic character of patient's tears offered us a targeted hypothesis for the *in vitro* studies and thus helped determine a link between UV radiation and the tissue damage observed in CDK. We showed that UVB radiation induces a prompt (4 to 6 hours post-stimulation) pro-inflammatory response from corneal epithelial cells, followed by a progressive secretion of gelatinases (MMP-2 and MMP-9). Initially the MMP secretion is balanced by their inhibitors, in a second stage, however, the destructive increase in MMP-9 secretion was unmatched by TIMP-1 production, a possible consequence of cytokine signalling.

Expanding the range of environmental stress stimuli, we observed that apart from proteinases, HCE cells release also lipid-modifying enzymes into the tear fluid or culture medium, highlighting the complex role played by the epithelium in tear film homeostasis. Our attention was drawn to the components of the sphingolipid metabolism, a ubiquitous signalling pathway with essential functions in the cellular stress response. Stimuli, such as UVB or hyperosmolarity, induced stress-proportional release of extracellular vesicle-associated sphingolipid enzymes. These enzymes maintain the balance between the different bioactive lipids in the sphingolipid pathway, the key to the intensity-modulated response to stress. Their definite function in the tear fluid remained elusive, we could conclude, however, that extracellular vesicle-lipids downregulated IL-8 expression and secretion in non-stressed HCE cells. Additionally, the degree of enzyme/extracellular vesicle release could provide a non-invasive measure of stress at the ocular surface.

UV radiation as well as LPS were well-known inducers of bioactive sphingo-lipids. We showed for the first time that also hyperosmolarity activates the sphingomyelinase-dependent production of ceramides. The engagement of the sphingolipid signalling pathway is mediated by NSM2 enzyme and controls the hyperosmolarity-induced IL-8 secretion. Upstream from NSM2 we identified a group VI phospholipase A – iPLA2b – while p38 MAPK was suggested as a

downstream effector. Besides inflammatory signalling through the IL-8 chemokine, the two phospholipases, iPLA2b and NSM2, additionally regulated the intracellular synthesis of triglycerides stimulated by the high osmolarity of the medium. All cells possess the machinery for triglyceride storage, and lipid droplet formation was associated with numerous cellular functions. Their production was considered both detrimental as well as protective. The first is illustrated by triglyceride hydrolysis to the pro-inflammatory eicosanoids, while the prolonged cell-survival under stress emphasized their protective function. The iPLA2b involvement in this process suggested membrane phospholipid hydrolysis as source of triglyceride building blocks. We hypothesized that the membrane lateral stress, resulted from the rapid volume change imposed by hyperosmolarity, is released by NSM2 and iPLA2 mediated membrane recycling. The increased influx of phospholipids is neutralized by the ER synthesis of triglycerides.

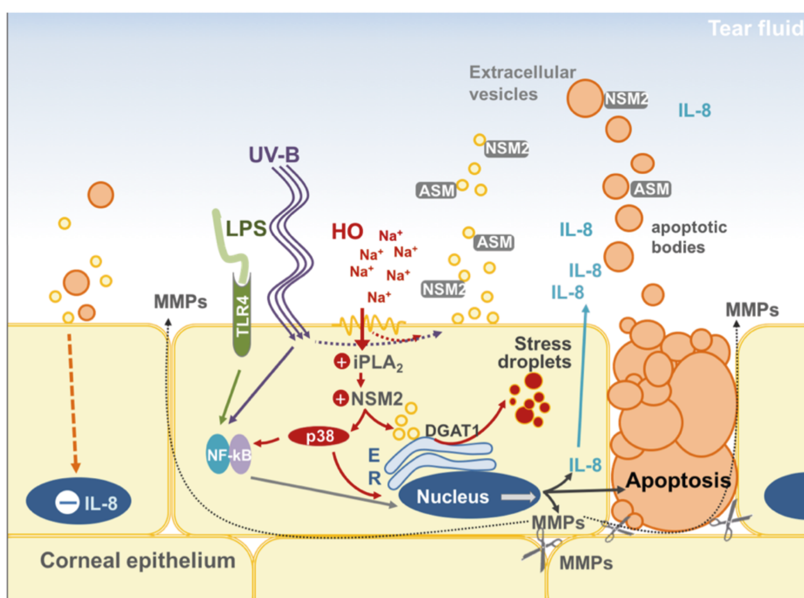


Figure 18: Schematic representation of the major findings of the thesis project.

The ocular surface is vulnerable to numerous threats from the surrounding environment and its stress response is a thin balance between tissue protection and maintenance of function. One of the most relevant clinical signs of distress is ocular surface inflammation, a priority for the clinician attempting to treat disease. The major aim of this thesis project has been to understand more about the corneal stress response to different stimuli and to identify better targets for anti-inflammatory therapies that would seek to restore tissue homeostasis.

Acknowledgements

The studies included in this thesis project were carried out in the Helsinki Eye Lab at the Department of Ophthalmology, University of Helsinki and in the Genomics and Biomarkers Unit, National Institute of Health and Welfare, between 2010 and 2014. I would like to thank Docent Anu Jalanko and Professor Tero Kivelä for providing great research facilities and good working environment. The Sigrid Juselius Foundation, Finnish Academy, Finnish Eye Foundation, Eye and Tissue Bank Foundation and the University of Helsinki are recognised here for providing financial support.

I wish to express my deepest gratitude to my supervisors, the late Professor Juha Holopainen, and Docent Matti Jauhiainen. Matti always brought enthusiasm and optimism in every aspect of the scientific process. His door is always open for all kinds of troubles. Juha has been my mentor in science and in life, his enthusiasm, professionalism and good humour will always be something I will try to emulate. I am so happy I was one of the lucky ones to share his time, wisdom, and laughs.

The reviewers, Docent Maria Vartiainen and Professor Kai Kaarniranta are kindly thanked for their time and dedication on improving this thesis.

I would like to acknowledge all my co-authors without whom this work would not have been completed. I thank Professor Timo Sorsa and Taina Tervahartiala from the Dentistry Department and Tuulia Hyötyläinen for very pleasant and fruitful collaborations. A huge thank you to my wonderful colleagues, the members of the Helsinki Eye Lab, Antti Rantamäki, Riku Paananen and Pipsa Kulovesi. Thank you for our memorable lunch and coffee break discussions. Our group dynamic steered by Juha made coming to work feel like a vacation and only the "downs" in science brought dark clouds on our perpetual parade. Thank you, Antti, for putting up with me and my weird humour and for becoming my best friend. You were there even in my lowest moments and you always helped me fight on.

I am thankful for my wonderful collaborators Susanne Wiedmer, Suvi-Katriina Ruokonen and Joanna Witos from the Chemistry Department, Niko Setälä, Riku Arvola, Ilkka Immonen, Asta Hautamäki, Sirpa Loukovaara, and Jukka Moilanen from the Eye Clinic. I would also like to thank Olli Mattila and Corinna Sandwald for their kindness and friendship. Jari Metso and Sari Nuutinen have always helped with the practical problems in the lab and had a friendly chat to spare. I thank Seikku and Ritva for their kindness to me and my family from our very first months in Finland. My friends at home Madalina, Cosmin, Adrian and Anca who have showed me how fun science can be from the very beginning. A big thank you

to my wonderful friend Outi, I am very lucky to have met you and you will always have my love and admiration.

Many thanks to all those I have met and interacted with over the years for enriching my experience both as a scientist and as a person.

Finally, I would like to thank my mother who believed in me and always saw more in me than I did myself. She raised me as a single parent since I was three and made it look effortless. I have always admired you for your strength and determination. I would like to thank my late grandparents, my aunt and uncle, and George who made our small family big at heart. Madi, it is ultimately thanks to you that I chose biology and science as my road in life and I am grateful. You've been (and still are) a tough act to follow but I am doing my best.

Marius, thank you for your love and for sharing your courage and strength with me. It is thanks to you that I am here and I became the someone I always wanted to be. Our journey hasn't been always smooth but we have always managed to turn the obstacles into stepping stones and hopefully we'll keep doing that for a long time to come. Ana, you will always be my most wonderful achievement, thank you for being you.

Alexandra

Helsinki, June 2017

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Helsinki Eye Lab 2017

ISBN 978-951-51-3519-3 (PAPERBACK)
ISBN 978-951-51-3520-9 (PDF)

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