INNOVATIONS FOR THE PREVENTION OF OCULAR SURFACE SCARRING

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Abstract

Background

Corneal scarring from various diseases is a leading cause of blindness globally and considered a priority by the World Health Organisation. Microbial keratitis (MK) is the commonest cause, often associated with regionally distinctive risk factors such as trauma in agricultural workplaces in lower- and middle-income countries versus ocular surface disease in upper- and middle-income countries. MK's impact on working-age people means the burden of disease is vast considering it impacts the productivity. There are no approved medical therapies to prevent or reverse corneal scarring, and the only sight restoring options involve corneal transplantation or implantation, which necessitate access to specialised eye-care infrastructure and long-term healthcare service engagement. As such, there is an unmet need for non-invasive and easily accessible treatments for MK.

Aims

To investigate a novel mechanism through which decorin inhibits fibrosis in corneal fibroblasts *in vitro*; horizon scanning for other approved or developmental therapies for corneal scarring; to conduct a human clinical trial of decorin to prevent corneal scarring in patients with MK.

The COVID-19 pandemic considerably delayed the development of this intervention, therefore additional aims were added to determine the impact of the pandemic on the project. These oriented understanding the UK public's perception and health seeking behaviours with regards to eye symptoms in the pandemic, and finally understanding the impact of the pandemic on MK patients attending a tertiary eye care centre.

Methods

A mixed methods study including a systematic scoping review, an *in vitro* investigation of decorin's mechanism, a survey of public perceptions, a clinical cohort study and an early phase clinical trial.

Results

Eye health was a primary concern for the UK population who demonstrated proportionality of health seeking behaviour in response to eye symptoms of varying severity. The severity of MK during the pandemic was found to be no different compared to pre-pandemic times. No approved treatment for

corneal fibrosis exists. Decorin is one such candidate which is imminently due to enter human clinical trials, with the potential to change the corneal fibrosis landscape. Decorin did not induce or inhibit autophagy in primary human corneal fibroblasts however the results helped to generate novel hypotheses for further investigations.

Conclusions

Although some of decorin's mechanisms of action are yet to be elucidated, understanding the significant mechanistic checkpoints in corneal fibrosis are key, thus mechanistic studies have been incorporated into the clinical trial. Well-designed clinical trial outcome measures are required to generate robust evidence for the adoption of novel therapeutics that have the potential to change lives around the world. The public were reportedly apprehensive about volunteering for eye research, therefore researchers must be proactive in reassuring patients.

Impact of COVID on proposed thesis

The COVID-19 pandemic considerably impacted the implementation of the trial, which remains indefinitely delayed. Moreover, the university laboratories were closed for several months shutting down any in vitro work. Finally, I was seconded onto the wards to deal with patients of the pandemic. Therefore, additional project aims were required to determine the impact of the pandemic on the trial, ophthalmic conditions and research. The first new objective was therefore to understand the UK public's perception of eye symptoms and health seeking behaviours with regards to eye symptoms in the pandemic, as well as their willingness to volunteer for research. The second was to determine the impact of the pandemic on MK patients' clinical outcomes at a tertiary eye care centre.

Contents

List of abbreviations

AMSTAR 2 - A measurement Tool Assess systematic Reviews 2

BCL – bandage contact lens

bFGF – basic Fibroblast growth factor

BK – bacterial keratitis

CCP - Cytochrome c peroxidase

DNA – deoxyribonucleic acid

ECM – extra cellular matrix

EGF - Epidermal growth factor

FML - fluorometholone

HBO – hyperbaric oxygen

HSK – herpes simplex keratitis

IVCM – in-vivo confocal microscopy

LASEK – laser assisted epithelial keratomileusis

LASIK - Laser-assisted in situ keratomileusis

LRS – Laser refractive surgery

MK - Microbial Keratitis

MMC – Mitomycin C

NAC – N-acetyl cysteine

NGF - Nerve growth factor

NK – neurotrophic keratitis

NSAID – non-steroidal anti-inflammatory drugs

OCT – optical coherence tomography

PED – persistent epithelial defect

PRISMA – Preferred reporting items for systematic reviews and meta-analyses

PRK – Photorefractive keratectomy

PRO – patient reported outcome

RCT – randomised controlled trial

RGTA – matrix regenerating agent

SCUT – steroids for corneal ulcers trial

VKC – vernal keratoconjunctivitis

x-CMHA - Crosslinked thiolated carboxymethyl hyaluronic acid liquid-gel

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List of figures

List of tables

List of supplementary figures and tables

1. Chapter 1: Introduction

1.1 The burden of corneal fibrosis

Corneal blindness is one of the most prevalent causes of visual impairment globally. Estimates of prevalence range from approximately 3.2% to 5.1% of all blindness cases (WHO definition of Snellen acuity less than 3/60 in the better eye), amounting to approximately 1.2 to 1.9 million people worldwide, not accounting for those estimated 161 – 216 million with moderate to severe visual impairment (WHO definition worse than 6/18 and better than 3/60 in the better eye) [1–3]. Further still, these estimates do not account for the unilateral visual impairment occurring in many corneal pathologies [3]. This is difficult to estimate globally, however, based on the comparison of bilateral:unilateral blindness ratios in India, this may be estimated at 23 million [4,5], not taking into consideration those with unilateral visual impairment. Corneal blindness accounts for 20% of childhood blindness [6] and nearly 80% of corneal blindness is considered avoidable [7].

As it more commonly affects younger people, its impact on the socio-economic attainment [8] and quality [9]of life are of significance to broader society. It is also associated with an increased risk of death. [10,11]Even unilateral vision loss leads to poorer quality of life in terms of increased risk of falls, help to perform ADLS and loss of independence [12]. It is also an important consideration for children, where in addition to the loss of stereopsis, motion processing and oculomotor behaviour can be adversely affected [13].

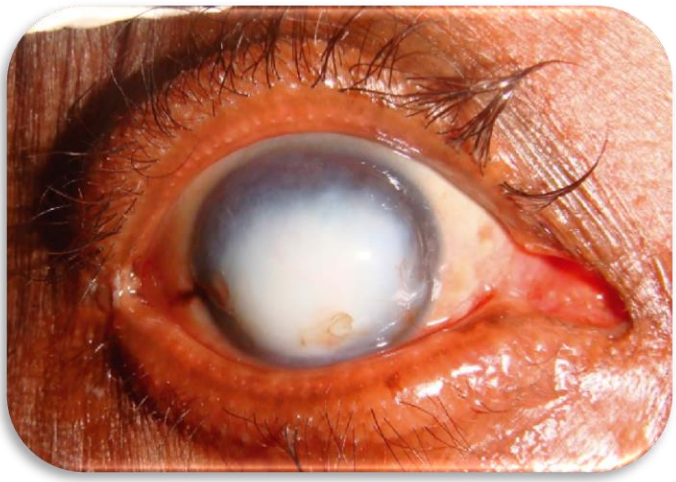


Figure 1-1. Extensive corneal scarring causing blindness

This picture depicts the right eye of a patient who has chronic corneal scarring following microbial keratitis. Adapted from [14].

It disproportionately impacts lower- and middle-income countries (LMICs) due to a combination of aetiological factors and access to healthcare [7,15]. For example, trachoma is of greater prevalence due to sanitation facilities, while ocular trauma related ulceration is more prevalent due to agricultural work related eye injuries and delayed presentation to health services [15,16]. Broadly, management approaches include prevention, treatment of the cause, and rehabilitation. Whilst preventive interventions have been beneficial [17], occupational risks will remain due to social circumstances. Despite effective treatment of the cause, scarring can lead to sight loss. Rehabilitation refers to corneal transplantation/implantation, which are the only sight-restoring strategies available, and is hugely hindered by donor tissue shortages globally and infrastructure variability between countries [18].

Corneal blindness is often associated with terms like “avoidable- or preventable- blindness” when considering its aetiological factors. This concept is born of the fact that many of these are acquired causes and that holistic healthcare strategies can effectively prevent and/or treat these conditions. However, the global burden of corneal blindness evidences otherwise.

Following insult, the cornea has limitations in its regenerative capacity, whereby healing leads to irreversible changes in its optical properties (curvature and clarity). The resultant visual impairment depends largely on the severity of the injury, with loss of vision termed corneal blindness. The most

common causes of corneal scarring can be broadly categorised as infective, inflammatory, chemical or mechanical trauma and congenital, with infection and trauma being particularly prevalent [19,20].

Corneal opacification is a term that more broadly refers to the loss of clarity of the cornea, which may or may not be transient or reversible in nature. However, the term corneal fibrosis, sometimes used interchangeably with corneal scarring, refers to established chronic structural changes causing opacification of the cornea. Fibrosis itself is part of the healing process in the majority of tissues, and as its name infers, is characterised by the deposition of fibrous ECM causing structural changes in tissues that may cause dysfunction. These biological and pathophysiological details are discussed next.

1.2 The Cornea and Fibrosis

1.2.1 Cornea

The human cornea is a biological lens characterised by its highly organised collagenous extracellular matrix (ECM) responsible for its optical clarity [21]. Its precise spherical curvature provides approximately two-thirds of the refractive power of the eye. It is continuous with the sclera making it structurally integral to the globe. As it forms the interface between the external world and the intraocular contents, it is an incredibly well-maintained site of immune activity. To preserve clarity, the cornea is avascular and derives its nutrients from three sources; the aqueous humour within the anterior chamber; the limbal vasculature; and the tear film on the ocular surface, which is critical for oxygen diffusion and also contains antimicrobials [22]. Although it is avascular, the branches of the anterior ciliary arteries terminate at the limbus, forming arcades that deliver nutrients to the peripheral cornea [23]. It is one of the most densely innervated tissues in the body, with a heterogeneous supply of sensory nerves from the ophthalmic division of the trigeminal nerve, capable of detecting various stimuli [24]. The corneal nerves play an important role in several processes, including tear production, blinking and the maintenance of corneal health through the release of trophic factors.

The cornea is composed of epithelial and endothelial cell layers, on either side of a well organised and densely packed collagenous stromal layer, which is sparsely populated by keratocytes. It is approximately 11mm in diameter and 550 um in thickness, with the stroma constituting approximately 90% of the thickness.

The corneal epithelium is characterised by its impermeability which is attributed to its stratification, the presence of cell junctions and strong anchoring to a partially permeable basal membrane (Bowman's layer) primarily composed of type IV collagen and laminin [25]. The epithelium is stratified into five layers of cells which transition from a cuboidal morphology basally to squamous superficially. These are renewed by the limbal epithelial stem cell niche every 7-10 days [26]. Mucous in the tear film adheres to interdigitations of the epithelial cell surface membrane – microplicae - and their glycoprotein coating, the glycocalyx, enabling it to resist the pull of gravity and spread over the ocular surface with each blink of the lid.

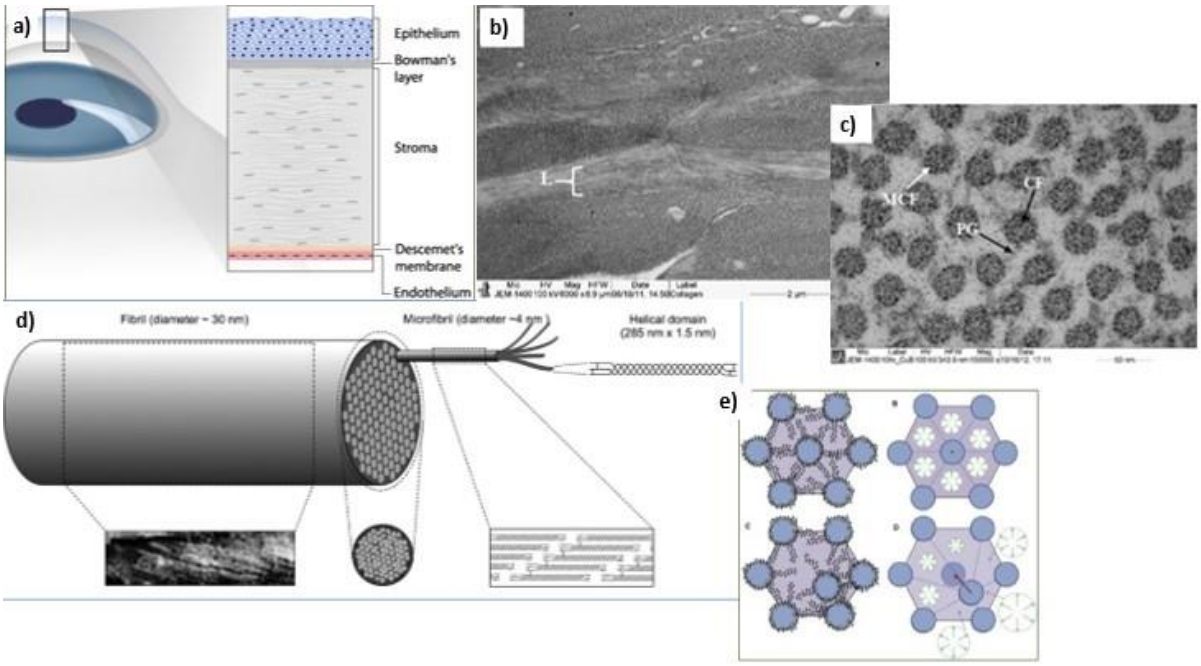
The corneal endothelium is a single layer of five to seven-sided cuboidal cells with limited self-renewing potential. The endothelial cell density is highest at birth, ranging from 3500 to 7000 cells/mm2 in a normal cornea. These cells are separated from the stroma by their basement membrane – Descemet's membrane [27]. Endothelial cells contain ion transport systems within that establish a chemical and osmotic gradient between the relatively hypo-osmotic stroma and hypertonic aqueous [28]. This osmotic gradient produces a net fluid flux from the stroma to the aqueous, which maintains a constant percentage of water (78% H2O) in the stroma, necessary for corneal clarity and transparency. This process is referred to as deturgescence [29]. Disruptions to deturgescence, such as inflammation and hypoxia, can result in corneal oedema.

The stroma is a largely acellular layer conferring the cornea's optical and mechanical properties. Keratocytes constitute a small proportion of this layer, the majority consisting of collagen, proteoglycans and glycoproteins in a milieu of ions which help to maintain the hydrostatic pressure in the tissue [30,31]. The regularly spaced fibrillar collagen, primarily collagen I and IV, is arranged into over 200 orthogonal lamellae, maintaining superior, inferior and naso-temporal orientations. These lamellae are also more densely packed and interconnected antero-centrally [21,32], critical for maintaining cornea curvature [33]. Collagen fibrils demonstrate hierarchical structuring. The base collagen molecule consists of a polypeptide triple helix arranged in a right-hand superhelix. These units can self-assemble in a staggered array of five molecules to form the next order of structure - a microfibril. Approximately 70 microfibrils combine in a spiral to give rise to the collagen fibril, associated with proteoglycans which aid the regular spacing and hydration of the fibrils [21].

When light arrives at the cornea, it may be transmitted, absorbed, or scattered. In a healthy, transparent cornea, visible light is not absorbed, and scattering is minimal. Corneal scattering can increase in cases of corneal oedema, relaxation of collagen fibrils, haze caused by extracellular matrix production by keratocytes, or irregularities due to surgery. This scattering leads to a decrease in the quality of vision and,

if severe enough, blindness. Clinically this can be observed as a loss in the clarity of the cornea. Figure 1-2 depicts the corneas ultrastructure, the relationship of GAG chains and how they space collagen fibrils. Figure 1-2 ii illustrates the way back scattered light is generated when collagen fibril structure is disrupted.

i)



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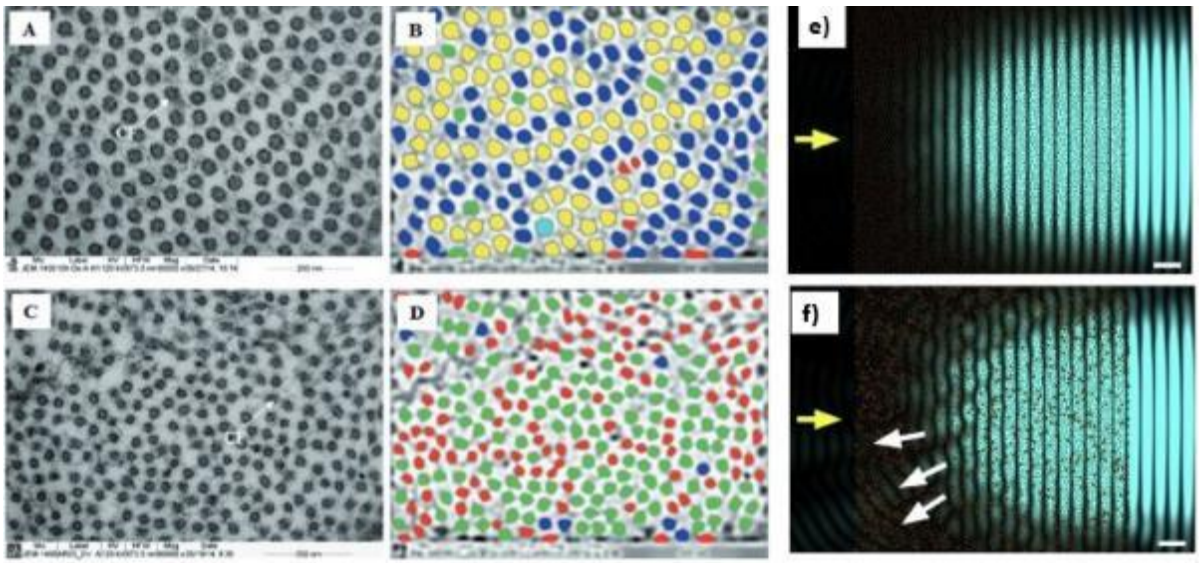


Figure 1-2. Corneal ultrastructure and optical clarity

i) depicts the structure of the cornea and corneal stromal constituents (i-a); (b) electron micrograph of healthy cornea showing interlacing lamellae (L) and (c) further magnified cross sectional view of collagen fibrils (CFs) and microfibrils (MCFs) with associated proteoglycans (PG); d) Structural hierarchy of collagen fibrils α-helices comprise the classic triple helix (top right) which self-assembles (bottom right) into microfibrils which comprise the 30nm fibrils (c), micrographs of a coiled fibre from the side (bottom left) and in cross section (bottom middle); e-a) Healthy fibril organisation with associated glycosaminoglycan chains, and the electrostatic forces (e-b) maintain the lattice organization. IF a single fibril is displaced it has a considerable effect on the rest of the unit (e-c, e-d).

ii) Electron micrograph and image analysis of collagen fibril (CF) of normal (a, b) demonstrating uniform size and arrangement, and 2hr hydrated cornea (c, d) with significantly more irregular structure. The impact of these can be seen in light scatter experiments with simulated fibrils of differing structure. e) The highly regular structure of collagen in the cornea leads to destructive scatter with light propagating in the incidental direction (left to right, yellow arrow).No backwards secondary can be seen. f) Irregularly sized and spaced fibrils result significantly more scattered light (white arrow) seen in the interference patterns of the light. Scale bar in (e & f) 500 nm. Adapted from [34–36].

1.2.2 Corneal homeostasis

In general, the eye is an organ vulnerable to insult and injury due to its site and the complexity of its anatomy. This is particularly true for the intricately structured cornea that is exposed to the outer world. There exist several mechanisms to protect the eye. These range from the bony confines of the orbit to the lids and lashes, which are intimately associated with corneal nerves through reflexive blinking and tear production. Additionally, the eye maintains a complex relationship with the immune system as much of the eye is maintained as a site of immune privilege.

Superficial to the sclera, the cornea is continuous with the conjunctiva, and these together contribute to the ocular surface microenvironment and the lids and tear film. Immune activity commences at the tear film, which, similar to other mucosal fluids, contains antimicrobial proteins such as lysozyme and lactoferrin and Immunoglobulin a (IgA) [22]. Of the cornea itself, the epithelial cells and keratocytes can recognise and react to pathogens through the expression of Toll-like receptors, resulting in the release of antimicrobial molecules such as human Beta-defensins and immunomodulatory molecules such as interleukin 6, 8 and TNF alpha [37–39]. Epithelial cells also contribute to managing excessive inflammation through the expression of Fas ligand, programmed death ligand 1 and vascular endothelial growth factor traps [40–42]. One important group of cytokines released during epithelial injury are the Transforming growth Factor Beta (TGFβ) family. These molecules involve a broad range of cellular processes and are

central to fibrotic healing in many organs, including the cornea. Within the quiescent corneal and conjunctival epithelium, antigen presenting dendritic cells and Langerhans cells are distributed, most abundantly near the limbus, decreasing in number up to the paracentral cornea [43]. With activation, these resident cells trigger the inflammatory cascade leading to the recruitment of circulating leukocytes from the limbal vasculature.

1.2.3 Corneal wound healing and fibrosis

During epithelial-stromal injury, the corneal epithelium releases a range of growth factors and cytokines to initiate wound-healing. Mediators that are crucial for regulating both epithelial and stromal healing are usually prevented from entering the stroma by corneal epithelial cells and an intact Bowmans layer [44]. Following an injury, these factors, such as TGFβ, tumour necrosis factor (TNF), epidermal growth factor (EGF), interleukin (IL)-1, bone morphogenic proteins 2 and 4 (BMB), and platelet-derived growth factor (PDGF) can enter and reach the keratocytes [45–48]. Similar to the epithelium and Bowman's layer, Descemet's membrane is thought to regulate the development of posterior corneal myofibroblasts and fibrosis by limiting the release of pro-fibrotic growth factors from the aqueous humour and endothelium [49].

Following an injury to the cornea, numerous cytokines, chemokines and growth factors are released, which modulate resident and circulating cells to promote wound healing. In the anterior stroma, early keratocyte apoptosis occurs due to the IL-1 released by epithelial cells [50], which induces the cells to produce Fas ligand that binds to the Fas in an autocrine fashion [51]. Twelve to 24 hours after the initial injury, keratocytes proliferate and migrate to repopulate the stromal areas that suffered a loss [46]. These cells differentiate into corneal fibroblasts that migrate to the wounded area and can further differentiate into myofibroblasts [52]. Selectins and integrins facilitate bone marrow-derived cell infiltration from the limbal vasculature, including polymorphonuclear cells, monocytes, and T-cells, while chemokines direct these cells to the injury [53]. Figure 1-3 illustrates these events.

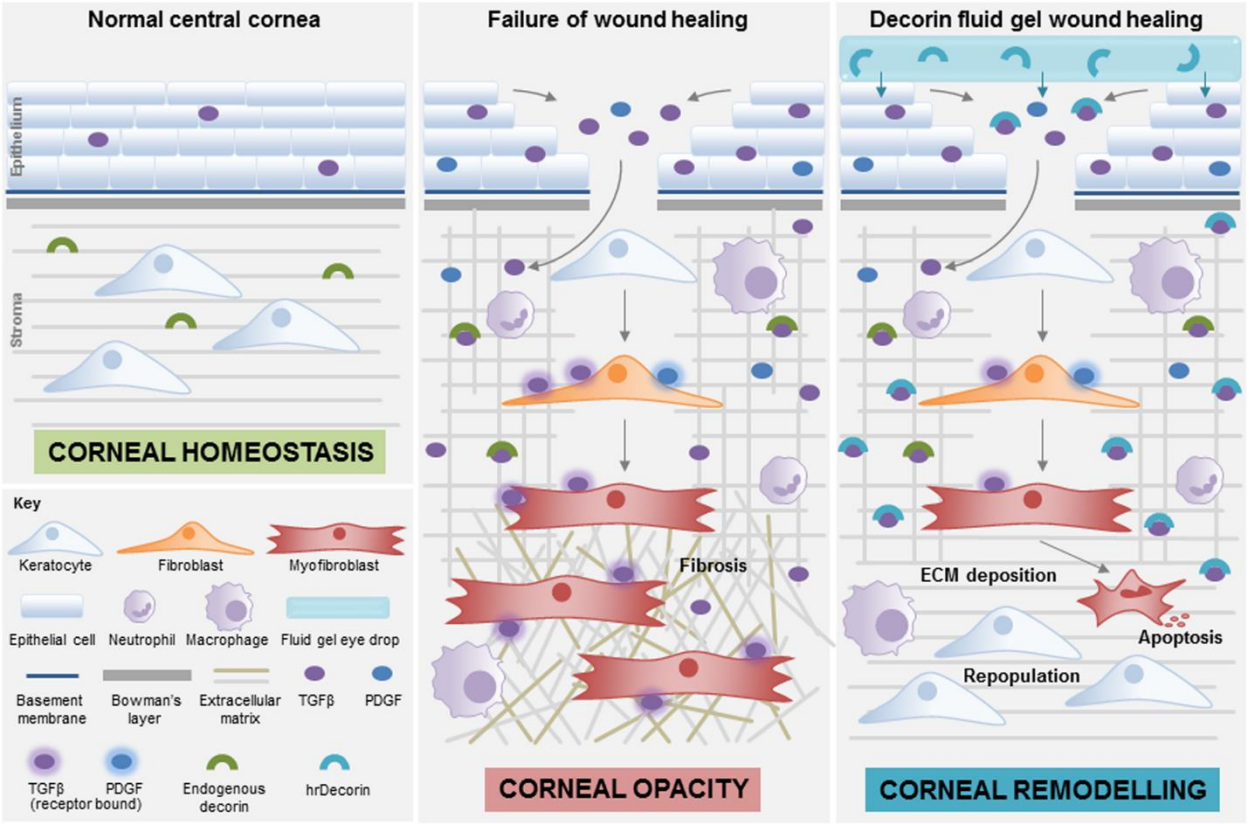


Figure 1-3 Schematic overview of corneal fibrosis.

A schematic overview of the steps involved in corneal fibrosis Adapted [54].

Corneal myofibroblasts may be derived from resident corneal keratocytes or monocyte-derived mesenchymal cells from the blood circulation, termed fibrocytes in the latter case [55,56]. These cells produce an extracellular matrix and express intermediate filament (IF) proteins such as alpha-smooth muscle actin (αSMA), which affords them contractile strength to achieve wound closure [57,58]. In Ifs, vimentin (V), α-SMA (A) and desmin (D) are expressed sequentially, each in addition to the last, with increasing maturity, which correlates to time from wounding and the severity of corneal haze. Therefore, this has been used in grading criteria for the ordered differentiation of stromal cells, where keratocytes or bone-marrow-derived precursors undergo differentiation into V-type, the VA-type and finally, VAD-type myofibroblasts [59]. The contractile effect of these cells increases the tension within the cornea. Increased matrix rigidity has been found to enhance fibroblast differentiation in several different tissue fibroblasts, including corneal fibroblasts [60,61]. Differentiation causes a loss of intracellular crystalline

protein, which causes a concurrent loss in the transparency of fibroblasts [62]. Thus the loss of cellular crystallins combined with disordered extracellular matrix deposition and increased wound tension can propagate fibrosis and lead to loss of corneal opacification.

Enzymes including the collagenolytic metalloproteinases and plasminogen-activator/plasmin system, are released to degrade and remove damaged tissue [63]. However, this can also lead to loss of stromal tissue, also termed “corneal melting”, which can result in perforation of the cornea and compromise of the globe.

1.2.3.1 Transforming Growth Factor β 1

A number of signalling molecules have been identified as key mediators of the above events in the fibrotic pathway. The largest body of work exists on the role of Transforming Growth Factor β (TGFβ)1 in the differentiation of keratocytes to myofibroblasts [64].TGFβ1 molecules are critical components of the wound healing response in all organs, where they influence cell differentiation, proliferation, and ECM production and interact with the immune system [65]. There are three isoforms of TGFβ, of which TGFβ 1 and 2 are pro-fibrotic, and TGFβ 3 is anti-fibrotic [66].

In the cornea, TGFβ1 release from the epithelium into the stroma is found to confer stromal cells resistance to apoptosis induced by IL1 [67]. It is released in an inactive complex with latent TGFβ binding protein (LTBP), which has several activators including proteases, reactive oxygen species and integrins [68–70]. TGFβ1 binds to the type II TGFβ receptor (TbRII), an autophosphorylated receptor, which activates the type I TGFβ receptor (TbRI). This can then propagate downstream signalling through several pathways including the Smad-dependent (Figure 1-4) and –independent signalling pathways [71]. Smad stands for small mothers against decapentaplegics and comprises a family of signal-transducing proteins closely associated with the TGFβ family. Activated TbRI phosphorylates the intracellular receptor-regulated SMADs (R-Smads). Of the many R-Smads, in this particular pathway, Smad2 and Smad3 are the targets [72]. These complex with Smad4, a co-Smad that enables nuclear translocation and subsequent transcription of TGFβ responsive genes, causing, for example, differentiation and ECM production [73]. This pathway is negatively regulated by Smad7, an inhibitory Smad that promotes the internalisation of TbRI and competes with Smad2/3 for the TbRI biding [74,75].

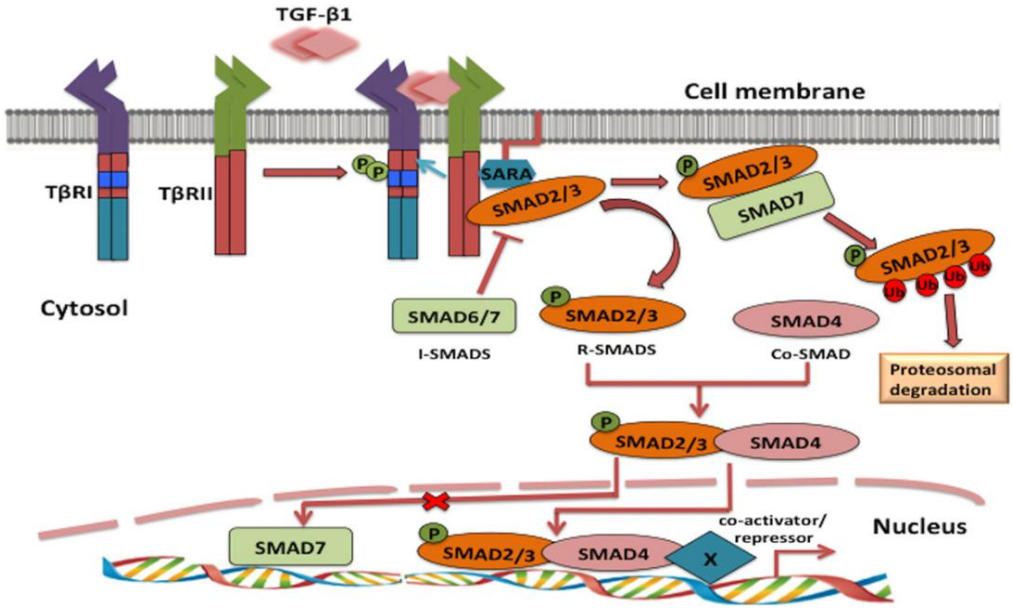


Figure 1-4. The SMAD pathway

A schematic overview of the TGFβ1- Smad dependant signalling pathway, adapted from [76].

1.2.4 Causes of corneal blindness

Globally the most prevalent conditions causing corneal blindness include trachoma, microbial keratitis, trauma, inflammatory disease and nutritional deficiencies [15] (Table 1-1). However, their prevalence varies geographically due to environmental and socio-political factors. For example, trachoma, work related trauma, nutritional deficiencies and ophthalmia neonatorum are more prevalent in LMICs [77]. In upper and middle-income countries (UMICs) with better infrastructure and easier access to healthcare, instead, microbial keratitis, trauma and inflammatory pathologies are more prevalent [78]. Whilst the single most prevalent condition varies with region, microbial keratitis is a globally significant problem such that it has been referred to as a "silent epidemic" in developing countries as well as a "neglected tropical disease (NTD)" due to the under-recognition of its impact [20,79].

Table 1-1. Causes of corneal blindness
Infectious
Bacterial
Fungal
Viral
Trachoma
Onchocerciasis
Leprosy
Ophthalmia neonatorum
Nutritional
Vitamin A deficiency
Inflammatory
Mooren's ulcer
Stevens-Johnson syndrome
Ocular mucous membrane pemphigoid
Inherited
Corneal stromal dystrophy
Fuch's endothelial dystrophy
Degenerative
Keratoconus
Trauma
Penetrating Trauma
Chemical injury
Iatrogenic
Pseudophakic bullous keratopathy

1.3 Microbial keratitis

1.3.1 Introduction

Microbial keratitis (MK) has been identified as a leading cause of corneal blindness worldwide, both in UMICs and LMICs [20,80] and has been referred to as a "silent epidemic" in developing countries [79] because its impact on the young working population affects their employment and ability to provide for their dependents, thereby having a much greater societal impact beyond the individual patient. Furthermore, implicated in this are the barriers to healthcare and the ongoing need for an agricultural workforce as contributing factors contingent on a nation's broader infrastructure. It is characterised by replicating microorganisms in the compromised corneal tissue, resulting in inflammation and tissue changes, including corneal melting (loss of stroma) and scarring. These include bacteria, fungi and parasites [81]. If left untreated, catastrophic sequalae may ensue, such as perforation, endophthalmitis and loss of the eye. Given the potential for vision loss, microbial keratitis requires aggressive management. Even when successfully treated, stromal inflammation can lead to irreversible corneal fibrosis and consequent loss of visual acuity. This is dependent on multiple factors, including the size, location and optical density of the fibrosis.

1.3.2 Microbial keratitis epidemiology and aetiology

In the United Kingdom, microbial keratitis is the most encountered non-surgical ophthalmic emergency, with an estimated incidence rate of 40 to 52 individuals/100,000 persons per year [82]. Globally, its incidence is quite variable, with estimates in LMICs ranging from 113/100,000 in South India [83] to a considerable 799/100,000 reported in Nepal [84]. This variation is thought to be due to geographical variations in risk factors. Non-surgical trauma is responsible for 48.6% to 65.4% of corneal ulcer cases in developing countries like Nepal and India. In the United States, 27% of cases were accounted for by non-surgical eye trauma [85]. In contrast, in developed countries, contact lens (CL) wear is a prominent risk factor [86]. Broadly, environmental or host factors that compromise homeostasis of the cornea all risk development of MK. In particular, trauma, foreign bodies or contact lens wear, ocular surface disease, diabetes and immunocompromise (pathological or iatrogenic) are significant contributors to the development of MK [86–93] (Table 1-2).

All groups of microbes can infect the cornea, however, the most common are bacteria, fungi and parasites [94,95]. Geographical variation in microbes is associated with certain risk factors. Bacterial keratitis is far

more common in the west, while studies conducted in India and Nepal found the highest fungal infections because of agricultural-related corneal trauma. In the UK, coagulase-negative *staphylococcus*, *Staphylococcus aureus* and *epidermidis*, *streptococcus* and *pseudomonas aeruginosa* are the most prevalent bacteria [86–94].

Table 1-2. Microbial keratitis risk factors [86–93]
Contact lens wear
Trauma
Foreign body
Pre-existing ocular surface or other corneal disease e.g. neurotrophic keratitis, viral keratitis
Eye lid disease e.g. blepharitis, trichiasis
Immunosuppressant medication systemic or topical, e.g. topical steroids
Corneal oedema
Systemic disease Diabetes Immune mediated disease e.g. rheumatoid arthritis Other immunocompromise

1.3.3 The burden of microbial keratitis

The National Health Service (NHS) gathers clinical activity data via coding. Still, there is limited data on admissions for MK due to the lack of a specific Healthcare Resources Group (HRG) code for admitting patients in hospitals who have presented with MK for intensive therapy.

Collier et al. estimated a direct healthcare cost of $175 million for treating MK in the USA in 2010, including almost 1 million outpatient department attendances [96]. NHS Digital estimated roughly 1388 annual admissions for MK between 2021 and 2022 nationally [97]. Moussa et al. estimated the average direct cost to be £357,075 per admission, amounting to over £495 million, not including further outpatient or surgical care nor other indirect costs of disease [98,99].

Unilateral and bilateral visual impairment cause significant morbidity discussed earlier in the chapter (see section: Global burned of corneal blindness). In addition, MK causes acute pain and visual impairment, which themselves impact patients' vision-related QOL [100,101]. The psychological impact of MK is associated with the fear of losing one's eyes and the social stigma associated with it [100].

1.3.4 Pathophysiology of bacterial keratitis

Pathogenesis shares many similarities with pathogens. Broadly, the sequence of events involves:

(1) Bacterial adhesion, invasion and colonisation. Unique bacterial virulence factors confer different potency of different steps of this initial phase [102,103]. For example, *Staphylococcus aureus* has a specific group of surface adhesins termed microbial surface components recognising adhesive matrix molecules (MSCRAMMS) that mediate its adherence with host extracellular components, fibronectin, elastin, collagen, laminin, and fibrinogen [104]. Once the bacteria have entered the tissue, they must evade host defences and colonise it. Here P. aeruginosa has exceptional ability, as it forms biofilms and uses its mucoid glycocaylyx to evade immune cells [105].

(2) Initially, the innate and then adaptive host immune responses attempt to address the growing issue. There is a surprising similarity in the host's innate immune response to bacterial keratitis. Neutrophils comprise the primary initial response cells, although macrophages are also present. Polymorphonuclear cells (PMNs) produce inflammatory cytokines, engulf pathogens, and release reactive oxygen species and neutrophil extracellular traps (NETS) to combat infection [103,106]. This action is, however, also highly toxic, leading to the corneal stromal melting, which is potentiated by the upregulation of metallo-matrix-proteases (MMPs) [107,108]. Regarding the adaptive immune response, antigen-specific T and B lymphocytes are upregulated, which aid in removing microbes and antigens [109]. Langerhans cells also contribute to the adaptive immune system through their antigen presenting function, expression of toll-like receptors and pro-inflammatory cytokines, e.g. interferons, Tumor Necrosis Factor-α and interleukin-1β [110].

(3) The final broad phase involves the long-term stromal remodelling that starts in the acute phase and can be clinically seen to evolve up to 12 months post-infection [111,112].

1.3.5 Diagnosis of microbial keratitis

To diagnose and determine the cause of microbial keratitis (MK), microscopy, culture, and sensitivity are the most informative tests [113]. They can also help guide treatment by identifying effective antibiotics against strains of bacteria. However, cultures are identified in less than half of the cases where samples are taken, indicating that this is a technically challenging [91–93,114–116].

Polymerase chain reaction (PCR) is an effective supporting investigative technique which involves amplifying DNA from corneal samples to identify the offending pathogen. Multiplex PCR allows for testing multiple pathogens from one sample simultaneously.

Next-generation sequencing (NGS) is a set of advanced technologies designed for efficient DNA sequencing. In current times, such technologies have been used in combination with bioinformatic analysis to compare DNA samples with extensive reference genome sequence databases. This development has the potential to identify the cause of infection quickly and precisely, as well as the antimicrobial susceptibility properties of the identified pathogen. One of the primary benefits of genome sequencing is that it overcomes the limitation of PCR, which requires a pre-existing clinical suspicion to identify the appropriate primer sets for detecting a suspected microbe.

Emerging technology in this field is rapid genome sequencing for bedside diagnosis of infections. Oxford Nanopore Technologies have developed its technique which uses untargeted metagenomic sequencing to identify the causative organism. It overcomes some of the limitations of gene sequencing for diagnosis such as portability, cost and time. Its utility has been demonstrated in human studies of infection and recently in an invitro model of microbial keratitis, and it awaits further field validation [117].

1.3.6 Management

The most important step of initial management is the commencement of intensive topical broad-spectrum antimicrobials to initiate wound sterilisation. In the subsequent phase, therapies facilitate the cessation of inflammation and support the healing of the wound. These include intense lubrication to decrease trauma from eyelid rubbing, the systemic administration of sub-antimicrobial doses of tetracyclines to inhibit MMPs [118], and supplements like vitamin C that promote tissue remodelling by acting as antioxidants and free-radical scavengers [119]. Importantly, topical corticosteroids are administered to control destructive inflammation and reduce the severity of corneal scarring. Their use is contentions with few well-designed studies with limited evidence for their effectiveness [120–124].

The development of novel anti-infective agents, particularly those that circumvent anti-microbial resistance, are vital for the progression of the field. One such advancement is using ultraviolet (UV) assisted corneal-cross linking for sterilisation. More commonly used to treat corneal ectasia, the anti-microbial effect of this technology is through disruption of microbial DNA and free radical production. Its

clinical evidence to date has not identified it as an alternative to conventional microbial therapy, but further studies are awaited, and it nevertheless holds potential for corneal ulcerations [125].

The use of antimicrobial peptides is another promising development in the management MK [126]. Naturally released by host immune cells during infection, have broad antimicrobial activity [127]. Whilst experiments of exogenous antimicrobial peptides have demonstrated benefit in preclinical studies, translation of the technology to clinic stages study is limited by the complex protein structure-activity relationships, toxicity to host tissues and limited half-life due to host proteases [128]. However, the study of hybrid peptides to subvert these limitations holds promise for their entry into clinical study [129].

Where treatment is not successful, complications such as persistent epithelial defects, corneal perforations, endophthalmitis and phthisis bulbi can occur, all devastating to sight [91–93,114–116]. Such complications often require further specific management steps for example, temporary tarsorrhaphy or amniotic membrane transplantation, to facilitate healing. Where a perforation occurs, the patient may require corneal gluing or tectonic corneal grafting [130]. Even after successfully eradicating the infection, patients often experience significant corneal opacity and astigmatism that can compromise their visual acuity, especially if it affects the visual axis. In this scenario, only surgical options can currently restore vision.

1.3.7 Steroids for microbial keratitis

The use and timing of steroids for corneal ulcers is contentious due to the need to balance the risks of exacerbating infection against the benefits of reducing inflammation and scarring. The body of literature on human studies on the topic is relatively small. Only one large-scale clinical trial – the Steroids for Corneal Ulcers (SCUT) trial – has been completed, among other smaller studies [112,121,122,131,132].

The SCUT RCT recruited 500 patients and divided them into either topical corticosteroid (prednisolone phosphate 1%) or placebo commenced 48 hours after the commencement of the antibiotics. Their primary outcome measure was visual acuity, which did not differ between the two main groups at three months. However, subgroup analyses revealed a small benefit in the vision for those with worse starting acuity (vision worse than counting fingers at presentation). Rates of corneal perforation did not differ between the main groups either, indicating that the treatment was safe.

Three main discussions came from the studies in question, the first being about the true value of corticosteroids in reducing scarring, as the trial did not demonstrate a resounding effect. The second regarding the conduct of the trial and, more specifically, the outcome assessment utilised. The difficulty with studying corneal fibrosis is that MK lesions are heterogenous and visual acuity offers limited insight into the nature of the scarring. For example, if the lesion is far from the visual axis it may not interfere with vision. In contrast a small central lesion can cause profound morbidity. Interestingly SCUT trial patients with *Nocardia* infection demonstrated a propensity for larger infiltrate/scars without corresponding differences in visual acuity, indicating that 1) visual acuity is not an adequate assessment of corneal fibrosis and 2) the differential microbial response to treatment must be factored into therapeutic strategies.

1.3.8 Evaluating corneal fibrosis

Fibrosis causes light-scatter of incident light passing through the cornea to the retina. Vision can be impaired to varying degrees dependent on the precise morphology of the lesion. Vision is a complex cortical function with components related to cognition [133–135], and comprehensive assessments of its functions are of limited value in the clinical environment. Assessments can be broadly categorised into functional assessment, subjective assessment, objective assessments, patient-reported outcome measures and a combination of the above. Visual acuity is perhaps the most utilised outcome assessment in ophthalmology; however, in assessing corneal fibrosis, this offers limited insight into the disease process. This section discusses the relevant commercially available techniques available.

The quality of one's vision can be characterised through assessments of visual function. Perhaps the most reported outcome measure in ophthalmology is central visual acuity. The most used measures include the Snellen visual acuity and LogMAR chart-based tests, in which sequentially smaller optotypes are presented to the patient until they can no longer discern the target [136]. In the context of corneal fibrosis, contrast sensitivity is of great interest. It is helpful in assessing disorders of optical media opacity which may cause increased light scatter. It is found to be impaired even when visual acuity is not significantly affected [137–139], suggesting that it may help differentiate individuals with milder corneal opacities. Figure 1-5 illustrates the impact of mild corneal opacity on vision.

a) **b)**

Figure 1-5. The impact of mild corneal opacity causing light scatter.

Mild degrees of corneal opacity can cause debilitating visual symptoms, as demonstrated by the increased glare in (b) compared to (a). Adapted from Oculus [140].

Visual acuity assessments generally examine central vision, which may not be involved if a corneal lesion is eccentric to the visual axis. In such cases, assessments of lesion morphology are more helpful in monitoring lesion change. Basic slit-lamp (SL) biomicroscopic examination with measurements of lesion size is useful but suffers from observer-dependent subjective variation.

Small variations in the amount of fibrosis across a lesion may not be easily observable and are difficult to quantify. Imaging devices can generate data less prone to observer subjectivity and offer a degree of rigour that is preferable for research purposes. Corneal light scatter can be objectively measured using light scatter meters. These have mostly been used in research as purpose-built devices. However, Oculus has released a device for commercial use, which has been used in many studies to demonstrate the impact of media opacity [141,142].

Two techniques which maybe be particularly beneficial in measuring smaller changes in corneal lesions are optical coherence tomography (OCT) and Scheimpflug imaging. Such devices are commonly available in ophthalmic departments. Both have shown much better reliability and repeatability than SL examination at reporting corneal lesions [143]. These methods measure the lesion in 2-dimensional images. Oculus' Pentacam Scheimpflug camera has an inbuilt 'densitometry' module, which computes an

averaged value of ‘density’ from a corneal scan, which can be used to monitor changes in lesions morphology [144]. This rapidly generates a global estimate of the optical changes in cornea, making it more comfortable from the patient’s perspective, however it does not differentiate areas of interest in the cornea, thus limiting its application.

Even though both OCT and Scheimpflug imaging have significant potential, currently, no widely adopted technology exists that measures the 3-dimensional characteristics of corneal lesions. Imaging techniques have the advantage of their data being amenable to post-acquisition image analysis techniques, which then lead to novel approaches to the patient care [145].

The challenge for the researcher is to be able to quantify changes in scarring when it is both severe and mild. However, there may be scenarios where the benefits are quantified by other means. Severe scarring will cause significant vision loss. However, it is not known if minor improvements in scarring that equate to small changes in vision (as measured on an acuity chart) would meaningfully improve patients' lives. While there may be some proxy measures of the impact of the disease on the patient's life, e.g. rate of trips/falls, without specifically engaging the patient, the true impact cannot be determined.

Patient-reported outcome measures (PROMs) are an increasingly important parameter in clinical trials, particularly for health technology assessments where the benefit of treatments are considered in direct and more broad terms [146]. One difficulty with PROMs is that they must be designed and validated to measure specific features in a reliable and repeatable manner, which is challenging when measuring an individual's subjective experience. No validated questionnaires exist for MK. However, a number of general vision-related quality-of-life measures have been adopted for use in studies [147]. The most widely known and used is the 25-item National Eye Institute Visual Functioning Questionnaire (VFQ-25) which has also been translated into many other languages [148].

1.4 Developing novel strategies for the prevention of corneal fibrosis

1.4.1 Current and prospective approaches to the management of corneal fibrosis

As discussed earlier, MK is a globally significant disease with complicated socio-political and medical factors influencing its management. These create many potential points of intervention in the patient journey (e.g., prevention). Discussed here are the themes relevant to this thesis, namely in regeneration.

Two broad approaches may be adopted in managing corneal fibrosis: 1) prevention and 2) management of established fibrosis. Preventative strategies orient around modulation of the wound microenvironment by reducing inflammation (e.g., corticosteroids) and creating a pro-regenerative environment (e.g. amniotic membrane grafts). For established fibrosis, functional interventions such as optimisation of refractive error and contact lens use can improve vision in some milder cases [149]. However, definitive management involves surgical removal of the scarred tissue. This can be done with laser ablation for small scars, however, where the scar involves a significant volume of tissue, stromal replacement may be indicated. This involves the replacement of scarred and opaque corneal tissue with healthy donor corneal tissue that is optically clear. Alternatives to allogenic corneal grafts include synthetic protheses such as the Boston keratoprosthesis, comprised of polymethacrylate optic and titanium housing plates [150]. Although corneal transplant and implant techniques are well established, they entail a risk of operative complications and failure. As such, they require access to specialised medical services, donor tissue (of which there is a global shortage), long-term medication, and the ability to engage with medical services long-term [151,152].

The chosen intervention may vary dependent on the pathological context and the risk-benefit trade-off. For example, topical corticosteroid drops, as mentioned in the earlier section, are judiciously used in infective keratitis due to their risk profile. An extensively investigated therapy is Mitomycin-C (MMC), an anti-metabolite cancer therapeutic, which is administered intraoperatively during laser refractive surgery (LRS) to minimise post-operative haze. Its application causes cell death, decreasing the number of corneal stromal cells contributing to the fibrotic response post-operatively. However, it is not considered a routine anti-fibrotic treatment in many other clinical scenarios due to concerns about its toxicity [153–155].

There is a pressing need for a non-invasive and versatile anti-fibrotic treatment. Although several novel candidate strategies have been successful in preclinical studies, none have yet been licensed for treating corneal fibrosis. Furthermore, only two anti-fibrotic drugs in the UK have been approved for human clinical use, pirfenidone and nintedanib, both for pulmonary fibrosis. The body of literature regarding

corneal fibrosis therapeutics dates back several decades and is heterogeneous regarding clinical context, therapeutic mechanism and study design.

Newer approaches aim to direct the host injury response towards regeneration of the damaged cornea by modulating immune and corneal cell responses across all phases of the injury. A number of approaches have been used and are summarised in Figure 1-6. Of note are molecules with broad complex interaction partners with pleiotropic effects, able to influence multiple pathways towards a pro-regenerative environment. In a similar vein, stem cell therapies offer potential to modulate multiple pathways, additionally they may be able to prevent as well as treat corneal scarring [156–159]. The ability of these cells to differentiate into keratocytes and regenerate stromal matrix is central to their role in scar prevention. Work by S.Basu at LV Prasad Eye Institute and J.Funderburgh at the Pittsburgh School of Medicine has demonstrated the ability of these cells to improve corneal stromal scarring in animal models following injury [156,157] and whilst also having good safety profiles in rabbit models [156].

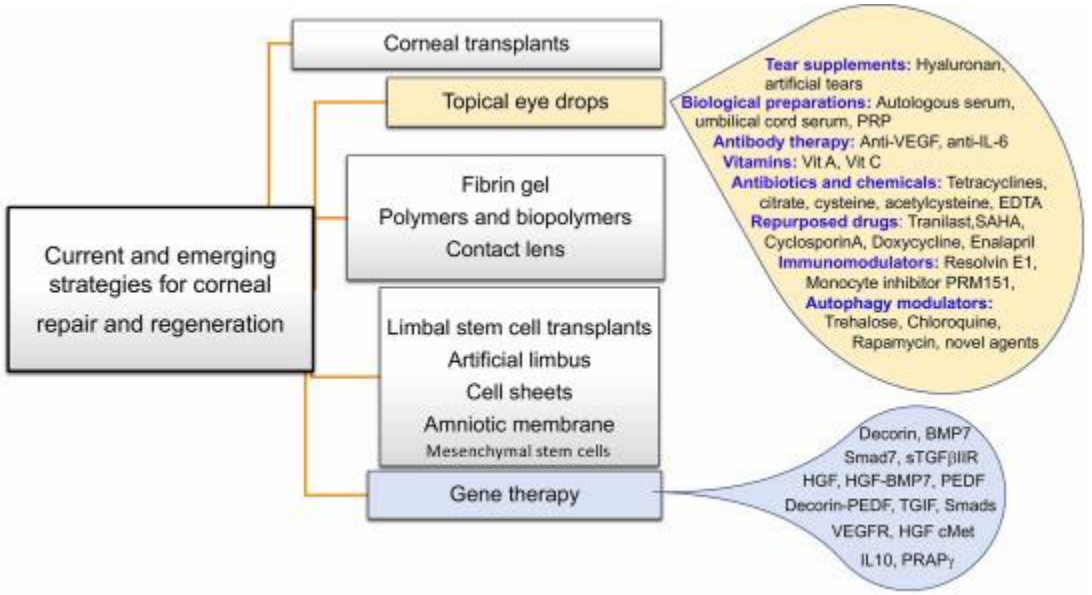


Figure 1-6. Current and emergent therapies in corneal regeneration

Adapted from [160].

1.4.2 Decorin

Decorin is a member of the small leucine-rich proteoglycan (SLRP) family, which are a group of extracellular matrix molecules that are structurally similar through their leucine-rich protein core and linked glycosaminoglycan (GAG) side chain [161]. Several members of the family are expressed within the human cornea; however, decorin and the closely related biglycan are the two most prominent members with regards to the corneal collagen fibrillogenesis [162]. Decorin has been found in most connective tissues and is involved in wound healing. It has numerous binding partners (Figure 1-7) [163], including several growth factors such as TGFβ1, and has been found to contribute to various cellular processes including cell-cycle progression [162].

Decorin contains a core protein with 10-12 leucine-rich repeats [164,165] that give it a horseshoe shape which facilitates its interactions with other proteins and receptors [166] (Figure 1-7). The core protein is capped by cysteine residues at both ends. The carboxyl terminus then resides beyond one cysteine residue, and in the other direction beyond the cysteine residue, one chondroitin sulfate or dermatan sulfate side GAG chain may be attached, covalently bound to the amino terminus [164,165]. Such GAG chains can also be found at the cell surface and throughout the ECM, where they have multiple structural functions [165]. The broad interactivity of the GAG chains and core protein confer this molecule many opportunities for interaction. The core protein portion of decorin can directly interact with collagen independent of the GAG chain [167]. Through this interaction, it is able to promote the formation of homogenous collagen fibrils with a smaller diameter [168]. In addition, the GAG chain helps to regulate the fibril spacing [169]. Thus, decorin facilitates organised construction of the cornea. Decorin has also been found to decrease the susceptibility of collagen I and III to cleavage by metallomatrix proteins 1 and 13, potentially helping to preserve corneal architecture during inflammatory reactions [170]. Where these interactions or structuring are interrupted then irregularities in corneal ultrastructure occur (Figures 1-2 and 1-8.

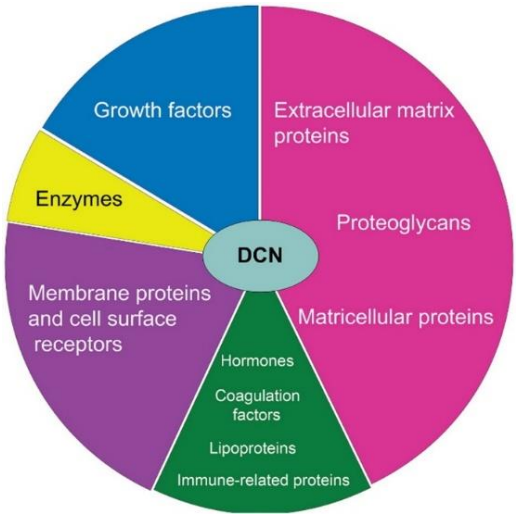


Figure 1-7 Decorin binding partners. *Adapted from* [166].

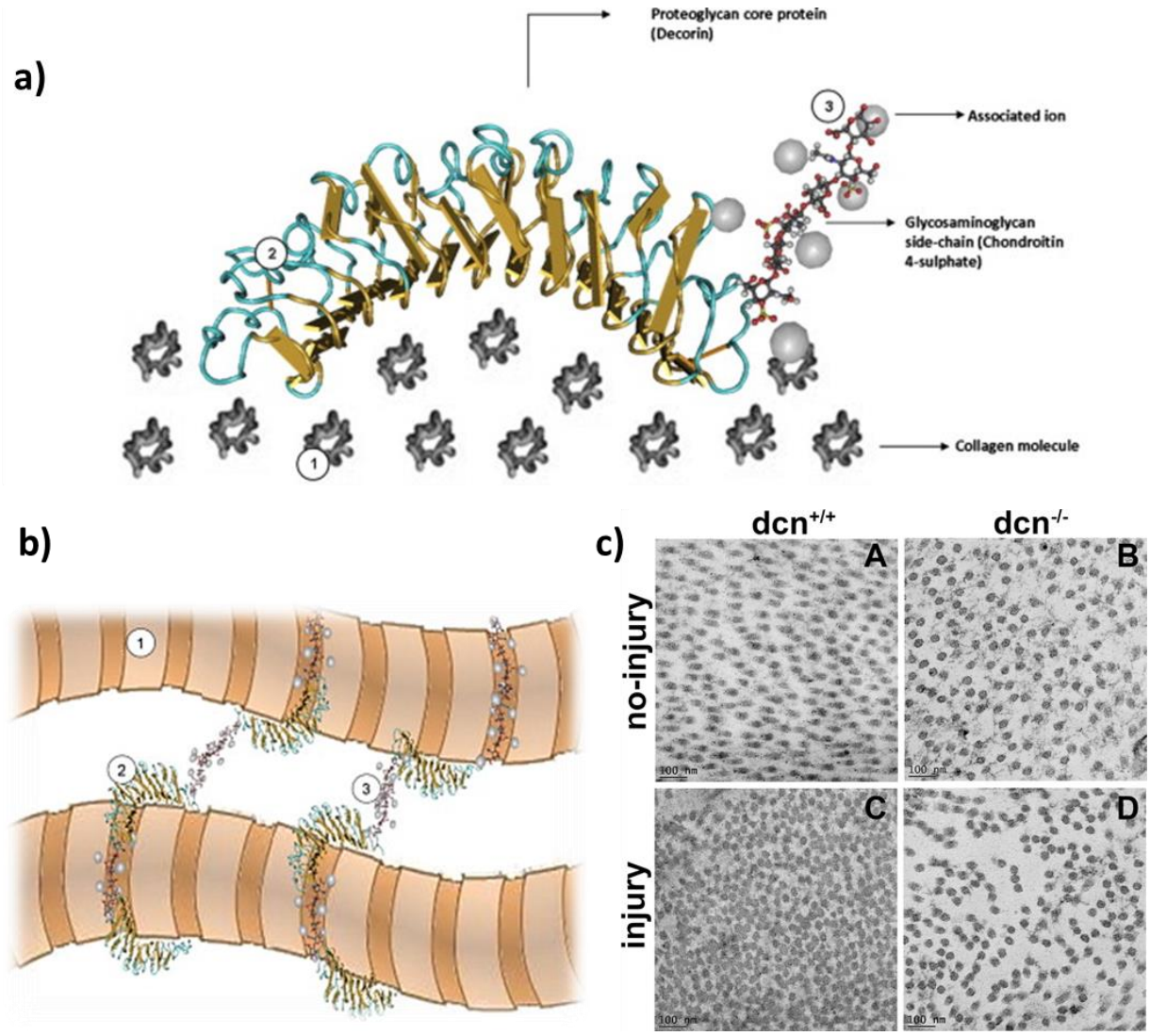


Figure 1-8. Decorin and its interaction with collagen

a) Diagram of decorin's structure [171]*. The collagen fibrils (1) and core protein (2) are not to scale, however it represents their interaction. The gag-chain (3) helps regulate spacing between fibrils diagrammatically represented (b). (c) - transmission electron microscopy images from wild type (dcn+/+) and decorin null (dcn−/−) mice with and without injury* [172]*. At day 21 post injury collagen fibrils in cross section can be seen to vary in size and organisation, worse following injury and lacking decorin. Magnification x25,000, scale bar = 100 nm. Adapted from* [173].

1.4.2.1 Decorin in corneal disease

In corneal wound healing, decorin is upregulated in corneal stromal cells at the wound edge [174]. Its absence in gene knock-out decorin null mice leads to impaired stromal collagen arrangement following injury (Figure 1-8). In inflammation, decorin's effect is found to be cell and context dependant. For example, it promotes macrophagy mediated inflammation via TLR 2 and 4 whereas its downregulation of TGFβ1signalling is found to lead to decreased production of the anti-inflammatory IL-10 [175]. Its role in fibrosis is largely thought to orient around decreasing TGFβ1 signalling. The core protein can bind TGFβ1, however other mechanisms of TGFβ1 signalling interference have been postulated, including the modulation of TGFβ1 regulators (e.g. myostatin) [164,176–179].

Congenital stromal dystrophy is an autosomal dominant disorder characterized by bilateral stromal opacities at or shortly after birth with an abnormally thick cornea. A frame shift mutation leads to the expression of a truncated decorin protein and resultant opacification due to disorganised collagen production and decorin aggregation [180,181]. Decorin transfection of human corneal fibroblasts has been found to reduce αSMA expression in response to TGFβ stimulation [182]. In a rabbit model of laser-induced corneal fibrosis, the same group reported the benefit of decorin transfection demonstrated through a reduction in αSMA, F-actin and fibronectin expression [183].

1.4.3 Decorin therapy

1.4.3.1 Decorin enables scarless regeneration of the cornea after microbial keratitis: a preclinical study

Our group was the first to demonstrate the potential of topical human recombinant decorin in a murine model of *Pseudomonas* aeruginosa keratitis, a paper on which I was co-author [184]. It was formulated into a novel ocular dressing composed of a gellan gum fluid gel, termed gel-plus, which is able to persist on the ocular surface longer than conventional eye drops [185]. Decorin 0.24 mg/ml in gellan was applied four times a day alongside gentamicin (1.5%) and prednisolone phosphate (0.5%) and compared to a hydrogel only (gel-minus), and a control group who received only the gentamicin and prednisolone. At 16 days post infection the gel-plus group had significantly lower corneal opacification areas on anterior segment photography compared to the other two groups. Histologically the structure and organisation of the cornea and stromal extracellular matrix were also significantly improved at the end of the study at 16 days (Figure 1-9).

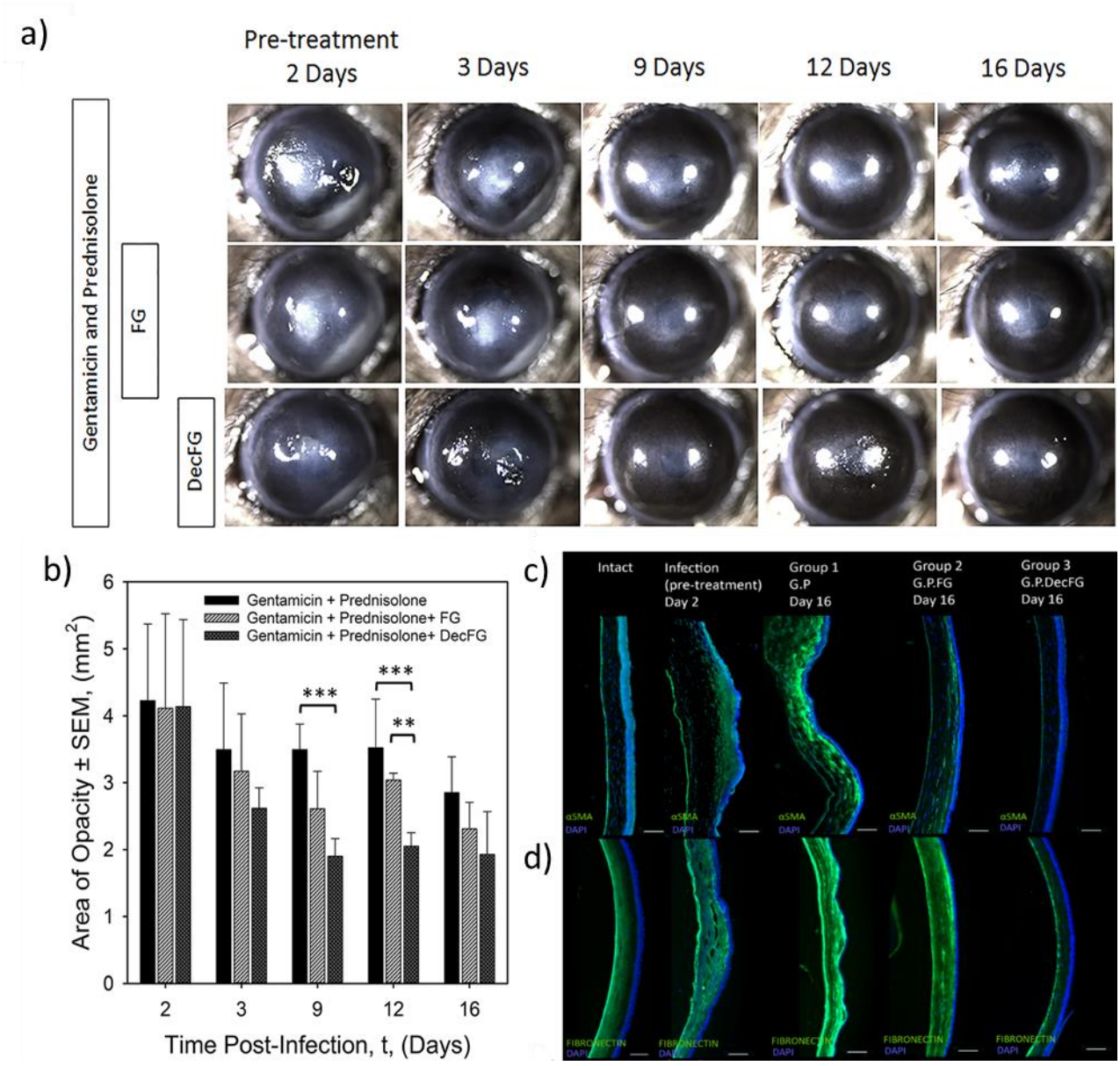


Figure 1-9. Effects of standard-of-care, decorin and gellan fluid gel treatment in a mouse model of P. aeruginosa keratitis.

*a) Representative photographs taken at days 2, 3, 9, 12, and 16 post Pseudomonas infection and treatment b) Graph to show the mean area ± SEM (mm^2) of opacity as measured by two independent masked ophthalmologists from photographs taken from each individual mouse per group (n = 6; **p < 0.01, ***p < 0.001).*

c & d) Fibrotic protein expression in cells and extracellular matrix following treatmentRepresentative immunofluorescence images of (c) αSMA (green to stain myofibroblasts), (d) fibronectin (green to stain fibronectin in the ECM). Adapted from [54].

Abbreviations G – gentamycin 1.5%, P – prednisolone 1%, Dec- decorin, FG – fluid gel.

1.4.3.2 Decorin gene therapy

Gene therapy is an exciting approach because inducing host tissue to express therapeutic proteins bypasses many of the difficulties of producing these proteins synthetically [186]. Professor Rajiv Mohan's lab has been at the forefront of many advances in the field and continues producing interesting results. They have tested a range of viral vectors, candidate therapies and alternate vectors [160] and have demonstrated encouraging results transfecting genes for SMAD-7, decorin and human leukocyte antigen G, among other candidates, in various animal models, with promising efficacy and safety [183,187]. The entry of such treatments into the clinical phase is an appealing prospect.

1.4.3.3 A systematic search of Medline for other animal trials

In vivo studies of decorin are numerous, however, few have investigated it in the cornea. Table 1-3 summarises all the studies in the literature found through a systematic search of the Medline database. Two groups, Professor Rajiv Mohan's lab, University of Missouri) [183,187–189] and ours [54,185,190–192], were found to contribute to all the studies. The pathological context (injury model) and treatment regimens vary across the studies, as do the follow up periods. Generally, decorin therapy is efficacious and safe. No toxicity was reported. This is particularly interesting to note in the 6-month safety study of decorin gene therapy in healthy mice.

1.4.3.4 Ocular dressing for the Prevention of Corneal Scarring in Microbial Keratitis – OPtiCS MK

In light of the potential of decorin to treat corneal blindness in patients with MK, OPtiCS MK was devised as the early phase trial of this therapy and is discussed in detail in Chapter 6.

Table 1-3 Results of a systematic search of the Medline database for the use of decorin in corneal pathology trailed in animal

Study		Treatment	Formulation	Dosing	Arms	N per arm	Final follow up (post injury)	Injury model	Animal	Main target	Outcomes
Galacorin											
Sustained release of decorin to the surface of the eye enables scarless corneal regeneration. [193]	1	Galacorin 4.76 mg/ml	Gellan	X4/day for 14 days	Gel + vs gel – vs soc	6	16 days	*P aeruginosa* keratitis	C57bl/6 mice	Fibrosis	• AS photos • IHC
A self-healing hydrogel eye drop for the sustained delivery of decorin to prevent corneal scarring. [185]	2			X3/day for 1 day	Decorin sol vs saline vs gel - vs gel +	3	2 hours	Healthy	Rats (anesthetised)	Retention time	• Gel retention using oct
The neuroregenerative effects of topical decorin on the injured mouse cornea. [190]	3		Gellan or saline	X3/day for 7 days	Decorin sol vs saline vs gel - vs gel +	8	7 days	2mm burr	C57bl/6j mice	Neuroprotection	• OCT for CCT • Confocal for nerves • Whole-mount
The effect of topical decorin on temporal changes to corneal immune cells after epithelial abrasion. [191]	4	Galacorin 1.07 mg/ml (dose escalation at 0.24, 1.07 and 4.76 mg/ml)	Saline 0.9%	** X3/day for 7 days	3 x doses expts; main expt decorin vs saline vs no injury control	8	7 days	2mm burr	C57bl/6j mice	Epithelium and inflammation	• OCT for CCT • Confocal for nerves • Whole-mount
Topical decorin reduces corneal inflammation and imparts neuroprotection in a mouse model of benzalkonium chloride-induced corneal neuropathy. [192]	5	Galacorin 1.07 mg/ml	Saline 0.9%	X3/day for 7 days	Decorin vs saline vs no injury control	7	7 days	BAK neuropathy	Mouse	Inflammation and neuroprotection	• OCT for CCT • Confocal for nerves • Whole-mount
AAV5 vector Gene therapy											
Targeted decorin gene therapy delivered with adeno-associated virus effectively retards corneal neovascularization in vivo. [187]	6	Aav5-decorin Single topical application of aav5 (100 µl; 6.5×10^{12} µg/ml) onto the bare stroma for 2 minutes			Aav5-decorin vs aav5-gfp	Not clear	14 days	VEGF pellet implantation	New zealand white rabbits	Neovascularisation	• AS photos • IB, IHC, PCR
Significant inhibition of corneal scarring in vivo with tissue-selective, targeted aav5 decorin gene therapy. [183]	7				Aav5-decorin vs aav5-gfp	12	28 days	PRK fibrosis		Fibrosis	• Haze score, Stereo • IHC. IB, Tunel assay
Six-month in vivo safety profiling of topical ocular aav5-decorin gene transfer. [194]	8				Aav5-decorin vs aav5	6	6 months	Healthy		Safety	• Toxicity score* • SL/stereo, IVCM, IOP, CCT, tears • PCR, IHC
The functional role of decorin in corneal neovascularization in vivo. [188]	9				Decorin deficient mice	10	21 days	Alkali burn injury	C57bl/6j decorin-deficient mice	Neovascularisation	• SL/stereo • IHC/IMF, PCR

C57BL/6 mice (Jackson Laboratory, CA, USA); C57BL/6J decorin-deficient (Dcn−/−) mice were generated from a breeding pair kindly provided by Professor Renato Iozzo, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA); New Zealand White rabbits (Myrtle Laboratories Inc., Thompson's Station, TN); IVCM – in vivo confocal microscopy; IHC – immunohistochemistry; AS photos – anterior segment photos; CCT – central corneal thickness; PRK – photorefractive keratectomy; BAK – benzylalkonium chloride; OD – once daily; SOC – standard of care; Topical BAK, od, 7/7 - neuropathy model; IOP – intraocular pressure (using tonopen); CCT using pachymeter or OCT – optical coherence tomography; VEGF – vascular endothelial growth factor; IMF – immunofluorescence; SL/Stereo – Slitlamp / Stereomicroscopy; Tears – measured using Schrimer's test; Haze score – qualitative scoring of corneal haze; Whole-mount – IMF for immune and nerve cells; IB – immunoblot

** Modified Hackett-McDonald ocular scoring system for toxicology profiling validated in rabbits*

*If multiple experiments were described in the study, then it is indicated by ** and the regime with the highest decorin exposure is described*

1.5 Research question, aims and objectives of the thesis

1.5.1 Research question

1.5.1.1 Primary research question

What is the role for decorin in the management of corneal fibrosis secondary to microbial keratitis?

1.5.1.2 Additional research question

Given that this clinical trial has been delayed indefinitely due to the COVID-19 pandemic, a further research question was devised to advance the development of this study.

What have we learnt from the COVID-19 pandemic about caring for MK patients and conducting clinical research on patients with corneal fibrosis?

1.5.2 Aims and objectives

1) Review the landscape of treatments for corneal fibrosis trialled in humans.
2) Confirm the novel association of decorin, autophagy and corneal fibrosis.
3) Conduct the first into-man clinical trial of decorin in a gellan hydrogel for the prevention of corneal scarring in microbial keratitis patients.

1.5.3 Thesis overview

The purpose of this thesis is to evaluate the following:

i) Chapter 2: A systematic scoping review of medical therapies for corneal fibrosis trialled in humans was performed to characterise the literature, assess for competitors, understand the therapeutic mechanisms being leveraged and to understand the gaps in the literature. It also reviews the landscape with regard to study design, population and outcome measures in order to inform optimal study design for future researchers.

ii) Chapter 3: An *in vitro* study of the novel association of decorin in autophagy and corneal fibrosis in primary human cornea fibroblasts.

iii) Chapter 4: Forms the first part of a study exploring the impact of the pandemic on the broader development of decorin for human study. This was a web based survey study of the public's perceptions of eye symptoms, health seeking behaviours and willingness to volunteer for ophthalmic research. I was the first author in the peer-reviewed publication of this study [195].

iv) Chapter 5: forms the second study of the impact of the pandemic on this body of work. This study of MK patient clinical features at presentation and final follow up specifically explored whether the pandemic had negatively impacted these patients. I was the first author in the peer-reviewed publication of this study [196].

v) Chapter 6: describes the design and discusses the key considerations and implications of the OPtiCS MK clinical trial, which was unable to open and continues to be delayed because of the disruption caused by the COVID-19 pandemic. It covers:

- Regulatory and design consideration
- Clinical trial design with regards to safety

- How early phase trials can be optimally utilised to generate insights for further drug development what experimental samples can be taken to evaluate the mechanism of disease

2. Chapter 2: A systematic scoping review of medical therapies for corneal fibrosis trialled in humans

2.1 Introduction

Recently Kwowk et al. published a systematic review (SR) summarising the literature regarding therapeutics for corneal fibrosis since 2010, which included studies in humans, animals and in vitro [197]. They identified 12 papers that met their inclusion criteria including conventional therapies such as MMC and steroids as well as a number of preclinical candidates such as gene- and cell-therapies. Their study was evaluated using "A measurement Tool Assess systematic Reviews 2" (AMSTAR 2) for its reliability [198], and found to score poorly (Supplementary table 1), largely due to a miss-match between study aims and study design. Whilst not strictly adhering to the definition of a SR, their insightful summary identified important and valid questions about the field, such as the translational potential of various therapeutics. The field of corneal regeneration therapies is broad and there is not a singular treatment of choice, nor is there a consensus regarding the optimal therapy(ies) for corneal fibrosis. By characterising and summarising the field, much can be understood about the features of the studies and gaps in the literature, thereby informing future investigations of candidate treatments. Taking into consideration the heterogenous nature of the literature, and the limitations of previous studies, particularly regarding the search date range, databases and inclusion/exclusion criteria, an appropriate knowledge synthesis approach must be adopted.

A systematic scoping review (SScR) is the preferred methodological approach to capture the breadth of the field where the aim is to identify the types of available evidence, identify knowledge gaps, characterise the key features and conduct of studies. They differ from systematic reviews/meta-analyses which are designed to rigorously assess the available evidence in order to answer a specific question on topics such as treatment efficacy, diagnostic accuracy etc. SScR's may be performed as a precursor to SRs, as they aim to qualitatively report on broad field of study and are best as mapping investigations [199].

SRs and SScRs have some methodological similarities in defining a study question, establishing a search strategy, assessing study eligibility, and analysing and synthesising. Whilst the first few steps are similar, their considerations for study inclusion differ based on the analysis and synthesis needs. For example, this chapter will explore the literature regarding all treatments for corneal fibrosis trialled in humans. One aim of this chapter is to find out what therapies trialled for all the different pathologies. This is to better

understand the competitor landscape and in the range of pathologies these have been trialled. A SR would be better suited if the aim was to compare the efficacy of treatments. In that case the study would have to define outcomes that might be comparable across studies, the homogeneity of the patient populations, underlying pathology, interventions, study designs and other features that may impact the result. Therefore, a SR does not address the broad aims of this chapter.

2.1.1 Considerations for study design

Since the aim was to characterise the breadth of literature, the search strategy and screening process included studies in which corneal fibrosis was not the primary outcome. For example, a study of epidermal growth factor may consider 're-epithelialisation' as the phenomenon of interest, but corneal fibrosis may also be beneficially impacted, and reported in the study. Therapies were included if they were thought to act either directly on corneal fibrotic pathways or indirectly to improve fibrosis through other pro-regenerative mechanisms. However, such studies had to report an assessment corneal fibrosis to be included. Since visual acuity is a widely used outcome which is not a specific measure of corneal fibrosis and is variably affected dependant on the location of the corneal opacity; studies were included if they reported a corneal fibrosis specific outcome measure in addition to, or instead of visual acuity. As optimal wound healing and/or regeneration are the focus of this study, surgical procedures that physically remove and replace tissue e.g. ocular surface reconstructions and keratoplasties were excluded from analysis. Whilst multiple databases must be searched, only peer-reviewed papers with available full-texts, will be included for analysis, to reduce the reporting of less significant studies which have little impact on the field.

Terminology referencing corneal fibrosis varies significantly in the literature. Whilst all having some overlap in meaning their differences implicate differing phenomenon. For example, LRS studies consistently refer to stromal changes as "corneal haze" and find it to be bimodal. This terminology refers to the optical change in the cornea's appearance, becoming less clear. In the early post-operative phase this may be contributed to by cellular infiltration and corneal oedema as well as fibrotic protein deposition, whereas in the late phase it is more likely extracellular matrix (ECM) change causing the change in optical appearance [200]. Similarly, corneal opacification refers to the optical phenomenon where there is loss of clarity of the cornea, which may be due to a number of aforementioned reasons. By contrast cornea fibrosis specifically refers to, or matters related to, the process of fibrosis within the cornea. This change may be of varying severity, thereby impacting corneal optical clarity to a greater or lesser degree. The specific shape of a lesion (or lesion morphology) may determine the precise distribution

of this optical abnormality. As such the optimised literature search strategy will be designed to account for this breadth in terminology.

2.1.2 Aims

The aim of this review was to systematically characterise the literature regarding therapies for corneal fibrosis trialled in human patients. The range of therapies tested are the primary interest. Features of secondary interest include the study design and outcomes used, and the pathological contexts within which these therapies have been trialled.

2.2 Methods

2.2.1 Protocol

This study's design was guided by the Joanna Briggs Institute recommendations for scoping review conduct [201] and followed the Preferred Reporting Items for Scoping reviews reporting guideline [202].

2.2.2 Research Question/area:

What are the characteristics of the existing research on therapeutics for corneal fibrosis in human participants following corneal stromal injury?

2.2.3 Eligibility Criteria

To define the breadth of the study, including the inclusion criteria and search terms, a "population, concept/intervention, pathological concept, study design" approach was used, adapted from the "population, intervention, comparator, outcome" (PICO) approach for systematic reviews [199,201].

2.2.3.1 Inclusion criteria

Population

- Humans

Concept - Intervention

- Therapeutic to manage specifically corneal fibrosis, which may act either directly on corneal fibrotic pathways or indirectly improve fibrosis through other pro-regenerative mechanisms

Pathological context

- Any acquired pathology causing stromal injury and consequent fibrosis
- Any treatment, including small molecule, biologic, cell, or genetic therapies

Study design

- All study designs (prospective and retrospective, interventional and observational studies)
- All published peer-reviewed studies
- Any date

2.2.3.2 Exclusion Criteria

Population

- Non-human studies

Concept - Intervention

- Lubricants and antimicrobials which may be given in parallel to other treatments
- Surgical procedures involving the removal of tissue
- Surgical procedures that transplant or implant replacement stromal tissue or prostheses
- Ocular surface reconstructive procedures (e.g. limbal transplants)
- Mechanical interventions – ptosis + bandage contact lenses etc

Pathological contexts

- Congenital opacities, dystrophic corneal disorders, genetic disorders causing metabolic abnormalities which are managed by enzymatic replacement
- Pathologies not primarily corneal stromal for example adenoviral infiltrates and salzman nodular degeneration
- Limbal epithelial cell failure of any cause, leading to corneal fibrosis for example chemical injury, Stevens Johnson syndrome, ocular mucous membrane pemphigoid however treatments for chemical injuries in the acute phase (first 7 days) will be included

Study design

- No corneal fibrosis specific outcome assessment reported (in addition or instead of visual acuity)
- No language restrictions will be applied in the literature searching phase, however only studies in English will be used for data collection and analysis

2.2.4 Information Sources

Relevant studies will be sought through comprehensive searches of databases and repositories (listed below). Where articles are not accessible or further information is required, authors will be contacted for further information. A time limit of three weeks will be permitted for a response following which the study will progress to the next stage. These databases were selected as they index the majority of scientific journals globally [203]. The major clinical trials databases were included to ensure thorough exploration of human clinical trials.

Bibliographic databases:

- EMBASE
- MEDLINE
- CINAHL
- SCOPUS

Web of Science Core Collection + supplementary collections

- Clinical trials registers:
- Cochrane
- ClinicalTrials.gov
- EduraCTR

2.2.5 Search Strategy

A comprehensive search strategy was developed using the Ovid portal and refined for EMBASE and MEDLINE, taking into consideration the use of MeSH and EMTREE terms. This was derived from the terms and concepts identified in the eligibility criteria using a "population, concept/intervention, pathological concept, study design" approach.

Studies of any design were eligible. The strategy combined the concepts of corneal fibrosis and any "treatment" for it. Since treatments may have been administered for specific pathologies (e.g. acyclovir for herpetic keratitis) a second component of the search strategy listed possible causes of corneal fibrosis and combined these with the concept of "treatment" (e.g. "herpetic keratitis" and "treatment"). All possible iterations of such terms were included (e.g. (("herpes simplex" and "keratitis") or "herpetic keratitis") and ("treatment" or "therapy")). Truncations were used to make the strategy more efficient

and to help with capturing frequent potential typographic errors that could be made by the author (e.g. “therap*” to capture “therapy”, “therapies” or “therapes”).

This was then adapted and optimised for the remaining sources. Where a database used standardised indexing terminology, this was utilised to optimise the search for those databases respectively. Supplementary figure 1 is an example search strategy, in this case from MEDLINE with corresponding results. All results from all the databases were obtained from their earliest work up till 17th March 2021.

2.2.6 Data management

Mendeley was used to manage references.

2.2.7 Study Selection and Data Extraction

Initial screening for suitability was performed using the title and abstract, followed by full text screening and then data extraction. Two independent observers, GB and JW, performed each of these stages. Discrepancies were discussed and where required a third adjudicator made a final decision about any disagreements.

Data were extracted into a piloted form by the observers independently. The extracted study characteristics included publication year and type, study location, study design, details of the population, intervention, the pathological context and the outcomes assessed. Where data was not available from the publication, the authors were contacted for further details. A time limit of 3 weeks was given for responses, after which the study progressed to the next stage.

2.2.8 Quality assessment statement

Since a systematic scoping review aims to characterise the literature, as opposed to appraising it, evaluation of study quality does not serve the aim of this methodology and therefore were not performed [201].

2.2.9 Data Synthesis

Data was tabulated and a narrative synthesis of all included studies was made. Summary tables for the studies were generated with studies grouped according to design, interventions and pathological context for comparative analyses [201].

2.2.10 Reporting

The review and its findings were reported in accordance with the PRISMA guidelines [202]. The implications of the review findings will be discussed. The full data in the extraction table has been made publicly available to view online at the following web address: https://figshare.com/articles/dataset/A_systematic_scoping_review_of_therapies_for_corneal_fibrosis_trialed_in_humans/22320622

2.3 Results

2.3.1 Search results

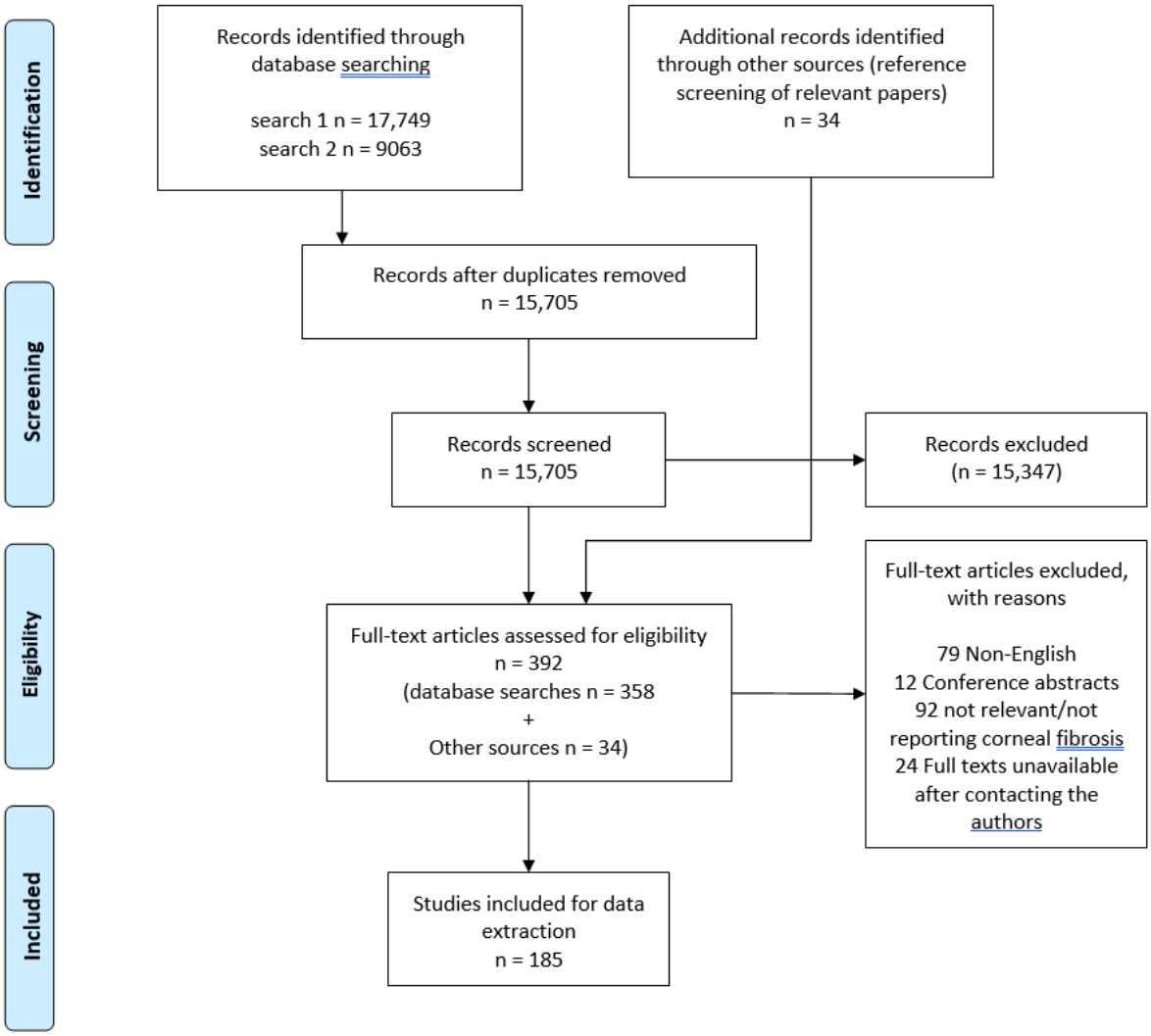


Figure 2-1. PRISMA flow diagram of study selection

Database searching yielded 15,705 unique records which were entered into the study. The PRISMA record flowchart describes the outcomes of the screening stages, Figure 2-1. Data were extracted from 185 published peer-reviewed papers. Whilst the majority of papers reported the results of single studies, there were instances of papers reporting sub-analyses of larger studies as in the case of the 'Steroids for corneal ulcers trial' series. Studies originated from Australia (n=5), Asia (n=31), Europe (n=38), the Middle East (n=25), South America (n=5) and North America (n=81). Regarding the year of presentation, one study was published prior to 1990, 18 were published between 1991-2000, 75 between 2001-2010 and 91 from 2011 to 2021.

Over 40 different therapeutics were identified including small-molecules, growth-factors, immunotherapies, blood, tissue, and cell therapies (Table 2-1). The full list of included papers and their summaries can be found at this repository: https://figshare.com/articles/dataset/A_systematic_scoping_review_of_therapies_for_corneal_fibrosis_trialed_in_humans/22320622. The majority of studies were of Mitomycin-C (MMC) in the context of scar prevention following laser refractive surgery (LRS). The greatest number of randomised controlled trials (RCTs) were for MMC and corticosteroids. Whilst all the RCTs for MMC were in LRS, steroids were investigated in bacterial keratitis (BK), herpes simplex keratitis (HSK) as well as LRS (Tables 2-2 and 2-3).

Table 2-1. Potential therapies for corneal fibrosis

Therapy		**Number of studies**
Amnion		
	Amniotic membrane extract	2
	AMT	24
	PROKERA	6
Blood product		
	Serum	3
	Plasma	4
Cell therapies		
	Cell therapies	3
Growth factors		
	bFGF	1
	EGF	1
	NGF	1
Immunotherapy		

Table 2-1. Potential therapies for corneal fibrosis		
Therapy		**Number of studies**
	Cyclosporine	3
	Interferon	3
	Tacrolimus	1
Mitomycin-C		64
NSAIDs		
	Acular	1
	Diclofenac	3
	Flubriprofen	1
	Indometacin	2
	Ketorolac	2
	Nefapenac	1
Steroids		
	Dexamethasone	5
	FML	10
	Loteprednol	4
	Prednisolone	12
	Triamcinolone	1
Other	Ascorbate	4
	Cacicol/RGTA	5
	CMHA	1
	Cooled saline	4
	Cytochrome c peroxidase	1
	Heparin	1
	N-acetyl cysteine	1
	Oxygen	2
	Plasmin inhibitor	1
	Co-Q10	1
	Thiotepa 0.05 or 0.075%	1
	Umbilical cord patch	1
	Vit a	2
	Vit e	2

Abbreviations: MT – amniotic membrane transplant; BCL – bandage contact lens; bFGF – basic Fibroblast growth factor; CMHA - thiolated carboxymethyl hyaluronic acid liquid-gel; EGF - Epidermal growth factor; FML – fluorometholone; NGF - Nerve growth factor; NSAID – non-steroidal anti-inflammatory drugs; RGTA – matrix regenerating agent.

Table 2-2. An overview of therapy categories and study designs

	Meta-analysis	Randomised Controlled trial	Controlled Trial	Fellow Eye study	Single arm trial	Cohort study	Case controlled	Case Series	Case Report	Other
Amnion	1	1	2	1	3	0	1	15	8	1
Blood product	0	1	1	0	0	0	2	3	0	1
Cell therapy	0	0	1	0	2	0	0	0	0	0
Growth factor	0	3	3	0	1	0	0	0	1	0
Immunotherapy	0	3	3	0	1	0	1	0	1	0
Mitomycin C	2	7	11	12	7	6	12	17	6	1
NSAIDs	0	3	3	2	0	0	1	0	0	1
Other	0	2	5	5	1	0	5	5	3	0
Steroid	3	10	11	2	1	3	4	3	1	6

Table 2. Fellow eye studies were encountered in laser refractive surgery and were defined as prospective trials where each of the patient's eyes received a different treatment, thereby acting as experiment and control arms. Other study designs for Amnion, blood product and NSAIDs was a mixed study design with retrospective and prospective groups. Other study designs for steroid – all are Steroids for Corneal Ulcers Trial sub-analyses.

Table 2-3. A summary of therapy categories and pathology investigated

	Amnion	Blood product	Cell therapy	Growth factor	Immunotherapy	Mitomycin C	NSAIDs	Other	Steroid
Acute chemical injury	6	2	0	0	0	0	0	2	1
AK	1	0	0	0	0	0	0	1	2
BK	10	0	0	0	0	0	0	1	14
Fungal keratitis	1	0	0	0	0	0	0	0	0
HSK	3	0	0	0	2	0	0	1	1
Keratitis	1	2	0	0	1	0	0	1	1
LASEK/LASIK	1	1	0	0	1	9	1	2	0
MK	3	0	0	0	0	0	0	2	0
NK	0	1	0	1	0	0	0	1	0
Other	2	0	2	0	1	3	1	0	2
PED	2	0	0	1	0	0	0	2	0
PRK	2	1	1	3	1	49	5	11	12

Abbreviations: AK – acanthamoeba keratitis; BK – bacterial keratitis; HSK – Herpes simplex keratitis; LASEK – Laser epithelial keratomileusis; LASIK – Laser-assisted in situ keratomileusis; MK – microbial keratitis; NK – neurotrophic keratitis; PED – persistent epithelial defect; PRK – photorefractive keratectomy.

Studies reported a range of corneal pathologies (Table 2-4) of which LRS was the most common. The vast majority of interventions aimed to prevent corneal fibrosis. Only seven of the included reports investigated a therapy in established corneal fibrosis. These comprised: two reports in keratoconus patients receiving stem cell therapy [204,205]; a report on corticosteroids in LRS upon the onset of haze 4-12 months post-operatively [206]; a report on interferon alpha-2b in vernal keratoconjunctivitis [207]; a report on sub-conjunctival triamcinolone and bevacizumab in blepharoconjunctivitis [208]; a report on topical indomethacin in patients with established fibrosis of various causes [209]; and a report on nerve growth factor in a neurotrophic cornea [210].

The pathologies varied considerably in their disease course. For example LRS and bacterial keratitis are typically a single event, however the majority of the other pathologies, such as NK and HSK, have protracted or recurrent-remittent disease courses. All studies investigated interventions within the context of a single episode (as opposed to multiple episodes). This is significant when considering the timing of interventions and suitability of study design. For example, in HSK corneal scarring may increase over the course of years following recurrent episodes. Thus an understanding of patients' pre-morbid corneal clarity and visual acuity are important in order to avoid confounding from significant pre-existing morbidity skewing the results.

The follow-up periods varied significantly across the included studies (range 4 days – 5 years). Where follow-up was short, it significantly impacted the generalisability of study results, although this varied somewhat with the pathological context. For example, in LRS NSAIDs were used primarily for post-operative pain but also reported corneal haze.

Table 2-4. List of pathologies in which potential treatments for corneal scarring were trialled
Acute chemical injury
Acanthamoeba keratitis
Bacterial keratitis Varying severity and progression
Herpes Simplex Keratitis Stromal, necrotising/ non-necrotising
Fungal keratitis
Keratitis - other Idiopathic interstitial keratitis Sterile ulcer Sterile diffuse lamellar keratitis (post LASIK)
Neurotrophic keratitis

Table 2-4. List of pathologies in which potential treatments for corneal scarring were trialled
Various aetiologies
Persistent epithelial defect Various aetiologies
Laser refractive Surgery PRK (Including repeat procedures in complicated, scarred, and regressed cases), LASEK, LASIK (including EpiLASIK)
Other Local anaesthetic toxicity Keratoconus Vernal keratoconjunctivitis Corneal crosslinking Post-pterygium surgery Blepharokeratoconjunctivitis Established corneal scar

2.3.2 Treatments

2.3.2.1 Amniotic Membrane

Human amniotic membrane has pro-regenerative properties and is used to aid healing in many different tissues [211]. Amnion was used in 34 studies, of which 1 was a meta-analysis [212], 3 controlled trials [213,214] (1 RCT [215]), 1 prospective single arm trial [216], 1 mixed prospective/retrospective case-controlled study [217], and the remaining majority comprised of case series and case reports [124,218–243]. Most studies described the use of amniotic membrane sheets. Six studies used the PROKERA device [214,232,239–242], a pre-prepared amniotic membrane on a conformer ring which can be applied to the eye like a contact lens in an office setting. In two studies amniotic membrane was formulated into a solution for topical application [219,220].

The pathologies treated with amnion were generally severe with 21 studies in infectious keratitis [124,212,215–218,221,223,224,226–229,231,234–236,240,241,243,244]. Three studies targeted patients of LRS, two were in local anaesthetic toxicity, six were in chemical/thermal injuries, and MK with 4 studies specifically targeting severe MK with significant stromal thinning (+/- descemetocele, +/- perforation).

AMT was found to help prevent corneal perforations in patients with severe ulcers. These studies typically used AMT as a structural scaffold in melted stroma. Although quantitative measures of corneal scarring were sparse, other healing metrics such as corneal re-epithelailisation time were reported

[215,217,223,224,226]. Anterior segment photographs were used in one study to assess the cornea [217], and IVCM was used in two studies however it was used to demonstrate integration of AMT tissue with the host stroma by one paper [124] and inflammatory cell density in the other [215].

Of the three LRS studies two were controlled trials, one in laser-epithelial-keratomileusis (LASEK) utilising only a strip of AMT sutured to the limbus [213], the other in PRK patients used the PROKERA device post-operatively [214]. Interestingly, while the LASEK study reported better post-operative haze in patients receiving the amnion at the early post-operative and 6-month time points, the PRK study did not find a significant difference between the amnion and control groups. The final study, a case series in patients undergoing PRK for post-operative regression and persistent corneal haze utilised an amnion sheet laid over the ablated corneal bed. This study reported no further recurrence of the post-operative haze among these patients, who had previously been unresponsive to medical therapy [238].

Endpoints of these studies typically oriented around qualitative descriptions of "resolution" of corneal healing, however quantitative measures such as time to re-epithelialisation and lesion morphology were also used (8 and 5 studies respectively), albeit infrequently. Subjective grading scales of corneal haze were used in were used in 5 studies, including all 3 LRS studies, one BK and one HSK. LRS studies were generally better at reporting some sort of measure of corneal haze/opacification/scarring. The other studies more commonly discussed the promotion of healing with little quantification corneal scarring. This is likely due to a) the majority of papers being case reports or series without predefined protocols, b) severe infective keratitis generally has poor outcomes with regards to corneal clarity and visual acuity and the aim of treatment is to prevent a catastrophic outcome such as perforation and as such studies were more likely to report corneal clarity as a secondary outcome.

2.3.2.2 Corticosteroids

As a broad acting anti-inflammatory, corticosteroids have been trialled in a wide variety of ocular pathologies. Thirty-four relevant publications were identified [111,112,121–123,131,206,208,209,245–270], of which the majority were BK or LRS. Other pathologies included chemical injuries and keratitis of other aetiologies. Whilst a significant proportion were RCTs, retrospective studies also featured, particularly where less-common pathologies were investigated. Nine of the papers were secondary analyses of the Steroids for Corneal Ulcers Trial (SCUT), making the number of unique RCTs lower [111,112,123,245,249,251,253,260,261]. A Cochrane review about corticosteroids in bacterial keratitis was identified, and its update seven years later.

Several different steroids have been utilised (Table 2-1), with some drug-pathology associations being identified, for example dexamethasone and prednisolone were more frequently used for keratitis, whereas several less potent steroids such as FML and loteprednol were trialled in LRS, to reduce drug-related side effects such as raised intra ocular pressure.

Steroids for LRS constituted approximately one third of the studies, of which 9 were prospective comparative clinical trials (6 RCTs). Dexamethasone, loteprednol and FML were the most frequently investigated steroids. Studies administered steroids post-operatively in all but one single armed prospective study by Marques et al. [206] where they treated refractive regression and haze 4-12 months post-operatively.

Only one meta-analysis was identified. Despite the design limitations and heterogeneity of reported outcomes in the literature, this concluded that corticosteroids were beneficial at reducing haze in the early post-operative period (3-6 months post-operatively). This benefit was less apparent at 12 months [256].

The use of steroids for corneal ulcers is controversial in view of the risk of immunologically unopposed infection. Although morbidity from BK is significant globally, only four well-designed RCTs have explored this topic, of which three met the inclusion criteria for this study and only one, the SCUT trial, was a large-scale RCT. Whilst the results of the included RCTs did not demonstrate a statistically significant benefit in the main study groups with regards to visual acuity and lesion size at final follow up, they did indicate a potential benefit and highlighted the importance of optimal study design (discussed in the outcomes section). In the SCUT trial, sub-group analyses of severe ulcers (vision worse than counting fingers at presentation) did identify a statistically significant improvement in the visual acuity of in the steroid treated patients at 3 months [123]. Further analyses also revealed that initiation of steroids up to 3-days after antibiotic initiation may improve the final visual acuity compared to those patients where steroids were initiated after 3 days, an effect primarily seen in those with severe ulcers [253]. Patients with *Nocardia* infection demonstrated a propensity for larger infiltrate/scars without corresponding differences in visual acuity, prompting the thought that atypical slow growing bacteria may fare worse with steroid treatment [253].

2.3.2.3 Mitomycin C

MMC, an alkylating agent that inhibits DNA replication and cellular proliferation. In LRS it is applied to tissues the corneal stroma to reduce the number of stromal keratocytes that may cause haze post-

operatively. Risk factors for haze include large corrections [271], ethnicity [272] and post-operative UV exposure [273].

MMC was reported in 64 studies of various study design, including two meta-analyses [262,274–337]. In the overwhelming majority of papers it was used in the context of LRS, with only two studies in alternate settings, one using it as an adjunct in corneal cross linking [304] and the other following pterygium surgery with assessment of corneal haze post-operatively [330]. Whilst most LRS studies were in naïve eyes, MMC was also trialled in related scenarios such as repeat LRS for regression, persistent and/or recurrent post-operative haze, and laser-assisted in-situ keratomileusis (LASIK) flap complications, as well as correcting refractive error in lamellar and penetrating keratoplasties.

Almost half were retrospective and non-comparative in design. The follow-up periods ranged from 3 to 24 months (mean=12).

In LRS MMC is applied for varying lengths of time before being washed off. Studies have reported the effects of varying concentrations and exposure times, among other factors influencing the final outcomes in these patients. The most frequently used concentration in the included studies was 0.02% (range 0.002% to 0.04%).

In one of the largest observational studies in the field, Kaiserman et al. identified that shorter exposure times were associated with an increased incidence of post-operative haze, particularly in those patients undergoing larger refractive corrections. In their cohort of over 9000 patients, the incidence of haze in high myopia was 2.1% and in low to moderate myopia 1.1% at 15 weeks post-operatively. With such low incidence rates, well-designed RCTs need to consider how to detect clinically meaningful differences. A further methodological consideration is the follow up period. Haze intensifies at around 1-2 months post-operatively and regresses over the following months [338], its not clear if those with haze at 15 weeks continued to have clinically significant haze at 1 year. However, in their meta-analysis Chang et al. convincingly demonstrated that haze at 1 year also reduced with MMC treatment [295].

In addition to consistently reporting corneal clarity using a range of subjective haze-scores, these studies also incorporated the largest variety of scanner/device assessments of corneal fibrosis (Table 2-5).

2.3.2.4 Cell therapies

In two papers by J L Alio's group, adipose derive stem cells were used in keratoconic patients, the first study being a small single arm clinical trial of 5 patients, and the second being a randomised pilot of 14

patients in 3 arms. The studies utilised objective imaging measures (optical coherence tomography [OCT] and in-vivo confocal microscopy [IVCM]) to demonstrate the benefit of this treatment with the primary outcomes focussing on safety and survival of the transplanted cells. Although the recruitment numbers were low, they were able to demonstrate new stromal tissue production by the transplanted cells, and in 1 case in the randomised pilot, the improvement of a pre-existing scar.

2.3.2.5 Blood products

Various combinations of blood products were encountered in the literature.

Matsumoto et al. investigated autologous serum eye drops in NK in a retrospective case series and noted improvements in scarring as per slit-lamp examination. Geremicca at al [339] reported their case series of platelet rich plasma (PRP) lysate in nonresponsive NK with modest improvement in corneal health.

Yoon et al. [340] reported their case controlled study using umbilical cord serum eye drops after LASEK in which they found improvements in re-epithelialisation and haze scores at 2 and 4 weeks. They only reported haze to this relatively early time-point. Semeraro et al. [341] presented their case series in patients with various ocular surface disorders and found improvements in corneal opacity (using a custom-scale) at 12 months.

Sanchez-Avila et al. [342] compared plasma rich in growth factors to MMC in a retrospective case controlled study and found it to be similar in terms of refractive predictability, and to have fewer complications including keratitis and haze, which was reported according to Fantes et al.'s scale.

Arcana Del Cid et al. [343] administered subconjunctival injection of regenerative growth factor rich plasma in chemical injury patients. They vague report a shorter time to corneal scarring, which didn't give much insight as to the state of the cornea. They did report some thinning in patients with the worst injuries, raising questions about the safety of this treatment.

Panda et al. [344] performed an RCT using PRP in chemical injuries and found a significant improvement in corneal clarity (reported using a custom scale) at 3 months compared to the control group.

2.3.2.6 Growth factors

Three different growth factors were identified: basic fibroblast growth factor (bFGF); epidermal growth factor (EGF); and nerve growth factor (NGF).

bFGF is released by corneal cells and modulates corneal wound healing in an autocrine/paracrine fashion [345]. It promotes proliferation of epithelial and stromal cells and increases the rate of re-epithelialisation [346]. Three RCTs investigated the use of bFGF in PRK patients, of which two co-administered oral L-cysteine (for its ability to improve re-epithelialisation [347]). The follow-up ranged from 3-12 months, and each study used a subjective haze-scoring system. All studies reported improved re-epithelialisation with bFGF, without differences in haze.

One single arm pilot trial by Holland et al. reported the use of a human recombinant epidermal growth factor (rh-EGF) soaked bandage contact lens (BCLs) in 9 patients with persistent epithelial defects (PEDs) of various aetiology. These patients had previously received prolonged topical treatments and BCLs. In 7 of these patients, re-epithelialisation occurred within 8 days. The remaining 2 patients were noted to have significantly more inflamed and vascularised cornea in failing corneal grafts, which the authors suggested may have decreased the efficacy of the treatment. The observation of corneal fibrosis was not a main aim of this study and a few qualitative statements reported the post-treatment state in some patients.

One paper reported on the use of topical NGF in a 5-month-old child with trigeminal insufficiency leading to corneal ulceration with stromal opacification. Topical NGF is well studied in ocular surface disease however only this paper reported on corneal clarity in addition to epithelial health. Eighteen-weeks into the 6-month trial of NGF the patient's epithelium recovered and stromal opacity diminished and following cessation only mild stromal scarring remained [210]. Long term data were not reported.

2.3.2.7 Immunotherapies

2.3.2.7.1 Calcineurin inhibitors

Calcineurin inhibitors, such as ciclosporin and tacrolimus, inhibit interleuking-2 production, thereby inhibiting the T – lymphocyte mediated immune responses [348]. They are used for ocular surface inflammatory conditions such as immune-mediated dry eye, particularly when corticosteroids treatment is undesirable [349].

Ciclosprin was used in one case report of interstitial keratitis and one case control study in post-LASEK patients. One randomised control trial used tacrolimus in HSK patients. In all cases corneal clarity was reported as a secondary outcome.

Lee et al.'s study subjectively rated post-LASEK corneal haze according to the Faergolm et al.'s system, in 40 treated and 40 control eyes. In their 8 week follow up period an effect of ciclosorin on corneal haze

was not demonstrable. Miserocchi et al., qualitatively reported the resolution and sequalae of the acute inflammation in a case of idiopathic interstitial keratitis, and the corneal sequalae 16 months post-treatment.

Akbari et al.'s RCT of tacrolimus in stromal HSK with 25 patients in each of the tacrolimus and control groups., demonstrated promising results. They co-administered topical prednisolone 1% and oral acyclovir, in the acute phase and followed up the patients for 28 days, concluding that tacrolimus was effective in reducing inflammation, neovascularisation and scarring. These positive results indicate that adjunctive Tacrolimus alongside steroids is beneficial for these patients, however the short follow up raises questions about the long term effects of the treatment and whether changes in fibrosis or stromal haze from inflammation were observed.

2.3.2.7.2 Interferon

Interferons are cytokines named for their ability to 'interfere' with viral replication. They have been trialled in viral keratitis as well as other ocular inflammatory conditions. Three such studies met the inclusion criteria for this review. Interferon alpha 2b an antiviral and anti-neoplastic medication was investigated in one single arm trial for vernal keratoconjunctivitis (VKC) patients, and in one RCT for PRK patients. In 28 eyes with VKC, corneal inflammation, infiltration and opacity regressed significantly upon interferon treatment [207]. Gillies et al. conducted an RCT using interferon in PRK patients who were followed-up for a year. In their cohort of patients requiring correction for high myopia, they noted that corneal haze was significantly better in the 15 patients receiving interferon alpha2B, over the course of the study compared to the control group, however at 12 months no difference existed between groups. Interestingly, post-procedural re-epithelialisation was delayed by 2 days (mean) in the interferon group, although this was not found to impact haze development.

2.3.2.8 Non-Steroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs primarily act through the inhibition of cyclooxygenase, which reduces inflammation by inhibiting the formation of endogenous prostaglandins [350]. This mechanism suggests that these medications have potential in a range of ocular inflammatory disorders. The present study identified 7 relevant publications, with a high proportion of RCTs (4 out of 7). Six of the seven studies investigated NSAIDs in LRS, with the primary aim of reducing post-operative pain and inflammation. They formed part of the of the few studies

utilising PROMs. NSAIDs were effective at reducing post-operative pain. Post-operative corneal haze was a secondary outcome, and results for its reduction of haze were variable. Since NSAIDs were typically used for a few days post-operatively and only 2 of the 7 studies had follow up periods of 6 months or longer the impact of NSAIDs remains unclear.

The final study incorporated a self-designed PROM (non-validated) in their assessment of indomethacin in patients with established corneal scars of various aetiology. Although this study was primarily exploring the change in symptomatology following drug administration, they qualitatively reported no changes in the established lesions of any of their patients.

2.3.2.9 Ascorbate

Four studies regarding the use of ascorbate (Vitamin C) were identified. Three were in LRS [337,351,352]of which two studies were retrospective and one was a prospective controlled trial. Treatment regimens were similar for LRS studies where patients were prescribed 500mg orally, twice a day usually commencing prior to surgery and finishing a few days post operatively. Although the controlled trial in patients undergoing PRK had a relatively short follow up (4 days) because it was primarily investigation post-operative symptoms, they did report corneal haze to be indifferent between the treatment arms. However, the two retrospective case-controlled studies in patients undergoing PRK and LASEK followed up patients for 26 and 52 weeks respectively. While the former study identified a statistically significant improvement in post-operative corneal haze at early and late time points, this was not the case in the LASEK study. The final paper was retrospective comparative study in BK comparing oral and intravenous ascorbate in the acute phase of infectious keratitis which reported smaller corneal scars, as measured on photographs, at 3 months [353].

2.3.2.10 Cacicol

Cacicol is a matrix regenerating agent (RGTA) containing poly-carboxymethylglucose sulfate, a heparan sulphate analogue. Its role in corneal wound healing is complex in view of its numerous binding partners. It replaces damaged heparan sulphate as an extracellular scaffold and stabilises matrix proteins and growth factors that it binds, reducing proteolytic loss [354,355]. By preserving the natural microenvironment of the cells and the endogenous factors it promotes tissue regeneration [356]. Although described as a matrix regenerating therapy, the focus of RGTA studies in the literature have

been epithelial healing. The present study included 5 studies investigating Cacicol in persistent epithelial defects and ulcers of various aetiology including herpetic keratitis, acanthamoeba keratitis and neurotrophic keratitis. Epithelial healing was the primary outcome in these studies, with corneal fibrosis specific outcomes in all cases being either a qualitative description of corneal scarring, photographs, or both. None of the studies were comparative, making it difficult to infer the efficacy of Cacicol for corneal fibrosis.

Although promoting re-epithelialisation of persistent ulcers itself may reduce corneal fibrosis [357], mechanistically RGTA is of great interest as it has many binding partners with potential to impact fibrotic cascades downstream. Further well designed studies are required to determine the potential of RGTA as an anti-scarring therapy.

2.3.2.11 Crosslinked thiolated carboxymethyl hyaluronic acid liquid-gel (x-CMHA)

Hyaluronic acid (HA) is a common component of ocular lubricants. Durie et al. utilised an altered cross-linked HA hydrogel to create an ocular dressing that can persist on the ocular surface for an extended period of time. Although simple lubricants were excluded from this study, the formulation of a hydrogel ocular dressing represents a significant advancement above and beyond the relatively simple HA lubricant solutions which are typically at low concentrations (up to approximately 0.4%). In their study Durrie et al. used this formulation to treat 26 patients undergoing PRK compared to 13 control patients. In this pilot RCT patients were followed up for 28 days. Despite stating the intention to observe post-operative corneal haze using two validated qualitative scoring criteria (Fantes et al. [358] and Hanna et al. [359]) data regarding haze were qualitatively reported. Therefore no conclusions can be drawn about the efficacy of this formulation with regards to corneal fibrosis, however it represents a novel approach to corneal wound healing.

2.3.2.12 Cytochrome c peroxidase

Cytochrome c peroxidase (CCP) is a water-soluble haem containing antioxidant enzyme involved in the prevention of intracellular peroxide accumulation and maintenance of mitochondrial health. In their 2005 study Scalinci et al. evaluated the utility of a 7 day course in 36 eyes of 36 post-PRK patients, whilst their fellow eyes acted as controls receiving the standard of care. As the primary outcome was re-

epithelialisation time, the final follow up was at 7 days. Although it was long enough to demonstrate a clear improvement in re-epithelialisation with the use of CCP, no difference was noted in corneal haze.

Reactive oxygen species are increased following PRK [360] and contribute to keratocyte dysfunction leading to corneal fibrosis. Although no such effect was demonstrable in the study by Scalinci et al. the mechanism remains plausible and future clinical studies must be specifically designed to explore this.

2.3.2.13 Heparin

Heparin is a glycosaminoglycan clinically used as an anti-coagulant, which also has anti-inflammatory and anti-angiogenic properties [361]. It is also available in eye drop formulations for dry-eye [362] and has been found to reduce posterior capsular opacification following cataract surgery in humans [363]. Jian-wei et al. reported its use in acute chemical injuries secondary to parquat, a dipyridylium ammonium salt herbicide. Although their retrospective case-controlled study reported no corneal scarring to have occurred in the treatment group, (15 eyes) this was also the case for the control group (16 eyes). Across both groups only one patient had an injury categorised as severe (according to a custom severity scale based on limbal injury). As such, no answer can be inferred about the efficacy of heparin eye drops for corneal fibrosis.

2.3.2.14 N-acetyl cysteine (NAC)

NAC is clinically used for its mucolytic properties for example in filamentary keratitis [364], but is also a potent antioxidant [365], and anti-inflammatory molecule [366]. Urgancioglu et al. [367] conducted a non-randomised controlled trial of post-operative NAC in LASEK patients (16 NAC eyes vs 10 control eyes). They reported a significant improvement in post-operative corneal haze subjectively graded according to the Hanna et al. classification, and IVCM analysis at 1 month after surgery. However they did not find there to be a significant difference between treatment and control groups at 3 months. Thus adverse early stromal changes, were found to be ameliorated using NAC. Since stromal haze peaks at 1-2 months post-operatively and has a relatively low incidence, the long term outcomes following this treatment are uncertain.

2.3.2.15 Oxygen

Oxygen therapy is thought to aid wound healing by improving oxygen delivery to wounded tissue which becomes more metabolically active when damaged. The present study identified two papers utilising therapeutic oxygen to improve corneal wound healing [368,369]. In their non-randomised controlled trial Sharifipour et al. prescribed 100% inhaled oxygen for one hour, twice a day in patients with acute chemical injuries and reported promising results with regards to subjectively graded corneal clarity and vascularisation (according to a custom scale) six months after the injury.

Chong et al. report a case of *Pseudomonas* keratitis treated with one session of adjunctive inhaled hyperbaric oxygen (HBO) (at 2.0 atmospheres absolute pressure) for 90 minutes. HBO is an established treatment of *Pseudomonas aeruginosa* causing malignant otitis externa [370] and certain other bacterial infections [371]. In this case a 4mm x 4mm superior corneal ulcer with visual acuity of 6/12 rapidly progressed to a visual acuity of counting fingers overnight under hourly dual antibiotic therapy. After no significant response on day three, HBO therapy was administered after which a steady recovery was made, and the patient was left with 'mild scarring and thinning'.

Although corneal fibrosis may not be specifically targeted by oxygen therapies the indirect effects may be beneficial for wound healing and in the case of high flow oxygen, this represents a relatively simple and important supportive therapy to be considered in clinical practice.

2.3.2.16 Plasmin inhibitor

Plasmin, a plasminogen derived serine protease, is a component of the tear-film [372]. It degrades numerous matrix proteins such as fibronectin and laminin, and also activates a number of enzymes such as pro-collagenase [373]. Its tear-film concentration increases in pathological states [374]. Fibronectin is essential for epithelial cell migration and its breakdown may inhibit corneal wound healing [375].

O'Brart et al. conducted a randomised controlled trial of the plasmin inhibitor aprotinin (for 3 weeks) compared to fluorometholone (FML) (for 6 months) and control (no treatment) groups undergoing PRK [257]. In their comprehensive assessment or corneal clarity, they included objective measures of forward and backward light scatter, and by using a halo-meter. They discovered their plasmin inhibitor protocol actually increased corneal haze at 9- and 12-months post-procedure compared to untreated controls. The authors postulated that plasmin's inhibition prevented ECM breakdown in the early phase, but the matrix

remodelling and deposition occurring in the late phase continued, thus leading to an excess of ECM and increased corneal haze.

2.3.2.17 Thiotepa

Thiotepa is an alkylating agent used to treat neoplastic disease. It was investigated in one case series of 5 patients, who received topical Thiotepa following PRK, all of whom had previously undergone multiple PRK treatments and at enrolment were suffering from recalcitrant post-operative corneal haze [376]. In this cohort of patients with a strong tendency to develop late post-operative haze, steroids alone were ineffective at preventing haze. Thiotepa treatment was administered for 3 months post-operatively, with reported side effects of mild ocular surface irritation and a bacterial keratitis in one case. Of the 5 eyes, only 2 experienced regression of their refraction and none had recurrence of haze. In such a high-risk cohort, this finding is interesting for some scenarios of corneal fibrosis, however, similar to MMC, concerns about toxicity may limit its application.

2.3.2.18 Vitamin A, E and Co-Q10

Two studies using vitamin A were identified. One RCT of vitamin A in a commercially available formulation VitAPOS (now available as HyloNight, URSAPHARM Arzneimittel GmbH) in PRK [377], and one RCT of vitamin A and E in PRK [378]. Chelala et al. did not find their formulation to be impactful with regards to pain, time to healing or corneal haze at 3 months. However, Vetrugno et al. administered high dose vitamin A and E systemically, and found significant improvements in re-epithelialisation rate, visual acuity and incidence of corneal haze, up to 1-year post-op.

One case series of six patients with non-healing ulcers received Coenzyme Q10 (ubiquinone Q10, CoQ10) and vitamin E (TPGS) topically [379]. Co-Q10 is a well-known electron transporter and part of the mitochondrial respiratory system that also has anti-inflammatory, antioxidant and anti-apoptotic effects [380,381]. Vitamin E TPGS is synthetic form of vitamin E often used as an excipient in formulations of lipophilic compounds [382]. Although the case series was non-comparative, they qualitatively reported healing of all ulcers within in 4-8 weeks, and presented a time series of photos from which the improvements in corneal opacity were surmised. No side effects were noted during the 6 month follow up period.

2.3.3 Outcomes

Studies used a range of outcomes (Table 2-5) broadly categorised as: clinical examination features; subjective grading of clarity/opacity/haze; device-related outcomes; PROMs; and 'other' outcomes not falling under the preceding categories. A significant number of studies reported qualitative descriptions, ranging from sparse to more detailed descriptions of the lesions. However, these lacked the rigour required to be valuable outcomes for clinical studies of anti-scarring efficacy.

Examination features were predominantly lesion morphology metrics. Some studies utilised subjective measurements such as the longest and longest perpendicular axis of the lesion taken at the slit-lamp, often used in daily clinical practice. Several studies built on this method and obtained more accurate lesion metrics, such as lesion area or lesion area as a percentage of the corneal area. For this, anterior-segment photographs were measured with digital tools like imageJ to calculate the lesion area.

Several subjective haze rating scales were identified. Where studies used a scale but a validating-study was not referenced, they were reported as adopting a custom scale [Table 2-5]. Most of these were used for post-LRS haze, however some generic and custom scales for non-LRS assessments were also found, for example, the nebula-macula-leukoma description. The scales used in LRS grade the degree of opacification caused by the lesion. Although they are subjective, clear definitions of the grades and relative ease of use in daily clinical practice make them a convenient outcome measure. The two most common scales were those by Fantes et al. [358] and Hanna et al. [359] utilised in studies regarding post-LRS corneal haze.

Contrast sensitivity was used to assess the impact of corneal haze. Increased ocular light scatter was found to correlate with poorer contrast sensitivity even when visual acuity is not significantly affected [137,383]. This suggests that contrast sensitivity assessment may be helpful for differentiating milder corneal haze severities, as supported by its use in studies of post-LRS corneal haze. Another group of patients in whom this assessment may be helpful are those patients with lesions encroaching or partially covering the visual axis. As the pathology in these patients resolves, the edge of the lesion may regress away from the visual axis, which may be detected as an improvement in their visual acuity for denser scars, and perhaps contrast sensitivity function for fainter ones.

Corneal clarity was measured objectively by devices such as the Pentacam (OCULUS ®) densitometry module, C-Quant ocular stray light analyser (OCULUS ®) and halo meter. The objectivity of these devices holds significant potential and represents a more robust analytic system compared to subjective scoring. These techniques have potential to be utilised in a range of pathological contexts. However, these must

be validated in the clinical setting to determine their value as robust measures in clinical trials of investigative medicinal products.

Histopathological changes were qualitatively and quantitatively analysed using the IVCM by assessing cell hyperreflectivity and deriving a value for the degree of light scatter/reflectance caused by fibrosis. IVCM is excellent for offering microscopic detail which may be useful in some study settings, and it may provide valuable insight about stromal cell density in different pathological scenarios. However, it is less useful for macroscopic assessments of the lesion. In contrast, OCT is quick, non-contact and can generate raster-volume scans of the anterior segment. In this study OCT was found to be used to measure lesions in 2-dimensions.

PROs were used very little. Various pain scores were used in LRS studies investigating therapies with analgesic effects. Two custom tools were identified enquiring about subsets of ocular surface disease symptoms most relevant to their condition. The visual function questionnaire 14, the only validated ophthalmic PRO, appeared in once, in an RCT regarding corticosteroids in BK.

Table 2-5. Summary of outcome measures

Examination features	Subjective haze grading	Scanners & other devices	Patient Reported Outcomes	Other
Lesion Diameter	Alio et al	Corneal volume by topographer	Custom Tool About OSD Symptoms	Tear fluid samples
Lesion Size Mmxmm	Braunstein et al	C-Quant Ocular stray light measurement	Custom Symptom Questionnaire	
Lesion Area Mm2	Custom scale	Corneal light scatter	NRS 11	
Lesion Area % Of Cornea	Durrie et al	Halo meter	Pain Score	
Lesion Depth	Fagerholm et al	Higher order aberrations iTrace	Pain VAS	
Contrast Sensitivity (MCT 8000 (Vis Tech))	Fantes et al	IVCM - Anterior stroma intensity	VAS Pain Score	
Contrast Sensitivity (CVS1000)	Heitzmann et al	IVCM (stromal keratocyte density)	Visual Function Questionanire-14	
Contrast Sensitivity (Pelli Robson)	Farah et al	IVCM (stromal keratocyte count)		
Contrast Sensitivity (Vector Vision 100)	Greensteins et al	IVCM for langerhans cell density		
Stromal Thickness Restoration Time	Hanna et al	keratometry		
imageJ to analyse lesion area	Helena et al	OCT 2 dimensional lesion measurement		
	Kim et al	OCT - CCT		
	Lohman et al	Pachymetry		
	Maldonado et al	Scheimpflug densitometry		
	Nebula-macula-leukoma	Specular microscopy		
	O'Keefe et al	Tomography		
	Panda et al	Topography		
	Qualitatively reported			
	Salz et al			
	Siganos et al			
	Summit US FDA guidelines			

Abbreviations: CCT – central corneal thickness; IVCM – invivo confocal microscopy; NRS11 – Numerical Rating Scale 11; OCT – optical coherence tomography; OSD – ocular surface disease; VAS – visual analogue scale.

2.4 Discussion

This is the first study to adopt a rigorous knowledge synthesis approach to characterise the literature about potential therapies for corneal fibrosis in this way. The results help to identify the variation and commonalities between the pathologies, treatments, and study designs within the field.

2.4.1 Therapies

Prevention was overwhelmingly the most investigated strategy, as opposed to strategies for managing established fibrosis. The most used therapeutics, such as corticosteroids, MMC, NSAIDs and amnion, were also the most frequently investigated. Corticosteroids are the most versatile treatment, being investigated in the broadest range of conditions. MMC was the least diversified, explored exclusively in LRS or related patients. Studies investigated the treatments in the context of a single episode of illness, however no studies investigated the impact of treatments longitudinally over multiple episodes in conditions such as HSK where the disease is recurrent and is a significant contributor to the rates of corneal transplantation in the UK [78].

Several candidates have shown promising results in limited clinical scenarios. Further investigations are warranted to elucidate the potential of their broader application. Treatments like inhaled oxygen therapy, vitamin & amino acid supplements, topical wound dressings (CMHA) and NAC could conceivably be safely trialled in most pathologies and could then supplement existing clinical protocols. Amnion was explored in severe corneal ulcers, largely to restore the structural integrity of melted corneas. However, its potent regeneration capacity would be beneficial for all ulcers. While it has been implanted on infected cornea, concerns about risks may be allayed by adopting alternative delivery modes such as the amniotic membrane extract studies, where tissues were processed into an eye-drop solution. Therefore, new applications of therapies must also involve an adequate risk assessment to ensure safe translation.

Of note were the studies of adipose derive stem cells in keratoconus patients. The entry of cell-based studies in human clinical trials represents a significant advancement in the field of corneal stromal regeneration, and more diverse applications of this therapy are eagerly awaited. Furthermore, these were used in patients with some degree of established fibrosis and may hold promise for millions of patients with corneal blindness.

There was considerable breadth in the investigated pathologies. Corneal opacity following injury may occur from oedema, immune cell and pathogen infiltration, and from abnormal ECM components. The

optimal protocol for managing corneal fibrosis would take into consideration the nuance of each disease pathophysiology and direct it towards regeneration.

For example, NAC was found to improve corneal haze at 1-month post-LRS. However, there was no difference between the groups at three months. NAC has been investigated in a broad range of corneal pathologies and is purported to work through a number of mechanisms including anti-inflammatory, anti-oxidant and anti-collagenase activity [384]. It is possible that one or more of these mechanisms contributed to the rapid recovery following LRS, and that no effect is detected at the later time points as the degree of injury in LRS is naturally overcome by corneal homeostatic mechanisms. Indeed, in those LRS studies that observed a benefit with treatment, it tended to be early post-operatively, apart from MMC, which demonstrated benefit at 12 months. Another speculation may be that early opacification is driven by oedema and inflammation which are expedited with NAC treatment, but the later changes occur from modulation of stromal cell behaviour due to changes in stromal mechanical properties. Additionally, most of the studies only administered the medication for short timeframes, and so may have lost their impact when late haze tends to occur. This does raise interesting questions about what drives fibrosis at the later stage, as the major contributions from the damaged epithelium have ceased by then.

Examining the effects of NAC in other pathologies would be interesting, as in the example of microbial keratitis where stromal lysis is a significant concern, NAC's anti-collagenase activity would be beneficial.

Typically, only antimicrobials are utilised to eliminate pathogens and "cease" the injury in microbial keratitis, however consideration should be given to the fact that corneal injury leads to increased metabolic stress and hypoxia in this avascular tissue, which in turn case tissue injury [385]. This also leads to corneal endothelial dysfunction and oedema [386]. This study identified oxygen therapy as a potentially beneficial adjunct in the management of microbial keratitis. Hyperbaric O_2, which increases oxygen free-radicals in hypoxic tissue to improve neutrophil action and kill bacteria, may aid in clearing microbes from corneal tissue and reduce stress on the host cells, thereby reducing inflammation [387]. It raises questions about the role of corneal hypoxia on the incidence of corneal fibrosis and corneal neovascularisation in MK, and if O_2 therapy would improve corneal outcomes.

Generally, these studies tested potentially beneficial molecules in isolation. The absence of a widely accepted singular treatment amongst the breadth of candidates is suggestive that a multifactorial approach may be required. AMT offers a multifactorial approach to corneal wound healing as it contains numerous pro-regenerative factors. Although out of the time-window of this study's conduct, in their

meta-analysis Ting et al. found that adjuvant AMT improved healing time, visual acuity and corneal vascularisation in patients with infectious keratitis [388]. In both the present study and Ting et al. . Administration of AMT is typically in theatre as it needs to be sutured onto the cornea, presenting a logistical challenge. The PROKERA device, in which amnion is fixed in a conformer ring, is less complicated in terms of preparation and administration however, it is no longer in production. Interestingly, Sheha et al. processed amnion into a solution to administer as drops which may represent a more straightforward approach in terms of administration. However, commercial-scale production may be challenging [219,220].

Blood products would be expected to contain numerous growth factors and serum eye drops are an establish treatment for sever ocular surface disease, however they are used judiciously in infective keratitis [389]. In lab studies with corneal stromal cells, sera are seen to promote a fibrotic phenotype from cells (see Chapter 3) and so their modestly beneficial results in the trialled scenarios here is curious. Larger scale well designed studies are required to elucidate their true value.

This study has characterised the mechanisms of therapies used to manage corneal injuries and supports the concept of multi-mechanistic approaches, as afforded by stem cell therapies. By adopting such a mechanism-based perspective to corneal wound healing, one may consider the different treatment options along the disease timeline. An ideal protocol may aim to expedite the abolition of the injury, followed by optimisation of the wound microenvironment and immune response to injury, with subsequent and timely cessation of inflammation. Activation of tissue remodelling/regeneration may follow, with the maturation of new tissue and restoration of homeostasis.

2.4.2 Outcomes

A wide range of outcome assessments were used in these studies including PROMs, functional assessments (e.g., visual acuity, and contrast sensitivity), objective assessments of lesion with macroscopic (corneal photographs, corneal OCT, Scheimpflug densitometry etc) and microscopic (IVCM) resolution. Few studies utilised PROMs, and fewer still were validated. Patients' lived experience of their disease is critical in evaluating the effectiveness of novel therapies. This is particularly important when considering the cost of novel treatments, as the value they create may not be measurable in terms of visual acuity or lesion size.

Numerous different subjective corneal haze grading scales were utilised, largely in the LRS studies. Whilst this is a time-efficient approach to recording corneal haze, it is of limited value where there is significant

heterogeneity in lesion morphology among patients. Therefore, their translation to other pathologies is limited. Furthermore, such scales lack the quantifiability, objectivity, accuracy and sensitivity offered by other identified modalities from the scanners & devices category.

Contrast sensitivity may be affected by corneal haze in the presence of unaffected visual acuity [137,139,383]. As such, imperceptible changes in vision from milder or off-axis may be better assessed using contrast sensitivity, thus forming a valuable addition to the assessment panel.

Whilst functional assessments and PROMs give us a better understanding of the impact of pathology, lesion morphology assessments are also helpful for monitoring and quantifying responses to treatment. Objective, quantifiable measures of corneal scarring represent an ideal outcome measure. The present study identified techniques of interest however, no single method broadly applied to all pathological scenarios. IVCM can offer cellular-level insights although it has a narrow field of view. Research groups [390] have performed montage imaging techniques to visualise large areas of cornea however such protocols are laborious and may increase the risk of measurement errors. Scheimpflug imaging can provide rapid whole corneal imaging and the densitometry analysis that OCULUS's Pentacam offers can be used as a numerical outcome measure. However, little is known about how the technique calculates these values, as it is a proprietary software. Furthermore, morphological information is not obtained from commercially available software, although this may be possible in post-acquisition analyses. Perhaps the greatest potential is demonstrated by anterior segment OCT devices, many of which have versatile scanning protocols that can provide data from which 3-dimensional volume scans can be created. However, the present study found OCT utilised in 2-dimensional analyses only.

2.5 Future directions

This work has identified several potential avenues for future investigation. Novel anti-scarring drug-discovery is a large and active field. An optimally designed treatment may consider the frequency and method of delivery, length of treatment, initiation time and cost of treatment. However, the drug-discovery-development lifecycle is resource and time intensive. Those investigators in a position to do so, may consider trialling any number of already commercially available treatments in isolation or combination for conditions leading to corneal blindness.

Future investigators must consider the optimal combination of outcome measures as part of the design of their clinical studies. The specific pathology heavily influences the choice of investigation, but the

outcome measures must be specific and quantifiable, nevertheless. The ideal battery of assessments should be resource efficient and combine objective quantitative evaluations with subjective PROMs that capture the impact of disease and treatment on patients' lives. Additionally, PROMs must be able to capture the patient experience through the natural history of the disease and their response to treatment. Since they must be designed and validated for specific purposes, investigators should consider searching for a suitable existing tool prior to considering non-validated self-designed tools. Although not currently commercially available, Scheimpflug and OCT image analysis techniques could be utilised to produce metrics of corneal lesions morphology.

There much development and validation must be done in the range of conditions leading to corneal blindness.

2.6 Limitations

Inherently, the design of a scoping review means that in addition to characterising a field it also raises specific questions about the field. As such this study has several limitations. To ensure adequately broad coverage of the literature the search strategy had to be comprehensive. This was complicated by the facts that 1) corneal fibrosis may occur secondary to many pathological processes, whilst also taking into consideration 2) the different possible treatments of these conditions, and 3) different terminologies have been adopted in reference to the process, for example corneal scarring, opacification and haze. A significant number of studies were excluded at the full text screening phase because of language reasons or that that full texts were unavailable following messages to the author (n=24). Although no grey literature was included in this study to avoid the inclusion of lower impact investigations, it is possible that the grey literature contains valuable information and may identify therapies that have not progressed to larger studies. It would be helpful to explore which trialled treatments have had poor results and for what reasons, to aid future investigations. Since only human studies were included, no information is available about candidate therapies in the preclinical phases of development. This body of literature would be fascinating and may offer insight into the field's future direction.

Reporting the outcomes from these studies was a secondary aim, thus the search strategy may not have been optimised for detecting the full range of outcomes available for use in humans. This may be an avenue to explore in future investigations.

The aim of this study was not to assess the efficacy of investigated medicinal products, and as such it cannot make recommendations for clinical practice. However, it serves as a point of reference for clinicians and researchers interested in considering all treatment possibilities and aids those conducting future systematic reviews and clinical trials. The inclusion criteria of this study necessitated the reporting of an outcome in addition to visual acuity – this may have led to the exclusion studies utilising investigational products of interest. Yet, many studies were included by virtue of presenting anterior segment photographs or making some descriptive comments about corneal scarring, which led to the inclusion of studies which did not offer significant insight into the efficacy of their treatments. Since this study did not plan to report efficacy, the poor reporting of efficacy was not in conflict with the aims of the study, and in fact, yielded richer results to draw from (e.g. O2 studies).

2.7 Conclusion

This study adopted a systematic approach to comprehensively collate the relevant literature as a point of reference for future investigators. It highlights the gaps in the literature as well as opportunities for translational researchers. Of the numerous strategies identified in this study, no single approach has proven to be ubiquitously efficacious and safe. Corneal wound healing is a complex multifactorial process, and promoting scarless regeneration is likely to require effective modulation with multiple therapies. Many strategies were investigated where anti-fibrosis was not the primary aim, such as oxygen therapy, yet they may be beneficial in reducing corneal scarring. While clinical protocols typically incorporate a number of the treatments discussed, there may be scope to adopt other relatively low-risk strategies, such as oxygen therapy, l-cysteine supplements and NAC, in more pathologies and warrant further investigation. Such investigations must judiciously select the optimal pathological context, outcome assessments and study design to demonstrate efficacy explicitly.

3. Chapter 3: An in vitro investigation of decorin's role in fibrosis and autophagy in primary human corneal fibroblasts

3.1 Introduction

Corneal fibrosis is the natural healing response to corneal stromal injury. Key steps in its pathogenesis are described in Chapter 1, however all its regulatory mechanisms have not yet been elucidated. Corneal keratocyes are considered the most important effectors in this process as they are responsible for ECM homeostasis in health and disease. In response to injury, they differentiate into fibroblasts and myofibroblasts, migrate to the wound, contract and produce ECM components. The wound inflammatory milieu directly impacts the function and persistence of these cells, the latter being correlated to the corneal opacity.

One cellular process that may link these activities is autophagy, which is a dynamic catabolic process that can be triggered by context and cell-specific stimuli. Broadly these can be categorised as macroautophagy, chaperone mediated autophagy and microautophagy, although many forms do not fit this categorisation, such as mitochondrial autophagy (or mitophagy) [391] (Figure 3-1). Whilst multiple autophagic pathways have been characterised they are conceptually similar in that they involve the fusion of isolation membranes called phagophores which engulf cargo in what are termed autophagophores. These in turn fuse with lysosomes forming autolysosomes, where cargo is degraded. Autophagic degradation can be non-selective for bulk cytosolic materials or selective via adaptor proteins for specific cargo (e.g., mitochondria, aggregation-prone proteins, pathogens) [391].

Autophagy is involved in many cellular functions, including peculiar dichotomies such as survival and cell death during stress. Its homeostatic functions include cell protein quality control and waste management, hence it has an important role in disease states of abnormal protein production and aggregation [392].

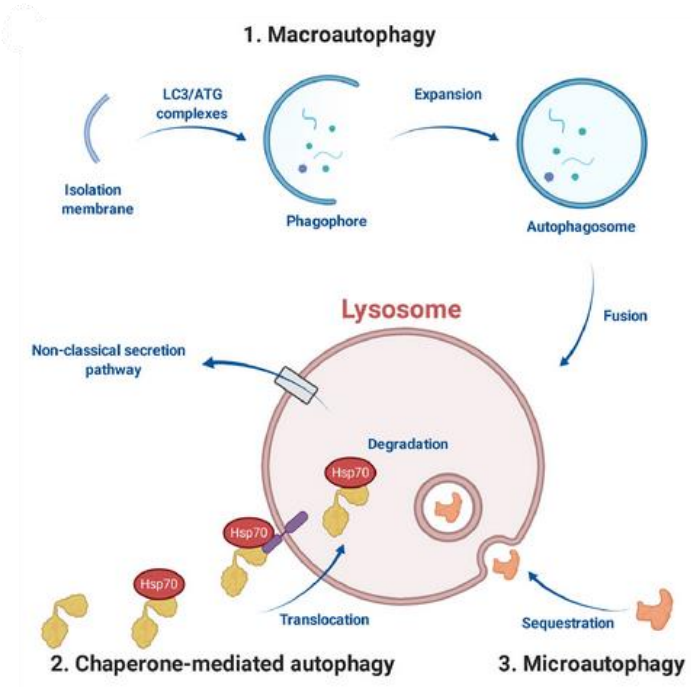


Figure 3-1 Diagrammatic overview of macro, micro and chaperone-mediated autophagic pathways

The schematic overview of autophagic pathways depicts three of the most common forms of autophagy. Other forms of specific autophagy also exist and contribute to cell homeostasis. Adapted from [393].

It is a relatively new concept in fibrosis and found to be associated with key pathogenic steps including stromal cell differentiation, survival, senescence, ECM synthesis & remodelling, and fibrogenesis. Evidently, it's role in fibrosis is context dependant.

In oral mucosa cells, wound healing autophagy leads to pro-fibrotic signals through the promotion of myofibroblast differentiation and is required for successful healing [394].

In liver fibrosis, both increasing and decreasing autophagy attenuate fibrosis dependant on the cell and context. Increased autophagy in hepatic stellate cells, key effectors of liver fibrosis, leads to fewer extracellular vesicles being released and attenuated fibrosis in vitro [395]. Whereas autophagy inhibition with carvedilol in these cells can promote their apoptosis and reduced fibrosis also in vitro [396].

In the lung, insufficient autophagy has been linked with epithelial cell senescence and induction of myofibroblast differentiation [397] perhaps representing the failure of a compensatory stress response.

Characterisation of autophagy in the eye is similarly eclectic. For example, ocular surface cells and tears demonstrated elevated autophagy pathway proteins, suggesting dysregulation, or more likely a response to stress [398]. In the cornea, autophagy is found to be involved in corneal opacification in granular dystrophy-2 where abnormal TGFB1 accumulates in the stroma. Although its precise contribution is not clear, it is thought basal autophagy is overwhelmed by the aggregates [399]. In vitro models of subconjunctival fibroblast driven fibrosis reveal that increasing autophagy inhibits cell differentiation and attenuates fibrosis [400].

Decorin's role in autophagic regulation has been characterised in other tissues. It is induced in cardiac tissue of nutrient deprived mice, where it also regulates autophagy, as demonstrated when its genetic deletion leads to abnormal autophagy flux [401]. In the vascular endothelium cells, it inhibits the release of vascular endothelial growth factor [402,403], and in carcinoma cells it causes mitochondrial membrane depolarisation leading to mitophagy [404].

The relationship of decorin and autophagy in corneal fibrosis is not known. Considering the heterogeneity of cells, and mechanisms involved, its role is likely to be complex. If it does have a regulatory role then it could potentially be leveraged to promote regeneration.

3.2 Aims & Objectives

This study aimed to explore the relationship of decorin, fibrosis and autophagy in an in vitro model of corneal fibrosis.

Objectives:

- Establish cultures of primary human corneal fibroblasts (PHCFs) and an invitro model of fibrosis
- Characterise the anti-fibrotic effect of decorin on PHCFs
- Determine whether modulation of autophagy alters fibroblast behaviour
- Determine whether treatment with decorin modulates autophagy

3.3 Methods

3.3.1 Primary cell culture, passage, and storage

Primary human corneal fibroblasts were cultured from corneal transplantation tissue surplus to surgical requirement [405], which was obtained under the ethics *Ocular Microenvironment in Health and Disease* (LREC 06/Q2702/44). Experiments were conducted in accordance with the Declaration of Helsinki. Atleast three intra-experimental replicates and three experiment repeats were performed unless stated otherwise.

Using aseptic technique, corneal stromal tissue was isolated by debridement of the epithelial and endothelial layers. Following which the superficial lamella of the stroma was debrided further. Stromal tissue was removed from the corneoscleral rim, cut into segments, placed in complete media (CM) in a 6-well plate and stored in an incubator at 37°C/5% CO_2. Fibroblasts were allowed time to grow until at least 30% (visual estimate) of the well surface was populated prior to their first passage. Cell media was replaced every two to three days. Following initial culture, routine passage was performed once cells reached at least 90% confluence. CM consisted of RPMI 1640 medium with 1% glutamate, penicillin & streptomycin (GPS, Sigma-Aldrich, Cambridge, UK) and 10% fetal bovine serum. Serum free media (SFM) consisted of RPMI 1640 medium with 1% GPS.

Passage was performed by washing the cells with Phosphate Buffered Saline (PBS) solution three times prior to application of 1x Trypsin-EDTA (TE, Sigma-Aldrich, Cambridge, UK) for up to 5 minutes. The TE and cell solution was neutralised by adding at least an equal volume of CM. The solution was centrifuged at 1500 rpm for 7 minutes to obtain a cell pellet. The supernatant was discarded and the cell pellet was resuspended in CM, with subsequent distribution of cells into the next container. If a specified number of cells were required for experimental purposes, they were counted using a haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Konigshofen, Germany) after being resuspended in fresh CM and distributed accordingly.

Cultured cells were stored at -80 °C in freezing solution if they were not required for experimentation. This was done after trypsinisation and centrifugation; the cell pellet was suspended in a solution of 50% heat inactivated fetal bovine serum (FBS, Gibco TM, ThermoFisher scientific, USA), 40% serum free media and 10% dimethyl sulfoximine (DMSO, Sigma-Aldrich, Cambridge, UK) at a concentration of approximately

$1x10^6$ cells per 1 ml of freezing solution. To re-culture these cells, the storing media and cell solution was diluted in CM and centrifuged at 1500rpm for 7 minutes prior to continuing cell culture as above.

3.3.2 Cell treatments

Reagents were made up in serum free medium (SFM) (unless stated otherwise) to the desired concentrations. Controls included CM or SFM depending on the experimental set up.

TGF β 1 (human recombinant, 100-21, Peprotech)

The experimental dose of 10 ng/ml was determined based on the literature [406]. It was prepared as recommended by the manufacturer. The stock solution should be further diluted to 1-10ng/ml in the appropriate cell culture immediately before use. Reconstitute in sterile 10mM Citric Acid, pH 3.0 to a concentration of 1.0 mg/ml (i.e. to 10µg add 10µl). To prepare 10mM citric acid, pH 3.0 add 192mg to 100ml sterile deionised water and then adjust pH of the solution with 1N Hydrochloric acid and/ or Sodium Hydroxide to pH 3.0 using a calibrated pH meter. Dilute to 100µg/ml by adding 90µl of sterile 0.1% bovine serum albumin in D-PBS in small, single use aliquots at -20 to -80□C for up to 12 months.

Decorin (Galacorin, human recombinant, Catalent Pharmaceuticals)

The dose of 1 µg/ml was determined based on the highest dose (Figure 3-4). This was in the range of doses in the literature with biological activity in vitro [407] and our experimental data regarding collagen fibrillogenesis (figure 2) [54].

Trehalose (PHR1344, Sigma-Aldrich)

The experimental dose was determined based on the literature [408]. For experiments 113.5mg of trehalose was dissolved in 3ml of media to give a 100mM solution and filtered using 0.2 µm syringe filter into a sterile vessel. It was diluted with further sterile media if appropriate. Trehalose solution was used on the day of preparation and stored in the fridge only for short periods of time if necessary (2-8°C).

Baffilomycin A1 (196000, Sigma-Aldrich)

A 100mM stock solution of bafilomycin in DMSO (10 µg in 160.6µl of DMSO) was prepared and stored at -20°C and should be defrosted thoroughly before opening the vial. Dilute 3 µl of 100mM solution in 3ml of media to give a 100µM solution. This was filtered through a 0.2 µm syringe filter and further diluted by 1000 fold to give a 100nM solution, typically used for autophagy experiments [408]. The diluted solution was not utilised after 4 weeks following preparation.

Rapamycin (553210, Sigma-Aldrich) experimental dose 100 nM.

Stock solutions of 200µM solution in DMSO were kept at -20°C for later use. These were diluted to 100nM solution in cell media for experiments. The were filter sterilised through 0.2 µm syringe filter. Typically 100nM is used to promote autophagy [409]. The diluted solution is stable for the duration of the specific complete cell culture media, typically 1-4 weeks after preparation.

3.3.3 CyQUANT proliferation assay

The CyQUANT (Thermo Fisher, USA) assay was used to assess cell proliferation as per the manufacturer protocol. It provides a measure of DNA content in samples, thereby giving a measure of cell number and overcoming limitations of metabolic activity-based cell assays. This was particularly important in the case of decorin administration because of its numerous binding partners and their potential to impact cell metabolism [163].

Cells were grown in 24-well plates (Corning, USA) and desired treatment were administered for 16 hours. Following this the treatments were washed off with PBS, and 200 µL of the CyQUANT® GR dye/cell-lysis buffer (prepared in Preparing the Reagent) was added to each well and incubated with the cells for 2-5 minutes in the dark prior to being measured on the microplate reader microplate reader (Spark, Tecan, USA) with filters set at ~480 nm excitation and ~520 nm emission.

3.3.4 MTT assay

The MTT assay (Abcam, USA) was used to determine cell viability following administration of various treatments, as per the manufacturer protocol. Cell were seeded into 96-well flat clear bottom black wall culture plates (Corning, NY, USA) and treatments applied when cells reached a confluency of 70%, typically 24 hours later. Treatments were applied for 16 hours before cell viability analysis was performed. Discard media from cell cultures. Cell media and MTT solution was combined in a 1:1 ratio and added onto cells which were incubated at 37°C for 3 hours.

After incubation 150 µL of MTT solvent was added into each well, the plate wrapped in foil and agitated on an orbital shaker for 15 minutes. To ensure adequate dissolution of the dye, each well was pipetted 5-10 times. The plates wear analysed on a microplate reader (Infinite M Nano, Tecan, USA) , with absorbance at OD=590 nm, within 1 hour.

3.3.5 Immunofluorescent studies

Fluorescent antibodies were utilised to characterise the cells and various cellular proteins. Targets included cell nuclei, the intermediate filaments vimentin, desmin and a-smooth muscle actin (αSMA), and the extracellular matrix proteins collagen I and fibronectin. Anti-FGFR4 and Anti-cytokeratin 3 were used as markers for and epithelial cells respectively [410,411].

Cells were seeded into eight-chambered culture slides, Corning Falcon (Corning, NY, USA) and incubated for 48 hours in media then incubated for a further 24 hours with the respective treatments prior to fixation, blocking and immunolabelling.

Once ready for immunolabelling, the media was removed and cells were fixed using 4% paraformaldehyde (PFA, Sigma-Aldrich, Cambridge, UK) at room temp for 15 minutes. PFA was subsequently removed and the cells were treated with 100µL of ice cold methanol and placed in a -20 ^{o}C freezer for 10 minutes. After the removal of methanol and three washes with PBS, blocking solution made of 5% FBS in PBS was applied to the cells, which were incubated at 37^{o}C/5%CO_2 for 30 minutes. After three further washes with PBS the cells were treated with 100µl of primary antibody solution for one hour at room temperature. The concentration ratio of the primary antibody solution was 1:100 (stock:PBS) for confirmation of cell culture purity, and 1:200 for the drug treatment experiments. Following this, the primary antibody was removed and each well was washed with 100µl of PBS x3 times. The secondary antibody alexa fluor 488 was then

applied in a solution with Hoechst nuclear stain for one hour in light-restricted conditions. These are diluted in PBS at a dilution of 1:100 and at 1:25 for Alexa Fluor 488 and Hoechst respectively. These are then removed and the cells are washed with 100µL PBS x3 times. The chamber moulds were removed using the kit's key and a coverslip coated in Vectamount (Fischer Scientific, USA) was placed over the slide and imaged or stored in light-restricted conditions.

For the cell culture characterisation images were taken using the Leica DM6000 microscope (Leica, Wetzlar, Germany) on the LasX software, at 80x-200x magnification. The green and blue filter cubes were used for Alex 488 and Hoechst respectively. Image acquisition settings were kept constant between samples.

For the immunofluorescence quantification experiments cell were grown in 96-well Greiner Screenstar plates (Greiner Bio-one, Germany), and imaged using the CQ1 Yokogawa confocal microscope (Yokogawa, Japan). Following the desired treatment and for 24 hours, cells were washed with PBS (Sigma-Aldrich, Cambridge, UK) once, before being fixed with 4% paraformaldehyde in PBS. Ice cold Methanol was used to permeabilise the cells in a -20 freezer for 20 minutes. Samples were subsequently blocked with solution consisting of 10% FBS in PBS to reduce non-specific fluorescent antibody staining. The samples were incubated with the antibodies overnight, then washed with PBS and submerged in PBS for imaging. The antibodies were suspended in a solution consisting of 0.05% Tween20 (Sigma-Aldrich, Cambridge, UK) in PBS with 3% w/v Bovine Serum Albumin (Sigma-Aldrich, Cambridge, UK).

3.3.6 Image analysis on Fiji

The expression of targets of interest were assessed by quantifying the fluorescence of cell samples. Two fields of the central portion of each well were sampled for fluorescence. The separate fluorescence channel images were analysed for surface area and mean intensity. To calculate the surface area, first the image was binarized using the Otsu threshold technique was used [412], and then all the occupied area was measured for further analysis. This was automated for high throughput analysis using a script on Fiji.

Table 3-1. List of antibodies and stains

Antibodies	Host species	Target species	dilution	provider	catalogue number
LC3	mouse	human	1:4000	Novus Biologicals	NB100-2220
Beclin-1	mouse	human	1:1000	Cell Signaling Technology	3738
ATG-5	mouse	human	1:400	Nanotools	0262-100
Tom20	mouse	human	1:5000	Santa Cruz Biotechnology	SC-17764
p62	mouse	human	1:1000	BD Biosciences	610832
β-actin	mouse	human	1:10,000	Sigma-Aldrich	A2228
Secondary antibodies					
Mouse IgG (Alexa Fluor® 488)	donkey	mouse	1:10,000	Abcam	ab150105
Mouse IgG horse raddish peroxidase	donkey	mouse	1:10,000	Vector Labs	PI-2000-1
Primary conjugated antibodies					
αSMA primary conjugated PERCP	mouse	human	1:100	RND systems	IC1420C
Desmin antibody [Y66] (Alexa Fluor® 488)	mouse	human	1:100	Abcam	ab185033
Vimentin Alexa Fluor® 594-conjugated Antibody	mouse	human	1:100	RND systems	IC2105T
Collagen I alpha 1 Antibody [FITC]	mouse	human	1:150	Novus Biologicals	NBP2-46874
Fibronectin (phytoerythrin)	mouse	human	1:150	MD systems	IC1918P
Other stains					
DAPI (4',6-diamidino-2-phenylindole)			1:500	Invitrogen	D1306

3.3.7 Wound healing assay

The wound healing assay was used to assess the behaviour of PHCFs in response to various treatments [413]. PHCFs were seeded into 12 – well plates (Corning, NY, USA) and grown until a confluent mono layer was formed. A single scratch was made in the monolayer using a 200 ul natural pipette tip (Sigma-Aldrich, UK) whilst the media was still in the well. The media was removed and after one gentle wash with PBS the cells were incubated with their respective treatments. The samples were imaged using the live cell imaging

microscope Cell IQ Analyser (Version 2.2.1 Chip-Man Technologies Ltd). Image analysis was semi-automated and was performed using the Cell IQ proprietary software. Outcomes included wound width and percentage wound closure.

3.3.8 Contraction assay

The contraction assay was used to evaluate fibroblast contractility in response to various treatments [414]. Cells were constituted with collagen solution which gelated in wells in preparation for the assay. To achieve this cells were harvested from culture containers and made up into a solution of 3 x 10^5 cells/ml of cell culture media. A 400 µl sample of cell solution was added to 200 µl of collagen I solution at 3mg/ml (Bovine collagen I, Thermo Fisher, USA) and 6 µl of NaOH was added to neutralise the pH of the mixture. Further NaOH was added in increments of 0.5 µl if the solution was not adequately neutralised. To create each gel 500 µl of the mixture was transferred into a well on a 24-well plate (Corning, NY, USA) and allowed to solidify for 20 minutes in a cell culture incubator at 37°C. The gel was then released from the edges by scraping around the edge with a 200 µl natural pipette tip (Sigma-Aldrich, UK) 3 times. To this 500 µl of culture media was added into the well with the respective treatments in solution and incubated for the duration of the experiment. The GelDoc (BioRad, UK) imaging system was used to obtain images of the gels at various time points and images were analysed using Fiji. The freehand draw tool was used to delineate the circumference of each gel following which the area of the upper surface was calculated. The diameter of the 6-well plate was used to scale each image for the calculation.

3.3.9 Polymerase Chain Reaction for analysis of protein transcription

3.3.9.1 RNA extraction

To determine cell response to various treatments their RNA expression was analysed. The RNeasy kits (Qiagen, USA) were used to extract RNA samples. Cells were seeded in 6 well plates at a density of 200,000 cells per well and treated 24-48 hours post seeding, following which they were harvested for RNA extraction.

RLT buffer from the kit was added directly to the cells after removal of the media in order to detach them from the plate and commence the lysis process. In accordance with manufacturer recommendations, β-mercaptoethanol (Gibco, Thermo Fisher scientific, USA) was added to the RLT buffer 10µl to 1ml. This

solution was then passed through the QiaShredder and RNA extracted according to the manufacturers protocol. DNase (RNase-Free DNase Set, Qiagen, USA) was used in the RNA spin columns in order to reduce sample contamination.

RNA quality was quantified using a spectrophotometer (Nanophotometer, Implen, Germany). Sample absorbance was measured at 260nm and 280nm and ratio determined for nucleic acid purity/contamination. Samples with a ratio between 2.1 and 1.8 were used for experiments. Lower ratios were deemed to be contaminated with protein and unsuitable for analysis. RNA was stored in -20^{o}C for short term storage and -80^{o}C if it was required to be stored for more than 1 day. RNA samples were converted to cDNA within 24 hours of extraction.

3.3.9.2 cDNA Synthesis

The SensiFAST cDNA Synthesis Kit (Meridian Bioscience, USA) was used according to the manufacture's guidance. Briefly, to produce cDNA, 4µl of 5xTranAmp Buffer and 1µl of ReverseTranscriptase were added to the RNA sample. The volume of the RNA sample was made up to 15 µl in RNAse free water so that a final reaction volume of 20µl was achieved. Typically between 600ng to 1000ng was used to make cDNA. This was determined by the concentration obtained at the quantification step.

PCR tubes containing the RNA sample and synthesis kit solution were loaded into a T100 thermal Cycler (BioRad, UK), which was programmed with the settings below. Following completion of the reaction the produced cDNA was assumed to be equivalent to the amount of RNA in the reaction. The cDNA was stored at -20 ^{o}C for short term and -80 ^{o}C for longer term storage.

Thermal Cycler Settings:

25 ^{o}C for 10 minutes (primer annealing)

42 ^{o}C for 15 minutes (reverse transcription)

48 ^{o}C for 15 minutes (inactivation)

10 ^{o}C to hold

3.3.9.3 Quantitative Real Time PCR

The QuantStudio 5 Real-Time PCR system (Applied Biosystems, Netherlands) was used to analyse PCR-reactions in real time using Power Up SYBR Green Master Mix (Applied Biosystems, Netherlands). The lay out of the reactions was planned on the Thermofisher Cloud website and the reactions were performed on a 384 well PCR plate. A final volume of 10ul per reaction was utilised, consisting of 2µl of cDNA at a concentration of 12.5-25 ng/µl, and 8 µl of primer and sybrgreen mix (conditions previously optimised in the group).

The QuantStudio 5 machine was programmed as per the manufacturers guidelines as follows:

Table 3-2 PCR thermal cycler settings

Cycle Step	No. of Repeats	Duration/Temperature
1	1	2 minutes/ 50 °C
2	1	10 minutes/ 95 °C
3 (Real Time)	40	a) 1 second/ 95 °C
		b) 30 seconds/ 60 °C
4 (Melt Curve)	1	a) 15 seconds/95 °C (ramp 1.6 °C/second)
		b) 1 minute/ 60 °C (ramp 1.6 °C/second)
		c) 15 seconds/95 °C (ramp 0.15 °C/second)

Table 3-3. Primer sequences	
Target RNA Primer	Primer Sequence (5'-3')
α-SMA forward Sigma-Aldrich	CCGACCGAATGCAGAAGGA
α-SMA reverse Sigma-Aldrich	ACAGAGTATTTGCGCTCCGAA
Fibronectin forward Sigma-Aldrich	CGGTGGCTGTCAGTCAAAG
Fibronectin reverse Sigma-Aldrich	AAACCTCGGCTTCCTCCATAA
Collagen I forward Sigma-Aldrich	GAGGGCCAAGACGAAGACA
Collagen I reverse Sigma-Aldrich	CAGATCACGTCATCGCACA
GAPDH Primer Design HK-SY-hu-1200	

Primers were kindly designed by Dr. Ghazala Begum [415].

Table 3-4. Gene sequences				
Gene of Interest	**Forward (5' to 3')**	**Reverse (5' to 3')**	**Elongation time (seconds)**	**Recommended RNA per assay (ng)**
SMAD 3	ACTCAAGAAGAC GGGGCAG	GGCACCAACAGG AGGTAG	25	20
SMAD 4	CCCATCCCGGAC ATTACTGG	GCACACCTTTGCC TATGTGC	25	20
SMAD 7	GGACGCTGTTGG TACACAAG	GCTGCATAAACTC GTGGTCATTG	25	20
SMAD 2	CGTCCATCTTGCC ATTCACG	CTCAAGCTCATCT AATCGTCCTG	25	20
GAPDH	Primer Design Cat. No. HK-SY-Hu	23 or 25	20 to 1.25	

3.3.10 Western blots

Primary human corneal fibroblasts were plated at a seeding density of approximately 26,000 cells per cm^2 in 6-well plates and treated when reached 70% confluence. Cells were scraped and cell pellets stored at -80°C until use. Protein was extracted in ice-cold RIPA lysis buffer (ThermoFisher, UK) supplemented with a protease inhibitor cocktail (Halt-100 Sigma, Poole, UK). Protein was quantified using a Bradford protein assay kit 1 (BioRad, UK).

Extracts were denatured by heating to 90°C for 5 mins and equivalent amounts of protein were used per lane for gel electrophoresis on a 12% Tris-glycine SDS gel. A fast transfer kit was used to transfer the protein onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Watford) using the Trans-Blot Turbo transfer system (BioRad, UK).

To probe for specific proteins on the band. Specific protein bands were detected through probing with appropriate primary and secondary antibodies (Table X) and visualised using chemiluminescence on G:Box Chemi XX6 (Syngene, Synoptics, UK).

The membranes were first blocked using 5% (w/v) dry milk powder (Marvel, UK) solution for 1 hour at room temperature. Then the membrane was segmented and treated with primary antibody solution in a 50ml test tube (ThermoFischer, UK) on a roller overnight in 4°C. Following this, the membrane was washed three times for 5 mins each with x1 Tris buffered saline with 0.1% tween20 (TBS/T, Cell signalling technology, Netherlands), before application of the Horse-radish-protein conjugated secondary antibody for 1 hour. Following application of the secondary antibody the membrane was washed three times as

before imaging. Antibodies were made up in a solution containing 1X Tris buffered saline with 0.1% tween20 (Cell signalling technology, Netherlands) and 5% dry milk. Antibodies and their dilutions are given in table xyz.

Protein chemiluminescence imaging was performed in a G:Box Chemi XX6. Membranes were incubated with LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water, Cell signalling technology, Netherlands) for 1 minute at room temperature and sequentially imaged over 10 minutes.

Treatments were run in triplicate and repeated on three independent occasions. Integrated band density was measured using Fiji and displayed as a percentage of loading control (β-actin). Error bars represent standard error of the mean (SEM).

3.3.10.1 Statistical analysis

Where appropriate, descriptive statistics were used. Pairwise comparisons of groups were performed using the Student's-T test for parametric data and Mann-Whitney U test for non-parametric data. The means of multiple groups were compared using the one-way analysis of variance (ANOVA) test, and post-hoc paiwise comparisons with Bnferoni's correction. All graphs were generated in, and all analyses performed using GraphPad Prism version 9.5.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3.3.11 Equipment and Reagent List

- 12 – well plates (Corning, NY, USA)
- 200 µL natrual pipette tips (Sigma-Aldrich, UK)
- 24-well plate (Corning, NY, USA)
- 96-well Greiner Screenstar plate (Greiner Bio-one, Germany)
- Baffilomycin A1 (196000, Sigma-Aldrich)
- Bovine Serum Albumin (Sigma-Aldrich, Cambridge, UK).
- Bradford protein assay kit 1 (BioRad, UK).
- Cell IQ Analyser (Version 2.2.1 Chip-Man Technologies Ltd).
- Collagen I solution (3mg/ml) (Bovine collagen I, Thermo Fisher, USA)
- CQ1 Yokogawa confocal microscope (Yokogawa, Japan).
- CyQUANT assay kit (Thermo Fisher, USA)
- Decorin (Galacorin, 16012, Catalent Pharmaceuticals, USA)
- Dimethyl sulfoximine (DMSO, Sigma-Aldrich, Cambridge, UK)
- DNase (RNase-Free DNase Set, Qiagen, USA)
- Eight-chambered culture slides, Corning Falcon (Corning, NY, USA)

- Fetal Bovine Serum (Gibco TM, ThermoFisher scientific, USA)
- G:Box Chemi XX6 (Syngene, Synoptics, UK).
- GelDoc (BioRad, UK)
- Glutamate, penicillin & streptomycin (GPS, Sigma-Aldrich, Cambridge, UK)
- Haemocytometer (Paul Marienfeld GmbH & Co. KG, Germany)
- Halt-100 (Sigma, Poole, UK)
- LasX microscope operating system software for (Leica, Germany)
- Leica DM6000 microscope (Leica, Germany)
- LumiGLO® (20X LumiGLO®, Cell signalling technology, Netherlands)
- Microplate reader (Infinite M Nano, Tecan, USA)
- Microplate reader (Spark, Tecan, USA)
- MTT assay kit (abcam, USA)
- Nanophotometer (Implen, Germany).
- Paraformaldehyde (PFA, Sigma-Aldrich, Cambridge, UK)
- PBS (Sigma-Aldrich, Cambridge, UK)
- Polyvinylidene difluoride (PVDF) membrane (Millipore, Watford)
- Power Up SYBR Green Master Mix (Applied Biosystems, Netherlands).
- QuantStudio 5 Real-Time PCR system (Applied Biosystems, Netherlands)
- Rapamycin (553210, Sigma-Aldrich)
- RIPA lysis buffer (ThermoFisher, UK)
- RNeasy kits (Qiagen, USA)
- SensiFAST cDNA Synthesis Kit (Meridian Bioscience, USA)
- T100 thermal Cycler (BioRad, UK),
- TGF β 1 (human recombinant, 100-21, Peprotech)
- Trans-Blot Turbo transfer system (BioRad, UK)
- Trehalose (PHR1344, Sigma-Aldrich)
- Tris buffered saline with 0.1% tween20 (Cell signalling technology, Netherlands)
- Trypsin-EDTA (TE, Sigma-Aldrich, Cambridge, UK)
- Tween20 (Sigma-Aldrich, Cambridge, UK)
- Vectamount (Fischer Scientific, USA)
- B-mercaptoethanol (Gibco, Thermo Fisher scientific, USA)

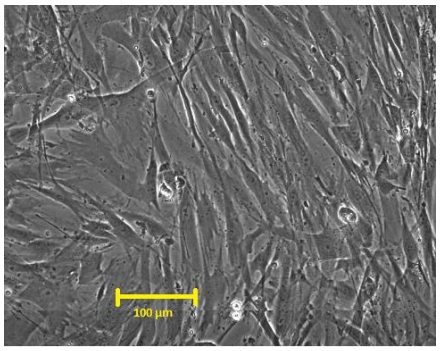
3.4 Results

3.4.1 Establishing primary human corneal fibroblast cell culture

To confirm the culture of isolated primary human corneal fibroblasts (PHCFs), cells were plated in eight-chamber culture slides and investigated immunocytologically. Positive staining for vimentin in the absence of contaminants confirmed pure PHCFs, whereas the presence of either FGFR-4 or cytokeratin indicated contamination of cultures by corneal epithelial cells respectively [410,411,416]. Controls included cells incubated with no anti bodies and secondary antibody only (anti-mouse Alexa fluor 488) alone to assess for any non-specific staining. To investigate whether culture cells were behaving normally [417], cultures were stained for proteins typically expressed by PHCFs including α-SMA, fibronectin and collagen and found to do so, Figure 3-2.

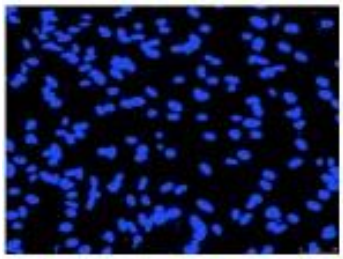
To optimise cell culture protocols, experiments to determine the ideal concentration of FBS were performed. A dose dependant proliferative response was seen with increasing FBS concentrations and so the mid-range dose was selected as it is also the most frequently observed in the literature Figure 3-3.

a)



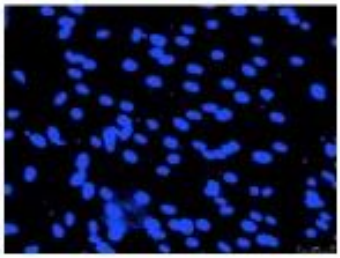
b)

c)



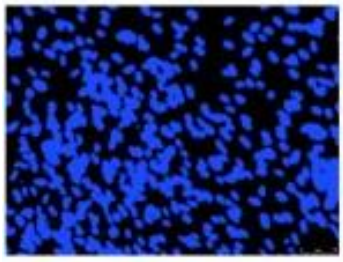
Control (no antibodies)

d)



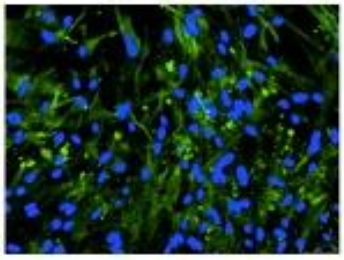
Anti-FGFR-4

e)

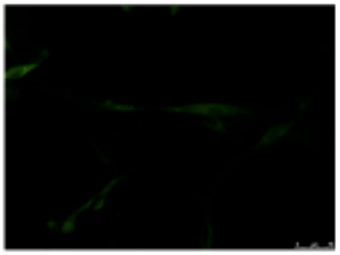


Cytokeratin

f)

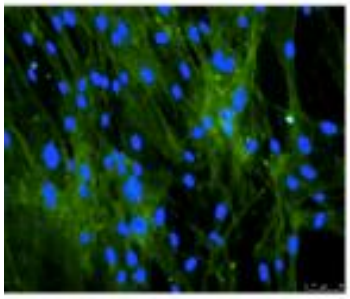


Vimentin



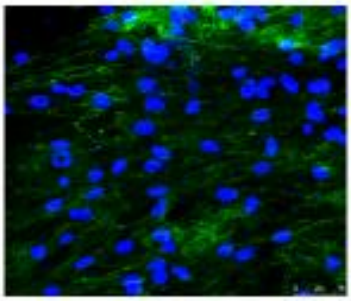
g) Secondary antibody Alexa 488 only (no primary antibody)

h)



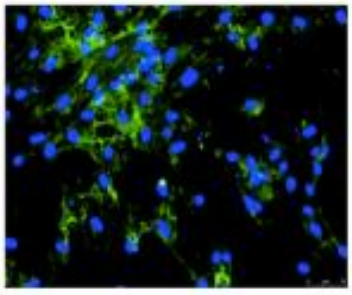
α-SMA

i)



Fibronectin

j)



Collagen-I

Figure 3-2 Establishing primary human corneal fibroblasts cell cultures.

To confirm the culture of isolated primary human corneal fibroblasts (PHCFs), cells were plated in eight-chamber culture slides for immunofluorescence. Brightfield and fluorescent microscopy was performed. Cells were then fixed and stained with primary and secondary antibodies to confirm culture purity and that cells were behaving as expected. A) Bright field image of confluent PHCFs demonstrating their typical fusiform shape and connections with nearby cells. B) Brightfield image (60x magnification) of cells left in serum free media for 2 weeks immediately after harvesting, demonstrate little proliferation.

Fluorescent microscopy was performed on a Leica DM6000 microscope at 80x magnification. PHCF nuclei were stained with DAPI (blue). Primary antibodies for various targets were then stained with a fluorescent secondary antibody (conjugated with Alexafluor 488 – green). C) No background staining is demonstrated in the absences of fluorescence antibodies. F) Positive staining for vimentin in the absence of FGFR-4 (d) or cytokeratin (e), confirmed that cultures were not contaminated. G) The secondary antibody demonstrated some but insignificant, non-specific binding in cell samples. PHCFs expressed α-SMA (h), fibronectin (i) and collagen (j).

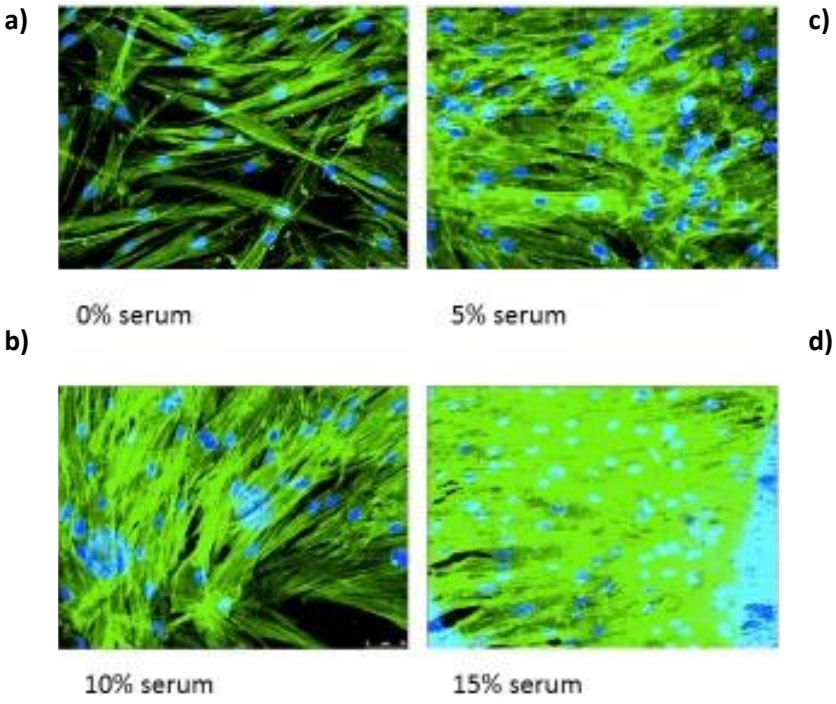


Figure 3-3. Establishing optimal cell culture conditions

To optimise cell culture conditions with specific regard to FBS, primary human corneal fibroblasts (PHCFs), were plated in eight-chamber culture slides, incubated with culture media containing varying concentrations of foetal bovine serum (FBS) for immunofluorescence. Cell nuclei were stained with DAPI (blue), primary antibodies to αSMA and secondary fluorescent antibodies (Alexaflour 488) and imaged on the Leica DM6000 at 80x magnification to observe for cell responses. High foetal bovine serum (FBS) concentrations of culture media led to excessive proliferation and αSMA expression. At 0% FBS cells did not proliferate and cultures were unable to be expanded for further experiments. This identified 10% FBS.

3.4.2 The Effect of Decorin on the PHCF fibrotic behaviour

3.4.2.1 Decorin does not affect cell proliferation within the treatment range, however super-doses impair proliferation.

To determine if decorin affected cell viability or proliferation, in order to inform dosing for later experiments, the Cy-Quant proliferation assay (Thermo Fisher Scientific, USA) was performed, investigating a broad dosing range Figure 3-4. Cells were treated for 16 hours with serial dilutions of decorin ranging from 0.001 µg to 100 µg (made up in SFM) alongside CM and SFM controls. No discernible differences were noted in cell survival in response to these doses of decorin apart from the highest dose of 100 µg where there was slight decrease compared to 1 and 10 µg as well as the SFM and CM controls, indicating mild toxicity at this super dose. This confirmed the safe range of decorin dosing for further experiments. It also clarified that PHCFs did not significantly proliferate in response to decorin treatment.

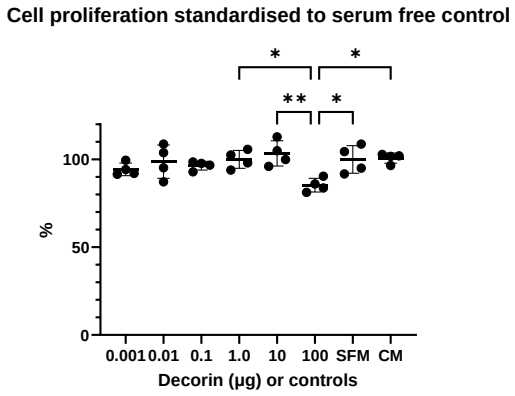


Figure 3-4. Primary human corneal fibroblast cell proliferation following 16 hours of treatment with various doses of decorin does not cause toxicity.

Primary human corneal fibroblast cells were seeded into 96 well plates and treated with various doses of decorin and serum free media (SFM) and complete media (CM) controls, for 16 hours. Their proliferation was then assessed using the Cy-Quant assay. Treatment with various doses of decorin does not cause toxicity except at 100 µg. One-way ANOVA p=0.008, Bonferroni's multiple comparisons tests 100 vs 1.0 p= 0.045, 100 vs 10 p=0.006, 100 vs SFM p=0.047, 100 vs CM p=0.029.

3.4.2.2 Decorin does not alter cell migration, serum promotes PHCF migration

Cell proliferation and migration are key components of wound healing. The *in vitro* scratch assay models wound closure using a monolayer of cells. Figure 3-5 demonstrates the progressive migration of PHCFs into the wound over time (a, b and c). Upon application of the standard fibrosis panel (SFM, CM, decorin and TGFβ1, both separate and combined in SFM) revealed little effect of the treatments on cell migration apart from that of CM which led to more rapid wound closure, Figure 3-5 d . At 40 hours the wound width was significantly smaller than the other groups (Student T-test's: CM vs TGFB1 p=0.003; CM vs decorin p=0.002; CM vs SFM p= 0.01; CM vs TGFb1+Decorin not-significant p=0.13).

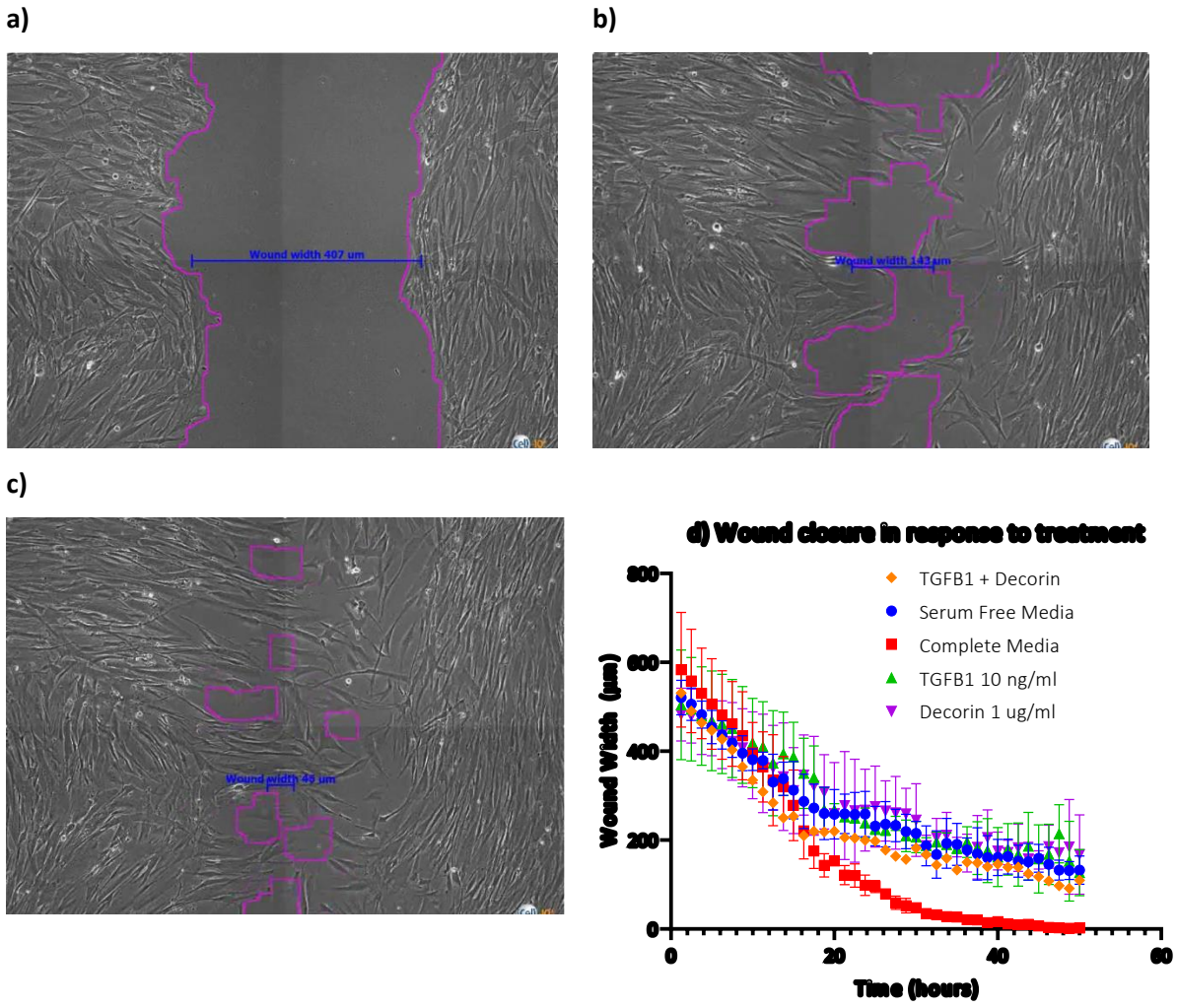


Figure 3-5. Scratch assay set up and time course of images demonstrate wound closure by primary human corneal fibroblasts.

Primary human corneal fibroblasts were seeded into 12 well plates and entered into experiments when 95-100% confluence was reached. They were then scratched with a 200µL pipette tip, carefully washed, and treated with the standard fibrosis panel for 48 hours. They underwent sequential imaging in the atmosphere-controlled Cell-IQ microscope over the incubation period (37oC/5% CO2). These images can then be analysed over time to determine the degree and rate of wound closure e.g. time zero (a), 8 hours (b) and 16 hours (c) post wounding.

PHCFs were treated with decorin and TGFβ1 in SFM, both separately and combined, alongside SFM and CM as controls. After 48 hours of incubation, no treatments impacted cell migration, apart from CM which significantly increased the rate of wound closure, leading to complete closure by 40 hours. At 40 hours the wound width was significantly smaller than the other groups (Student T-test's: CM vs TGFB1 p=0.003; CM vs decorin p=0.002; CM vs SFM p= 0.01; CM vs TGFb1+Decorin not-significant p=0.13).

3.4.2.3 Contraction assay set up and time point determination and TGFB1 cell contraction dose response

Wound contraction, a key step in corneal healing, is mediated by corneal fibroblasts. The contraction assay was performed to assess the contractility of PHCFs in response to experimental treatments.

Figure 3-6 a demonstrates the experimental set-up for the contraction assay on the G:Box XX6. Time course experiments revealed that PHCFs contracted the collagen gel up till around 24 hours after which there was little change. Further experiments were therefore after 24 hours. There was little observable impact of the TGFB1 treatments and SFM control, however the CM group demonstrated a profound contractile effect, Figure 3-6 b. At 9 hours' data revealed a significant difference between CM and all other groups (CM vs 100µg p = 0.0005; CM vs 10µg p=0.0003; CM vs 1µg p=<0.0001; CM vs SFM p=<0.0001). A trend for a TGFB1 dose response was noted. Using the 24 hours timepoint, TGFB1, decorin, in SFM and CM and SFM were compared (Figure 3-6 c). CM was significantly more contracted than the other groups (one-way ANOVA p=0.001; Bonferroni's tests CM vs SFM p= 0.011, CM vs TGFB1 p= 0.020, CM vs decorin p=0.002; CM vs TGFB1+decorin p=0.001).

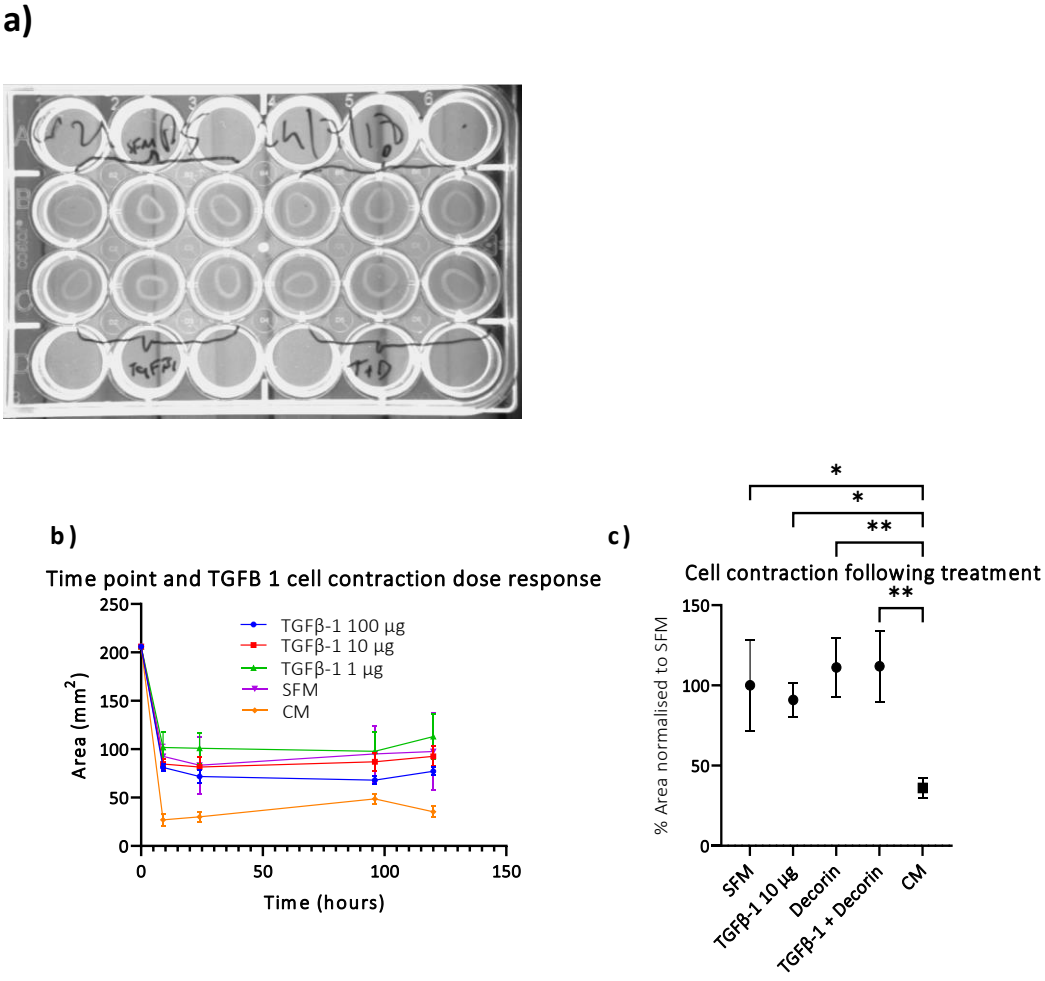


Figure 3-6. Collagen contraction assay results

Collagen I and primary human corneal fibroblast gels were created and incubated in 24-well plates (a) with the standard fibrosis panel. The plates were sequentially imaged in the G:Box xx6 at regular time intervals. PHCFs contracted the collagen gels up till around 24 hours, after which the response plateaued (b). TGFB1 demonstrated a dose response (not significant). Treatment with the standard fibrosis panel assessed at 24 hours revealed that CM was significantly more contracted than the other groups (one-way ANOVA p=0.001; Bonferroni's tests CM vs SFM p= 0.011, CM vs TGFB1 p= 0.020, CM vs decorin p=0.002; CM vs TGFB1+decorin p=0.001).

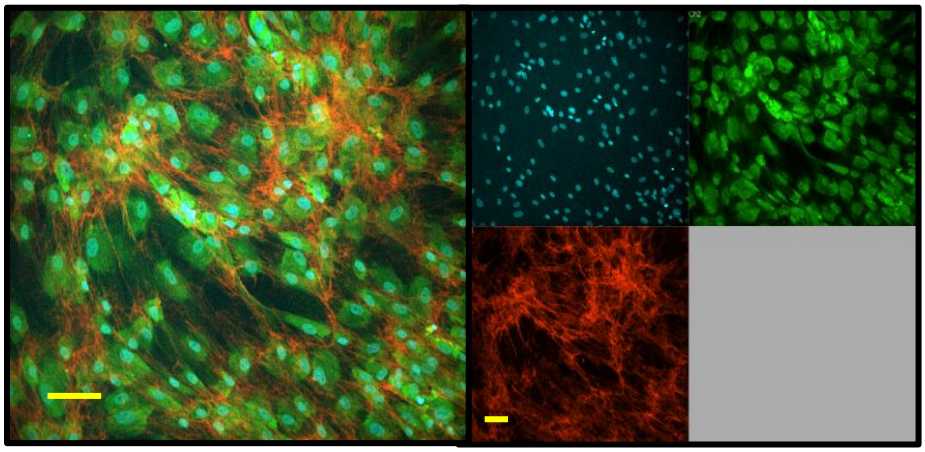
3.4.2.4 Cellular protein expression:

Corneal fibroblasts increase expression of various proteins as they partake in wound healing. Collagen I and fibronectin are expressed to repair lost ECM. The intermediate filaments vimentin, αSMA and desmin

are expressed sequentially, each in addition to the last, where keratocytes differentiate into fibroblasts and then myofibroblasts [59].

Fluorescent microscopy was performed to identify and quantify these proteins in response to PHCF incubation with the various treatments for 24 hours. Two images were acquired for each sample with 3 technical repeats of each experimental condition. Images of the individual channels were exported and analysed in Fiji (image j) to calculate the area of fluorescence and mean signal intensity of each image. To calculate the area the Otsu thresholding technique was used to binarise the images following which the area was calculated. A One-way ANOVA was performed to assess statistical significance, where indicated, post-hoc pairwise comparisons were performed using Bonferroni's correction. All images were also reviewed qualitatively to ensure validity for entry into quantification analyses, Figure 3-7.

a)



b)

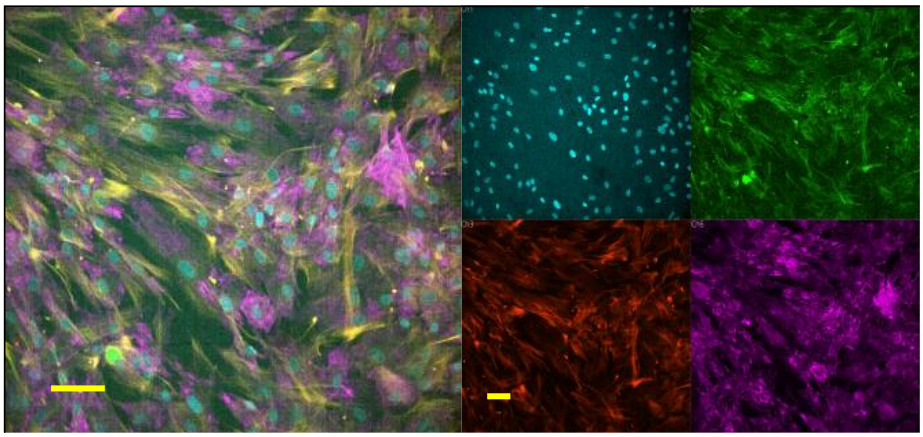


Figure 3-7. Primary human corneal fibroblast immunofluorescence images of fibrotic proteins and intermediate filaments.

Primary human corneal fibroblasts (PHCFs) were seeded into 96-well Greiner screenstar plates and following any treatments, were stained for (a) fibrotic proteins – cell nuceli (blue channel – DAPI), collagen I (Green channel – FITC), fibronectin (red channel – phycoerythrin) and (b) intermediate filaments, desmin (green – Alexafluor 488), vimentin (red -Alexafluor 524) and αSMA (purple – Percp). Example of the separate channels and overlay images of primary human corneal fibroblasts (PHCFs) stained for fibrotic proteins and intermediate filaments using multiplex analysis. Scale bar is 100 µm. Images were acquired on the CQ-1 spinning disc confocal microscope (Yokogawa, Japan). A fluorescence marker and scanning protocol (see appendix) was optimised to minimise risk of contaminant fluorescence.

3.4.2.5 TGFB1 in serum-free media increases extracellular matrix protein expression (collagen and fibronectin) in PHCFs, however decorin does not inhibit this.

PHCFs treated with SFM expressed comparatively less collagen I and fibronectin than CM and TGFb1 cells, Figure 3-8. When these images were quantified, both the percentage area of the image field and the mean intensity of antibody staining were increased in cells treated with TGFB1 compared to the SFM control. A similar result was seen for cells treated with the CM control suggesting that the growth factors within serum promote a fibrotic phenotype. Decorin itself had no impact on the degree of fibrotic protein expression, nor did it impact the effect of TGFB1 when they were co-administered, Figure 3-9. Nuclei for all fields were counted and compared to determine if differential cellular proliferation in the wells may bias the results, no differences were observed (Figure 3-8 d), One-way ANOVA p=0.46.

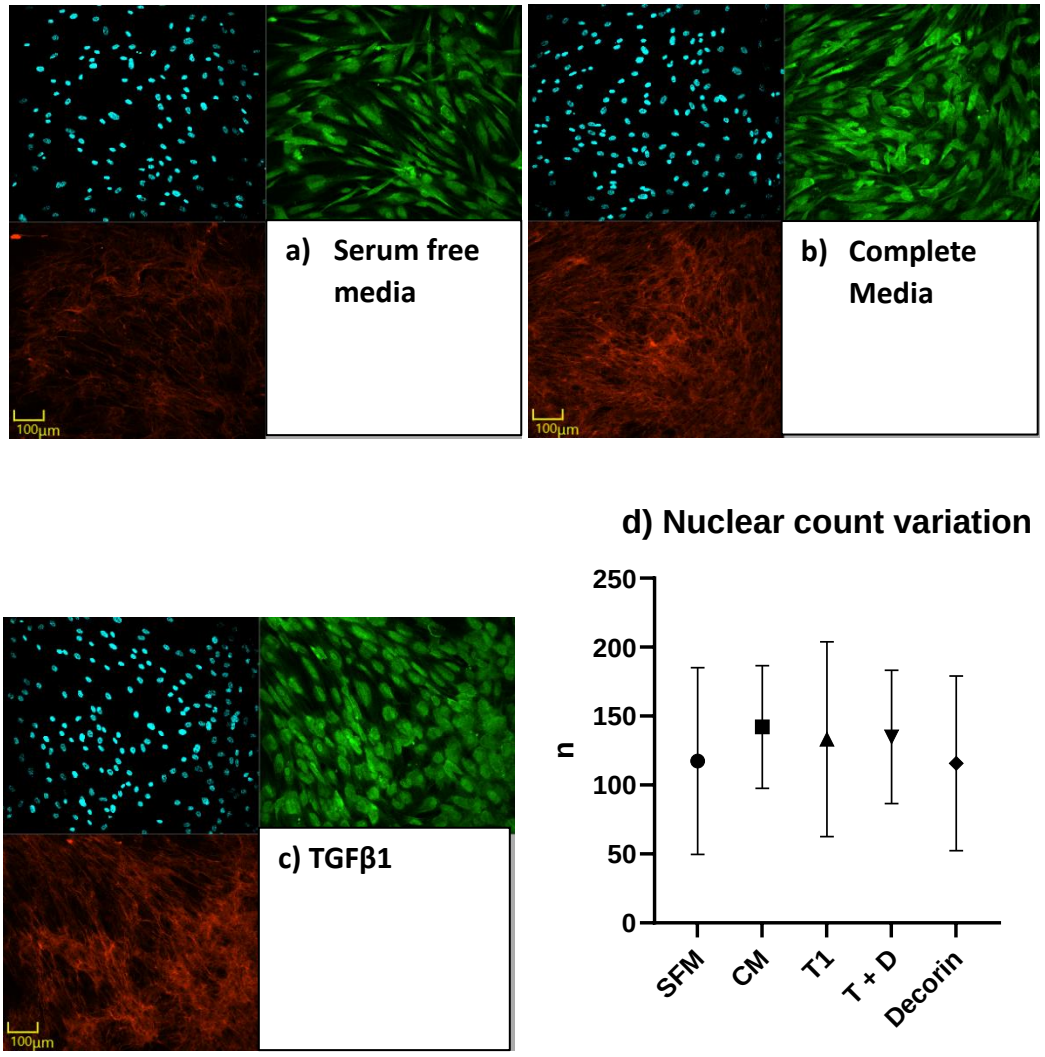


Figure 3-8. Immunofluorescence images of primary human corneal fibroblasts following different treatment.

Primary human corneal fibroblasts (PHCFs) were seeded into 96-well plates for 24 hours before being administered various treatments, incubated for a further 24 hours then stained for fibrotic proteins collagen I (Green - FITC), fibronectin (red - phycoerythrin), and cell nuceli (blue -DAPI). PHCFs treated with complete media(b) and TGFB1(c) demonstrate greater fibrotic protein expression than serum free media (a). Nuclei for all fields were counted and compared to determine if differential cellular proliferation in the wells may bias the results, no differences were observed (d), One-way ANOVA p=0.46. Abbreviations SFM – serum free media CM complete media; T1 – TGFβ1; T+D - TGFβ1 + decorin.

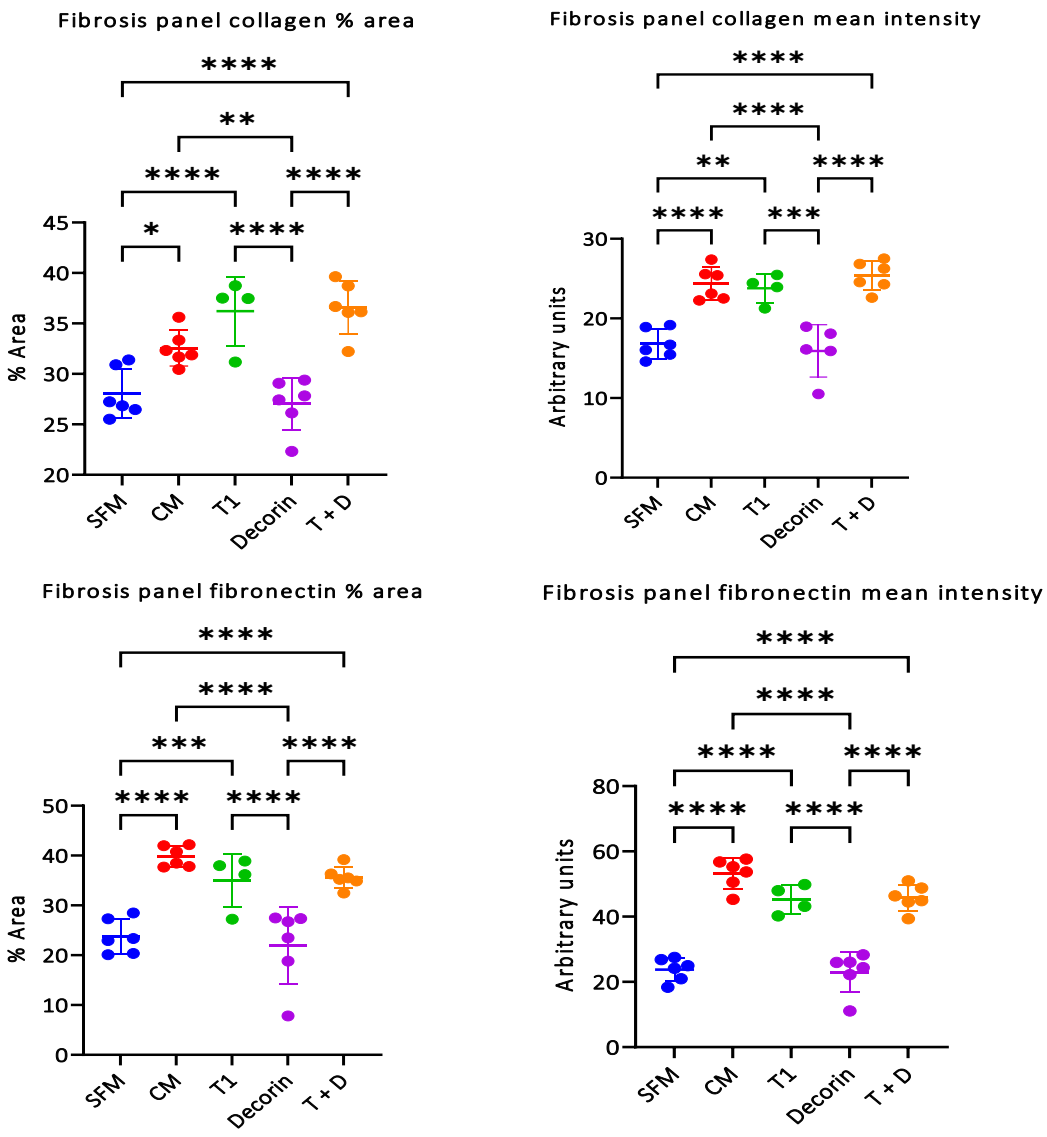


Figure 3-9. Immunofluorescence quantification of collagen I and fibronectin area and mean intensity in primary human corneal fibroblasts treated with the fibrosis panel treatments

Primary human corneal fibroblasts (PHCFs) were seeded into 96-well plates for 24 hours before being administered the standard fibrosis panel (SFM – serum free media; CM – complete media; TGFB1; decorin; and T+D -TGFB1 + decorin) and then incubated for a further 24 hours. They were then immunolabelled targeting the fibrotic proteins collagen I and fibronectin in a multiplex analysis. Post acquisition image analysis of the separate channels was performed on Fiji, in order to determine the percentage area and

mean-intensity of the target proteins in the imaging field. Treatment with CM, TGFB1, TGFB1+decorin all induced a pro-fibrotic response. Differences between the groups were tested for using a one-way ANOVA with post-hoc pairwise comparisons using Bonferroni's. Table 3-6 lists the highlighted significant results above.

Table 3-5. Fibrosis panel ANOVA significance results	
Collagen % area – ANOVA p = <0.0001	Adjusted P Value
SFM vs. CM	0.036
SFM vs. T1	<0.0001
SFM vs. T + D	<0.0001
CM vs. Decorin	0.0018
T1 vs. Decorin	<0.0001
T + D vs. Decorin	<0.0001
Collagen mean intensity ANOVA p= <0.0001	Adjusted P Value
SFM vs. CM	<0.0001
SFM vs. T + D	<0.0001
SFM vs. T1	0.0015
CM vs. Decorin	<0.0001
T1 vs. Decorin	0.0003
T + D vs. Decorin	<0.0001
Collagen mean intensity ANOVA p= <0.0001	Adjusted P Value
SFM vs. CM	<0.0001
SFM vs. T1	0.0006
SFM vs. T + D	<0.0001
CM vs. Decorin	<0.0001
T1 vs. Decorin	<0.0001
T + D vs. Decorin	<0.0001
Fibronectin mean intensity ANOVA p= <0.0001	Adjusted P Value
SFM vs. CM	<0.0001
SFM vs. T1	<0.0001
SFM vs. T + D	<0.0001
CM vs. Decorin	<0.0001
T1 vs. Decorin	<0.0001
T + D vs. Decorin	<0.0001

The table reports the results of the one-way ANOVAs and post hoc Bonferroni's tests of the collagen and fibronectin data following cell treatment with the standard fibrosis panel.

3.4.2.6 TGFB1 in serum-free media and decorin do not affect the expression of the intermediate filaments alpha-smooth muscle actin, vimentin and desmin

None of the standard fibrosis panel treatments, including SFM, impacted PCHF expression of intermediate filaments, Figure 3-10.

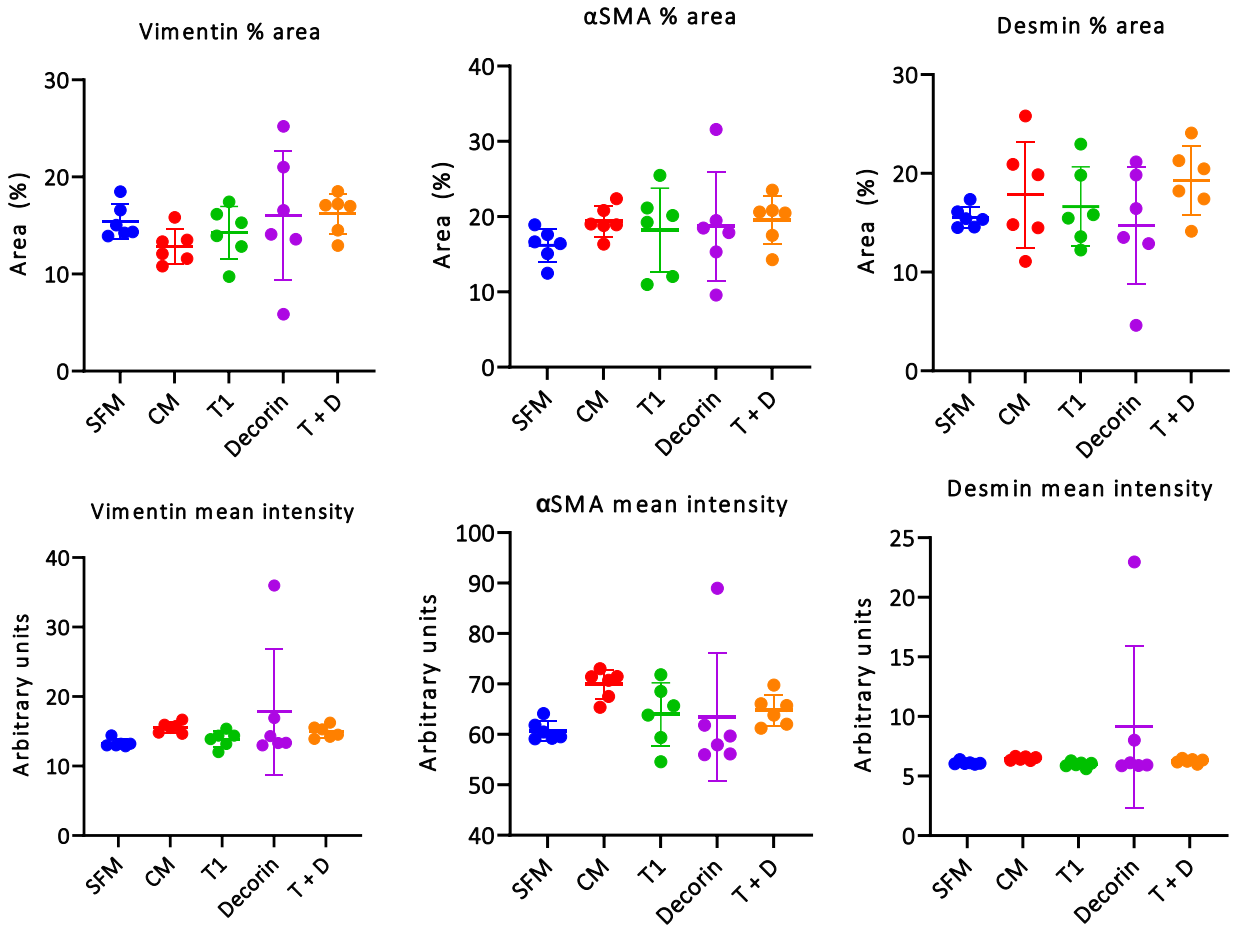


Figure 3-10. Immunofluorescence quantification of the intermediate filament area and mean intensity in primary human corneal fibroblasts treated with the fibrosis panel.

Primary human corneal fibroblasts (PHCFs) were seeded into 96-well plates for 24 hours before being administered the standard fibrosis panel (SFM – serum free media; CM – complete media; TGFB1; decorin; and T+D -TGFB1 + decorin) and then incubated for a further 24 hours. They were then immunolabelled targeting the intermediate filaments vimentin, αSMA and semin, in a multiplex analysis. Post acquisition

image analysis of the separate channels was performed on Fiji, in order to determine the percentage area and mean-intensity of the target proteins in the imaging field.

No significant differences between the treatment groups were noted using a one-way ANOVA.

3.4.2.7 RNA expression of fibrotic proteins and the SMAD signalling pathway – TGFB1 induces SMAD 7 however decorin does not inhibit this.

TGFB1 works through a number of cell signalling pathways, the SMAD pathway being particularly relevant in fibrosis [418]. TGFB1 PHCFs were again treated with the fibrosis panel and the cell samples were harvested for real time quantitative PCR analysis to measure changes in RNA expression, measured as fold- change compared to the housekeeping gene. Treatments were administered in SFM, which was also used on its own as a control group. Fibronectin and collagen-I demonstrated little response to any of the treatments. A trend for TGFB1 to increase αSMA was noticed, however this was not statistically significant. Decorin did not appear to significantly impact this trend, Figure 3-11.

Of the SMADs involved in transducing TGFB1 signalling none appeared to have increased expression after 24 hours of treatment with TGFB1. Decorin did appear to increase SMAD 3 expression. However, the expression of SMAD 7, an inhibitory SMAD, was increased by 24 hours of treatment with TGFB1 alone and with decorin, but not by decorin alone.

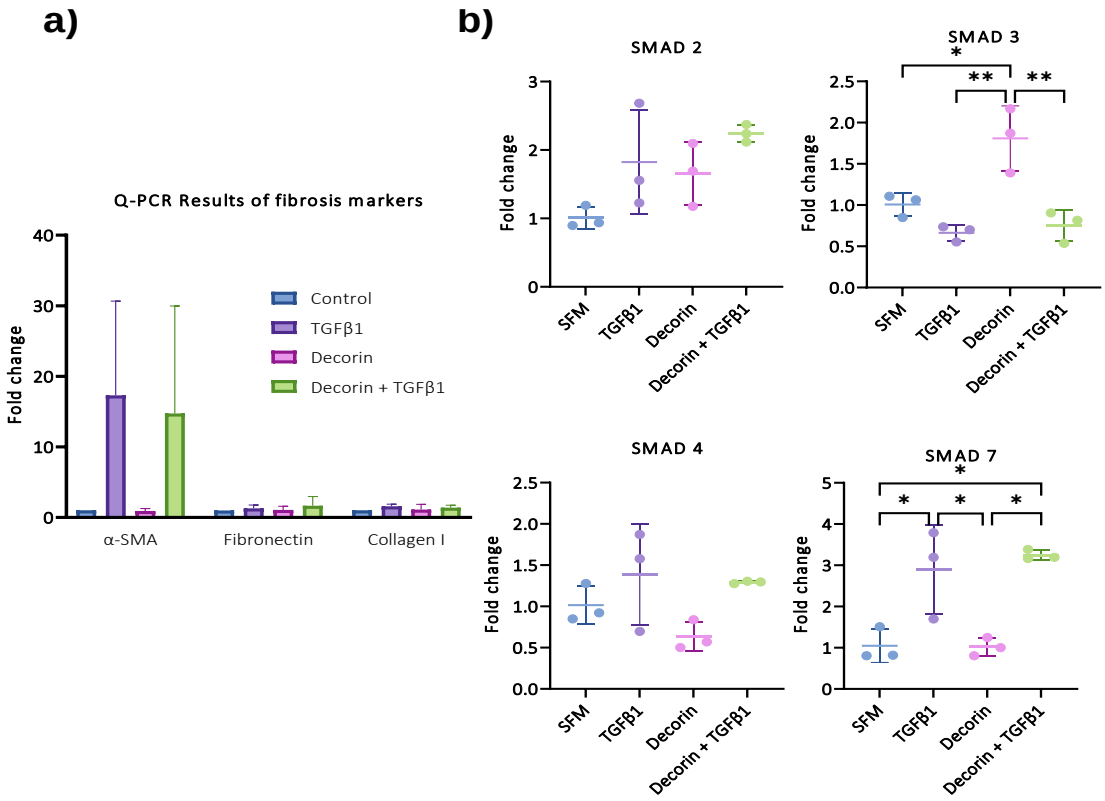


Figure 3-11. Q-PCR results of fibrotic protein and SMAD genes in primary human corneal fibroblasts treated with the fibrosis panel.

Primary human corneal fibroblasts were grown in six-well plates and treated with the standard fibrosis panel (SFM – serum free media, CM – complete media, decorin and TGFB1 both separate and combined) for 24 hours. Samples were interrogated for their differential RNA expression of collagen-I, fibronectin (a), and SMAD genes (b), presented as fold-change compared to the control gene, GAPDH.

3.4.3 Decorin, autophagy and fibrosis in vitro

In this section, decorin's role in PHCF autophagy was investigated to determine if it modulates corneal fibrosis through this pathway. A number of known autophagy inducers were tested in parallel to compare their impact on the behaviour of PHCFs. Functional cellular assays (scratch and IHC) were followed by western blot analysis of autophagy induction in treated PHCF samples.

TGFB1, Trehalose and rapamycin are all autophagy inducers, which are known to work through different pathways. Since these and decorin are all inducers working through different pathways they were all investigated to determine if they would differentially impact PHCF fibrotic phenotype, thereby implicating a role for autophagy in corneal fibrosis.

3.4.3.1 Autophagy modulators do not impact PHCF wound closure, CM has the most significant effect

PHCFs were treated with decorin and TGFβ1, both separately and combined; trehalose (100mM); rapamycin (dose); and TGFβ1 with trehalose, alongside SFM and CM controls.

After 48 hours of incubation, all treatments appeared to impact cell migration equivocally, apart from CM which significantly increased the rate of wound closure, leading to complete closure by around 40 hours. At 40 hours the difference in wound widths was compared across all the treatment groups using a one-way ANOVA with post-hoc pairwise comparisons and Bonferroni's correction (ANOVA p= 0.0056, CM vs SFM p= 0.014, CM vs TGFβ1 p= 0.002, CM vs decorin p=0.002, CM vs rapamycin p=0.026), Figure 3-12.

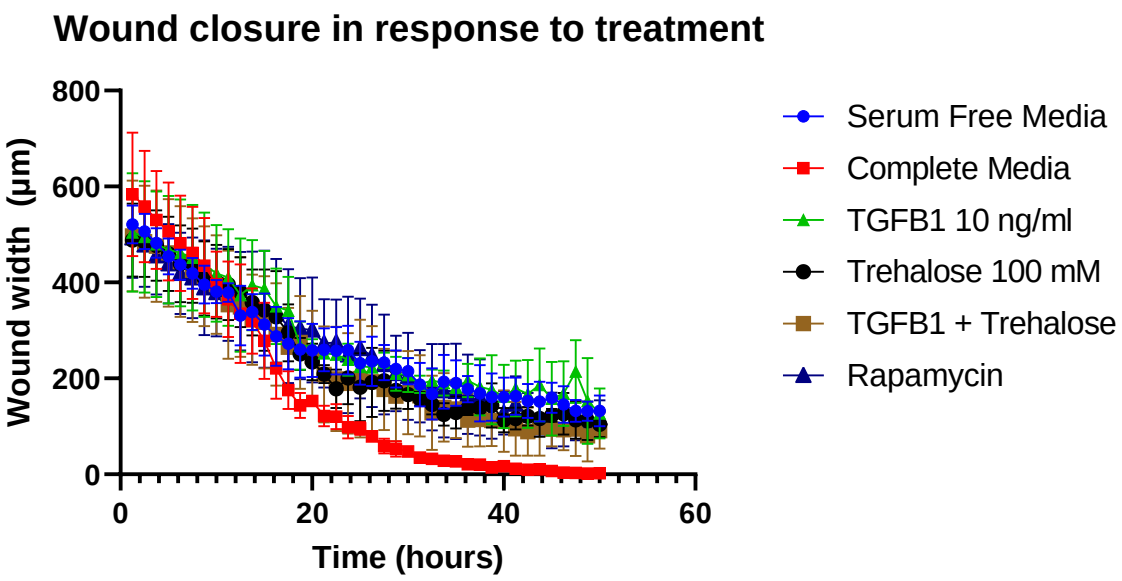


Figure 3-12. Wound closure in response to the autophagy panel of treatment

Primary human corneal fibroblasts were seeded into 12 well plates and entered into experiments when 95-100% confluence was reached. They were then scratched with a 200µL pipette tip, carefully washed, and treated with the standard fibrosis panel for 48 hours. They underwent sequential imaging in the atmosphere-controlled Cell-IQ microscope over the incubation period (37oC/5% CO2). These images can then be analysed over time to determine the degree and rate of wound closure.

PHCFs were treated with decorin and TGFβ1 in serum free media (SFM), both separately and combined, alongside SFM and complete media as controls. The autophagy panel (bafilomycin, rapamycin, trehalose and trehalose and TGFB1). After 48 hours of incubation, no treatments impacted cell migration, apart from CM which significantly increased the rate of wound closure, leading to complete closure by 40 hours.

3.4.3.2 IHC quantification of PHCFs treated with the autophagy panel reveals that TGFβ1 increases fibrosis which was not attenuated by decorin, but was by trehalose.

Quantification of the ECM proteins revealed that PHCFs increased expression of collagen-I and fibronectin in response to treatment with CM and TGFB1 but none of the other autophagy inducers. Interestingly, starvation of nutrition and growth factors is known to induce autophagy and SFM had amongst the lowest expression of the ECM proteins. Whilst trehalose did not significantly increase or decrease ECM protein production, it attenuated the effects of TGFB1 when co-administered Figure 3-13. One-way ANOVA with post-hoc pairwise comparisons and Bonferroni's correction were conducted on each experiment and the significant results are list in Table 3-7. With regards to intermediate filaments no convincing pattern of expression was observed although upon statistical testing a number of comparisons were significant, Figure 3-14 Table 3-8. Cell nuclei were compared between groups to indicate if this was affecting results and no differences were found Figure 3-13. Figure 3-15 is an illustrative IHC image of th effect of trehalose and TGFβ1 coadminstration.

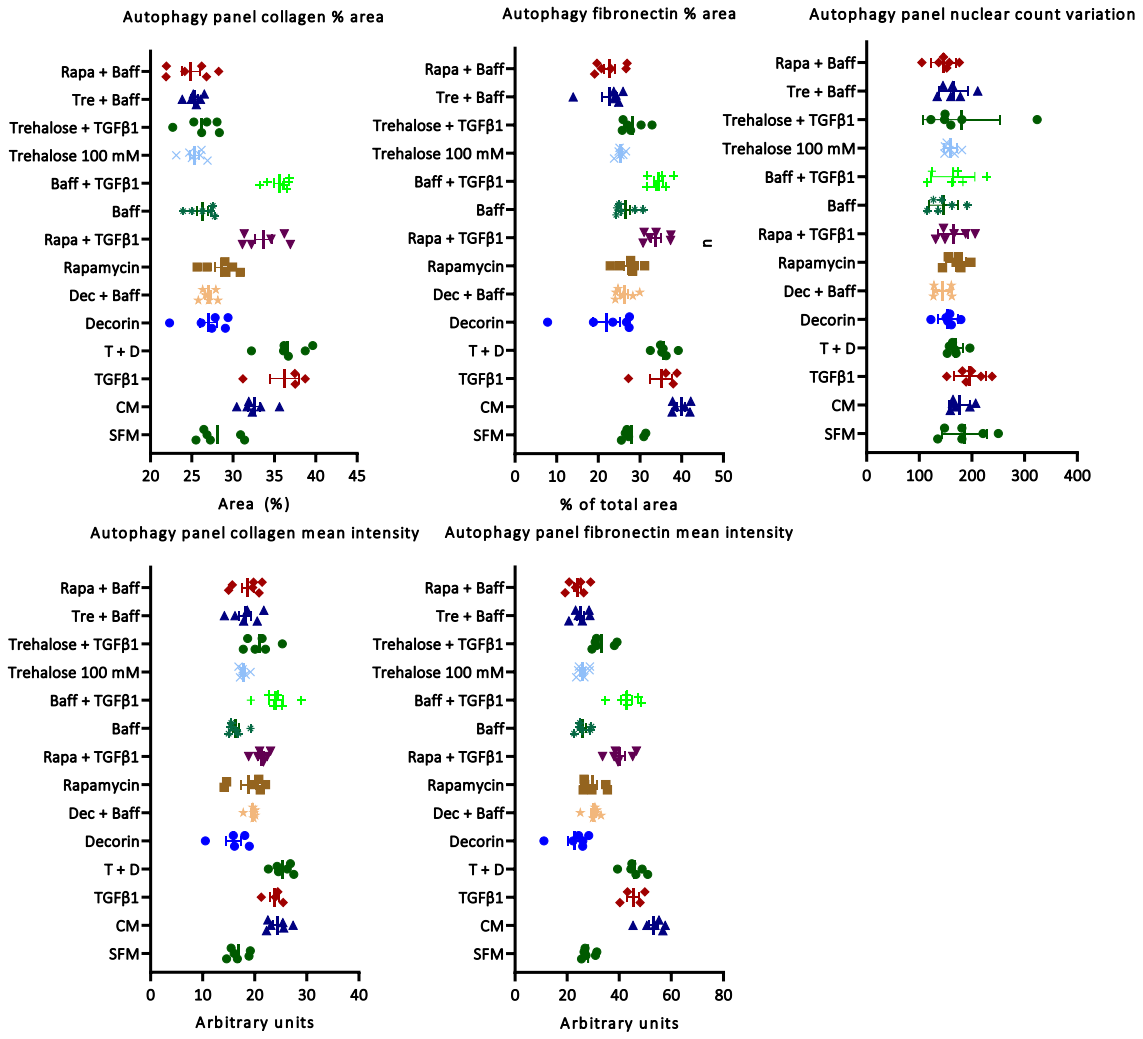


Figure 3-13. Immunofluorescence quantification of collagen and fibronectin in primary human corneal fibroblasts treated with the autophagy panel.

Primary human corneal fibroblasts (PHCFs) were seeded into 96-well plates for 24 hours before being administered the standard fibrosis panel (SFM – serum free media; CM – complete media; TGFB1; decorin; and T+D -TGFB1 + decorin, bafilomycin, rapamycin and trehalose, as well as combinations of them) and then incubated for a further 24 hours. They were then immunolabelled targeting the fibrotic proteins collagen I and fibronectin in a multiplex analysis. Post acquisition image analysis of the separate channels was performed on Fiji, in order to determine the percentage area and mean-intensity of the target proteins in the imaging field. Nuclei for all fields were counted and compared to determine if differential cellular proliferation in the wells may bias the results, no differences were observed (d), One-way ANOVA p=0.75.

Treatment with CM, TGFB1, TGFB1+decorin all induced a pro-fibrotic response. Differences between the groups were tested for using a one-way ANOVA with post-hoc pairwise comparisons using Bonferroni's. Table 3-7lists the highlighted significant results above.

Abbreviations SFM – serum free media CM complete media; T1 – TGFβ1; T+D - TGFβ1 + decorin.

Table 3-6. Autophagy panel ANOVA significance results

A one-way ANOVA was performed on the ombnibus, followed by post-hoc analysis with Bonferroni's test. Significance is denoted as follows: not significant p>0.05, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p≤ 0.0001.

Collagen % area ANOVA ****		Collagen mean intensity ANOVA ****		Fibronectin % area ANOVA ****		Fibronectin mean intensity ANOVA ****	
Baff + TGFB1 vs. Rapa + Baff	****	Baff + TGFB1 vs. Rapa + Baff	*	Baff + TGFB1 vs. Rapa + Baff	****	Baff + TGFB1 vs. Rapa + Baff	****
Baff + TGFB1 vs. Tre + Baff	****	Baff + TGFB1 vs. Tre + Baff	**	Baff + TGFB1 vs. Tre + Baff	****	Baff + TGFB1 vs. Tre + Baff	****
Baff + TGFB1 vs. Trehalose + TGFB	****	Baff + TGFB1 vs. Trehalose 100 mM	**	Baff + TGFB1 vs. Trehalose 100 mM	**	Baff + TGFB1 vs. Trehalose + TGFB	*
Baff + TGFB1 vs. Trehalose 100 mM	****	Baff vs. Baff + TGFB1	****	Baff vs. Baff + TGFB1	*	Baff + TGFB1 vs. Trehalose 100 mM	****
Baff vs. Baff + TGFB1	****	CM vs. Baff	****	CM vs. Baff	****	Baff vs. Baff + TGFB1	****
CM vs. Baff	***	CM vs. Decorin	****	CM vs. Dec + Baff	****	CM vs. Baff	****
CM vs. Dec + Baff	**	CM vs. Rapa + Baff	**	CM vs. Decorin	****	CM vs. Baff + TGFB1	**
CM vs. Decorin	**	CM vs. Rapamycin	**	CM vs. Rapa + Baff	****	CM vs. Dec + Baff	****
CM vs. Rapa + Baff	****	CM vs. Tre + Baff	**	CM vs. Rapamycin	****	CM vs. Decorin	****
CM vs. Tre + Baff	****	CM vs. Trehalose 100 mM	***	CM vs. Tre + Baff	****	CM vs. Rapa + Baff	****
CM vs. Trehalose + TGFB	***	Decorin vs. Baff + TGFB1	****	CM vs. Trehalose + TGFB	****	CM vs. Rapa + TGFB1	****
CM vs. Trehalose 100 mM	****	Decorin vs. Rapa + TGFB1	*	CM vs. Trehalose 100 mM	****	CM vs. Rapamycin	****
Dec + Baff vs. Baff + TGFB1	****	Rapamycin vs. Baff + TGFB1	*	Dec + Baff vs. Baff + TGFB1	*	CM vs. Tre + Baff	****
Dec + Baff vs. Rapa + TGFB1	****	SFM vs. Baff + TGFB1	****	Dec + Baff vs. Rapa + TGFB1	*	CM vs. Trehalose + TGFB	****
Decorin vs. Baff + TGFB1	****	SFM vs. CM	****	Decorin vs. Baff + TGFB1	****	CM vs. Trehalose 100 mM	****
Decorin vs. Rapa + TGFB1	****	SFM vs. T + D	****	Decorin vs. Rapa + TGFB1	****	Dec + Baff vs. Baff + TGFB1	****
Rapa + TGFB1 vs. Baff	****	SFM vs. T1	**	Rapa + TGFB1 vs. Rapa + Baff	****	Dec + Baff vs. Rapa + TGFB1	**
Rapa + TGFB1 vs. Rapa + Baff	****	T + D vs. Baff	****	Rapa + TGFB1 vs. Tre + Baff	***	Decorin vs. Baff + TGFB1	****
Rapa + TGFB1 vs. Tre + Baff	****	T + D vs. Dec + Baff	**	Rapa + TGFB1 vs. Trehalose 100 mM	**	Decorin vs. Rapa + TGFB1	****
Rapa + TGFB1 vs. Trehalose + TGFB	****	T + D vs. Decorin	****	SFM vs. Baff + TGFB1	***	Decorin vs. Trehalose + TGFB	**
Rapa + TGFB1 vs. Trehalose 100 mM	****	T + D vs. Rapa + Baff	***	SFM vs. CM	****	Rapa + TGFB1 vs. Baff	****
Rapamycin vs. Baff + TGFB1	****	T + D vs. Rapamycin	***	SFM vs. Rapa + TGFB1	***	Rapa + TGFB1 vs. Rapa + Baff	****

Rapamycin vs. Rapa + TGFB1	**	T + D vs. Tre + Baff	***	SFM vs. T + D	****	Rapa + TGFB1 vs. Tre + Baff	****
SFM vs. Baff + TGFB1	****	T + D vs. Trehalose 100 mM	****	SFM vs. T1	***	Rapa + TGFB1 vs. Trehalose 100 mM	****
SFM vs. CM	*	T1 vs. Baff	***	T + D vs. Baff	**	Rapamycin vs. Baff + TGFB1	****
SFM vs. Rapa + TGFB1	**	T1 vs. Decorin	***	T + D vs. Dec + Baff	**	Rapamycin vs. Rapa + TGFB1	**
SFM vs. T + D	****	T1 vs. Tre + Baff	*	T + D vs. Decorin	****	SFM vs. Baff + TGFB1	****
SFM vs. T1	****	T1 vs. Trehalose 100 mM	*	T + D vs. Rapa + Baff	****	SFM vs. CM	****
T + D vs. Baff	****			T + D vs. Rapamycin	*	SFM vs. Rapa + TGFB1	****
T + D vs. Dec + Baff	****			T + D vs. Tre + Baff	****	SFM vs. T + D	****
T + D vs. Decorin	****			T + D vs. Trehalose 100 mM	***	SFM vs. T1	****
T + D vs. Rapa + Baff	****			T1 vs. Baff	*	SFM vs. Trehalose + TGFB	*
T + D vs. Rapamycin	****			T1 vs. Dec + Baff	*	T + D vs. Baff	****
T + D vs. Tre + Baff	****			T1 vs. Decorin	****	T + D vs. Dec + Baff	****
T + D vs. Trehalose + TGFB	****			T1 vs. Rapa + Baff	****	T + D vs. Decorin	****
T + D vs. Trehalose 100 mM	****			T1 vs. Tre + Baff	***	T + D vs. Rapa + Baff	****
T1 vs. Baff	****			T1 vs. Trehalose 100 mM	**	T + D vs. Rapamycin	****
T1 vs. Dec + Baff	****					T + D vs. Tre + Baff	****
T1 vs. Decorin	****					T + D vs. Trehalose + TGFB	***
T1 vs. Rapa + Baff	****					T + D vs. Trehalose 100 mM	****
T1 vs. Rapamycin	****					T1 vs. Baff	****
T1 vs. Tre + Baff	****					T1 vs. Dec + Baff	****
T1 vs. Trehalose + TGFB	****					T1 vs. Decorin	****
T1 vs. Trehalose 100 mM	****					T1 vs. Rapa + Baff	****
						T1 vs. Rapamycin	****
						T1 vs. Tre + Baff	****
						T1 vs. Trehalose + TGFB	**
						T1 vs. Trehalose 100 mM	****
						Trehalose + TGFBvs. Rapa + Baff	*

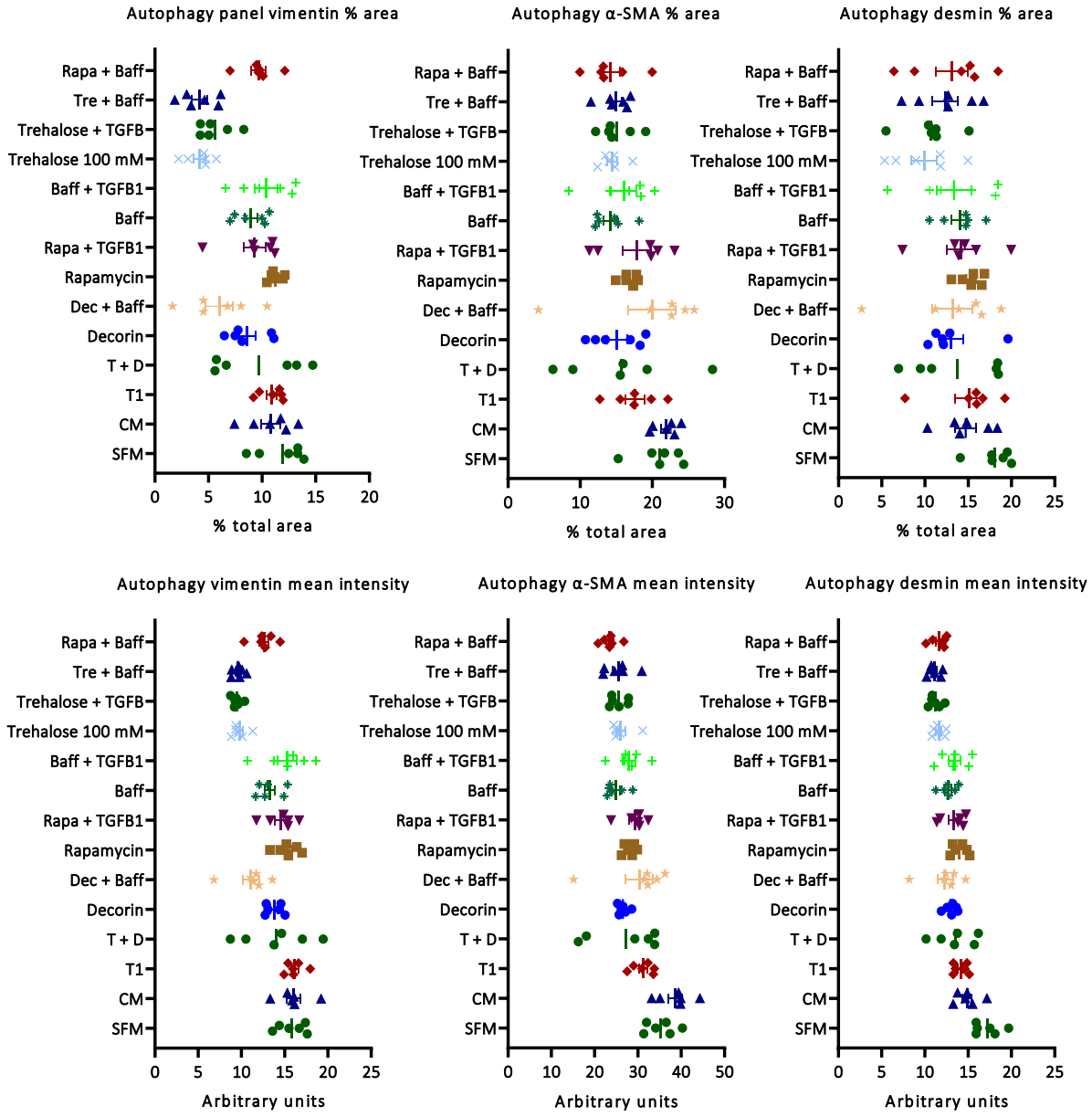


Figure 3-14. Immunofluorescence quantification of the intermediate filaments in primary human corneal fibroblasts treated with the autophagy panel.

Primary human corneal fibroblasts (PHCFs) were seeded into 96-well plates for 24 hours before being administered the standard fibrosis panel (SFM – serum free media; CM – complete media; TGFB1; decorin; and T+D -TGFB1 + decorin) and then incubated for a further 24 hours. They were then immunolabelled targeting the intermediate filaments vimentin, αSMA and desmin, in a multiplex analysis. Post acquisition image analysis of the separate channels was performed on Fiji, in order to determine the percentage area and mean-intensity of the target proteins

in the imaging field. One-way ANOVA was performed and post hoc analysis with Bonferroni's testing where indicated, which revealed some significant comparisons, see Table 3-8.

Table 3-7. Autophagy panel significance results for intermediate filaments.
A one-way ANOVA was performed on the ombnibus, followed by post-hoc analysis with Bonferroni's test. Significance is denoted as follows: not significant p>0.05, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p≤ 0.0001.

Desmin % area ANOVA NS	Desmin mean intensity ANOVA ****		Vimentin % area ANOVA ****		Vimentin mean intensity ANOVA ****		αSMA % area ANOVA *	αSMA mean intensity ANOVA NS	
SFM vs. Trehalose 100 mM	*	SFM vs. T1	*	CM vs. Dec + Baff	*	T + D vs. Trehalose 100 mM	*		
		T1 vs. Trehalose + TGFB	*	CM vs. Trehalose + TGFB	*	Decorin vs. Trehalose 100 mM	*	SFM vs. Decorin	*
		T1 vs. Tre + Baff	*	T1 vs. Dec + Baff	*	Decorin vs. Trehalose + TGFB	*	CM vs. Dec + Baff	*
		Rapamycin vs. Trehalose + TGFB	*	Rapa + TGFB1 vs. Trehalose 100 mM	*	Decorin vs. Tre + Baff	*	SFM vs. Baff	**
		Rapamycin vs. Tre + Baff	*	Rapa + TGFB1 vs. Tre + Baff	*	Dec + Baff vs. Rapamycin	*	SFM vs. Trehalose 100 mM	**
		SFM vs. Rapamycin	**	Baff vs. Trehalose 100 mM	*	Dec + Baff vs. Baff + TGFB1	*	SFM vs. Trehalose + TGFB	**
		CM vs. Trehalose 100 mM	**	Baff vs. Tre + Baff	*	SFM vs. Dec + Baff	**	SFM vs. Tre + Baff	**
		CM vs. Rapa + Baff	**	Baff + TGFB1 vs. Trehalose + TGFB	*	CM vs. Dec + Baff	**	CM vs. Rapamycin	**
		SFM vs. T + D	***	SFM vs. Dec + Baff	**	T + D vs. Trehalose + TGFB	**	CM vs. Rapa + TGFB1	**
		SFM vs. Rapa + TGFB1	***	T1 vs. Trehalose + TGFB	**	T + D vs. Tre + Baff	**	SFM vs. Rapa + Baff	***
		SFM vs. Baff + TGFB1	***	T + D vs. Trehalose 100 mM	**	Rapa + TGFB1 vs. Trehalose 100 mM	**	CM vs. T + D	***
		CM vs. Trehalose + TGFB	***	T + D vs. Tre + Baff	**	Rapa + TGFB1 vs. Tre + Baff	**	CM vs. Baff + TGFB1	***
		CM vs. Tre + Baff	***	Dec + Baff vs. Rapamycin	**	T1 vs. Dec + Baff	***	CM vs. Decorin	****
		SFM vs. Decorin	****	Rapamycin vs. Trehalose + TGFB	**	Rapamycin vs. Trehalose 100 mM	***	CM vs. Baff	****
		SFM vs. Dec + Baff	****	Trehalose 100 mM vs. Rapa + Baff	**	Rapamycin vs. Tre + Baff	***	CM vs. Trehalose 100 mM	****
		SFM vs. Baff	****	Tre + Baff vs. Rapa + Baff	**	Rapa + TGFB1 vs. Trehalose + TGFB	***	CM vs. Trehalose + TGFB	****
		SFM vs. Trehalose 100 mM	****	SFM vs. Trehalose + TGFB	***	Baff + TGFB1 vs. Trehalose 100 mM	***	CM vs. Tre + Baff	****
		SFM vs. Trehalose + TGFB	****	CM vs. Trehalose 100 mM	***	Baff + TGFB1 vs. Tre + Baff	***	CM vs. Rapa + Baff	****
		SFM vs. Tre + Baff	****	CM vs. Tre + Baff	***	SFM vs. Trehalose 100 mM	****		
		SFM vs. Rapa + Baff	****	T1 vs. Trehalose 100 mM	***	SFM vs. Trehalose + TGFB	****		
				T1 vs. Tre + Baff	***	SFM vs. Tre + Baff	****		
				Baff + TGFB1 vs. Trehalose 100 mM	***	CM vs. Trehalose 100 mM	****		
				Baff + TGFB1 vs. Tre + Baff	***	CM vs. Trehalose + TGFB	****		
				SFM vs. Trehalose 100 mM	****	CM vs. Tre + Baff	****		
				SFM vs. Tre + Baff	****	T1 vs. Trehalose 100 mM	****		

		Rapamycin vs. Trehalose 100 mM **** Rapamycin vs. Tre + Baff ****	T1 vs. Trehalose + TGFB **** T1 vs. Tre + Baff **** Rapamycin vs. Trehalose + TGFB **** Baff + TGFB1 vs. Trehalose + TGFB ****		

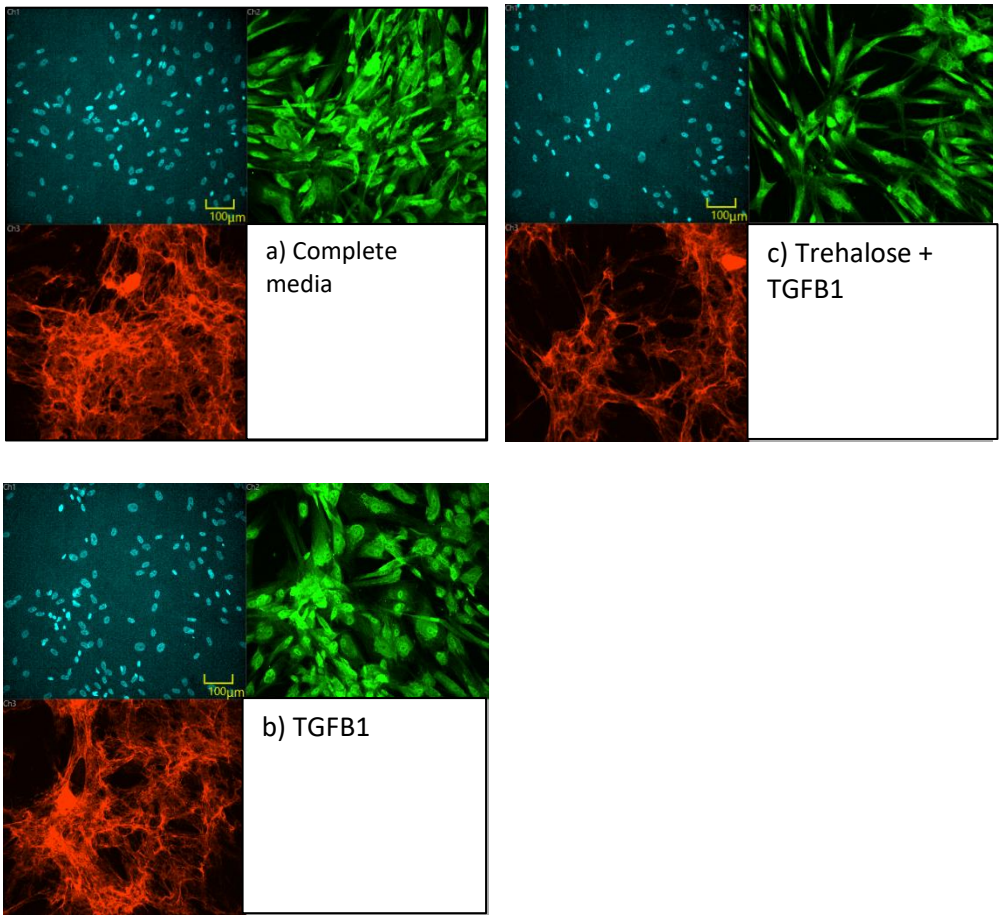


Figure 3-15. IHC images of PHCFs treated with trehalose and controls

Primary human corneal fibroblasts (PHCFs) were seeded into 96-well plates for 24 hours before being administered various treatments, incubated for a further 24 hours then stained for fibrotic proteins collagen I (Green - FITC), fibronectin (red - phycoerythrin), and cell nuceli (blue -DAPI). PHCFs treated with complete media(a) and TGFβ1 (b) demonstrate greater fibrotic protein expression than Trehalose+TGFβ1 (c). Qualitative assessment of the IHC images revealed that trehalose treated cells appear to have more fusiform cell bodies and to be more separated than CM or TGFB1 treated cells.

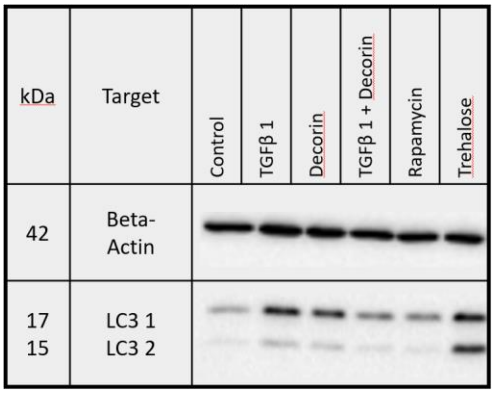
3.4.3.3 Investigating autophagic flux with western blots – decorin does not affect LC3B but does increase p62 in primary human corneal fibroblasts

To investigate the change in autophagic flux the proteins LC3 I, II and P63 were analysed using western blots. LC3 I is a 17kDa cytosolic protein that is conjugated into autophagosome membranes when it becomes LC3 II seen at 15 kDa. An increased ratio of LC3 II:I is indicative in increased autophagosome formation or decreased downseam degradation causing a backlog of autophagosomes. P62 is a ubiquitin binding scaffold protein which facilitates the degradation of proteins by autophagy. It is degraded through autophagy and its protein levels decrease when autophagy is increased.

In these experiments PHCFs were seeded into 6 well plates at 330,000 cells/well, cultured for two days in CM and then cultured in fibroblast growth media (FGM) (ATCC, USA) for 3 days prior to treatment. This growth factor fortified media was used because the concentrations of the supplementary factors in the media (e.g., TGFB1, ascorbate etc) are controlled, therefore given a more consistent cell culture environment compared to supplementation with FBS which may vary unpredictably between samples [419]. Cells were treated for 24 hours before harvesting for analysis.

Figure 3-16b revealed a trend for SFM, trehalose and trehalose + TGFB1 to lead to an increased LC3 II:I ratio, although these were not statistically significant. P62 was actually increased by trehalose with a number of pairwise comparisons being statistically significant Figure 3-16b. Rapamycin, a known inducer and bafilomycin an inhibitor, of autophagy did not appear to change autophagic flux in these cell samples.

a)



b)

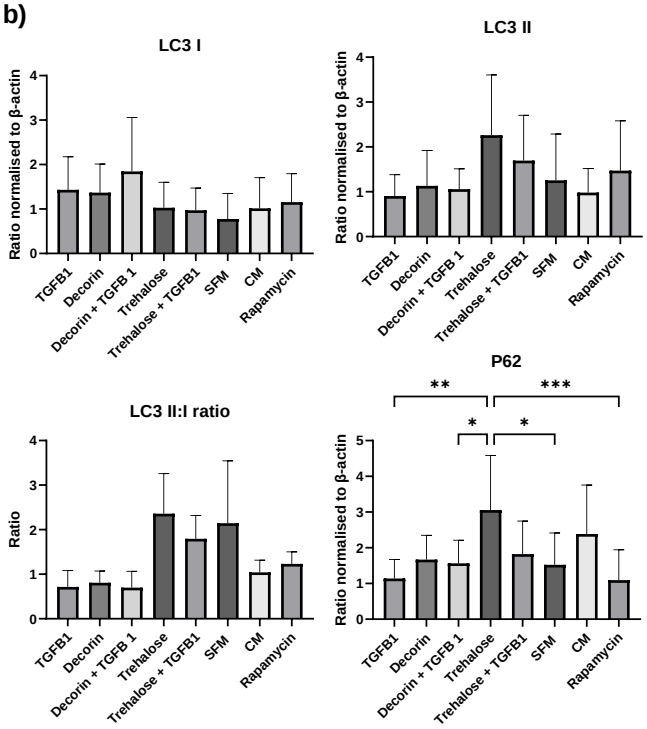


Figure 3-16. Western blot experiments and densitometry analysis of autophagy markers expressed in primary human corneal fibroblasts treated with the fibrosis panel and autophagy modulators.

Primary human corneal fibroblasts were seeded into 6 well plates at 330,000 cells/well, cultured for two days in complete media and then cultured in fibroblast growth media (ATCC, USA) for 3 days prior to treatment in order to have a consistent culture media that was consistent between experiments. Following 24 hours of treatment with he autophagy panel, cells were harvested and protein extracted. Western blot analysis of cell samples was used to interrogate for core autophagy markers LC3 I & II and p62. A) An example immunoblot for LC 3 I & II proteins illustrates the protein expressions in response to the various treatments. B) The immunoblots underwent semi-quantitative densitometry analysis on Fiji and were plotted as the ratio of the target:control protein (β-actin). Trehalose with and without TGFβ1 as well as serum free media (SFM) increased the LC3 II:I ratio although these were not statistically significant changes. Trehalose also increased P62. Decorin did not induce any protein changes. P62 ANOVA p=0.0001, Trehalose vs TGFB1 p=0.001, D+T vs trehalose p=0.039, trehalose vs SFM p = 0.028, trehalose vs rapamycin p=0.001.

3.5 Discussion

3.5.1 Summary

In this study, successfully isolated PCHFs were entered into a series of experiments to interrogate the relationship of decorin, fibrosis and autophagy in these crucial effector cells. Cultures were successfully isolated and expanded from donor human corneas. A pro-fibrotic phenotype was inducible with treatment with TGFB1 but CM itself appeared to induce a considerable profibrotic effect. Decorin did not appear to inhibit the effect of TGFB1 in various experiments. Neither TGFβ1 nor decorin were found to increase autophagy in the samples. However there was an emergent trend for trehalose, TGFB1 combined with trehalose and SFM, to all increase the LC3 II:I ratio, which is indicative of increased autophagic flux. Since TGFB1 did not appear to increase this ratio alone, it is probable that the trend seen for co-administered TGFB1 and trehalose is due to the trehalose independently, and TGFB1 may actually impairing this effect. Trehalose reduced the production of ECM proteins as detected in the IHC experiments, implying a potential anti-fibrotic role for this molecule in corneal fibroblasts. This is consistent with human subconjunctival fibroblasts in vivo and should be investigated further [400].

3.5.2 Discussion

TGFB1 treatment induced pro-fibrotic behaviours like increased contractility, ECM protein collagen-I and fibronectin production. It also demonstrated trends for increasing αSMA RNA and inducing SMAD signalling. Pro-fibrotic behaviour was also promoted by CM, which also increased fibroblast-mediated wound closure. TGFB1 did not impact wound closure in keeping with Gallego et al., who demonstrated its effect on cell motility was less than that of PDGF, whilst its other pro-fibrotic effects were greater [406].

The decorin core protein is thought to inhibit downstream TGFB1 signalling, through a number of possible mechanisms including binding and sequestration [420]. However, in these experiments decorin did not impact the activity of TGFB1. Human decorin is gagylated with either a chondroitin or dermatan sulfate side chain. Studies have demonstrated the importance of this gag-chain in the mediation of decorin's pro-inflammatory activation of TLR 2 and 4 and that may interfere with some core protein interactions between TGFB1 and decorin [175]. However, the decorin used in these experiments (galacorin), lacks the gag chain. GLP grade galacorin was used for all experiments. It is non-gagylated, which should permit it to

interact with TGFB1. However, since this is not the primary mechanism of anti-fibrotic activity, it raises a question about the involvement of the gag-chain in other pathways related to fibrosis. Conversely, evidence also exists to support the necessity of the gag-chain in decorin-TGFB1 interactions. In their experiments with vascular smooth muscle cells, Yan et al. revealed that gagylated decorin triggered TGFB1 signalling, observed as increased SMAD 2 phosphorylation, which did not occur without the gag-chain [421]. Thus is it conceivable, that non-gagylated galacorin is less effective in vitro because of this.

The results of this chapter demonstrated corneal stromal cells were cultured without contamination by epithelial cells. The addition of serum to culture media induced stromal cell proliferation seen immunocytochemically as increased numbers of cells stained with αSMA. This effect of serum was profound even at 5%, the lowest concentration assessed. For cell culture, cultivation and experiment purposes, 10% serum was used in the media to facilitate cell proliferation. Cells that were kept in serum-free conditions appeared to enter a quiescent state with no increase in cell numbers.

Stimuli in the culture process such as serum [422], culture substrate stiffness and topography, lead to fibroblast differentiation [423,424]. Undergoing cell passages in these conditions leads to evolutionary pressures that can cause phenotypic divergence from the initially cultured cells [425]. Since fibroblasts are the key effector cells in corneal fibrosis, it is necessary for some experimental paradigms to study these cells in a fibrotic phenotype as in the presence of serum.

Murray et al. examined mesenchymal stem cells for the fractions of vimentin that were insoluble and in filaments, and compared this to the soluble fraction in the cytosol. The7 found that at physiological ranges of substrate rigidity, the soluble fraction of vimentin varied significantly. However, on very soft or very rigid substrates the soluble fraction was low, and vimentin was likely polymerised into the cytoskeleton [426]. This may explain the findings of this study with regards to the lack of an observable change in intermediate filaments. If cells were stressed and at the upper limit of a response due to environmental conditions, such as intermediate filament expression in this example, then additive effects of treatments could be masked [427].

Two-dimensional models of fibrosis are simpler representations of the complex wound healing environment. Mechanistic studies, in particular those of pleiotropic molecules, must consider the trade-off between model practicality and the significance of the observed results. Direct TGFB1 inhibition by decorin is thought to be only one of several possible mechanisms of signalling interference, the majority of others occurring through indirect mechanisms [178]. Thus, looking for decorin's anti-fibrotic effect

without the wound microenvironment components such as inflammatory cells and ECM, may not fairly represent its potential. Indeed, the literature is very convincing of decorin's antifibrotic effect, as are our group's mouse studies [54]. One contributing mechanism to decorin's success in vivo may be that exogenous decorin interacts with some of the binding partners in the wound microenvironment, freeing more endogenous-gagylated decorin. Besides TGFB1 signalling inhibition, decorin can promote regeneration through other mechanisms such as collagen fibrillogenesis [420,428], which has not been examined here.

A further consideration is the experimental condition exposure and timing of protein expression. Vimentin is an IF, which is critical in numerous cellular functions including migration, and is differentially expressed during stress. Whilst it's integration and re-organisation into the cytoskeleton can occur over very short time frames (e.g. seconds to minutes [429]) the results of Chapter 3 did not identify a change in vimentin expression. Although staining for both vimentin and α-SMA was plentiful in the earlier experiments establishing the model (see figure 3-2), no change in IF was detected during the immunofluorescence experiments.

It was hypothesized the other IFs involved in migration and wound healing would also change expression in a similar time frame to vimentin and a-SMA based on the initial experiments establishing the culture. Although in-vivo vimentin is expressed as early as 1 week post injury, it peaks with α-sma and desmin at 4 weeks as demonstrated in Chaurasia et al's study [59]. Thus it would be beneficial to conduct similar experiments at different points to determine if this is similar in 2-d culture.

Simplified models help to isolate the interactions between key experimental variables. In this screening study examining the impact of decorin on autophagy, no modulatory association was observed. Decorin induces autophagy in endothelial cells via vascular endothelial growth factor receptor 2 (VEGFR2)[403]. Following corneal injury in rabbits, stromal cells at the leading wound edge have been found to stain positively for VEGFR2, but not keratocytes away from the wound [430]. Besides experimentally excluding granulocytes, the exact origin of these cells was not confirmed. Since different cells contribute to the stromal fibroblast population following injury [431] this finding suggests that the different populations may have different roles and may also be differentially susceptible to treatments. Gan et al. found these cells to be in a relatively discrete area, implicating a role for spatially constrained environmental factors. It is unknown if this cell population would be amenable to decorin mediated autophagy and of what physiological relevance it would be. However, since decorin is found to be upregulated in corneal stromal cells at the wound edge [174], it may be involved in modulation of cells in this region. Fibroblast

heterogeneity is well recognised in other organs [432] and needs to be understood further in the cornea. Spatially resolved single cell techniques such as RNASeq in pathogenic models would help characterise these subpopulations of effector cells, in an effort to uncover their roles and how they may be leveraged therapeutically.

Autophagy is a dynamic constitutive cellular process. Measuring it experimentally requires a multifaceted approach. In these experiments, LC 3 I, II and P62 levels were measured at a single time point. These proteins can all be increased or decreased in response to both increased and decreased autophagy. For example, where autophagy is increased, the LC 3II:I ratio can be increased due to the conversion of LC3 I into II as more is incorporated into autophagosomes. However, the ratio can also be decreased where the autophagosomes are being degraded more rapidly, which is similar for P62. Thus a triangulation approach is required to explain experimental observations.

Bafilomycin is an autophagy inhibitor that prevents the autophagosomes from downstream degradation, causing a backlog of intracellular vesicles. Bafilomycin can be used experimentally to demonstrate this backlog of vesicles such that those cell samples experiencing rapid autophagic flux (due to an experimental condition) will demonstrate a greater build-up of vesicles than control samples, which will be detectable on western blot. Such experiments should form the immediate next steps of this line of investigation.

Other autophagic experimental paradigms must also be considered. For example, decorin is found to have a regulatory role in autophagy. Gubiotti et al. demonstrated that decorin deficient mice had impaired autophagy in response to autophagy inducing starvation, whereas their basal autophagy was comparable to wild type mice [401]. Thus, its absence led to dysfunction. However, it is not known whether its abundance would confer beneficial properties in terms of autophagic flux or any associated autophagic functions such as cell survival or even cell death.

3.5.3 Conclusion

These findings and the literature support the idea that decorin's most prominent function and mechanism are context dependant, and therefore must be investigated in representatively complex models in order to determine its impact. This is something that needs consideration if decorin is to be applied to other corneal scarring disorders particularly if they have different inflammatory cascades, such as HSK, which is another major cause of corneal fibrosis in developed countries. The literature supports the association of

autophagy and fibrosis, however the relationship is contextual and complicated, and its relevance in corneal fibrosis is yet to be elucidated.

Interventional clinical trials of novel treatments are vital opportunities to undertake parallel mechanistic studies that may inform future drug development.

Despite these results, the literature regarding decorin is clear, with multiple animal studies demonstrating definitive benefit (see Chapter 1), so transition into human study is warranted.

4. Chapter 4: Public perceptions of eye symptoms and hospital services during the first UK lockdown of the COVID-19 pandemic: A web-survey study

4.1 Publication statement

This study was published upon completion [195]. I was the first author responsible for design, data collection, analysis and write up, with help from my supervisors and a statistician, who were also co-authors on the paper.

4.2 Introduction

During the early stages of the COVID-19 (SARS-CoV-2) pandemic in the UK, attendances to Emergency Departments (ED) and emergency admissions decreased from 1,969,691 in February 2020 to 916,581 in April 2020 [433] despite the Academy of Medical Royal Colleges [434] and the UK Government prompting people to continue seeking medical help where necessary.

A similar and equally alarming pattern has been described for the ophthalmic emergency department, where reductions in attendances have been reported [116,435–442].

The ED at the Birmingham and Midland Eye Centre (BMEC) is a major ophthalmic ED for the West Midlands, which records approximately 120 patient attendances a day, comprising community, secondary care, as well as walk-in self-referrals. Emergency and urgent referrals constitute a critical part of the ophthalmic service [443]; however, due to the COVID-19 pandemic, there was a significant reduction in face-to-face appointments and an increase in telephone consultations at the BMEC ED, in efforts to safely minimise patient-patient and patient-staff exposure [444]. Each year approximately 100 emergency admissions are made to the BMEC for microbial keratitis (MK) [445], which constitutes the most common non-surgical emergency and indication for admission in eye care services [115]. Prompt management of MK is essential as, if untreated, MK can rapidly lead to profound irreversible sight-loss [446].

Rosenstock's health belief model describes how the act of seeking help is consequent to the interplay of core factors, including the individual's perceptions of disease severity, susceptibility, benefits, and barriers to action [447]. Whilst the driving factors (severity of disease and benefits of seeking help) must outweigh the barriers to action for healthcare-seeking to occur, prompt symptom recognition underpins timely engagement with health services. While the general public consider eye health to be critical to overall

health [448], knowledge about eye disease and eye care services is poor [449–452], which could contribute to a misunderstanding of risk. As such, the pandemic may be considered a barrier to seeking care in Rosenstock's health belief model.

Amidst concerns about the change in public healthcare-seeking behaviours and service utilisation, this study aimed to explore the public's perception of eye symptoms, self-management strategies, hospital services and clinical research, and to better understand how the pandemic has affected healthcare-seeking behaviour. The Care Quality Commission confirmed that research is a priority for improving patient care that is embedded in the well-led framework [453]; changes in patient attitudes regarding participation in research will disrupt recruitment and delivery of future clinical research. Such changes may result from the public's concern about the greater risk of exposure at healthcare facilities [454,455]. Moreover, news of poorer outcomes in the elderly and ethnic minority COVID-19 patients [456,457] has heightened anxieties in these individuals [458–460]. Thus, the pandemic's influence on health and healthcare services is widespread, and disproportionally impacts some members of society.

4.3 Aims

Amidst concerns about the changes in the public's health seeking behaviours, this study aimed to explore the public's perception of eye symptoms, self-management strategies, hospital services and clinical research, and to better understand how the pandemic impacted these.

4.4 Methods

This cross-sectional study was conducted between June and August 2020, using an anonymous online survey to collect opinions of the first national lockdown in the UK. The study adhered to the principles of the declaration of Helsinki. A proportionate approach to consent was adopted, and participants taking time to complete the questionnaire were deemed to have provided implied consent.

The open survey, available in English, was administered through the web-tool Research Electronic Data Capture® (REDCap) v9.6.3 (© 2020 Vanderbilt University, Nashville, TN, USA), and a convenience sample was obtained through dissemination via social media (Facebook, Twitter, Instagram and LinkedIn);

University of Birmingham and local community group mailing lists; General Practice patient participation groups from around England; and the 1000 Elders research participation group, University of Birmingham UK. A short video, produced using Doodly® (Bryxen Inc, Ohio, USA), accompanied and explained the survey (S1 Video, https://www.youtube.com/watch?v=a-42fFn5meQ&feature=youtu.be).

The survey was refined through multiple rounds of testing for face validity by clinical and lay volunteers, who provided feedback on language, content, style and length [461]. The complete survey is summarised below.

Demographic data were collected, namely: gender, age (as ranges, e.g. 18-25 years), ethnicity, occupation, and postcode. Individuals were categorised by their occupation into either the 'working' group (in full-time or part-time education or employment), or 'not-working' group (retirees, homemakers, unemployed and individuals not working due to health reasons). Postcodes were used to determine the Index of Multiple Deprivation (IMD) score, using the 2019 English and Welsh government data [462,463]. The IMD score is based on seven domains: income, employment, health deprivation and disability, crime, barriers to housing and services, and living environment deprivation. For partially completed postcodes (e.g., SW1A), an averaged IMD was derived from all the corresponding postcodes. The national deciles of the resulting scores were used for further analyses, with decile 1 being the most and 10 the least deprived.

Section I asked four questions about six hypothetical clinical scenarios (Table 4-1), that were answered on a five-point Likert scale (Not at all, Not very, Somewhat, Moderately, Very; scoring 1-5 points, respectively). The first two questions asked about the seriousness of the scenario, and how it would impact daily life. The other two questions asked how quickly participants would seek medical attention for the scenario, both if the COVID-19 pandemic was not a factor, and after taking the COVID-19 pandemic into account.

Scenarios 1 to 3 represented combinations of ocular surface disease symptoms of progressively increasing severity. Scenario 1 symptoms were typical of mild dry eye, scenario 2 was consistent with conjunctivitis, and scenario 3 with MK. Scenario 4, painless loss of vision, was consistent with multiple differential diagnoses requiring urgent review (e.g., retinal detachment or retinal vascular occlusion). Two additional overtly serious non-ophthalmic scenarios were included, to benchmark the ophthalmic scenario responses against conditions that participants could identify as serious and requiring urgent medical attention [464]. Accordingly, Scenarios 5 and 6 described symptoms of rectal bleeding (consistent with bowel cancer) and chest pain (consistent with angina), respectively.

Table 4-1. Summary of clinical scenarios

Potential Diagnosis	Description of Scenario Given in Survey
Scenario 1 - Eye mildly red and gritty	
Dry eye disease	Over the previous week you have noted that your right eye feels gritty as though you have sand in it. The eye looks minimally red, it is not sticky, and your vision is unaffected. You have not experienced this symptom before.
Scenario 2 - Eye red, sticky and blurred	
Conjunctivitis	Over the previous week your right eye is red and sticky. It is slightly uncomfortable and your vision is slightly blurred, but not all the time. You have not experienced these symptoms before.
Scenario 3 - Eye red, painful, photophobia, sticky, blurred, white spot	
Microbial keratitis	Over the previous two days your right eye is red, painful and sensitive to light. It is sticky and your vision is blurred. You also notice there is a white area on your eye. You have not experienced these symptoms before.
Scenario 4 - Painless loss of vision	
Retinal detachment / retinal vascular occlusion	Over the previous day the vision in your right eye becomes very blurred. The eye is NOT red, painful or sticky. You have not experienced these symptoms before.
Scenario 5 - Rectal bleeding	
Bowel Cancer	Over the previous week you visit the bathroom and notice that there is blood in your stools. This has happened several times over the last couple of weeks. Recently you've been going to the toilet more often and have had some diarrhoea. You have also noticed that you have been losing weight, which is unusual because your appetite has been normal, and you have not been exercising more than normal. You are also feeling run down and very tired.
Scenario 6 - Chest pain	
Angina	Over the previous week whenever you undertake physical activity you experience a pain across your chest. The pain feels like a heaviness and tightness in the chest area. You also experience light-headedness and a slight shortness of breath. The symptoms subside after a few minutes, but start again when you engage in strenuous activities or when you experience emotional upset and stress.

Section II explored scenario 3 (MK) further, by evaluating the influence of six factors on healthcare-seeking ideation, and of five factors on the decision to agree to hospital admission. A five-point Likert scale was used (Strongly Agree, Agree, Somewhat Agree, Disagree, Strongly Disagree; scoring 1-5 points, respectively). An additional set of questions inquired about the likelihood of using seven different self-management strategies for scenario 3.

Section III asked participants if they had a contact (family/friend/work colleague/other, where 'other' may be in any capacity, e.g. at work in a clinical setting) with the conditions discussed in the survey. Further questions asked about the sources of information that participants would access about the COVID-19 pandemic, and before seeking an ophthalmologist. In each case, participants ranked their top three sources from a list (See: S2).

Section IV asked whether participants would be willing to volunteer for an ophthalmic research study, both if the COVID-19 pandemic was not a factor, and after taking the COVID-19 pandemic into account.

Returned surveys where Section I was incomplete were excluded from analysis. For the remaining sections, where questions were not completed, participants were excluded from analysis for that specific question.

4.4.1 Statistical Analysis

Due to the established differences of COVID-19 impact and outcomes in the ethnic minority community, comparisons were initially made between participants of white and non-white ethnicity. The aim of this comparison was to test for any differences in demographics between ethnicities, which may have acted as confounders in the analysis, as well as to test how perceptions varied with ethnicity. These comparisons were performed using Fisher's exact tests for nominal variables, and Mann-Whitney U tests for ordinal variables.

Responses to questions were then compared across the six scenarios using Freidman's test, followed by post-hoc pairwise comparisons, where applicable. For each of the scenarios, questions that were answered with COVID-19 both being and not being a factor were compared using Wilcoxon's signed rank tests. Spearman's rank correlations were used to describe the relationship between the answers to Section I and the demographic factors: age, ethnicity, IMD, occupation, and gender. All analyses were performed using IBM SPSS 22 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be statistically significant.

4.4.2 Patient and Public involvement

Patient and lay volunteers were engaged in the development of the survey, as well as assisting in the dissemination of the survey by means of sharing the web-link and video. Once the study is published, the results will be disseminated through the same channels as the original survey.

4.5 Results

4.5.1 Cohort characteristics

Over the eight weeks the survey was accessible, 524 responses were generated, of which 402 completed Section I and so were included in the analysis. Participants were predominantly female (253; 63.1%) and of white ethnicity (348; 87.7%), with a mean age of 61.6 years. Most participants were retired (60.4%), with 36.6% in either employment or education. The postcodes provided by participants were distributed around England, albeit with a larger number around the University of Birmingham, BMEC and the surrounding areas (Supplementary Figure 2). Consequently, participants were from the full range of IMD deciles, with a preponderance around the midpoint (17.8% in decile 5). Over half of the cohort knew someone who had COVID-19 (57.7%), with the majority also knowing someone with the diseases described in the questionnaire (eye disease, bowel cancer, angina; Table 4-2).

Table 4-2. Cohort characteristics	**Total N**	**N (%)**
Gender (% Female)	401	253 (63.1%)
Age (Years)	400	*Mean: 61.6**
18-25		24 (6.0%)
26-35		45 (11.3%)
36-45		18 (4.5%)
46-55		20 (5.0%)
56-65		49 (12.3%)
66-75		159 (39.8%)
76+		85 (21.3%)
Ethnicity	397	
White		348 (87.7%)
Asian or Asian British		33 (8.3%)
Black or Black British		7 (1.8%)
Mixed		5 (1.3%)
Other		4 (1.0%)
Employment	402	
Full-Time Employment		89 (22.1%)
Part-Time Employment		35 (8.7%)
In Education		23 (5.7%)
Retired		243 (60.4%)
Homemaker		7 (1.7%)
Not Working Due to Illness/Disability		3 (0.7%)
Unemployed		2 (0.5%)
IMD Decile	371	
1 (Most Deprived)		23 (6.2%)
2		15 (4.0%)
3		37 (10.0%)
4		47 (12.7%)
5		66 (17.8%)
6		38 (10.2%)
7		40 (10.8%)
8		36 (9.7%)
9		38 (10.2%)
10 (Least Deprived)		31 (8.4%)
Do You Know or Have Known Someone with the Following Conditions?		
Eye Disease	402	264 (65.7%)
Bowel Cancer	402	207 (51.5%)
Angina	402	233 (58.0%)
COVID-19	402	232 (57.7%)

*Total N represents the number of participants that answered the stated question. *The mean age was estimated by assigning each participant to the midpoint of the age range that they had specified. IMD – index on multiple deprivation.*

Due to the established differences of COVID-19 impact and outcomes in the ethnic minority community, comparisons of the white (87.7%; N=348) and non-white (12.3%; N=49) subgroups were performed (Supplementary Table 3). This found white participants to be significantly older (mean 65.1 vs. 36.5 years, p<0.001) and, hence, more likely to be retired (p <0.001). White participants were also significantly more likely to be female (65.2% vs. 49.0%, p=0.039) and had lower levels of deprivation (16.1% vs. 53.8% in IMD deciles 1-3, p<0.001). Both groups were similarly likely to know contacts with eye disease (p=0.264). White participants were significantly more likely to know contacts with a history of bowel cancer (55.7% vs. 22.4%, p<0.001) and angina (60.9% vs. 40.8%, p=0.009), whilst non-white participants were more likely to know contacts with a history of COVID-19 (77.6% vs. 55.2%, p=0.003).

4.5.2 Seriousness of symptoms

The reported seriousness differed significantly between the six scenarios (p<0.001, Fig 4-1a). A progressive increase in the severity score was observed over the first two scenarios (1=dry eye disease, 2=conjunctivitis), with 90 (22.4%) and 257 (63.9%) of participants, respectively, reporting these as "moderately" or "very" serious. Scenario 3 (MK) was reported as moderately/very serious by 376 (93.5%), which was similar to the 358 (89.1%), 370 (92.0%), and 362 (90.0%) in the more overtly serious scenarios: 4=painless loss of vision, 5=bowel cancer, 6=angina, respectively.

4.5.3 Impact of symptoms

Responses about the impact of the symptoms on daily life demonstrated similar trends to those described for seriousness (p<0.001, Fig 4-1b), with 115 (28.6%), 264 (65.7%) and 372 (92.5%) participants rating the impactfullness as moderately/very for the first three scenarios, respectively. The response to scenario 4 was similar to that for scenario 3, with 366 (91.0%) stating this to be moderately/very impactful (p=1.000). However, symptoms of both Scenarios 3 and 4 were reportedly more impactful than scenarios 5 and 6 (bowel cancer and angina), for which 321 (79.9%) and 346 (86.1%) responded moderately/very (all pairwise comparisons p<0.001).

4.5.4 Urgency of medical attention

Similar to the previous questions, a progressive increase in urgency across scenarios 1-3 (all pairwise comparisons, p<0.001), and comparable responses for scenarios 3-6 were observed (all pairwise comparisons p>0.05). This was true for responses both during the pandemic and during 'normal' conditions (Fig 4-1c). Within each scenario, participants rated urgency significantly lower when asked to take the COVID-19 pandemic into consideration (all p<0.001). For example, in scenario 3 (MK), the proportion of participants rating urgency as "very" fell from 78.4% to 65.7% when taking the COVID-19 pandemic into consideration.

The full Likert score results for all scenarios are available as Supplementary table 2.

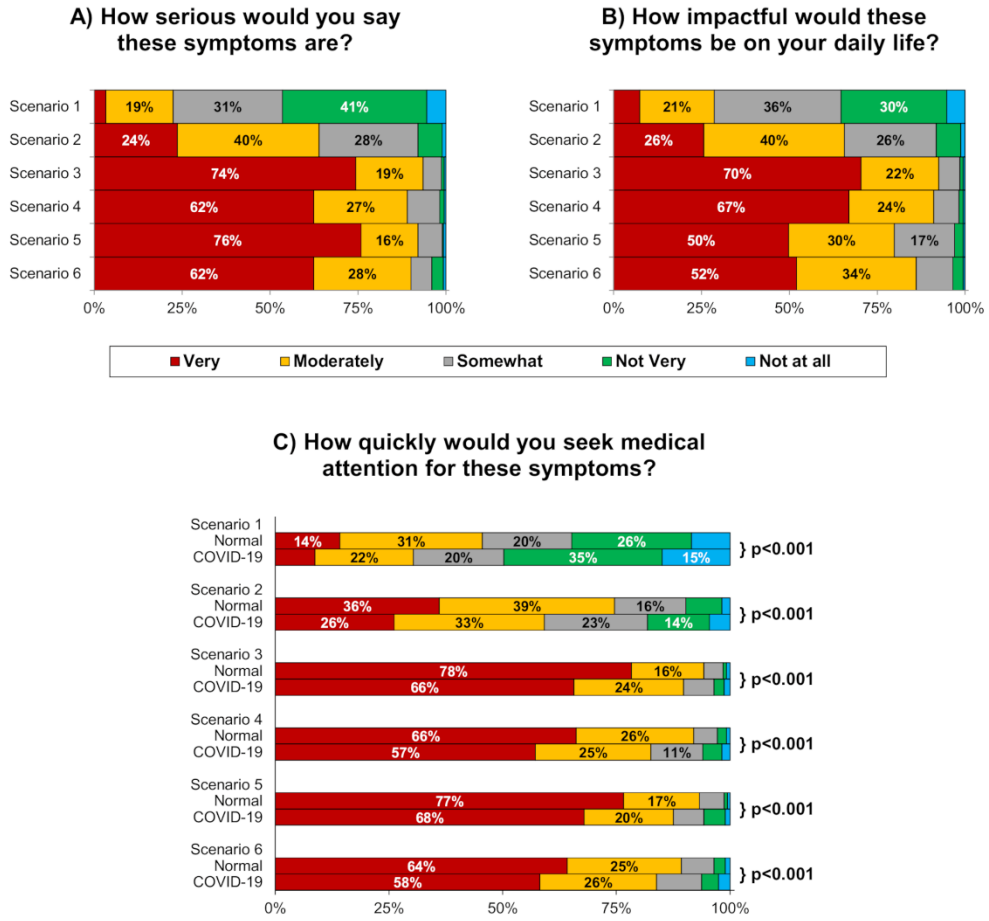


Figure 4-1. **Clinical scenarios perceived seriousness, impact and urgency of medical attention.**

Scenarios are as described in table 1. For each scenario, participants were asked to indicate how serious (A) and impactful (B) the symptoms would be, and how quickly they would seek medical attention (C) if the COVID-19 pandemic was not currently a factor ('Normal'), and after taking into consideration the COVID-19 pandemic ('COVID-19'). For all three figures, responses were found to differ significantly across the six scenarios (Friedman's test, p<0.001 in each case). In (C), p values are from Wilcoxon signed-rank tests, comparing Normal versus COVID-19 for each scenario. Unlabelled bars each consist of <10% of participants.

4.5.5 Associations of age, gender, ethnicity and deprivation

Responses were compared across demographic factors (Supplementary Table 3). The strongest associations were observed in scenarios 5 and 6 (bowel cancer and angina), where older participants tended to rate seriousness, impact and urgency more severely. Non-white participants and those from more deprived backgrounds tended to give lower scores for these outcomes. Subgroup analysis of the non-white participants found the relationship between deprivation and the reporting of lower seriousness, impact and urgency of symptoms to be stronger than for the cohort as a whole. No significant associations with age were observed in this subgroup (Supplementary Table 4).

4.5.6 Responses to symptoms of scenario 3 (microbial keratitis)

Participants were asked how they would react if they developed the symptoms of scenario 3 (MK). The first question related to self-management, with the preferred strategies being over-the-counter eye drops, painkillers, and hot compress, reported as likely/very likely by 50.5%, 38.6% and 36.1% respectively (Fig 4-2a). Participants were also asked how likely they would be to seek medical attention for scenario 3 in a range of different situations (Fig 4-2b). Of these, worsening of symptoms and spreading to the other eye elicited the strongest responses (strongly agree: 85.6% and 71.6%, respectively), with the COVID-19 pandemic being the lowest-rated factor (strongly agree: 42.5%). Finally, participants were asked whether they would agree to hospital admission in a range of situations (Fig 4-2c). Responses were generally similar for all situations, apart from the COVID-19 pandemic, for which participants were significantly less likely to agree to admission (p<0.001 for all pairwise comparisons).

4.5.7 Sources of Information about eye problems and COVID-19

A total of 358/392 (91.3%) of participants indicated that they would seek information regarding eye problems before visiting an ophthalmologist. Of these 319/358 (89.1%) completed the questions relating to their top three sources of information. The internet (211; 66.1%), a general practitioner (195; 61.1%) and an optometrist (148; 46.4%) were the most common (Supplementary figure 3a).

Regarding information sources about the COVID-19 pandemic (Supplementary figure 3b), for the 364 participants that completed this section, government briefings (262; 72.0%), the internet (235; 64.6%) and the TV/radio (205; 56.3%) were most frequently ranked within the top three sources. Comparisons by ethnicity found that white participants were significantly more likely to state that they use government briefings (75.0% vs. 51.1%, p=0.002) or TV/radio (59.5% vs. 37.8%, p=0.009) for information, as compared to non-white participants.

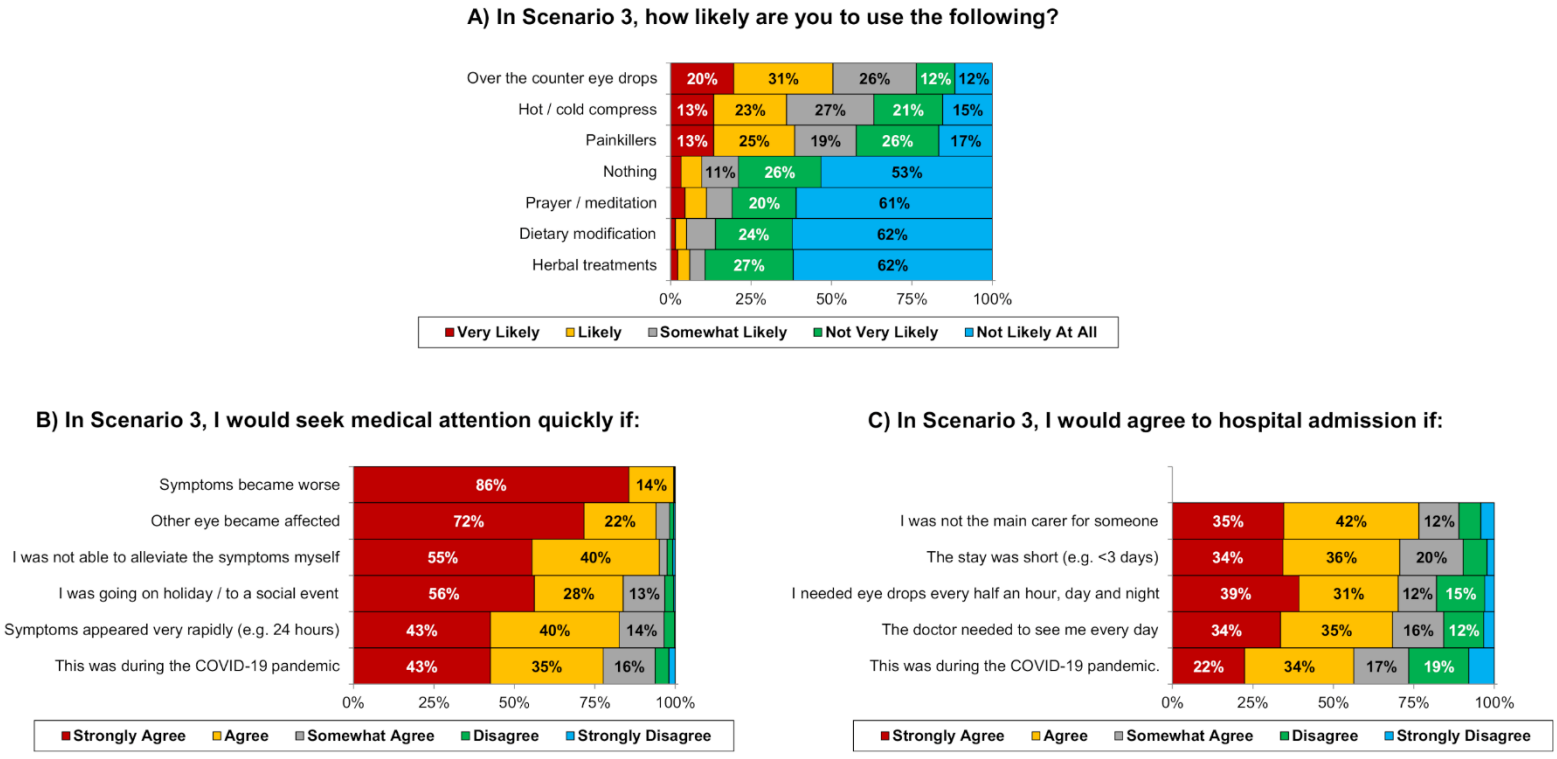


Figure 4-2. Participant responses to further questions relating to scenario 3 (microbial keratitis)

For scenario 3 (microbial keratitis), participants were asked how likely they would be to use various self-management strategies (A), and how likely they would be to seek help (B) or agree to a hospital admission (C) in a range of situations. For both (B) and (C), Friedman's test found significant differences in responses across the situations (both p<0.001). Unlabelled bars each consist of <10% of participants.

4.5.8 Volunteering for Research

If COVID-19 was not a factor, 65.9% of participants stated that they would be likely/very likely to volunteer for a research project. However, after considering the COVID-19 pandemic, this dropped to 36.3% ($p<0.001$, Fig 4-3).

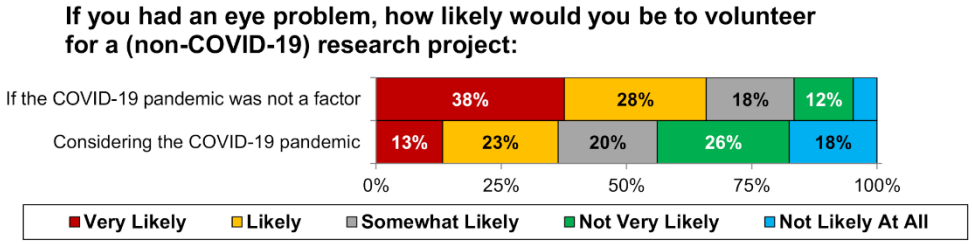


Figure 4-3. Participant willingness to be involved in ophthalmic research.

Responses were found to differ significantly when comparing the pandemic to pre-pandemic conditions.

4.6 Discussion

The COVID-19 outbreak has presented a far greater threat than other recent pandemics, for which the prevention of transmission is a very important management strategy. The ability of individuals to risk assess their needs is of the utmost importance to ensure adherence to transmission mitigation measures. The present study demonstrates the public's ability to recognise the severity of ocular symptoms and seek medical attention accordingly; offers insight about the significance of various factors when deciding to seek help; and evidences the impact of the COVID-19 pandemic on reported healthcare-seeking behaviour and the decreased willingness of individuals to volunteer for non-COVID-19 research.

Appropriate healthcare-seeking behaviour is contingent on understanding one's own health, which is influenced by profession, knowledge, social relationships and circumstances. Out of the conditions discussed in section III of the survey (eye disease, bowel cancer, angina and COVID-19), participants were most likely to state that they knew someone with eye disease. Although public knowledge of eye health has been found to be poor [449–452] this study's participants were able to identify the increasing severity of the eye-related scenarios correctly. This supports the notion that knowledge of pathology is but one of the components in the process of seeking healthcare. Visual impairment was a differentiating symptom in the first three scenarios, and this commonality between scenarios 3 (MK) and 4 (painless loss of vision) may explain the pattern of responses. Vision is considered as the most important sense [465], and its impairment is considered worse than heart disease [448]. This was also echoed in these results, with scenarios 3 and 4 being reported by participants to be of similar severity, impact and urgency, and both

were reported to be either similar to, or worse than scenario 6 (chest pain). One key difference between the ophthalmic scenarios were symptoms of pain and vision loss. Both vision [448] and pain [466] have been identified as key health concerns, and as such, their presence or absence may go some ways to explaining the responses to the scenarios. Furthermore, the presence of both pain and vision loss in Scenario 3 may explain why it was of greatest seriousness, urgency and impact.

Across all scenarios, the COVID-19 pandemic was associated with a significant reduction in the urgency of healthcare-seeking behaviour. Further investigation of scenario 3 (MK) also found the COVID-19 pandemic to be the factor that would make participants the least likely to seek urgent medical attention, or to agree to admission, in comparison with the other factors discussed in the survey. This illustrates the public's concerns, and the potential altered healthcare-seeking behaviour that individuals may adopt, in view of the risks of attending healthcare services [455,467,468]. The decrease in likelihood was more pronounced for seeking care than for admission to hospital, which may be due to the greater seriousness implicated by the need for admission, as well as it being the health-professional's suggestion. As such, apprehensions about engaging with medical services appear to have a considerable impact on the decision to seek help. The disproportionately greater mortality and morbidity in ethnic minority groups [456] has heightened health anxieties in these individuals [458,459] while the national lockdowns have worsened isolation, and compromised the public's financial and personal wellbeing [467,469–471].

In this sample, comprised mostly of white elderly retirees, greater age correlated with a greater perception of seriousness and urgency, particularly for the mild dry eye, bowel cancer and angina scenarios. These associations with age are curious, and likely reflect the higher prevalence of these conditions in these groups. Young UK citizens perceive eye disease as a concern for older life [450], hence younger individuals may be less inclined to seek healthcare and consider symptoms to be less severe. They may also rationalise symptoms, for example, chest pain on exertion to a non-cardiac cause. In this regard, the associations of younger age and lower perceived seriousness and urgency in the present study would be, to an extent, expected. However, due to the sample demographics (i.e., the close association of younger age, non-white ethnicity, and greater deprivation), it is difficult to discern the influence of these factors independently.

The relatively small non-white subgroup in this study was significantly more likely to know someone with COVID-19, and less likely to report the use of the government briefings as a main source of information about the pandemic. Greater deprivation, particularly in the non-white group, correlated with lower reported severity, impact and urgency of medical attention of select scenarios. These results are in agreement with other work describing the increased concerns and health anxiety in relation to the

pandemic [459,460,472], and raise the possibility of altered healthcare seeking in these individuals. Although this study did not explore the specific work roles of participants, it is important to consider that individuals from greater deprivation may be less financially resilient, and the nature of their occupation may not permit working from home. Thus, differing beliefs and priorities reflecting their circumstance may influence their responses. The lower likelihood of using government briefings as a major source of information about the pandemic suggests this communication medium inadequately serves all demographic groups. The underlying reasons for these results are likely complex and, whilst the results from 49 non-white participants must be interpreted with caution, it is an indicator of the need for further consideration.

Healthcare information seeking is an integral component of healthcare-seeking behaviour [473]. During this pandemic, alternate channels such as social media took a more prominent, and frequently negative role in information dissemination [474–477]. A tragic example from Iran in March 2020 occurred following social media posts about alcohol ingestion as a preventive measure, resulting in hundreds of deaths around the country [478]. In the present study, the internet and clinical staff were the most preferred sources of information for eye symptoms, whereas the internet and traditional media (TV/radio/newspapers) were the most preferred for COVID-19, in keeping with other recent work [479,480]. Social media did not feature highly, which is possibly related to the predominant elderly demographic of the study being less inclined to use social media. Effective utilisation of the internet for health information can be challenging [481–483]. Lower proficiency with the internet and related technologies is associated with increased susceptibility to misinformation [484,485].

From the ophthalmologists perspective, it is important to consider how ophthalmic patients may be more vulnerable than the general public in order to plan service adaptations for existing patients. Vision impairment is associated with a reduced quality of life and various negative health outcomes [486,487]. Shalaby et al. identified that amongst visually impaired patients, visual impairment itself was perceived as a risk factor for contracting COVID-19 [488]. These individuals also reported greater COVID-19 related health anxiety and more difficulty adhering to standard preventive measures compared to healthy controls. Similarly Ting et al. identified that the lockdown disproportionately impacted the mental health and health anxiety of individuals with visual impairment [489]. These findings highlight the increased vulnerability of the visually impaired population as a consequence of lockdown measures. As such patients with visual impairment may not engage with hospital services during lockdown conditions and may be at greater risk of the complications from delayed presentation to health care services.

Ophthalmic emergency services have been amongst the most disrupted around the globe as a result of the pandemic [116,435,437–442,490]. MK is the most common non-surgical ophthalmic emergency requiring admission. Even with successful treatment of the acute infection, post-infective scarring can lead to permanent visual impairment, meaning that early initiation of treatment is critical for preserving vision. Although the feature composition of scenario 3 was typical of MK, real world symptomatology may vary, particularly in the early stages of the disease where symptoms may be more akin to scenarios 1 and 2, or to other conditions predisposing MK. In keeping with the Rosenstock's health belief model [447], this study demonstrates that milder symptoms are perceived to require medical attention less urgently, particularly during the pandemic. In the case of innocuous conditions such as dry eye, this behavioural adaptation may help to decrease the transmission rate of COVID-19. However, in the event of a predisposing pathology or early MK, a delay that permits the disease to progress may lead to increased severity of MK by the time of presentation and, consequently, poorer final outcome. Such a phenomenon has been reported in patients with retinal detachments in this pandemic [439], as well as other hospital services that are being utilised less [491,492] and have patients presenting later with more severe disease [493]. This warns us of how a 'Swiss-cheese' model of accident causation [494] might arise in patients with early and mild symptoms of MK who, due to concerns regarding the pandemic, delay presentation and consequently have worse disease and final outcomes.

Public health literacy is vital to combat the pandemic. Scenarios 3-6 were all sight- or life-threatening conditions, yet still 1-6% of participants did not consider them serious, impactful or urgent. As such, accurate information about the pandemic, as well as increasing awareness of eye health must remain important public health priorities.

In the wake of the pandemic, those responsible for health service design may glean insights for future practice. For example, the importance of tele-health technologies was highlighted by many medica disciplines, including ophthalmology as seen at Moorfields eye hospital who adopted a triaging system in order to minimise risk of viral transmission and deliver eye care safely [495–497].

Developing pathways to ensure all who require healthcare are able to receive it is essential and adaptations should be considered for vulnerable groups. For example, those with chronic eye diseases who require ongoing management such as diabetic macular oedema and wet age related macular degeneration, specific clinics could be set up with additional safety precautions. This could be combined with personalised communications with these patients to understand their level of concern and willingness to attend, and should they be unable to attend for any reason, they could be considered for urgent management, following the relaxation of lockdown restrictions.

Patient experiences with tele-medicine are generally positive and satisfaction is high, they report a preference for the time and money saved through remote appointments and it reduces their time away from work [498,499].

Such a personalised approach to non-attendance in the future could be adopted to increase service efficiency. With increasing technology literacy, a multimodal approach could be used to contact a range of demographics thereby obtaining a broad patient perspective, and open the possibility of using interventions to avoid non-attendance [500].

Whilst many specialties may benefit from this development, and pockets of ophthalmic services such as virtual clinics in glaucoma, may benefit from tele-medicine, ophthalmic care is unique in many ways compared to other medical disciplines as visual assessments with various devices are required to make diagnoses, which necessitate some degree of in-person interaction. Thus there are some practical considerations that may limit the application of tele-medicine.

The main strength of the current study is the relatively large sample size, comprising participants spread across England and with a range of socioeconomic backgrounds. In addition, the data were collected prospectively, using a standardised and well-refined survey. However, the study also had several limitations, which largely resulted from the challenges of investigating public perceptions during a pandemic. Barriers to public engagement, in particular face-to-face interactions, meant that it was only feasible to collect data based on a convenience sampling approach, using an online questionnaire. This was distributed using several channels, to maximise its reach, and with a view to including a diverse range of participants. However, despite this, there was a preponderance of participants within the areas surrounding the University of Birmingham and BMEC. In addition, the average age of participants was relatively high, and correlations between demographic factors were observed, with those of white ethnicity tending to be considerably older and less deprived than non-white participants. As such, the demographics of the included participants may not be the optimal representation of the UK as a whole; hence the generalizability of the findings cannot be guaranteed. In addition, the observed associations between age, ethnicity and deprivation make it difficult to isolate the effect of these factors; hence the observed effects of each of these on participants' views may be confounded by other factors. Finally, the use of an online survey precluded participants who either did not have internet access, or were not computer literate, which may have introduced selection bias, particularly for the questions relating to preferred sources of information.

As a result, future work should aim to investigate the demographics less well represented in the current study, for example, by targeting promotion of the survey using communication media more utilised by these demographics, and adopting a purposive sampling methodology. This would yield more generalizable results, and would help to validate the current findings. In addition, the current study was only intended to identify the beliefs of the participants, not to explore the underlying reasons for why these beliefs were held. A future study that further investigated the reasoning behind participants' beliefs would help to further explain the findings of the current study, as well as potentially highlighting areas that could be targeted in future to disseminate health education. Such a study would need to collect more detailed and qualitative responses, which would likely require a different format of investigation (e.g. by telephone or face-to-face structured interviews).

In conclusion, the results of this study offer insight into the healthcare-seeking attitudes adopted by the public during the lockdown period. Highlighted here is the importance of accurate health information and adequate public education, so that individuals may risk assess their own needs and act accordingly. Continually assessing the public's understanding of health campaigning is useful for decision makers. COVID-19 exacerbates the gap in health inequality, and raises concerns about safely accessible healthcare. The case for large scale lockdown is compelling; however, the implications of this behavioural adaptation must be carefully considered by policy makers, to avoid potential deleterious consequences. Following on from this work, clinical departments are encouraged to audit their services to investigate the extent of local impact with regard to patient outcomes.

5. Chapter 5: The Impact of the COVID-19 Pandemic on Microbial Keratitis Presentation Patterns

5.1 Publication statement

This study was published upon completion [196]. I was the first author responsible for design, data collection, analysis and write up, with help from my supervisors and a statistician, who were also co-authors on the paper.

5.2 Introduction

During the UK's first national lockdown in 2020 due to the COVID-19 pandemic, the official parliamentary advice in the UK, and of bodies such as the *Academy of Medical Royal Colleges* [434] urged the public to seek timely medical attention, where required, to avoid unnecessary delay and complications. However, Emergency Departments (EDs) across England experienced a significant decrease in activity during the national lockdown period [501], raising concerns about whether patients were seeking help appropriately. Concurrently, rising numbers of reports described public apprehensions about attending hospital or seeking medical attention [455,468]. The pandemic impacted non-COVID patients in manifold ways, for example, with reduced patient attendances for emergency and urgent services, and disruptions to cancer services [502–504]. This has raised concerns globally about the need to mitigate these effects.

Ophthalmology departments provide a highly specialised emergency service and were amongst the most disrupted by the pandemic. During the pandemic, the reported emergency eye care workload increased in severity and complexity, as did the demand for emergency surgical procedures, despite an overall reduction in attendances [116,437,490]. The public's apprehensions about engaging clinical services during the pandemic may potentially be causing delays in presentation, diagnosis, and implementation of appropriate management. For example Poyser *et al.* reported fewer cases of retinal tears, but increased cases of macular-off retinal detachments [439]. Similarly, Babu et al. described the inability to meet the demand for emergency corneal transplants for patients presenting with perforated corneal ulcers [435], also highlighting the secondary impact of the pandemic on supporting services, such as organ donation and retrieval [505].

The Birmingham and Midland Eye Centre (BMEC) is a tertiary referral unit providing seven-days-a-week Emergency and in-patient eye services for the West Midlands, UK. The BMEC ED records approximately 120 attendances per day, with over 450 emergency admissions annually (Supplementary table 6); almost 25% of these are for microbial keratitis (MK) [506], requiring intensive eye drops and daily reviews. MK is rapidly progressive and potentially blinding. It constitutes the most common non-surgical emergency and reason for admission in eye care services in the UK [115]. MK is typically managed by hospital eye services following presentation either by self-referral or through community-based services, such as community opticians and general practitioners. However, during the pandemic, these community healthcare services were severely impaired, with more patients instead utilising alternate pathways, such as the NHS non-emergency phoneline 111 [507].

Our group previously characterised public perceptions of eye symptom severity and consequent health seeking behaviour [508]. The comparison of various clinical scenarios in normal and pandemic contexts revealed that respondents felt materially less of an impetus to seek in-person clinical help during the pandemic. For mild self-limiting disease, this study highlighted the potentially beneficial adaptive behaviours that may reduce the risk of COVID-19 exposure; however, this difference was also noted in response to serious conditions such as MK, raising concerns about potential delays in presentation and consequent poorer clinical outcomes. To investigate this further, we explored whether the first UK national lockdown during the SARS-CoV-2 pandemic impacted upon the clinical presentation, causative organisms, admission rates or outcomes of MK. We hypothesized that the presentation and outcomes of patients with MK would be affected by the first UK national lockdown in 2020, compared to previous years.

5.3 Methods

This retrospective study of medical records was conducted in accordance with the Declaration of Helsinki, and in accordance with local institutional policy. All data were anonymised prior to analysis, and the need for consent was waived. Approval was obtained from Sandwell and West Birmingham Hospitals NHS Trust Department of Clinical Effectiveness (registration #1512) to undertake this project as a service evaluation.

5.3.1 Study design & population

All cases of MK requiring corneal scrapes, presenting between the 23rd March and 30th June 2020 (Y2020) were identified through the regional microbiology service (Black Country Pathology Services Supporting Sandwell and West Birmingham Hospitals NHS Trust) database, and cross-checked with BMEC ED electronic medical records. Patients presenting during the equivalent time windows in the preceding three years (2017, 2018, and 2019) were also identified, and included as the comparator cohort (pre-C19), to reflect the variation of the disease. During the period being studied in 2020, all first-time face to face appointments were replaced with an initial telephone consultation, followed by a face-to-face consultation, where indicated. This had the effect of reducing the overall number of face-to-face appointments [444].

5.3.2 Routine clinical practice

Clinical assessments and decisions, such as the need for investigations, admission and follow ups, were undertaken by the BMEC ED attending ophthalmologists, in accordance with local guidelines. During the first national lockdown period, the BMEC in-patient ward was closed to allow nursing staff to be redeployed to specified medical wards to undertake general nursing duties, as well as to provide specialist care for admitted ophthalmic patients. Patients requiring admission for urgent care were initially admitted to amber wards (COVID status unknown), before relocating to specified Green (COVID-Free) and Red (COVID positive) wards with designated ophthalmic beds. The decision to admit patients was based on factors including clinical severity, risk of adverse events (e.g., perforation), social care needs (e.g., the

ability to diligently administer all drops, proximity to clinic, and ability to attend for daily visits), with the final decision being taken by the lead clinician for any given session.

Where MK was suspected on presentation, corneal scrapes were taken to confirm the diagnosis. The typical corneal sampling kit consisted of a sterile needle (e.g. 23G) or scalpel blade for corneal tissue acquisition, one each of chocolate and blood agar plates, and a Sabouraud's agar plate for fungus. Nutrient depleted agar seeded with Escherichia coli was used for Acanthamoeba cultures, where indicated. Samples were placed on glass slides for microscopy and Gram staining. Dry swabs were also acquired for microbe polymerase chain reaction (PCR) typing. All cultures were incubated according to departmental protocols for at least one week.

A positive isolate was defined as a growth along the line of inoculation on solid media, and poly-microbial keratitis was confirmed if more than one clinically significant organism was isolated. Significant isolates were tested against antibiotics, in accordance with local protocols, using both disc diffusion (the British Society of Antimicrobial Chemotherapy methodology; www.BSAC.org.uk) and Vitek AST systems (www.biomerieux.co.uk). Isolates identified as contaminants in the microbiology reports were excluded from analysis.

5.3.3 Data collection

All data were recorded in an adaptation of a validated data collection proforma used in a previous study[506], using the secure web application Research Electronic Data Capture (REDCap© v9.6.3 2020 Vanderbilt University, Nashville, TN, USA). Data collected included patient demographics (sex, age, ethnicity and Index of multiple deprivation [IMD] score) and clinical details (presenting features, underlying risk factors, past ocular history, medications). The IMD score combines information from seven differentially weighted domains, to classify the relative deprivation of small areas around the UK; and scores were obtained from a government website [462].

Underlying risk factors were grouped as follows: contact lenses wear; active ocular surface disease (complete list in Supplementary Table 7); previous keratitis (infective and marginal); previous trauma (healed before the onset of MK) or previous ocular surgery; concurrent trauma (related to the onset of MK); foreign bodies associated with the current episode; as well as the systemic conditions: diabetes mellitus; rheumatoid arthritis; thyroid eye disease; and the use of systemic immunosuppression medication.

Details of clinical assessments were also recorded. The best corrected Snellen visual acuity (VA) at presentation was collected, and converted to LogMAR VA for analysis [509]. In addition, the final VA was also recorded, based on assessments performed at clinical follow up appointments (1, 3, 4 and 12 weeks after presentation). Where patients attended a clinical follow up at week 12, the VA at this appointment was used, with the latest available assessment used instead in patients that were discharged from the service prior to this.

Slit-lamp biomicroscopy was used to assess the size of the epithelial defect, infiltrate, or scar, using standardised methodology adapted from the Herpetic Eye Disease Study [510], by measuring the longest and the longest perpendicular dimensions. The area was then calculated by multiplying these readings together. Epithelial defect, infiltrate, and scar size were not differentiated, henceforth this measurement is referred to as the "ulcer area", which was also the summation of all single areas of involvement in the cornea.

The corneal involvement score (CIS) was retrospectively derived from the clinical notes, based upon the validated corneal opacification score described by Ong *et al.* [511]. Briefly, the locations of the corneal ulcer are documented according to the number of quadrants involved (temporal, superior, nasal, inferior), which are each assigned 1 point, with involvement of the central 4mm zone being assigned 5 points. The numbers of points are then added, to give a final CIS out of 9.

5.3.4 Statistical analysis

Comparisons of patient characteristics by presentation period (Y2020 or pre-C19) were performed, using Fisher's exact tests for nominal variables, and Mann-Whitney U tests for ordinal and continuous variables. Continuous variables were reported as mean ± standard deviation if approximately normally distributed, with median (interquartile range; IQR) used otherwise. Cases with missing data were excluded from the analyses of the affected variables, and the sample sizes included in each analysis are reported in the associated tables. All analyses were performed using IBM SPSS 26 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance throughout.

5.4 Results

5.4.1 Included cases

A total of 230 MK patients were identified, comprising 63, 50, 68 and 49 patients from the time windows in the years 2017, 2018, 2019 and 2020, respectively. Total numbers of attendances to the BMEC for any indication were 12,128 during the time window in 2018 and 12,239 in 2019, compared to only 5,759 in 2020 (accurate data were not available for 2017). As such, MK comprised 0.5% of attendances in 2018-19, which increased significantly to 0.9% in 2020 (p=0.001). Comparisons between the years 2017-2019 found no significant differences in patient characteristics (Supplementary Table 8). As such, the 181 cases from these three years were combined into a single cohort for subsequent analysis (pre-C19), and compared to the 49 cases from the year 2020 (Y2020).

5.4.2 Patient characteristics

Comparisons between Y2020 and pre-C19 found no significant differences in the age, sex, laterality of eye, ethnicity or IMD scores between the groups (Table 5-1). The duration of symptoms at presentation was also similar in the Y2020 and pre-C19 groups, with medians of 4 days (IQR 2-7) and 3 days (1-6), respectively (p=0.201). Of the risk factors considered, concurrent ocular trauma (16.3% vs. 5.5%, p=0.030) and systemic immunosuppression (12.2% vs. 1.7%, p=0.004) were both significantly more prevalent in the Y2020 group. The full list of causes of concurrent trauma is reported in Supplementary Table 9.

Table 5-1. Characteristics of patients presenting with microbial keratitis					
	Pre-C19		**Y2020**		
	N	***Statistic***	***N***	***Statistic***	**p-Value**
Age (years)	181	55.5 ± 21.1	49	53.3 ± 17.8	0.503
Sex - male (%)	181	95 (52.5%)	49	31 (63.3%)	0.198
Ethnicity	164		38		0.864
White		112 (68.3%)		27 (71.1%)	
Asian		38 (23.2%)		7 (18.4%)	
Black		9 (5.5%)		3 (7.9%)	
Mixed / Other		5 (3.0%)		1 (2.6%)	
IMD decile	178		49		0.839*
1-3		96 (53.9%)		26 (53.1%)	
4-7		58 (32.6%)		19 (38.8%)	
8-10		24 (13.5%)		4 (8.2%)	
Laterality - right (%)	181	89 (49.2%)	49	27 (55.1%)	0.521
Duration of symptoms at presentation (days)	121	3 (1-6)	37	4 (2-7)	0.201
Risk factors					
Contact lens	181	60 (33.1%)	49	14 (28.6%)	0.608
Underlying OSD (active)**	181	80 (44.2%)	49	26 (53.1%)	0.333
Previous keratitis***	181	20 (11.0%)	49	7 (14.3%)	0.616
Previous surgery/trauma	181	31 (17.1%)	49	10 (20.4%)	0.674
Concurrent trauma	181	10 (5.5%)	49	8 (16.3%)	**0.030**
Foreign body	181	3 (1.7%)	49	2 (4.1%)	0.289
Diabetes mellitus	181	17 (9.4%)	49	7 (14.3%)	0.304
Rheumatoid arthritis	181	6 (3.3%)	49	1 (2.0%)	1.000
Systemic immunosuppression	181	3 (1.7%)	49	6 (12.2%)	**0.004**
Thyroid eye disease	181	1 (0.6%)	49	0 (0.0%)	1.000

*Table 5-1. Abbreviations: Pre-C19, Pre-COVID-19 Years (2017,2018,2019); Y2020, year 2020; OSD, ocular surface disease; IMD, index of multiple deprivation; MK, microbial keratitis. Continuous variables are reported as mean ± SD or median (interquartile range), with p-values from Mann-Whitney U tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests, unless stated otherwise. Bold p-values are significant at p<0.05. For risk factors, "previous" denotes that the risk factor had healed prior to onset of MK. *p-Value from Mann-Whitney U test, as the factor is ordinal. **Ocular*

*surface disease, such as dry eye, affecting the patient at the time of presentation – a full list of included diseases is reported in Supplementary Table 7. ***Viral/bacterial/fungal/parasitic/marginal disease.*

5.4.3 Clinical assessments

The severity of disease at presentation was quantified using the VA, ulcer area and CIS, none of which were found to differ significantly between the Y2020 and pre-C19 groups (Table 5-2, Figure 5-1a). These comparisons were also repeated after excluding the 67 patients with pre-existing visual impairment at presentation (based on their ophthalmic history), with the difference between groups remaining non-significant.

Table 5-2. Clinical assessments and outcomes

	Pre-C19		**Y2020**		
	N	***Statistic***	***N***	***Statistic***	**p-Value**
All Patients (N=230)					
Visual acuity at presentation (LogMAR)	181	0.60 (0.18 - 2.10)	49	0.60 (0.18 - 2.40)	0.785
Final visual acuity (LogMAR)	181	0.30 (0.00 - 1.78)	49	0.30 (0.18 - 1.48)	0.990
Ulcer area at presentation (mm^2)	112	3.0 (1.0 - 9.5)	36	4.0 (1.7 - 9.7)	0.520
Corneal involvement score	72	2 (1 - 5)	29	5 (2 - 5)	0.120
Excluding patients with pre-existing visual impairment in affected eye (N=163)					
Visual acuity at presentation (LogMAR)	126	0.30 (0.18 - 1.00)	37	0.48 (0.18 - 1.00)	0.608
Final visual acuity (LogMAR)	126	0.18 (0.00 - 0.60)	37	0.18 (0.10 - 0.78)	0.458
Ulcer area at presentation (mm^2)	78	2.0 (1.0 - 6.3)	27	2.9 (0.8 - 12.0)	0.575
Corneal involvement score	54	2 (1 - 5)	23	5 (2 - 5)	0.167

Table 5-2. Abbreviations: Pre-C19, Pre-COVID-19 Years (2017,2018,2019); Y2020, year 2020. Data are reported as median (interquartile range), with p-values from Mann-Whitney U tests. Bold p-values are significant at $p<0.05$.

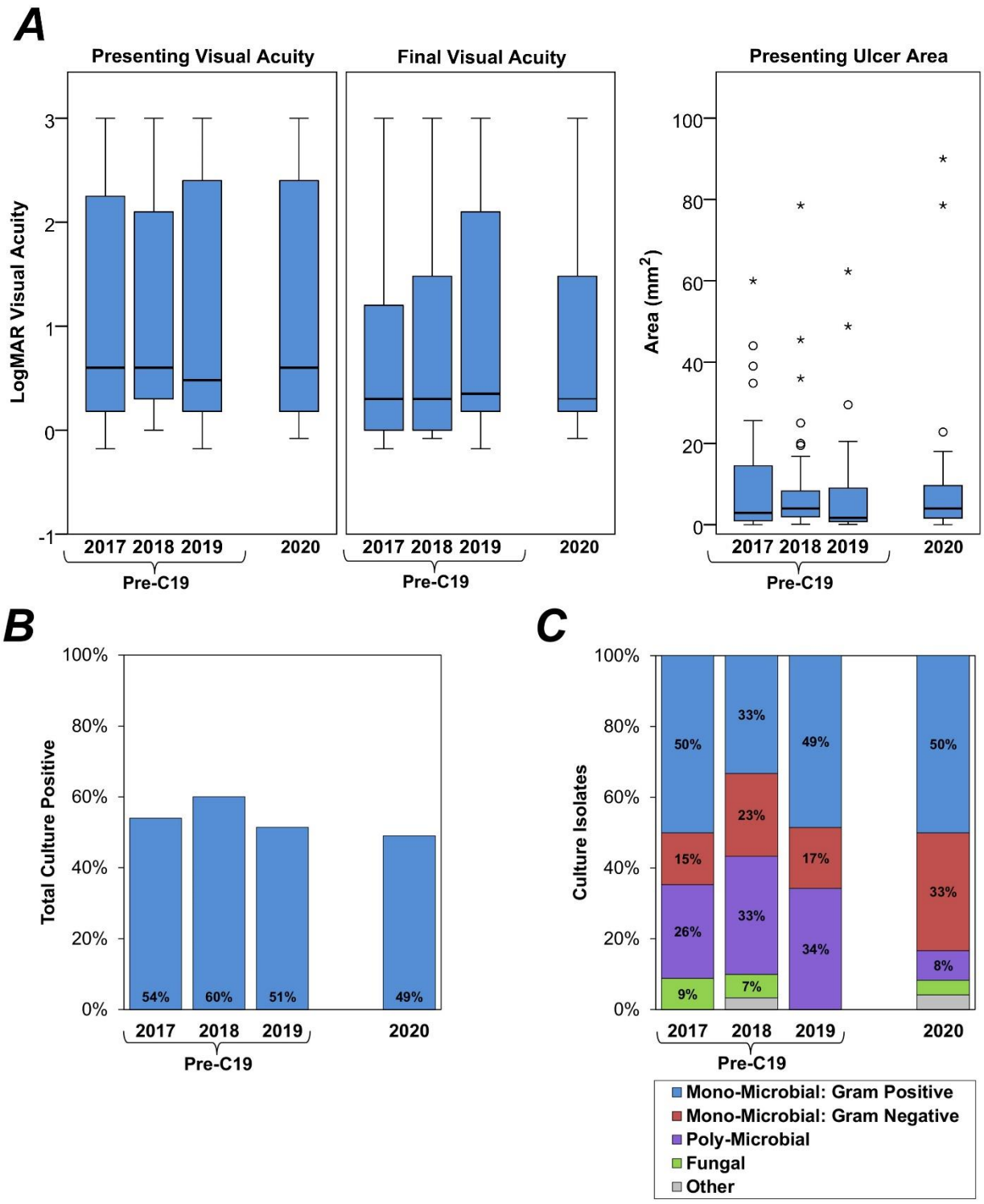


Figure 5-1. Clinical assessments and microbiology by year of admission

Clinical assessments (a) are summarised using boxplots, with outliers indicated with circles or asterisks for those outside the box by 1.5- or 3-times the interquartile range, respectively. Microbiology is summarised

as the total proportion of the cohort that were culture positive (b), and the distribution of culture isolates from these positive cases (c); unlabelled bars consist of <5% of cases. Further details of the definitions used for the microbial cultures are reported in Table 5-4.

Despite the similarities in patient characteristics, admission rates were found to be significantly lower in Y2020, at 8.2% compared to 32.6% for the Pre-C19 group (p<0.001, Table 5-3). However, the disease course was found to be similar in the two groups, with no statistically significant differences noted in the final VA (Figure 5-1a), or in complication or intervention rates (Table 5-3).

Table 5-3. Clinical sequalae

	Pre-C19	**Y2020**	**p-Value**
Admissions	59 (32.6%)	4 (8.2%)	**<0.001**
Complications			
Re-admission*	1/59 (1.7%)	0/4 (0.0%)	1.000
Corneal perforation	12 (6.6%)	2 (4.1%)	0.740
Endophthalmitis	2 (1.1%)	1 (2.0%)	0.514
Phthisis	1 (0.6%)	1 (2.0%)	0.381
Other complication**	3 (1.7%)	1 (2.0%)	1.000
Interventions			
Therapeutic lens	11 (6.1%)	0 (0.0%)	0.126
Corneal biopsy	0 (0.0%)	1 (2.0%)	0.213
Botulinum toxin ptosis	2 (1.1%)	1 (2.0%)	0.514
Corneal gluing	11 (6.1%)	1 (2.0%)	0.469
Evisceration	1 (0.6%)	0 (0.0%)	1.000
Repeat scrape	5 (2.8%)	2 (4.1%)	0.643
Tectonic corneal transplant	2 (1.1%)	0 (0.0%)	1.000
Temporary surgical tarsorrhaphy	0 (0.0%)	1 (2.0%)	0.231
Amniotic membrane graft	2 (1.1%)	0 (0.0%)	1.000
Other intervention***	2 (1.1%)	3 (6.1%)	0.066

*Table 5-3. Abbreviations: Pre-C19, Pre-COVID-19 Years (2017,2018,2019); Y2020, year 2020. All analyses are based on N=181/N=49 in the two groups, unless stated otherwise, with p-values from Fisher's exact tests. Bold p-values are significant at p<0.05. *In the subgroup of patients who were admitted on initial presentation. **Consisted of one retinal detachment, one case of corneal graft failure and one iatrogenic corneal perforation at the time of corneal scraping in the pre-C19 cohort, and a retinal detachment in the*

*2020 cohort. ***Consisted of one count of suture removal and one count of anterior chamber reformation following persistent aqueous humour leakage in the pre-C19 cohort, and two counts of corneal suture removal and one retinal detachment surgery in the Y2020 cohort.*

5.4.4 Microbiology

Rates of culture positivity were similar in the two groups, at 49.0% in Y2020 and 54.7% in Pre-C19 (p=0.520, Table 5-4, Figure 5-1b). However, the distribution of culture isolates was found to vary between the groups (Fig 5-1c), with a significantly lower rate of poly-microbial infections in Y2020, compared to pre-C19 (8.3% vs. 31.3%, p=0.022), and a non-significant tendency for higher rates of gram-negative mono-microbial infections in Y2020 (33.3% vs. 18.2%, p=0.160). Fungal infections comprised similar proportions of culture positive cases in both groups (4.2% vs. 5.1% in Y2020 vs. pre-C19, p=1.000).

Table 5-4. Microbiology results summary

	Pre-C19	**Y2020**	**p-Value**
Total culture positive	99/181 (54.7%)	24/49 (49.0%)	0.520
Culture isolates			
Mono-microbial - gram positive	44/99 (44.4%)	12/24 (50.0%)	0.654
Mono-microbial - gram negative	18/99 (18.2%)	8/24 (33.3%)	0.160
Poly-microbial (bacterial only)	31/99 (31.3%)	2/24 (8.3%)	**0.022**
Fungal	5/99 (5.1%)	1/24 (4.2%)	1.000
Other*	1/99 (1.0%)	1/24 (4.2%)	0.353
Most frequent gram-positive isolates**			
Cutibacterium acnes	19/99 (19.2%)	1/24 (4.2%)	0.119
Staphylococcus epidermidis	18/99 (18.2%)	2/24 (8.3%)	0.359
Staphylococcus aureus	11/99 (11.1%)	6/24 (25.0%)	0.099
Streptococcus pneumoniae	10/99 (10.1%)	0/24 (0.0%)	0.207
Most frequent gram-negative isolates**			
All *Moraxella* species	17/99 (17.2%)	6/24 (25.0%)	0.389
Pseudomonas Aeruginosa	11/99 (11.1%)	0/24 (0.0%)	0.120
All *Serratia* species	5/99 (5.1%)	3/24 (12.5%)	0.187
Haemophilus influenzae	2/99 (2.0%)	1/24 (4.2%)	0.482

Table 5-4. Abbreviations: Pre-C19, Pre-COVID-19 Years (2017,2018,2019); Y2020, year 2020. p-Values are from Fisher's exact tests and bold p-values are significant at p<0.05. Bacterial isolates are presented as

*the number of cases in which they were isolated, and as a percentage of the total number of culture positive cases in the respective group. Poly-microbial bacterial infections were defined as more than one pathogenic bacterial isolate. *Other cultures consisted of a mixed parasitic-bacterial case in the pre-C19 group, and a mixed fungal-bacterial infection in the Y2020 group. **Most frequent isolates are calculated as the total frequency of isolation of each species in all the samples, accounting for both mono- and poly-microbial isolates.*

Assessment of the most frequent gram-positive isolates found a preponderance of *Staphylococcus aureus* infections in the Y2020 group, being isolated in 25.0% of those with positive cultures, compared to 11.1% in pre-C19 (p=0.099). Of the gram-negative isolates, it was notable that no cases of *P. aeruginosa* were detected in Y2020, compared to 11.1% in previous years (p=0.120). However, neither of these differences reached statistical significance, largely as a result of the small sample sizes in these subgroups.

5.5 Discussion

The pandemic's negative impact on ophthalmic services [116,439,505,512,513] has raised concerns about patients' well-being. This study evaluated the impact of the first COVID-19 lockdown on the outcomes of patients with MK. In a survey completed by the British public, our group identified how concerns about the pandemic would lead individuals to consider seeking healthcare for their eye symptoms less urgently than if there was no pandemic [514]. The present study demonstrates a strong similarity between patients with MK in the first UK lockdown and those from previous years, with respect to time-to-presentation, presenting VA and ulcer area, complications, interventions, and final VA. However, the prevalence of concurrent trauma and use of systemic immunosuppression were greater than in previous years, while fewer poly-microbial infections and ward admissions occurred. Thus, patients presenting to this centre during the lockdown appear to be accessing services on time, did not have worse MK, and perhaps had milder disease in a more vulnerable group of patients.

Disease epidemiology and health care services vary geographically. Whilst Agarwal *et al.* [515] reported an increase in MK incidence during the lockdown at their unit in India, Poyser *at al.*[116] report a decrease in contact lens associated keratitis of more than 50%, compared to the same period in 2019, although the proportions remained similar in both study periods. The present study's results identified an increase in the proportion of MK patients seen in the department compared to previous years, whilst fewer patients were seen in the department overall [444]. The average time-to-presentation and number of patients

attending in Y2020 compared to pre-C19 indicate no change in the patterns of the public accessing services for MK. Both time to presentation and the broader profile of the pre-and COVID-19 groups may be influenced by the occurrence of pain symptoms as a driver for presentation [466].

Although no specific restrictions were placed on ward admissions, the decrease in 2020 is likely influenced by clinicians' concerns about their patients being exposed to COVID-19 in hospital. It is interesting to note that this did not appear to have a significant impact on the measured outcomes in these patients. Before the COVID-19 pandemic, severe MK ulcers (e.g., ulcer >3mm in diameter) were admitted to the ward for intensive topical medication. However, the adjustment to self-administration of drops in 2020 appears safe and effective. The economic burden of managing MK as an in-patient is considerable [506]. While other factors (e.g. social care) may drive the need for hospital admission, judiciously increased outpatient management would help to reduce the risk of COVID-19 exposure and be significantly more cost-efficient. In this case, an estimated £150,000 of direct patient cost-savings were made in the 2020 study period [506].

Cultured isolates identified as contaminants by the microbiology department were excluded from analysis in this study; however, it can be challenging to discern contaminants from pathogenic isolates, due to the high prevalence of commensal bacteria known to cause MK [114,115,516,517]. Corneal sample culture contamination may be influenced by face-mask wear. In their interesting study, Samarawickrama *et al*. demonstrated the impact of study participants speaking out-loud for 30 seconds at 30cm from an open culture dish, with and without wearing a surgical mask [518]. They found a significantly higher culture rate in the no-mask group. However, as acknowledged by the authors, their simulation likely over-estimates contamination rates compared to real-world practice, as culture plates are unlikely to have such prolonged direct exposure, and the scrape needle (or knife) surface area is considerably smaller. Although this may explain the decrease of some oral-cavity commensals such as *Streptococcus*, it is not supported by the results of the present study when considering the prevalence of others isolates like *Staphylococcus epidermis* and the increase in *Staphylococcus aureus*. Furthermore, if mask wear reduced contamination, it would be expected that the culture positive rate in Y2020 would have reduced, relative to the earlier period. This did not occur in the present study, with the pre-C19 and Y2020 rates being similar, and comparable to other UK studies [114,115,516,517].

Poor hand hygiene is a known risk factor for developing MK [519]. The bacterial diversity of the hands is greater and more dynamic than other body areas, and is considerably influenced by factors such as sex, environment and hand washing [520]. Following handwashing, although the bacterial load is decreased, its diversity is retained [520]. Since bacteria have innately varying transmission potentials, hand washing

may have a differential effect on the prevention of transmission of different species [521]. Thus, the microbiological findings of this study may be influenced by the increased handwashing and the impact of campaigns advising against excessive hand-face contact during the pandemic [522], which may have altered the autoinoculation of pathogenic microbes onto the ocular surface.

The urgency with which individuals seek medical attention may differ considerably between pathologies, and delays in presentation for some conditions may be more clinically significant than for others. Mild ocular surface disease symptoms are considered by the public to be of low seriousness, and to require medical attention less urgently during lockdown, compared to normal circumstances [514]. Ocular trauma, specifically occurring at home, has increased during the lockdown, with delays in medical review also being reported [512,523]. This is reflected in the higher prevalence of concurrent trauma in patients from the Y2020 group. Although uncomplicated mild trauma is relatively easily managed with lubricants and topical antibiotic prophylaxis, delays in initiating treatment may permit progression to infective keratitis. An explanation for the increased prevalence of systemic immunosuppression as a risk factor among the Y2020 cohort is less apparent.

The strength of this study is in its use of real-world data over four years in a large unit serving an out-of-hours population of up to 3.5million (5.25% of the UK population), where 72% of emergency room referrals are out of the local catchment area. This has helped to generate well-documented cohort of patients, thus reducing any variability introduced by dissimilar geography and clinical practice at different departments that may confound results. In this study, presenting VA, ulcer area, CIS, and final VA were utilised as proxy measures of severity. This represents an inherent limitation within the study; although severity scales for MK have been proposed [524,525], there is currently no widely accepted severity stratification system that adequately covers the entire spectrum of the disease. Complications and interventions are indicators of severity; however, these relatively rare events occurred too infrequently to offer insight here. Further assessments of disease state, including a detailed time-course of lesion morphology, assessment of final optical state (corneal scarring), and patient-reported outcomes, were desirable, but not possible here. Further limitations of this study include its external generalisability, since these observations are of one centre, hence further work from multiple centres across the UK is required to validate these findings.

This study compared the features of MK patients from the first UK lockdown to previous years. These results demonstrate the considerable similarity in the presenting severity and clinical outcomes of the two groups, despite fewer patients being admitted for care in 2020. This finding is significant, considering the persisting need to safely adapt clinical practice to manage the risk of COVID-19 transmission. While

other literature supports the link between increased ocular trauma and lockdown-related lifestyle changes, an explanation for the microbiology findings is less readily identifiable. Increased handwashing practices, as well as changes in environmental factors, such as reduced contact lens wear, may have contributed to this. However, these findings must be validated on a larger scale; therefore, future work aims to connect the corneal clinician network in the UK to investigate this nationally.

6. Chapter 6: Ocular dressing for the Prevention of Corneal Scarring in Microbial Keratitis (OPtiCS-MK)

6.1 Introduction

Corneal blindness is a disease of increasing burden in a globally growing and ageing population (discussed in Chapter 1). Health inequalities and difficulties in access to transplant tissues mean there is a significant need for a broadly applicable anti-scarring agent for the cornea. To date, no corneal fibrosis-specific candidate therapies have made it to the market, despite it being a blinding complication of many disorders. In Chapter 2, a systematic scoping review of corneal fibrosis treatments trialled in humans, gaps were identified in the clinical landscape. Despite many candidates being investigated in the preclinical setting, no approved therapy has yet arrived on the market.

In addition to having limited specificity in terms of mechanistic action in corneal fibrosis, eye drop formulations suffer from certain generic limitations (Table 6-1). In contrast, eye drop formulation benefit from the ease of access to the eye and limited systemic absorption (which can be reduced further by obstructing the tear duct at the time of administration), thereby minimising systemic side effects. Thus, the ideal candidate must aim to leverage these limitations and advantages to its benefit.

Table 6-1. Eye drop formulation limitations

- Short period of persistence on the ocular surface leading to issues with dosing and efficacy
- Administration difficulties due to bottle design or patient factors (e.g., frailty or pathological hands)
- Loss of product from inaccurately placed drops
- Maintaining sterility of the product and dropper bottle
- Maintaining stability of product in household storage environments (e.g., fridge, room temperature) and implications for travel
- Ocular surface irritation and toxicity due to components of the formulation e.g., preservatives

One such prospect, decorin, has been extensively investigated in the literature and by our group. Its efficacy and safety in preclinical studies has been well characterised (Chapter 1 section 4). The lab study in Chapter 3 was conducted to investigate a novel mechanism for decorin's action. Although this

mechanism was not confirmed, strong evidence remains in favour of decorin's potential benefit in humans, and further investigations are indicated.

Early phase trials focus on safety prior to establishing dosing ranges and then efficacy in larger later stage trials. Very early-stage trials, sometimes termed phase 0, are conducted in small numbers of humans, and are focussed investigations of a product, for example the pharmacokinetics, which help indicate proof of concept and potential in further trials [526]. This chapter describes the intended clinical trial of decorin in patients with MK, based on the regulations and design requirements identified in the preceding studies.

6.2 Aims

This early phase study primarily aims to determine the safety profile of the gellan dressing with and without decorin, in patients with microbial keratitis. Secondary aims of the study include the collection of samples for mechanistic analysis and preliminary efficacy data to guide the design of the next phase of clinical study.

This chapter covers key details and considerations of the regulatory process and other factors that have led to the trial design. It presents the consequent trial protocol and several trial documents.

6.3 My contributions

The development of novel therapeutics takes considerable effort from a dedicated multidisciplinary team. I have been involved with the project since the preclinical study phase where I contributed to study design, conduct and data analysis in the mouse corneal image analysis study. In anticipation of the trial, I was involved in setting up many clinical and governance aspects of the trial. These included but were not limited to: the iterative development of the trial and clinical pathway; drafting documents including the protocol, standard operating procedures, patient information leaflets, case report forms, product labels and elements of the lab manual. Other related activity included grant proposals to support the trial including the Barbara-Mary Wilmot Trust Fund grant for research equipment, which was used to acquire the CASIA II anterior segment OCT machine that will be used as a trial outcome.

6.4 The regulatory pathway

6.4.1 Device vs medicinal product

Medical devices (MD) are regulated under the Medical Devices Regulations 2002 (SI 2002 no 618, as amended) whilst medicinal products (MP) are regulated under separate legislation, Humans Medicine Regulations 2012 (SI 2012/1916). The classification of novel therapeutics as an MD or MP has numerous implications regarding its regulatory route, its marketing and post-marketing safety surveillance.

Ophthalmic lubricants are classified as MDs, as their chief mechanism of action is through lubrication of the ocular surface, with their intended purpose of preventing the mechanical abrasive effect of the eyelids. MDs typically work by mechanical action, as physical barriers or physical supports to the body's function. A therapeutic may be considered an MP where its action is typically achieved through pharmacological, immunological, or metabolic means. Medical devices may contain medicinal substances which act on the body in a manner ancillary to the device. Where they do, the product is regulated as a medicinal product rather than a medical device [527].

The developmental journey of MPs generally differs from MDs as well. MPs are initially evaluated for feasibility and efficacy theoretically, then in the laboratory and animals, following which they enter safety and efficacy clinical trials of varying scale, in which design features of the MP continue to be refined. In contrast, MDs are more mature at the point they enter the human testing phase, which is typically shorter than for MPs. The Medicines and Healthcare products Regulatory Agency (MHRA) regulates both MPs and MDs in the UK, however, the approval regulatory package (supporting evidence, administration, product marking and assessments) significantly differs. The MHRA also monitors the safety of both after their launch on the market.

MDs are categorised by their features (Table 6-2) and their relative risk (Figure 6-1). As the eyelids are considered an orifice, eye drops are therefore considered to be internally applied. This means lubricants as devices are categorised among the highest-risk devices (IIb) [528], thus making their regulation more stringent than for example, spectacles which are the lowest class. Whilst Gel-PLUS can be classified as an MP, the classification of Gel-MINUS is less clear. Due to the novel production method of gellan in this formulation, it is possible its physico-chemical properties may interact with the wound healing environment and confer properties in addition to its mechanical occlusive effect.

Table 6-2. MHRA categorisation of medical device categories		
Category	**Definition**	**Examples**
1. Non-invasive	Devices which do not enter the body	Plasters, walking sticks, wheelchairs, artificial kidneys (external dialysis)
2. Invasive	Devices inserted into the body's orifices	Contact lenses, enemas, examination gloves
3. Surgically invasive	Devices used or inserted in surgery	Needles, scalpels, cardiovascular catheters
4. Active	Devices requiring an external source of power	X-ray equipment, ultrasound, TENS devices
5. Implantable	Devices implanted into the body	Breast implants, orthopaedic implants, intraocular lenses

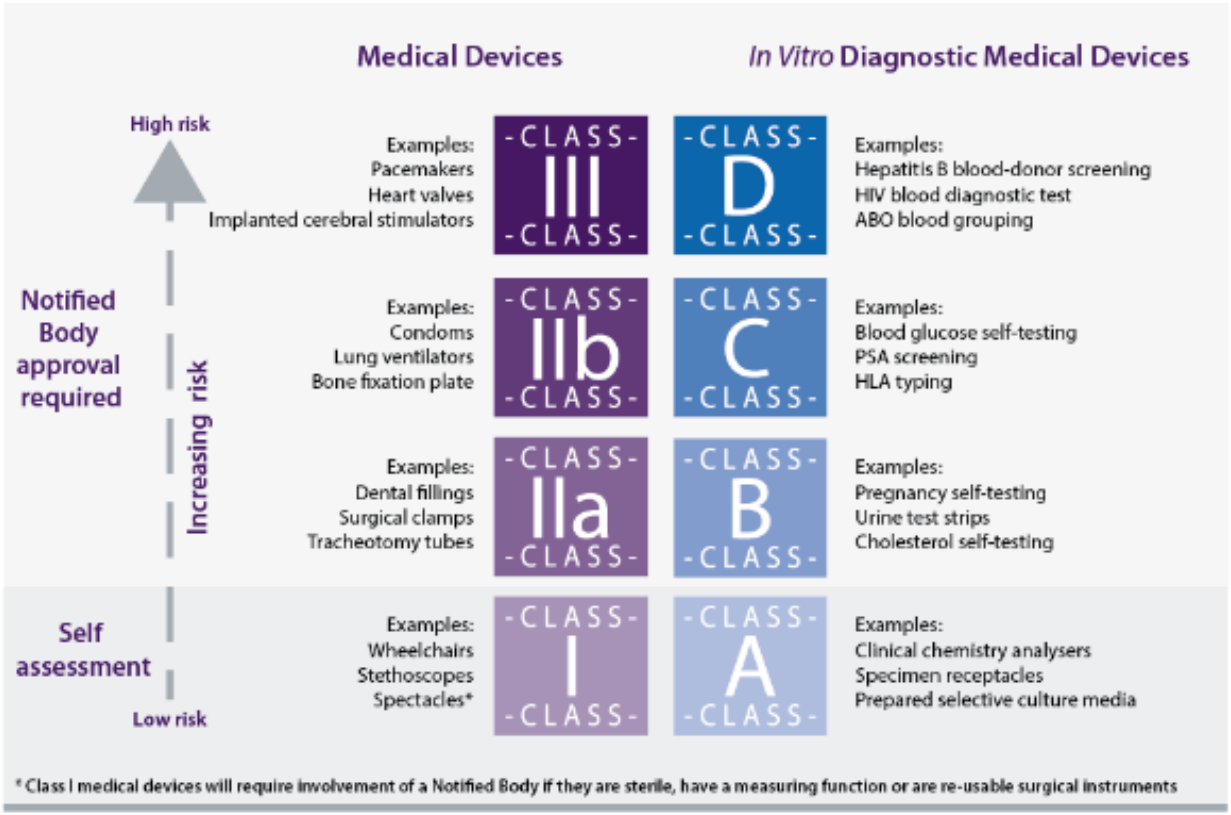


Figure 6-1. Device classification conceptual overview. Adapted from the MHRA Handbook

6.4.2 The formulation – physical and chemical properties

6.4.2.1 Decorin (Galacorin)

Galacorin is a proprietary formulation of human recombinant decorin without a glycosaminoglycan side chain, that is supplied by Catalent Pharmaceuticals (New Jersey, USA). Galacorin is a 362 amino acid comprising only the protein core containing leucine repeats as shown in the 3-D structure below (Figure 6-2).

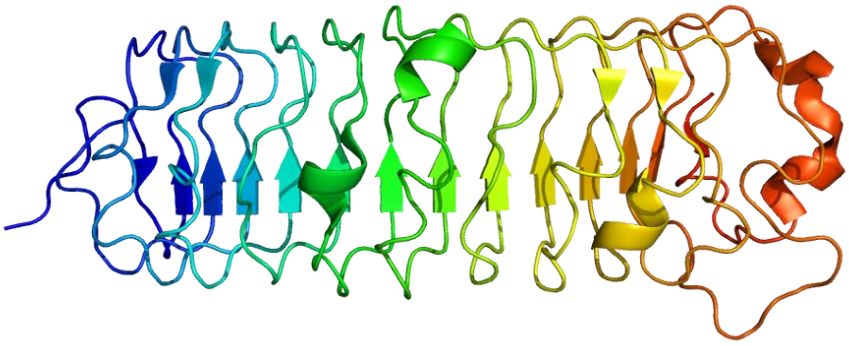


Figure 6-2: 3D Structure of Galacorin

6.4.2.2 Gellan fluid gel

Gellan Gum is a water-soluble anionic polysaccharide produced by the bacterium *Sphingomonas elodea* (formerly *Pseudomonas elodea*). It was initially approved as a food additive, also known as E418, where it acts as a thickener, emulsifier, and stabiliser. Gellan gum has subsequently been approved for non-food, cosmetic and pharmaceutical uses by many countries, including the US and EU. It is also used in timolol gel-forming eye drops (Santen, UK) [529], although its formulation significantly differs.

The fluid gel is created by cooling a high-temperature gellan-water mixture under shear forces, which results in the polysaccharide molecules forming elongated polymer ribbons, Figure 6-3. The size of these ribbons can be tuned by altering the shear forces and cooling rates. The specific size of these polymers gives the fluid gel its physical properties. Under shear, for example whilst being expelled from an eye dropper, the gel can flow, however at rest, the gel stiffens and solidifies.

This biphasic property was designed into the carrier to increase ocular surface retention. The expectation of the drop is that each blink introduces a shear force that spreads the drop across the ocular surface, and

whilst the lids are separated, gel-stiffening maintains an occlusive dressing over the ocular surface. These properties enable the gel to persist on the ocular surface for extended periods improving drug delivery.

Thus, this purposely designed formulation has the potential to overcome the limitations with current treatments for corneal scarring. Furthermore, gellan fluid gel has the potential as a platform technology in the management of many ocular diseases, if combined with other therapeutics.

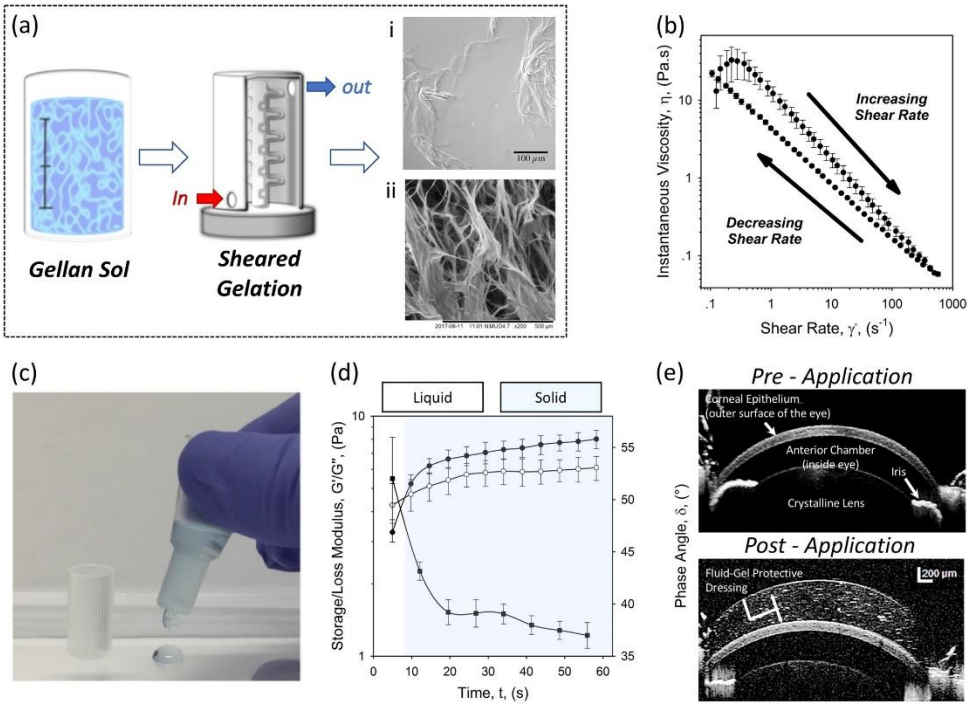


Figure 6-3. Gellan fluid gel properties

Processing and properties of the fluid gel. ***a)*** *The fluid gel solution is mixed under shear and slowly cooled to obtain "ribbon-like" structures seen on transmission microscopy (i) and scanning electron microscopy (ii).* ***b)*** *Viscosity profile of the fluid gel product highlighting its increase in viscosity unagitated, and rapid decrease in viscosity under shear. The fluid gel being dispensed from the eye dropper packaging (gel has been stained blue to be visible in the photograph).* ***d*** *Small deformation rheology data obtained at a single frequency (1 Hz, 0.5% strain) as a function of time. Data show the evolution of an elastic network post-shearing resulting in a transition from liquid to solid-like behavior.* ***e*** *Anterior segment OCT images showing the ocular surface before fluid gel application (top image) and post application (bottom image). Images demonstrate a uniform layer that covers the entirety of the ocular surface. Adapted from* [54].

6.4.3 Formulation manufacture and packaging

Initially the primary container of choice was a single-use, 1mL low-density polyethylene ampoule, which was produced within the Blow Fill Seal (BFS) machine and was outputted from the BFS machine in multiple connected containers as shown in Figure 6-4.

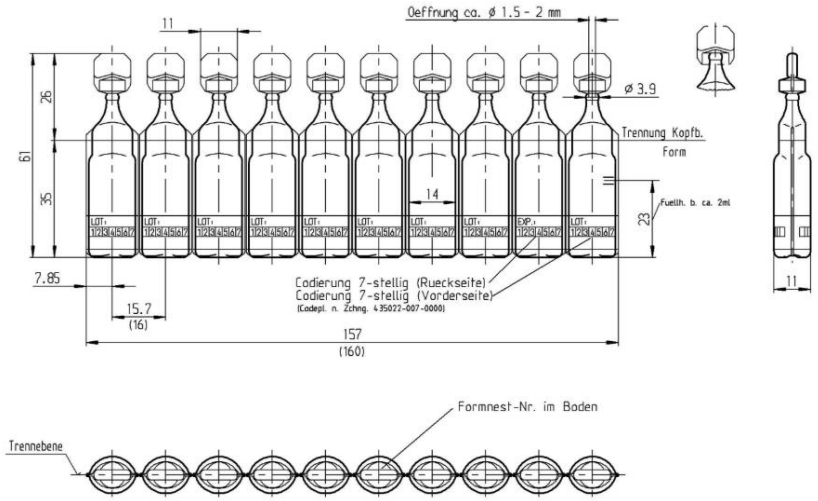


Figure 6-4. Initial single dose unit packaging for the investigative products

The initial single dose unit packaging conferred many benefits. They eliminated the need for preservatives, were easy to carry, and the soft plastic enabled easier administration for weak hands. However, the MHRA stated that this was not a metered device and therefore impossible to confirm consistent drop/drug quantity was being administered each dose. Despite the tear film volume being approximately 6 µL [530], and which would be saturated by the application of a single drop, the MHRA insisted on alternate packaging or demonstration of manufacturing validity using the desired bottle system. An alternative multidose bottle was identified. However, the manufacturer did not have approval as a device in the UK, thus, the remaining MHRA-approved option was the multidose APTAR Ophthalmic Squeeze Dispenser (OSD) (Figure 6-5).

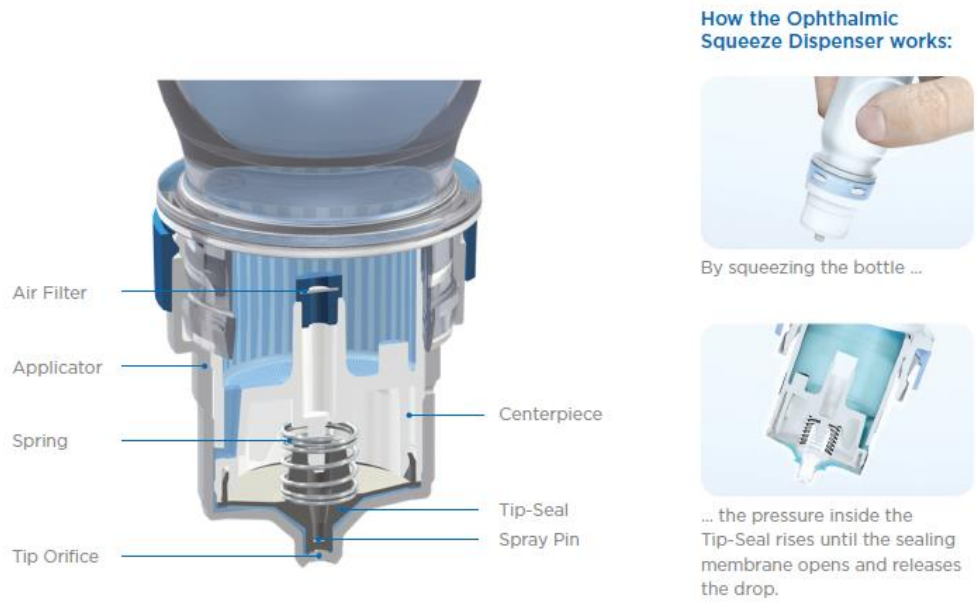


Figure 6-5. Ophthalmic squeeze dispenser by Aptar (Aptar crystal lake, USA)

Ophthalmic squeeze dispenser (OSD) diagram adapted from Aptar Pharma's brochure.

6.4.4 Preclinical trials decorin for the cornea – a safety perspective

Chapter 1 (Section 1.4) discusses the literature published over the past six years regarding this decorin-gellan fluid gel formulation, and other in vivo ocular studies of decorin treatment. Various dosing regimens have been trialled for differing lengths and in different injury and healthy models (Table 1-3). Although these studies were not primarily examining safety or toxicity, their findings are reassuring as no acute toxicity was reported nor was any detected upon histological analyses compared to controls. Of most relevance is our group's study in mice with MK. In this acute condition, corneal inflammation was not worsened, no perforations occurred nor did any deaths.

Of interest was Mohan et al.'s long-term safety of healthy rabbit cornea treated with their AAV5-decorin therapy and followed up for 6 months. Through their clinical evaluations and final histological evaluations, they determined that their formulation caused no toxicity as measure by a clinical assessment panel [531]. Stromal cell counts were no different compared to control AAV5 vector only and naïve control groups.

Furthermore, tissue decorin levels were found to be >30% greater in the treated group, while αSMA mRNA was almost half of the naïve control cornea, and lower still compared to the vector-only animals [194]. However, what is not known are the impacts of chronically raised decorin within the eye and systemically in response to stressors, and whether these corneas would respond favourably to pathology.

Decorin has also been trialled in a host of non-ocular injury in vivo models with good safety, tolerability, and efficacy. These include murine muscle injuries, skin, and the CNS where it also reduces scarring and encourages regeneration [532–537].

6.5 Clinical trial design requirements

For the safe transition of candidate therapeutics from bench to bedside, several factors must be taken into consideration, and regulatory requirements met [538]. Potential investigative products must be demonstrably safe and reproducibly manufactured to a high standard (The Good Laboratory Practice Regulations SI 1999 no.3106) before permittance into human testing, which itself needs to be quality assessed prior to commencement [539]. The previous chapters in this body of work have highlighted the gaps in the literature and generated discussions pertinent to the field. For example, the mechanism of action of decorin in MK. Based on this, it is incumbent on the trial to investigate the mechanisms involved in corneal regeneration and to mitigate the methodological limitations learnt through previous trials.

6.5.1 Patient population

Selection of the patient population has knock-on implications for trial design and conduct. An anti-scarring treatment for the cornea has potential applications in very many disorders. However, not all conditions are easily investigated due to the natural history of the disease course. For example, herpetic corneal disease is another leading cause of corneal transplantation [78]. It is a chronic condition which progresses in a relapsing and remitting pattern, impairing vision over the course of years. Flares are unpredictable and heterogenous in their severity and response to treatment. Individual baselines differ and typically time to corneal transplant varies in the order of years. This poses clear pragmatic difficulties in designing an intervention trial. By contrast MK patients make good candidates for such studies as their pathology occurs as a single episode, lesions can be discrete within the cornea, and they may also have fewer

ophthalmic comorbidities, such as uveitis or glaucoma which can complicate chronic Herpes simplex infection, which may complicate their entry into a clinical trial.

In terms of risk mitigation, careful patient selection helps to prevent vulnerable patients from being entered into the trial and reduces the chance of patient factors impacting the trial results. For example, patients with significant co-morbidities that may not be able to take part for the entire duration of the trial will not be entered into this trial. The full inclusion and exclusion criteria can be found in the protocol in the next subsection.

6.5.2 Safety

Safety is central to this study. Toxicity assessments were defined based on features of ocular surface inflammation and assessment criteria described in the literature [540]. Safety and toxicity were defined through discussions with the technical project and clinical trial teams' input taking into consideration side effects from systemic absorption, risk to corneal ulcer, limitations of our assessment techniques and acceptable margins of safety [541]. To support rigorous and unbiased assessments of data, an external trial Data Monitoring Committee was established to review trial safety data and determine progression at each data review checkpoint [542].

Dosing is informed by the preclinical studies with this formulation. As this is the first human trial, dosing is not being explored and a mid-range dose has been selected considered as likely to be efficacious and to explore proof of such.

6.5.3 Exploratory outcomes

A number of exploratory outcomes were added into the panel of assessments for this trial following the work in the preceding chapters.

6.5.3.1 Anterior segment image analysis

Outcomes used in the preclinical portion of the project [54] were considered with the human clinical trial in mind. I was responsible for the design and conduct of this study. Cornea of mice with MK, receiving

either Gel-PLUS, Gel-MINUS or standard of care, were assessed using imageJ to quantify treatment effects [54]. Two observers annotated a series of images for both reliability testing and assessment of the animal data for efficacy. The same test data images were repeatedly mixed among study data in order to determine the reliability of the method. While trends in efficacy were observed in the main paper, only few pairwise comparisons demonstrated statistically significant differences (Chapter 1 Figure 1-9). Despite being analysed by two experienced ophthalmologists there was considerable variation between the observers (Figure 6-6). These were thought to be due to the variation in image quality (e.g. position, lighting, reflections) impacting the interpretation of results. Limitations of such a method would be undesirable in a large-scale clinical trial, and optimisation of the photography protocol was a key consideration. Thus, in the absence of validated alternatives, this technique will be used as one of several exploratory outcomes of efficacy.

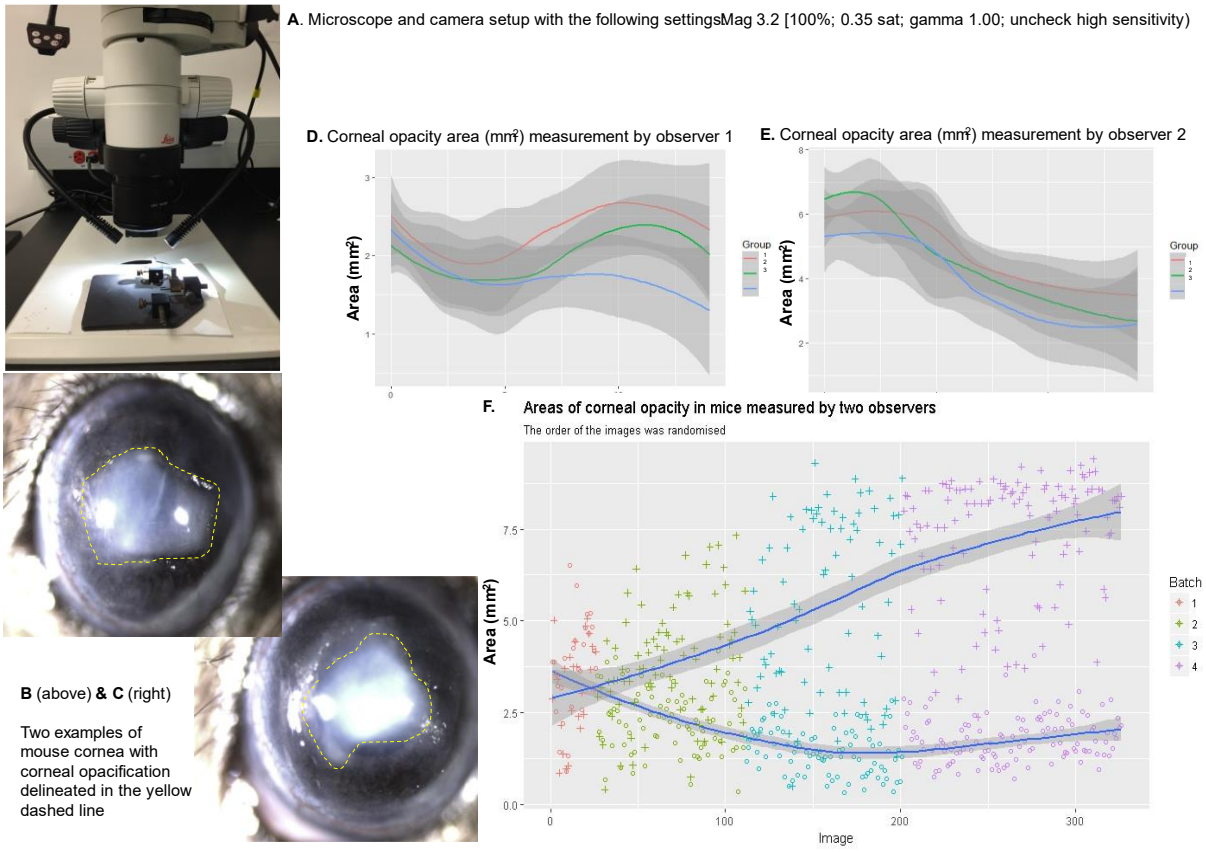


Figure 6-6. Corneal scar quantification using anterior segment photos and imageJ.

Mouse model and sampling: mice had one eye inoculated with Pseudomonas aeruginosa and were allocated into 1 of 3 treatment arms: Group 1 - standard treatment; Group 2 - vehicle control; Group 3 - vehicle+ novel treatment. En-face 24-bit colour photographs were captured over the subsequent 14 days, using a SPOT RTKE camera (Diagnostic Instruments) connected to a Leica MZF III stereo Microscope (A). Two ophthalmic clinicians acting as masked independent observers and analysed 326 images, in 4 batches in the same randomised order. The area of opacification was delineated and measured using Fiji, an open-source image-processing package based on imageJ (B & C). Definitions of corneal opacification, adequate and inadequate images were agreed upon prior to commencement of image analysis by the observers.

Measurements, in mm2 were plotted using ggplot2 in R, and loess smoothers fit to the chronological series for each assessor. The randomised order dictated that there should be no time-trend in the measured areas. Analysing absolute values (F), initially the observers were in reasonable agreement but this decreased with time. While the actual values differed, both observers demonstrated similar trends (D & E). The vehicle (group 2) and vehicle+novel treatment (group 3) demonstrated less opacification, in mm2, compared to the conventional treatment (group 1) (D & E) by the end of the study. The mean measured area was hypothesised to remain constant throughout the experiment for each assessor, and the assessors to show reasonable agreement, however this was only partially demonstrated. Adapted from [543].

6.5.3.2 Anterior segment OCT

Anterior segment OCT is another assessment measure in the trial. OCT has been utilised for quantifying keratitis in the past [544,545], however its use has focussed more on 2-dimensinal analyses.

To determine its potential a proof-of-concept study was performed. OCTs of six patients with corneal opacities were used to develop a technique on MatLab (The Mathworks inc, Version 9) to quantify images in 3-d. Briefly, entire corneal volume scans acquired on the CASIA II (Tomey, Japan) were deconvolved, high-pass filtered (to optimise contrast), and then segmented, following identification of a region of interest. The lesion was reconstructed and their volume measured. Figure 6-7 illustrates the findings from this proof-of-concept work.

Patient	Lesion volume	3-dimensional image

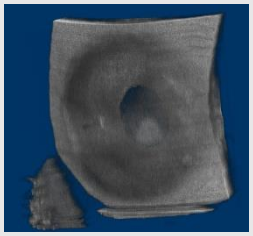
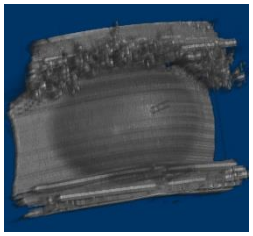
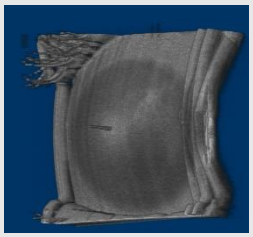
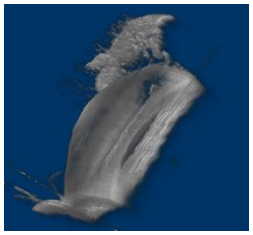
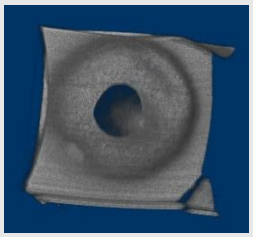
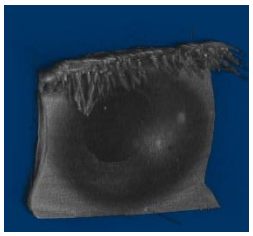
Patient 1	0.884 mm^3	
Patient 2	0.216 mm^3	
Patient 3	0.572 mm^3	
Patient 4	0.114 mm^3	
Patient 5	0.837 mm^3	
Patient 6	0.114 mm^3	

Figure 6-7. Anterior segment OCT image analysis.

Images of six patients were analysed and the volume of the corneal opacities derived on MatLab version 9. Thanks to the help from Dr F Menduni, Aston University, with MatLab.

OCT forms part of the exploratory outcomes in this study as it has not been formally validated in a reliability study for this application. Whilst the primary focus of this trial is safety, the OCT assessments of lesion morphology will contribute to the development of new outcome assessment techniques for trials in the future.

6.5.4 Sample for Nanostring and other mechanistic studies

Understanding the mechanistic pathways that are elicited in response to novel therapeutics is important for the development of effective treatment strategies. To generate mechanistic insights, a sampling strategy was incorporated in the trial (Table 6-3). Samples will be obtained for proteomics (tear washings), ocular surface inflammatory molecule profile (Luminex bio assay https://www.luminexcorp.com/eu/), ocular surface gene expression (Nanostring analysis) and ocular surface microbiome assessment using the Nanopore sequencer (Chapter 1 section 3.5)

The NanoString nCounter technology can perform multiplex gene analysis in order to generate insights about the elicited pathways in a pathological context [546]. For this study, the human fibrosis panel, which interrogates 770 relevant genes will be used. Data are analysed using ROSALIND (https://rosalind.bio/), in accordance with criteria provided by NanoString. The abundance of various cell populations will be calculated on ROSALIND using the Nanostring Cell Type Profiling Module (www.nanostring.com). By analysing relative gene up and down regulation, information about the activated pathway can be surmised. Longitudinal analysis using Nanopore will provide insights as to the dynamics of corneal wound healing in response to the novel treatments.

Table 6-3. Overview of mechanistic samples.

Samples will be taken according to the schedule of events at various time points through the study.

Outcome	Sample	Volume (x Repeats)	Quantity	Measurement	Specifics

Impact of fluid-gel therapy on the ocular surface inflammatory profile						
		Tear washings (70-100 µL)	10µL (x1)	30 patients x 2 eyes x 6 visits	Custom Luminex Bioassay™ IL-1, IL-2. IL-4, IL-5, IL-6, IL-13, IFNγ, TNFα, TGFβ1, EGF, VEGF, PDGF, MMP8, MMP9,	Bio-Plex Custom Assay [BioRad]
		Serum (5ml)	10µL (x1)	30 patients x 6 visits		Bio-Plex Custom Assay [BioRad]
Changes in fibrotic and immunoregulatory gene expression						
	Nanostring fibrosis and inflammatory panels	Polyester swab (estimated RNA 75-125ng)	Single Swab each eye (X1)	30 patients x 2 eyes x 3 visits		Fibrosis Panel Immunology panel
Proteomics						
		Tear washings (70-100 µL)	70-100µL (x1)	30 patients x 2 eyes x 6 visits		
			Single swab each eye (x1)	30 patients x 2 eyes x 6 visits x 2 eyes		
Ocular surface microbiome						
		SurgiSpear tarsal/fornix swab: Microbiome	Single swab each eye (x1)			
		SurgiSpear tarsal/fornix swab: Microbiome	Single swab each eye (x1)			

6.5.4.1 Other notable adjustments

The study of public perceptions in the pandemic (Chapter 4) identified patient reluctancy to volunteer for research in view of the risk of infection associated with presenting to hospital services. Patient safety is

always a priority, and it is important that they feel safe when engaging with clinical services [547]. To facilitate this, adaptations were made to the trial design. A patient information video was developed, and the protocol was adapted to enable patients to consider the trial information remotely; if they didn't want to attend hospital, this could be facilitated by a virtual meeting if required. Many trial team meetings will now be conducted in a hybrid format to facilitate the team to continue safely. The original plan for the safety stages was for the patients to remain in hospital for the duration of the five days of treatment, which is now being conducted on an outpatient basis.

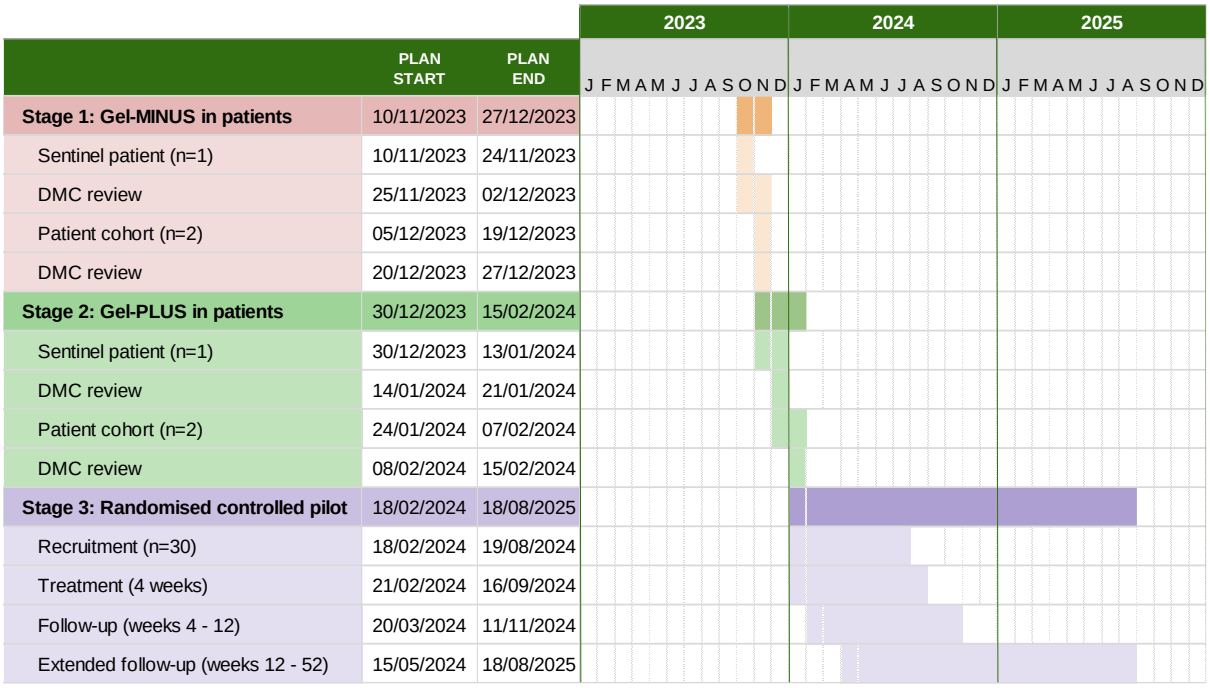
6.6 Clinical trial protocol

6.6.1 Trial overview

This is a single centre early phase human clinical trial with the primary objective of assessing safety. The study is comprised of 3 stages and is expected to take approximately 22 months to complete, Figure 6-8. The initial stages will be safety and toxicity 'run in' stages (Stages 1 and 2) evaluating Gel-MINUS in three volunteers and then Gel-PLUS in three volunteers. The trial will be monitored by an independent Data Monitoring Committee (DMC) who will review the results after each stage. If during Stages 1 or 2 any gel-limiting-toxicity (GLT) (criteria defined in the 'outcomes' section) occurs in the first cohort of 3 patients, a second cohort of 3 patients will be recruited for that stage. If any GLT occurs in the second cohort of 3 patients this will confirm end of trial.

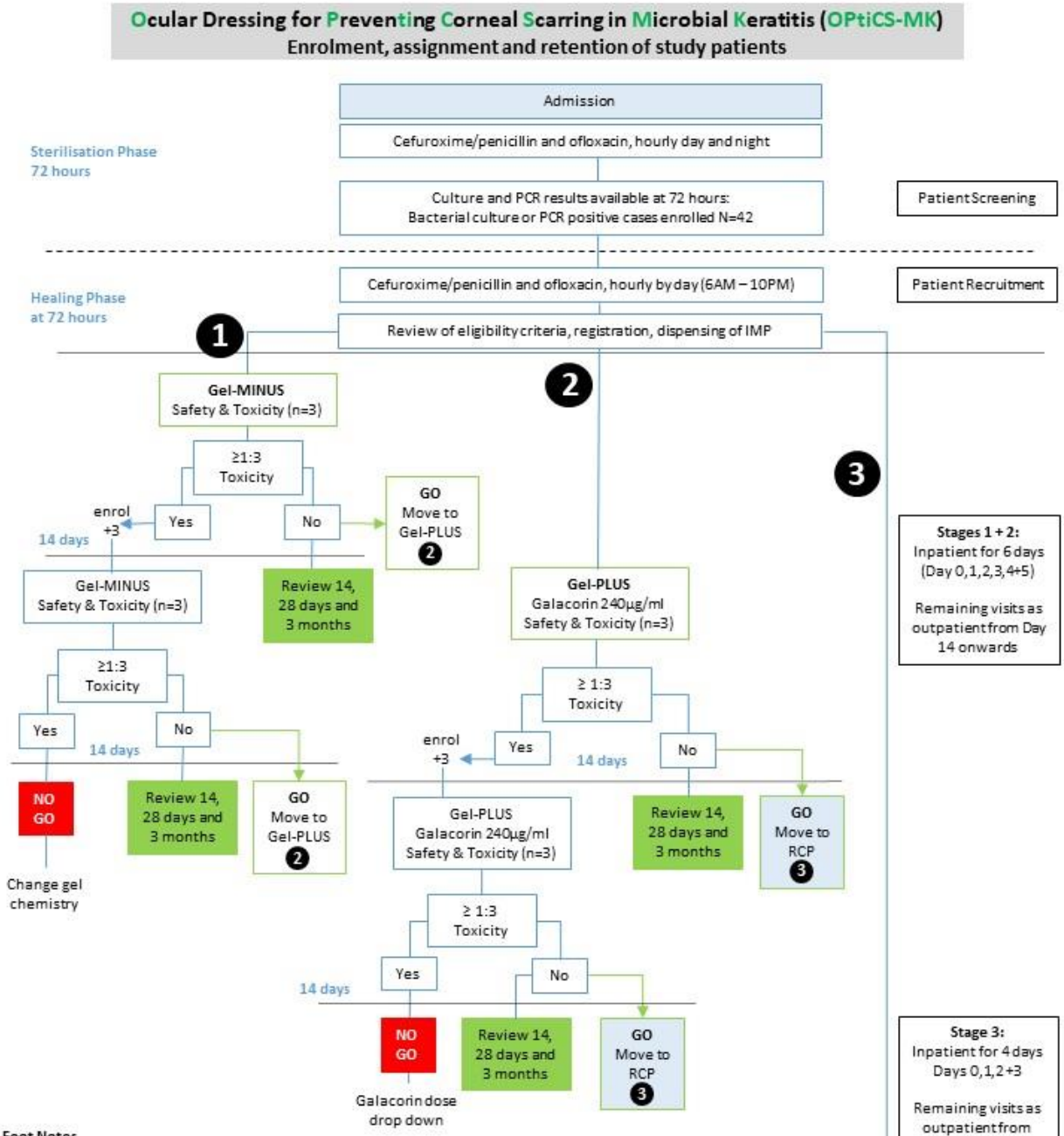
Once safety of the interventions has been confirmed an open label randomised controlled trial pilot (Stage 3) will commence. Patients will be randomised into one of three treatment arms resembling the preclinical study; No-Gel (standard of care), Gel-MINUS (gellan gum fluid gel only with standard of care) and Gel-PLUS (decorin with gellan gum fluid gel and standard of care). Gel-MINUS and Gel-PLUS groups will remain on the intervention for 4 weeks from commencement of treatment. The trial schema in Figure 6-9 illustrates the workflow and details of each stage.

OPtiCS-MK Timelines



	PLAN START	PLAN END
Stage 1: Gel-MINUS in patients	10/11/2023	27/12/2023
Sentinel patient (n=1)	10/11/2023	24/11/2023
DMC review	25/11/2023	02/12/2023
Patient cohort (n=2)	05/12/2023	19/12/2023
DMC review	20/12/2023	27/12/2023
Stage 2: Gel-PLUS in patients	30/12/2023	15/02/2024
Sentinel patient (n=1)	30/12/2023	13/01/2024
DMC review	14/01/2024	21/01/2024
Patient cohort (n=2)	24/01/2024	07/02/2024
DMC review	08/02/2024	15/02/2024
Stage 3: Randomised controlled pilot	18/02/2024	18/08/2025
Recruitment (n=30)	18/02/2024	19/08/2024
Treatment (4 weeks)	21/02/2024	16/09/2024
Follow-up (weeks 4 - 12)	20/03/2024	11/11/2024
Extended follow-up (weeks 12 - 52)	15/05/2024	18/08/2025

Figure 6-8. Clinical trial timeline Gantt chart with anticipated opening date



Foot Notes

* **All patients:** continue topical antibiotics as clinically directed and receive dexamethasone sodium phosphate (0.1%) 4x/day, 3x/day, 2x/day and 1x/day each for 1 week (4 weeks total)
* **Stage 1 - Gel-MINUS**[1]**:** comprises 0.9% w/v Gellan without Galacorin, 4x/day for 5 days
* **Stage 2 - Gel-PLUS**[1, 2]**:** comprises 0.9% w/v Gellan plus Galacorin 240µg/ml, 4x/day for 5 days
* **Stage 3 Randomised Controlled Pilot**[2]**:**
  1. **NO-Gel:** standard care lubricant: sodium hyaluronate (0.4%) 6x/day for 4 weeks
  2. **Gel-MINUS:** 4x/day for 4 weeks
  3. **Gel-PLUS:** 4x/ day for 4 weeks

[1] A sentinel patient will complete the 14 day GLT period prior to recruitment of further patients; continuation of recruitment will be dependent on successful DMC review.

[2] Progression through to Stages 2 and 3 are dependent on successful independent DMC review

Abbreviations

AS-OCT: Anterior Segment-Optical Coherence Tomography
DMC: Data Monitoring Committee
EQol-5D: European Quality of Life – 5 Dimensions
GLT: Gel Limiting Toxicity
LogMAR: Log_{10} of the Minimal Angle of Resolution
NEI-VFQ-25: National Eye Institute Visual Function Questionnaire 25
NRS-11: Numerical Rating Scale – 11 points

Figure 6-9. Clinical trial schema. Overview of the clinical trial stages and patient pathway.

6.6.2 Schedule of Events

STAGE 3														
		Wk 1					Wk 2	Wk 3	Wk 4	Wk 8	Wk 12	Extended follow up		
	Screening D -3 to -1	D 0	D1	D 2	D 3	D 7	D 14	D 21	D 28	D 56	D 84 ± 1 week	Wk 26	Wk 52	
						±1 D			± 3 Ds			±1 month		
Informed Consent	☑													
Inclusion and Exclusion Criteria	☑													
Demographics	☑													
Medical History [5]	☑													
Adverse Events		☑	☑	☑	☑	☑	☑	☑	☑	☑	☑			
Concomitant Medications	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	☑	
Vital Signs [6]	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	
Clinical Examination [7]	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	
Study Drug Dispensing [8]		☑			☑	☑	☑	☑						
Treatment Education [9]		☑	☑	☑	☑									
Assess Participant Adherence [10]			☑	☑	☑	☑	☑	☑	☑					
Randomisation		☑												
Ophthalmic Examinations														
Microbiology Samples	☑													

STAGE 3														
		Wk 1					Wk 2	Wk 3	Wk 4	Wk 8	Wk 12	Extended follow up		
	Screening D - 3 to -1	D 0	D1	D 2	D 3	D 7	D 14	D 21	D 28	D 56	D 84	Wk 26	Wk 52	
						±1 D			± 3 Ds		± 1 week	D 182	D 364	±1 month
Polymerase Chain Reaction	☑													
LogMAR BCD Visual Acuity [11]	☑	☑	☑	☑	☑		☑		☑	☑	☑	☑	☑	
Electronic Tablet-Based Functional Vision Tests		☑	☑	☑	☑		☑		☑	☑	☑	☑	☑	
External Ocular Examination	☑	☑	☑	☑	☑		☑		☑	☑	☑	☑	☑	
Periocular Erythema	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	
Intraocular Pressure	☑	☑	☑	☑	☑		☑		☑	☑	☑	☑	☑	
Fundus Ophthalmoscopy	☑	☑	☑	☑	☑		☑		☑	☑	☑	☑	☑	
Photography without Fluorescein	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	
Photography with Fluorescein	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	
Slit-Lamp Biomicroscopy														
Slit-Lamp Examination	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	
Location of Abscess/Scar [12]	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	
Corneal Abscess/Scar Size [13]	☑	☑	☑	☑	☑	☑	☑		☑	☑	☑	☑	☑	

STAGE 3														
		Wk 1					Wk 2	Wk 3	Wk 4	Wk 8	Wk 12	Extended follow up		
												Wk 26	Wk 52	
	Screening D - 3 to -1	D 0	D1	D 2	D 3	D 7	D 14	D 21	D 28	D 56	D 84 ± 1 week	D 182	D 364	
						±1 D			± 3 Ds			±1 month		
Corneal Epithelial Defect Size [13]	☐	☐	☐	☐	☐	☐	☐		☐	☐	☐	☐	☐	
Hypopyon (mm)	☐	☐	☐	☐	☐	☐	☐		☐	☐	☐	☐	☐	
Fibrin and Cellular Activity [14]	☐	☐	☐	☐	☐	☐	☐		☐	☐	☐	☐	☐	
Laboratory Tests														
Pregnancy Test [15]	☐													
Clinical Laboratory Tests [16]	☐	☐☐							☐	☐	☐			
Blood for Anti-Decorin Antibodies		☐							☐	☐	☐			
Research bloods for future research [17]		☐							☐					
Tear Washings		☐							☐					
Conjunctival swabs for ocular surface microbiome analysis		☐							☐					
Conjunctival swabs for ocular surface nanopore sequencing		☐							☐					
Quality of Life Questionnaires [18]														

STAGE 3														
	Screening D -3 to -1	Wk 1					Wk 2	Wk 3	Wk 4	Wk 8	Wk 12	Extended follow up		
												Wk 26	Wk 52	
		D 0	D1	D 2	D 3	D 7	D 14	D 21	D 28	D 56	D 84 ± 1 week	D 182	D 364	
						±1 D			± 3 Ds			±1 month		
NEI VFQ-25		?					?		?	?	?	?	?	
NRS-11		?	?	?	?		?		?	?	?	?	?	
Ocular Tolerability VAS		?	?	?	?		?		?					
EQ-5D-5L		?					?		?	?	?	?	?	
Imaging														
OCT Imaging		?		?		?	?		?	?	?	?	?	

Figure 6-10. OPtiCS - MK clinical trial schedule of events for Stage 3 the randomised controlled pilot

The figure depicts the schedule of all patient-staff contact points and the activities that will be conducted at those times. This includes the time of consent, the investigations, drug dispensing etc.

6.6.3 Treatment Details

6.6.3.1 Standard of care

Broad spectrum dual antimicrobial therapy comprised of cefuroxime 4% w/v and levofloxacin 5% w/v. Topical corticosteroid eye drops (dexamethasone phosphate 0.1%) commenced after the sterilisation phase (as determined by the patients physician), on a tapering regime starting at x4 per day reducing by one drop each week. Sodium hyaluronate 0.2% lubricant administered along the other drops.

6.6.3.2 Gel-Minus

The Gel-MINUS formulation is composed of 0.9%w/v gellan gum aqueous solution.

6.6.3.3 Gel-Plus

A single dose, defined as a 60µL droplet will deliver 14.4µg of Galacorin in 0.9% w/v gellan gum aqueous solution.

6.6.3.4 Rescue protocol

In the event of any GLT encountered by the patient they will be withdrawn from the trial and commenced on prednisolone 1% eye drops at a frequency determined by clinical need.

6.6.4 Trial design

This is a four-stage, open-label trial consisting of three safety run-in stages prior to a randomised-controlled stage. Stages 1 (Gel-MINUS in patients with microbial keratitis) and 2 (Gel-PLUS in patients with microbial keratitis) are early phase safety and toxicity studies of Gellan without (Gel-MINUS) and with the addition of Galacorin (Gel-PLUS). Stage 3 is a Phase IIa Randomised Controlled Pilot (RCP) evaluating safety, toxicity and efficacy of the interventions. The adaptive trial design will enable safety and toxicity testing prior to randomisation.

6.6.4.1 Stage 1 – Gel MINUS Safety in patients with microbial keratitis

Stage 1a will comprise a cohort of three participants, with potential for an additional cohort of three participants if toxicity is encountered in a volunteer. Participants in Stage 1 will receive Gel-MINUS , 4x per day for 5 days as outpatients at BMEC. All patients will also receive protocol defined standard care topical steroids (dexamethasone sodium phosphate 0.1%) and topical antibiotics.

Following the safe completion of stage 1, stage 2 will commence in the same format using Gel-PLUS in place of Gel-MINUS.

If, in the first cohort of either stage 1 or 2, one or more of the first cohort of three patients demonstrate GLT, a second cohort of 3 patients will be recruited. If one or more patients from the second cohort show

GLT, this will confirm NOGO (end of trial for that formulation) and the need to review the formulation. If Gel-MINUS demonstrates GLT then the trial will close.

6.6.4.2 Stage 2 – Gel PLUS Galacorin Safety in patients with microbial keratitis

Stage 3 will repeat the safety profiling in Stage 2 for the Gel-PLUS (Gellan with Galacorin) treatment. A cohort of three participants, will receive Gel-PLUS 4x per day for 5 days as outpatients at BMEC. All patients will receive protocol defined standard care topical steroids (dexamethasone sodium phosphate 0.1%) and topical antibiotics.

If in the first cohort ≥1 out of 3 patients demonstrate GLT, a second cohort of 3 patients will be recruited to Gel-PLUS treatment. If after assessing the second Gel-PLUS cohort, ≥1 in 3 participants demonstrate GLT, this will confirm NOGO (end of trial) and the need to reduce Decorin dosing.

Preclinical results indicated that Gel-MINUS was efficacious even without decorin, therefore if Gel-PLUS demonstrated toxicity, but Gel-MINUS did not, then the next stages of the study would continue with Gel-MINUS alone.

6.6.4.3 Stage 3 – Randomised-controlled pilot (RCP)

The RCP is an open-label, three arm, randomised-controlled trial, with participants being randomised (1:1:1) into three arms of 10 patients. Patients will be managed on an outpatient basis.

The treatment arms are as follows:

(1) Control: Patients will receive protocol defined standard care of:

- Lubricant eye drops (Sodium hyaluronate 0.2%) 6x per day for 4 weeks
- Topical steroids (dexamethasone sodium phosphate 0.1%) 4x per day for 1 week, 3x per day for 1 week, 2x per day for 1 week and 1x per day for 1 week and
- Topical antibiotics as clinically directed

(2) Gel-MINUS: Patients will receive application of Gel-MINUS dressing 4x per day for 4 weeks alongside protocol defined standard care topical steroids (dexamethasone sodium phosphate 0.1%) and topical antibiotics as listed above, excluding the lubricant drops.

(3) Gel-PLUS: Patients will receive application of Gel-PLUS dressing containing 240µg/ml Galacorin applied 4x per day for 4 weeks alongside protocol defined standard care topical steroids

(dexamethasone sodium phosphate 0.1%) and topical antibiotics as listed above, excluding the lubricant drops.

6.6.4.4 Randomisation process – Stage 3

Patients will be randomised via the online electronic remote data capture (eRDC) system (https://www.cancertrials.bham.ac.uk).

6.6.5 Eligibility

6.6.5.1 Inclusion Criteria - Stages 1, 2 and 3 - Patients with microbial keratitis

1. ≥16 years of age
2. Best corrected distance visual acuity (BCDVA) score of worse than or equal ≤68 ETDRS letters (≥+0.3 LogMAR or ≤6/12 Snellen) in the affected eye.
3. Corneal ulcer ≥2.5mm in smallest dimension
4. Stromal thickness >67% (two thirds) in the affected eye
5. In patients with bilateral disease, only the worse eligible eye should be entered provided it fulfils VA criteria
6. Culture and or PCR positive for bacterial infection of the cornea
7. Must initiate standard of care sterilisation treatment of broad spectrum antibiotic therapy hourly day and night for 72 hours prior to enrolment
8. Patients must be willing and able to provide written Informed Consent before any study-related procedures are performed.

6.6.5.2 Exclusion Criteria - Stages 1, 2 and 3 - Patients with microbial keratitis

1. Evidence of fungal, acanthamoebal, or herpetic keratitis
2. History of systemic immune mediated ocular surface disease (acne rosacea, eczema, mucous membrane pemphigoid). (Exclusion 2. Applies to Stages 2 and 3 only)
3. Use of systemic corticosteroids for an underlying health condition during the course of the present episode

4. Patients with a complicated corneal transplant history or; with a corneal transplant performed <1 year ago or; with episodes of rejection (patients with stable functional corneal transplants are not excluded).
5. Affected eye is the patient's only seeing eye
6. Visual acuity less than 6/60 (LogMAR 1.0) in the fellow eye
7. Previous entry into the trial (unless fellow eye)
8. Patients being treated with chemotherapy for cancer
9. Known hypersensitivity to any of the components of the study or procedural medication (e.g. fluorescein)
10. History of drug, medication or alcohol abuse or addiction
11. Use of any investigational agent within 4 weeks of screening visit
12. Participation in another interventional clinical study at the same time as the present study
13. Patients who are not willing or able to comply with study procedures and/or schedule
14. Patients with evidence of significant acute or chronic medical or psychiatric condition that, in the judgement of the investigator, would compromise the patient's safety or ability to complete the study
15. Females of childbearing potential, who are not surgically sterilised or post-menopausal for at least one year, and meet any one of the following conditions:
    i. are currently pregnant or,
    ii. have a positive result on the urine pregnancy test at the screening visit or,
    iii. intend to become pregnant during the study period or,
    iv. are breast-feeding or,
    v. are not willing to use highly effective birth control measures during heterosexual intercourse, such as: Hormonal contraceptives –oral, implanted, transdermal, or injected and/or Mechanical barrier methods –spermicide in conjunction with a barrier such as a condom or diaphragm or IUD from screening through to 30 days after the last dose of study treatment
16. Men if not vasectomised, who do not agree to use barrier contraception (condom plus spermicide) during heterosexual intercourse from screening through to 30 days after the last dose of study treatment.

6.6.6 Outcomes

6.6.6.1 Primary Outcome Measure – Gel limiting toxicity

The incidence of gel limiting toxicity will be analysed as a binary variable. In the components of the primary outcome, epithelial defect size and abscess size will be measured as the geometric mean of the product of the perpendicular diameters [251], match the method used in the SCUT trial. A 5% type I error rate will be used in comparisons as the threshold for significance.

6.6.6.1.1 Stages 1, 2 and 3 – patients with microbial keratitis

Safety will be assessed by the rate of occurrence of Gel Limiting Toxicity (GLT), defined as the occurrence of any of the following during 14-day assessment window that is determined to be related to Gel-MINUS or Gel-PLUS:

- 50% increase from baseline (and absolute increase ≥ 1mm^2) in epithelial defect size
- 50% increase from baseline (and absolute increase ≥ 1mm^2) in subjacent infiltrate and abscess size
- Loss of one third of the total corneal thickness (one third of the total thickness)
- 50% increase from baseline (and absolute increase >= 1mm) in hypopyon
- Occurrence of corneal perforation
- Prolongation of hospitalisation relating to trial treatment
- Increase of periocular erythema of more than 1 grade on the erythema scale [548]
- Occurrence of allergic reaction recorded as Grade 3 or higher as per CTCAE V4.0

6.6.6.2 Secondary outcome measures

6.6.6.2.1 Stages 1, 2 and 3 – patients with microbial keratitis

- Best-corrected distance visual acuity (LogMAR)
- Corneal epithelial defect size
- Infiltrate size
- Corneal scar size
- Stromal thickness
- Location: periphery, partially covers 4mm circle, completely fills 4mm circle (SCUT Study)
- Electronic tablet based functional vision tests: Contrast sensitivity; reading speed test

- National Eye Institute Visual Functioning Questionnaire – 25 (NEI-VFQ-25)
- Numeric rating scale 11 (NRS-11) – patient reported pain score
- Quality of life by EuroQol-5D (randomised pilot only)
- Visual analogue scales for ocular tolerability symptoms
- Incidence and severity of adverse events

6.6.6.3 Exploratory outcome measures

6.6.6.3.1 Stages 1, 2 and 3 – patients with microbial keratitis

- Gel retention time and ocular surface dynamics - OCT
- Lesion area analysis - photographs
- Lesions volume analysis – OCT

6.6.6.4 Assessment parameters

Where a technique or sample is concerned, standard operating procedure was complied to ensure standardisation.

6.6.6.4.1 Medical History, clinical examination & vital sign observations

A full ophthalmic and medical history will be taken including details about medications, family history, social and work history. A full clinical examination will be carried out at screening with subsequent clinical examinations being symptom directed. Vital sign observations include blood pressure, heart rate, oxygen saturation, temperature and respiratory rate.

6.6.6.4.2 Patient Compliance

In Stage 3 treatment compliance will be assessed at each visit within the treatment period. A treatment diary will be kept by the patients and must be reviewed by the trial team at each visit.

6.6.6.4.3 Microscopy, culture and sensitivities

Corneal scrapes must be taken at screening for microscopy, culture and sensitivities as per local standard practice. If these have already been taken as part of patients' standard care; they do not need to be repeated.

6.6.6.4.4 Polymerase Chain Reaction (PCR)

PCR sample must be taken at screening as per local standard practice and sent to Micropathology Ltd. Coventry. The following must be requested for testing: bacteria, virus, fungi and acanthamoeba. If these have already been taken as part of patients' standard care; they do not need to be repeated. Results will be required to confirm eligibility prior to enrolling the patient on the trial.

6.6.6.4.5 LogMAR BCD Visual Acuity (DVA)

Best Corrected Distance Visual Acuity (BCDVA) testing must precede the administration of any eye drops to dilate or anesthetise the eye, or any examination requiring contact with the eye. If a patient reads fewer than 20 letters at 4m, acuity is measured at 1m. If fewer than 10 letters are read at 1m, low vision is assessed by counting fingers (CF), hand motions (HM), perception of light (PL), and no perception of light (NPL).

6.6.6.4.6 Electronic tablet based functional vision tests

Aston Radner read speed mobile app (courtesy of Prof Wolffsohn, Aston University, Birmingham, UK) must be completed by the patient prior to administration of any eye drops to dilate or anaesthetise the eye. The Aston Radner read speed mobile app will consist of the following tests.

6.6.6.4.6.1 Aston Radner reading speed test

Wearing their refractive correction for near vision, recruits will perform the reading test on the app at 40cm. The app presents sentences from the Radner reading speed test at decreasing size, objectively measuring reading speed using voice recognition and allows manual correction for incorrect word recognition. The output of the assessment is the reading speed across a range of print sizes.

6.6.6.4.6.2 Contrast sensitivity

Contrast sensitivity is assessed using a sine wave grating presented on the tablet screen across which the recruit will draw (Figure 6-11).

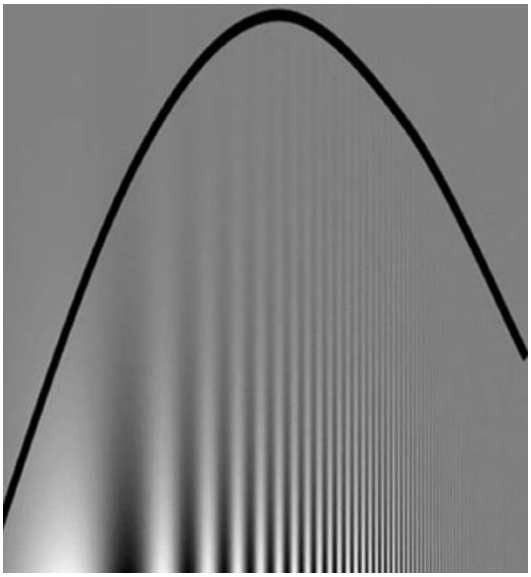


Figure 6-11. Contrast sensitivity assessment app interactive screen.

The recruits will use their finger to draw across the perceptible limit at each spatial frequency (example black line).

6.6.6.4.7 Ocular examination

A complete ocular examination will be performed at screening including:

- External ocular examination
- Ocular motility
- Anterior segment examination
- Posterior segment examination
- Intraocular pressure
- Lesion morphological assessment according to the Herpetic Eye Disease Study [549]
    - Location (assessed as periphery; partially covers central 4mm circle or completely fills central 4mm circle),

  - Size (length of greatest dimension and greatest perpendicular dimension in mm; with measurements to the closest 0.1mm)
  - Percentage depth of thinning.
- Epithelial defect assessment
  - Size (length of greatest dimension and greatest perpendicular dimension in mm; with measurements to the closest 0.1mm)

6.6.6.4.8 Photography without Fluorescein

Anterior segment photography of the affected eye with and without fluorescein will be taken at each visit during the trial. Photographs will then be assessed by 2 observers masked to treatment allocation, who will measure the ulcer area using imageJ [54].

6.6.6.4.9 OCT Imaging

The Tomey Casia II anterior segment optical coherence tomographer will be used to assess the cornea using a raster scan pattern encompassing the entire cornea. The scanning parameters were optimised to balance acquisition speed and, resolution and contras. This data set will be analysed for the trial and used to aid the development of an algorithm to assess lesion morphology, including lesion volume and density. Over the course of the study this will enable assessment of treatment efficacy.

6.6.6.4.10 Patient reported outcome measures (PROMs) - Quality of Life (QoL) questionnaires

6.6.6.4.10.1 National Eye Institute Visual Functioning Questionnaire – 25 (NEI-VFQ-25)

The NEI-VFQ is one of the most commonly used patient reported outcome measures in ophthalmology which has undergone multiple iterations, translations and rationalisations to reduce its size. It is designed to assess health related quality of life in patients with visual impairment. Its utility in infectious keratitis was characterised by Li et al. [550] and is considered the standard to assess vision-related impact on functioning [551].

6.6.6.4.10.2 Numeric rating scale 11 (NRS-11) – Pain analogue score

Pain analogue score will be used to assess pain that is experienced by the patient. This consists of an 11 point analogue scale of 0-10, 0 being no pain and 10 being the most intense pain.

6.6.6.4.10.3 VAS – Visual Analogue Scale for Ocular Tolerability

A VAS will be used to determine ocular tolerability. It will be assessed by the patient using a self-administered 100mm VAS on which 0 means no symptoms and 100 means the worst possible discomfort. The scales will be used for 7 categories as follows:

- foreign body sensation
- burning/stinging
- itching
- ocular pain
- sticky feeling
- blurred vision
- photophobia.

6.6.6.4.10.4 EuroQol-5D (EQ-5D)

EQ-5D will be used in Stage 3 of the trial only. The EQ-5D is a standardized measure of health status developed by the EuroQol Group in order to provide a simple, generic measure of health for clinical and economic appraisal.

6.6.6.4.11 Clinical laboratory tests

Blood sampling should be performed after the vital signs have been taken.

The following blood samples will be taken at baseline and follow up intervals to screen for gross changes as a result of the treatment.

- Full blood count (FBC)
- Liver function tests (LFT)
- Urea and electrolytes (U&E)

- Erythrocyte sedimentation rate (ESR)
- C-Reactive protein (CRP)

6.6.6.4.12 Mechanistic study samples

The following samples will be taken at baseline and then points along the patient's journey (see schedule of events).

Tear washings 70-100 µL of normal saline stored at -80oC from both eyes.

Polyester swab, one per eye, for Nanostring pathway analysis of elicited genes in corneal wound healing.

6.6.6.4.13 Pharmacokinetic (PK) blood collection

Blood samples for PK analysis will be taken on Days 0 - 5 for Stages 1 and 2 only. Samples must be taken prior to the first dose, 60 minutes after the first dose and daily until Day 5.

6.6.6.4.14 Anti-decorin antibodies blood collection

Blood samples for anti-decorin antibody analysis will be taken on Day 0 and at weeks 4, 8 and 12. Samples must be processed and shipped to the central laboratory as per the current laboratory manual.

6.6.7 End of trial definition

Data will be collected from participants up till 12 months post start of trial treatment. This will allow sufficient time for the completion of protocol procedures, data collection and data input. The OPtiCS-MK Trial Office will notify the MHRA and main REC that the trial has ended and will provide them with a summary of the clinical trial report within 12 months of the end of trial.

6.6.8 Analysis

Continuous Variables

For approximately-normally distributed data, means and standard deviations will be calculated for each arm. Comparisons of multiple treatment arms against the control arm will be made by two-tailed Dunnett's test. This procedure handles multiplicity, whilst taking into account that all the comparisons are correlated since they all use the same control data. It is more powerful than methods performing all possible pairwise comparisons. It will not compare the two active treatments. For non-normally distributed data, medians and IQRs will be calculated for each arm, and comparisons will be made by Kruskal-Wallis tests.

Binary Variables

For binary outcome measures, success rates and 95% confidence intervals using Wilson's method will be presented for each group. Comparisons will be conducted by chi-squared test.

Power Calculations

If we observe one or more GLTs with Gel-MINUS in a cohort of 3, we can reject, at the 3% significance level, our hypothesis that the rate of GLT associated with Gel-MINUS is 1%. If the rate of GLT under Gel-MINUS is 1%, the treatment will be approved at this stage with probability 94.1%.

If we observe one or more GLTs with Gel-PLUS in a cohort of 3, we can reject at the 5% significance level our hypothesis that the rate of GLT associated with Gel-PLUS is 1.5%. If the rate of GLT under Gel-PLUS is 1.5%, the treatment will be approved at this stage with probability 91.3%.

For continuous outcomes in the randomised study, 10 patients per arm provides 80% power to detect a difference in means of 1.35 standard deviations at a 5% significance level using a two-sided t-test.

6.6.9 Adverse Events

Definitions:

Adverse Event (AE): Any untoward medical occurrence in a participant or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment.
Adverse reaction: All untoward and unintended responses to an IMP related to any dose administered.

Serious Adverse Event (SAE): Any untoward medical occurrence or effect that at any dose:

Serious Adverse Reaction (SAR): An Adverse Reaction which also meets the definition of a Serious Adverse Event.

Suspected Unexpected Serious Adverse Reaction (SUSAR): A Serious Adverse Reaction that is unexpected i.e., the nature, or severity of the event is not consistent with the applicable product information. A Suspected Unexpected Serious Adverse Reaction should meet the definition of an Adverse Reaction, Unexpected Adverse Reaction and Serious Adverse Reaction.

Unexpected Adverse Reaction (UAR): An Adverse Reaction, the nature or severity of which is not consistent with the Reference Safety Information. When the outcome of an Adverse Reaction is not consistent with the Reference Safety Information, the Adverse Reaction should be considered unexpected

All medical occurrences which meet the definition of an Adverse Event should be reported. This includes all abnormal laboratory findings (defined as outside the local normal range) and any pre-existing conditions that worsen by at least one CTCAE grade from baseline. Details of Adverse Events will be captured on an Adverse Events Form. AEs will be reported in accordance with CTCAE guidelines, version 5.

The investigator will exercise their medical judgment in deciding whether an abnormal laboratory finding or other abnormal assessment is clinically significant. However, if in the opinion of the investigator, the frequency or severity of the event is greater than would be expected then it must be reported.

6.6.9.1 Serious Adverse Events

Investigators should report Adverse Events that meet the definition of a Serious Adverse Event (see Appendix 4 for definition).

6.6.9.2 Unexpected Pregnancy

If a female participant becomes pregnant during the course of the trial, trial treatment should be discontinued immediately.

In the event that a participant or their partner becomes pregnant during the Serious Adverse Event reporting period please complete a Pregnancy Notification Form (providing the participant's details) and return to the Trial Office as soon as possible. If it is the participant who is pregnant provide outcome data on a follow-up Pregnancy Notification Form. Where the participant's partner is pregnant, consent must first be obtained and the participant should be given a Release of Medical Information Form to give to their partner. If the partner is happy to provide information on the outcome of their pregnancy they

should sign the Release of Medical Information Form. Once consent has been obtained provide details of the outcome of the pregnancy on a follow-up Pregnancy Notification Form. If appropriate also complete a Serious Adverse Event Form as detailed below.

6.6.9.3 Post-trial Suspected Unexpected Serious Adverse Reactions

Serious Adverse Events that are judged to be at least possibly related to the Investigational Medicinal Product and are unexpected must still be reported in an expedited manner irrespective of how long after Investigational Medicinal Product administration the reaction occurred.

6.6.9.4 Reporting Period

Details of all Adverse Events will be documented and reported from the date of Informed Consent until resolution or the participant's last trial visit (whichever occurs sooner), as long as the start date is within the timeframe above.

6.6.10 Informed Consent

It is the responsibility of the Investigator and/ or their delegated clinical research team to obtain written informed consent for each participant prior to performing any trial related procedure. A Participant Information Sheet (PIS) is provided to facilitate this process. The informed consent process is expected to involve an interview between the investigator team and the participant which should facilitate two-way communication. It is possible for this interview to be conducted remotely. Where this occurs, the participant will view the Participant Information Video and will be sent the Participant Information Sheet in advance by email or in the post. The Informed Consent Form should be wet ink signed by the participant and the Investigator or designate be prior to their entry onto the trial.

The participant should be given ample time (e.g. at least 24 hours) to view the Patient Information Video, read the Participant Information Sheet and to discuss their participation with others outside of the site research team.

Once the participant is entered into the trial the participant's trial number should be entered on the relevant Informed Consent Form maintained in the Investigator Site File.

Details of the informed consent discussions should be recorded in the participant's medical records.

6.6.11 Trial organisation structure

6.6.11.1 Sponsor

The trial is sponsored by the University of Birmingham.

6.6.11.2 Coordinating Centre

The trial is being conducted under the auspices of the Cancer Research UK Clinical Trials Unit (CRCTU), University of Birmingham according to their local procedures.

6.6.11.3 Trial Management Group

A Trial Management Group (TMG) will be established, and will include the Chief Investigator, the Trial Management Team Leader (or delegate), the Trial Biostatistician and the Trial Coordinator. Other key trial personnel (manufacturer representative(s), pharmacy, co-investigators, clinical coordinators, PPI representatives, the Monitor) may be invited to join the TMG meetings as appropriate to ensure representation from a range of professional groups.

The TMG will operate in accordance with a trial specific charter based upon the template created by the Damocles Group [542] and be responsible for the day to day running of the trial.

6.6.11.4 Trial Steering Committee

The clinical trial is part of a larger project grant which has its own Project Steering Committee. An independent Trial Steering Committee will form part of the Project Steering Committee and will oversee the conduct of the trial. The Committee will be chaired by an independent Chair. Membership will include independent clinicians and two participant advocates. Selected members of the Trial Management Group including the Chief Investigator, Trial Biostatistician and the Trial Management Team Leader (or delegate) will report to the Trial Steering Committee. A secretariat will be provided by the Trial Coordinator. The Trial Steering Committee will operate in accordance with a trial specific charter based upon the template created by the Damocles Group.The Trial Steering Committee will supervise the conduct of the trial, monitoring progress including recruitment, data completeness, losses to follow-up, and deviations from the protocol. They will make recommendations about conduct and continuation of the trial to the sponsor.

6.6.11.5 Data monitoring committee

Data analyses will be supplied in confidence to an independent DMC, which will be asked to give advice on whether the accumulated data from the trial, together with the results from other relevant research, justifies the continuing recruitment of further participants.

The DMC will operate in accordance with a trial specific charter based upon the template created by the Damocles Group.IT will meet regularly and at the specified review time points. It will meet after any SUSAR or other reason necessitating an emergency if any other safety issues are identified.

The DMC will report to the Trial Steering Committee who will convey the findings of the DMC to the Medicines for Healthcare products Regulatory Agency, Research Ethics Committee and the funders. The Data Monitoring Committee may consider recommending the discontinuation of the trial if the recruitment rate or data quality are unacceptable or if any issues are identified which may compromise participant safety.

6.6.12 Ethical considerations

The trial will be performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects, adopted by the 18th World Medical Association General Assembly, Helsinki, Finland, June 1964, amended at the 48th World Medical Association General Assembly, Somerset West, Republic of South Africa, October 1996 (website: http://www.wma.net/en/30publications/10policies/b3/index.html).

The trial will be conducted in accordance with: the UK Policy Framework for health and social care research, 2017; the applicable UK Statutory Instruments (which include the Medicines for Human Use (Clinical Trials) regulations 2004 and subsequent amendments; and the Data Protection Act (2018); and Human Tissue Act (2008) and Good Clinical Practice (GCP)).

This trial will be carried out under a Clinical Trial Authorisation in accordance with the Medicines for Human Use Clinical Trials regulations. The protocol will be submitted to and approved by the main Research Ethics Committee (REC) prior to circulation.

6.6.13 Patient and public involvement

Patients and public have been involved in various stages of the broader project including, practicalities of the trial. Patients contributed to the testing and selection of the eye-drop container, the patient facing documents such and the information leaflet and the information video.

6.7 Clinical trial documents

These documents were developed in conjunction with the clinical trials unit at the University of Birmingham.

Patient facing documents were submitted for *Clear Print Review* assessment from the Royal National Institute for the Blind (RNIB), who provided feedback on how to ensure the documents met their established standards. All the documents, including the report can be accessed at the following website: https://figshare.com/account/articles/22491967

1) Consent forms
2) Patient information leaflet (PIL)
3) RNIB *Clear Print Review* response
4) Patient information video – derived from the PIL leaflet https://www.youtube.com/watch?v=eLGZdJSIfQk
5) Letter to the family doctor
6) Patient instructions for using the drops
7) Example CRF

6.8 Discussion

The aim of this trial is to determine the safety of a novel ocular dressing formulation. The pilot RCT element of the trial is designed to compare standard of care treatment to the gellan fluid gel both with and without decorin, to avoid confounding and explore the safety and therapeutic potential of the dressing itself. Following the successful completion of this trial, the investigative products will enter the next phase of study.

This formulation is a significant development and a first-in-class treatment with the potential to change the landscape of ocular scarring disorder management. Fibrosis occurs in many of the most prevalent chronic and debilitating ocular disorders (glaucoma, diabetic retinopathy, and age-related macular degeneration) thus alternate applications of this therapy make it an exciting prospect.

Strengths of the design of this trial include the investigation of two novel formulations addressing a major clinical need; a carefully considered and needful target population in whom the natural history of the disease lends itself well to investigation; and recruitment of these patients at the largest tertiary eye care centre in the region, increasing recruitment potential.

Important novel features have been adopted into the trial design, including the outcome measures, that aim to drive forward the standards of conduct in this field. Chapter 2 identified the range of outcome measures used in studies of corneal fibrosis, from which three important categories of outcomes were identified: lesion morphology, functional assessments, and patient experience, all assessed over an appropriate time frame. Building on previous work in the field [122,123,131,132], this trial incorporates a comprehensive array of outcome measures with a final follow-up at 12 months post-recruitment. It is vital to understand the patient experience fully. One example when patients with poor vision at presentation have an improvement in their vision which is not significant on the visual acuity chart; however, the improvement leads to better function. Such findings are essential and not easily captured without the use of PROMs. In addition to using existing validated techniques, this trial will use OCT for 3-dimensional assessments of corneal lesions, representing a novel outcome not previously used in a trial of this kind.

Some design features limit this study in practice, while others raise important questions for future studies. For example, patients are being followed up to 1 year to make the design comparable to other eminent studies in the field (namely the SCUT trial). Since this is a safety trial and the treatment regime is relatively short compared to the follow-up period, this element of the design may be considered inefficient. However, as this is the first time such treatment is being trialled in humans and since corneal stromal

remodelling is known to continue until 12 months post-MK [111,260], it is reasonable to consider this an appropriate follow up time frame both in terms of safety and efficacy (to detect and regressive effects in stromal remodelling).

This trial will recruit patients with large ulcers, as they suffer the greatest morbidity. This raises two issues in terms of their recruitment. The first is that there is no ceiling to the size of the ulcer for recruitment. As such, indiscrete lesions in the study may be more challenging to monitor for changes. Secondly, patients with larger ulcers may inherently be more vulnerable to complications by virtue of having worse disease. The GLT safety criteria are designed to detect worsening of ulcers and prevent the continuation of the trial in such an event. However, this could be confounded in scenarios where an ulcer is resistant to treatment due to pathophysiological factors rather than the experimental treatments. Since the period between recruitment and commencement of the trial medication is short, it could mean that those with slow-responding ulcers are recruited into the trial before they are recognised as being so. This has the potential to impact measures of efficacy in the event of delayed healing, and measures of safety if encountered by complications such as perforation. The latter is mitigated by including patients with adequate stromal thickness at the time of recruitment, however predicting the patient trajectory in these terms is difficult, and these patients typically have poor visual outcomes.

Despite the inbuilt RCT, safety is the primary outcome assessment. As such, this study is not powered for outcomes of efficacy and is therefore limited by its design in this regard. Although the eligibility criteria have been designed with the intention of optimising patient homogeneity and recruitment potential, variations in their demographics, risk factors, pathogen and lesion morphology at presentation, entail that some heterogeneity will remain. This is advantageous for exploring the safety of the treatment in differing populations and lesions. Although such heterogeneity may influence the efficacy outcome measures with such low numbers of recruits, this will be overcome in future larger-scale trials.

The preclinical animal work informed the study treatment and dosing regime. In that study, steroids were given alongside the experimental treatments. This implicates the need for the co-administration of corticosteroids. If the novel treatment is necessarily coupled with steroid treatment, then the treatment will remain subject to the limitations of steroid treatment. In the case of MK this means waiting for adequate sterilisation of the infection, and potential delays in the anti-scarring treatment. However, this may be considered an acceptable limitation if the treatment regime is truly effective at reducing corneal scarring. This may also be addressed in future trials.

Both Gel-PLUS and Gel-MINUS have demonstrated efficacy in animal studies of microbial keratitis. However, other causes of corneal fibrosis remain to be investigated. Gel-MINUS holds the potential to deliver any number of therapeutic molecules in isolation or as a composite of multiple therapies. Thus, the investigative products in this trial represent a platform technology with broad potential.

Fundamental to this study is sampling for interrogation of pathway and mechanistic analysis of patients in this trial. Conclusions from Chapter 3 and the literature regarding decorin's pleiotropic contextual effect suggest that much remains to be uncovered about our understanding of the impact of this novel formulation on corneal wound healing. Nanopore is an exciting analytical technique that will help identify the crucial pathways involved in MK and regeneration. Indeed, a better understanding of the mechanism of action would strengthen its case for other disease applications. As such, this trial pragmatically approaches the key questions for developing this technology at this stage in its developmental life cycle.

6.9 Conclusion

This chapter described the considerations and planned conduct of an early-phase clinical trial of a first-in-class anti-scarring treatment for corneal fibrosis. With a design based on safety assessment, other priority design features included the acquisition of samples for mechanistic studies and the development of appropriate outcomes for future efficacy studies. The COVID-19 pandemic prompted several management adaptations to the trial that will hopefully help patients engage with the trial and impact recruitment positively. It explains the project's progress thus far and acknowledges its strengths and weaknesses in anticipation of opening for recruitment.

7. Discussion

7.1 Summary of results

This thesis presented a body of work regarding the development of a novel therapeutic strategy for the management of corneal fibrosis. It has shown that the public recognised eye symptoms of varying severity and acted proportionately (Chapter 4). There was no impact on the presentation patterns of microbial keratitis and the severity of the disease was similar to pre-pandemic levels (Chapter 5). The management of corneal infections involves eradication of infection and scarless wound healing. A number of strategies are used to facilitate healing and reduce scarring, however there are no licensed agents for the prevention or management of corneal scarring (Chapter 2). Therefore there remains an unmet clinical need. Decorin is one candidate which has potential to change the corneal fibrosis landscape. It's pluripotency means much remains to be discovered about its actions. It was not found to modulate autophagy in a novel concept linking decorin and fibrosis (Chapter 3). Nevertheless considerable *in vivo* evidence exists to support its progression into human study. OPtiCS MK will be the early phase clinical trial of this novel formulation, which has evolved over the past few years, in order to optimally utilised this opportunity to generate insights for further drug development.

7.2 Public perceptions, clinical presentations and lessons from the pandemic - the evolving burden

The COVID-19 pandemic challenged health and social care systems tremendously, whilst exposing their shortcomings. It also highlighted the importance of health literacy (HL) and how the public consume health information [552,553]. During the pandemic, poorer HL was associated with increased anxiety about the pandemic [554,555]. As healthcare departments around the country reported decreased attendances [501], the cause for this altered health seeking behaviour became a primary concern as delayed presentations could lead to worse disease.

HL impacts health seeking behaviour and positively correlates with health outcomes [556]. Chapter 4 revealed the wide range of health information sources utilised by the public and their responses to differing groups of eye symptoms. Reassuringly, respondents were able to discern symptom clusters by severity and indicated that they would take proportionate action. However, it was hypothesised that mild symptoms of early MK may be overlooked until the condition had become more serious, which could lead to delayed presentation and clinical outcomes. This was found not to be the case in the clinical study in Chapter 5, which demonstrated MK presentation patterns to be similar in and pre-pandemic. This finding suggests that pandemic associated health anxiety did not incite adverse behaviours with regard to presenting to ophthalmic services for MK in the UK, although the evidence for the adverse impact on other conditions is clear [557,558]. Despite eye health literacy being low [449–452], these study findings implicate factors other than literacy in driving eye-health seeking behaviour, which may also differ from other medical conditions. This indirectly supports the previously recorded notion that eye health is a major health priority for the public [448]. Whilst this was the case for the majority of respondents, Chapter 4 also identified that a significant proportion of the public (1-6%) did not consider the most serious symptom clusters to warrant urgent attention. This raises concerns about the health literacy of individuals at fringes of the population. Recognition of symptoms may be influenced by individual experience [559], so variation in HL must be expected because of these factors as well. On a national scale, this represents many individuals potentially at risk of significant morbidity. Whilst the study was unable to identify the reasons for these opinions, it highlighted the importance of further investigations to understand the perspective of all demographic groups of the public, and thereby reduces barriers to healthcare.

There was a preponderance of respondents of greater age in this study. Whilst this doesn't explain why a proportion did not indicate appropriate urgency to some scenarios, it does highlight the need to adopt a campaign strategy that will reach all members of society. Health educational campaigns remain a challenge in ophthalmology, particularly in the developing world [560]. For example, since the widespread adoption of contact lenses (CL), the incidence of CL related MK has remained stable despite improvements in CL materials[561]. This is thought to be due to the prevalence of compliance issues despite more access to information than ever before [561].

Health is intrinsically and complexly linked to socioeconomic status, which was sadly highlighted in the pandemic when certain demographics were forced to continue working in public spaces and put themselves at risk [562]. HL and socioeconomic status correlate with each other [563–565], and it has been suggested that actively improving one can benefit the other, and consequently overall health

outcomes as well [564]. Whilst many clinical services have returned to “business as usual” in the NHS, the impact of the pandemic has increased backlogs, for example outpatient waiting lists. Thus, optimisation of the broader health care system, including health education is vital. Access to and utilisation of information impact HL, however a holistic understanding of HL must also consider the societal structures that impact public decisions, as in the case of zero-hour employees working during the lockdown, and the corporate influences in society that nudge consumers to take potentially unhealthy decisions [566]. Therefore, the positive influence of HL may be outweighed by social circumstances, and sustainable improvements may require concurrent change in both.

Respondents were less likely to volunteer for ophthalmic research during the pandemic. This is understandable as volunteers would have considered it to be placing them in social situations and therefore increase risk of COVID-19 exposure. For research systems to become resilient, the research community must consider adaptations that ensure patients that their safety is paramount and that patients understand this to be a priority. Had the clinical trial already been running as the pandemic erupted, it would have likely faced even greater difficulties and so contingencies must be in built.

In the UK the pandemic was estimated to have led to an increase of two million people on the 18-week wait list for treatment, and an additional 2986 cases of sight loss, which added a cost of £2.5 billion to eye care services in the NHS [567]. By September 2020, 41% of research sites across the UK were still not recruiting [568].

Similar patterns of MK presentation and outcomes during the pandemic compared to previous years were identified in this thesis (Chapter 5). Interestingly this was also reported by other units around the country [569,570]. One study from Dublin reported a surprising decrease in MK incidence during the pandemic. As MK does not spontaneously resolve, this finding is curious and could be due to a number of reasons such as decreased contact transmission, avoidance of risk (i.e. less trauma), increased hand washing, successful self-treatment with over the counter drops, or over investigation or diagnosis of cases pre-COVID.

Similar to findings in Chapter 5, other units had reduced hospital admissions for MK during the pandemic, whilst experiencing clinical outcomes comparable to the pre-COVID-era. The corroboration of this finding supports the concept that MK can be managed safely on an out-patient basis in the event of a future pandemic, and more broadly considering the NHS funding landscape. This also sheds light on the debate of in-patient versus out-patient management on MK in general. Whilst home based care is not suitable

for all patients, there may be a specific cohort that may be managed safely from home. With the boost in advancement and adoption of tele- or virtual-health care services during the pandemic, it is conceivable that more ophthalmic care could leverage these technologies to address future public health crises [495].

Whilst patients were not found to present with worse disease, MK patients were not entirely unaffected. Pandemic related disruption exacerbated the global shortages of corneal transplant tissue which were already scarce pre-COVID [505]. Patients who require visual rehabilitation by way of corneal transplant, now have less access to sight restoring treatment. Furthermore, MK is more severe and has poorer outcomes in patients of lower socioeconomic status [571] and considering the negative impact of visual impairment on socioeconomic attainment, these factors may negatively feedback to each other, worsening health and poverty in a vicious cycle. There is, therefore, an unmet and urgent need for medicinal therapies to decrease the burden of corneal scarring.

7.3 Approaches to corneal regeneration following corneal infections for the near future and beyond

There are a number of intervenable points in the MK patient pathway. Broadly, these involve a) prevention and control of risk factors, b) timely diagnosis c) initiation of effective antimicrobials and d) regenerative therapies, and finally e) rehabilitative strategies which orient around surgical options. Preventive strategies may be helpful in reducing some specific causes of MK and corneal blindness on a global scale, however some (e.g. trachoma) are more inherently intervenable than others (e.g. underlying immune disorders).

Rapid diagnosis is helpful in differentiating the pathogen in order to direct medical therapy, in particular where pathogens are resistant to first-line therapies. Patients are commenced on broad spectrum antibiotics which they complete in the absence of complications. However, increasing rates of resistant strains in MK [572] necessitate the development of more rapid diagnostics to inform treatment [117], in parallel to the development of strategies that circumvent anti-microbial resistance. Novel repurposing of existing therapies such as corneal crosslinking and topical iodine for MK [573] hold significant potential. But their value in changing the landscape is yet to be determined as these studies have not demonstrated superiority to conventional treatments [125].

Discussion

To chart the landscape of medicinal therapies for corneal fibrosis trialled in humans, Chapter 2 systematically reviewed the literature. Although most therapies were well established (e.g. steroids, vitamin C etc.), the study revealed some innovative therapies, and new hypotheses regarding novel applications of established treatments. Whilst no large trials of novel therapies were found, those tested in smaller trials may soon progress to larger scale studies.

The majority of therapies aimed to prevent corneal scarring in the acute injury phase. In MK this is complicated by the need to simultaneously eradicate infection, unlike other pathology such as trauma. Furthermore, the risk of corneal melting presents another challenge for therapeutic strategies. Besides opacification, irregularities of the corneal surface significantly impact vision [574]. Late-phase surgical strategies are oriented around the correction of these physical parameters and were not the focus of this work. However, it was interesting to note that autologous adipose derived stem cells therapies have entered human studies, which represent a major advancement in the field. They were applied in keratoconus patients with established scarring and found to make ECM changes. Such cell therapies can potentially create a pro-regenerative environment more complex than simple pharmaceutical drug-based therapies. However, there may be significant variation between treatments and as such standardisation for regulatory purposes poses a challenge [575]. Furthermore, procedural logistics and costs hinder their delivery and generalisability for the globe. It is helpful to consider the relative merits and weaknesses of experimental therapies, and how they progress through their development, as they offer important insights for future development.

Realising novel applications of established treatments was a strength of this chapter. Interesting candidates included O2 therapy (hyperbaric and high flow), topical N-acetyl cysteine's anti-inflammatory potential and vitamin supplementation (e.g. Co-Q 10). These are low-cost and currently available, making them interesting prospects which could enter human trials in a relatively short time frame. Such options deserve consideration, particularly as combination approaches that may impact multiple intervenable mechanisms simultaneously. Addressing each of the involved pathological mechanisms is important for holistic strategies to be impactful. Chapter 3 brought to attention the potential involvement of cellular energy regulating systems in the wound microenvironment. As such, therapies addressing hypoxia (e.g. oxygen) and mitochondrial stress (e.g. Co-Q 10) may hold potential in MK induced corneal fibrosis. However, their roles must be carefully considered. For example, although hypoxia modulates autophagy, it can lead to both cell survival and cell death [576]. A similar dichotomy may be encountered with regards to oxygen therapy which eradicates bacteria through free radicals [387] which may themselves potentiate

damage and fibrosis in the cornea [360,577,578]. These may be addressed by the co-administration of treatments such as NAC and Co-Q10 which may support cellular functions, in a multi-mechanistic approach. Thus, reflections across these chapters have helped generate new hypotheses.

Chapter 2 also identified the breadth of outcome assessments used in clinical studies of corneal fibrosis, spanning functional, morphological and PRO assessments. The ideal panel of outcomes must be able to capture robust measures of disease activity and treatment response. Due to lesion heterogeneity in MK, measuring visual acuity alone may be insufficient to determine response to treatment. Imaging modalities offer objective measure but to date no commercially available technique is optimised for quantitative analysis of corneal scarring. Oculus' pentacam has a densitometry module which can generate a measure of corneal opacity for the cornea as a whole, however other measurements such as lesion size, shape and change over time are not automated. Anterior segment OCT holds significant potential, and the results from preliminary work (Chapter 6) indicate good potential for the technique. The next steps for this would be to optimise it and validate it in a large cohort. Another challenge for researchers is being able to identify change in lesions that are on the very mild and very severe ends of the spectrum, where significant changes in vision may not be noticed. In mild corneal opacity, contrast sensitivity has been found to be impacted before visual acuity is and so this represents an essential item. Whereas in severe scarring other approaches may be required. PROs give insight into the lived experience of a disease and tend to be for specific purposes e.g. post-operative pain, however some general visual function related PROs were also encountered. One potential use of PROs in the MK patient group is to give insight into the impact of treatment in patients with severe scarring. Quality of life is found to improve in patients who despite having macular degeneration, undergo cataract surgery [579]. This suggests that smaller improvements in the quality of vision may still be perceptible to the patient and would be important to capture in clinical trials.

The MHRA released a strategy statement regarding patient involvement, emphasising the importance of involving patients in all steps of the regulatory process, in which they describe the central role for PROs in all licencing decisions [580]. This was echoed by the International Network of Agencies for Health Technology Assessment (INAHTA) in their statement of intent in 2021 [581]. As a significant proportion of healthcare involves the private sector, a trend towards greater consumer-driven design will hopefully lead to more novel research and better outcomes.

Horizon scanning is essential for translational research. A limitation of Chapter 2 was that the preclinical candidate landscape was not captured nor is there any similar study in the literature that could enable a

comparison of the range of candidates and their mechanisms of action. It is increasingly evident from the study of fibrosis pathophysiology that a multifactorial approach is likely to achieve the best outcomes for patients, whether that be through multiple medications or a single therapy with multiple effects (e.g. stem cells) [432,582]. Screening approaches to multi-mechanism therapies have indeed been trialled with results of interest on both conceptual levels regarding the combination of drugs and also the potential of such research approaches [583]. Therefore this study has helped identify potential combinations that could be explored in corneal disease in the near future.

7.4 Decorin

Decorin demonstrates many of the advantages mentioned above and has the potential to circumvent some of the drawbacks. As a single molecule, its dosing can be easily adjusted for research and clinical purposes. It is pleiotropic, safe and efficacious in studies thus far, and novel features continue to be discovered about its mechanisms. It can be manufactured consistently and in large volumes, leveraging economies of scale. Once its path has been paved into the market, other similar biologics could be developed and delivered alongside it, to offer nuanced manipulation of the wound environment. Gellan hydrogel is a versatile, cost effective and sophisticated carrier that could be a multipurpose carrier for bespoke drug combinations, including those already available. Although its compatibility with other additives needs to be determined, it has the potential to improve drug delivery in many scenarios.

Decorin is anti TGFβ1 through multiple mechanisms [420]. In Chapter 3, decorin was not observed to exert anti-fibrotic or autophagy modulating effects, which was surprising given previous published results (Chapter 1). There is also a rapidly growing body of *in vivo* murine models supporting the use of decorin [188,190–194,584,585]. In these models, decorin is both safe and efficacious. In vitro experiments create a simplified model of pathophysiological environments; therefore, researchers must carefully consider implications and generalisability of experiments. This is particularly relevant for decorin, which has very many binding partners and its net effect in any given pathological system is context dependent. For example, decorin is both pro- and anti-inflammatory in the tumour microenvironment [586]. The *in vitro* system used in Chapter 3 is limited by the fact that it did not receive physiological contributions from the immune system and ECM.

Alternate experimental paradigms should also be considered in further experiments, since it is possible that decorin does not act as a simple stimulator or inhibitor of autophagy. In cardiac cells, decorin is necessary for normal autophagic flux, where its absence leads to pathology [587]. Such a metabolic regulatory role for decorin was not investigated and requires consideration.

However, these results inform the critical next steps of the investigation. Several mechanistic hypotheses can be generated from the results of these studies. For example, decorin upregulates autophagy through VEGFR2 in endothelial cells, but was not found to influence autophagy in PHCFs. Corneal stromal cells expressing these receptors have been characterised at the leading edge of wounds in rabbit cornea, but not in the entire tissue [430]. This differential expression and evidence of heterogeneity of the stromal cell population during injury [56,588] combined with the differential expression of VEGFR2 suggests that these sub-populations have different roles in the corneal wound and may be variably susceptible to the effects of decorin. Further investigations are warranted to elucidate this relationship and the differential role of the various sub-populations. Moreover, single cell techniques combined with more complex *in vitro* models such as corneal organoids or *in vivo* models of disease may well provide a more natural target to assess potential treatments [589]. Its *in vivo* or human application must also consider the collateral effect of treatment. For example, increased autophagy in conjunctival fibroblasts decreases differentiation and reduces fibrosis [590] which may be beneficial in some scenarios, whereas the pro-inflammatory effects on macrophages may be less desirable [175]. Thus a successful design will need to consider an element of cellular specificity into its design.

7.5 Considerations for trials of decorin in microbial keratitis and other conditions

Chapter 5 revealed that fewer MK patients were admitted onto the eye ward at the BMEC during the pandemic. Since OPtiCKS MK is planned to be a single-centre trial based at the BMEC, disruptions to patient flow, such as the national lockdown, had the potential to impact trial recruitment. Indeed, this was reported by other research facilities, and increased losses to attrition [591] would be very impactful in this relatively small early phase trial.

Other considerations include the disproportionate impact of the pandemic on those from lower socioeconomic backgrounds [592], who also tend to have worse MK [98]. Diversity and representation, or lack thereof, is a well-recognised historical issue in clinical research [593], which has led to knowledge

gaps [594]. Whilst there are many reasons contributing to this, such as zero-hour employment contracts, this demographic represents a potentially vulnerable group, thus systems must give them the same opportunity to volunteer for research without inadvertently targeting them [595].

The pandemic devastated the global economy, and consequently caused the contraction of charitable funds for research [596], constituting one of many reasons the research community must refine its approach to clinical trial design [597]. It has brought to light the importance of uniformity in trial conduct and outcomes and prompted initiatives such as the WHO's core master protocol for COVID-19 [598], a blueprint on how to make studies comparable and avoid expensive equivocal results. Intimately linked to this, is the concept of open data, that can help facilitate the generation of new insights between studies and from old data, although this has typically been hampered by accrediting contributions and commercial interests.

The benefit of anti-fibrosis drug development in the cornea, particularly in MK, is that the nature of the organ being exposed to the surface facilitates intervention and assessment, whilst the natural history of MK enables recruitment of patients at similar points in the progression of the disease. Nevertheless, effective trial design must balance a thorough approach against the practicalities of conducting a trial. Appropriate outcome assessments need to be simple and specific. Chapter 2 revealed much heterogeneity in the literature regarding this, with significant scope for development. In 2019 the European Vision Institute (EVI) gathered a panel to discuss anterior eye and cornea relevant developments. Whilst imaging and outcome measures formed a significant proportion of the agenda, corneal fibrosis assessments featured minimally [599]. The importance of a corneal scarring-specific measure is perhaps under recognised and may be due to the fact that there is no treatment driving the need for it. As macular OCT significantly improved clinical practice in parallel to anti-VEGF therapy, anterior segment OCT is anticipated to impact the management of corneal fibrosis alongside novel treatments like decorin.

Other major causes of corneal blindness in the UK include HSK, chemical injury, immune-mediated keratitis and trauma. A pathology specific approach is essential for clinical trials in these conditions. For example, HSK occurs in a relapsing-remitting pattern, requiring long term monitoring to determine the efficacy of treatments. In addition to morphological assessments of lesions, studies will need to consider rates of complications, such as corneal transplantation, secondary MK, medical service usage and impact on quality of life. Glaucoma is one of the most globally prevalent causes of blindness in which fibrosis results in failure of filtration surgery. Although this is currently managed by a combination of intra-

operative MMC, post operative steroids, surgical scar disruption and further injections of anti-metabolites, each treatment has its own limitations [600].

Different pathological scenarios necessitate appropriate study design and may benefit from similarly matched therapeutic strategies. For example, it was interesting to note Mohan et al.'s six-month safety data of AAV5-decorin gene therapy in healthy rabbits. In particular, the increased tissue decorin at six months is an exciting therapeutic prospect and may be useful for more slow-progressive corneal scarring disorders such as HSK. What the study does not explain is whether there are any local or systemic impacts of chronically raised decorin that may lead to disadvantageous responses to pathology. The pharmacokinetics are also not certain. For example, it is unknown whether the vector is persistent in the tissue at this timepoint, and host cells continue to produce decorin, or whether the decorin has been produced initially and remains gathered. As the stromal cell population is dynamic and experiences considerable turnover, it is unclear whether similar results would be seen in pathological scenarios where there is a high turnover of cells, such as that in MK. Corneal injury models used by Mohan et al. (neovascularisation, chemical injury and PRK injury) (Chapter 1, table 1-3) are less severe injuries compared to MK, which would cause significantly more, and longer lasting inflammation (i.e. one laser treatment and one exposure to the chemical to induce injury at a single time point compared to bacterial colonisation and persistency for days). Nevertheless the advent of competing technologies helps to feed the knowledge pool and will help refine strategies for the various aetiologies.

Decorin's pluripotency and complex binding partner relationships raise questions about its action in different scenarios. Its context dependant pro- and anti-inflammatory characteristics [586] must be completely understood in pathological scenarios if it is to be broadly adopted in the clinic. Future trials should aim to optimise resource efficiency and incorporate mechanistic studies into their funding request and study design. For example, samples for multi-omics assessments could be taken in parallel and analysed separately without biasing the trial. Other disciplines could consider piggy-backing with existing trials to obtain relevant sample if feasible, for example conjunctival RNA swabs from patient receiving decorin, may be of value to those interested in glaucoma, ocular surface neoplasia and cicatrising disorders.

7.6 Development and market entry

MK is sometimes referred to as the “silent epidemic” because its incidence in developing countries causes profound morbidity in patients and loss of productivity, which impacts their dependants and broader society [601]. Limited resources and access to healthcare are two huge barriers in the management of these patients.

Based on studies of FDA approved drugs, it costs an estimated $161 million to $4.54 billion (2019 US$) to bring a drug to market [602]. Subsequent entry into other markets then incurs further costs to pharmaceutical companies, which ultimately work for profit. Thus, another barrier to patient care is introduced. Galacorin is under patent held by its supplier Catalent Pharmaceutical, and is very expensive [603,604]. Its incorporation into formulations significantly raises the cost of treatment and may preclude vast numbers of patients globally. This is particularly relevant considering the global economic outlook and the fact that many countries are now enlisting cost containment measures to control healthcare spending; for example, Germany has reduced the free drug pricing window from 12 to 6 months post access [605]. Following generation of evidence of its efficacy, the next major challenge for the scientific community would be to optimise the efficiency of its manufacturing process.

In this regard, development of biosimilar drugs could lead to cheaper treatments reaching the market. This strategy may incur financial and time costs of its own, due to litigation from patent enforcement, as many patents are designed to give broad coverage [606]. It is often enough to simply engage in expensive legal proceedings to delay biosimilar market entry even in the defence of relatively low-quality patents [607]. However, this is more commonly experienced in the USA because of their patent law legislation [608]. The pandemic has also raised discussions about alternate big-pharma pricing and payment models [609,610], but whether these will truly enable new treatments to have global reach is to be seen.

7.7 Strengths of the thesis

This was a mixed methods study in the development of a treatment for corneal fibrosis which draws insights from multiple perspectives. Chapter 2 was conducted in accordance with rigorous guidelines of conduct and act as a reference point in its own right that will help guide future researchers and clinicians in the field. Chapter 3 screens the novel association of corneal fibrosis, autophagy and decorin, and forms the foundational work to develop this line of investigation. It also emphasised the importance of

mechanistic studies in the developmental pathway and was the impetus to incorporate these into the trial design.

The results of Chapter 4 offers important insights into eye-related health seeking behaviour which can help inform future service adaptations and public health campaigns going forward. Combined with the results of Chapter 5, they highlight the importance of health services understanding their users perspective in order to adequately design clinical and research services, and to remain resilient during unpredictable times.

Chapter 6 explores the proposed methodology of the OPtiCS-MK trial and offers useful recommendations for its implementation in the future. It crystalises these ideas and considerations of an early phase trial of a first in class intervention. By paving this path, it will facilitate the more rapid development of other similar therapies.

7.8 Limitations of the study

Chapter 2 did not include preclinical studies thus offering a limited view of the drug development horizon. Clinical recommendations cannot be made as this study did not quantitatively compare efficacy of treatments. A comparison of their mechanisms of action may help to uncover key similarities and differences for future investigators and novel drug combination development.

Chapter 3 combined concepts in a novel study however, due to factors such as model and experimental design, the generalisability of these results is limited.

Whilst Chapters 2 and 3 enable inferences to be made about the benefits of a multi-mechanistic approach to corneal wound healing, further evidence is required to support this hypothesis.

A limitation of Chapter 5 is that visual acuity is a poor indicator of severity – as small lesions at the visual axis will be more impactful than a large lesion peripherally. Whilst the risk of the latter causing a blinding complication is greater, the former (a large peripheral ulcer) may cause greater morbidity (and risk of complications) even if successfully treated. Morphological assessments of ulcers and patient reported

outcomes would provide a better understanding of the patients' pathology and experience. However, this was a real-world study, and these are not yet in routine clinical use.

7.9 Recommendations for future practice & research

Pre-Clinical studies

Decorin's pluripotency continues to be unravelled. An efficient approach to consolidating such a broad range of binding partners is to utilise *in silico* techniques to screen for interactions, in order to generate hypotheses in corneal and other complex wound environments [611]. This will facilitate the design of future mechanistic studies, which are clearly needed. Such investigations should adopt a multifaceted approach by drawing on insights from *in vitro*, *in vivo* and human studies, as planned in the next phase of this work.

Clinical studies

A number of therapies already available (see Chapter 2) could be entered into clinical trials, in isolation or in combination with each other. The OPtiCKS-MK trial is eagerly awaited and forms the next major step in this line of study. Parallel research priorities include the development of a better objective quantitative outcome measure than is currently available commercially. The intended mechanistic studies are important aspects of the trial, second only to decorin itself.

To better characterise corneal fibrosis for outcome assessments, long term multi-modality studies of corneal fibrosis will help generate data for future comparisons. Furthermore, normative datasets and standardisation assessment techniques should be hastened to ensure comparability between trials of other candidates in the future. If the community regulates itself with this in mind, then secondary analyses, meta-analyses and linked meta-analyses could take place much more readily and ideal candidates can be identified more efficiently.

7.10 Closing remarks

Discussion

Corneal blindness is a leading cause of blindness globally. Many therapies have been tested in this context however no widely accepted and effective solution exists. Understanding the key mechanistic checkpoints in corneal fibrosis will inform management strategies for fibrotic disorders in the cornea and beyond. Although MK is different to many other fibrotic conditions which typically involve chronic inflammation and slow progressive fibrosis, treatments acting through common pathways shared amongst other organs have greater potential for application in other conditions.

Decorin is one such candidate which is imminently due to enter human clinical trials. Its results have the potential to change the landscape of corneal fibrosis management. As the field is still in its infancy in many ways, robust clinical outcome measures are yet to be established. The OPtiCKS-MK trial was delayed by the COVID-19 pandemic. Although the presentation patterns and outcomes of MK patients in the UK were largely unchanged during the pandemic, the global disruption has exacerbated the pre-existing corneal transplant tissue shortages, meaning access to sight restoring surgery for corneal blindness is now scarcer than before. Unexpected global events like the pandemic continue to threaten the progress of society, which must learn to adapt ways of living to continue progressing safely and unquestionably. As such there is an urgent, unmet need for the development and implementation of a non-invasive, easily accessible, and cost effective therapy to prevent corneal fibrosis in patients with microbial keratitis, a disease with significant cost to society that disproportionately affects disadvantaged peoples.

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9. APPENDIX

Supplementary table 1. AMSTAR 2 assessment of Kwok et al. Systematic Review on Therapeutic Strategies to Minimize Corneal Stromal Scarring after Injury (2019)

	AMSTAR 2 Question	**Score (1 = yes; 2 = partial yes; 3 = no; 91=not applicable)**
1	Did the research questions and inclusion criteria for the review include the components of PICO?	3
2	Did the report of the review contain an explicit statement that the review methods were established prior to the conduct of the review and did the report justify any significant deviations from the protocol?	3
3	Did the review authors explain their selection of the study designs for inclusion in the review?	3
4	Did the review authors use a comprehensive literature search strategy?	3
5	Did the review authors perform study selection in duplicate?	3
6	Did the review authors perform data extraction in duplicate?	3
7	Did the review authors provide a list of excluded studies and justify the exclusions?	3
8	Did the review authors describe the included studies in adequate detail?	1
9	Did the review authors use a satisfactory technique for assessing the risk of bias (RoB) in individual studies that were included in the review?	3
10	Did the review authors report on the sources of funding for the studies included in the review?	3
11	If meta-analysis was performed did the review authors use appropriate methods for statistical combination of results?	91
12	If meta-analysis was performed, did the review authors assess the potential impact of RoB in individual studies on the results of the meta-analysis or other evidence synthesis?	91
13	Did the review authors account for RoB in individual studies when interpreting/ discussing the results of the review?	3
14	Did the review authors provide a satisfactory explanation for, and discussion of, any heterogeneity observed in the results of the review?	3
15	If they performed quantitative synthesis did the review authors carry out an adequate investigation of publication bias (small study bias) and discuss its likely impact on the results of the review?	91
16	Did the review authors report any potential sources of conflict of interest, including any funding they received for conducting the review?	1

APPENDIX

Supplementary figure 1. Example search strategy developed on OVID and optimised for the Medline R, and In-Process, In-Data & Other non-indexed Citations 1946-2021.

Ovid®

My Account | Admin Tools | Support & Training | UNIVERSITY OF BIRMINGHAM | Help | Feedback

Search | Journals | Books | Multimedia | My Workspace

▼ Search History (92)

# ▲	Searches	Results	Type	Actions	Annotations
1	therapy/ or therap*.mp.	5701809	Advanced	Display Results More	
2	drug/ or drug therapy/ or drug.mp.	5488764	Advanced	Display Results More	
3	treat*.mp.	5194640	Advanced	Display Results More	
4	management.mp. or management/	1083714	Advanced	Display Results More	
5	agent*.mp.	2618108	Advanced	Display Results More	
6	prevent*.mp.	2169441	Advanced	Display Results More	
7	1 or 2 or 3 or 4 or 5 or 6	11851582	Advanced	Display Results More	
8	(cornea* adj3 fibro*).mp.	1202	Advanced	Display Results More	
9	(cornea* adj3 scar*).mp.	1820	Advanced	Display Results More	
10	(cornea* adj3 haz*).mp.	923	Advanced	Display Results More	
11	(cornea* adj3 opac*).mp.	5727	Advanced	Display Results More	
12	(cornea* adj3 cloud*).mp.	522	Advanced	Display Results More	
13	(cornea* adj3 transparency).mp.	822	Advanced	Display Results More	
14	(cornea* adj3 blind*).mp.	574	Advanced	Display Results More	
15	cornea*.mp. or cornea/	105004	Advanced	Display Results More	
16	scar free.mp.	209	Advanced	Display Results More	
17	(scar less or scarless).mp.	922	Advanced	Display Results More	
18	wound healing/ or wound healing.mp.	118233	Advanced	Display Results More	
19	regeneration.mp. or regeneration/	144725	Advanced	Display Results More	
20	healing/ or healing.mp.	197010	Advanced	Display Results More	
~~21~~	~~16 or 17 or 18 or 19 or 20~~	~~324674~~	~~Advanced~~	Display Results More	
22	16 or 17	1116	Advanced	Display Results More	
23	18 or 19 or 20	324106	Advanced	Display Results More	
24	22 and 23	548	Advanced	Display Results More	
25	15 and 24 scarless AND wound healing AND cornea	6	Advanced	Display Results More	
26	case control study/ or case control stud*.mp.	304704	Advanced	Display Results More	
27	case report/ or case report*.mp.	2033002	Advanced	Display Results More	
28	case study/ or case stud*.mp.	2057066	Advanced	Display Results More	
29	clinical study/ or clinical stud*.mp.	130054	Advanced	Display Results More	
30	cohort analysis.mp. or cohort analysis/	265485	Advanced	Display Results More	
31	Cohort Studies/ or cohort stud*.mp.	356217	Advanced	Display Results More	
32	correlational study/ or correlational stud*.mp.	2323	Advanced	Display Results More	
33	cross-sectional study/ or cross sectional stud*.mp.	346436	Advanced	Display Results More	
34	epidemiologic* stud*.mp.	80568	Advanced	Display Results More	
35	family study/ or family stud*.mp.	7751	Advanced	Display Results More	
36	follow up/ or follow* up.mp.	1238331	Advanced	Display Results More	
37	follow* up stud*.mp.	659116	Advanced	Display Results More	
38	hospital based case control study/ or hospital based case control stud*.mp.	2428	Advanced	Display Results More	
39	longitudinal study/ or longitudinal* stud*.mp.	160738	Advanced	Display Results More	
40	observational study/ or observation* stud*.mp.	141552	Advanced	Display Results More	
41	population based case control study/ or population based case control stud*.mp.	6576	Advanced	Display Results More	
42	prospective study/ or prospectiv* stud*.mp.	593681	Advanced	Display Results More	
43	retrospective study/ or retrospectiv* stud*.mp.	843120	Advanced	Display Results More	
44	nonconcurrent prospectiv*.mp.	62	Advanced	Display Results More	
45	(clinic* adj1 trial*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	955998	Advanced	Display Results More	
46	clinical trial.mp. or clinical trial/	682580	Advanced	Display Results More	
47	randomized controlled trial/ or randomized controlled trial*.mp.	667218	Advanced	Display Results More	
48	(random* adj1 assign*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	97549	Advanced	Display Results More	
49	(random* adj1 allocat*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	128125	Advanced	Display Results More	
50	(control* adj1 clinic*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	129538	Advanced	Display Results More	
51	(control* adj1 trial).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	535115	Advanced	Display Results More	
52	placebo/ or placebo*.mp.	208066	Advanced	Display Results More	
53	(quantitat* adj1 stud*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	16149	Advanced	Display Results More	
54	(quantitat* adj1 stud*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	16149	Advanced	Display Results More	

55	(control* adj1 stud*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	368018	Advanced	Display Results More	
56	(random* adj1 stud*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	41283	Advanced	Display Results More	
57	(random* adj2 trial*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	717148	Advanced	Display Results More	
58	(singl* adj1 blind*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	34840	Advanced	Display Results More	
59	(singl* adj1 mask*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	457	Advanced	Display Results More	
60	(doubl* adj1 blind*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	186337	Advanced	Display Results More	
61	(doubl* adj1 mask*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	3035	Advanced	Display Results More	
62	(tripl* adj1 blind*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	748	Advanced	Display Results More	
63	(tripl* adj1 mask*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	52	Advanced	Display Results More	
64	(trebl* adj1 blind*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	0	Advanced	Display Results More	
65	(trebl* adj1 mask*).mp. [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	0	Advanced	Display Results More	
66	cross over.mp.	58952	Advanced	Display Results More	
67	meta-analysis.pt.	115410	Advanced	Display Results More	
68	meta-analysis.sh.	115410	Advanced	Display Results More	
69	(meta-analys* or meta analys* or metaanalys*).tw,sh.	166756	Advanced	Display Results More	
70	(systematic* adj5 review*).tw,sh.	155944	Advanced	Display Results More	
71	(systematic* adj5 overview*).tw,sh.	1716	Advanced	Display Results More	
72	(quantitativ* adj5 review*).tw,sh.	6953	Advanced	Display Results More	
73	(quantitativ* adj5 overview*).tw,sh.	270	Advanced	Display Results More	
74	(quantitativ* adj5 synthesis*).tw,sh.	2322	Advanced	Display Results More	
75	(methodologic* adj5 review*).tw,sh.	5203	Advanced	Display Results More	
76	(methodologic adj5 overview*).tw,sh.	22	Advanced	Display Results More	
77	(integrative research review* or research integration).tw.	125	Advanced	Display Results More	
78	reference list*.ab.	15325	Advanced	Display Results More	
79	bibliograph*.ab.	15450	Advanced	Display Results More	
80	hand-search*.ab.	5847	Advanced	Display Results More	
81	relevant journal*.ab.	1134	Advanced	Display Results More	
82	manual search*.ab.	3742	Advanced	Display Results More	
83	26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34 or 35 or 36 or 37 or 38 or 39 or 40 or 41 or 42 or 43 or 44 or 45 or 46 or 47 or 48 or 49 or 50 or 51 or 52 or 53 or 54 or 55 or 56 or 57 or 58 or 59 or 60 or 61 or 62 or 63 or 64 or 65 or 66 or 67 or 68 or 69 or 70 or 71 or 72 or 73 or 74 or 75 or 76 or 77 or 78 or 79 or 80 or 81 or 82	6253261	Advanced	Display Results More	
84	8 or 9 or 10 or 11 or 12 or 13 or 14	10362	Advanced	Display Results More	
85	24 and 84 scarless AND wound healing AND cornea fibrosis	4	Advanced	Display Results More	
86	7 and 84 cornea fibrosis AND treatment/prevention	5777	Advanced	Display Results More	
87	25 or 85 or 86 3 strands	5780	Advanced	Display Results More	
88	exp animals/	23214247	Advanced	Display Results More	
89	exp humans/	18509293	Advanced	Display Results More	
90	88 or 89	23214247	Advanced	Display Results More	
91	83 and 90 human or animal trials	6192630	Advanced	Display Results More	
92	87 and 91 3 strands limited to human or animal trials	2925	Advanced	Display Results More	

Supplementary figure 2. Participant Map

Participants who provided a valid postcode (N=371) were grouped based on their postcode district. Points are plotted at each of these postcode districts, with the size of the point representing the number of participants; the maximum number of participants within a district was N=40.

Participant map. The district component was extracted from all postcodes provided by participants, the longitude and latitude of which was extracted from https://www.doogal.co.uk/PostcodeDistricts.php. The R package "maps" was then used to generate a world map, which was cropped to only display the UK. The "ggplot2" package was then used to plot points representing each included postcode district onto this map. The size of these points represented the total numbers of participants within each district.

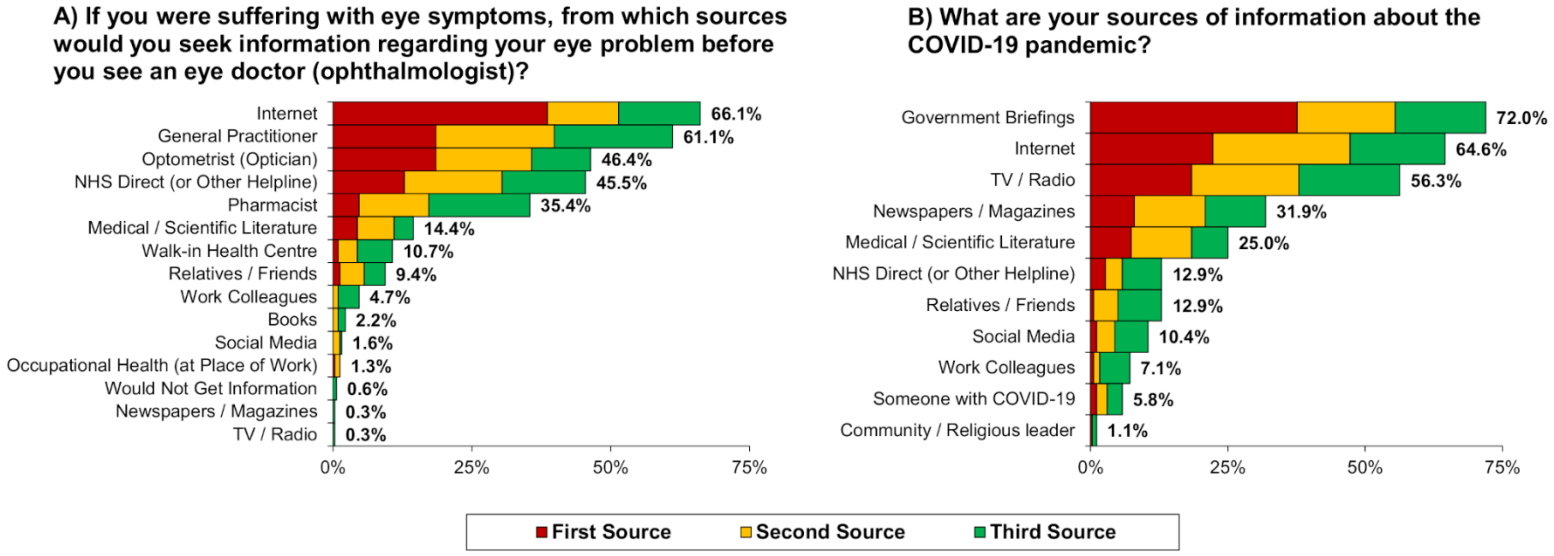
Supplementary table 2. Full Likert data for participants views on scenarios

	How Serious are These Symptoms	How Impactful are These Symptoms on Daily Life	How Quickly Would You Seek Attention If COVID-19 was not a Factor?	How Quickly Would You Seek Attention Considering COVID-19?
Scenario 1 - Eye mildly red and gritty				
Very	13 (3.2%)	30 (7.5%)	57 (14.2%)	35 (8.7%)
Moderately	77 (19.2%)	85 (21.1%)	126 (31.3%)	87 (21.6%)
Somewhat	125 (31.1%)	145 (36.1%)	79 (19.7%)	80 (19.9%)
Not Very	165 (41.0%)	121 (30.1%)	106 (26.4%)	140 (34.8%)
Not at all	22 (5.5%)	21 (5.2%)	34 (8.5%)	60 (14.9%)
Scenario 2 - Eye red, sticky and blurred				
Very	95 (23.6%)	103 (25.6%)	145 (36.1%)	105 (26.1%)
Moderately	162 (40.3%)	161 (40.0%)	155 (38.6%)	133 (33.1%)
Somewhat	113 (28.1%)	105 (26.1%)	63 (15.7%)	91 (22.6%)
Not Very	28 (7.0%)	28 (7.0%)	32 (8.0%)	55 (13.7%)
Not at all	4 (1.0%)	5 (1.2%)	7 (1.7%)	18 (4.5%)
Scenario 3 - Eye red, painful, photophobia, sticky, blurred, white spot				
Very	299 (74.4%)	283 (70.4%)	315 (78.4%)	264 (65.7%)
Moderately	77 (19.2%)	89 (22.1%)	64 (15.9%)	97 (24.1%)
Somewhat	21 (5.2%)	24 (6.0%)	17 (4.2%)	27 (6.7%)
Not Very	3 (0.7%)	4 (1.0%)	3 (0.7%)	9 (2.2%)
Not at all	2 (0.5%)	2 (0.5%)	3 (0.7%)	5 (1.2%)
Scenario 4 - Painless loss of vision				
Very	251 (62.4%)	269 (66.9%)	266 (66.2%)	230 (57.2%)
Moderately	107 (26.6%)	97 (24.1%)	104 (25.9%)	102 (25.4%)
Somewhat	37 (9.2%)	29 (7.2%)	21 (5.2%)	46 (11.4%)
Not Very	5 (1.2%)	5 (1.2%)	8 (2.0%)	17 (4.2%)
Not at all	2 (0.5%)	2 (0.5%)	3 (0.7%)	7 (1.7%)
Scenario 5 - Rectal bleeding				
Very	305 (75.9%)	200 (49.8%)	308 (76.6%)	273 (67.9%)
Moderately	65 (16.2%)	121 (30.1%)	67 (16.7%)	79 (19.7%)
Somewhat	28 (7.0%)	69 (17.2%)	22 (5.5%)	27 (6.7%)
Not Very	1 (0.2%)	10 (2.5%)	3 (0.7%)	19 (4.7%)
Not at all	3 (0.7%)	2 (0.5%)	2 (0.5%)	4 (1.0%)
Scenario 6 - Chest pain				
Very	251 (62.4%)	209 (52.0%)	258 (64.2%)	234 (58.2%)
Moderately	111 (27.6%)	137 (34.1%)	101 (25.1%)	103 (25.6%)
Somewhat	24 (6.0%)	42 (10.4%)	29 (7.2%)	40 (10.0%)
Not Very	13 (3.2%)	12 (3.0%)	10 (2.5%)	15 (3.7%)
Not at all	3 (0.7%)	2 (0.5%)	4 (1.0%)	10 (2.5%)

Supplementary table 3. Respondent characteristics by ethnicity

	Ethnicity		
	White	***Non-White***	**p-Value**
Age (Years)	*Mean: 65.1**	*Mean: 36.5**	**<0.001****
18-35	37 (10.6%)	32 (65.3%)	
36-65	74 (21.3%)	12 (24.5%)	
66+	237 (68.1%)	5 (10.2%)	
Gender			**0.039**
Male	121 (34.8%)	25 (51.0%)	
Female	227 (65.2%)	24 (49.0%)	
Employment			**<0.001**
Employed / In Education	102 (29.3%)	44 (89.8%)	
Unemployed / Retired	246 (70.7%)	5 (10.2%)	
Index of Multiple Deprivation [N=368]			**<0.001****
Decile 1-3 (Most Deprived)	53 (16.1%)	21 (53.8%)	
Decile 4-7	179 (54.4%)	11 (28.2%)	
Decile 8-10 (Least Deprived)	97 (29.5%)	7 (17.9%)	
Do You Know or Have Known Someone with the Following Conditions?			
Eye Disease	234 (67.2%)	29 (59.2%)	0.264
Bowel Cancer	194 (55.7%)	11 (22.4%)	**<0.001**
Angina	212 (60.9%)	20 (40.8%)	**0.009**
COVID-19	192 (55.2%)	38 (77.6%)	**0.003**
Sources of Information about Eye Problems [N=316]***			
Internet	180 (64.7%)	30 (78.9%)	0.099
General Practitioner	171 (61.5%)	23 (60.5%)	1.000
Optometrist (Optician)	139 (50.0%)	8 (21.1%)	**<0.001**
Sources of Information about COVID-19 [N=361]***			
Government Briefings	237 (75.0%)	23 (51.1%)	**0.002**
Internet	199 (63.0%)	33 (73.3%)	0.188
TV / Radio	188 (59.5%)	17 (37.8%)	**0.009**

Supplementary figure 3. Preferred sources of information on eye problems and the COVID-19 pandemic



Plots are based on the N=319 and N=364 that answered the questions relating to eye problems and COVID-19, respectively. Percentages represent the proportion of participants that classified the stated information source as being within their top three.

Supplementary table 4. Scenarios 1 to 6 correlation with demographic factors

Question	Age (Years)	Gender (Female)	Ethnicity (Non-White)	Employment (Unemployed/Retired)	Index of Multiple Deprivation
Scenario 1					
How Serious are Symptoms	**Rho: 0.15 (p=0.003)**	Rho: -0.03 (p=0.554)	Rho: -0.01 (p=0.870)	Rho: 0.08 (p=0.124)	**Rho: 0.11 (p=0.041)**
How Impactful are Symptoms	Rho: 0.02 (p=0.704)	Rho: 0.02 (p=0.688)	Rho: 0.02 (p=0.710)	Rho: 0.00 (p=0.983)	**Rho: 0.14 (p=0.006)**
How Quickly (Non-COVID)*	**Rho: 0.13 (p=0.009)**	Rho: -0.05 (p=0.299)	Rho: -0.06 (p=0.229)	**Rho: 0.12 (p=0.017)**	**Rho: 0.12 (p=0.025)**
How Quickly (COVID)*	**Rho: 0.11 (p=0.023)**	**Rho: -0.12 (p=0.014)**	Rho: -0.03 (p=0.523)	Rho: 0.07 (p=0.148)	Rho: 0.09 (p=0.074)
Scenario 2					
How Serious are Symptoms	Rho: 0.06 (p=0.209)	Rho: 0.01 (p=0.845)	Rho: 0.02 (p=0.631)	Rho: 0.02 (p=0.654)	Rho: 0.02 (p=0.654)
How Impactful are Symptoms	Rho: -0.09 (p=0.063)	Rho: 0.08 (p=0.116)	Rho: 0.09 (p=0.083)	**Rho: -0.11 (p=0.033)**	Rho: 0.06 (p=0.241)
How Quickly (Non-COVID)*	Rho: 0.03 (p=0.609)	Rho: 0.00 (p=0.931)	Rho: 0.04 (p=0.449)	Rho: 0.05 (p=0.286)	Rho: 0.09 (p=0.078)
How Quickly (COVID)*	Rho: 0.05 (p=0.311)	Rho: -0.07 (p=0.190)	Rho: 0.04 (p=0.463)	Rho: 0.03 (p=0.492)	Rho: 0.07 (p=0.152)
Scenario 3					
How Serious are Symptoms	Rho: -0.06 (p=0.207)	Rho: 0.04 (p=0.409)	Rho: 0.04 (p=0.471)	Rho: -0.06 (p=0.202)	Rho: 0.00 (p=0.947)
How Impactful are Symptoms	Rho: -0.07 (p=0.185)	Rho: 0.06 (p=0.249)	Rho: 0.04 (p=0.411)	Rho: -0.04 (p=0.369)	Rho: 0.02 (p=0.645)
How Quickly (Non-COVID)*	Rho: -0.07 (p=0.180)	Rho: 0.05 (p=0.361)	Rho: 0.06 (p=0.273)	Rho: -0.03 (p=0.505)	Rho: 0.06 (p=0.235)
How Quickly (COVID)*	Rho: 0.02 (p=0.707)	Rho: 0.03 (p=0.617)	Rho: 0.04 (p=0.434)	Rho: -0.01 (p=0.835)	Rho: 0.03 (p=0.593)
Scenario 4					
How Serious are Symptoms	Rho: 0.07 (p=0.149)	Rho: -0.04 (p=0.376)	**Rho: -0.14 (p=0.007)**	Rho: 0.09 (p=0.072)	Rho: 0.13 (p=0.015)
How Impactful are Symptoms	Rho: -0.03 (p=0.505)	Rho: -0.01 (p=0.804)	Rho: -0.09 (p=0.079)	Rho: 0.02 (p=0.694)	**Rho: 0.12 (p=0.022)**
How Quickly (Non-COVID)*	Rho: 0.08 (p=0.090)	Rho: -0.08 (p=0.132)	**Rho: -0.11 (p=0.032)**	Rho: 0.08 (p=0.106)	Rho: 0.10 (p=0.053)
How Quickly (COVID)*	Rho: 0.09 (p=0.072)	**Rho: -0.11 (p=0.028)**	**Rho: -0.14 (p=0.007)**	Rho: 0.07 (p=0.191)	Rho: 0.05 (p=0.362)
Scenario 5					
How Serious are Symptoms	**Rho: 0.13 (p=0.010)**	Rho: -0.03 (p=0.485)	**Rho: -0.20 (p<0.001)**	**Rho: 0.14 (p=0.004)**	Rho: 0.08 (p=0.118)
How Impactful are Symptoms	Rho: 0.06 (p=0.238)	Rho: -0.08 (p=0.120)	Rho: -0.07 (p=0.154)	Rho: 0.09 (p=0.066)	**Rho: 0.11 (p=0.031)**
How Quickly (Non-COVID)*	**Rho: 0.20 (p<0.001)**	**Rho: -0.11 (p=0.027)**	**Rho: -0.21 (p<0.001)**	**Rho: 0.23 (p<0.001)**	Rho: 0.07 (p=0.151)
How Quickly (COVID)*	**Rho: 0.17 (p=0.001)**	Rho: -0.09 (p=0.085)	**Rho: -0.10 (p=0.041)**	**Rho: 0.17 (p=0.001)**	Rho: 0.03 (p=0.609)
Scenario 6					
How Serious are Symptoms	**Rho: 0.27 (p<0.001)**	Rho: -0.04 (p=0.371)	**Rho: -0.14 (p=0.006)**	**Rho: 0.24 (p<0.001)**	**Rho: 0.11 (p=0.039)**
How Impactful are Symptoms	**Rho: 0.26 (p<0.001)**	Rho: -0.08 (p=0.120)	**Rho: -0.12 (p=0.014)**	**Rho: 0.21 (p<0.001)**	**Rho: 0.13 (p=0.013)**
How Quickly (Non-COVID)*	**Rho: 0.27 (p<0.001)**	Rho: -0.09 (p=0.081)	**Rho: -0.17 (p=0.001)**	**Rho: 0.27 (p<0.001)**	Rho: 0.09 (p=0.078)
How Quickly (COVID)*	**Rho: 0.29 (p<0.001)**	Rho: -0.09 (p=0.065)	**Rho: -0.14 (p=0.006)**	**Rho: 0.23 (p<0.001)**	Rho: 0.06 (p=0.259)

*Analysis was performed using Spearman's (Rho) correlation coefficients, with positive coefficients representing greater agreement with the question with increasing values of ordinal variables, or for the stated category relative to the reference for nominal variables. Bold values are significant at p<0.05. The details of the questions are abbreviated, and are detailed in full in the text. *The question asked how quickly the respondent would seek medical attention for symptoms either if the COVID-19 pandemic was not a factor (Non-COVID), or taking this into consideration (COVID).*

Supplementary table 5. Scenarios 1 to 6 correlation with demographic factors in non-white subgroup

	Age (Years)	Gender (Female)	Employment (Unemployed/Retired)	Index of Multiple Deprivation
Scenario 1				
How Serious are Symptoms	Rho: -0.02 (p=0.899)	Rho: 0.06 (p=0.669)	Rho: -0.11 (p=0.459)	Rho: 0.19 (p=0.243)
How Impactful are Symptoms	Rho: -0.08 (p=0.562)	Rho: 0.07 (p=0.638)	Rho: 0.04 (p=0.796)	Rho: 0.23 (p=0.158)
How Quickly (Non-COVID)*	Rho: 0.03 (p=0.836)	Rho: 0.00 (p=0.976)	Rho: 0.22 (p=0.121)	Rho: 0.19 (p=0.245)
How Quickly (COVID)*	Rho: 0.16 (p=0.283)	Rho: -0.16 (p=0.285)	**Rho: 0.29 (p=0.042)**	Rho: 0.22 (p=0.185)
Scenario 2				
How Serious are Symptoms	Rho: -0.21 (p=0.155)	Rho: 0.19 (p=0.197)	Rho: -0.11 (p=0.443)	Rho: 0.27 (p=0.103)
How Impactful are Symptoms	Rho: -0.13 (p=0.361)	Rho: 0.04 (p=0.794)	Rho: -0.12 (p=0.425)	**Rho: 0.34 (p=0.037)**
How Quickly (Non-COVID)*	Rho: -0.14 (p=0.323)	**Rho: 0.35 (p=0.013)**	Rho: 0.07 (p=0.626)	**Rho: 0.32 (p=0.046)**
How Quickly (COVID)*	Rho: -0.07 (p=0.609)	Rho: 0.03 (p=0.835)	Rho: 0.06 (p=0.680)	**Rho: 0.38 (p=0.017)**
Scenario 3				
How Serious are Symptoms	Rho: -0.11 (p=0.464)	Rho: -0.01 (p=0.966)	Rho: -0.14 (p=0.353)	**Rho: 0.36 (p=0.026)**
How Impactful are Symptoms	Rho: -0.09 (p=0.534)	Rho: 0.11 (p=0.448)	Rho: 0.18 (p=0.216)	**Rho: 0.41 (p=0.009)**
How Quickly (Non-COVID)*	Rho: -0.15 (p=0.318)	Rho: 0.15 (p=0.298)	Rho: -0.04 (p=0.789)	**Rho: 0.39 (p=0.014)**
How Quickly (COVID)*	Rho: -0.06 (p=0.668)	Rho: 0.07 (p=0.654)	Rho: -0.06 (p=0.681)	Rho: 0.24 (p=0.143)
Scenario 4				
How Serious are Symptoms	Rho: 0.24 (p=0.103)	Rho: -0.11 (p=0.435)	Rho: 0.32 (p=0.024)	**Rho: 0.33 (p=0.038)**
How Impactful are Symptoms	Rho: 0.18 (p=0.218)	Rho: -0.12 (p=0.408)	Rho: 0.27 (p=0.061)	Rho: 0.31 (p=0.052)
How Quickly (Non-COVID)*	Rho: 0.09 (p=0.534)	Rho: -0.13 (p=0.362)	**Rho: 0.29 (p=0.042)**	Rho: 0.15 (p=0.359)
How Quickly (COVID)*	Rho: 0.17 (p=0.247)	Rho: -0.23 (p=0.105)	Rho: 0.27 (p=0.064)	Rho: 0.13 (p=0.443)
Scenario 5				
How Serious are Symptoms	Rho: -0.01 (p=0.955)	Rho: -0.26 (p=0.072)	Rho: 0.19 (p=0.185)	Rho: 0.26 (p=0.115)
How Impactful are Symptoms	Rho: 0.20 (p=0.176)	Rho: -0.21 (p=0.144)	Rho: 0.26 (p=0.076)	**Rho: 0.34 (p=0.034)**
How Quickly (Non-COVID)*	Rho: 0.20 (p=0.176)	Rho: -0.20 (p=0.172)	**Rho: 0.29 (p=0.043)**	**Rho: 0.42 (p=0.008)**
How Quickly (COVID)*	Rho: 0.21 (p=0.156)	**Rho: -0.29 (p=0.042)**	Rho: 0.27 (p=0.062)	Rho: 0.27 (p=0.097)
Scenario 6				
How Serious are Symptoms	Rho: -0.03 (p=0.817)	Rho: -0.08 (p=0.603)	Rho: 0.08 (p=0.574)	**Rho: 0.38 (p=0.017)**
How Impactful are Symptoms	Rho: 0.03 (p=0.845)	Rho: -0.12 (p=0.408)	Rho: 0.10 (p=0.501)	**Rho: 0.43 (p=0.006)**
How Quickly (Non-COVID)*	Rho: 0.02 (p=0.872)	Rho: -0.12 (p=0.392)	Rho: 0.10 (p=0.497)	Rho: 0.25 (p=0.122)
How Quickly (COVID)*	Rho: 0.12 (p=0.418)	Rho: -0.16 (p=0.270)	Rho: 0.13 (p=0.378)	Rho: 0.25 (p=0.124)

Analysis was performed using Spearman's (Rho) correlation coefficients, with positive coefficients representing greater agreement with the question with increasing values of ordinal variables, or for the stated category relative to the reference for nominal variables. Only respondents of non-White ethnicity were included (N=49). Bold values are significant at p<0.05. The details of the questions are abbreviated, and are detailed in full in the text. *The question asked how quickly the respondent would seek medical attention for symptoms either if the COVID-19 pandemic was not a factor (Non-COVID), or taking this into consideration (COVID

Supplementary table 6. Birmingham & Midlands Eye Centre – Eye Ward Activity 2015 – 2016				
	Ambulatory/Day Case	**Elective**	**Emergency**	**Total**
Patients (n)	255	491	449	1195

Supplementary table 7. Complete list of risk factors		
Active ocular surface disease (OSD)	**Previous keratitis**	**Previous Surgery/Trauma**
Conjunctivitis	Keratitis (Viral)	Corneal Crosslinking
Corneal Anaesthesia (Neurotropic)	Keratitis (Bacterial)	Corneal Transplant (previous)
Corneal Decompensation	Keratitis (Fungal)	Corneal Trauma (old)
External Disease: Acne Rosacea)	Keratitis (infectious, non-specified)	Laser Refractive Surgery
External Disease: Anterior Lid	Marginal keratitis	Ocular Surgery (>6 month)
External Disease: Atopy		Ocular Surgery Recent (<= 6 month)
External Disease: Ectropion		
External Disease: Entropion		
External Disease: Floppy Eye Lid Syndrome		
External Disease: Generalised BKC		
External Disease: Immune OSD		
External Disease: Posterior Lid		
External Disease: Toxic OSD (e.g. drop related))		
External Disease: Trichiasis		
Keratitis - Viral (Active)		
Keratitis (Acanthamoeba)		
Keratoconus		
Marginal keratitis (active)		

Supplementary table 8. Trends in patient characteristics across the pre-C19 period					
	2017	**2018**	**2019**	**p-Value 2017-19**	**2020**
Age (years)	55.3 ± 21.9	56.6 ± 21.3	54.8 ± 20.5	0.922	53.3 ± 17.8
Sex – male (%)	34 (54.0%)	27 (54.0%)	34 (50.0%)	0.879	31 (63.3%)
Ethnicity				0.299	
White	46 (75.4%)	32 (71.1%)	34 (58.6%)		27 (71.1%)
Asian	11 (18.0%)	10 (22.2%)	17 (29.3%)		7 (18.4%)
Black	2 (3.3%)	1 (2.2%)	6 (10.3%)		3 (7.9%)
Mixed / Other	2 (3.3%)	2 (4.4%)	1 (1.7%)		1 (2.6%)
IMD decile				0.238*	
1-3	31 (50.0%)	26 (52.0%)	39 (59.1%)		26 (53.1%)
4-7	16 (25.8%)	20 (40.0%)	22 (33.3%)		19 (38.8%)
8-10	15 (24.2%)	4 (8.0%)	5 (7.6%)		4 (8.2%)
Laterality – right (%)	32 (50.8%)	28 (56.0%)	29 (42.6%)	0.351	27 (55.1%)
Duration of symptoms (Days)	3 (1-6)	3 (1-7)	3 (2-5)	0.965	4 (2-7)
Risk factors					
Contact lens	20 (31.7%)	20 (40.0%)	20 (29.4%)	0.476	14 (28.6%)
Underlying OSD (active)**	31 (49.2%)	17 (34.0%)	32 (47.1%)	0.217	26 (53.1%)

Previous keratitis***	10 (15.9%)	4 (8.0%)	6 (8.8%)	0.362	7 (14.3%)
Previous ocular surgery/trauma	12 (19.0%)	7 (14.0%)	12 (17.6%)	0.801	10 (20.4%)
Concurrent trauma	6 (9.5%)	0 (0.0%)	4 (5.9%)	0.064	8 (16.3%)
Corneal foreign body	1 (1.6%)	0 (0.0%)	2 (2.9%)	0.780	2 (4.1%)
Diabetes mellitus	6 (9.5%)	8 (16.0%)	3 (4.4%)	0.110	7 (14.3%)
Rheumatoid arthritis	2 (3.2%)	1 (2.0%)	3 (4.4%)	0.879	1 (2.0%)
Systemic immunosuppression	2 (3.2%)	1 (2.0%)	0 (0.0%)	0.378	6 (12.2%)
Thyroid eye disease	0 (0.0%)	1 (2.0%)	0 (0.0%)	0.276	0 (0.0%)

*Abbreviations: OSD, ocular surface disease; IMD, index of multiple deprivation; MK, microbial keratitis). Continuous variables are reported as mean ± SD or median (interquartile range), with p-values from Kruskal-Wallis tests. Categorical variables are reported as N (column %), with p-values from Fisher's exact tests, unless stated otherwise. All p-values represent comparisons across the three Pre-C19 years (2017, 2018 and 2019), and bold p-values are significant at p<0.05. Data from the year 2020 are also reported, for reference. For risk factors "previous" denotes that the risk factor had healed prior to onset of MK. *p-Value from Kruskal-Wallis test, as the factor is ordinal. **Ocular surface disease, such as dry eye, affecting the patient at the time of presentation – a full list of included diseases is reported in Supplementary Table 1. ***Viral/bacterial/fungal/parasitic/marginal disease.*

Supplementary table 9. Causes of concurrent ocular trauma	
Pre-C19	**Y2020**
Garden related x 4 Work related x 3 Finger x 2 Eye dropper x 1	Finger x3 Not specified x 2 Fist (assault) x1 Wall/pillar x1 Elastic band from mask x 1

Abbreviations: Pre-C19, Pre-COVID-19 Years (2017,2018,2019); Y2020, year 2020

APPENDIX