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1 **The Role of Fungi in Fungal Keratitis**

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20

21 **Abstract:**

22 Fungal keratitis (FK) accounts for approximately half of the microbial keratitis encountered in low
23 middle income countries (LMICs) and predominantly affect the working rural-poor. FK causes
24 significant morbidity with the majority of patients left with moderate or worse visual impairment
25 and approximately 25 % requiring expensive and often unsuccessful surgical interventions. The
26 severity of FK and the resultant corneal damage or resolution can be attributed to i) the virulence
27 and bioburden of the fungal pathogen, ii) the host defense mechanism and immune response and iii)
28 sub-optimal diagnostics and anti-fungal treatment strategies. This review provides a comprehensive
29 overview of the multifaceted components that drive FK progression and resolution, highlighting
30 where knowledge gaps exist and areas that warrant further research.

31 **Keywords:**

32 Cornea; Fungi; Keratitis; Virulence; Host-pathogen interactions; Diagnosis; Anti-fungal Treatment

33 **Abbreviations:**

34 CXL Corneal cross-linking

35 FK Fungal keratitis

36 IVCM *In vivo* confocal microscopy

37 LMIC Low middle income country

38 MBP Mannose binding protein

39 MMP Matrix metalloprotease

40 MUTT Mycotic Ulcer Treatment Trial

41 NETs Neutrophil extracellular traps

42 NGS Next generation sequencing

43 PAMP Pathogen associated molecular pattern

44 PDT Photodynamic therapy

45 PRR Pattern recognition receptor

46 PTX3 Pentraxin 3

47 ROS Reactive oxygen species

48 SigA Secretory Immunoglobulin A

49 SLIPI Secretory leucocyte protease inhibitor

50 SP-A/D Surfactant protein A/D

51 TLR Toll-like receptor

52 TPK Therapeutic keratoplasty

53 ZAG Zinc-α-glycoprotein

54

55 **1. Introduction**

56 The incidence of microbial keratitis in the developing world has increased to epidemic proportions
57 prompting cornea specialists to recommend the status of 'Neglected Tropical Disease' to this
58 condition (Ung et al., 2019a). Fungi have been implicated in the disease with increasing frequency,
59 accounting for 1-45 % of infectious keratitis depending upon the geographic distribution (Garg,
60 2012; Gower et al., 2010). This is most evident in regions where a significantly high proportion of the
61 populations are involved in agrarian activities (Lalitha et al., 2015; Whitcher et al., 2001). Whilst
62 filamentous fungi (*Fusarium* and *Aspergillus*) are the most common etiological agents causing fungal
63 keratitis (FK) in tropical regions, yeasts like *Candida* play an important role in temperate climates
64 (Srinivasan, 2004). The clinical outcomes of FK are worse than bacterial keratitis due to delayed
65 diagnosis, inappropriate use of antibiotics and/or steroids, the virulence of the organism, as well as a
66 limited choice of clinically approved antifungal drugs (Prajna et al., 2012). There have been no new
67 FDA approved treatments for this condition since the introduction of Natamycin in the 1960's
68 (Austin et al., 2017). Even where adequate treatment is provided, a quarter of the patients with FK
69 will fail medical treatment, be left with moderate or worse visual impairment and/or require surgical
70 interventions like therapeutic keratoplasty (TPK) (Khor et al., 2018; Prajna et al., 2012).

71 At least 166 genera and 144 species of fungi have been reported to cause human FK including over
72 100 genera of filamentous fungi, 18 genera of yeasts or yeast-like fungi, and 6 genera of dimorphic
73 fungi (Jones et al., 1970; Karsten et al., 2012, Thomas and Kaliamurthy, 2013). These fungi may be
74 newly introduced from the environment, or potentially arise from the ocular microbiome. Next
75 Generation Sequencing (NGS) is beginning to enable novel insights into the host fungal ocular
76 microbiome in both health and FK (Prashanthi et al., 2019, Shivaji et al., 2019, Wang et al., 2020). In
77 health, up to 94 distinct fungal genera have been identified at the ocular surface through NGS. In
78 these studies, the predominant phyla identified were *Basidiomycota* and *Ascomycota*, which were
79 present in all positive samples, and thus may constitute the core fungal microbiome at the ocular

80 surface within these populations. Prashanthi *et al* recently conducted NGS analysis on samples
81 obtained from FK and healthy control ocular swabs. They found that in FK, the relative abundance of
82 *Ascomycota* increased, whilst that of *Basidiomycota* decreased, and that overall the alpha diversity
83 indices decreased in the FK samples compared to healthy controls (Prashanthi et al., 2019). The
84 implications of this dysbiosis on FK progression are not yet well understood, and are beyond the
85 scope of this review.

86 Of importance to ophthalmologists in the diagnosis and treatment of FK are the differences in
87 geographic prevalence, risk factors, pathogenesis, distinctive signs of keratitis and antifungal
88 susceptibility of filamentous fungi and yeasts. Here we will consider the biology of FK, examining the
89 virulence mechanisms of the pathogen (Section 2), the host defense mechanisms (Section 3), host-
90 pathogen interactions (Section 4), the clinical features of disease (Section 5), diagnostic methods
91 (Section 6), and treatment strategies (Section 7).

92 **2. Fungal Virulence**

93 In contrast to systemic fungal infections which typically affect immunocompromised hosts, FK is able
94 to develop in both the immunocompromised and immunocompetent (Karthikeyan et al., 2011). The
95 virulence characteristics of the fungal pathogen and stages of disease progression can be broadly
96 grouped as i) immune evasion, ii) adhesion, iii) invasiveness, iv) toxin production, and v) biofilm
97 formation (Figure 1).

98 2.1 Immune evasion

99 All of the major fungal pathogens produce asexual spores (conidia), which are introduced to the
100 tear-film and ocular surface from the environment. The cell-surface of *Aspergillus* and *Fusarium*
101 conidia are covered by a protective hydrophobin and rodlet layer which aids in shielding of the
102 highly immunogenic fungal cell-surface proteins β-glucan and α-mannon (known as pathogen
103 associated molecular patterns (PAMPs)) from immune cell recognition (Aimanianda et al., 2009;

104 Carrion Sde et al., 2013; Fuchs et al., 2004). Absence of the gene (*rodA*) which encodes the rodlet

105 proteins has been shown to increase the susceptibility of fungi to the immune response (Hohl and

106 Feldmesser, 2007; Thau et al., 1994). However, despite the shielding layer, conidia may still be

107 recognized by host soluble mediators such as complement factor C3, SP-A and SP-D, as discussed

108 below (Section 3.2) (Aimanianda et al., 2009; Blango et al., 2019).

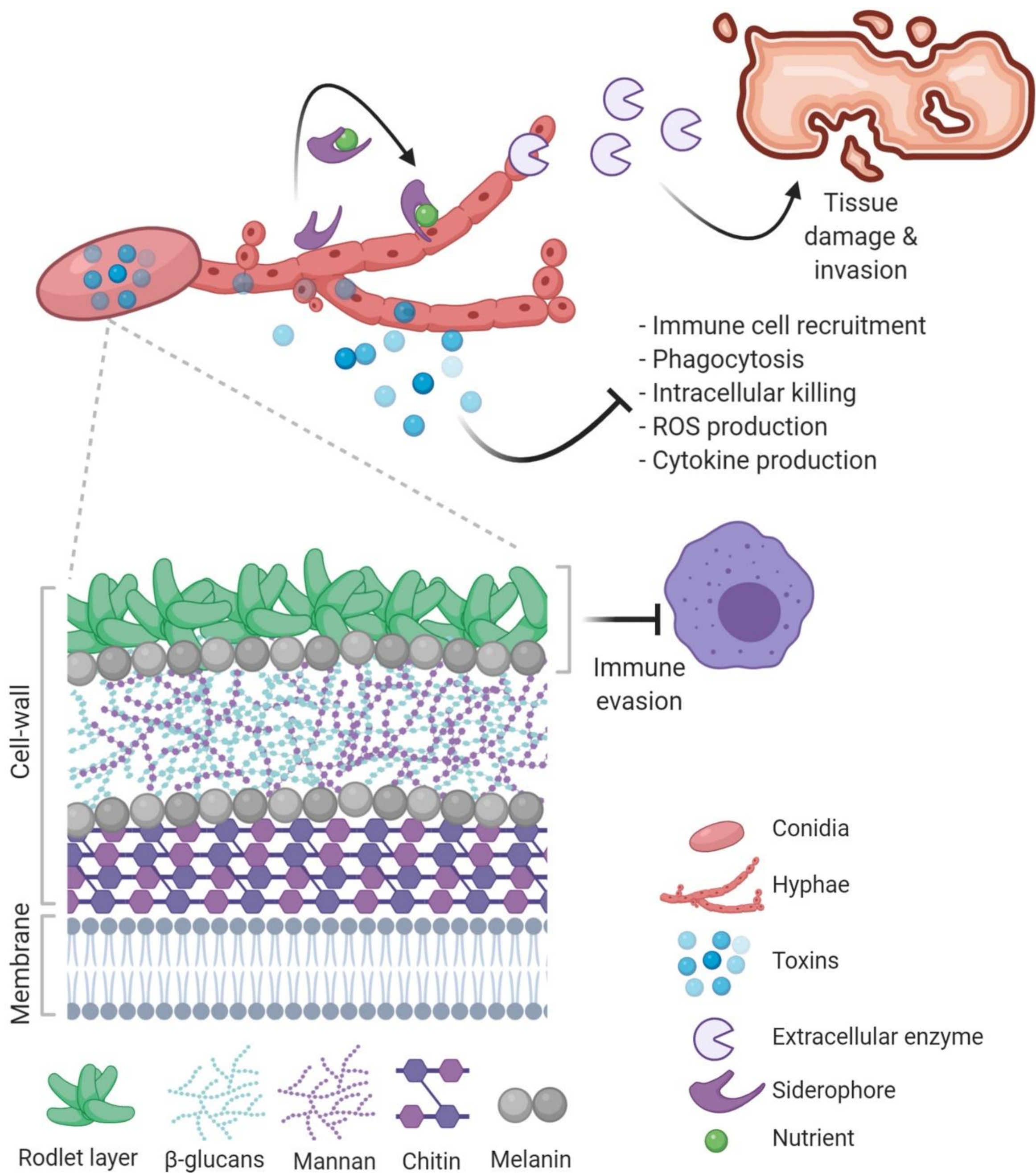


Figure 1. Mechanisms of fungal virulence

109 Additionally, fungi have melanin pigments in their cell-wall adjacent to the rodlet layer (Langfelder et
110 al., 2003). Dihydroxynaphthalene (DHN)-melanin and dihydroxyphenylalanine (DOPA)-melanin are
111 the two main types of melanin pigments in the fungal cell wall (Butler and Day, 1998; Wheeler and
112 Bell, 1988), and *in vitro* and *in vivo* studies have shown that the presence of these pigments provides
113 protection against environmental UV radiation damage and immune cell phagocytosis (Jahn et al.,
114 1997; Thywißen et al., 2011). Melanin can also block complement factors, such as C3 from binding to
115 fungal antigens and thus reducing complement mediated opsonization (Brakhage and Liebmann,
116 2005; Tsai et al., 1998). These pigments can also resist the fungicidal effect of antifungal drugs like
117 terbinafine (Almeida-Paes et al., 2016) and amphotericin B (Mario et al., 2016). The concentration of
118 melanin pigments decreases with the germination of conidia (Youngchim et al., 2004), thus the
119 rodlet layer and fungal melanin production play a crucial role in the survival in the conidial stage of
120 growth.

121

122 2.2 Adhesion

123 Hydrophobins and other conidia cell-surface proteins mediate host cell adherence. The outer fibril
124 layer of yeast and filamentous fungal conidia is comprised of the lectin-like proteins mannan and
125 galactomannan, which recognize mannose glycoproteins within the corneal epithelial cell membrane
126 (Blango et al., 2019). It has been demonstrated that corneal epithelial abrasion leads to increased
127 expression of cell-surface mannose glycoproteins as part of the wound healing response, thus
128 enhancing the availability of these cell-surface receptors (Zieske and Gipson, 1986). The corneal
129 epithelium also has other fungal binding sites, such as fibronectin, collagen and laminin (Coulot et
130 al., 1994).

131 2.3 Morphogenesis

132 Morphogenesis is the ability of the fungal pathogens to switch from yeast form to hyphal form.
133 While the pathogen can disseminate more efficiently during the yeast stage, the hyphal forms are
134 well adapted for invading and damaging tissues (Saville et al., 2003; Vila et al., 2017). Following
135 adhesion to the corneal epithelium and a favorable microenvironment, such as nutrient availability
136 and temperature, the conidia swell and begin germination, producing fungal hyphae (Beauvais and
137 Latgé, 2018). The hyphae are able to grow and pass through the epithelium, into the stroma and
138 eventually gain access to the anterior chamber if left unabated. *Candida* and filamentous fungi have
139 also been shown to invade the corneal epithelium via endocytosis, which is mediated by invasion
140 proteins and through the disruption of epithelial cell tight-junctions by proteolytic digestion
141 (Sheppard and Filler, 2014). Fungal invasiveness is related to fungal load and inversely proportional
142 to the host immune response (Vemuganti et al., 2002). Whilst conidia are relatively inert to host
143 immune surveillance mechanisms, the protective outer layers are disrupted during germination,
144 exposing the inner polysaccharides, which are much more immune-stimulatory (discussed in Section
145 4.2).

146 2.4 Production of mycotoxins and extra cellular enzymes

147 In addition to the physical disruption of the corneal epithelium, stroma and endothelium caused by
148 the fungal hyphal growth, fungi are also able to produce virulence factors (extracellular enzymes and
149 secondary metabolites (Hohl and Feldmesser, 2007)) with a broad range of roles, which ultimately
150 contribute to their invasiveness, primarily through tissue degradation (Mellon et al., 2007; Park et
151 al., 2013; Shibuya et al., 2006). Mycotoxin production and action is known to vary between isolates
152 and may be differentially expressed between *in vitro* and *in vivo* conditions (Naiker and Odhav,
153 2004). A study by Selvam *et al* identified 637 extracellular proteins across their *Aspergillus* clinical FK
154 isolates grown *in vitro* (Selvam et al., 2015). The majority of identified secreted proteins are
155 proteases which can degrade host tissue, such as MMPs (matrix metalloprotease, collagenase) and
156 serine and cysteine proteases (Balakrishnan Sangeetha et al., 2020; Monod et al., 2002; Yike, 2011;

157 Zhu et al., 1990). Other extracellular enzymes include nucleases, oxidases, catalases, phosphatases,
158 and peptidases (Ibrahim-Granet et al., 2008). Together these degrade complex macromolecules and
159 provide nutrients (such as amino acids, lipids and metals such as iron, zinc, manganese and copper)
160 for fungal growth which are sequestered through siderophores (high affinity metal binding
161 compounds) secreted by the fungi (Cassat and Skaar, 2013). Proteases secreted by fungi may also
162 induce the production and recruitment of pro-inflammatory cytokines and host proteases, affecting
163 the protease/anti-protease balance resulting in enhanced tissue damage (Yike, 2011).

164 The toxins produced by *Aspergillus* spp. include aflatoxins, gliotoxin A, fumagillin and helvolic acid
165 (Hedayati et al., 2007). *Fusarium* spp. produces nivalenol, T-2 toxin, deoxynivalenol,
166 diacetoxyscirpenol, fusaric acid and zearalenone (Aboul-Nasr et al., 2013; Raza et al., 1994). These
167 toxins inhibit phagocytosis, intracellular killing, cytokine production, antigen presentation and the
168 production of reactive oxygen species (ROS) by macrophages. In addition, they may play a role in
169 inhibiting the function of T-cell (Cusumano et al., 1990; Kupfahl et al., 2008).

170 Aflatoxin B1 is an important mycotoxin produced by *Aspergillus* and is acutely and chronically toxic
171 to animals and humans. Its production by *A. flavus* isolates obtained from FK patients has been
172 shown to be highly variable, but increased compared to *A. flavus* collected from the environment
173 when grown *in vitro* (Leema et al., 2010). Proteases from *Candida* have been shown *in vitro* to
174 degrade complement component C3 in human serum (Kaminishi et al., 1995). Furthermore, fungal
175 serine proteases have recently been shown to cleave Dectin-1, an important immune cell-surface
176 receptor of β-glucan, which is highly abundant in the fungal cell wall (Griffiths et al., 2018).

177 Despite the abundance and broad range of fungal secondary metabolites and extracellular enzymes
178 identified and characterized within *in vivo* studies, caution must be taken when extrapolating to
179 what may be happening within active FK. It is known that strains differentially express proteases,
180 and these expression levels may differ between *in vitro* and *in vivo* characterization. For example, a
181 study by Gopinathan *et al* demonstrated that whilst the filamentous fungi included in their study

182 secreted high levels of serine proteases *in vitro*, serine proteases were not detectable from the
183 cornea of infected rabbits (Gopinathan et al., 2001).

184 2.5 Biofilm formation

185 Biofilms are three-dimensional structures formed by a single or multiple microbial flora by producing
186 an extracellular polymer matrix on biotic or abiotic surfaces (Sandai et al., 2016). There are several
187 genera of fungal pathogens capable of forming biofilms (Sardi Jde et al., 2014) such as *Candida* spp.
188 including *C. albicans* (Al-Fattani and Douglas, 2006; Dongari-Bagtzoglou et al., 2009), *C. tropicalis*, *C.*
189 *parapsilosis*, and *C. glabrata* (Harriott et al., 2010), *Aspergillus* spp. (Silva et al., 2011) and *Fusarium*
190 spp. A major outbreak of contact lens associated *Fusarium* keratitis in 2005-2006 was attributed to
191 *Fusarium* biofilm formation on contact lens (Chang et al., 2006; Donnio et al., 2007; Dyavaiah et al.,
192 2007; Saw et al., 2007).

193 The role of fungal biofilm and the extracellular matrix is manifold. Biofilms promote fungal adhesion
194 and structural stability, whilst protecting the fungi from external threats. When compared to free-
195 living cells, biofilms are exhibit different phenotypic behaviors in growth rate, changes in gene
196 expression and are often highly resistant to antifungal treatments and the host immune system
197 (Hirota et al., 2017; Mukherjee and Chandra, 2004; Sandai et al., 2016, Ranjith et al., 2018).

198 The biofilm extracellular matrix is comprised of complex and heterogeneous mixtures of proteins,
199 carbohydrates, lipids and nucleic acids, with interspecies variation in composition and function of
200 individual components – although many of the macromolecule functional roles in biofilm are
201 currently poorly understood (Zarnowski et al., 2014, Gulati and Nobile, 2016). Biochemical analysis
202 of the extracellular matrix of *C. albicans* biofilm by Zarnowski *et al* has shown that it comprised
203 primarily of proteins (55 %). Proteomic analysis determined that these extracellular proteins were
204 primarily involved in metabolism and metabolic pathways, and thus may digest extracellular
205 biopolymers as an energy source within the biofilm. The same study determined that 25 % of the
206 matrix biomass was comprised of carbohydrates, including those from *Candida* (e.g. polysaccharides

207 β-1,3-glucan, β-1,6-glucan, branched mannan), but primarily from the animal host, highlighting the
208 importance of host derived factors in biofilm formation. Lipids were present at 15 % and nucleic acid
209 at 5 %. The nucleic acid present is largely non-coding DNA, which provides a structural scaffold and
210 protection from external threats, including antifungals. The role that lipids play in biofilm matrix has
211 been largely unexplored (Nett and Andes, 2020).

212 The protein expression profile of a *Fusarium falciforme* FK isolate in biofilm compared to planktonic
213 growth has recently been reported (Calvillo-Medina et al., 2019). 19 proteins were overexpressed in
214 biofilm, and 6 were expressed uniquely in biofilm. Several of the enzymes identified are involved in
215 glycolysis/gluconeogenesis and pentose phosphate pathways, and a number of proteins identified
216 have been shown to act as ligands to host cellular components, and as such, may promote
217 angiogenesis, adhesion, nutrient acquisition and immune evasion. This study also characterized six
218 distinct stages of biofilm; i) adhesion, ii) filamentation of conidia, iii) elongation of hyphae, iv)
219 formation and thickening of matrix, v) conidiation and further biofilm formation and vi) maturation.
220 The final three stages occurred following nutrient depletion from the microenvironment.

221 Similarly, these stages of biofilm development and maturation were recently characterized for a
222 *Fusarium solani* FK isolate (Córdova-Alcántara et al., 2019). It was shown that specific inhibitors of
223 matrix constituents (carbohydrates, proteins, lipids and nucleic acids) reduced biofilm formation,
224 and that mature biofilm conferred resistance to antifungals and UV irradiation.

225 Interestingly, not all FK fungal isolates are able to form biofilms. Recent studies by Ranjith *et al*
226 (Ranjith et al., 2017, 2018) determined that 42-47% of *Candida* FK isolates were unable to form
227 biofilm, underscoring the heterogeneity of pathogens causing FK. In a study of 7 *C. albicans* isolates,
228 four were able to form biofilm and only one exhibited multi-antifungal resistance, whereas the other
229 three remained susceptible (Ranjith et al., 2018). 27 genes involved in virulence and biofilm
230 formation were found to be temporally upregulated in the biofilm-forming *Candida* compared to the

231 non-biofilm formers, thus targeting of these genes across the stages of biofilm development could
232 serve as a therapeutic strategy.

233

234 ## 3. The Host Defense Mechanism

235 The cornea is exposed to the external environment and continuously comes into contact with
236 irritants and potential pathogens. This does not lead to infection in the vast majority of cases due a
237 complex system of host defenses, including physical, chemical and host immune derived factors and
238 cells.

239 ### 3.1 Physical defenses

240 The physical barriers exist to mechanically prevent injury to the ocular surface and intraocular space.
241 The physical barriers include i) the eyelids and blinking action, ii) the tear film and iii) the corneal
242 epithelium. The tear film has multiple roles to play as an effective defense mechanism; acting as a
243 lubricant to aid blinking, which washes microbes away from the cornea through shear stresses (the
244 mechanics of which are discussed elsewhere (Masterton and Ahearne, 2018; Pflugfelder and Stern,
245 2020)); it prevents the corneal epithelium from drying out, and is a major source of nutrients and
246 oxygen to the avascular cornea. Additionally the tear film has antimicrobial properties and can
247 modulate the innate response of the corneal epithelium (discussed below).

248 The corneal epithelium is the outermost layer of the cornea, and serves as the primary structural
249 barrier directly protecting the visual apparatus against invading pathogens. It is approximately 50
250 µm thick (5-7 cells deep) and comprises of tightly packed non-keratinized stratified squamous
251 epithelial cells (Ehlers et al., 2010; Sridhar, 2018). The corneal epithelium protects the underlying
252 corneal layers (the stroma and endothelium) from abrasions (including from eyelid motion, tear
253 fluid, contact lens use, eye rubbing), and from infiltration of microbes, whether they be from the
254 host bacterial or fungal ocular microbiome (Huang et al., 2016; Prashanthi et al., 2019) or

opportunistic pathogens. The corneal epithelium is not only a physical barrier to invading microorganisms; the cells are able to directly generate and secrete molecules which are both antimicrobial and modulate the immune response.

The most common risk factor for the development of FK is a breach of the corneal epithelium, usually sustained by corneal trauma or abrasion (often by vegetative matter). This not only alters the structural profile of the corneal surface, but also leads to an alteration in the expression of surface and secreted proteins and immune modulators, and thus skews the fine balance of host defenses at the cornea surface and within the tear film. The use of topical corticosteroids is also another major risk factor.

### 3.2 Chemical Molecular Defenses

The ocular surface is constantly exposed to the environment, and thus to opportunistic and pathogenic bacteria and fungi. Tear fluid is a complex aqueous solution, and as already described serves a number of roles. It comprises of three layers; closest to the cornea is the mucin layer, then the middle aqueous layer, which together form the bulk of the tear-film, and finally the superficial lipid layer (Mantelli and Argüeso, 2008). Whilst an antimicrobial role for tear-lipids has been demonstrated *in vitro* (Mudgil, 2014), the anti-fungal role of lipids has not been studied and will not be discussed further.

The aqueous layer of the tear film is highly proteinous, with over 2500 unique proteins and almost 100 metabolites identified within samples collected from healthy eyes, abundant from pg $mL^{-1}$ to mg $mL^{-1}$ (Ananthi et al., 2013; Chen et al., 2011; Kandhavelu et al., 2017; Zhou et al., 2012). The liquid of the aqueous layer, which contains the majority of tear film proteins, is secreted from the lacrimal gland, with other proteins arising directly from the corneal and conjunctival epithelia, serum and from neutrophils, which are resident within closed-eye tears (Prashar, 2019).

278 The proteins found within the tear film have a diverse range of mechanisms by which they protect
279 the cornea from invading microorganisms. These range from pathogen aggregation, to decoy
280 receptors, to direct killing, to nutrient scavenging, and to immune cell recruitment, and the
281 expression of many of these proteins has been found to be under or over expressed during FK
282 (Azkargorta et al., 2017; Kuo et al., 2019). It is also important to consider that many of the proteins
283 found within the tear film are likely to have dual roles, and/or act synergistically together to protect
284 the cornea from microbial invasion. Antimicrobial peptides (AMPs) are a major class of protective
285 proteins present within the healthy tear film with immunomodulatory effects as well as direct
286 antimicrobial action, and have the ability to work against both bacteria and fungi (McDermott, 2013;
287 Mohammed et al., 2017; Oshiro et al., 2019). They are positively charged and thus they are able to
288 interact directly with the negatively charged surface of fungi, causing disruption through
289 electrostatic actions. AMPs can be classified as membrane or non-membrane disruptive, and often
290 the direct mechanism of action is not yet fully understood (Choi et al., 2012; Oshiro et al., 2019).

291 Lysozyme, lipocalin and lactoferrin are the most abundant proteins within the tear film, together
292 accounting for 80 % of total protein content and are functionally interdependent (Prashar, 2019).
293 The action of **lysozyme** on fungi is two-fold. Firstly it is able to hydrolyze N-glycosidic linkages within
294 the cell wall, and secondly, through cationic-protein interactions is able to disrupt the fungal cell
295 membrane (Hanstock et al., 2019; Marquis et al., 1982; Samaranayake et al., 2001), together these
296 actions lead to cell lysis, and thus cell death.

297 **Lactoferrin** and **lipocalin** both interfere with the ability of fungi to acquire iron. Iron is essential for
298 the redox reactions of fungal antioxidants, and poor availability of iron prevents conidia from
299 germinating, and thus lactoferrin and lipocalin have fungistatic effects. Whilst lactoferrin chelates
300 iron within the environment directly (Fernandes and Carter, 2017; González-Chávez et al., 2009),
301 lipocalin is able to bind directly to siderophores secreted by fungi (Fluckinger et al., 2004; Leal et al.,
302 2013). Lactoferrin has also been shown to have direct antifungal action by a similar mechanism to

303 lysozyme, whereby lactoferrin interacts directly with the fungal cell-surface, resulting in damage to
304 the cell membrane, membrane permeability and a fungal apoptosis-like process (Andrés et al., 2016;
305 Farnaud and Evans, 2003).

306 **α** and **β-defensins** are small, cysteine rich, non-glycosylated cationic and amphipathic peptides.
307 Whilst β-defensins are present at the ocular surface, α-defensins are released by neutrophils (which
308 are present in closed-eye tears, or recruited following initiation of FK). Their activity has mostly been
309 studied in the context of *Candida* spp*.* infections, and their mechanisms of action are not fully
310 understood, however defensins have been shown to block adhesion to human epithelium, and
311 prevent *Candida* biofilm formation (Chairatana et al., 2017; Haynes et al., 1999).

312 **Histatins** are a group of histidine rich, small peptides with fungicidal activity. Their modes of action
313 again are not fully understood, however it is known that the histadin-5 peptide is internalized into
314 the *Candida* cell, where it causes a decrease in mitochondrial ATP synthesis, the efflux of ATP and
315 other ions, and promotes the generation of reactive oxygen species (ROS) (Swidergall and Ernst,
316 2014). It is important to note that *C. albicans* has been shown to evade the fungicidal action of
317 histatins through influx/efflux pumps, activation of stress response pathways and secretion of
318 proteases which degrade histatin (Swidergall and Ernst, 2014).

319 **Psoriasin** is a member of the S100 family of calcium binding proteins. It has been shown to have
320 differing effects against *Candida* and filamentous fungi. Psoriasin contributes to inhibition of *Candida*
321 adhesion to epithelial cells, but does not directly kill it (Brauner et al., 2018). However, in its
322 cysteine-reduced form, Psoriasin is able to kill filamentous fungi. This is due to selective
323 internalization, followed by intracellular zinc binding and the subsequent initiation of apoptosis-like
324 cell death (Hein et al., 2015). Psoriasin is not seemingly internalized by *Candida*.

325 Secretory leukocyte protease inhibitor (**SLIPI**) and **elafin** are low molecular weight inhibitors that
326 control the enzymatic activity of neutrophil serine proteases. Specifically, SLIPI inhibits human

327 neutrophil elastase and cathepsin G, whereas Elafin is able to inhibit human neutrophil elastase and
328 proteinase 3 (Zani et al., 2009). Whilst SLIPI and Elafin have a role in protecting the ocular surface by
329 dampening these hydrolytic, tissue destructive proteases, SLIPI and Elafin also have been shown to
330 have direct anti-fungal roles (Baranger et al., 2008; Sallenave, 2010). The anti-fungal mode of action
331 of the two proteins is most likely due to cationic disruption of the fungal membrane, although this is
332 not yet fully understood.

333 **Secretory Immunoglobulin A** (sIgA) is the major antibody present in tear fluid. sIgA binds directly to
334 lectin-type adhesion molecules on the fungal cell-surface, preventing binding of the cell to the
335 corneal epithelium and promotes pathogen aggregation, leading to direct removal by the tear film
336 (McDermott, 2013; Prashar, 2019).

337 **Mucins**, present in the mucus layer of the tear film, are secreted from specialized goblet cells in the
338 conjunctival epithelium and from the corneal and conjunctival epithelium directly. Transmembrane
339 mucins are able to anchor to corneal epithelium and act not only as a support to stabilize the tear
340 film, but also act as decoy receptors on the cornea surface for invading organisms, thus trapping
341 pathogens to facilitate their removal (Dartt and Willcox, 2013; Mantelli and Argüeso, 2008). It has
342 also been reported that positively charged proteins, such as lysozyme and SLIPI, along with sIgA may
343 also accumulate within the mucus layer of the tear film, forming a protective antimicrobial coat
344 (Sack et al., 2001); i.e. these proteins work synergistically. The pathogen is trapped by mucin, which
345 is then killed by AMPs or other proteins outlined here, or aggregated for mechanical clearance by
346 blinking.

347 As well as the multitude of proteins within the tear film which may act directly to kill or clear the
348 invading organism, there are also a number of mechanisms which aid in fungal recognition and
349 subsequent killing or removal through downstream pathway initiation. The examples of C-type
350 lectins and complement component 3 are described below.

351 **C-type lectins** are proteins which bind to carbohydrates. Their role is to moderate inflammatory
352 responses and thus limit infections at the ocular surface. The most abundant lectins within the tear
353 film which can affect fungal responses are mannose binding proteins (MBP) and surfactant proteins
354 (SP-A and SP-D). These lectins recognize pathogen associated molecular patterns (PAMPs) on the
355 yeast-like and filamentous fungi cell-surface, such as mannose (Pandit et al., 2012), eliciting a wide
356 range of responses, including aggregation and opsonization, increasing killing efficacy during
357 phagocytosis, and complement activation (Brummer and Stevens, 2010; Gupta and Surolia, 2007).

358 **Complement component 3** (C3) plays an essential role in the control of opportunistic fungal
359 infections, and it is the most abundant complement protein found in tears. It may be activated by all
360 three of the complement pathways - classical, alternative and lectin, with the latter two the most
361 significant in this context. Whilst fungi are resistant to complement mediated killing directly,
362 activation of C3 leads to fungal opsonization, and ultimately fungal clearance (Tsoni et al., 2009). To
363 prevent excessive tissue damage, complement must be tightly regulated, and a number of
364 complement inhibitors are also found in tears. Moreover, both lysozyme and lactoferrin have been
365 shown to inhibit the complement pathway to varying degrees (Willcox et al., 1997).

366

367 **4. The Organism - Host interplay**

368 4.1 Risk Factors

369 The morbidity associated with FK is often the consequence of shifted organism - host interplay; with
370 the breakdown of the host defense due to anatomical and physiological factors, allowing access of
371 the fungi to the denuded corneal epithelial surface (Srinivasan et al., 1997). Ocular trauma and
372 corneal abrasion, commonly attained whilst performing agrarian activities is a major risk factor
373 encountered in more than half of filamentous FK cases (Shah et al., 2019). Agricultural activity, like
374 thrashing of paddy, releases a high amount of viable fungal spores, which when coupled with a

375 corneal trauma sets up a perfect situation for the disease to occur (Atluri and Murthy, 2002; Uddin
376 and Chakraverty, 1994). Indeed, there is a higher incidence of filamentous FK occurring during the
377 windy and harvest seasons (July and January) in South India (Lin et al., 2012). Whilst filamentous FK
378 associated with agricultural ocular injury predominantly affects workers in low-middle income
379 countries (LMICs), there have been instances of outbreaks of filamentous FK in high resource
380 settings through use of contaminated contact lens solutions (Chang et al., 2006; Saw et al., 2007).

381 While the bulk of filamentary FK occurs in immunocompetent individuals with ocular injury, there
382 have been studies reporting increased incidence in an immunocompromised patient setting.
383 Filamentous fungi are the most common FK fungi associated with HIV infection as reported from
384 African countries (Burton et al., 2011). In a study from Tanzania, 77 % of patients with FK were
385 positive for HIV infection (Mselle, 1999), whilst HIV was reported as the most common risk factor
386 identified in approximately 25 % of the FK cases in a study from New York (Ritterband et al., 2006).

387 Infections due to *Candida* spp. are more commonly seen in immunocompromised patients, or those
388 with systemic illness, such as diabetes (Sengupta et al., 2012). Those with pre-existing ocular
389 conditions, such as decompensated corneas and post keratoplasty scenarios especially in interface
390 keratitis following lamellar keratoplasties are also at risk (Qiao et al., 2020; Sun et al., 2007).
391 Furthermore, Brothers *et al* demonstrated that tissue warming during endothelial keratoplasty
392 processing may be responsible for promoting *Candida* growth in donor rims (Brothers et al., 2017).
393 The topical use of corticosteroids and prior ocular surgery are also significant risk factors for both
394 filamentous and yeast-like FK development.

395 4.2 <u>Immune response to fungal invasion</u>

396 In normal eye health, the cornea is avascular and has relatively few resident macrophages and
397 dendritic cells dispersed throughout the epithelium and stromal layers, these are present in a
398 gradient from the lower density in the central cornea and increasing towards the limbus (Brissette-

399 Storkus et al., 2002; Hamrah and Dana, 2007; Mobeen et al., 2019; Palomar et al., 2019). The
400 resident leukocytes express a number of pattern recognition receptors (PRRs) on their cell-surface to
401 detect PAMPs on invading pathogens. Binding of these receptors to targets initiates signaling
402 cascades which ultimately increase the immune response, including neutrophil recruitment (Figure
403 2). During fungal infection, neutrophils comprise 95 % of the cellular infiltrate (Karthikeyan et al.,
404 2011; Leal et al., 2013), and infected corneas are characterized by progressive erosion and necrosis
405 of corneal tissue, a reduction in corneal epithelium and a disordered corneal stroma (Zhang et al.,
406 2018b).

407 The large size of fungal hyphae precludes from killing through **neutrophil** phagocytosis (Dursun et
408 al., 2003). Rather, the recruited neutrophils exert their anti-fungal activity by a number of other
409 ways. These include i) the regulation of hyphal growth through the generation of ROS, ii) neutrophil
410 extracellular traps (NETs), iii) iron acquisition through mechanisms such as lipocalin secretion, and

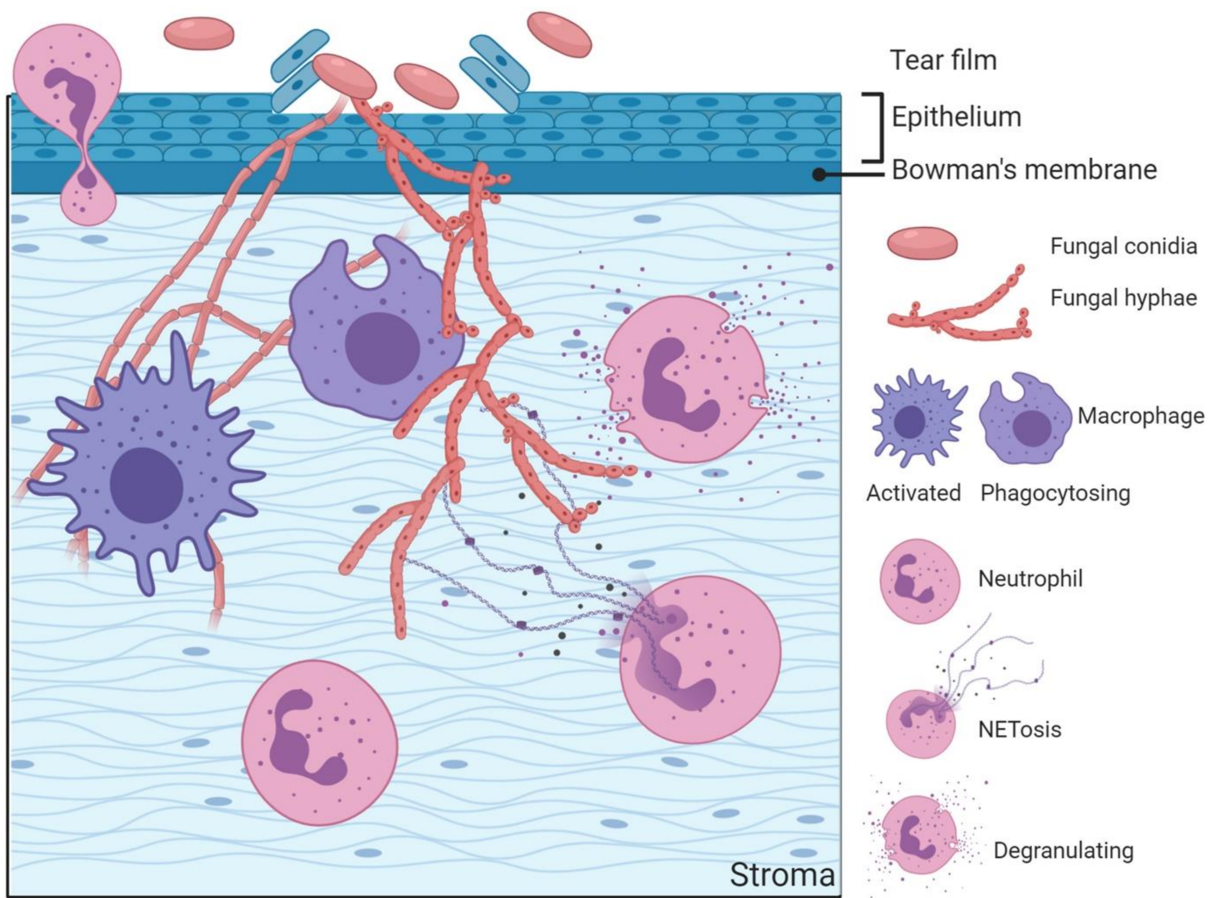


Figure 2. Fungal infiltration and immune cell recruitment/activation following epithelial damage. Not to scale.

411 metal chelation through calprotectin (Clark et al., 2016; Leal et al., 2013; Leal et al., 2012; Taylor et
412 al., 2014). Calprotectin accounts for approximately 40 % of cytosolic protein within neutrophils, and
413 upon neutrophil release (through degranulation and specific secretion), it is able to chelate zinc and
414 manganese from the environment, negatively impacting fungal germination and growth (Clark et al.,
415 2016). Furthermore, a subset of neutrophils have been shown to express IL-17, and these
416 demonstrate enhanced ROS generation and thus fungal killing compared to their counterparts
417 (Taylor et al., 2014). iv) Neutrophils may also mediate fungal killing through the release of acidic
418 mammalian chitinase (AMCase), which is able to hydrolyze chitin, the major fungal cell wall
419 component (de Jesus Carrion et al., 2019).

420 Membrane bound PRRs include **Toll-like receptors** (TLRs) and C-type lectins. TLR2 recognizes glucans
421 present within the cell wall of yeast-like and filamentous fungi, and TLR4 recognizes mannan on the
422 cell-surface of filamentous fungi (Redfern and McDermott, 2010; Yuan et al., 2010). Whilst activation
423 of TLR2 and TLR4 results in pro-inflammatory chemokine release, including IL-1β and IL-6, and
424 leukocyte recruitment from peripheral and limbal blood vessels (Guo and Wu, 2009) (Figure 3), TLR2
425 activation by *Candida* zymosan has also been shown to incite an anti-inflammatory response via IL-
426 10 production, and may be another fungal defense mechanism (Netea et al., 2006).

427 **Dectin-1** is a C-type cell-surface lectin of pivotal importance in the immune fungal response (Leal et
428 al., 2010; Salazar and Brown, 2018). Dectin-1 is expressed on the surface of macrophages and
429 dendritic cells, and binds to β-glucan exposed on the cell-surface of germinating conidia within the
430 corneal stroma (Figure 3). Dectin-1 then activates a Syk-CARD9-NFκβ intracellular signaling pathway
431 which ultimately triggers IL-1β and other pro-inflammatory cytokine release, NLRP3 inflammasome
432 activation, and ultimately results in neutrophil recruitment (Drummond and Brown, 2011;
433 Karthikeyan et al., 2011; Liu et al., 2015; Snarr et al., 2017). In addition to the intracellular signaling
434 cascade, Dectin-1 and TLR4 activation may induce phagocytosis of the ligand, and elicit a respiratory
435 burst through activated NADPH-oxidase, thus killing the pathogen (Leal and Pearlman, 2012).

436 Polymorphisms in TLR4 and Dectin-1 have both been shown to increase susceptibility of the host to
437 fungal infections (Bochud et al., 2008; Marakalala et al., 2011).

438 **Pentraxin 3** (PTX3) is a PRR expressed by corneal epithelial cells, as well as macrophages, dendritic
439 cells and neutrophils following *Aspergillus* exposure (Zhang et al., 2018a; Zhang et al., 2018b). PTX3
440 is able to recognize fungal spores, and upregulation of PTX3 within the corneal epithelium correlates
441 with the severity of infection. Expression of PTX3 has shown to be independent of Dectin-1
442 activation, but dependent on Syk signaling pathways. A role for TLR4 in the signaling pathway of
443 PTX3 has also been shown, however this has not yet been demonstrated in the context of FK (Jaillon
444 et al., 2014). Ultimately, PTX3 upregulation leads to the production of IL-1β, activation of other pro-
445 inflammatory cascades, increased phagocytic clearance by macrophages, and is thought to have a
446 non-redundant role in early infection.

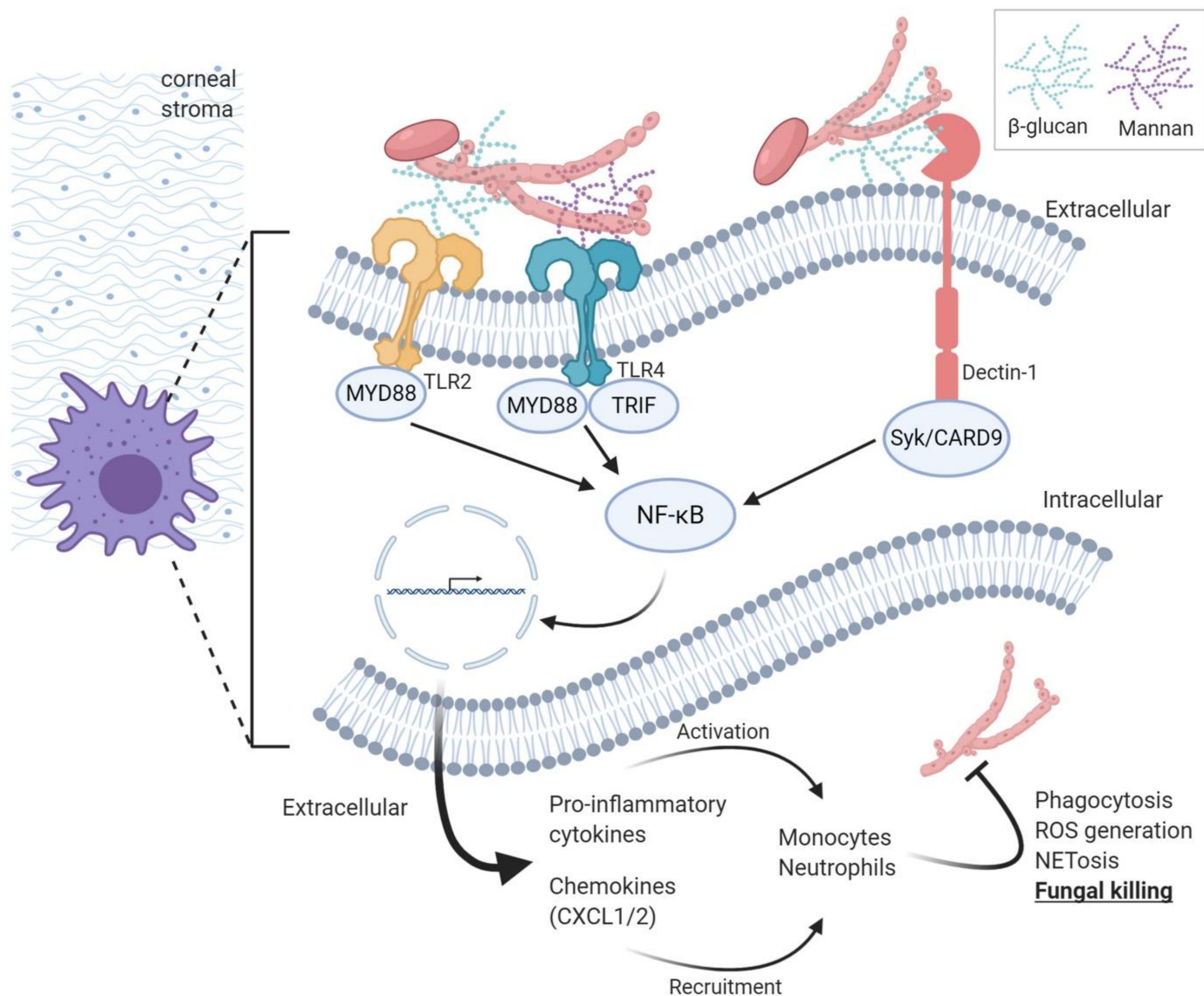


Figure 3. Signalling cascade by macrophage following fungal PAMP recognition. Not to scale.

447 4.3 Omics approaches to elucidating the clinical state-of-play

448 Much of our understanding of the host-pathogen interaction during FK has come from studying cell-
449 lines and clinical isolates *in vitro*, from *in vivo* animal models, or from extrapolating data acquired
450 from fungal-mucosal surface interactions at other mucosal epithelia within the human. In recent
451 years, “omics” approaches have emerged as an important tool for studying gene and protein
452 expression from human clinical samples, and have broadened our molecular understating of FK, and
453 served to validate some of the findings from the afore mentioned models. These have recently been
454 reviewed elsewhere (Azkargorta et al., 2017; Kuo et al., 2019), but some of the most significant
455 findings are outlined below.

456 Chidambaram *et al* examined gene expression within tears during late stage FK through
457 transcriptomics and reported that 291 genes were upregulated and 90 downregulated compared to
458 healthy controls (Chidambaram et al., 2017a). Unsurprisingly, the genes with the highest
459 upregulation were proinflammatory, involved in the immune response and specifically, neutrophil
460 chemotaxis. These included those encoding for *IL-1β*, the inflammasome *NLRP3*, *TNF*, multiple
461 chemokines, PRRs, including *TLR2* and *TLR4*, and *SYK.* Genes encoding complement proteins were
462 also upregulated, whereas complement regulator *Complement Factor H* was downregulated. Genes
463 involved in microbial killing were also upregulated, particularly those encoding ROS generation
464 processes. MMP9 is responsible for collagen degradation and is highly destructive to tissue; the gene
465 for *MMP9* was the most upregulated of all *MMP* genes. Genes involved in epithelial cell adhesion,
466 such as for the formation of cellular tight junctions were also downregulated.

467 Proteomics has enabled comparisons between infected and non-infected human eyes by identifying
468 and quantifying the levels of protein expression in tears, demonstrating translation of the
469 transcriptome, and can provide information about what is functioning within a snapshot of time. For
470 example, Kandhavelu *et al* identified 1223 proteins uniquely expressed in *A. flavus* FK tears
471 compared to healthy controls, 177 of which could be quantified with high confidence (Kandhavelu et

472 al., 2017). They found that all three complement pathways were represented at high levels in patient
473 tears, along with inhibitors and negative regulators of the complement pathway. Coagulation
474 cascade and wound healing proteins were only found in infected tears. Proteins associated with
475 NETs (neutrophil extracellular traps), MMPs (destructive proteases which degrade collagen and
476 basement membranes) and plasminogen were also present at higher levels in patient tears
477 compared to controls. They also saw upregulation of serine protease inhibitors, membrane attack
478 complex inhibitors and acute phase proteins. Parthiban *et al* also recently studied the proteome of
479 patients with *A. flavus* FK, and found similar expression profiles, with haptoglobin (a plasma
480 glycoprotein which binds free hemoglobin), alpha-1-antitrypsin (which controls activity of many
481 proteolytic enzymes), human serum albumin (indicating leakage from nearby blood vessels),
482 lactoferrin (iron binding) and apolipoprotein (lipid binding for transportation) all upregulated. They
483 saw downregulation of zinc-α-glycoprotein (ZAG), serotransferrin precursor (iron binding transport
484 protein), lipocalin (transport of small hydrophobic molecules), lacritin (an iron binding transfer
485 protein) and cystatin SN (an inhibitory protein which regulates proteolytic cathepsins) (Parthiban et
486 al., 2019).

487 ZAG has been characterized as a multidisciplinary protein, and has been implicated in lipid
488 metabolism (Hassan et al., 2008; Russell and Tisdale, 2011). Whilst it has been shown to be down
489 regulated in *A. flavus* FK (Ananthi et al., 2011; Parthiban et al., 2019), as well as other disease states
490 (Ihnatko et al., 2013; Lema et al., 2010), interestingly, it has been shown to be upregulated within
491 tears of *Fusarium* FK patients (Ananthi et al., 2013). Although Ananthi *et al* saw a differential
492 expression profile of ZAG in *Fusarium* FK compared to *A. flavus*, they similarly demonstrated
493 upregulation of haptoglobin, alpha-1-antitrypsin, apolipoprotein, lactoferrin and albumin; and
494 downregulation of cystatin SA, lipocalin and lacritin (Ananthi et al., 2013).

495 Together this data supports the findings from *in vitro* and *in vivo* models and indicates a highly pro-
496 inflammatory, proteolytic microenvironment from early to late stage FK. It is clear that tight

497 regulation of pro-inflammatory pathways to active resolution is required to strike the balance
498 between fungal clearance, and mitigation of permanent tissue damage, and this process is not yet
499 well understood.

500

501 **5. Clinical Features**

502 In contrast to bacterial keratitis, the symptoms of FK are often disproportionately less severe than
503 might be expected considering the size of the ulcer. This may be one of the reasons why patients
504 often present late to treatment centres, commonly with an advanced fungal corneal ulcer. Feathery
505 margins (Figure 4a) are the most characteristic clinical feature of FK and are well appreciated in the
506 early stages of infiltration (Dalmon et al., 2012). Other clinical features include a raised surface,
507 endothelial plaque, dry texture, and satellite lesions. While ring infiltrates can occur in fungal and
508 bacterial keratitis, it is 10 times more likely to indicate acanthamoeba keratitis, and multifocal
509 lesions are more commonly seen in acanthamoeba keratitis than fungal keratitis. In ulcers caused by
510 the dematiaceous fungi, there may be macroscopic pigment deposition over the surface (Kumar et
511 al., 2019). Keratitis caused by *Candida* may be more localized and have a collar button configuration,
512 often with a small ulceration and an expanding infiltrate (Sun et al., 2007). Infectious crystalline
513 keratopathy has also been reported with *Candida* spp. (Rhem et al., 1996). Interface keratitis in
514 lamellar keratoplasty due to *Candida* often presents with minimal inflammatory signs and
515 symptoms. In the initial stages, slight ocular pain and redness may be the only symptoms reported
516 by patients, with unaffected visual acuity. The cornea is usually clear with small (0.5-2 mm) single or
517 multiple whitish infiltrates seen at the graft–host interface. The anterior chamber is usually quiet
518 with no inflammation. Hypopyon is a common accompaniment with larger ulcers (Fontana et al.,
519 2019). The classical morphological changes of FK may not be appreciated in larger sized ulcers, and
520 microbial distinction based on clinical features is more challenging (Dahlgren et al., 2007, Dalmon et
521 al., 2012).

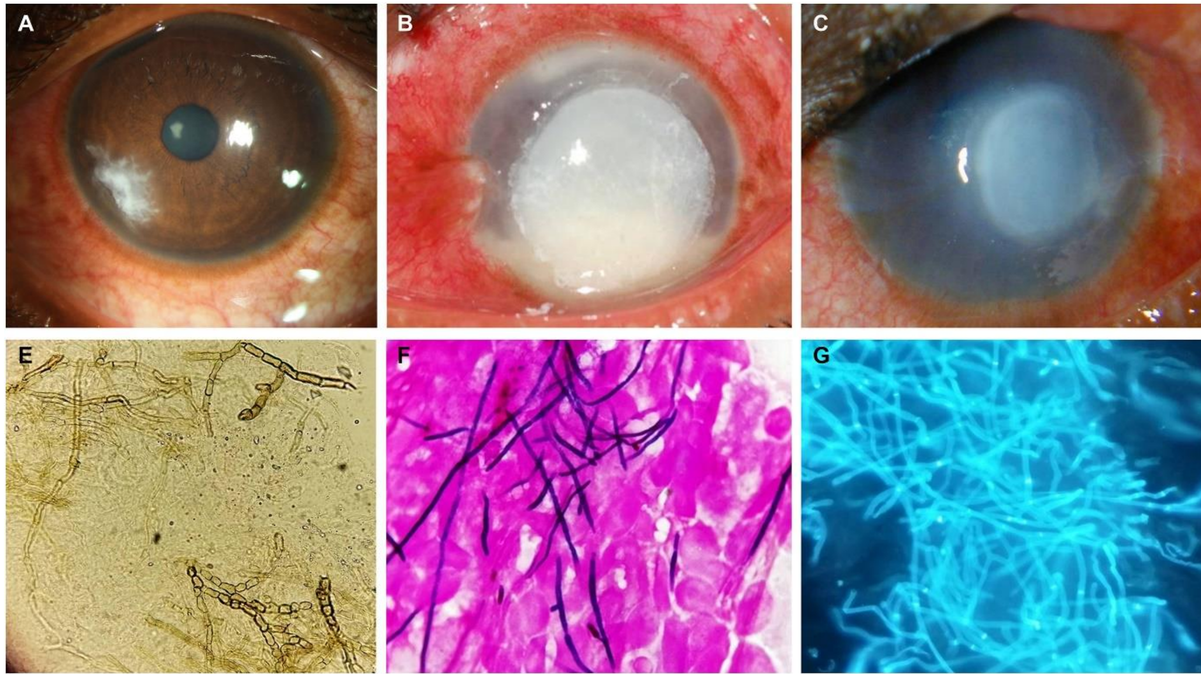


Figure 4. Clinical picture of A. early fungal keratitis with characteristic feathery margin, B. Late fungal keratitis indistinguishable from C. Bacterial keratitis. Microbial smear examination of branching fungal hyphae seen in D. Potassium hydroxide wetmount, E. Grams stain, F. Calcoflourwhite stain.

522 In spite of appropriate treatment, FK has higher odds for perforation and longer healing time than
523 bacterial keratitis (Prajna et al., 2013a). The poor prognostic factors identified are larger infiltrate
524 size at presentation, larger epithelial defect, ulcers caused by *Aspergillus*, presence of hypopyon and
525 smear positivity in spite of prior antifungal treatment (Lalitha et al., 2006). The MUTT (Mycotic Ulcer
526 Treatment Trial) II study defined a high risk case with high chances of perforation and TPK
527 requirement as an ulcer with geometric mean infiltrate size more than 6.63 mm, involving the
528 posterior one third of cornea with associated hypopyon (Prajna et al., 2017b). Polymicrobial keratitis
529 with fungus and bacteria are more challenging to treat, with a poorer outcome than FK and may
530 need early surgical intervention (Fernandes et al., 2015). In a longitudinal study comparing the visual
531 outcomes of bacterial and fungal corneal ulcers, the best corrected vision of 20/400 or worse at 4
532 years from the onset of infection was more common in patients with scars due to fungal ulcer
533 compared to scars of bacterial ulcer, even after successful antimicrobial treatment (Menda et al.,
534 2019).

535 **6. Diagnosis**

536 6.1 *In vivo* confocal microscopy

537 *In vivo* confocal microscopy (IVCM) has been used to identify fungal hyphae in the corneal stroma.
538 Fungal hyphae are seen as high contrast filaments with 4-6 µm thickness and 60-400 µm length
539 (Brasnu et al., 2007). Apart from the direct visualization of the fungal filaments, honeycomb
540 distribution of anterior stromal inflammatory cells in the absence of stromal bullae was significantly
541 associated with FK compared to bacterial keratitis (Chidambaram et al., 2018). IVCM is unable to
542 differentiate between *Aspergillus* and *Fusarium* based on branching angle, adventitious sporulation
543 or dichotomous branching characteristics. However, *Aspergillus* ulcers were associated with stromal
544 dendritiform cells, and *Fusarium* ulcers were associated with stellate appearance of interconnected
545 cell processes with nuclei (Chidambaram et al., 2017b; Chidambaram et al., 2018). IVCM enables the
546 depth of the corneal stromal infiltration with fungal hyphae and the response to treatment to be
547 monitored (Takezawa et al., 2010).

548 6.2 Microbiological Investigations

549 While clinical features may offer a clue, it may not be enough to differentiate fungal and bacterial
550 keratitis in all instances (Figure 4b and c), without the aid of microbiological investigations (Chang
551 and Chodosh, 2011; Dalmon et al., 2012; Kaufman and Wood, 1965; Thomas et al., 2005). This
552 assumes significance in a LMIC setting, where bacteria and fungi can cause infectious keratitis in
553 almost equal proportions. A careful and adequate specimen collection and an immediate access to
554 smear examination are the most important steps to get reliable microbiological confirmation.
555 Specimens are obtained by scraping the base and the edges of the ulcer under topical anesthesia
556 using a Kimura spatula. In deeper lesions, a corneal biopsy using 2-3 mm trephine may be required
557 for obtaining adequate specimen.

558 6.2.1 Conventional microbiology techniques: direct microscopy and culture

559 Direct microscopic examination and culture remain the gold standard for the aetiological diagnosis
560 of FK (Ficker et al., 1991; Sharma et al., 2002). Commonly used direct examination of corneal
561 scraping material are 10 % potassium hydroxide (KOH) wet mount, Gram stain (Bharathi et al., 2006;
562 Sharma et al., 1998; Vajpayee et al., 1993), Giemsa (Rosa et al., 1994), calcoflour white, periodic acid
563 Schiff, Gomori methenamine silver stain and lactophenol cotton blue (Chander et al., 1993; Chang
564 and Chodosh, 2011; Garg, 2012) (Figure 4). The 10 % KOH is a rapid, simple and inexpensive
565 procedure for detection of fungi. It has sensitivity in a range from 61-94 % and specificity of 91-97 %
566 for detection of fungi (Bharathi et al., 2006; Garg, 2012; Rathi et al., 2017; Revankar and Sutton,
567 2010). Gram stain has been reported to yield an accuracy of 35-90 % in detection of fungi in culture
568 positive cases (Badiee et al., 2010). For culture, the corneal scraping material is generally inoculated
569 onto culture plates in the form of multiple 'C'; only growths on the 'C' streaks are considered as
570 significant. Commonly used media for culture include 5 % sheep blood agar (incubated at 37 °C) and
571 potato dextrose agar (incubated at 22-25 °C) (Benson and Lanier, 1992; Wilhelmus et al., 1994). The
572 growth of fungi usually occurs in 3-4 days but culture media may require incubating for longer
573 periods of up to 4-6 weeks. In addition, culture is often necessary to identify the fungi and anti-
574 fungal susceptibility patterns to optimize the treatment. In clinically suspicious cases of FK, culture
575 showed positive results 25-59 % of the time (Moshirfar et al., 2019).

576 6.2.2 Molecular diagnostic methods

577 Genome-based tests for diagnosing FK are highly sensitive, less-time consuming than cultures, and
578 are ideal for ocular surface samples where the volume of the samples are in low quantities. Different
579 types of molecular techniques based on amplification, such as nested polymerase chain reaction
580 (PCR), real-time PCR, direct PCR, loop-mediated isothermal amplification and dot hybridization are
581 being developed for the detection of fungal pathogens (Zhao et al., 2014). The different targets of FK
582 detection include highly conserved ribosomal RNA (rRNA) genes (18S, 5.8S, and 28S rRNA genes),
583 internal transcribed spacer region (ITSs 1 and 2), elongation factor1-alpha gene and the

584 mitochondrial cytochrome b gene (Kuo et al., 2019). Genome-based tests for diagnosing FK have
585 reported a sensitivity of nearly 90 % or higher, but specificity of these techniques is highly variable,
586 ranging from 17-97 % (Kuo et al., 2019).

587 6.2.3 Recent advance in the diagnosis of FK

588 Recent techniques like next generation sequencing (NGS), deep sequencing and metagenomics have
589 advanced the field of genomic research and might help in identification of fungal pathogens causing
590 FK. Shigeyasu *et al*., reported a case of FK, which could not be identified by routine microscopy
591 where metagenomic shotgun NGS analysis with corneal scraping sample proved to be confirmatory
592 (Shigeyasu et al., 2018). Metagenomic NGS methods mark greater advances in rapid detection of
593 rare pathogens, and are also suitable for identifying slow growing, fastidious and unculturable fungal
594 pathogens (Lalitha et al., 2020). A wide range of pathogens have been identified by NGS analysis
595 from formalin-fixed corneal specimens (Li et al., 2018).

596 The use of fluorescent real-time optical molecular SmartProbes have recently been explored as a
597 novel method for detecting microbial isolates in corneal smears from microbial keratitis patients.
598 Gunasekaran *et al* demonstrated that this technique exhibited an equivalent or higher degree of
599 sensitivity and specificity than gold-standard culture and Gram stain techniques (Gunasekaran et al.,
600 2020), and offers an exciting new direction for low cost point-of-care diganostics.

601 **7. Treatment of FK**

602 7.1 Anti-fungal drugs

603 The treatment of FK is prolonged, often running into weeks. Topical 5 % natamycin drops remain the
604 drug of choice for filamentous FK while topical 0.15 % amphotericin is preferred for *Candida*
605 keratitis. Azoles and triazoles have been used as adjuncts or alternatives to natamycin or
606 amphotericin. A number of studies have examined how alternative treatment strategies compare to
607 these gold-standards, however none have yet proven superior.

608 **Topical natamycin vs voriconazole:** The Mycotic Ulcer Treatment Trial I (MUTT I) was a National Eye
609 Institute supported, randomized, active comparator controlled, double-masked, multi-center clinical
610 trial comparing outcomes in patients with filamentous fungal corneal ulcers receiving topical
611 natamycin (5 %) and topical voriconazole (1 %). This study concluded that, natamycin was superior in
612 terms of visual improvement and prevention of complications, and that voriconazole should not be
613 recommended as a monotherapy for filamentous FK (Prajna et al., 2013a). A subgroup analysis of
614 MUTT I showed that irrespective of the organism, patients randomized to voriconazole had higher
615 culture positivity on repeat scraping at day 6 of treatment than in the natamycin group, thereby
616 concluding that voriconazole was inferior to natamycin in the treatment of all fungi. Higher culture
617 positivity at day 6 was also associated with a poorer visual outcome (Ray et al., 2017). A Cochrane
618 review on medical treatment for FK concluded that patients treated with natamycin had a lower risk
619 of corneal perforation (FlorCruz and Evans, 2015).

620 **Oral voriconazole:** In a double masked randomized placebo controlled study (MUTT II), addition of
621 systemic voriconazole to topical antifungal therapy in deep stromal severe filamentous FK did not
622 show any added benefit. There was no difference in the rate of perforation and/or need for TPK,
623 visual acuity, scar size or rate of re-epithelialization. There were significantly more adverse events in
624 the oral voriconazole group, including elevations in liver enzymes and visual disturbances, than
625 patients in the placebo group (Prajna et al., 2016). However, a subgroup analysis in *Fusarium* ulcers
626 treated with oral voriconazole showed a reduction in the need for TPK and a reduced 3-month scar
627 size (Prajna et al., 2017a).

628 **Intrastromal voriconazole:** A randomized controlled trial was conducted by Narayana *et al* to
629 evaluate the effectiveness of intrastromal voriconazole in addition to topical natamycin application
630 for the treatment of moderate to severe FK. The trial concluded that there were no improvements in
631 microbiological cure rate at 3 or 7 days, visual acuity, the rate of perforation or the need for TPK
632 among those randomized to intrastromal voriconazole (Narayana et al., 2019). This was despite a

633 number of isolated case reports demonstrating the efficacy of intrastromal voriconazole in deep
634 fungal corneal ulcers (Sharma et al., 2011, Sharma et al., 2013).

635 **Natamycin vs amphotericin:** *In vitro* tests have shown no synergy or antagonism when natamycin
636 was added to amphotericin in the treatment of filamentous FK. A randomized controlled trial found
637 no difference in 24-hour culture positivity in moderate filamentous fungal corneal ulcers randomized
638 to amphotericin or natamycin (Lalitha et al., 2011). Furthermore combination therapy may increase
639 the risk of potential drug toxicity as well as the cost of therapy.

640 **Intracameral amphotericin:** A few case series have reported the efficacy of intracameral
641 amphotericin B as adjunctive treatment of FK unresponsive to conventional antifungal therapy
642 (Kaushik et al., 2001; Yilmaz et al., 2007). However a randomized controlled trial did not find any
643 additional benefit of intracameral amphotericin B over topical antifungal therapy when performed
644 alone or in combination with drainage of hypopyon in filamentous FK (Sharma et al., 2016).
645 Additionally anterior subcapsular cataract has been reported after intracameral amphotericin B
646 injection.

647 The major limitations for most of these studies were that they were conducted in India and most
648 infections were related to agricultural exposure and not to immunocompromised hosts, or contact
649 lens wear, such as those seen in developed countries. Therefore, it is possible that these differing
650 risk factors and/or genetic factors might modify the interaction between the infectious organism,
651 antifungal medications, and host responses.

652 **Newer Drugs:** Posaconazole is a newer triazole with broad spectrum activity against *Candida,*
653 *Fusarium and Aspergillus*. Oral posaconazole (200 mg four times a day, or 400 mg twice a day) alone
654 or in combination with topical formulation (4 mg – 10 mg/0.1 mL) has been used in the treatment of
655 recalcitrant *Fusarium* keratitis (Sponsel et al., 2002; Torres et al., 2005; Tu et al., 2007).

656 Echinocandins act on the fungal cell-wall by inhibiting the synthesis of (1,3)-D-glucan. The three
657 commercially available echinocandins are caspofungin, micafungin and anidulafungin (Patil and
658 Majumdar, 2017). Kamoshita *et al* reported a case of *Wickerhamomyces anomalus* FK that
659 responded to topical treatment with the antifungal micafungin (Kamoshita et al., 2015). *In vitro* and
660 animal studies have reported the efficacy of Capsulofungin in *Candida* spp. causing keratitis.

661 7.2 Corneal collagen crosslinking

662 Corneal collagen crosslinking (CXL) aims to strengthen and stiffen the cornea through the induction
663 of crosslinks in stromal collagen and is often used to treat keratoconus. No benefit in the treatment
664 of moderate filamentous fungal ulcers randomized to adjuvant crosslinking with riboflavin and UV-A
665 light was reported when compared to topical natamycin or amphotericin. There was no
666 improvement in microbiological cure, infiltrate and/or scar size, epithelization, and no difference in
667 adverse events including corneal perforation and the need for TPK. Additionally, in the patients
668 randomized to crosslinking, the visual acuity at 3 months was worse by 3 Snellen lines compared to
669 those who had not received CXL treatment (Prajna et al., 2020). CXL has also been found to have an
670 increased rate of perforation in recalcitrant deep stromal fugal keratitis (Uddaraju et al., 2015).

671 7.3 Rose Bengal Photodynamic therapy

672 Photodynamic therapy (PDT) in combination with a photosensitizer offers an anti-fungal free
673 approach to treating infection through the generation of ROS. PDT with 0.1 % rose bengal and green
674 light (518 nm) showed successful inhibition of growth of *Fusarium solani, Aspergillus fumigatus,*
675 *Candida albicans* (Arboleda et al., 2014), and has been used in successful treatment of multidrug
676 resistant *Fusarium* keratitis in a post keratoplasty patient (Amescua et al., 2017). A demarcation line
677 was seen in the anterior stroma following the procedure (Martinez et al., 2018). *In vitro* and *in vivo*
678 studies have also shown that PDT with rose bengal can arrest corneal melting and cause crosslinking
679 of the stromal lamellae and stiffening of the cornea (Fadlallah et al., 2016).

680 7.4 Therapeutic Keratoplasty

681 FK has a five times odds for perforation and longer healing time compared to bacterial keratitis
682 (Prajna et al., 2013b). The need for therapeutic keratoplasty (TPK) can vary from 15 % in mild-
683 moderate keratitis to 40 % in severe keratitis. The goals of the TPK are to primarily eliminate the
684 infection and restore the integrity of the globe. The cure rate of TPK for FK varies from 60-90 % with
685 a recurrence rate of 6-15 % (Sharma et al., 2010). Presence of hypopyon, corneal perforation, limbal
686 involvement and lens involvement are major risk factors for recurrence of FK after TPK (Shi et al.,
687 2010). Xie *et al* reported a recurrence rate of 7.8 % with lamellar keratoplasty for FK, with the risk
688 factors for recurrence being *Aspergillus* keratitis, pre-operative steroid use, endothelial plaque or
689 hypopyon (Xie et al., 2008).

690

691 **8. Critical gaps and Future Research directions:**

692 In spite of the growing evidence advocating the importance of ocular microbiology as an adjuvant to
693 clinical diagnosis, many corneal ulcers are still being treated empirically based on clinical features
694 alone, and this is contributing to poor prognosis and antimicrobial resistance. This is commonly due
695 to poor ocular healthcare-access, poor microbiology laboratory infrastructure, lack of trained
696 microbiologists, out-of-pocket costs, variations in patient sampling and prior antimicrobial use (Ung
697 et al, 2019b). Exciting new developments in this field including deployment of NGS and proteomics
698 in clinical practice will aid in the rapid detection and characterization of the invading fungi, and may
699 enable diagnosis from the tear-sample rather than invasive scrapes, although cost and infrastructure
700 may limit the appeal and uptake of these techniques. A simple, reproducible, point–of-care
701 deployable ocular microbiological diagnostic kit at an affordable cost would enhance the utilization
702 of microbiological techniques which will be critical to the appropriate therapeutic regimen to be
703 initiated.

704 Ocular morbidity in FK is a result of the interplay between the invading fungi and the defence
705 mechanism of the host. Even the best current therapeutic regimen is only directed towards killing
706 the invading fungus without taking into account the tissue destruction caused by an exaggerated
707 immune response. It is becoming clear that different fungi have different virulence patterns, with
708 this differentiation existing even amongst the same species. Adding to the complexity is that there
709 seems to be a difference in host response, and potentially the underlying ocular microbiome
710 between patients. Future therapeutic strategies should aim at personalized treatment regimen
711 which would include appropriate anti-infectives along with selective locally acting
712 immunomodulators which would curtail an unnecessary and a possibly harmful exuberant immune
713 response, thereby providing an enabling environment for the host responses to tackle the invading
714 fungus.

715 In order to achieve this personalised medicine approach, the paucity of molecular knowledge
716 surrounding host-pathogen interactions within the human eye must be addressed and fed into the
717 drug and diagnostic translational pipeline. This presents an exciting and dynamic area of research
718 and can focus a lens onto this neglected disease to improve patient outcomes.

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