

# Pathogen Induced Changes in the Protein Profile of Human Tears from *Fusarium* Keratitis Patients

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## Abstract

*Fusarium* is the major causative agent of fungal infections leading to corneal ulcer (keratitis) in Southern India and other tropical countries. Keratitis caused by *Fusarium* is a difficult disease to treat unless antifungal therapy is initiated during the early stages of infection. In this study tear proteins were prepared from keratitis patients classified based on the duration of infection. Among the patients recruited, early infection (n=35), intermediate (n=20), late (n=11), samples from five patients in each group were pooled for analysis. Control samples were a pool of samples from 20 patients. Proteins were separated on difference gel electrophoresis (DIGE) and the differentially expressed proteins were quantified using DeCyder software analysis. The following differentially expressed proteins namely alpha-1-antitrypsin, haptoglobin  $\alpha 2$  chain, zinc-alpha-2-glycoprotein, apolipoprotein, albumin, haptoglobin precursor -  $\beta$  chain, lactoferrin, lacrimal lipocalin precursor, cystatin SA III precursor, lacritin precursor were identified using mass spectrometry. Variation in the expression level of some of the proteins was confirmed using western blot analysis. This is the first report to show stage specific tear protein profile in fungal keratitis patients. Validation of this data using a much larger sample set could lead to clinical application of these findings.

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## Introduction

Corneal ulceration is the most common cause of corneal blindness in developing countries [1]. A retrospective population based study in the Madurai district of southern Tamil Nadu, India, estimated an annual incidence of corneal ulceration of 11.3 per 10,000 in the populations [2], which is ten times higher than the reported incidence from a study from the United States [3]. *Fusarium* and *Aspergillus*, are the predominant etiological agents responsible for 44% of all corneal ulcers [4]. Reports from Ghana [5] and northern Tanzania [6] also showed fungi as the etiological agent in over 50% of culture positive cases of keratitis. *Fusarium* species is more commonly associated with fungal keratitis in southern India, while *Aspergillus* species is more often implicated in northern India and Nepal [7]. The visual outcome following mycotic keratitis is generally poorer when compared to bacterial keratitis [8] and Natamycin, which is still the drug of choice for antifungal treatment is ineffective during late stages of the disease [9]. Therapeutic keratoplasty performed for mycotic keratitis is known to have a poorer prognosis [6,10–11]. *Fusarium* sp., sometimes invade the anterior chamber and form a lens-iris-fungus mass at the pupillary area, thereby interfering with the normal drainage of the aqueous humor and leading to a rise in intraocular pressure [12,13]. Predictive or diagnostic markers are not available to detect the preclinical infections, a stage that is

suitable for effective treatment. Neither the molecular mechanism underlying the susceptibility of the host and virulence of the pathogen examined.

In this study we have examined the tear profile of patients with keratitis caused by *Fusarium*. The tear proteins play an important role in maintaining healthy ocular surface, and changes in tear protein components have been shown to reflect the changes in the health of the ocular surface [14–20]. Proteomics is a valuable approach to find patterns of protein markers for a given disease [21]. Gel based proteomic approach has been used to show the alterations induced at the post translational level in an infectious disease [22,23]. Proteomic analysis can provide insights about protein expression patterns, which are associated with various pathological conditions and the identification of those tear proteins and their posttranslational modifications have the potential to reveal the mechanism of the disease [19,24–28]. The best method available at present to examine lower-abundance proteins and to quantify expression changes with high confidence is fluorescence two-dimensional difference gel electrophoresis (2D-DIGE) [29]. This multiplex technology allows the detection and quantitation of differences between samples resolved on the same gel, or across multiple gels, when linked by an internal standard [30]. 2D-DIGE circumvents the main drawbacks associated with conventional bidimensional polyacrylamide gel electrophoresis (2D-PAGE),

**Table 1.** The DIGE Experimental Design for Minimal Labeling with Cy Dyes used in this study.

Gel No	Cy 3	Cy 5	Cy 2
1	<i>Fusarium</i> early (30 µg)	Control(30 µg)	Pooled Internal standard
2	Control(30 µg)	<i>Fusarium</i> early(30 µg)	Pooled Internal standard
3	<i>Fusarium</i> intermediate(30 µg)	Control(30 µg)	Pooled Internal standard
4	Control(30 µg)	<i>Fusarium</i> intermediate(30 µg)	Pooled Internal standard
5	<i>Fusarium</i> late(30 µg)	Control(30 µg)	Pooled Internal standard
6	Control(30 µg)	<i>Fusarium</i> late(30 µg)	Pooled Internal standard

A total of 90 µg of labeled proteins were loaded on each gel for 2D electrophoresis; Tear sample from early, intermediate and late stage keratitis patients were used. Labeling, construction of pooled standards are described under materials and methods. 250 pmoles of dye per 30 µg protein was used.  
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such as low sensitivity, reduced dynamic range, and gel-to-gel variability, enabling more accurate and sensitive quantitation of proteins [31]. We have shown earlier variation in the expression level of tear proteins (prolactin inducible protein, serum albumin precursor, cystatin S precursor, cystatin SN precursor, cystatin, and human tear lipocalin) in fungal keratitis patients [15].

The present study aims to identify quantitative changes in the keratitis tear protein obtained from *Fusarium* keratitis patients using 2D DIGE. We have used tear fluid from different stages of *Fusarium* infection. As far as we know, this is the first report showing quantitative proteome level analysis of tear fluid from mycotic keratitis patients. Further these changes are fungal infection specific.

## Results

### 2D DIGE analysis of Tear Proteins

The experimental design of 2D DIGE is described in Table 1, and eighteen images from six gels of a typical experiment are shown in Figure 1. The spot position in the fluorescent images generated by the Typhoon trio system was comparable to the silver or coomassie stained gels (data not shown).

The protein co-detection of the three different CyDye labeled images in each gel was first performed by the DIA module of the software. To perform reliable quantification, there is a built-in normalization function (using the internal standard) to compensate for experimental variations, such as differences in laser power, fluorescence labeling, and sample loading. DIA module used in addition for quantitation of protein volume ratios between the co detected Cy3, Cy5 & Cy2 signals from a single gel. Cy2 forming the internal standard and the ratio of Cy3/Cy2 and Cy5/Cy2 were calculated in the DIA module. In this module variable of one ( $N = 1$ ) is considered. In Figure 2 protein spots were plotted with the log volume ratio on the  $x$ -axis and the left  $y$ -axis shows the number of spots and the right  $y$ -axis shows the spot volume. Table 2 shows the DIA data of the spot pairs detected, and the total number of spots used in the calculation is also given. In Figure 2, the spots that show less than two fold variation are within the two vertical lines. Spots outside the vertical lines represent the spots that show more than two fold variation. The normalized spot volume data against internal standard allowed a rapid overview of the differential protein expressions.

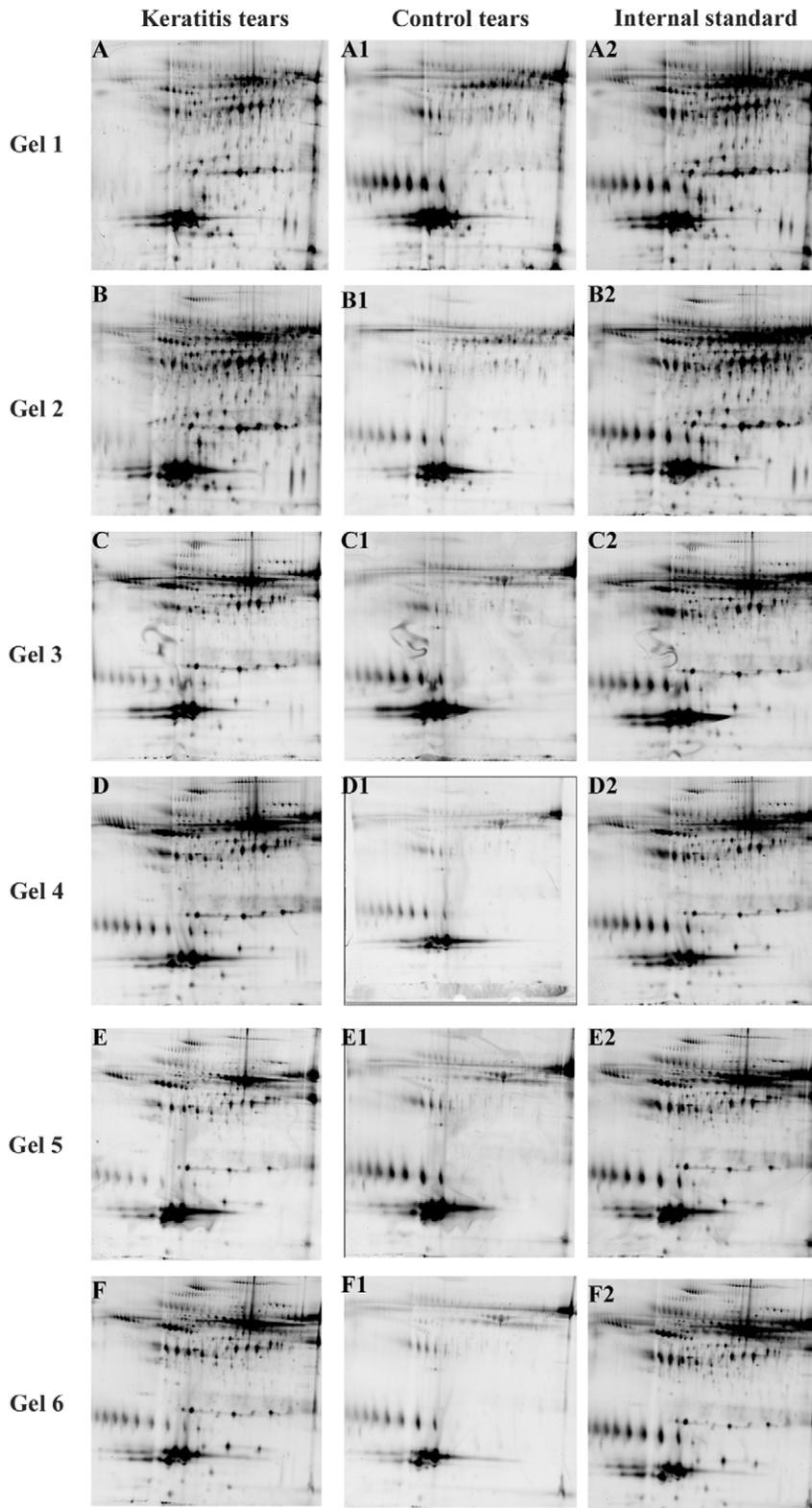
### Comparison of quantitative changes in protein expression across gels

Differential protein expression of those co-detected pairs between the keratitis and control tear across all six gels representing different stages of infection were quantified using

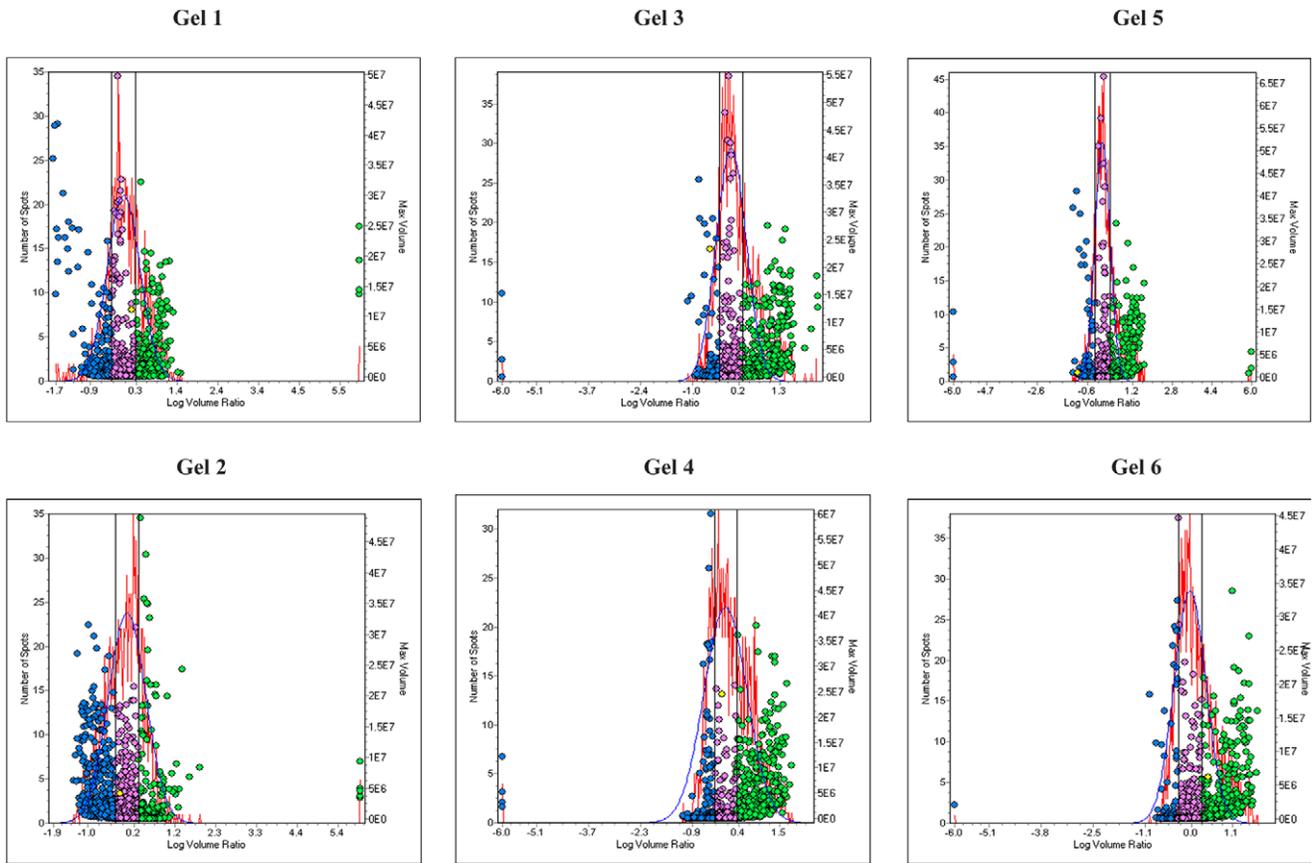
the BVA module. In order to avoid gel to gel variability, Isoelectric focusing and second dimension analysis were done using IPGphor III and Ettan Dalt six equipments, which allow simultaneous analysis of six samples. To detect variation in expression levels, relatively conservative selection criteria were used in image analysis. Differentially expressed proteins that fulfilled the following criteria alone were used for further analysis, 1) Minimum mean difference between the infected and control protein samples should be 1.5 fold or more, in terms of spot volume. 2) In all experiments an internal standard as described under materials and methods was included. 3) Built in t-test or one way ANOVA statistics software was used to detect significance of fold change ( $p \leq 0.05$ ). 4) These spots were checked manually to confirm that they were true protein spots and not artifacts. More than 281 protein pairs (showed 1.5 fold variation or more in expression) were statistically significant (ANOVA  $p < 0.05$ ). However, upon application of the above stringent criteria only 140 protein pairs could be selected for further analysis. Among these 140 protein pairs, 116 spots showed higher expression level and 24 spots were down-regulated. Therefore, up-regulation of proteins seems to be the dominant features in the expression profiles after fungal infection. Similar analysis procedure was followed for all the gel comparisons and Table 3 shows the data. The data for ten differentially expressed proteins are shown in Figure 3, 4 and 5. Protein expression changes of a particular spot in each of the six gels in terms of the spot volume between the keratitis and control tears are shown. In addition, the 3-D representations of the spot volume are also shown. The BVA data was analyzed individually for Cy3 & Cy5 labeled gels (Table S1) to confirm the EDA data.

### Image Analysis by DeCyder Extended Data Analysis (EDA)

*Extended Data Analysis* (EDA) module was used for principal component analysis and clustering studies. Matched spots that showed significant (ANOVA  $p \leq 0.01$ ) variation in their expression were transferred to the EDA module of the software. Default settings were used for intra and intergel statistical analyses allowing the characterization and classification of biological samples based on protein expression data. Principle component analysis (PCA), which is a statistical method to eliminate redundant variables and reduce data complexity, was also performed and the results are shown in Figure 6. An unsupervised clustering analysis was performed by selecting the hierarchical clustering algorithm available within the EDA software and Figure 7 shows the result. In all these analysis default settings of the software were used and hence the confidence of the data is assured.



**Figure 1. Fluorescent protein profiles of a set of six gels.** Experimental design is described in Table 1. A&B represents *Fusarium* early keratitis; C&D represents *Fusarium* intermediate keratitis; E&F represents *Fusarium* late keratitis; A1–F1 represents control tears; A2–F2 represents the pooled internal standard. Each gel contained 90  $\mu$ g of total protein separated by a pH 4–7 IPG strip in the first dimension and 12.5% polyacrylamide gel in the second dimension electrophoresis. Images were captured using a Typhoon Trio Variable Mode Imager.  
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**Figure 2. Discrimination of differentially expressed proteins in six gels.** The histograms were generated by DeCyder DIA software, plotting spot frequency (left y-axis) against log volume ratio (x-axis), and a normal distribution model (red line) was fitted to the main peak of the frequency histogram. A 2 fold variation in expression level was set as threshold. The threshold values are represented by vertical black lines in the histogram, and the differentially expressed spot features are shown as blue spots (volume decreased) and green spots (volume increased). Table 2 describes the details of spots which show variable expression.  
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**Dye swapping to minimize dye specific labeling efficiency**

It has been reported that there was preferential lysine labeling of Cy2 to particular protein spot which has not been identified [32]. Since DIGE technology has not been applied extensively to tear sample, we have examined preferential labeling using a reciprocal labeling of the same protein. Therefore, with the reciprocal

staining approach, any possible false positive results caused by the preferential labeling of dyes could be identified and corrected.

**Protein Identification by LC-MS/MS**

Sixty four tear proteins were identified (Figure 8) using mass spectrometry as described under materials and methods. Some of the protein spots were isoforms and hence a total of 23 different

**Table 2. Details of spot detection and spot quantitation using DIA software.**

GelNo	Cy3	Cy5	Spots detected	Max Volume Threshold	Decreased	Similar	Increased
1	<i>Fusarium</i> early	Control	1149	2	225(19.6%)	592(51.5%)	332(28.9%)
2	Control	<i>Fusarium</i> early	1455	2	316(21.7%)	682(46.9%)	457(31.4%)
3	<i>Fusarium</i> intermediate	Control	1578	2	253(16%)	824(52.2%)	501(31.7%)
4	Control	<i>Fusarium</i> intermediate	1533	2	350(22.8%)	641(41.8%)	542(35.4%)
5	<i>Fusarium</i> late	Control	1567	2	229(14.6%)	915(58.4%)	423(27%)
6	Control	<i>Fusarium</i> late	1525	2	242(51.9%)	792(51.9%)	491(32.2%)

DIA analysis was done as described in DeCyder software manual using default settings. Data for the threshold value of two alone is given. Dye swapping was performed to normalize variations in labeling.  
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**Table 3.** BVA analysis of the data.

S.No	Description	Control Vs <i>Fusarium</i> (early, intermediate & late) keratitis tears	Control Vs <i>Fusarium</i> early keratitis tears	Control Vs <i>Fusarium</i> intermediate keratitis tears	Control Vs <i>Fusarium</i> late keratitis tears	<i>Fusarium</i> early Vs intermediate keratitis tears	<i>Fusarium</i> early Vs late keratitis tears	<i>Fusarium</i> intermediate Vs late keratitis tears
(A)	Total no. of paired spots detected	1578	1578	1578	1578	1578	1578	1578
(B)	Among (A) differentially expressed spots > 1.5 fold difference in spot volume average ratio	1009	858	1129	854	750	551	496
(C)	Among (B) spots with ANOVA $\leq 0.05$	281	243	279	261	163	124	90
(D)	Among (C) manually confirmed true spots	140	132	140	133	68	60	30
(E)	Among (D) Total number up regulated spots	116	109	116	110	54	49	12
(F)	Among (E) total number of spots identified	11	18	23	22	20	25	4
(G)	Among (D) Total number down regulated spots	24	23	24	23	14	11	18
(H)	Among (G) total number of spots identified	14	14	14	14	1	0	2

doi:10.1371/journal.pone.0053018.t003

proteins from 64 spots were identified. The details of the identified proteins are shown in Table S2. Among them ten proteins were found to have statistically significant variation in expression level. Cystatin SA III precursor (spot ID 10), lacrimal lipocalin precursor (spot ID 24) and lacritin precursor (spot ID 30) were down regulated, while alpha 1 antitrypsin (spot ID 63), apolipoprotein (spot ID 32), haptoglobin (spot ID 18 & 19), albumin (spot ID 58), zinc  $\alpha$  2 glycoprotein (ZAG) (spot ID 38), lactoferrin (spot ID 52), were up regulated in the keratitis tear compared to control tear (Table 4 & Figure 8).

### Gene ontology annotation of differentially expressed proteins

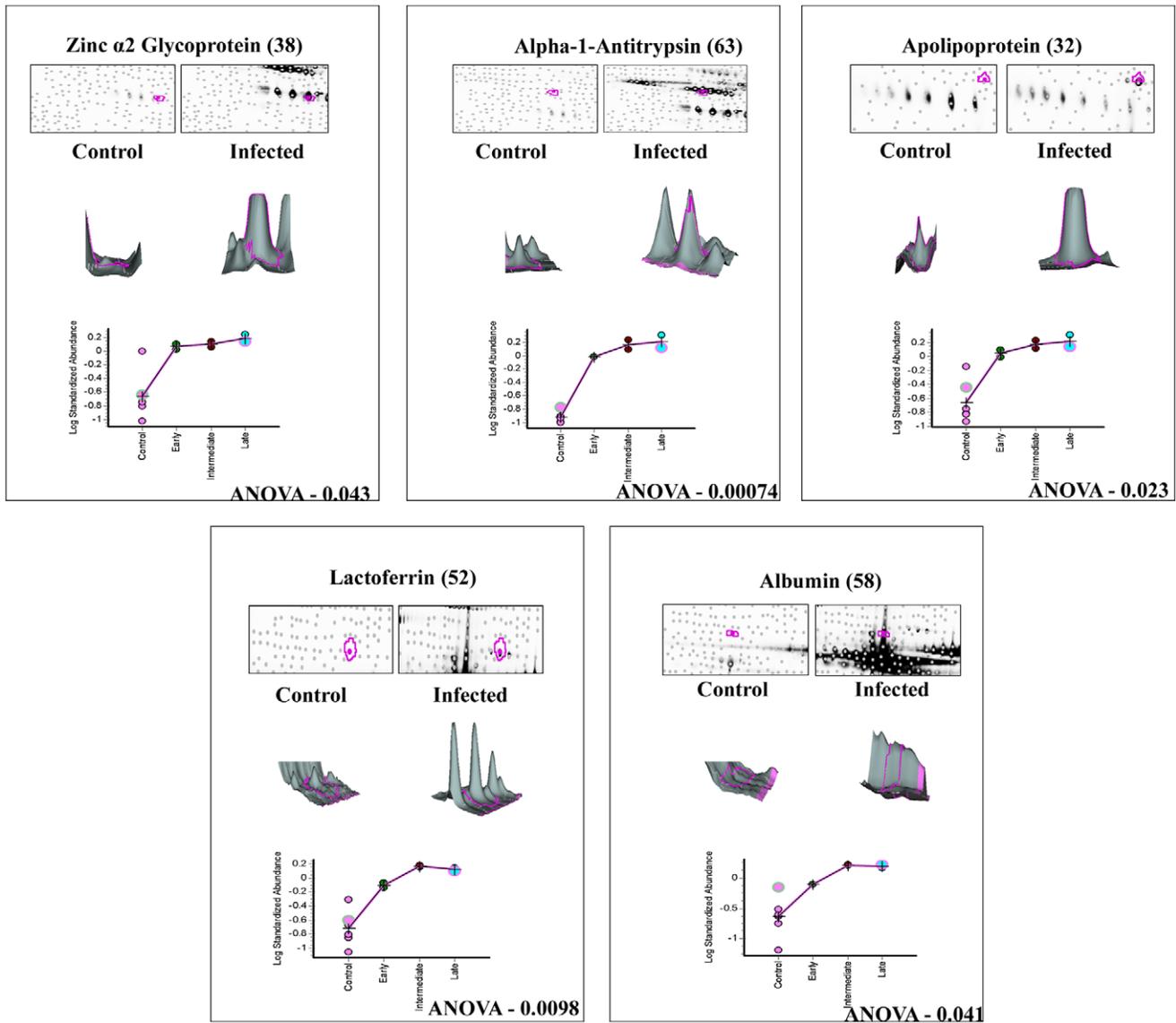
In order to better understand the biological function of differentially expressed proteins, gene annotation was performed using DAVID [33] 6.7 (a database for annotation, visualization and integrated discovery). Selected proteins were classified into three categories of cellular component, molecular function, and biological process (Table S3). All the proteins that showed altered expression levels were extracellular proteins. Proteins that have enzyme inhibitor activity (two were upregulated and two were downregulated) were the most common proteins in the category of molecular function. In the biological process category defense response proteins were most common and all of them were upregulated ( $n = 4$ ).

### Differential regulation of tear proteins at different stages of infection

The patients with keratitis were classified as early, intermediate and late as described under materials and methods. Differential expression of the proteins were observed in all the three infective stages (Table 5) and progressive up regulation of the seven proteins was observed as the disease progressed towards late stages of infection. Expression of cystatin SA III precursor and lacrimal lipocalin precursor declined during the late stage of infection. Lacritin precursor level was reduced to negligible amounts in the early infective stage itself compared to control (Figure 5). DIGE analysis shows that haptoglobin  $\beta$  chain (spots 40 & 43; Figure 8) and  $\alpha$ 2 chains (spots 18, 19, & 20; Figure 8) were upregulated in infected tear samples. Further two isoforms of beta chain and all three isoforms of  $\alpha$ 2 chains could also be detected. However, based on database entry all these are classified as haptoglobin precursors by MS/MS search as was shown earlier [22].

### Immunodetection of Lacritin, Lipocalin and Haptoglobin in Tear Fluid

Among the differentially regulated proteins, expression level of three proteins was confirmed using western blot analysis. 1D western blot analysis confirmed the reduction of lacritin and lipocalin levels and the increase of haptoglobin in tears from patients with *Fusarium* keratitis compared with control tears. The band volumes as determined using ImageQuantTL (GE Healthcare, Uppsala, Sweden) are shown in (Figure 9). 2D western analysis was performed to examine expression of isoforms. Western blot analysis confirmed that lacritin protein was totally absent in early stage of infection. Interestingly expression variation of lacritin and lipocalin isoforms in keratitis tears was also confirmed by western analysis (Figure 10). Upregulation of haptoglobin  $\alpha$  and  $\beta$  chains in fungal keratitis tears was also confirmed using western blot analysis.



**Figure 3. 3D and graphical representation of expression of up regulated proteins.** Graphic views show the standardized log abundance of spot volume (y-axis) against the changes of proteins between the control and infected groups (x-axis) in all six gels. 3-D view of control and late stage infection sample spots is also shown.  
doi:10.1371/journal.pone.0053018.g003

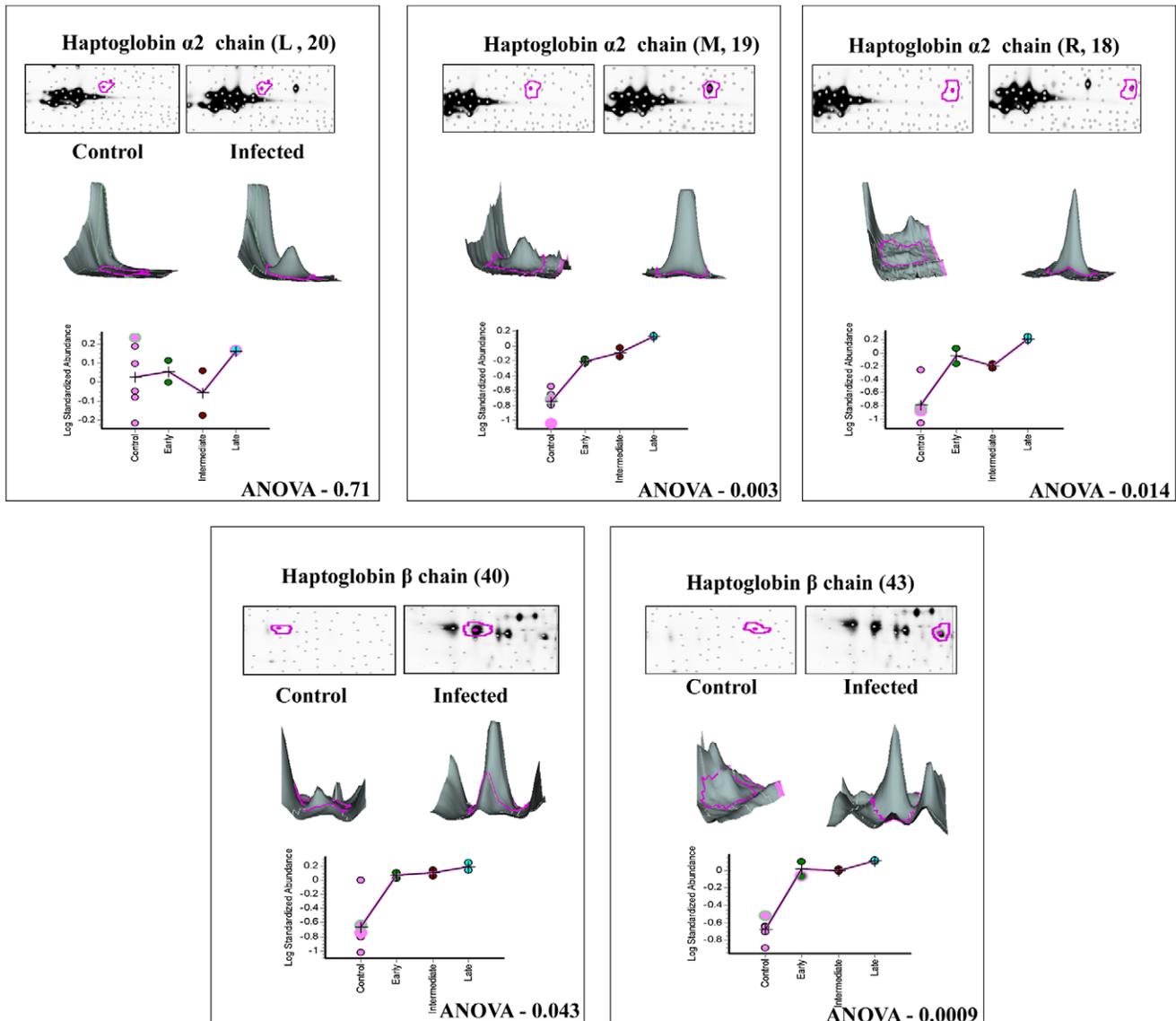
## Discussion

Tear proteins are derived from main and accessory lacrimal glands as well as proteins locally produced by corneal and conjunctival epithelial cells. Therefore tear is a useful sample to examine the expression of proteins in corneal infection.

One of the key steps that need careful attention in the tear proteomics is the method of collecting the tear. A recent study finds that there is no significant difference in the protein concentration in tears collected by capillary and schirmer collections, which are representatives of the reflex tear [34]. On loading the same amount of tear protein from normal and healthy control subjects, SDS PAGE reflected no significant change in the proteins as seen by the densitometry analysis. Most of the studies have used the capillary tear sample for the 2-D and MS analysis [35,15–16,28]. Capillary tear collection has been used for the 2-DE in disease conditions such as diabetic [20] and blepharitis

patients [19]. Since the tear secretion is profusing, the capillary collection has been used in pathological conditions like infectious keratitis.

Another study [36] aimed to compare methods of tear film collection (i.e., capillary collection versus Schirmer collection). There were 84 proteins identified from protein associated with the Schirmer method and 43 identified from the capillary method. Only 30 total proteins identified overlapped between the two collection methods. The study proposes that this difference arises through the Schirmer strip's interaction with the epithelium of the ocular surface (whereas the capillary method does not). To help examine this hypothesis, analysis of the various classifications/functions of the proteins identified were grouped based on their general function as follows transport, metabolism, immune response, structure, antioxidation, protease inhibitors, unclassified, cell signaling, and protein folding. There are several cellular proteins (i.e., not secreted) observed from the Schirmer method



**Figure 4. 3D and graphical representation of expression of Haptoglobin  $\alpha 2$  &  $\beta$  chain.** Graphic views show the standardized log abundance of spot volume (y-axis) against the changes of proteins between the control and infected groups (x-axis) in all six gels. 3-D view of control and late stage infection sample spots is also shown.  
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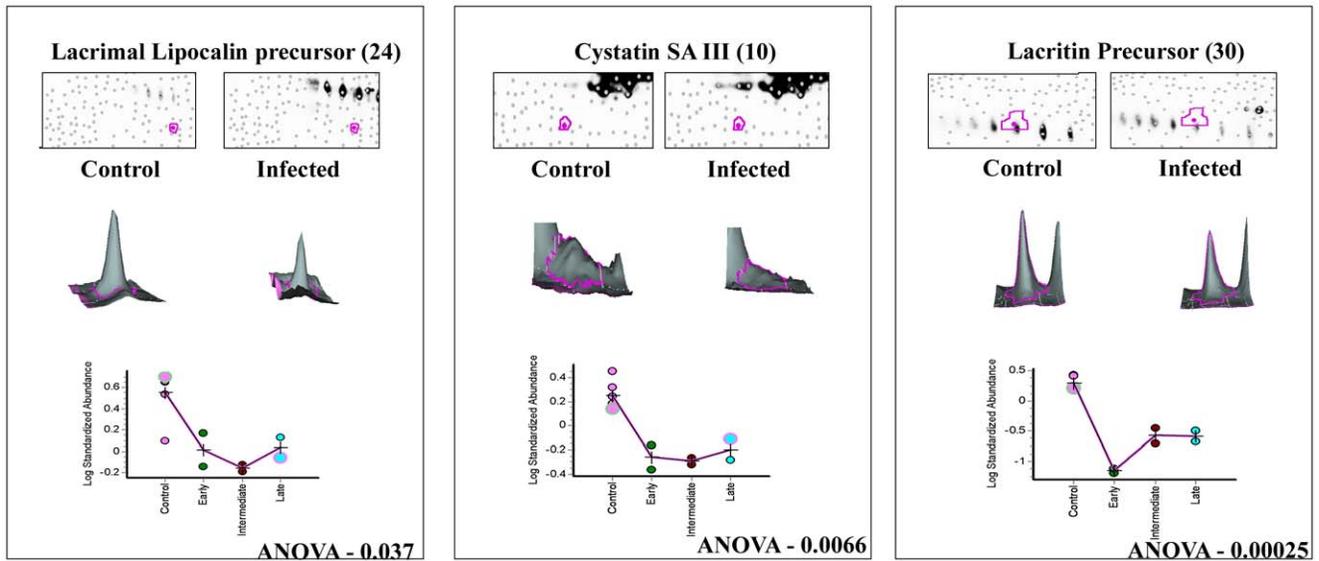
that were not found in tear film collected by capillary such as the S100 calcium binding series of proteins. Interestingly, serum albumin was detected at much higher levels in the tears collected using the Schirmer collection method.

Four fold decrease of lacrimal lipocalin precursor was observed in the tears of patients with fungal keratitis. Lipocalin-1, is a major tear protein secreted by lacrimal glands and acts as the principal lipid binding protein and is involved in the general protection of epithelial cell surfaces. Tear lipocalin protein level in human tears ranges between 0.5 and 1.5 g/l, making up 10%–20% of the total tear protein content in tears and is considered tear-specific [37,38]. Tissue specificity and concentration reflects the importance of lipocalin in the protection of the ocular surface [39]. Based on their possible role in lipid transport [40] lipocalin were called as tear-specific prealbumin [41].

Cystatins are cysteine proteinase inhibitors belonging to the cystatin superfamily. The role of cystatins as proteinase inhibitors

is well established. The balance between proteases secreted by the pathogen and protease inhibitor is believed to play a significant role in maintaining the normal ocular surface condition [42]. Human salivary cystatins are represented as Cystatin S. It contains three molecular species (cystatin S, cystatin SN and cystatin SA), which have distinct amino-acid sequences. Cystatin S binds cysteine proteases and inhibit their activity, thereby preventing uncontrolled proteolysis and tissue damages [43–46]. Cystatin S precursor was shown to be upregulated in healthy female tears compared to healthy male tears [47]. In tears of patients with blepharitis, significantly lower levels of cystatins SA have been reported [19]. The three fold reduction of cystatins in the tears of patients with fungal keratitis correlates well with their functional role. Cysteine protease inhibitors are reduced in several pathological conditions [48].

Ten fold reduction in the level of lacritin in the infected group compared to control may have relevance to resistance to fungal



**Figure 5. 3D and graphical representation of expression of down regulated proteins.** Graphic views show the standardized log abundance of spot volume (y-axis) against the changes of proteins between the control and infected groups (x-axis) in all six gels. 3-D view of control and late stage infection sample spots is also shown.

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infection. Lacritin is a glycoprotein found in the tears that is primarily produced by the lacrimal gland, but has also been shown to be produced by the meibomian glands, corneal and conjunctival epithelia. The protein is an extracellular growth factor thought to be associated with cell proliferation as a secretory mitogen [49,50]. It is a new lacrimal functional unit specific growth factor in human tears and flows through the ducts to target corneal epithelial cells on the ocular surface. This protein enhances unstimulated secretion and rapid tyrosine phosphorylation in lacrimal acinar cells, and it also stimulates corneal epithelial cell calcium signaling. Therefore, lacritin may play a key role in the function of the lacrimal gland-corneal axis and its deficiency could lead to cause some alterations in the defense mechanism. Lacritin might be important for tear secretion and maintaining normal tear conditions. Lacritin has been shown to be decreased in conditions altering tear film such as blepharitis [19] and dry eye, indicating the crucial role of this protein in infection and other conditions [34,51–52]. Reductions in the level of lipocalin precursor and lacritin have direct effect in the pathogenesis and susceptibility of host corneal tissue. Inhibition of the protease inhibitor cystatins may lead to better survival of the pathogen.

Alpha-1 antitrypsin is an enzyme that controls the activity of diverse proteolytic enzymes such as trypsin, chymotrypsin, collagenase, thrombin, fibrinolysin, granulocytic proteases and caseins by cleaving their catalytic sites [53]. This protein is found in a number of body fluids such as tears, perilymph, lymph, saliva, colostrum, breast milk, duodenal fluid, gallbladder bile, synovial fluid, cervical mucus, semen and amniotic fluid [54–57] and plays an important role in both physiological and pathological condition by inactivating enzymes activated by bacteria or other agents. This protein also regulates [58] the immune response by inhibiting the transformation and migration of lymphocytes. The increased level of tear alpha-1 antitrypsin in corneal ulcer and other diseases have been reported [59,60]. It has been shown that tear alpha-1 antitrypsin levels may increase in inflamed eyes in the absence of corneal involvement, for example, in cases of rosacea keratitis and allergic conjunctivitis [61,62]. All these studies imply that alpha-1-antitrypsin might be an acute-phase reactant [63]. In fungal

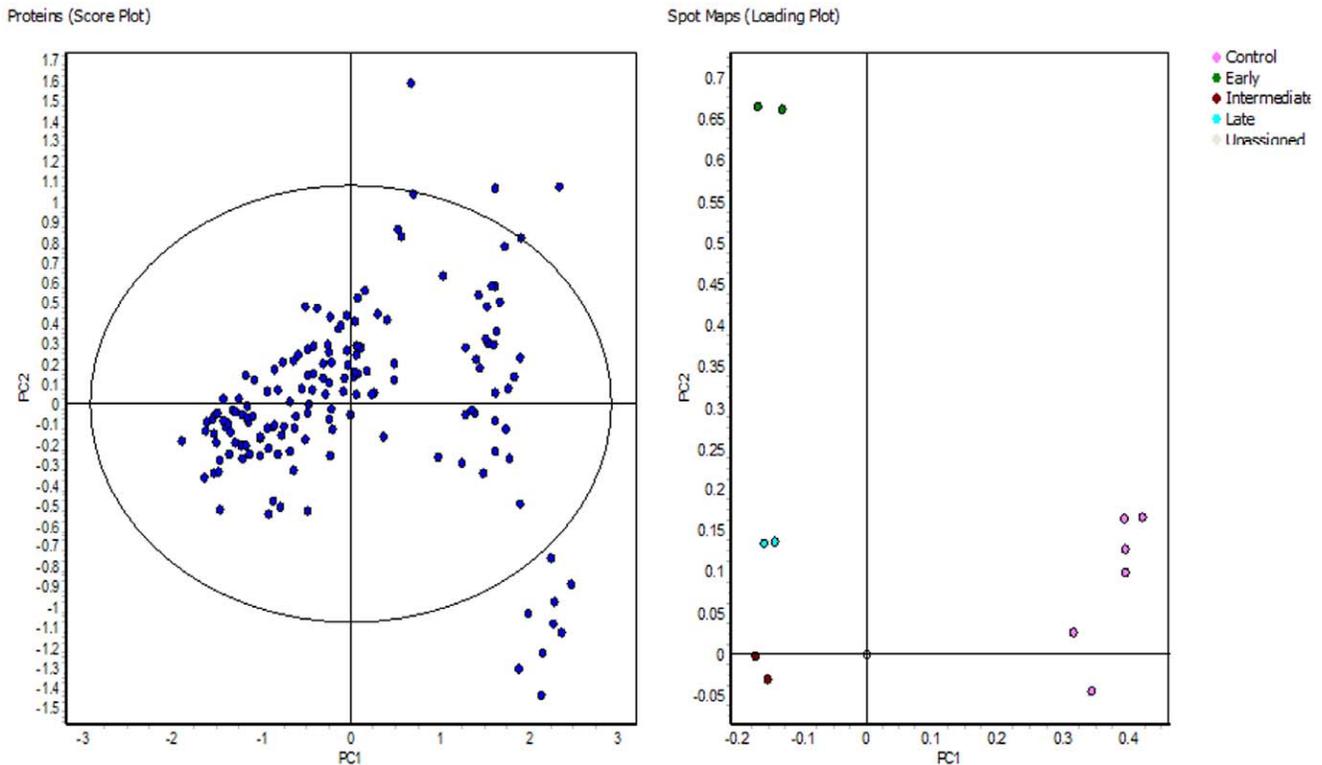
keratitis, the increase in alpha-1-antitrypsin could be due to the inflammation induced by the pathogen or a specific pathogen component may be involved.

Haptoglobin (Hp) is a plasma glycoprotein, the main biological function of which is to bind free hemoglobin (Hb). It is also a positive acute-phase protein with immunomodulatory properties [64–66]. Increase in tear haptoglobin alpha levels has been shown in conjunctivitis [65]. In our study haptoglobin  $\beta$  chain isoforms and  $\alpha 2$  chain isoforms were upregulated in infected tear samples but not in control tear. The fungal infection presumably leads to the increased synthesis and export into tear samples. Since both  $\alpha$  &  $\beta$  forms are found, it is interesting to examine whether functional  $\alpha 2\beta$  complexes are formed in the tear.

Zinc  $\alpha 2$ -glycoprotein (ZAG) is a 40-kDa single-chain polypeptide, which is secreted in various body fluids such as plasma, saliva, and tears [67]. It stimulates lipid breakdown in adipocytes [68] and is associated with the extreme weight loss that occurs in some cancers [69]. ZAG is shown to be increased in patients with Graves's ophthalmopathy [70]. The exact function of ZAG in tears and its possible role in lipid degradation are not demonstrated [70]. In our study, we detected significant upregulation of ZAG in keratitis patients, implying the alteration in the lipidome profile of tear in infection.

Apolipoproteins are proteins that bind to lipids (oil-soluble substances such as fat and cholesterol) to form lipoproteins, which transport the lipids through the lymphatic and circulatory systems. Apolipoproteins also serve as enzyme cofactors, receptor ligands and lipid transfer carriers that regulate the metabolism of lipoproteins and their uptake in tissues. Increased secretion of native apo A-I from the main lacrimal gland in patients with advanced diabetic retinopathy has been reported [71]. Apolipoprotein level was increased fivefold in all the three stages of fungal keratitis tears compared to control tears.

The mean albumin level in serum was reported as 44.7 mg/ml in the healthy individuals from India [72]. Albumin levels in tear fluids were reported to be 11.5 mg/l in healthy males and 12.7 mg/l in healthy females from Indian population [60]. In cases of allergic conjunctivitis tear levels of serum albumin reach



**Figure 6. Clustering analysis of fusarium infected tear samples.** Principal component analysis (PCA) has been performed across different experimental groups. A subset of those proteins whose expression varied within the 95th confidence level (ANOVA;  $p < 0.01$ ) was created. In this analysis the default settings of the software were used. doi:10.1371/journal.pone.0053018.g006

serum levels rapidly, indicating a direct leak from inflamed vessels. The albumin content of tears also increases in cases of corneal ulceration [59]. Our earlier report [15] and our present results show albumin upregulation in keratitis tear sample indicating that during fungal infection there might be a leakage of this protein from inflamed vessels.

Tear lactoferrin (formerly known as lactotransferrin) is an 82 kDa protein produced by the acinar cells of the lacrimal gland. Lactoferrin is present in normal tears of humans and its level was found to be invariant through age. Lactoferrin is a glycoprotein, and a member of the transferrin family, belong to those proteins capable of binding and transferring  $Fe_{3+}$  ions [73]. Due to the increase in its concentration during most inflammatory reactions and some viral infections, several reports classify lactoferrin as an acute-phase protein [74]. Its concentration increases in all biological fluids, but the highest levels have been detected in the nidus of inflammation [75]. Lactoferrin has a wide variety of biological functions, many of which do not appear to be connected with its iron binding ability [76]. Its role in fungal infection is not clear as of now.

#### Functional Implication of Differentially Expressed Tear Proteins at different infective stages

As the infective stage progressed seven acute phase proteins were upregulated. Interestingly 2D western analysis reveals lipocalin and lacritin isoforms are at a negligible level in the early stage of fungal infection, but their level increase marginally during intermediate stage. This increase could be due to the fungal elaboration and the induction of inflammatory response. Taken

together these observations imply that the treatment options needs to be tailored based on the stage of infection.

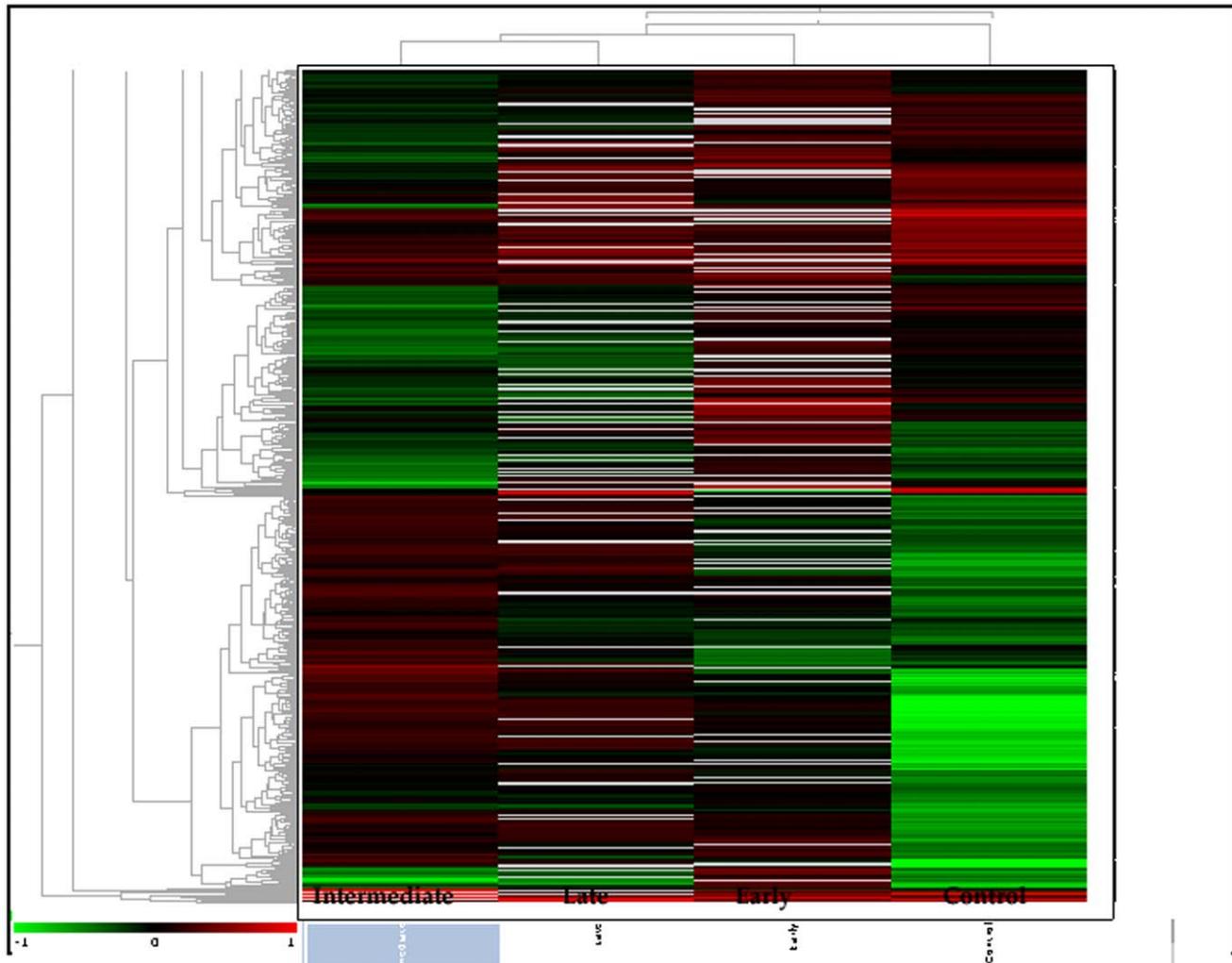
2D-DIGE analysis of tears from fungal keratitis patients allowed the confident quantification of several differentially expressed proteins in comparison to normal tears. Fungal infection of cornea clearly induce increased expression of alpha 1 antitrypsin, apolipoprotein, haptoglobin, albumin, zinc  $\alpha$  2 glycoprotein (ZAG), lactoferrin, haptoglobin precursor, proteins related to inflammatory events. Expression of proteins related to host immune response such as cystatin SA III potential precursor, lacrimal lipocalin precursor, lacritin precursor is reduced in infection. Quantitation of lacritin levels showed that this protein is down regulated significantly in fungal keratitis when compared to bacterial keratitis tears (data not shown). This information and other results (unpublished) show that the changes reported in this paper are specific to fungal keratitis.

Additional studies focusing on the functional properties of these proteins in the pathogenesis of fungal keratitis may contribute to a better understanding of the disease. In addition these proteins form putative candidate biomarkers that could aid in the diagnosis, prognosis and treatment of fungal keratitis.

## Materials and Methods

### Tear Samples

Written Informed consents were obtained from study subjects and the study was approved by the Institutional Review Board of Aravind Medical Research Foundation (Madurai, India). The Declaration of Helsinki was adhered to when enrolling subjects. Reflex tear samples (100–150  $\mu$ l) were collected from the keratitis eye of culture positive (*Fusarium*) patients who were categorized



**Figure 7. Unsupervised hierarchical of the expressed proteins.** Log-transformed normalized protein spot volumes were used to perform unsupervised hierarchical cluster analysis. Green indicates decreased expression; red indicates increased expression. Patient groups; control and fusarium infected (Early, Intermediate and Late). doi:10.1371/journal.pone.0053018.g007

based on the duration of the infection as early (within 7 days), intermediate (7–14 days) and late (after 14 days) stage of infection. Tears from healthy individuals (whose corneas were clear and devoid of any infection & inflammation) served as controls (60–80  $\mu$ l). All the tear samples were obtained from male individuals with the age range from 20–60 yrs. The total number of tear samples collected for each category of presentation is as follows, early –35; intermediate – 20; late – 11. Among them five samples from each category were included for this study. Tear samples were collected using 10  $\mu$ l-capillary tubes without touching the eye globe or the lid. The samples were centrifuged at  $7800 \times g$  for 10 min at  $4^{\circ}\text{C}$  to remove cellular debris and stored in liquid nitrogen until analysis. Samples were collected from patients before treatment. Protein concentration was estimated using Bradford's method [77]. Samples used in this study were described in table S4.

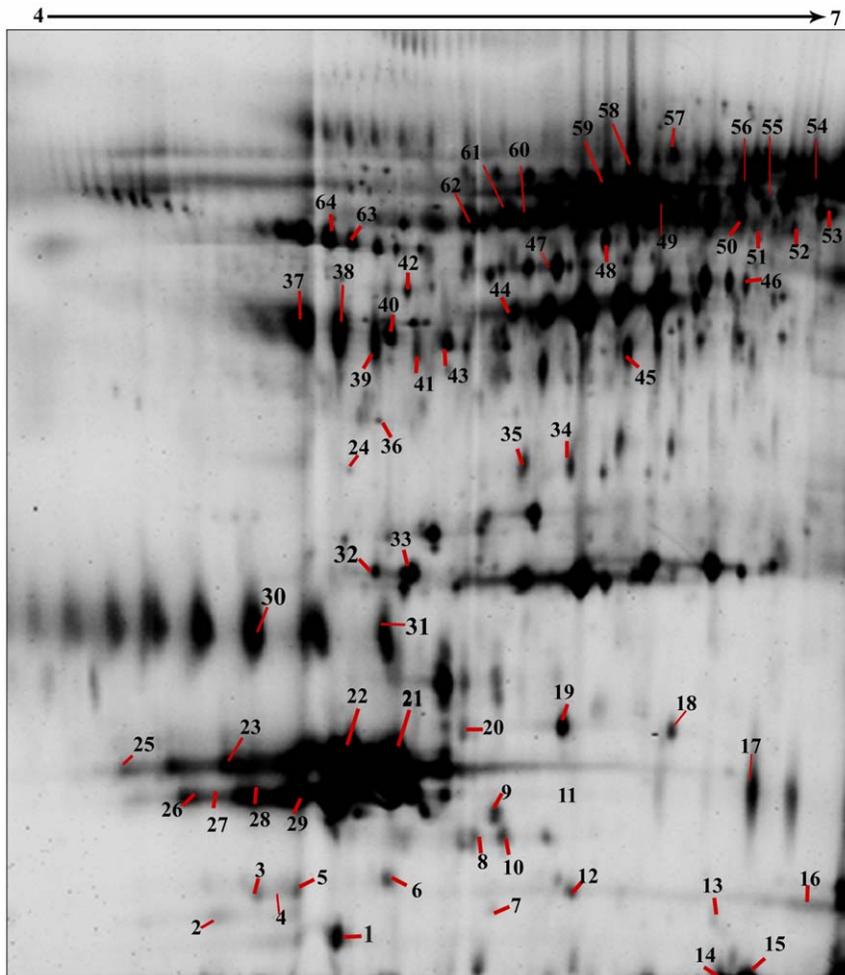
#### Two-dimensional difference gel electrophoresis

**Sample preparation.** Tears samples from *Fusarium* keratitis patients were pooled (250  $\mu$ g of protein per patient). Three pools each were made from early, intermediate and late stage infection samples. Each pool was made from tear from five patients. Tear

samples from 20 healthy individuals were pooled and used as control samples (100  $\mu$ g of protein per healthy individual). Pooled tear samples were concentrated using 3 kDa Amicon Ultra centrifugal device (MILLIPORE, County Cork, Ireland) as per manufactures instructions with minor modifications. Briefly, tear sample (2000–2500  $\mu$ g) was diluted to 600  $\mu$ l with MilliQ water and placed into a 3 kDa Amicon Ultra centrifugal device and centrifuged at  $13,000 \times g$  for 10 min; under these conditions the volume of the concentrate was reduced to 100  $\mu$ l. An additional 500  $\mu$ l of MilliQ water was added and the mixture was centrifuged as above.

#### Proteins Labeling with CyDyes

Lysine labeling protocol, (minimal labeling) was used in this study. Each dye (2 nmol) was reconstituted with 5  $\mu$ L anhydrous *N,N*-dimethylformamide (Sigma Aldrich chemical co.) and 400 pmol/ $\mu$ l stock dye was stored at  $-20^{\circ}\text{C}$ . The stock dye was further diluted to 100 pmol/ $\mu$ l and this working dye solution was used immediately. The processed tear proteins were then labeled individually with dyes Cy3 and Cy5 while the pooled tear proteins prepared by mixing equal aliquot of protein from all samples in an experimental set up were labeled with Cy2 and was used as



**Figure 8. Tear protein profile showing the annotations of the identified tear proteins.** 2D DIGE gel contained 30  $\mu\text{g}$  of Cy2 labeled internal standard as described in Table 1 was separated by a pH 4–7 IPG strip in the first dimension and 12.5% polyacrylamide gel in the second dimension electrophoresis. Image was captured using a Typhoon Trio Variable Mode Imager.  
doi:10.1371/journal.pone.0053018.g008

internal standard. A 30  $\mu\text{g}$  portion of each group was mixed with 2.5  $\mu\text{L}$  of dye and incubated on ice for 30 min in the dark. In the case of Cy2 labeling, total mix was labeled and 30  $\mu\text{g}$  aliquot was used in each gel. The labeling reactions were quenched with 2  $\mu\text{L}$  of 10 mM lysine and incubation was continued on ice for 10 min. Cy3 & Cy5 labeled samples were mixed with the Cy2-labeled samples, and an equal volume of lysis buffer containing 100 mM DTT and 2% (v/v) IPG buffer was added and incubated at room temperature for 10 min. The final volume for all preparations was adjusted to a total of 340  $\mu\text{L}$  with rehydration buffer (7 M urea, 2 M thiourea, 1% IPG buffer, 50 mM DTT, 4% CHAPS, and a trace amount of bromophenol blue). A reciprocal labeling experiment was also performed. The labeling protocols for the 2D DIGE experiments are shown in Table 1.

### Two dimensional gel electrophoresis

Two dimensional gel electrophoresis of Cy dye labeled proteins was done as described already [78] with the following modifications. Eighteen cm IPG strips of pH 4–7 (GE Healthcare, Uppsala, Sweden) were employed in the first dimension. Labeled proteins were focused for a total of 80,000 Vh at a constant temperature (20°C) under linear voltage ramp after an active IPG rehydration at 30 V in a IPGPhor III (GE Healthcare, Uppsala,

Sweden) apparatus with following IEF conditions 500 V step-n-hold for 1 h, 1000 V gradient for 1 h, 8000 V gradient for 3 h and 8000 V step-n-hold for 8 h. Following IEF, each IPG strip was placed in the equilibration buffer containing 2% DTT first followed by incubation in another buffer in which the DTT was replaced by 2.5% iodoacetamide. The second dimension PAGE (12.5%) was carried out in an Ettan DaltSix systems (GE Healthcare, Uppsala, Sweden) at 1 W/gel for 1 hr and 13 W/gel for 5 hr. All experimental procedures were performed in dim light or in the dark.

### Protein Visualisation and DeCyder image analysis

After second dimension electrophoresis, the gels were scanned with Typhoon TRIO Variable Mode Imager (GE Healthcare, Uppsala, Sweden). All laser power adjustments and image acquisition were done using default settings of the Typhoon Scanner Control program. Cy2, Cy3 & Cy5 images were captured using the settings recommended by the manufacturer. The scanned gels were saved as modified 16-bit.gel format which gave good gray-scale representation covering the dynamic range of protein spots. Prior to analysis, all gel images were cropped to identical size in ImageLoader module of DeCyder version 7.0 (GE Healthcare, Uppsala, Sweden) and then transferred to the

**Table 4.** Identified tear proteins and their function.

Spot ID	Identified Tear proteins	Database/Accession no.	Mowse score <sup>a</sup>	X & Y coordinate in DIGE gel	M/r/p/ 2-D <sup>b</sup>	M/r/p/ data base <sup>c</sup>	Peptides Matched <sup>d</sup>	Sequence Coverage (%) <sup>e</sup>	Function
10	cystatin SA-III = potential precursor of acquired enamel pellicle [human, Peptide, 121 aa]	NCBI/gi 235948	182(38)	484,835	16/5.4	14.1/4.7	R.IIPGGYDADLNDEWVQR.A R.RPQLVLR.A K.SQPNLDTCAFHEQPELQK.K K.SQPNLDTCAFHEQPELQK.K	35	Cysteine protease inhibitor
19	Haptoglobin OS = Homo sapiens GN = HP PE = 1 SV = 1 - $\alpha$ 2 chain (M)	Swissprot/HPT_HUMAN	212 (35)	555,724	20/5.9	45.8/6.1	K.NYYKLR.T K.LRTEGDGVYTLNNEK.Q R.TEGDGVYTLNNEK.Q R.TEGDGVYTLNNEKQWINK.A	6	Haeme binding protein
24	Lacrimal lipocalin precursor	MSDB/LCHUL	112 (36)	174,733	19/4.4	19.2/5.39	K.NNLEALEDFEK.A R.GLSTESILIPR.Q	12	Lipid scavenging and transport to outer tear layer
30	Lacritin Precursor	NCBI/gi 15187164	62 (38)	244,622	26/4.8	14.2/5.43	K.SILLTEQALAK.A K.KFSLKPPWA	14	Secretion, renewal of lacrimal & ocular surface epithelia
32	Pro Apolipoprotein	NCBI/gi 178775	204 (27)	367,575	30/5.2	28.9/5.45	K.LLDNWDVSTVTSFK.L R.THLAPYSDELRR.L K.ATEHLSLSEK.A K.AKPALEDLR.Q	18	Lipid profile regulator
38	Zn-alpha2-glycoprotein [Homo sapiens]	NCBI/gi 38026	607 (36)	293,317	51/4.8	34.7/5.71	R.YSLTYITGLSK.H K.SQPMGLWR.Q R.QVEGMEDWK.Q R.QVEGMEDWKDLSQLQK.A K.AREFIFMETLK.D R.EDIFMETLK.D K.YYYDGDYIEFNK.E K.QKWEAEPVYVQR.A K.WEAEPVYVQR.A K.AYLEECPATLR.K K.AYLEECPATLR.K.Y R.QPPSVVWTS HQAPGEK.K K.CLAYDFYPGK.I	37	Stimulates lipid degradation
40	Haptoglobin precursor - $\beta$ chain	NCBI/gi 306882	182(35)	379,336	39/5.6	45.1/6.24	R.ILGGHLDK.G K.GSFPWQAK.M K.DIAPTLTYVGGK.K K.QLVEIEK.V R.VGVYSGWGR.N K.FTDHLK.Y K.VTSIQDWVQK.T	15	Haeme binding protein
52	Lactoferrin	NCBI/gi 2104522	222 (38)	800,269	50/7	53.6/7.09	K.CGLVPVLAENYK.S R.SDTSLTWNSVK.G R.CLAENAGDVAFAVK.D	9	Antimicrobial activity
58	Serum albumin, chain A	MSDB/IAO6A	639 (36)	550,131	66/5.1	65.6/5.63	R.FKDLGEEFNK.A K.LVNEVTEFAK.T K.KYLYEYIAR.R18 K.YLYEYIAR.R K.VHTECCHGDLLEACADDDR.A K.VHTECCHGDLLEACADDDRADLAK.Y K.QNCELFEQLGEYK.F K.FQNALLLR.Y K.KVPQVSTPTLVEVSR.N K.VPQVSTPTLVEVSR.N K.QTALVELVK.H K.AVMDDDFAAFVEKC	18	Regulation of the colloidal osmotic pressure of blood
63	Alpha-1-antitrypsin	Swissprot/ A1AT_HUMAN	76 (28)	269,210	45/5.2	46.8/5.3	K.TDTSHHDDHPTFNK.I K.IVDLVK.E K.FLENERRR.S K.LSITGYDLK.S	9	Inhibitor of serine proteases

<sup>a</sup>MOWSE scores greater than the values given in the parenthesis are considered to be significant ( $p < 0.05$ ). All proteins were also searched across multiple databases to confirm their identity.

<sup>b</sup>Apparent/experimental molecular weight and pI of protein spot on 2-DE gels.

<sup>c</sup>Theoretical molecular weight and pI of the identified protein in database.

<sup>d</sup>Represents the peptides matched.

<sup>e</sup>Sequence coverage (SC) represents the % amino acid sequence covered in the protein by the matched peptides. 18R, 19M and 20L of  $\alpha 2$  isoforms are labeled according to Gupta<sup>22</sup> et al., 2007.

doi:10.1371/journal.pone.0053301.t004

**Table 5.** Differentially Expressed Proteins in Tears of keratitis patients.

Spot.ID	Protein Name	Gene ID	Pattern of regulation	Fold Variation	One way ANOVA p value	T - test p value
63	Alpha-1-antitrypsin	SERPINA1	Up regulation	12.04	0.00074	2.8e-005
19	Haptoglobin $\alpha$ 2 chain (M)	HP	Up regulation	4.93	0.0030	0.00050
38	Zinc-alpha-2-glycoprotein	AZGP1	Up regulation	4.37	0.043	0.0032
32	Apolipoprotein	APOA2	Up regulation	5.19	0.023	0.0014
58	Albumin	ALB	Up regulation	4.56	0.041	0.0059
40	Haptoglobin precursor - $\beta$ chain	HP	Up regulation	10.96	0.0053	0.00024
52	Lactoferrin	LTF	Up regulation	5.35	0.0098	0.00088
24	Lacrimal lipocalin precursor	LCN1	Down regulation	-4.04	0.037	0.0038
10	Cystatin SA III Precursor	CST4	Down regulation	-3.22	0.0066	0.00029
30	Lacritin Precursor	LACRT	Down regulation	-9.64	0.00025	0.00036

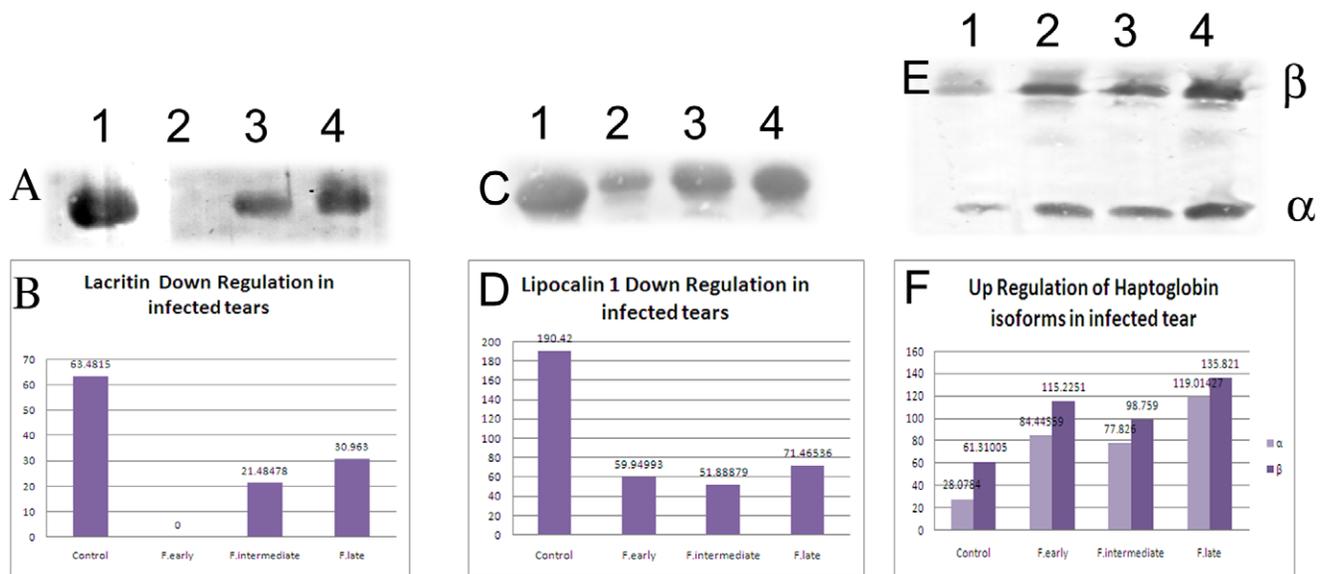
doi:10.1371/journal.pone.0053018.t005

DeCyder Differential Analysis Software, version 7.0. The standardized spot volume (the sum of pixel values within a detected spot boundary) was calculated as the ratio between the absolute volume of the individual spot and the total volume of all spots in the gel. The internal standard (Cy2) on each gel intrinsically linked all gel images (Cy3 and Cy5) within the same gels and across all the gels in the specific set of an experiment. A DeCyder differential in-gel analysis (DIA) module was used for image analysis between samples within the same gel while a DeCyder biological variation analysis (BVA) module was performed for pairwise image analysis among multiple gels. Ratios of differentially expressed proteins were shown as “fold” changes between the spots of keratitis tears and the control tears. An increase of protein abundance in the keratitis tear was expressed as a positive value while a negative value denoted a decrease in the protein abundance. Protein spot

has been normalized using the corresponding spot on the pooled internal standard on every gel. Student's *t*-test and ANOVA were used to compare the average spot volume and differences of protein abundance for all detectable spots between the keratitis and control groups. Reciprocal dye labeling was performed to normalize bias in labeling.

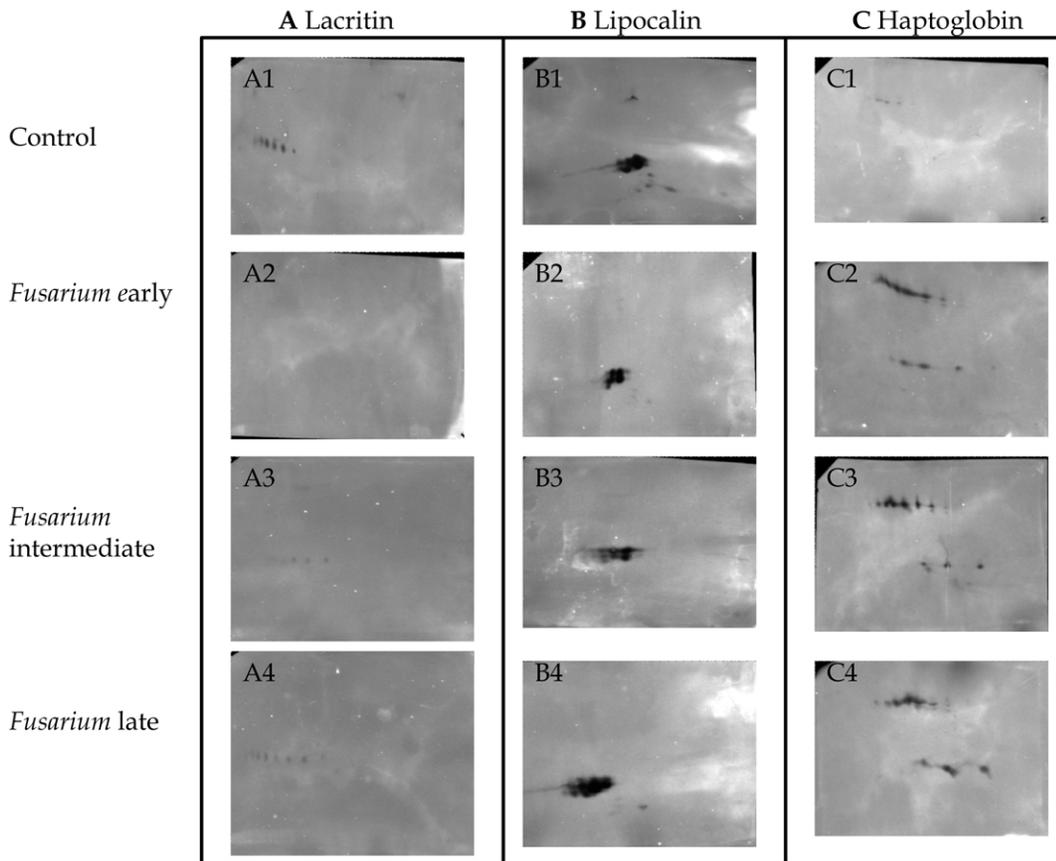
#### Protein Identification and Mass Spectrometry

Pooled proteins (300  $\mu$ g) from control tear group were separated on 18 cm IPG strips of pH 4–7 in the first dimension. First and second dimension electrophoresis were done as given under 2D DIGE method. The second dimension gels were stained with colloidal coomassie blue G-250 [79] and gel spots from this preparative gel were excised manually for in-gel trypsin digestion [15], and LC-MS/MS was performed. The resulting peptides were



**Figure 9.** 1-DE Immunoblot of keratitis patients tear proteins separated on 1D PAGE; control subject (1) and tears of fusarium keratitis early stage (2), intermediate stage (3) and late stage (4) patients. Forty  $\mu$ g tear proteins were separated on 13.5% SDS PAGE. Tear proteins were transferred to nitrocellulose membranes for subsequent immunodetection with appropriate primary antibody. Secondary antibody used was HRP labeled and the images were scanned using a Typhoon Trio scanner as described under materials and methods. B, D and F are histograms showing the spot intensities.

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**Figure 10. Immunoblot of tear proteins from control & keratitis patients separated on 2D PAGE.** For 2-DE western blotting, 40  $\mu$ g tear proteins was separated on pH 4–7 strip (7 cm), followed by second dimension separation on a 13.5% polyacrylamide gel. Tear proteins were transferred to nitrocellulose membranes for subsequent immunodetection with appropriate primary antibodies as described already.  
doi:10.1371/journal.pone.0053018.g010

separated by an electrospray ionisation (ESI) quadrupole time-of-flight (Q-TOF) Bruker MicrOTOF Q (Bruker Daltonics) coupled with a nano liquid chromatography system (Ultimate 3000, Dionex, Hongkong). After data acquisition, the generated XML files were used to perform database searches using the MASCOT software v. 2.2 (Matrix Science, London, UK) using the following parameters: peptide tolerance, 0.2 Da; MS/MS ion mass tolerance, 0.1 Da; one missed cleavage; variable modifications used were methionine oxidation and cysteine carboxyamidomethylation.

#### Gene Ontology by DAVID software

Analysis of functional enrichment of differentially regulated proteins was performed in DAVID 6.7 software [34] <http://david.abcc.ncifcrf.gov/> according to the standard protocol [80]. The combined list of official gene symbols corresponding to the identified proteins was used for input.

#### 1-D and 2-D Western Blot Analysis

Proteins (40  $\mu$ g) were resolved either by SDS-PAGE or 2-DE and transferred onto a nitrocellulose membrane (GE Healthcare, Uppsala, Sweden) by semidry blotting using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories). Membrane was presoaked and equilibrated in Towbin transfer buffer [25 mM Tris, 192 mM glycine (20% methanol)]. Nonspecific binding was blocked with 5% skim milk in phosphate buffered saline (PBS). Primary antibodies used for immunodetection were given in Figure legends. They were obtained from the following

sources Dako Denmark A/S, Denmark; Santa Cruz, USA; kindly provided by Robert McKown, James Madison University and Gordon Laurie, University of Virginia.

Immunodetection was performed by incubating the membranes in antibody solutions; The list of antibodies used include the following- Polyclonal rabbit anti-human haptoglobin (Dako Denmark A/S, Denmark), polyclonal rabbit anti lipocalin 1 (Santa Cruz, USA) and polyclonal rabbit anti lacritin (kindly provided by Robert McKown, James Madison University and Gordon Laurie, University of Virginia). The antibodies were diluted in 1% BSA prepared in Tween PBS (TPBS) and used for incubation with the membrane at RT for 2 h. After three washes with 0.5% TPBS, the membrane was exposed to secondary antibody conjugated with horseradish peroxidase Goat anti Rabbit Ig G (Jackson ImmunoResearch Laboratories, USA) diluted 1:2000 in 0.5% TPBS for one hour at RT. The membranes were then incubated with 4-Chloro-Naphthol substrate for 30 minutes. The membranes were rinsed with water thrice and documented using Typhoon Trio variable mode scanner (with TAMRA, Alexafluor 546) (GE Healthcare, Uppsala, Sweden) and Image analysis was done using ImageQuantTL software (GE Healthcare, Uppsala, Sweden) was used for image analysis.

#### Supporting Information

**Table S1** Differentially Expressed Proteins in Tears of keratitis patients.  
(DOCX)

**Table S2** Identified Tear proteins and their functions. (DOCX)

**Table S3** Classification of differentially regulated proteins according to biological process, cellular & molecular. (DOCX)

**Table S4** Tear sample details used in this study. (DOCX)

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## Author Contributions

Conceived and designed the experiments: SA NVP PL KD. Performed the experiments: SA MV. Analyzed the data: SA NVP PL KD. Wrote the paper: SA NVP PL KD.

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