New developments in analysis of ocular surface diseases|Nieuwe ontwikkelingen in analyse van ziekten van het oogoppervlak

Keijser, S.

CHAPTER 9

LACTOFERRIN GENE POLYMORPHISM GLU561ASP COULD BE ASSOCIATED WITH EPITHELIAL HEALING OF INFECTIOUS CORNEAL ULCERS

Submitted

S. Keijser[1], H. Dogterom-Ballering[2], D.T. Schoonderwoerd[2], R.J.W. de Keizer[1], M.J.A. van der Plas[2], J.T. van Dissel[2], A. van der Lelij[3], M.J. Jager[1], P.H. Nibbering[2]

1 Department of Ophthalmology, Leiden University Medical Center, The Netherlands
2 Department of Infectious Diseases, Leiden University Medical Center, The Netherlands
3 Department of Ophthalmology, University Medical Center Utrecht, The Netherlands

Abstract

Background/aims. Lactoferrin is found in high concentrations in human tears, and plays an important role in host defense against infections and in down-regulation of ocular inflammation. Since polymorphic variants of lactoferrin may differ in their antibacterial activities, we investigated whether lactoferrin gene polymorphism is associated with susceptibility to and outcome of infectious corneal ulcers.

Methods. Information about the various clinical aspects of the corneal ulcers was extracted from patient's records. Lactoferrin gene polymorphisms Ala11Thr, Lys29Arg, and Glu561Asp were determined by restriction fragment length analysis on PCR-amplified genomic DNA in 70 patients with an infectious corneal ulcer. Lactoferrin concentration in tears of 26 patients and 40 healthy controls was quantified by ELISA.

Results. The frequencies of genotypes did not differ between patients and controls. Lactoferrin gene polymorphisms Glu561Asp showed a trend towards delayed epithelial healing for patients homozygous for 561Glu. Polymorphisms Ala11Thr, Lys29Arg did not reveal any differences for clinical outcome in covariate analysis. Lactoferrin concentration in tears of the affected and contralateral eye of the patients did not differ, and were the same as the lactoferrin levels of healthy controls.

Conclusion. Lactoferrin gene polymorphisms Ala11Thr, Lys29Arg, and Glu561Asp are not associated with the susceptibility to infectious corneal ulcers, but patients with glutamic acid at position 561 of the lactoferrin protein showed a trend for delayed healing of the corneal ulcer. Thus, differences in the functionality of the lactoferrin variants may contribute to the clinical outcome of infectious corneal ulcers.

Introduction

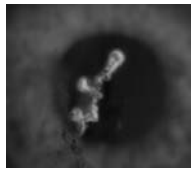
Infectious corneal ulceration is a rare but serious ophthalmologic disease that can cause corneal perforation[1,2] and blindness. In the Western World, the incidence of corneal ulceration is about 11 cases per 100.000 inhabitants;[3] and it has been estimated that worldwide each year 1.5 million people become unilaterally blind due to corneal ulcers.[4] The main risk factors for corneal ulceration are contact lens wear and trauma;[5] 30 % of all corneal ulcerations in the Western World are contact lens related.[6] Regarding the total number of individuals with extended-wear contact lenses, only a small minority develops an infectious corneal ulceration,[7] *Pseudomonas aeruginosa* being the most frequently cultured organism from contact lens associated ulcers.[8]

In general, the risk to develop an infectious corneal ulcer and/or its clinical outcome depends on the type of infectious etiologic agent, extent of precipitating trauma, the intrinsic antimicrobial capacity of the eye, and the inflammatory response of the host.[9] Among the factors determining the host response to eye infections, lactoferrin is a multifunctional protein with antimicrobial and anti-inflammatory activities, [10,11] and it is found in a high concentration in tears (approximately 2 mg/ml) (Keijser et al., submitted).[12] Recently, we investigated lactoferrin gene polymorphisms in patients with herpes simplex keratitis and found an association between polymorphism Glu561Asp and the susceptibility to herpes simplex keratitis. (Keijser et al. submitted). Two other lactoferrin polymorphisms causing amino acid substitutions at position 11 (Ala11Thr) and 29 (Lys29Arg) of the lactoferrin protein are associated with infections of the oral cavity.[13,14] In addition, the lactoferrin polymorphism Lys29Arg displayed differences in antibacterial and transcriptional activation activities.[15] Based on these findings, we investigated whether the lactoferrin genotypes are associated with the susceptibility and clinical outcome of infectious corneal ulcer.

Patients and methods

Patients

Seventy-six patients ages ≥18 years with an infectious corneal ulcer treated between 2004 and 2006 at the university eye clinics of LUMC and UMCU were invited to participate in the study. The study was approved by the local medical ethics committee (No p03-078), and followed the tenets of the Declaration of Helsinki. All patients provided a written informed consent. Patients with herpes simplex keratitis hepatitis C, or severe dry eyes as defined by a tear break up time (BUT) of less than 5 seconds and/or Schirmer test under 5 mm/5 min were excluded. Seventeen patients (36 male, 34 female, mean age 51 (range 18-91)) were included in the study. Twenty-nine patients (42 %) wore contact lenses at onset of the corneal infection, seven patients had diabetes (10%). Six patients were excluded from the study: three because they suffered from severe dry eyes, two with corneal decompensation, and one with a corneal ulceration after radiation of the eye for melanoma of the iris. Patient's records were used to extract demographic information, contact lens wear, best corrected visual acuity at onset, best corrected visual acuity after treatment, size of the ulcer, identification of the pathogen, duration of corneal defect, duration of treatment, and type of treatment. Visual acuity was measured with Snellen charts. All patients signed a written in-



formed consent before a tear sample and 10 ml of blood were collected. From 26 patients sufficient tears from the affected eye could be collected, of four of these patients no contralateral tear sample was available. Tears were acquired with Sugi Steril sponges (Kettenbach Medical, Eschenburg, Germany) which were kept in the inferior conjunctival fornix for a few minutes. Forty healthy subjects underwent a BUT and Schirmer test before a tear sample was collected. A blood sample was available from 145 control subjects without a history of severe systemic or eye infection; the characteristics of these patients have been reported earlier.[16]

PCR-restriction fragment length analysis of lactoferrin gene polymorphisms

We used PCR-restriction fragment length polymorphism analysis to determine the genotypes encoding the amino acids at positions 11, 29, and 561 as described earlier (Keijser et al., submitted). DNA was extracted from the blood samples using the Nucleospin® Blood L Kit (Macherey-Nagel A.G., Oensingen, Switzerland) and then amplified using a primer set for exon 1 and another set for exon 15 of the lactoferrin gene. Sequence for primers for exon 1 are: forward 5'-CTGTGTCTGGCTGGCCGTAGG-3' and reverse, 5'-AATGGCCTGGATACACTGGAT-3', for exon 15: forward 5'-ATTCCATTGCATGGACACAG-3', and reverse 5'-CCCACACAGCTAAGAAAGCA-3'.

PCR reactions were performed in ~50 µl comprising of 37 µl of H_2O, 3 µl of 25 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 1 µl containing 100 ng of DNA, 5 µl of 10x concentrated PCR buffer (Roche), 1 µl containing 10 pmol of each primer (Isogen Life Science, Maarssen, The Netherlands) , 1 µl of DMSO, 2 µl of 25 mM of dNTPs (Invitrogen, Carlsbad, CA, USA), and 0.16 µl of 5 U/ml Taq-polymerase (Promega®). The PCR reaction consisted of one denaturation step of 5 min at 95 °C and subsequently 35 cycles of 30 sec at 95°C followed by 30 sec at 55°C and 30 sec at 72°C with a final 10 min extension at 72°C. PCR products were detected after separation on a 2 % agarose gel (Invitrogen).

The following restriction enzymes: Hha1 (Gibco BRL, Paisley, Scotland), MBOII (Gibco BRL), and Hga1 (Biolabs, Hitchin, England) were used to detect the genotypes encoding the amino acids at position 11, 29, and 561, respectively. The PCR product (8 µl) was mixed with 1.5 µl of 2 U/ml of restriction enzyme and 1 µl of 10x buffer and then incubated for 3 hours at 37°C. To visualize the restriction fragments, the mixtures containing Hha1 or MBOII were run on spreadexgel (EL300, 50-200 bp, Elchrom, Scientific, Cham, Switzerland) and those containing HgaI on a 2% agarose gel.

ELISA for human lactoferrin

Tear lactoferrin concentrations were quantified by a human lactoferrin-specific ELISA as described earlier (Van Berkel et al.[17], and Keijser et al., submitted).

Statistics

Statistical analysis was performed using SPSS version 11 (SPSS, Chicago, IL, USA). Chi-

Square test was used to calculate the significance of the differences in frequencies of various lactoferrin genotypes between the patient group and control subjects. An oneway ANOVA and Cox regression mono- and covariate analysis were used to compare the lactoferrin genotypes with the clinical parameters. The ANOVA-test was also used to compare tear lactoferrin concentrations between the different genotypes. A P-value of 0.05 or less was considered significant.

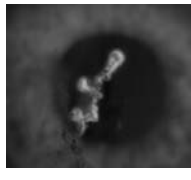
Results

The PCR analysis revealed no differences in the frequencies of the various genotypes of lactoferrin between the patients and the controls (Table 1). Next, we investigated whether the various genotypes may be associated with the clinical outcome of the infectious corneal ulcers. Patients homozygous for alanine at position 11 of the lactoferrin protein are suffering from a worse clinical outcome in monovariate cox regression analysis. The best corrected visual acuity at onset (p=0.028) and the duration of the corneal defect (p=0.017) differed significantly. However, when corneal ulcer size was introduced as a covariate in Cox regression analysis none of the clinical parameter reached significance (Figure 1). Furthermore, monovariate Cox regression analysis showed longer duration of epithelial defect in patients heterozygous for Glu561Asp (p=0.046). When corrected for corneal ulcer size a trend was still visible (p=0.075). In addition, patients with a 561Asp allele showed a faster epithelial healing than patients without the 561Asp allele (p=0.043), when corrected for corneal ulcer size a trend was still visible (p=0.06) (Figure 2). No differences were seen for clinical outcome in polymorphism Lys29Arg.

Table 1 Distribution of the various genotypes in exon 1 and exon 15 of the lactoferrin gene in patients with corneal ulcerations and in controls subjects. There are no significant differences.

		Patients		Control subjects	
Gene polymorphisms causing a substitution at position		Genotype frequencies	Allele frequencies	Genotype frequencies	Allele frequencies
11	Ala/Ala	57	75	55	74
	Ala/Thr	36		37	
	Thr/Thr	7	25	8	26
29	Lys/Lys	53	71	49	66
	Lys/Arg	37		34	
	Arg/Arg	10	29	17	34
561	Asp/Asp	10	40	16	40
	Asp/Glu	59		49	
	Glu/Glu	31	60	35	60

Data are from 70 patients and 145 controls. All genotypes are in Hardy-Weinberg equilibrium.



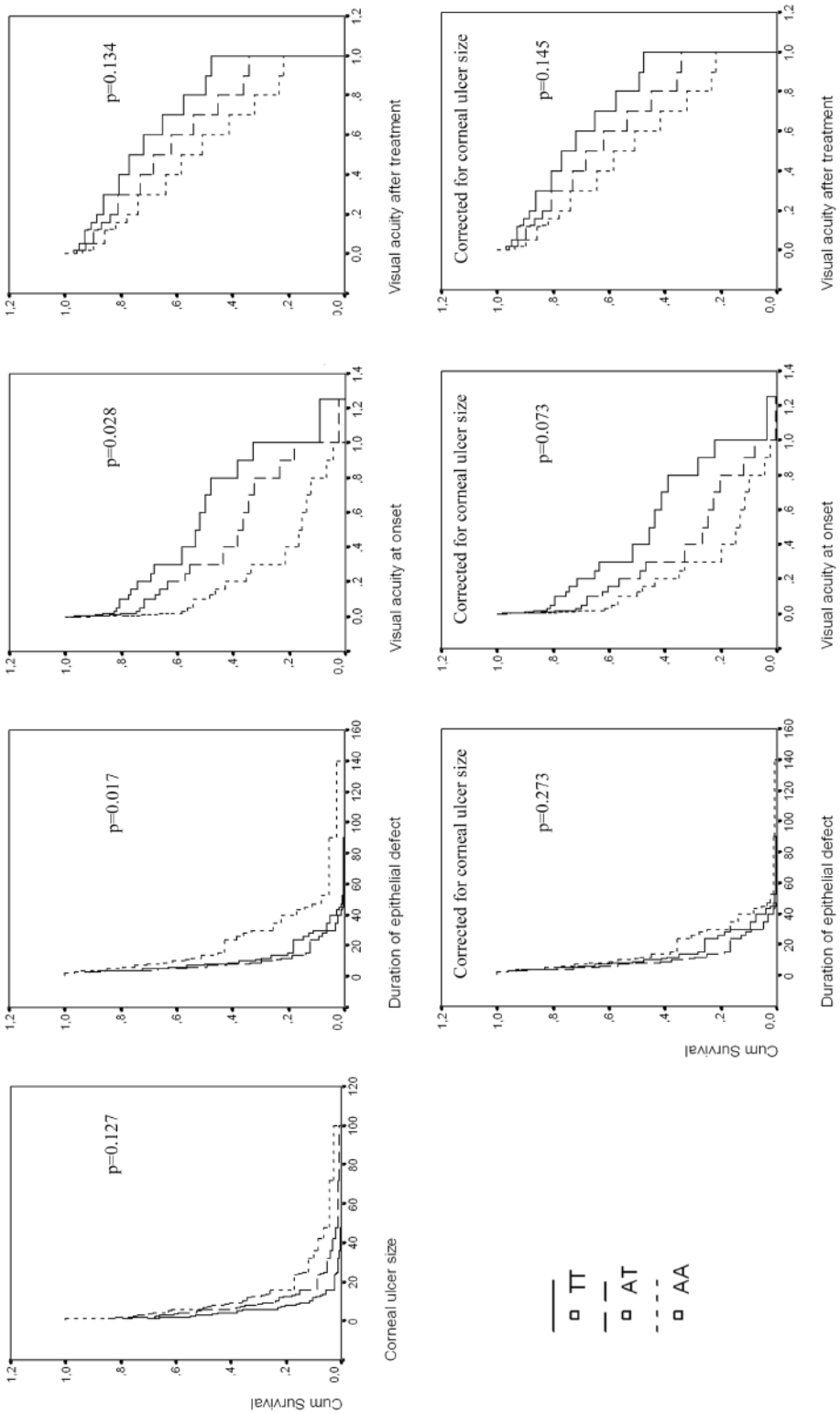


Figure 1. Cox regression analysis of lactoferrin polymorphism ala11thr and clinical outcome of infectious corneal ulcers. Note the reduced significance when corneal ulcer size was introduced as a covariate.

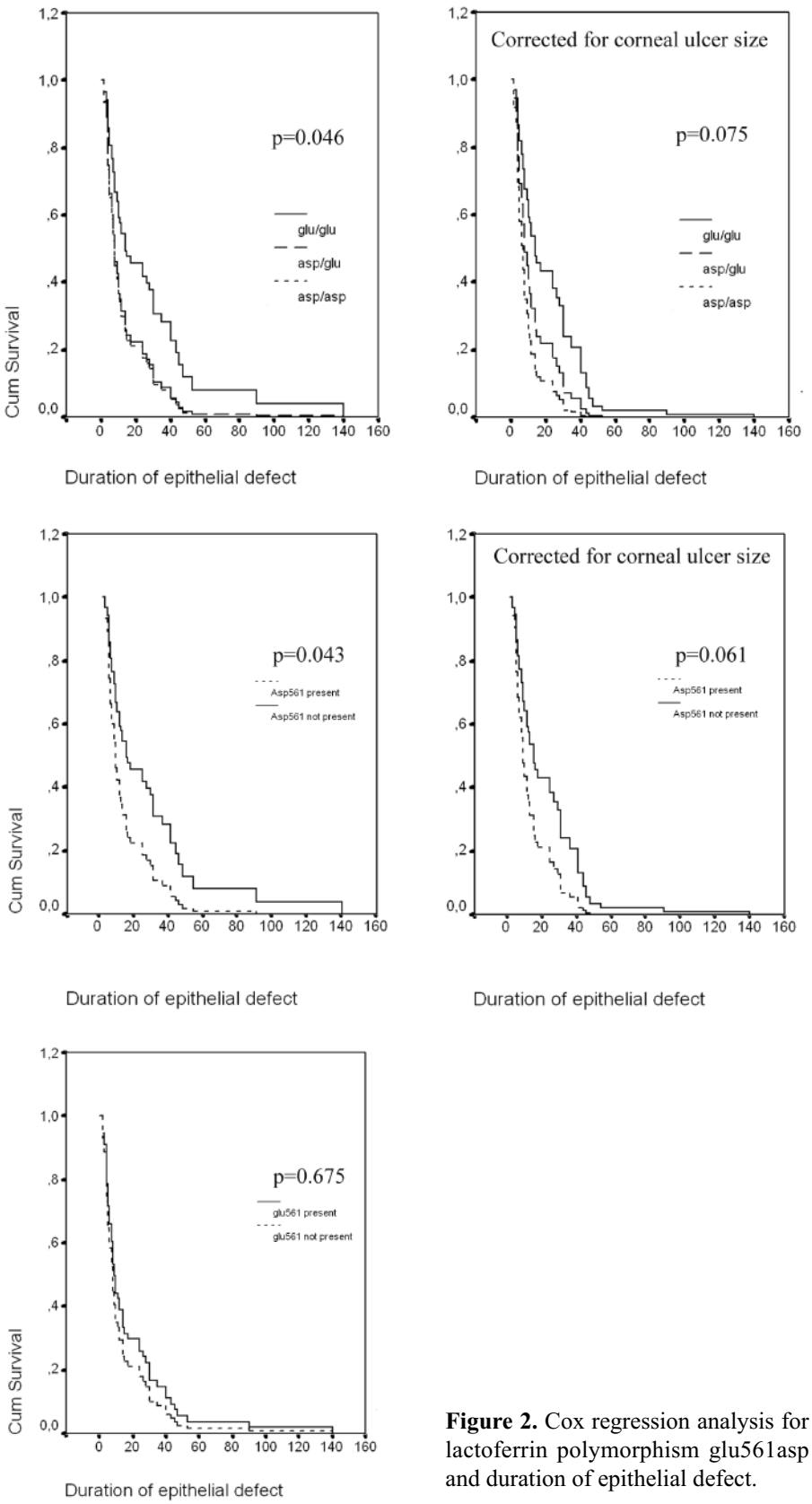


Figure 2. Cox regression analysis for lactoferrin polymorphism glu561asp and duration of epithelial defect.

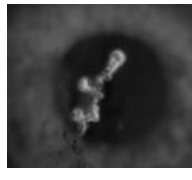
No differences in tear lactoferrin concentrations between the affected eye and the contralateral eye of patients (2.2 ± 0.3 mg/ml and 1.9 ± 0.2 mg/ml, respectively) were observed, and these values were about equal to lactoferrin concentrations in tears of healthy controls (2.1 ± 0.1 mg/ml).
Comparison between patients with and those without contact lenses revealed no differences in clinical outcomes, except the best corrected visual acuity after treatment in patients without contact lenses was significantly (p=0.018) lower than of patients with contact lenses. Patient with contact lenses were evenly distributed among the various lactoferrin genotypes.

Discussion

The main conclusion from the present study is that the lactoferrin gene polymorphism causing an glutamic acid/aspartic acid substitution at position 561 of the protein could be associated with the duration of epithelial defect of infectious corneal ulcers, but not with the susceptibility to this disease. Patients without the 561Asp allele seem to have a slower corneal epithelial healing than patients with 561Asp allele. This finding together with a previous report of an association with susceptibility to Herpes Simplex Keratitis and the Glu561Asp lactoferrin polymorphism (Keijser et al, submitted), indicates that the Glu561Asp polymorphism could influence the functionality of the lactoferrin protein. The Asp561 lactoferrin variant displays more flexibility in the C-lobe than Glu561 leading to the exposure of a hydrophobic domain (Araki-sasaki 2005), which could interfere with the interaction with other proteins. Further studies are required to elucidate the possible role of this amino acid substitution on the biological activities of lactoferrin.
Moreover, no differences in the tear lactoferrin levels among the various lactoferrin gene polymorphisms have been found (Keijser et al, submitted). In agreement with tear lactoferrin levels in Chinese patients with an infectious condition of the eye and healthy controls,[18] the tear lactoferrin levels in the affected and the contralateral eye of the patients were the same and did not differ from the levels in healthy controls, excluding the possibility that differences in tear lactoferrin levels affected the susceptibility to and clinical outcomes of infectious corneal ulcers in our study.
The genotype frequencies for the polymorphisms resulting in an amino acid substitution at positions 11, 29, and 561 of the lactoferrin protein did not differ between patients with infectious corneal ulcers and healthy controls. This observation may seem surprising, but it should be realized that events independent from the susceptibility to infections, such as microtrauma to the corneal epithelium, precede an infectious corneal ulcer. Although no association was found for infectious corneal ulcer patients and susceptibility between lactoferrin gene polymorphisms, others showed that lactoferrin gene polymorphisms causing a lysine/arginine substitution at position 29 and an alanine/threonine substitution at position 11 of the protein are associated with susceptibility to aggressive (bacterial) periodontitis.[19,20]
Obviously, the present lactoferrin gene polymorphisms do not explain susceptibility to infectious corneal ulcers. It is likely that that further polymorphisms within the lactoferrin gene[21] as well as the genes for other host-response factors, such as lysozyme[22] and cytokines like interleukin (IL)-10,[23] IL-12,[24,25] interferon-gamma,[26] and corticosteroids contribute to susceptibility to infectious corneal ulcers. Furthermore, other - yet unidentified - genes,

which control the presence and numbers of bacteria in corneal epithelium, may render some patients less susceptible than others. In addition, numerous factors related to the causative agent(s) could also be involved in the susceptibility. The kind of bacteria found in the cultures is related to the clinical outcome in patients with infectious corneal ulcers,[27] with commonly found bacteria such as *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. One possible confounding factor in our present data could be contact lens wear as it has been reported that this is a risk factor for the development of infectious corneal ulcers.[28] However, we found no difference in contact lens wear among the patient groups with the different lactoferrin gene polymorphisms. Although the number of patients included in our study may seem small, it is in full agreement with the results of our power calculations. Therefore, we believe that our data allow us to draw reliable conclusions.

The results presented here and those reported by others[29,30,31] and us (Keijser et al., submitted) revealing an association between lactoferrin gene polymorphisms and susceptibility to and clinical outcome of mucosal infections underline the notion that genes play a role in the predisposition to and progression of infections of the eye. Once the contribution of the genetic variations in the different genes to susceptibility/progression of infections of the eye have been documented, a better risk profile for patients can be made, which may in the future influence the treatment of infections to the eye.



References

1. Laibson PR. Cornea and sclera. *Arch Ophthalmol*. 1972;88:553-574.
2. Jones DB. Early diagnosis and therapy of bacterial corneal ulcers. *Int Ophthalmol Clin*. 1973;13:1-29.
3. Erie JC, Nevitt MP, Hodge DO, Ballard DJ. Incidence of ulcerative keratitis in a defined population from 1950 through 1988. *Arch Ophthalmol*. 1993;111:1665-1671.
4. Whitcher JP, Srinivasan M. Corneal ulceration in the developing world—a silent epidemic. *Br J Ophthalmol*. 1997;81:622-623.
5. Kanski JJ. Disorders of the cornea and sclera. In: Kanski JJ. eds. *Clinical ophthalmology*. Oxford: Butterwoth-Heinemann; 1999:103-105.
6. Mah-Sadorra JH, Yavuz SG, Najjar DM et al. Trends in contact lens-related corneal ulcers. *Cornea*. 2005;24:51-58.
7. Poggio EC, Glynn RJ, Schein OD et al. The incidence of ulcerative keratitis among users of daily-wear and extended-wear soft contact lenses. *N Engl J Med*. 1989;321:779-783.
8. Bosscha MI, Van Dissel JT, Kuijper EJ, Swart W, Jager MJ. The efficacy and safety of topical polymyxin B, neomycin and gramicidin for treatment of presumed bacterial corneal ulceration. *Br J Ophthalmol*. 2004;88:25-28.
9. Ma JJ, Dohlman CH. Mechanisms of corneal ulceration. *Ophthalmol Clin North Am*. 2002;15:27-33.
10. Ward PP, Uribe-Luna S, Conneely OM. Lactoferrin and host defense. *Biochem Cell Biol*. 2002;80:95-102.
11. Levay PF, Viljoen M. Lactoferrin: a general review. *Haematologica*. 1995;80:252-267.
12. Kijlstra A, Jeurissen SH, Koning KM. Lactoferrin levels in normal human tears. *Br J Ophthalmol*. 1983;67:199-202.
13. Velliyagounder K, Kaplan JB, Furgang D et al. One of two human lactoferrin variants exhibits increased antibacterial and transcriptional activation activities and is associated with localized juvenile periodontitis. *Infect Immun*. 2003;71:6141-6147.
14. Jordan WJ, Eskdale J, Lennon GP et al. A non-conservative, coding single-nucleotide polymorphism in the N-terminal region of lactoferrin is associated with aggressive periodontitis in an African-American, but not a Caucasian population. *Genes Immun*. 2005;6:632-635.
15. Velliyagounder K, Kaplan JB, Furgang D et al. One of two human lactoferrin variants exhibits increased antibacterial and transcriptional activation activities and is associated with localized juvenile periodontitis. *Infect Immun*. 2003;71:6141-6147.
16. Schippers EF, van ‘, V, van Voorden S et al. TNF-alpha promoter, Nod2 and toll-like receptor-4 polymorphisms and the in vivo and ex vivo response to endotoxin. *Cytokine*. 2004;26:16-24.
17. van Berkel PH, van Veen HA, Geerts ME, de Boer HA, Nuijens JH. Heterogeneity in utilization of N-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants. *Biochem J*. 1996;319 (Pt 1):117-122.
18. Chen WJ. [Tear lactoferrin content in normal Chinese adults and various ocular diseases]. *Chung Hua Yen Ko Tsa Chih*. 1989;25:292-295.
19. Jordan WJ, Eskdale J, Lennon GP et al. A non-conservative, coding single-nucleotide polymorphism in the N-terminal region of lactoferrin is associated with aggressive periodontitis in an African-American, but not a Caucasian population. *Genes Immun*. 2005;6:632-635.
20. Velliyagounder K, Kaplan JB, Furgang D et al. One of two human lactoferrin variants exhibits increased antibacterial and transcriptional activation activities and is associated with localized juvenile periodontitis. *Infect Immun*. 2003;71:6141-6147.
21. Teng CT, Gladwell W. Single nucleotide polymorphisms (SNPs) in human lactoferrin gene. *Biochem Cell Biol*. 2006;84:381-384.
22. Mehra KS, Singh R, Bhatia RP, Sen PC, Singh H. Lysozyme in corneal ulcer. *Ann Ophthalmol*. 1975;7:1470-1472.
23. Hazlett LD, McClellan SA, Barrett RP et al. Spantide I decreases type I cytokines, enhances IL-10, and reduces corneal perforation in susceptible mice after Pseudomonas aeruginosa infection. *Invest Ophthalmol Vis Sci*. 2007;48:797-807.
24. Hazlett LD, Huang X, McClellan SA, Barrett RP. Further studies on the role of IL-12 in Pseudomonas aeruginosa corneal infection. *Eye*. 2003;17:863-871.

25. Hazlett LD, Rudner XL, McClellan SA, Barrett RP, Lighvani S. Role of IL-12 and IFN-gamma in Pseudomonas aeruginosa corneal infection. *Invest Ophthalmol Vis Sci*. 2002;43:419-424.
26. Hazlett LD, Rudner XL, McClellan SA, Barrett RP, Lighvani S. Role of IL-12 and IFN-gamma in Pseudomonas aeruginosa corneal infection. *Invest Ophthalmol Vis Sci*. 2002;43:419-424.
27. Keay L, Edwards K, Naduvilath T, Forde K, Stapleton F. Factors affecting the morbidity of contact lens-related microbial keratitis: a population study. *Invest Ophthalmol Vis Sci*. 2006;47:4302-4308.
28. Keay L, Edwards K, Naduvilath T et al. Microbial keratitis predisposing factors and morbidity. *Ophthalmology*. 2006;113:109-116.
29. Jordan WJ, Eskdale J, Lennon GP et al. A non-conservative, coding single-nucleotide polymorphism in the N-terminal region of lactoferrin is associated with aggressive periodontitis in an African-American, but not a Caucasian population. *Genes Immun*. 2005;6:632-635.
30. Mohamed JA, DuPont HL, Jiang ZD et al. A novel single-nucleotide polymorphism in the lactoferrin gene is associated with susceptibility to diarrhea in North American travelers to Mexico. *Clin Infect Dis*. 2007;44:945-952.
31. Velliyagounder K, Kaplan JB, Furgang D et al. One of two human lactoferrin variants exhibits increased antibacterial and transcriptional activation activities and is associated with localized juvenile periodontitis. *Infect Immun*. 2003;71:6141-6147.