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Tear Cytokine Levels in Contact Lens Wearers with Acanthamoeba Keratitis
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Abstract:	ABSTRACT Purpose: To determine differences in key tear film cytokines between mild and severe cases of Acanthamoeba Keratitis (AK) and control contact lens (CL) wearers. Methods: This was a prospective study of CL wearers with AK attending Moorfields Eye Hospital (MEH) and control CL wearers from the Institute of Optometry, London. Basal tear specimens were collected by 10ul capillary tubes (Blaubrand intraMARK, Wertheim, Germany) and tear protein levels were measured with a multiplex magnetic bead array (Luminex 100, Luminex Corporation, Austin, TX) for cytokines IL-1β, IL-6, IL-8, IL-10, IL-17A, IL-17E, IL-17F, IL-22, and IFNγ and with ELISA (Abcam, Cambridge, UK) for CXCL2. Severe cases of AK were defined as having active infection for over 12 months and at least one severe inflammatory event. Results: One hundred and thirty two tear samples were collected from a total of 61 cases (15 severe and 46 mild-moderate) and 22 controls. IL-8, part of the TLR4 cytokine cascade, was found to be expressed at a detectable level more often in cases of AK compared to control CL wearers (p=0.003), and in higher concentrations in severe compared to milder forms of the disease (z=-2.35). IL-22, part of the IL-10 family, and a proinflammatory Th17 cytokine, was detected more often in severe compared to milder forms of AK (p<0.02). Conclusion: Profiling Acanthamoeba Keratitis patients during disease shows differences in cytokine levels between severe and milder disease that may inform clinical management. The TLR4 and IL-10/Th17 inflammatory pathways should be included in further investigations of this disease.

1   Tear Cytokine Levels in Contact Lens Wearers with Acanthamoeba Keratitis

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30   acanthamoeba, keratitis, contact lens, tear, cytokine

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40

41   ABSTRACT

42   Purpose: To determine differences in key tear film cytokines between mild and severe cases

43   of Acanthamoeba Keratitis (AK) and control contact lens (CL) wearers.

44   Methods: This was a prospective study of CL wearers with AK attending Moorfields Eye

45   Hospital (MEH) and control CL wearers from the Institute of Optometry, London. Basal tear

46   specimens were collected by 10ul capillary tubes (Blaubrand intraMARK, Wertheim,

47   Germany) and tear protein levels were measured with a multiplex magnetic bead array

48   (Luminex 100, Luminex Corporation, Austin, TX) for cytokines IL-1β, IL-6, IL-8, IL-10, IL-17A,

49   IL-17E, IL-17F, IL-22, and IFNγ and with ELISA (Abcam, Cambridge, UK) for CXCL2. Severe

50   cases of AK were defined as having active infection for over 12 months and at least one severe

51   inflammatory event.

52   Results: One hundred and thirty two tear samples were collected from a total of 61 cases (15

53   severe and 46 mild-moderate) and 22 controls. IL-8, part of the TLR4 cytokine cascade, was

54   found to be expressed at a detectable level more often in cases of AK compared to control CL

55   wearers (p=0.003), and in higher concentrations in severe compared to milder forms of the

56   disease (z=-2.35). IL-22, part of the IL-10 family, and a proinflammatory Th17 cytokine, was

57   detected more often in severe compared to milder forms of AK (p<0.02).

58   Conclusion: Profiling Acanthamoeba Keratitis patients during disease shows differences in

59   cytokine levels between severe and milder disease that may inform clinical management. The

60   TLR4 and IL-10/Th17 inflammatory pathways should be included in further investigations of

61   this disease.

62

63 INTRODUCTION

64 Acanthamoeba Keratitis (AK) is one of the most severe forms of corneal infection, with over

65 90% of cases occurring in contact lens (CL) wearers.[1] Vision loss occurs in 33% of patients,

66 with corneal transplantation required in around 26%.[2] Recent reports, and case monitoring at

67 our centre, show that the numbers of AK cases are increasing.[3, 4] AK generally affects a young

68 and otherwise healthy group of individuals[5] in whom lifetime disability costs are high. As well

69 as the long term effects, such as decreased quality of life, and loss of productivity due to

70 reduced vision, there are significant short term costs to sufferers and carers, such as loss of

71 wages and distress, in addition to symptoms such as severe pain and light sensitivity

72 experienced by sufferers.[6]

73 Some complications associated with CLs are somewhat controlled by the release of tear

74 inflammatory molecules, such as giant papillary conjunctivitis which is characterized by altered

75 levels of eotaxin[7] or corneal neovascularization which is mediated by vascular endothelial

76 growth factor (VEGF).[8] Moreover, it has been shown that CL wearers with CL-induced acute

77 red eye present higher concentrations of IL-8 than healthy subjects[9]. Others[10-13] have indicated

78 altered levels of tear cytokines such as interleukin (IL)-6, IL-8 and epidermal growth factor

79 (EGF) during CL wear. However, to this day little is known about the tear inflammatory

80 mediation in AK. Profiling AK patients during disease could show differences in cytokine levels

81 between severe and milder disease that may inform clinical management.

82 The aim of this study is to determine the differences in cytokine levels in CL-wearing patients

83 with AK compared to CL wearers without the disease. A secondary goal is to investigate

84 differences in cytokine levels between patients with severe forms of AK and those with mild-

85 moderate forms of this infection

86 MATERIALS AND METHODS

87   This was a prospective case control study of CL wearers with AK attending Moorfields Eye

88   Hospital (MEH) and control CL wearers from the Institute of Optometry, London. The research

89   protocol adhered to the tenets of the Declaration of Helsinki and was approved by the local

90   ethics committee. Written informed consent was obtained from all participants.

91   Participants

92   Severe cases of AK were defined as having active infection for over 12 months and having

93   had at least one severe inflammatory event such as scleritis, persistent non-healing defect (for

94   14 days or more) and/or pupil paralysis. Mild-moderate cases had recurrent disease in the

95   absence of severe inflammatory events or disease that required active treatment for less than

96   and up to 12 months.

97   Tear sample collection

98   Samples were collected from AK patients at follow-up visits during their treatment on a

99   convenience basis depending on the flow of the clinic visits. Collection times varied between

100   10am and 4pm. Samples were collected from the affected eye only. For bilateral cases, the

101   worst affected eye was sampled.

102   Samples were collected from control CL wearers at the conclusion of routine aftercare

103   appointments at the Institute of Optometry. Lenses may or may not have been worn according

104   to the patient preference at the end of the appointment. So as to not affect the equilibrated

105   tear milieu, that status remained for tear collection. For these control CL wearers, samples

106   were collected from the right eye, and switched to the left should no sample be obtained from

107   the right eye. In monocular wearers, the eye sampled was the CL wearing eye.

108   Tear samples for both AK cases and controls were basal tear specimens collected by 10ul

109   capillary   tubes   (Blaubrand   intraMARK,   Wertheim,   Germany)   and   stored   in

110   ethylenediaminetetraacetic acid (EDTA) coated 0.5ml Eppendorf tubes. Following collection,

111 the samples were kept cold using a standard cool box and ice packs. Upon delivery to the

112 laboratory on the same day, the samples were centrifuged at 1,600rpm for 5 minutes. The

113 cell-free supernatant was then pipetted into clean EDTA coated 0.5ml Eppendorf tubes and

114 stored at -80°C prior to analysis.

115 Analysis of tear molecules

116 Cytokines IL-1β, IL-6, IL-8, IL-10, IL-17A, IL-17E, IL-17F, IL-22, interferon (IFNγ) and

117 chemokine (C-X-C motif) ligand 2 (CXCL2) were chosen for analysis based on established

118 and hypothesised inflammatory pathways in AK. Tear protein levels were measured with a

119 multiplex bead array using the Luminex based platform (Luminex 100, Luminex Corporation,

120 Austin, TX) for all analytes apart from CXCL2. CXCL2 was measured with an enzyme-linked

121 immunosorbent assay (ELISA, Abcam, Cambridge, UK) as this protein was not compatible

122 with the chosen Luminex range of targets.

123 Samples were diluted with the respective kit reagent depending on the sample volume and

124 normalised for analysis. Standard curves using duplicate known dilutions were generated for

125 the Luminex and ELISA analysis. Luminex data were analysed with the instrument software

126 and raw scores of the ELISA optical density were converted to concentrations in Excel 2010

127 (Microsoft). Concentrations lower than the detectable limits were labelled as not detectable

128 (ND). Final concentrations above the minimum detectable limit were adjusted for the dilution

129 factor.

130 Data analysis

131 Statistics were analysed using Graphpad.com/online calculator and Microsoft Excel 2010

132 software.

133 Differences between cases and controls and between severe and mild-moderate cases were

134 determined as follow: Fishers exact test was performed to determine the proportions of

135 detectable samples and Mann-Whitney U test was used for the sample quantities over the

136 detectable levels.

137 P values less than or equal to 0.05 were considered statistically significant.

138 RESULTS

139 One hundred and thirty two tear samples were collected from a total of 61 AK cases (15 severe

140 and 46 mild-moderate) and 22 controls. There were no differences in gender distribution

141 between case and control groups (p=0.06), however significant differences in age were found

142 between the groups (p<0.001). In addition, there were more daily disposable wearers in the

143 control group compared to the AK cases (p=0.02). Descriptive data detailing age, gender and

144 lens type, are shown in Table 1.

145 Levels of IL-6, IL-8, IL-22 and IL-17E were readily detectable. The levels of IFNy, IL-17F, IL-

146 17A, IL-10, IL-27 and IL-1β were below the minimum detectable limit for all case and control

147 samples. The proportion of non detectable (ND) samples for each protein are detailed in Table

148 2.

149 Cases vs. controls

150 Figure 1 shows the proportion of cytokines for the cases and controls for each of the molecules

151 for which there was more than 1 positive sample (IL-1 β was detectable in only one sample,

152 and was considered "non detectable" for this study). There were more samples with detectable

153 levels of IL-8 in the cases compared to the controls (p=0.003). Almost half of the tear

154 specimens in both groups had detectable levels of IL-22, whereas IL-6 and IL-17E showed

155 very low frequencies of positivity. The one control with a positive sample for IL-6 was not the

156 same control that was the only control sample positive for IL-17E. There was no difference

157 between the CXCL2 levels for cases and controls with more than 75% of tear specimens

158 yielding detectable quantities of this molecule (cases 56/67, 84% and controls, 10/13, 77%).

159   Figures 2-5 show the concentrations of IL-6, IL-8, IL-22, and IL-17E, respectively in tears of

160   individual cases (by visit) and individual controls that measured above detectable limits by

161   Luminex. Figure 6 shows the concentrations CXCL2 in tears of individual cases (by visit) and

162   individual controls that measured above detectable limits by ELISA. There was no difference

163   between the median concentration of IL-8, IL-22 and CXCL2 in tears of cases and controls

164   (z=-0.57, z=0.97 and Z=0.05 respectively). Only one control sample was positive for IL-6 and

165   IL-17E and so Mann-Whitney U Test could not be performed.

166   Severe vs. mild-moderate cases

167   Figure 7 shows the proportions of detectable protein samples (IL-8, IL-22, IL-6 and IL-17E)

168   investigated with Luminex for severe compared to mild-moderate cases. IL-22 was less likely

169   to be detected amongst the mild-moderate cases compared to the severe cases of AK

170   (p=0.02), however there was no difference between mild-moderate cases and severe cases

171   for the proteins, IL-8, IL-6 and IL-17E (p=0.48, p=0.27 and p=1.0 respectively). There was also

172   no difference in CXCL2 levels between the severe and moderate/mild cases (23/29, 79.3%

173   compared to 33/38, 86.8%, p=0.41)

174   Table 3 shows the median tear protein concentrations for severe compared to the mild-

175   moderate samples. There was a higher level of IL-8 detectable in the tears of severe cases

176   compared to the mild-moderate cases of this infection (z=-2.31), however there was no

177   difference between tear protein levels of IL-22, IL-6, IL-17E and CXCL2).

178   DISCUSSION

179   The present study was the first to examine the cytokine levels in patients with mild compared

180   to more severe AK, and compare these to control CL wearers. This study has highlighted IL-

181   8 as a key molecule in the AK inflammatory response, and there is also some evidence for

182   cell mediated inflammatory response involving the IL-17 pathway, via IL-22.

183   IL-8 was found to be expressed at a detectable level measured by Luminex more often in

184   cases of AK compared to control CL wearers, and in higher concentrations in more severe

185   compared to milder forms of the disease. IL-8 is a key inflammatory chemokine that mobilises

186   and activates neutrophils.[7] Neutrophils are essential components of the early inflammatory

187   response to *Acanthamoeba*.[8] Furthermore, IL-8 is part of the toll like receptor 4 (TLR-4)

188   cascade which initiates the cytokine response in AK.[9] IL-8 also promotes angiogenesis in the

189   eye[7] and further characterisation of patients that develop neovascularisation in AK may reveal

190   differences in levels that may predict patients who go on to develop this complication, and

191   more targeted management such as frequent topical steroids may be advocated in these

192   cases. Neovascularisation is a contraindicated in corneal transplant candidates, often the last

193   resort to significantly improve vision in AK patients. Keratoplasty is required for visual

194   rehabilitation in around 12% of AK cases.[2]

195   IL-22, part of the IL-10 family, and a proinflammatory Th17 cytokine,[10] was detected more

196   often in severe compared to milder forms of AK. IL-22 may prolong the inflammatory response

197   and, in severe forms of disease, this may be beneficial to control infection but may also be

198   involved in tissue destruction due to inflammation.

199   Most of the IL-17 cytokines were not detected in levels high enough to be measured in the

200   tears in these subjects using Luminex technology. Since multiplex bead arrays are well

201   established as being one of the more sensitive methods of detection for low levels of analytes,

202   the specimens with no detectable levels were presumed negative. It may be useful to compare

203   the IL-17E cytokine, which was expressed by a small number of cases and one control, using

204   ELISA, in another cohort of samples. Like IL-22, IL-17 has been implicated in chronic

205   inflammatory conditions[11] and IL-17A has recently been shown to be protective against

206   *Acanthamoeba* keratitis severity in a mouse model.[12] This contrasts with keratitis caused by

207   Herpes Simplex Virus and *Pseudomonas* where IL-17A is associated with an increased

208   corneal inflammatory response.[13-15]. IL-17A is known as a "double sword" agent; in some

209   circumstances it protects the host and in others, it results in chronic inflammation and tissue

210   damage.[16]) IL-17A both initiates and activates neutrophils and is also produced by

211   neutrophils.[17] Recently, a novel population of neutrophils were characterized, that are capable

212   of autocrine IL-17A activity, which leads to increased death of fungal hyphae in a murine model

213   of *Aspergillus* corneal infection.[18]

214   CXCL2 (also known as macrophage inflammatory protein 2-alpha, MIP2-α) appears to be

215   constitutively expressed in AK cases and control CL wearers and not up- or down-regulated

216   in this disease. MIP2 has been shown to be important in animal models of AK.[19] Animal models

217   of disease do not exhibit the severe inflammatory complications of AK, such as scleritis [5], and

218   inflammatory pathways may vary somewhat between humans and animal models.

219   IL-6, a proinflammatory cytokine with several functions, was only detected in one control

220   sample; either this study did not have enough power to show differences between cases and

221   controls or IL-6 is not important in the inflammatory response in this disease. Furthermore it is

222   possible that there is a defect in IL-6 at the protein level. Our group has found that single

223   nucleotide polymorphisms (SNP) of IL-6 genes are implicated in the susceptibility and severity

224   of bacterial keratitis in CL wearers.[20] IL-6 is a key player in the IL-22 and IL-17 pathways[11] and

225   it would be prudent to further investigate this protein as a candidate in future immunological

226   analysis in AK.

227   Cytokine and chemokine profiles correlate with several inflammatory anterior eye disease

228   states such as dry eye,[21-24] allergic eye disease,[25, 26] the autoimmune condition, Sjogren's

229   syndrome,[27-29] vernal keratoconjunctivitis[30] and ocular rosacea.[31] Two studies have

230   highlighted tear protein profiles associated with bacterial[32] and fungal keratitis.[33]

231   In bacterial keratitis, cytokines and chemokines are upregulated in both the affected and

232   contralateral eye, and these changes have been correlated with cellular changes imaged on

233   the ocular surface [32]. Specifically, IL-1β, IL-6 and IL-8 were elevated in the 'infected' tears

234 compared to non-affected controls. Changes were also found in the contralateral eye of
235 bacterial keratitis patients, namely the upregulation of chemokine ligand 2 (CCL-2), IL-10 and
236 IL-17a. TREM-1 was also elevated in both the affected and contralateral eyes. Changes in
237 tear cytokines were correlated with dendritic cell and sub-basal nerve fibre presence and
238 morphology, as follows; tear concentrations of the proinflammatory cytokines, IL-1B, IL-6, IL-
239 8 and IL-17a were positively correlated with dendritic cell density, and IL-1B, IL-6, IL-8 and
240 TREM-1 were inversely correlated with sub-basal nerve density.

241 Proteomic analyses have been used in an Indian study of fungal keratitis patients compared
242 to controls to examine differences between tear proteins. Seven protein levels varied between
243 the cases and controls: Prolactin inducible protein and serum albumin precursor were up
244 regulated in the infected samples; Cystatin S precursor, cystatin SN precursor, cystatin, and
245 human tear lipocalin were downregulated in the infected samples; glutaredoxin-related protein
246 was found only in the infected samples[33].

247 Concentrations of the following cytokines for all subjects in this study fell below the detectable
248 limit for IFNγ, IL-10, IL-1β, IL-27 as well as IL-17F and IL-17A. Cross reactivity of the
249 antibodies and/or poor sensitivity of the array are unlikely to be implicated since bead-based
250 Luminex technology is one of the most sensitive assays available and has successfully
251 allowed detection of cytokines in tear fluids.[34] It is possible that these cytokines were masked
252 from detection in the tear specimens due to a build-up of protein and debris at the ocular
253 surface. Alternatively, these cytokines might not be involved in this disease but, until a larger
254 cohort of specimens and controls is investigated, this cannot be assumed.

255 The differences in cytokine levels found in this study may be due to the effects of the disease
256 on the immune system and/or due to differences in the individual's immune profile at the gene
257 level. Being such a rare disease, it is impossible to conduct a prospective study and compare
258 cytokine levels before and during AK disease, however future studies that assess variations
259 in the DNA structure of these genes in patients will provide more insight into this conundrum.

260 Furthermore the differences between mild/moderate and severe disease may be due to

261 differences in strains of Acanthamoeba organism. The majority of Acanthamoeba spp that

262 cause keratitis are from the T4 group based on 18s RNA genotyping that separates strains

263 into 17 evolutionary clades or groups (T1-T17). Preliminary information from one study

264 indicates that strains with non-T4 genotypes may cause more severe disease,[35] however, only

265 three cases of non-T4 AK were compared to 14 T4 genotypes and confirmation in a larger

266 study is required. As genetic profiling of Acanthamoeba spp. allows more refined typing, as

267 can be seen by the mitochondrial cytochrome oxidase (Cox) gene sequencing,[36] and greater

268 number of cases are reported from other T strains[37, 38] correlation between different strains

269 and the outcomes of AK may be found. Human biomarker profiling alongside in vitro and

270 animal models will be key to future understanding of the interplay between the host immune

271 system and organism virulence that is evidenced in some conditions such as malaria.[39]

272 A limitation of the present study could be that AK cases were younger than controls. Tear

273 investigations have generally been limited to normals or certain conditions affecting specific

274 age groups and differences between normals across a range of ages has not been shown.

275 Dry eye is more prevalent in older individuals, and increased levels of two cytokines measured

276 in this study, IL-6 and IL-8 have been found in elevated levels in dry eye patients.[21-24] The

277 controls in this study, although older than the cases, were successful CL wearers, and are

278 unlikely to have had significant dry eye disease. In any case, had some of the cases been on

279 the dry eye spectrum, this would have only potentially masked greater differences in IL-8 levels

280 and would not have affected the IL-6 results, in which only one control showed a reading

281 above the detectable level.

282 More daily disposable wearers were in the control group compared to AK patients in this study.

283 This likely reflects the evidence that AK is more often a disease that occurs in reusable lens

284 wearers, as the environmental contamination of lens cases supports the growth of

285 Acanthamoeba spp.[40] Only one study has evaluated the tear profile while wearing different

286   lens types; using lotrafilcon B (O2OPTIX; CIBA VISION, Duluth, Atlanta, GA) or senofilcon A

287   (Acuvue Oasys; Johnson & Johnson Vision Care, Inc., Jacksonville, FL), no differences in

288   levels on matrix metalloproteinase 9 (MMP-9), tissue inhibitor of metalloproteinases 1 (TIMP-

289   1) and neutrophil gelatinase-associated lipocalin (NGAL) during adapted daily wear were

290   found.[41] It is unlikely that even if lens wear type had an effect on tear cytokine/chemokine

291   levels that this would confound results in the present study as all the AK patients and a

292   proportion of the controls were not wearing lenses at the time of collection.

293   Another limitation of the study might be the time of the tear samples collection. The tear

294   collection time was scheduled between 10am and 4pm to minimise possible diurnal effect and

295   disruption to the MEH and IO clinics. While there are recent publications showing a diurnal

296   change of certain tear cytokines and chemokines they indicate a difference between daytime

297   and evening intervals (11am-1pm vs 5pm -7pm)[42]; 12am (midday) compared to 9-12pm

298   (midnight).[43] It is improbable that there would be a major variation in cytokine and chemokine

299   levels during the 6-hour daytime interval in which we sampled tears.

300   This study highlights key areas for future investigation of the pathogenesis of AK. We have

301   shown that in a clinical setting, we can collect tears from patients with AK that may indicate

302   the inflammatory status of the eye. Further investigation of cytokines not detected in this study,

303   and other candidates in the pathways indicated by this analysis, may define a wider spectrum

304   of cytokine changes. In association with careful tracking of patients during the disease

305   process, we may be able to predict when the inflammatory status is changing. This information

306   may help the clinician to better understand the clinical picture and make more informed

307   decisions on individual AK patient management.

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314 Hospital who helped coordinate the study.

315

316 REFERENCES

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318

319 Figure legends

320 Figure 1. The distribution of the detectable samples for each analyte tested with Luminex for

321 AK case samples and controls

322 Figure 2. IL-6 protein levels above minimum detectable for individual cases (by visit) and

323 individual controls measured by Luminex

324 Figure 3. IL-8 protein levels above minimum detectable for individual cases (by visit) and

325 individual controls measured by Luminex

326 Figure 4. IL-22 protein levels above minimum detectable for individual cases (by visit) and

327 individual controlsas measured by Luminex

328 Figure 5. IL-17E protein levels above minimum detectable for individual cases (by visit) and

329 individual controlsas measured by Luminex

330 Figure 6 CXCL2 protein levels above minimum detectable for individual cases (by visit) and

331 individual controls measured by ELISA

332 Figure 7. Detectable sample distribution for severe compared to mild-moderate AK cases

333 measured with Luminex (mod=moderate)

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446

Table 1

Table 1. Descriptive data of participants recruited for the study.

	AK Cases (n=61)	Controls (n=22)	p value
Age, years, mean (SD)	35.4 ±13.6	52.7±15.4	<0.001
Gender, n (%) Males Females	28 (45.9) 33 (54.1)	5 (22.7) 17 (77.3)	0.06
Type of CL worn, n (% known) Daily soft 2-4 weeks disposable soft >1 month replacement soft unknown	9 (20.0) 33 (73.3) 3 (6.7) 16	14 (63.6) 7 (31.8) 1 (4.5) 0	0.02

SD= standard deviation; CL= contact lens

Table 2

Table 2. Proportion of non detectable samples for cases and controls.

ND Analyte, n (%)	AK Cases	Controls
IL-6	104/120 (86.7)	10/11 (90.9)
IL-8	24/120 (20.0)	7 /11 (63.6)
IL-22	74/120 (61.7)	7/11 (63.6)
IL-17E	114/120 (95.0)	10/11 (90.9)
CXCL2	11/69 (15.9)	3/11 (27.3)
IL-1 β	119/120 (99.2)	11/11 (100)
IFNy	120/120 (100)	11/11 (100)
Il-17F	120/120 (100)	11/11 (100)
IL-17A	120/120 (100)	11/11 (100)
IL-10	120/120 (100)	11/11 (100)
IL-27	120/120 (100)	11/11 (100)

ND= non detectable

Table 3

Table 3. Median concentrations and 95% confidence intervals (CI) for cytokines in tear samples of severe compared to mild-moderate cases.

Cytokine	Severe			Mild-Moderate			Z value
	n	median	95% CI	n	median	95% CI	
IL-8	36	162.4	72.8-447.3	60	66.2	57.6-119.5	*-2.31*
IL-22	22	470.8	313.5-1237.0	24	671.6	214.9-1501.0	-0.44
IL-6	9	145.0	31.9-1361.5	7	80.9	16.8-391.0	1.27
IL-17E	2	7265.1	N/A	4	2587.1	N/A	-0.93
CXCL2	22	3173.3	1150.9-4110.7	34	3007.2	1847.5-3703.9	-0.23

n: number of samples; CI: confidence index; N/A: not applicable
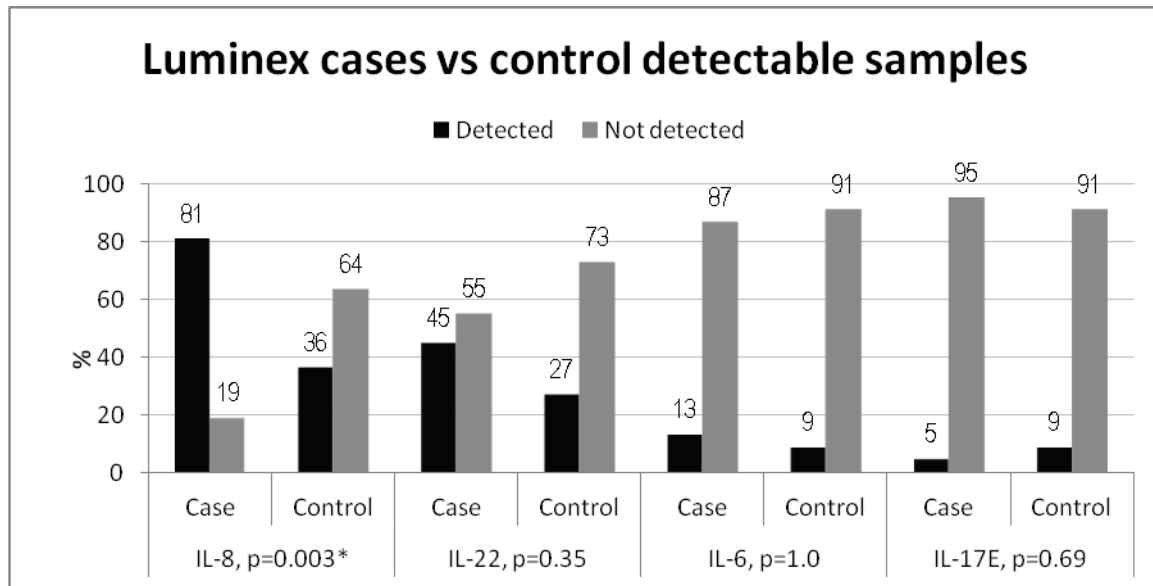
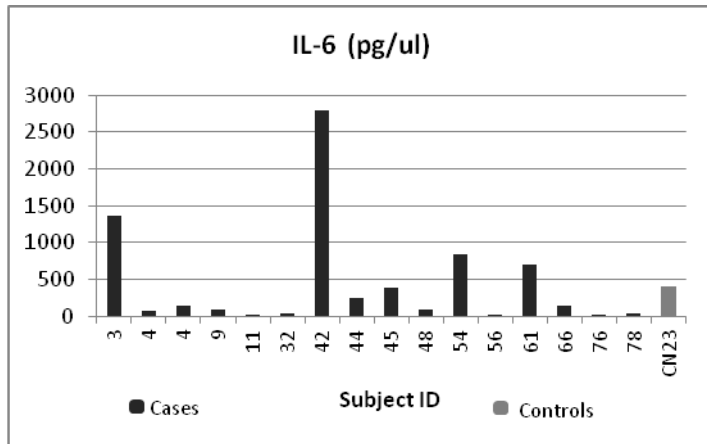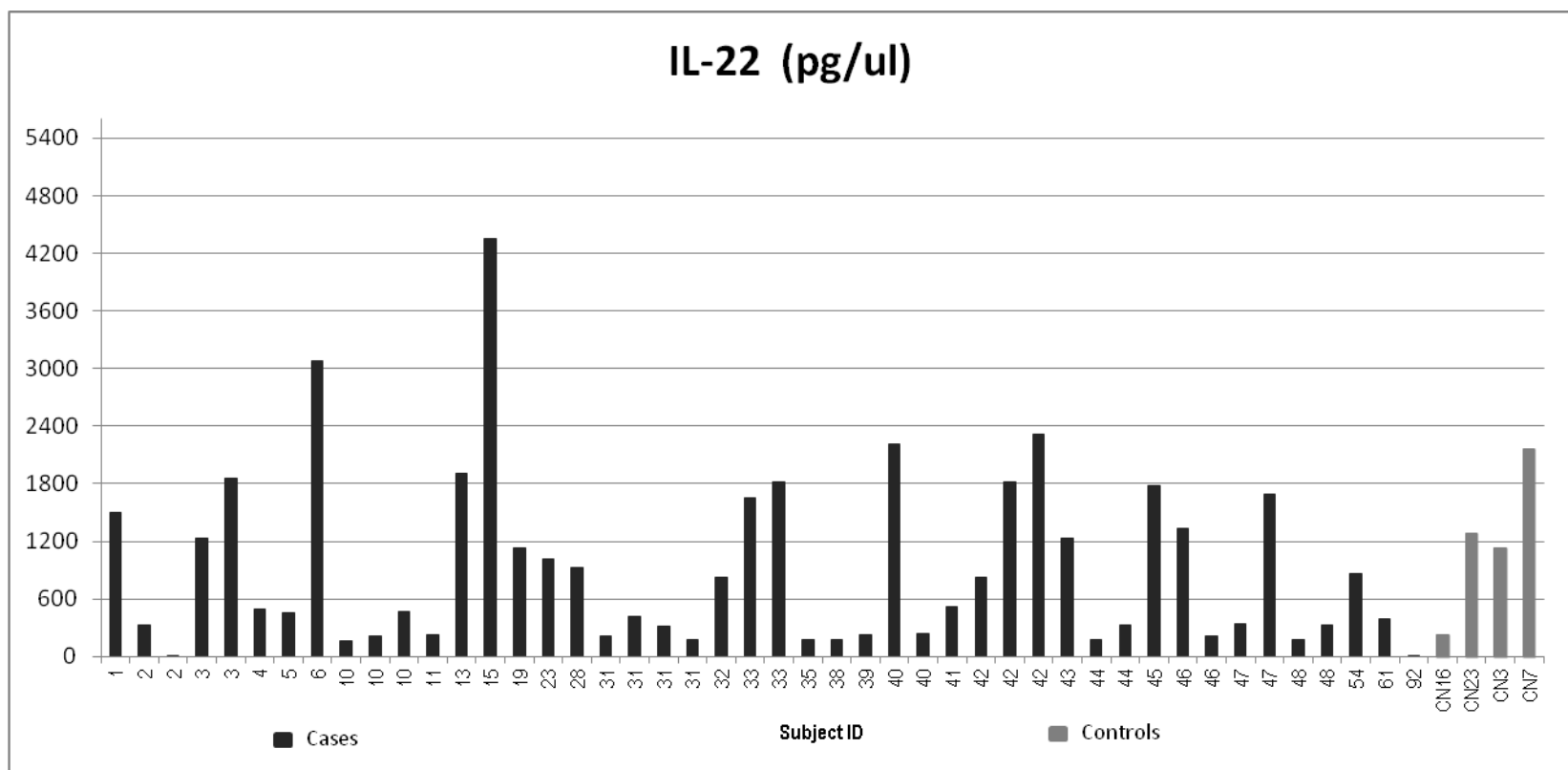Figure 1

Figure 2_previously fig 4

IL-6 (pg/ul)

Figure 3_previously fig 5
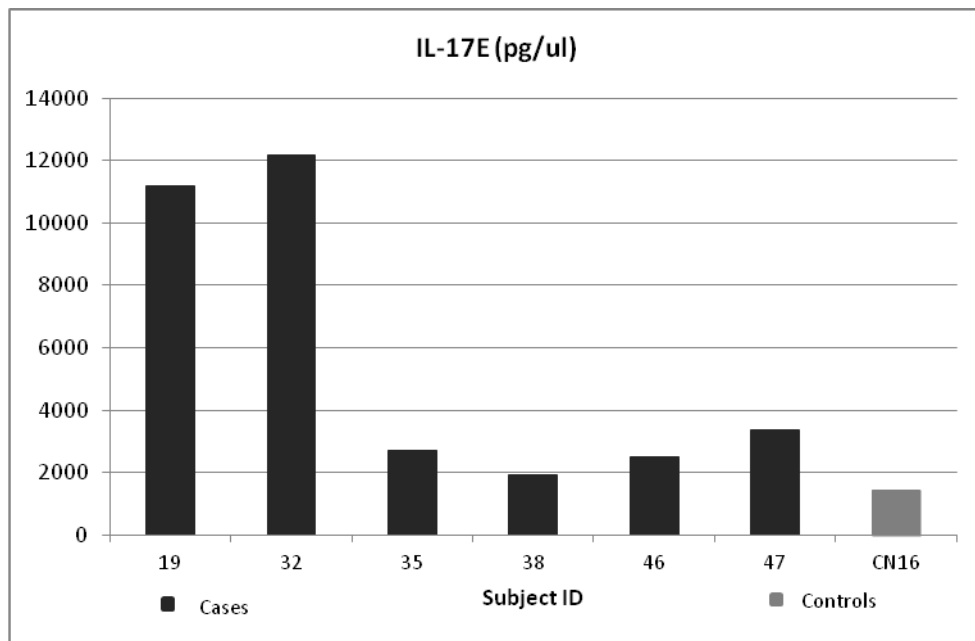

IL-8 (pg/ul)

Figure 4_previously fig 6

IL-22 (pg/ul)

Figure 5_previously fig 7

IL-17E (pg/ul)

Figure 6_previously fig 8

CXCL2 (pg/mL)

Figure 7_previously fig 9

Luminex Severe vs Moderate/Mild cases detectable samples

■ Detected ■ Not detected

	IL-8, p=0.48		IL-22, p=0.02*		IL-6, p=0.27		IL-17E, p=1.0	
	Severe	Mod/Mild	Severe	Mod/Mild	Severe	Mod/Mild	Severe	Mod/Mild
Detected	84	78	52	30	18	11	5	5
Not detected	16	22	48	70	82	89	95	95