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Tear Cytokine Levels in Contact Lens Wearers with Acanthamoeba Keratitis

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Tear Cytokine Levels in Contact Lens Wearers with Acanthamoeba Keratitis

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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.

INTRODUCTION

Acanthamoeba Keratitis (AK) is one of the most severe forms of corneal infection, with over 90% of cases occurring in contact lens (CL) wearers.[1] Vision loss occurs in 33% of patients, with corneal transplantation required in around 26%.[2] Recent reports, and case monitoring at our centre, show that the numbers of AK cases are increasing.[3, 4] AK generally affects a young and otherwise healthy group of individuals[5] in whom lifetime disability costs are high. As well as the long term effects, such as decreased quality of life, and loss of productivity due to reduced vision, there are significant short term costs to sufferers and carers, such as loss of wages and distress, in addition to symptoms such as severe pain and light sensitivity experienced by sufferers.[6]

Some complications associated with CLs are somewhat controlled by the release of tear inflammatory molecules, such as giant papillary conjunctivitis which is characterized by altered levels of eotaxin[7] or corneal neovascularization which is mediated by vascular endothelial growth factor (VEGF).[8] Moreover, it has been shown that CL wearers with CL-induced acute red eye present higher concentrations of IL-8 than healthy subjects[9]. Others[10-13] have indicated altered levels of tear cytokines such as interleukin (IL)-6, IL-8 and epidermal growth factor (EGF) during CL wear. However, to this day little is known about the tear inflammatory mediation in AK. Profiling AK patients during disease could show differences in cytokine levels between severe and milder disease that may inform clinical management.

The aim of this study is to determine the differences in cytokine levels in CL-wearing patients with AK compared to CL wearers without the disease. A secondary goal is to investigate differences in cytokine levels between patients with severe forms of AK and those with mild-moderate forms of this infection

MATERIALS AND METHODS

This was a prospective case control study of CL wearers with AK attending Moorfields Eye Hospital (MEH) and control CL wearers from the Institute of Optometry, London. The research protocol adhered to the tenets of the Declaration of Helsinki and was approved by the local ethics committee. Written informed consent was obtained from all participants.

Participants

Severe cases of AK were defined as having active infection for over 12 months and having had at least one severe inflammatory event such as scleritis, persistent non-healing defect (for 14 days or more) and/or pupil paralysis. Mild-moderate cases had recurrent disease in the absence of severe inflammatory events or disease that required active treatment for less than and up to 12 months.

Tear sample collection

Samples were collected from AK patients at follow-up visits during their treatment on a convenience basis depending on the flow of the clinic visits. Collection times varied between 10am and 4pm. Samples were collected from the affected eye only. For bilateral cases, the worst affected eye was sampled.

Samples were collected from control CL wearers at the conclusion of routine aftercare appointments at the Institute of Optometry. Lenses may or may not have been worn according to the patient preference at the end of the appointment. So as to not affect the equilibrated tear milieu, that status remained for tear collection. For these control CL wearers, samples were collected from the right eye, and switched to the left should no sample be obtained from the right eye. In monocular wearers, the eye sampled was the CL wearing eye.

Tear samples for both AK cases and controls were basal tear specimens collected by 10ul capillary tubes (Blaubrand intraMARK, Wertheim, Germany) and stored in ethylenediaminetetraacetic acid (EDTA) coated 0.5ml Eppendorf tubes. Following collection,

the samples were kept cold using a standard cool box and ice packs. Upon delivery to the laboratory on the same day, the samples were centrifuged at 1,600rpm for 5 minutes. The cell-free supernatant was then pipetted into clean EDTA coated 0.5ml Eppendorf tubes and stored at -80°C prior to analysis.

Analysis of tear molecules

Cytokines IL-1β, IL-6, IL-8, IL-10, IL-17A, IL-17E, IL-17F, IL-22, interferon (IFNγ) and chemokine (C-X-C motif) ligand 2 (CXCL2) were chosen for analysis based on established and hypothesised inflammatory pathways in AK. Tear protein levels were measured with a multiplex bead array using the Luminex based platform (Luminex 100, Luminex Corporation, Austin, TX) for all analytes apart from CXCL2. CXCL2 was measured with an enzyme-linked immunosorbent assay (ELISA, Abcam, Cambridge, UK) as this protein was not compatible with the chosen Luminex range of targets.

Samples were diluted with the respective kit reagent depending on the sample volume and normalised for analysis. Standard curves using duplicate known dilutions were generated for the Luminex and ELISA analysis. Luminex data were analysed with the instrument software and raw scores of the ELISA optical density were converted to concentrations in Excel 2010 (Microsoft). Concentrations lower than the detectable limits were labelled as not detectable (ND). Final concentrations above the minimum detectable limit were adjusted for the dilution factor.

Data analysis

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

Differences between cases and controls and between severe and mild-moderate cases were determined as follow: Fishers exact test was performed to determine the proportions of

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

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

RESULTS

One hundred and thirty two tear samples were collected from a total of 61 AK cases (15 severe and 46 mild-moderate) and 22 controls. There were no differences in gender distribution between case and control groups (p=0.06), however significant differences in age were found between the groups (p<0.001). In addition, there were more daily disposable wearers in the control group compared to the AK cases (p=0.02). Descriptive data detailing age, gender and lens type, are shown in Table 1.

Levels of IL-6, IL-8, IL-22 and IL-17E were readily detectable. The levels of IFNy, IL-17F, IL-17A, IL-10, IL-27 and IL-1β were below the minimum detectable limit for all case and control samples. The proportion of non detectable (ND) samples for each protein are detailed in Table 2.

Cases vs. controls

Figure 1 shows the proportion of cytokines for the cases and controls for each of the molecules for which there was more than 1 positive sample (IL-1 β was detectable in only one sample, and was considered “non detectable” for this study). There were more samples with detectable levels of IL-8 in the cases compared to the controls (p=0.003). Almost half of the tear specimens in both groups had detectable levels of IL-22, whereas IL-6 and IL-17E showed very low frequencies of positivity. The one control with a positive sample for IL-6 was not the same control that was the only control sample positive for IL-17E. There was no difference between the CXCL2 levels for cases and controls with more than 75% of tear specimens yielding detectable quantities of this molecule (cases 56/67, 84% and controls, 10/13, 77%).

Figures 2-5 show the concentrations of IL-6, IL-8, IL-22, and IL-17E, respectively in tears of individual cases (by visit) and individual controls that measured above detectable limits by Luminex. Figure 6 shows the concentrations CXCL2 in tears of individual cases (by visit) and individual controls that measured above detectable limits by ELISA. There was no difference between the median concentration of IL-8, IL-22 and CXCL2 in tears of cases and controls (z=-0.57, z=0.97 and Z=0.05 respectively). Only one control sample was positive for IL-6 and IL-17E and so Mann-Whitney U Test could not be performed.

Severe vs. mild-moderate cases

Figure 7 shows the proportions of detectable protein samples (IL-8, IL-22, IL-6 and IL-17E) investigated with Luminex for severe compared to mild-moderate cases. IL-22 was less likely to be detected amongst the mild-moderate cases compared to the severe cases of AK (p=0.02), however there was no difference between mild-moderate cases and severe cases for the proteins, IL-8, IL-6 and IL-17E (p=0.48, p=0.27 and p=1.0 respectively). There was also no difference in CXCL2 levels between the severe and moderate/mild cases (23/29, 79.3% compared to 33/38, 86.8%, p=0.41)

Table 3 shows the median tear protein concentrations for severe compared to the mild-moderate samples. There was a higher level of IL-8 detectable in the tears of severe cases compared to the mild-moderate cases of this infection (z=-2.31), however there was no difference between tear protein levels of IL-22, IL-6, IL-17E and CXCL2).

DISCUSSION

The present study was the first to examine the cytokine levels in patients with mild compared to more severe AK, and compare these to control CL wearers. This study has highlighted IL-8 as a key molecule in the AK inflammatory response, and there is also some evidence for cell mediated inflammatory response involving the IL-17 pathway, via IL-22.

IL-8 was found to be expressed at a detectable level measured by Luminex more often in cases of AK compared to control CL wearers, and in higher concentrations in more severe compared to milder forms of the disease. IL-8 is a key inflammatory chemokine that mobilises and activates neutrophils.[7] Neutrophils are essential components of the early inflammatory response to *Acanthamoeba*.[8] Furthermore, IL-8 is part of the toll like receptor 4 (TLR-4) cascade which initiates the cytokine response in AK.[9] IL-8 also promotes angiogenesis in the eye[7] and further characterisation of patients that develop neovascularisation in AK may reveal differences in levels that may predict patients who go on to develop this complication, and more targeted management such as frequent topical steroids may be advocated in these cases. Neovascularisation is a contraindicated in corneal transplant candidates, often the last resort to significantly improve vision in AK patients. Keratoplasty is required for visual rehabilitation in around 12% of AK cases.[2]

IL-22, part of the IL-10 family, and a proinflammatory Th17 cytokine,[10] was detected more often in severe compared to milder forms of AK. IL-22 may prolong the inflammatory response and, in severe forms of disease, this may be beneficial to control infection but may also be involved in tissue destruction due to inflammation.

Most of the IL-17 cytokines were not detected in levels high enough to be measured in the tears in these subjects using Luminex technology. Since multiplex bead arrays are well established as being one of the more sensitive methods of detection for low levels of analytes, the specimens with no detectable levels were presumed negative. It may be useful to compare the IL-17E cytokine, which was expressed by a small number of cases and one control, using ELISA, in another cohort of samples. Like IL-22, IL-17 has been implicated in chronic inflammatory conditions[11] and IL-17A has recently been shown to be protective against *Acanthamoeba* keratitis severity in a mouse model.[12] This contrasts with keratitis caused by Herpes Simplex Virus and *Pseudomonas* where IL-17A is associated with an increased corneal inflammatory response.[13-15]. IL-17A is known as a “double sword” agent; in some

circumstances it protects the host and in others, it results in chronic inflammation and tissue damage.[16]) IL-17A both initiates and activates neutrophils and is also produced by neutrophils.[17] Recently, a novel population of neutrophils were characterized, that are capable of autocrine IL-17A activity, which leads to increased death of fungal hyphae in a murine model of *Aspergillus* corneal infection.[18]

CXCL2 (also known as macrophage inflammatory protein 2-alpha, MIP2-α) appears to be constitutively expressed in AK cases and control CL wearers and not up- or down-regulated in this disease. MIP2 has been shown to be important in animal models of AK.[19] Animal models of disease do not exhibit the severe inflammatory complications of AK, such as scleritis [5], and inflammatory pathways may vary somewhat between humans and animal models.

IL-6, a proinflammatory cytokine with several functions, was only detected in one control sample; either this study did not have enough power to show differences between cases and controls or IL-6 is not important in the inflammatory response in this disease. Furthermore it is possible that there is a defect in IL-6 at the protein level. Our group has found that single nucleotide polymorphisms (SNP) of IL-6 genes are implicated in the susceptibility and severity of bacterial keratitis in CL wearers.[20] IL-6 is a key player in the IL-22 and IL-17 pathways[11] and it would be prudent to further investigate this protein as a candidate in future immunological analysis in AK.

Cytokine and chemokine profiles correlate with several inflammatory anterior eye disease states such as dry eye,[21-24] allergic eye disease,[25, 26] the autoimmune condition, Sjogren's syndrome,[27-29] vernal keratoconjunctivitis[30] and ocular rosacea.[31] Two studies have highlighted tear protein profiles associated with bacterial[32] and fungal keratitis.[33]

In bacterial keratitis, cytokines and chemokines are upregulated in both the affected and contralateral eye, and these changes have been correlated with cellular changes imaged on the ocular surface [32]. Specifically, IL-1β, IL-6 and IL-8 were elevated in the 'infected' tears

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

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

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

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

Furthermore the differences between mild/moderate and severe disease may be due to differences in strains of Acanthamoeba organism. The majority of Acanthamoeba spp that cause keratitis are from the T4 group based on 18s RNA genotyping that separates strains into 17 evolutionary clades or groups (T1-T17). Preliminary information from one study indicates that strains with non-T4 genotypes may cause more severe disease,[35] however, only three cases of non-T4 AK were compared to 14 T4 genotypes and confirmation in a larger study is required. As genetic profiling of Acanthamoeba spp. allows more refined typing, as can be seen by the mitochondrial cytochrome oxidase (Cox) gene sequencing,[36] and greater number of cases are reported from other T strains[37, 38] correlation between different strains and the outcomes of AK may be found. Human biomarker profiling alongside in vitro and animal models will be key to future understanding of the interplay between the host immune system and organism virulence that is evidenced in some conditions such as malaria.[39]

A limitation of the present study could be that AK cases were younger than controls. Tear investigations have generally been limited to normals or certain conditions affecting specific age groups and differences between normals across a range of ages has not been shown. Dry eye is more prevalent in older individuals, and increased levels of two cytokines measured in this study, IL-6 and IL-8 have been found in elevated levels in dry eye patients.[21-24] The controls in this study, although older than the cases, were successful CL wearers, and are unlikely to have had significant dry eye disease. In any case, had some of the cases been on the dry eye spectrum, this would have only potentially masked greater differences in IL-8 levels and would not have affected the IL-6 results, in which only one control showed a reading above the detectable level.

More daily disposable wearers were in the control group compared to AK patients in this study. This likely reflects the evidence that AK is more often a disease that occurs in reusable lens wearers, as the environmental contamination of lens cases supports the growth of Acanthamoeba spp.[40] Only one study has evaluated the tear profile while wearing different

lens types; using lotrafilcon B (O2OPTIX; CIBA VISION, Duluth, Atlanta, GA) or senofilcon A (Acuvue Oasys; Johnson & Johnson Vision Care, Inc., Jacksonville, FL), no differences in levels on matrix metalloproteinase 9 (MMP-9), tissue inhibitor of metalloproteinases 1 (TIMP-1) and neutrophil gelatinase-associated lipocalin (NGAL) during adapted daily wear were found.[41] It is unlikely that even if lens wear type had an effect on tear cytokine/chemokine levels that this would confound results in the present study as all the AK patients and a proportion of the controls were not wearing lenses at the time of collection.

Another limitation of the study might be the time of the tear samples collection. The tear collection time was scheduled between 10am and 4pm to minimise possible diurnal effect and disruption to the MEH and IO clinics. While there are recent publications showing a diurnal change of certain tear cytokines and chemokines they indicate a difference between daytime and evening intervals (11am-1pm vs 5pm -7pm)[42]; 12am (midday) compared to 9-12pm (midnight).[43] It is improbable that there would be a major variation in cytokine and chemokine levels during the 6-hour daytime interval in which we sampled tears.

This study highlights key areas for future investigation of the pathogenesis of AK. We have shown that in a clinical setting, we can collect tears from patients with AK that may indicate the inflammatory status of the eye. Further investigation of cytokines not detected in this study, and other candidates in the pathways indicated by this analysis, may define a wider spectrum of cytokine changes. In association with careful tracking of patients during the disease process, we may be able to predict when the inflammatory status is changing. This information may help the clinician to better understand the clinical picture and make more informed decisions on individual AK patient management.

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Figure legends

Figure 1. The distribution of the detectable samples for each analyte tested with Luminex for AK case samples and controls

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

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

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

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

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

Figure 7. Detectable sample distribution for severe compared to mild-moderate AK cases measured with Luminex (mod=moderate)

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446

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. Proportion of non detectable samples for cases and controls.

	AK Cases	Controls
ND Analyte, n (%)		
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. 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

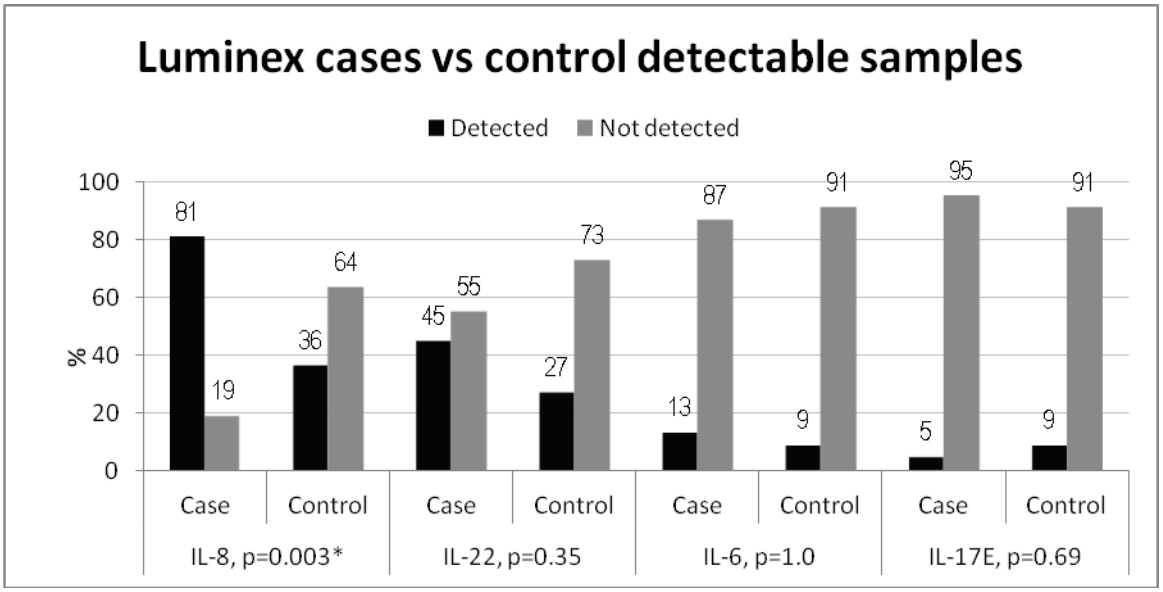
Luminex cases vs control detectable samples
Detected
Not detected
%
100
80
60
40
20
0
81
19
36
64
45
55
27
73
13
87
9
91
5
95
9
91
Case
Control
Case
Control
Case
Control
Case
Control
IL-8, p=0.003*
IL-22, p=0.35
IL-6, p=1.0
IL-17E, p=0.69

Figure 2_previously fig 4

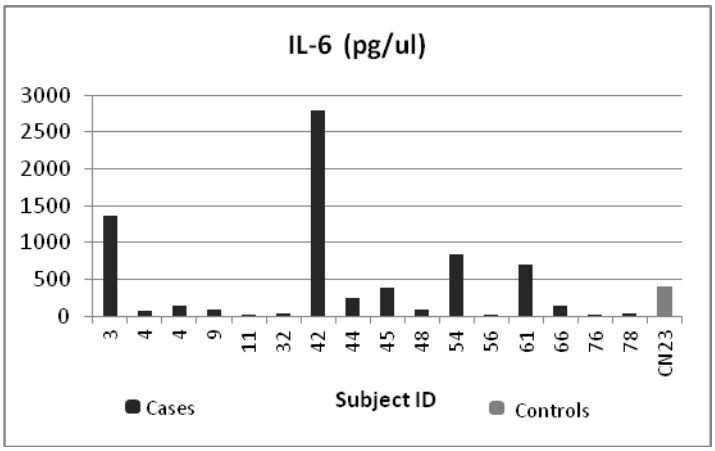

Figure 3_previously fig 5

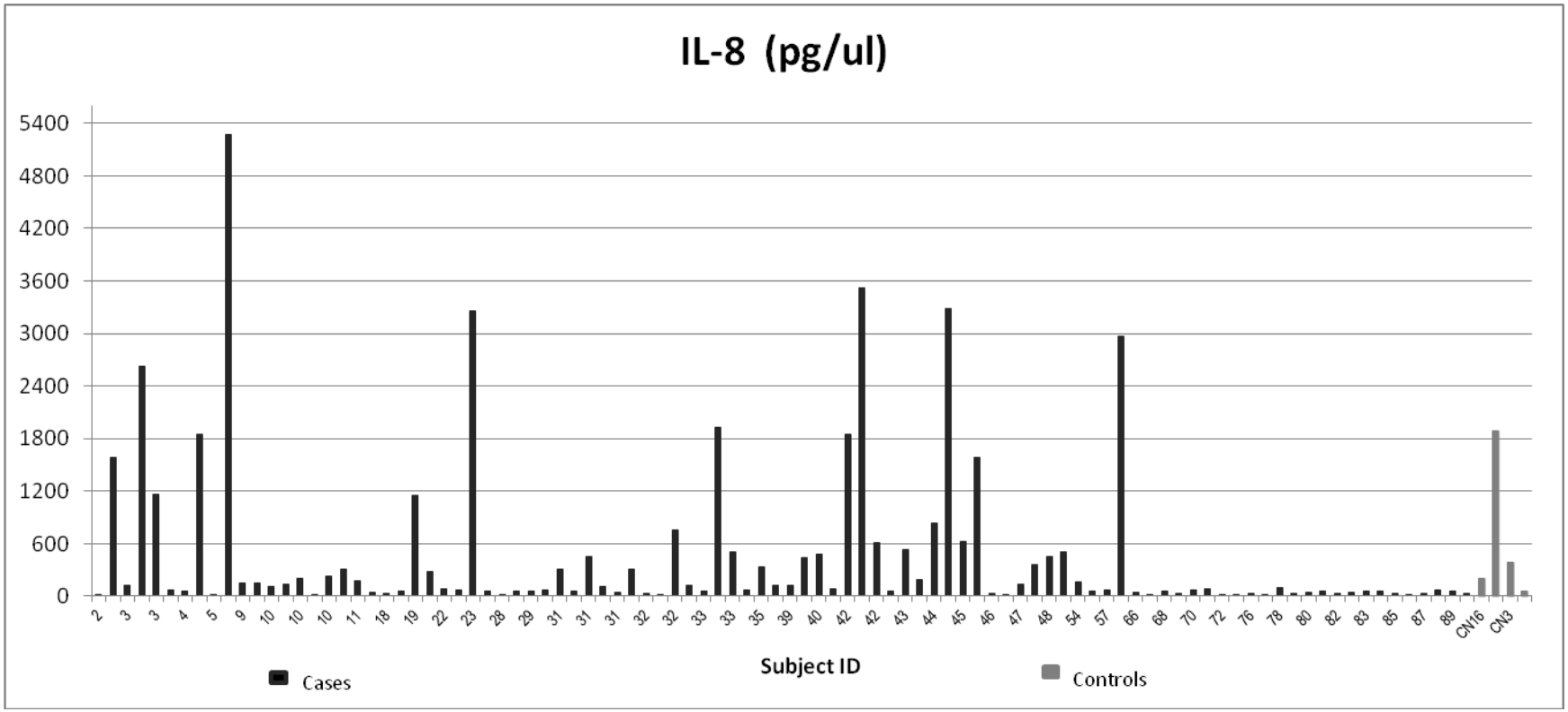

Figure 4_previously fig 6

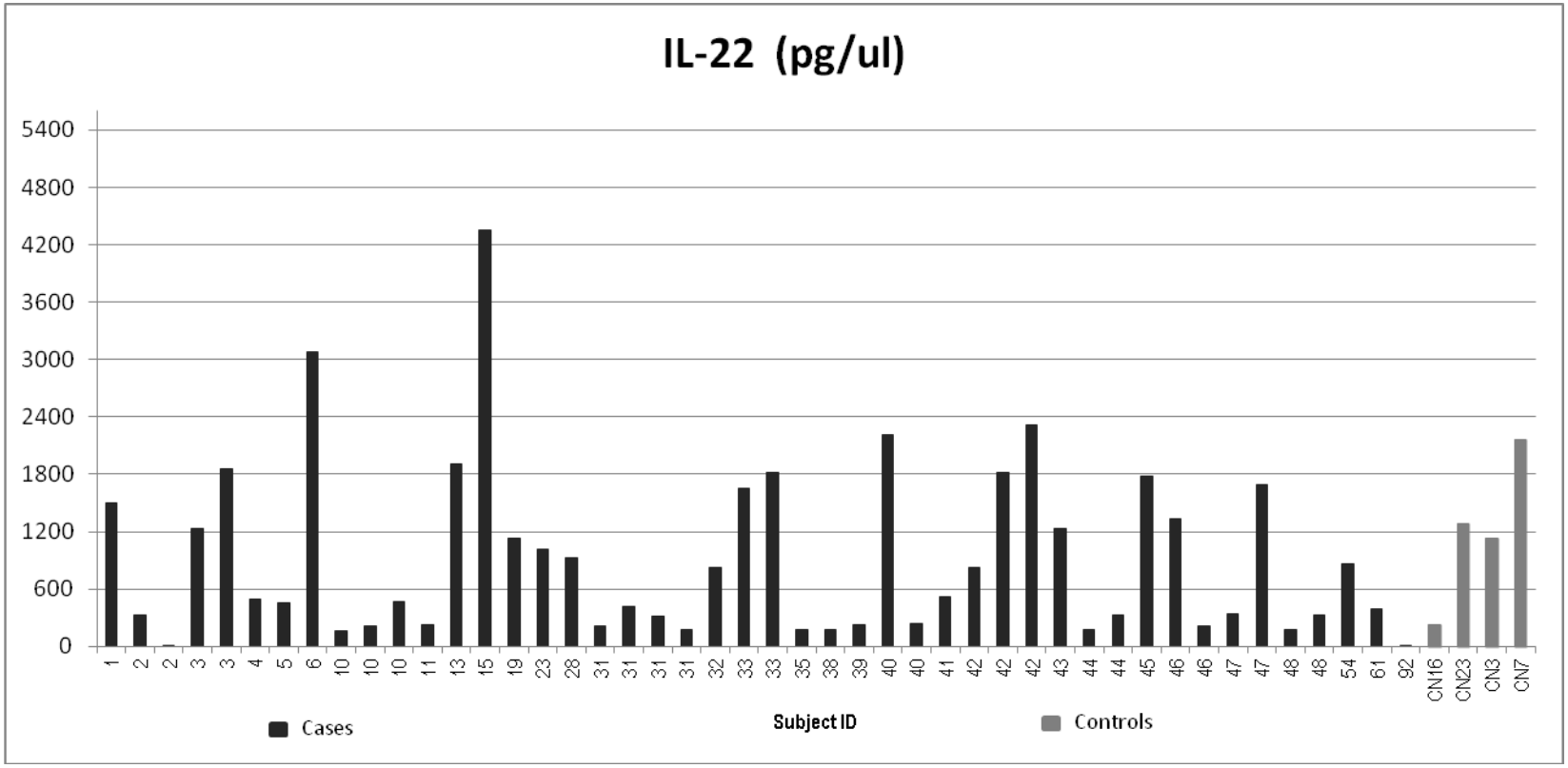

Figure 5_previously fig 7

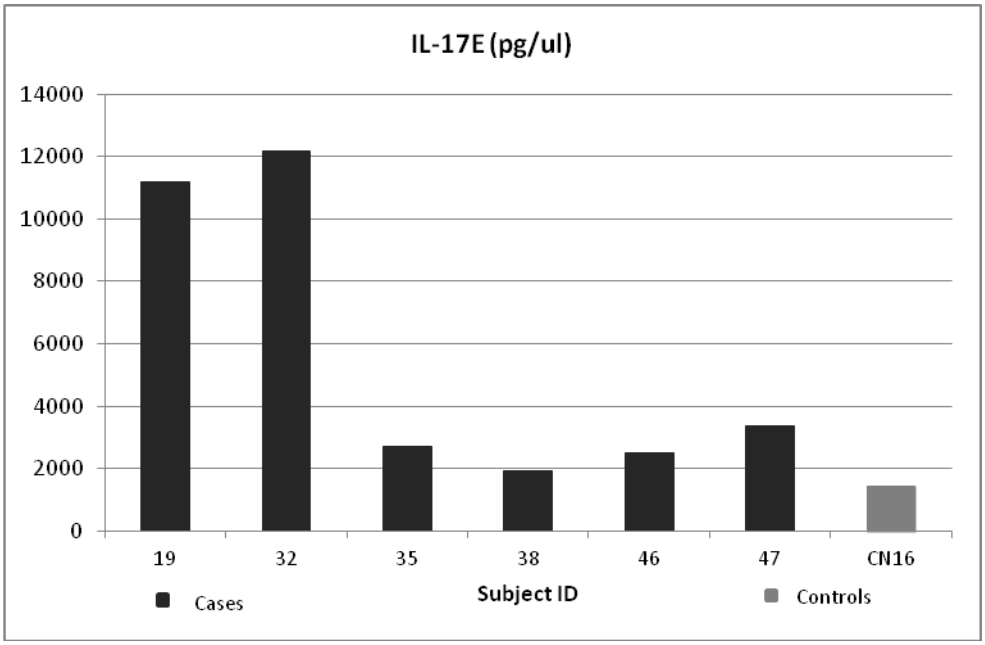

Figure 6_previously fig 8

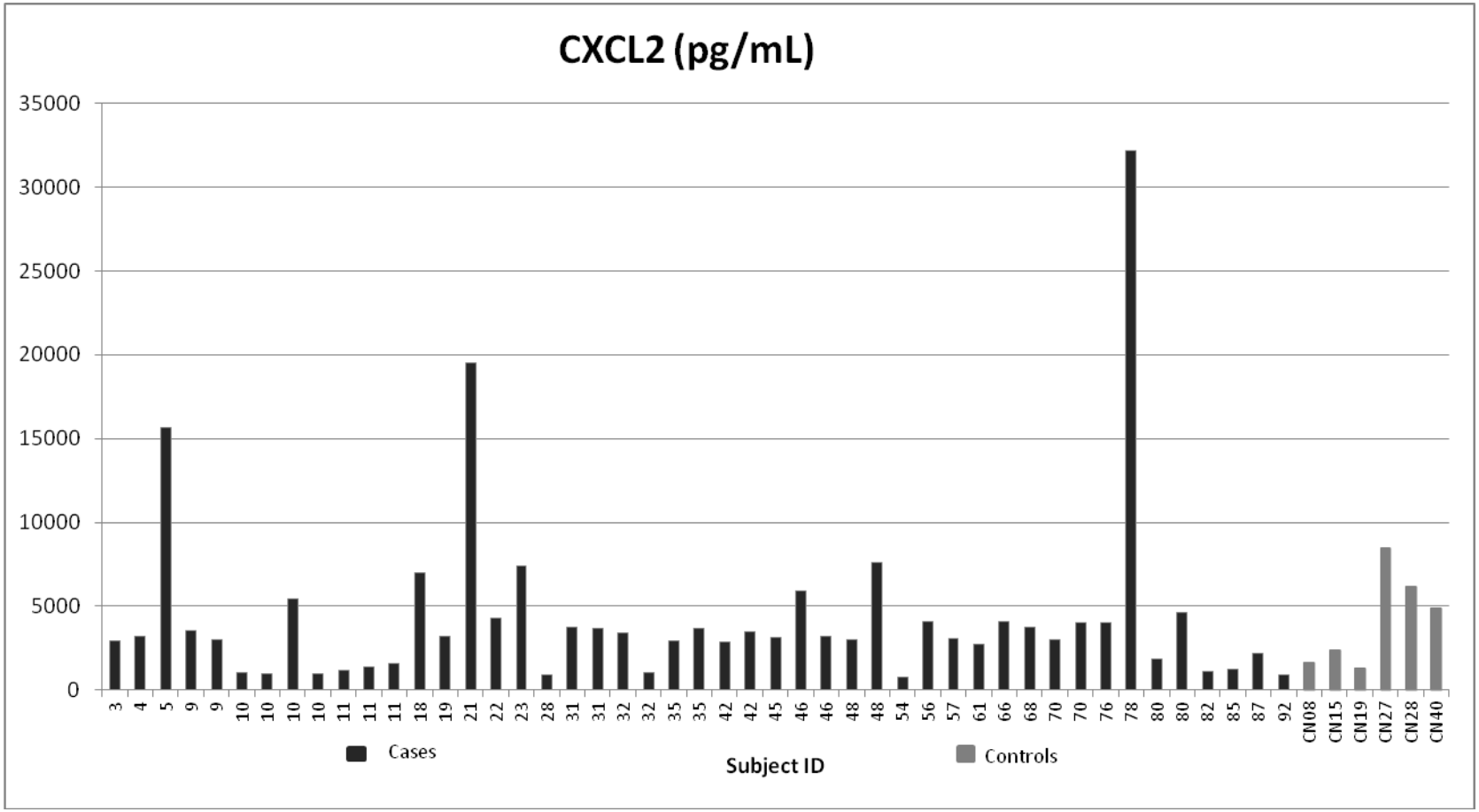

Figure 7_previously fig 9