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Effects of Daily Versus 30-Day Continuous Contact Lens Wear on Tear Cytokine Levels

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EFFECTS OF DAILY VERSUS 30-DAY CONTINUOUS CONTACT LENS WEAR ON TEAR CYTOKINE LEVELS

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ABSTRACT

The purpose of this investigation was to determine if 30 days of continuous contact lens wear produces a different ocular inflammatory response compared to 30 days of daily wear of Bausch & Lomb® PureVision™ silicone hydrogel contact lenses. Eighty-five individuals wore these lenses for 30 days continuously in one eye and daily in the contralateral eye. Relative concentrations of 27 cytokines were measured in tear samples collected from the subjects using cytometric bead-based assays. Clinical tests were also conducted to correlate changes in cytokine levels to changes in clinical profiles. This study contributes to a greater understanding of the impact of contact lens wear on the ocular surface.

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LIST OF ABBREVIATIONS

AKC	atopic keratoconjunctivitis
AMD	age-related macular degeneration
ANOVA	analysis of variance
APC	antigen-presenting cell
C	complement
CAB	cellulose acetate butyrate
CBA	cytometric bead-based assay
CD	cluster of differentiation
CLARE	contact lens induced acute red eye
CLDEQ	contact lens dry eye questionnaire
CLPC	contact lens induced papillary conjunctivitis
CLPU	contact lens induced peripheral ulcers
DTH	delayed-type hypersensitivity
EGDMA	ethylene glycol dimethacrylate
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FGF-b	fibroblast growth factor-basic
G-CSF	granulocyte colony stimulating factor
GMA	glyceryl methacrylate
GM-CSF	granulocyte-macrophage colony-stimulating factor

GPC	giant papillary conjunctivitis
HEMA	hydroxyethyl methacrylate
HSD	honestly significantly different
IFN	interferon
IL	interleukin
IP	interferon-gamma-inducible protein
KCS	keratoconjunctivitis sicca
LASIK	laser-assisted in situ keratomileusis
LTB	leukotriene B
MCP	monocyte chemotactic protein
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MHC	major histocompatibility complex
NIBUT	non-invasive break-up time
PDGF-BB	platelet-derived growth factor
PHEMA	poly hydroxyethyl methyacrylate
PE	phycoerythrin
PCR	polymerase chain reaction
PLA_2	phospholipase A_2
PMMA	polymethylmethacrylate
PMN	polymorphonuclear leukocytes
PMT	photomultiplier tube
PVA	polyvinyl alcohol

ra	receptor antagonist
RANTES	regulated on activation, normal T expressed and secreted
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEAL	superior epithelial arcuate lesions
sIgA	secretory immunoglobulin A
T_C	cytotoxic T-cells
TGF	transforming growth factor
T_H	helper T-cells
TMH	tear meniscus height
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
VKC	vernal keratoconjunctivitis

INTRODUCTION

Although contact lens wear offers a comfortable and convenient alternative to spectacles for individuals in need of vision correction, contact lens wearers have a higher incidence of complications including inflammation and infection of the ocular surface than non-wearers, particularly with continued overnight wear. Thirty years of contact lens research has been focused on ocular complications due to contact lens wear and the production of new materials, including silicone hydrogel with increased water content and oxygen permeability, to mitigate adverse effects. Research has shown that individuals who wear silicone hydrogel lenses overnight for an extended period have a marked decrease in corneal changes associated with hypoxia compared to those wearing older lens materials and designs [1, 2]. Nevertheless, several studies suggest that other pro-inflammatory events can be associated with continuous silicone hydrogel lens wear [3].

The composition of tear fluid is a key element in the health of the ocular surface environment. The pre-ocular tear film has several functions: it forms a smooth interface over the intrinsically irregular corneal surface for optimal transmission of light to the retina, flushes out debris, and lubricates and protects the cornea and conjunctiva.

A major component of the tear film is the aqueous phase. Aqueous tear fluid is secreted by the main lacrimal gland located superior-temporally to the globe of the eye, as well as by the accessory glands of Krauss and Wolfring in the upper and lower eyelids. The main lacrimal gland is under the control of the autonomic nervous system, secreting antimicrobial enzymes, most importantly sIgA, lipocalin, lysozyme and lactoferrin. During sleep or any prolonged period of eye closure, tear flow is stagnated and lower levels

of these protective proteins are secreted; leaving the eye more susceptible to infection. With overnight wear, the presence of a contact lens may contribute to an increased inflammatory response of the ocular tissue [4]. Tissue inflammation induces the release of inflammatory cytokines. In the ocular surface environment, the levels of these cytokines can be quantified in tear film [5].

BACKGROUND

Cytokines

Cytokines are signaling proteins released from the ocular surface and lacrimal system in response to inflammation, infection or mechanical injury to ocular tissue. Corneal keratocytes and epithelial cells, and conjunctival mast cells also release cytokines as well as other inflammatory mediators [6]. These proteins are involved in a range of activities in the inflammatory process. They bind to membrane receptors on various target cells which initiates signal transduction that alters the gene expression of the cells. This expression can result in greater production of surface receptors or other cytokines. Cytokines are responsible for modulating the secretion of antibodies and other cytokines, thereby regulating the activation and growth of inflammatory cells. Some cytokines exhibit endocrine action, binding to distal cells in other parts of the body, while most have either autocrine (binding to the same cell that secreted it) or paracrine (binding to a neighboring cell) activity. Most cytokines are pleiotropic, meaning that their function is determined by the target cell they bind to. Multiple cytokines can also perform similar tasks; this is referred to as redundancy. Some are inhibitory; they down-regulate activity or production of other cytokines or cells, while other cytokines act synergistically, potentiating their effects. The array of cytokine functions include chemotaxis, activation and suppression of various inflammatory mediators [7].

Corneal and conjunctival epithelial cells release cytokines into tears to recruit and activate corneal polymorphonuclear leukocytes (PMNs). The PMNs migrate through

blood vessels, into corneal stroma and conjunctival epithelial surfaces and enter the tear film to phagocytose bacteria [8].

Many cytokines have been identified in human biological fluid and inflammatory diseases in various types of tissue. Names, sources and functions of key cytokines, as well as each of the cytokines included in the commercially available cytokine assay kits that were used in this study are described in Table 1.

Table 1

Cytokines, Sources and Functions

Cytokine	Source	Function/Activity [9-11]
IL-1α	Macrophages, T cells, B cells, endothelial cells, corneal and conjunctival epithelial cells, keratinocytes	Membrane bound or intracellular—induction of acute phase response, stimulation of neutrophil production, stimulates MMP production
IL-1β	Macrophages, T cells, B cells, endothelial cells, corneal and conjunctival epithelial cells, keratinocytes	Secreted—induction of acute phase response, promotes dendritic cell activation and maturation
IL-1ra	Macrophages, endothelial cells, epithelial cells, neutrophils	Receptor agonist; Inhibits IL-1α and IL-1β activity
IL-2	Th1 cells	Stimulates growth and differentiation of B and T lymphocytes.
IL-3	T_H2 cells, mast cells	Stimulates growth and differentiation of T cells and B cells
IL-4	Mast cells, T_H2 cells and eosinophils	Promotes growth and development of B and T cells, T_H2 lymphocyte differentiation
IL-5	Mast cells, T_H2 cells and eosinophils	Induces eosinophil formation and differentiation, B cell chemotaxis
IL-6	T cells, B cells, macrophages, endothelial cells	Regulation of B and T cell functions, induces acute phase response
IL-7	Stromal cells	Maintenance of T cells
IL-8	Monocytes, lymphocytes, endothelial cells	Chemoattraction and activation of neutrophils and some subpopulations of lymphocytes.
IL-9	T_H cell populations	Stimulates growth of T cells
IL-10	Some T_H and T_C cell populations	Inhibits MHC II expression, inhibits IL-1β and IL-12

IL-12 (p70)	Dendritic cells and macrophages	Promotes T_H1 response
IL-13	T cells	Promotes growth and differentiation of B cells, inhibits production of macrophages and T_H1 cells
IL-15	Dendritic cells	Promotes growth of T cells and natural killer cells
IL-17	T cells	Chemoattraction of T_H cells and monocytes, induces IL-6 and IL-8 production
Eotaxin	Conjunctival epithelium	Chemoattraction of eosinophil
G-CSF	Endothelial cells, macrophages	Production of neutrophils
GM-CSF	Th1 cells, Th2 cells, macrophages, mast cells	Activation and growth of dendritic cells, macrophage production
IFN-γ	T cells and natural killer cells	Antiviral, induces MHC expression, attraction and activation of macrophages
IP-10	Monocytes, endothelial cells	Inhibits angiogenesis, T cell chemotaxis
LTB_4	Monocytes, mast cells, macrophages	Neutrophil chemotaxis
MCP-1	Corneal epithelium, keratocytes	Modulates T_H1 response, recruitment of monocytes and T-cells
MIP-1α/ β	Monocytes, T cells, B cells, mast cells, neutrophils	T cells, neutrophils and monocyte chemotaxis
PDGF-BB	Macrophages, platelets, endothelial cells	Neutrophils, epithelial and endothelial cell chemotaxis
RANTES	Corneal epithelium, keratocytes	Modulates T_H1 response, recruitment of monocytes
TNF-α	Monocytes, macrophages, T cells	Promotes growth and differentiation of many cell types
TNF-β	T cells, B cells, epithelial cells, endothelial cells	Promotes phagocytosis
TGF-β	Monocytes, T cells	Immunosuppression, inhibits cell growth
VEGF	Vascular and corneal endothelial cells, epithelial cells	Promotes angiogenesis, induces proliferation of vascular endothelium, promotes monocyte and macrophage chemotaxis
FGF-b	Vascular endothelial cells	Regulates corneal wound healing, induces proliferation of vascular endothelium

As markers of inflammation, levels of cytokines in tears may be used as indicators of ocular inflammation. For example, significantly higher levels of certain cytokines are detected in tears of individuals with ocular allergies versus normal individuals [12] and various ocular surface diseases have been found to have more or less characteristic tear cytokine profiles [13].

Immunity and Inflammation

The healthy human body has a well-developed system for resistance and defense against invading viruses, bacteria and other foreign agents. This involves an innate and adaptive immune system producing acute and potentially chronic inflammatory responses.

Inflammation is the localized or systemic response involving changes in temperature, blood flow, immune cell influx and activity, pathogen removal and damaged tissue repair [14]. Vasodilation is initiated upon microbial invasion into tissue which triggers increased vascular permeability and leakage of plasma proteins and leukocytes (white blood cells) into surrounding tissue, causing the tissue to swell (edema). Leukocytes traverse the walls of the blood capillaries to the inflamed tissue, ingesting and digesting invading microbes in a process called phagocytosis. The complement system is composed of plasma proteins (C1-C9) that are also involved in the innate and acute inflammatory responses. They aid in the destruction of foreign microbes through phagocytosis and proteolysis of infectious cell membranes.

The innate immune system is a non-specific barrier that exists intrinsically and is fully developed prior to exposure to harmful pathogens. In the ocular environment, epi-

thelial cell tight-junctions, the mucous layer, the mechanical flushing of debris in tears and antibacterial enzymes present in normal tear film are all elements of innate immunity. The phagocytic white blood cells involved in the innate immune response are granulocytes and macrophages [15]. In the innate response, these cells secrete interleukin (IL)-1, IL-6, and TNF-α. IL-1 and tumor necrosis factor-α (TNF-α) stimulate the expression of adhesion molecules on the vascular endothelium so that inflammatory cells can attach and migrate to the site of compromised tissue [16]. They also stimulate neutrophil production and migration among other functions.

The elevated levels and concerted activity of IL-1, IL-6 and TNF-α in the innate response lead to acute phase inflammation that is associated with fever, increased body temperature and liver production of acute-phase proteins. Acute inflammation is the initial, early phase response to tissue injury and invasion of microbes involving the production and recruitment of inflammatory mediators. Persistence of the acute-phase response results in the development of chronic inflammation. The chronic inflammatory response is the outcome of sustained accumulation and activation of macrophages and subsequent continuous release of cytokines. The key cytokines produced in chronic inflammation are interferon- γ (IFN-γ) and TNF-α. Strong correlations of the levels of these two cytokines in tears have been found in individuals with atopic keratoconjunctivitis (AKC), a chronic allergic ocular disease [13]. These cytokines indirectly induce the recruitment of inflammatory cells and their release of pro-inflammatory mediators. Chronic inflammation can be particularly detrimental to the ocular surface as it may lead to tissue damage, scarring and tumor formation. Several studies have investigated the cytokines that are

elevated in tears from patients with various ocular allergies and inflammatory conditions (summarized in Table 2).

Table 2

Elevated Cytokines in Chronic and Acute Allergic Responses

Allergy Type	Elevated cytokines [13, 17]
Atopic keratoconjunctivitis (AKC)	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IFN- γ, MCP-1, TNF-α
Giant papillary conjunctivitis (GPC)	IL-8, eotaxin
Seasonal allergic conjunctivitis (SAC)	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IFN-γ, MCP-1
Vernal keratoconjunctivitis (VKC)	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN- γ, MCP-1, TNF-α, eotaxin
Type I hypersensitivity	IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF, TNF-α
Type IV hypersensitivity	IL-3, IL-12, IL-17, GM-CSF, IFN-γ, TNF-β

Adaptive immunity is the second line of defense when the initial innate response is insufficient for protection against antigens. This system takes 4-6 days to develop and the response occurs after the initial localized inflammatory response. The adaptive immune response involves the recruitment of cells and proteins with high specificity for the invading antigen, and "remembers" the antigen after it has been eliminated. The result is a more rapid response to repeat invasion by the same antigen. IL-2, IL-4, IL-5, IFN-γ and transforming growth factor β (TGF-β) are the major cytokines normally secreted in the adaptive immune response [16].

Lymphocytes are the cells of the adaptive immune system. The bone marrow releases stem cells that evolve into one of two populations of lymphocytes in adaptive immunity: B lymphocytes (B cells) which grow and differentiate in the bone marrow and T lymphocytes (T cells) that mature in the thymus. The surface of the B cell is coated with antibodies (immunoglobulins) that bind to specific antigens. Upon initial contact with an antigen, a naïve B cell becomes activated and divides into plasma cells and memory cells. Plasma cells secrete large amounts of antibody with precise binding affinity to the antigen epitope into the blood. Memory B cells are clones of the progenitor cell, expressing the same antibody on the membrane surface as the parent B cell. It recognizes the same antigen so that when the body encounters the antigen again, the response is much more rapid than it was during initial contact.

T cells also have membrane-bound receptors; however, they do not recognize antigens unless they are associated with a major histocompatibility complex (MHC) bound to a target or an antigen-presenting cell (APC) displaying part of the digested antigen on its surface. There are two main types of T cells that differ in their surface clusters of differentiation (CD): cytotoxic T (T_C) cells typically have CD8+ and helper (or suppressor) T (T_H) cells display CD4+ surface glycoprotein. T_C cells recognize and destroy target cells that display MHC-I. T_H cells are activated once they are bound to APCs (dendritic cells, B lymphocytes and macrophages) displaying MHC-II. There are two subsets of T_H cells that are distinguished based on the cytokines they produce and secrete. T_H1 cells secrete IL-2, IFN-γ, TNF-β, granulocyte macrophage colony stimulating factor (GM-CSF), MCP-1 and IL-3 and function to activate macrophages. The cytokine primarily involved in the development of T_H1 cells is IL-4. In addition to GM-CSF and IL-3, T_H2

cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 increasing production of antibodies, eosinophils and mast cells. IFN-γ and IL-12 are required for the development of the T_H2 response [7, 16].

Chronic inflammation increases both T_H1 and T_H2 cell concentrations. The T_H1 cell activity is indicative of the delayed type hypersensitivity (DTH) response which is an inflammatory reaction with delayed development and recruitment of non-specific inflammatory cells; primarily macrophages. T_H2 activity is thought to regulate immediate hypersensitivity responses.

The major cytokines that are elevated and decreased in tears during immune and inflammatory responses are summarized in Table 3.

Table 3

Major Cytokines in Immune and Inflammatory Responses

Response Type	Cytokines	Increase (↑) or Decrease (↓)	Functions/Activity [16, 17]
Innate	IL-1, IL-6, TNF-α IL-1ra, IL-10	↑ ↓	Fever induction, increase vascular permeability, T and B cell activation
Acute	IL-1, IL-6, TNF-α IL-1ra, IL-10	↑↑ ↓	Stimulation of adhesion molecule expression
Adaptive	IL-2, IL-4, IL-5, TGF-β, IFN-γ	↑	Production of T and B cells
Chronic	IFN-γ, TNF-α, eotaxin	↑	Attraction and activation of macrophages, recruitment of inflammatory cells, release of pro-inflammatory mediators
T_H1	IL-2, IL-3, IFN-γ, TNF-β, GM-CSF	↑	Reduces type I hypersensitivity response
T_H2	IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF	↑	Enhances type I hypersensitivity response

Although inflammation is a host defense mechanism against infectious agents, it has the potential to have deleterious effects on the ocular surface and subsequently, on clarity of vision. As such, the corneal tissue is an area of immune privilege. Due to the lack of blood supply and the inability of the central cornea to produce APCs, immune responses to foreign bodies do not occur in this region.

Open Eye vs. Closed Eye

Investigators for many years have gained an increased understanding of the importance of the sources, structure and function of pre-ocular tear film and its importance to the health of the pre-ocular surface. Over the past two decades, the state of the ocular environment in periods of prolonged eye closure has received a great deal of attention. While the open and closed eye environments are disparate, contact lenses introduce yet another dimension to both.

The tear film is composed of an outermost lipid layer secreted by meibomian glands, an aqueous phase, a mucin component and the epithelial cell glycocalyx. A consensus on the actual thickness of the aqueous and mucin layers is yet to be established; however, their functions have been widely accepted. The lipid layer is composed of polar and non-polar lipids that organize themselves to retain the tear film over the ocular surface by slowing evaporation and spillage over the lower lid margin (epiphora). Non-polar esters are the predominant lipids in this layer. Adjacent to the lipid layer is the aqueous phase that was originally thought to comprise up to 98% of the tear film. Much of this is now being attributed to the mucin layer contribution. Lacrimal gland secretion of the aqueous phase is under neural control [15, 18-20]. Non-stimulated tears flow at a

rate of less than 1 μL/min [21]. Upon stimulation, reflex tearing occurs with a subsequent shift the concentration of many tear proteins: The regulated tear proteins, lactoferrin, lysozyme and lipocalin concentrations are relatively independent of flow rate while secretory immunoglublin-A (sIgA), the major constitutive protein in tears, is diluted. The aqueous phase transitions into a mucin layer that is produced by goblet cells in the conjunctiva. This layer, composed mainly of glycoproteins, traps and eliminates debris before it reaches the corneal and conjunctival epithelium. Collectively, in the open eye, all portions of the tear film hydrate and protect the ocular surface, and provide a clear, smooth surface for refraction of light by the cornea. Open eye tears also lower the temperature of the corneal surface to 32-34ºC [19] which is unfavorable for bacterial colonization [22].

In contrast, the closed eye has a thicker mucin layer and thinner or absent aqueous phase as a result of diminished lacrimal secretion. sIgA concentration also increases in the closed eye. One report suggests a 40-fold increase in sIgA compared to open-eye tears [23], making it the main constituent of the closed eye tear film. There is greater recruitment of PMNs due to the raised level of complement C3c [23]. This sub-clinical inflammatory condition is further exacerbated by the obstructed passage of oxygen and carbon dioxide due to prolonged lid closure. At the epithelium, the minimum oxygen partial pressure required to avoid corneal edema is 10% of atmospheric oxygen [24]. When the eye is open, oxygen availability is 21%, and falls to only 8% during sleep [25].

Contact lenses with insufficient oxygen transmissibility decrease oxygen transmission from the atmosphere to the open eye. Oxygen availability is extremely important for normal cell function and respiration in the corneal endothelium, stroma and epithe-

lium. Corneal stromal and epithelial cells undergo anaerobic glycolysis when the eye is deprived of oxygen which leads to lactic acid production resulting in swelling of the corneal tissue [8, 24, 26]. Contact lenses with low oxygen transmissibility are also likely to cause thinning of the epithelium and polymegathism of the endothelium. A contact lens in the open eye interrupts the aqueous phase of the tear film, dividing this phase into two parts; the pre-lens tear film made up of the aqueous and lipid layers, and the post-lens tear film composed of the aqueous and mucin layers. The stability of the mucin layer is of particular importance in successful contact lens wear because it protects the corneal and conjunctival epithelial cells from mechanical stress of the lens [8].

The presence of a contact lens in the closed eye poses additional complications, not only disrupting the organization of the layers of tear film, but also providing a surface for microbial adhesion and proliferation. Exfoliated epithelial cells and other debris that would have otherwise been flushed out in tear turnover during open-eye blinking become trapped during overnight lens wear. Rubbing of this debris on the ocular surface from eye movement could disrupt tight junctions of the apical corneal epithelial cells, introducing binding sites for bacteria that are part of the normal lid microflora or that were introduced to the lens by contamination from improper care or handling. The higher temperature of the closed eye (37ºC) is also more conducive to bacterial proliferation [25].

Inflammation of the ocular surface is particularly detrimental because corneal scarring and tissue damage could result in obstruction of vision. Contact lenses have been used for over a century as a means of visual correction. Unfortunately, they have presented disadvantages to the ocular surface from the very beginning, proven by the fact that wearers have always been more prone to ocular inflammation and other complica-

tions than non-wearers. The aim of each progressive development in contact lens materials has been to reduce the potential harm that these ocular devices pose. The following section presents a brief overview of the history of contact lens designs and the advantages and disadvantages of each.

Contact Lenses

Rigid contact lenses

In the late 1800s, haptic contact lenses were the first lenses developed for effective optical correction. Haptic lenses, now commonly referred to as scleral lenses were originally made of glass and designed to rest on the scleral surface [27]. Some of the advantages of the scleral lenses were attributed to their large size because they protected the eye from invasion by foreign bodies and retarded evaporation of the tear film, preventing corneal dehydration [28]. In 1934, polymethylmethacrylate (PMMA), a light-weight, durable plastic material offering optical clarity was introduced as an alternative to glass. Fenestrations were created in the corneo-scleral transition portion of the lens to permit sufficient tear flow [29]. Unfortunately, they also allowed air bubbles to form, causing visual disturbances for the wearer. Additionally, because the PMMA scleral lenses were so large, they reduced oxygen availability, resulting in long-term corneal changes [30].

The need for a smaller lens that did not cover such a large portion of the ocular surface was addressed with invention of a PMMA lens that only covered the central cornea in 1948. There was, however, some discomfort and inconvenience associated with the corneal PMMA design due in large part to upper and lower lid interaction with the lens edge during blinking, which sometimes caused lens dislocation. Presently, the rigid

corneal lens is becoming increasingly obsolete in the clinical contact lens practice due to its impermeability to gases and hypoxia-induced complications [27].

Realizing the importance of the oxygen and carbon dioxide transmission to the cornea, scientists developed the first rigid gas permeable (RGP) contact lens made of cellulose acetate butyrate (CAB) in 1973. Although they allow the passage of oxygen and carbon dioxide, CAB lenses are not as sturdy as PMMA lenses and have low wettability which is important for proper tear interaction with the lens [27, 29]. Over the next 20 years, several materials were developed for RGP lenses including fluoropolymer, silicone resin, siloxane-methacrylate and styrene. Although these materials have variable wetting characteristics, rigidity, and weights, they shared a common inflexible characteristic that increased the likelihood of corneal abrasions and corneal compression [27].

Soft contact lenses

Soft contact lenses were invented and developed in the late 1950s with a spin-casting production method used to shape the hydrogel material to match the shape of the cornea and part of the sclera. As the technology advanced, it created the opportunity for mass production of these new lenses by 1972. They quickly proved to be more a comfortable, pliable alternative to the rigid corneal lenses; however, there was still a demand for rigid lenses for the astigmatic population because at the time, only rigid lenses could correct for astigmatism.

Hydrogel lenses are larger than the rigid gas permeable corneal lenses, covering the entire cornea and a small portion of the sclera. Many soft contact lenses are made with hydroxyethyl methacrylate (HEMA) or polyhydroxyethyl methacrylate (PHEMA).

HEMA is a flexible polymer with high water content and oxygen permeability. Glyceryl methacrylate (GMA), polyvinyl alcohol (PVA) and ethylene glycol dimethacrylate (EGDMA) are some of the other materials used for hydrogel lenses with varying water content, stiffness, wettability and resistance to deposits [31]. Although soft lenses are more flexible, there are many complications associated with hydrogel lens wear, regardless of the material. These complications include, but are not limited to an increased risk of giant papillary conjunctivitis (GPC), polymegathism and polymorphism of the corneal endothelium, and corneal neovascularization compared to rigid gas permeable wear [31]. As soft contact lenses gained popularity and the number of soft lens wearers increased, the rate of bacterial infection (microbial keratitis) among soft lens wearers followed suit. Some incidents were an indirect result of hypoxia (it was later determined that hypoxia also increased expression of lectin-binding cell surface receptor for *Pseudomonas aeruginosa* on the corneal epithelium [32]. Still, many cases of microbial keratitis were a direct result of improper lens care and handling or inadequate hygiene on the part of patients. Subsequently, it became apparent that these soft lenses would need to be replaced more frequently. By 1990, disposable soft lenses were distributed. Unfortunately, this did not drastically reduce the rate of infection seen with soft lens wear.

Silicone Hydrogel. One of the more recent advancements in soft contact lens manufacturing was the development of the silicone hydrogel lens. In 2001, Bausch & Lomb's PureVision (balafilcon A) silicone hydrogel lenses were approved for up to 30 days of extended wear by the Food and Drug Administration (FDA) [33]. Silicone hy-

drogel lenses like PureVision have significantly higher oxygen transmissibility than older lens designs, permitting longer-term wear [1, 34-36].

Since the emergence of silicone hydrogel lenses, epidemiological clinical studies report that the incidence of hypoxia-induced complications (edema, inflammation and vascularization of the ocular surface) is reduced among wearers of silicone hydrogel lenses compared to other lens materials [2, 35]. Still, other non-inflammatory events [3, 37-39] are known to occur.

Increased tear evaporation rates [40-42] and decreased epithelial exfoliation [32, 43-45] are examples of conditions that can result from the mere presence of the lens on the eye and therefore, are not unique to silicone hydrogel lens wear. Any lens worn on the eye creates an irregularity in the otherwise smooth interface of the tear film by thinning the outer-most lipid layer along the lens edge. This introduces some instability of the lipid layer, which functions to retard evaporation of the underlying aqueous. Consequently, retardation is compromised and the rate of evaporation increases [46]. Several studies have demonstrated that there is a significant decrease in exfoliation of the corneal epithelium with soft and rigid contact lens wear alike, regardless of the material [44, 45]. Corneal homeostasis involves a balance of a series of events: cells of the corneal epithelium originate from limbal stem cells which undergo proliferation, differentiation, centripetal and upward migration, ending in apoptosis (programmed cell death) signaling exfoliation from the corneal surface into tear film [47]. Results of several investigations suggest that extended contact lens wear disrupts corneal homeostasis at the proliferation, migration and apoptotic/exfoliation stages. Corneal exfoliation, also referred to as “shed-

ding", "sloughing" or "desquamation", is the terminal step in the sequence of homeostasis events.

Exfoliation rate is typically determined with a corneal irrigation mechanism described by Fullard and Wilson (1986) [21]. Using this method, corneal cells are obtained, but are not isolated. A corneal irrigation sample is also likely to contain conjunctival cells as well as various other inflammatory cells. Ocular surface cells are visually distinguishable from inflammatory cells; however, staining must be done to distinguish conjunctival epithelial cells from corneal epithelial cells. Prior investigations do not indicate whether or not staining was done to ensure that the decreased exfoliation rate was truly indicative of decreased exfoliation of the corneal epithelium. The trends that were found may have been confounded by the presence of other cell types.

Other conditions are a result of mechanical trauma due to lens stiffness, like superior epithelial arcuate lesions (SEAL). It has been proposed that these lesions can form when the silicone hydrogel lens does not conform to the limbus. SEAL, detectable with fluorescein staining, are typically found on the superior cornea [48].

In contrast to the events that may be attributed to general contact lens wear, results of a meta-analysis suggest that 30 days of extended silicone hydrogel lens wear results in up to a two-fold increased risk of corneal inflammatory events compared to seven days of hydrogel lens wear. Still, these results cannot solely be attributed to the lens material, per se, but to the 30-day extended wear time [3].

Extended silicone hydrogel lens wear also appears to induce other changes in the ocular surface environment. Formation of mucin balls and microcysts and contact lens papillary conjunctivitis (CLPC) are among the phenomena that may occur with overnight

wear of these lenses. Overnight contact lens wear can often result in accumulation of trapped debris behind the lens. Mucin balls, which are not uncommon with overnight silicone hydrogel lens wear, are small (10-20 μm in diameter), translucent spheres consisting of mucus, lipid and other debris. Though they create small depressions in the surface of the cornea that can be seen with sodium fluorescein staining, they are generally inconsequential.

CLPC has been linked to the mechanical impact of a lens on the lid surface, but is generally thought to be an immunologic response to allergens and debris that accumulates on the surface of a lens worn for an extended period of time.

It has also been shown that there is increased corneal staining with extended wear, which may be attributed to the moving lens rubbing debris against the corneal surface [33]. The study by Covey (2001) suggests that the debris from the cornea may contain clumps of inflammatory cells and that abrasions due to mechanical rubbing of the debris against the corneal surface will also stimulate an inflammatory response. In addition, inflammatory events like CLPU and contact-lens associated red eye can occur with continuous wear of silicone hydrogel lenses [22].

It has been reported that there is also a higher incidence of microbial colonization in extended silicone hydrogel lens wearers (specifically, PureVision® lenses) [21], The higher levels of microbial colonization on balafilcon A have been attributed to its high hydrophobicity which is conducive for bacterial cell adhesion. Prolonged lid closure results in temporarily hypoxic conditions that increase expression of lectin-binding receptors on corneal surface cells, leaving the eye more susceptible to binding of various bacterial strains. Similar results are seen with contact lens-induced hypoxia. *Pseudomonas*

aeruginosa is the most common cause of contact lens-related microbial keratitis (MK). Silicone hydrogel lens wearers have a decreased likelihood of MK caused by *p. aeruginosa*, but these lenses do not eliminate the risk of infection [49].

Most previous comparative contact lens studies have evaluated various parameters of the ocular tissue and/or environment, for example, tear protein composition (tear albumin, lactoferrin, lysozyme, etc), tear meniscus height, tear break up time, corneal swelling, neovascularization, hyperemia, infiltrates and infection among many others [2, 34, 38, 50-52]. To date, relatively few have measured cytokine concentrations [34, 53].

Past Studies of Tear Components

In 1998, one of the earliest studies to explore cytokine levels in tears of contact lens wearers measured the levels of IL-1β, IL-6, IL-8, Leukotriene B_4 (LTB_4) and GM-CSF in tears of seven individuals with CLARE and eight individuals with CLPU [54]. Subjects were assigned to wear hydrogel lenses for six consecutive nights and tears were collected within two hours of symptoms of an adverse response to lens wear. Proteins were quantified using enzyme-linked immunosorbent assays (ELISA). Significantly higher levels of IL-8, LTB_4, and GM-CSF were found in tears of subject with CLARE while only LTB_4 levels were significantly higher in tears of subjects with CLPU compared to normal control reflex tears. There was no difference in the IL-8 and GM-CSF levels between the CLPU group and normal individuals. The authors attributed the disparity in cytokine profiles among the CLARE and CLPU groups to the nature of these diseases. The scarring associated with CLPU may be from bacteria or PMNs releasing

enzymes that are involved in scar tissue formation. There is less corneal damage associated with CLARE where there is an inhibition or lack of protease activity.

Investigators in this study compared non-stimulated tears from CLARE and CLPU patients to reflex tears of normal individuals. The author suggests that CLARE and CLPU increase the rate of lacrimation to the rate of reflex tears in normal individuals but, it has been established that non-stimulated or "basal" tears have markedly different protein compositions than reflex tears [21]. It could not be firmly concluded that the differences between the groups were not influenced by the nature of the tears produced. Greater contribution to the understanding of the cytokine profiles of patients with CLARE and CLPU would have been made if they were compared to non-stimulated tears of normal individuals.

In 2000, the same group measured certain polymorphonuclear leukocyte (PMN) chemoattractant levels in tears of subjects wearing etafilcon lenses on a daily wear basis and a group of subjects who wore them overnight. A third group of non-contact lens wearers served as controls [5]. Tear samples were assayed with ELISA. They reported lower levels of IL-8 and IL-6 in the tears of subjects who wore hydrogel lenses overnight during sleep compared to subjects who wore no lenses at all. The subjects who wore the lenses only during the day had higher levels of IL-6 and IL-8 than the other two groups. There was also a distinct diurnal trend in the IL-8 concentration as it increased significantly after 8 hours of sleep compared to 3 hours in all three groups.

Those who wore the lenses overnight were neophytes (non-adapted lens wearers). The daily wear group was made up of individuals who had worn contact lenses for at least 2 years on a daily wear schedule. Adaptation to contact lens wear may have had an

effect on the outcome of this study because the adapted wearers may have different cytokine profiles than neophytes. Relatively high levels of IL-6 and IL-8 have been detected in non-stimulated tears of normal individuals. These cytokines are thought to play a role in homeostasis and maintenance of the state of ocular surface [55]. The lower levels of these cytokines in the neophyte overnight wear group compared to the other groups [5] suggests that their state of homeostasis was disrupted by the introduction of a lens. The adapted daily wear group would have developed an adaptive-immune response to any stimuli consistently associated with contact lens wear while the neophytes responded to their primary exposure. If the aim of this study was to identify cytokine changes in daily wear versus overnight wear, a more direct comparison could have been made if both groups were either made up of neophytes or adapted wearers. Still, the results of this study suggest that contact lens wear alters levels of inflammatory mediators.

Another tear cytokine study investigated IL-6 in tears of contact lens wearers and non-wearers using sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) [53]. This group reported no IL-6 in non-wearers and about 45 pg/ml in samples from those who wore the lenses. Three of these individuals were found to have IL-6 profiles similar to the non-wearers after one week without wearing the lenses. However, 24 hours after reinsertion, IL-6 levels returned to the original level of the wearer. IL-6 was not detected in the tears of normal non-wearers.

A cytometric bead-based assay (CBA) was used in 2006 to measure relative concentrations of five pro-inflammatory cytokines, IL-1β, IL-6, IL-8, IL-12p70, and TNF-α and one anti-inflammatory cytokine, IL-10. Only IL-10 and IL-8 levels significantly decreased while IL-1β, IL-6, IL-12p70, and TNF-α concentrations were elevated after 3

hours of eye closure, supporting the classification of the closed eye as a sub-clinical inflammatory environment [56]. The 15 μL non-stimulated tear samples took 5-20 minutes to collect, indicating that the flow rate was not tightly regulated. It has been shown that the composition of stimulated and non-stimulated tears vary [21]. Additionally, there is no evidence that oxygen availability to the eye was consistent in this study. The closed eye was patched for 3 hours; however, the oxygen transmission to a patched closed eye will differ from the transmission to a patched open eye. In order to obtain a valid classification of the state of the eye based on the assay of the proteins in tears, it would be necessary to regulate the tear flow rate as well as the oxygen transmission to minimize potential confounding factors.

There is a need for further investigation employing a highly sensitive and quantitative technique to measure levels of multiple cytokines in tears collected at critical intervals during a 30-day period; one that will determine changes in inflammatory responses to daily and extended contact lens wear through variations in cytokine concentrations, and one that tightly regulates tear-flow rate. The objective of the following study is to accomplish these goals.

SPECIFIC AIMS

1. To determine if there is a difference in ocular inflammatory response between 30 days of continuous wear and 30 days of daily wear of silicone hydrogel contact lenses through analysis of tear cytokines using a cytometric bead-based assay.
2. To correlate any changes in tear cytokine profiles with clinical test results.

Null Hypothesis: There is no difference in ocular inflammatory response between 30 days of continuous wear and 30 days of daily wear of silicone hydrogel contact lenses.

Research Hypothesis: The ocular inflammatory response to 30 days of continuous wear of silicone hydrogel contact lenses differs from the response to 30 days of daily wear. There will be a more marked low-grade inflammatory response to continuous wear.

Based on previous findings, it is likely that there will be a change in the levels of certain pro-inflammatory cytokines in the tear film after 30 days of continuous wear compared to daily wear of the silicone hydrogel contact lens. Elevated levels of IL-6, IL-8 and IL-17 were expected in tears from eyes assigned to extended wear compared to daily wear: Elevation of IL-6 and IL-8 were anticipated because their concentrations are known to increase with overnight lid closure [5, 57]. Because IL-17 induces IL-6 and IL-8 production, it was also expected to increase [7]. The concentration of TNF-α was expected to rise in tears from eyes assigned to the extended wear regimen compared to daily wear as it is present in all immune and inflammatory responses and plays a major role in

the induction of other cytokines [58]. Also, anti-inflammatory cytokines like IL-1 receptor antagonist (ra) and IL-10 levels were expected to decrease over time.

It has been suggested that IL-1 is involved in repair of ocular tissue damage and that its concentration is likely to increase in disease and inflammatory states. Therefore, higher IL-1 levels were expected in tears with continuous wear due to increased likelihood of surface epithelial cell damage.

Vascular endothelial growth factor (VEGF) is another signaling protein that is expressed during neovascularization [59]. It has been shown vascularization recedes in eyes exposed to continuous wear of silicone hydrogel contact lenses [35, 50] compared to daily hydrogel lens wear. Due to the minimal likelihood of neovascularization with silicone hydrogel lens wear, no changes were expected in levels of VEGF in tears between daily wear and continuous wear during the course of the study. The same was predicted for basic fibroblastic growth factor (FGF-b), an inducer of vascular endothelial proliferation. If FGF-b levels were to increase in tear film, it would have been as a result of corneal wound healing, which could potentially have taken place at any time during the study as a result of abrasions due to trapped post-lens debris.

Predicted changes in levels of key cytokines at 3, 14 and 30 days of daily and continuous wear of silicone hydrogel lenses based on findings in prior investigations are illustrated in Table 4.

Table 4

Prediction of Cytokine Changes in Tears at 3, 14 and 30 Days of Daily and Continuous Wear of Silicone Hydrogel Lenses

	Daily Wear			Continuous Wear		
Days	3	14	30	3	14	30
↑	IL-1β IL-6 IL-8 TNF-α	IL-1β IL-6 IL-8 TNF-α	IL-1β IL-6 IL-8 TNF-α	IL-1β IL-6 IL-8 TNF-α	IL-1β IL-2 IL-4 IL-5 IL-6 IL-8 TNF-α	IL-1β IL-2 IL-4 IL-5 IL-6 IL-8 TNF-α FGF-b eotaxin
↓	IL-1ra IL-10	IL-1ra IL-10	IL-1ra IL-10	IL-1ra IL-10	IL-1ra IL-10	IL-1ra IL-10 VEGF
Unknown:	IL-7, IL-13, IP-10, GM-CSF, MIP-1β, IL-9, IL-15, PDGF-bb, MCP-1, RANTES, G-CSF, MIP-1α					

There are no tear-specific studies addressing cytokines in the "unknown" category of Table 4. However, potential changes in these cytokine concentrations may be observed during the course of this study as they are included in the Bio-Rad™ 27-Plex human cytokine assay kit. The following predictions were based on the knowledge of how these cytokines function in tissue in other parts of the body.

It was expected that an acute phase adaptive response marked by increased IL-1, IL-6 and TNF-α (key markers of acute phase inflammation), IL-2, IL-4, IL-5 and IFN-γ (key markers of adaptive immunity) would be detected in tears after 14 days of extended wear compared to 0 and 3 days.

A chronic inflammatory response may have occurred as a result of 30 days of continuous wear and may also have caused increases in IFN-γ and TNF-α compared to 30 days of daily wear.

IL-7, IL-9, IL-13 and IL-15 regulate T-cell maintenance and growth, and MCP-1 and IP-10 are involved in T-cell chemotaxis. It was expected that a marked increase in these cytokines would be observed in tears collected at 14 days of daily and continuous lens wear compared to tears collected before lens insertion on day 0 because the adaptive immune response which involves T-cell activation would be fully developed by that time.

Granulocyte macrophage colony stimulating factor (GM-CSF) and G-CSF promote macrophage and neutrophil production while RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted), PDGF-bb and MIP-1α and -1β are involved in chemotaxis and recruitment of various inflammatory cells (monocytes, neutrophils and macrophages). It was predicted that higher levels of these cytokines would be detected in tears collected on days 3, 14 and 30 days, representing an acute response to daily and continuous wear, compared to tears collected on day 0.

This study will provide a better understanding of the impact of contact lens wear on the ocular environment and the ocular response to daily and extended wear. If extended wear of silicone hydrogel lenses causes changes to the ocular surface, early stages of events leading to inflammation or infection through tear cytokine levels may be detected using the methods described in the following section.

EXPERIMENTAL DESIGN AND METHODS

Overview

Eighty-five individuals between the ages of 19 and 55 with no systemic diseases, ocular infection or inflammation were recruited for study participation. Each individual had successfully worn soft contact lenses without complications for at least 30 days prior to enrollment. Informed consent was obtained from all participants. All procedures were conducted with approval of the University of Alabama at Birmingham Institutional Review Board for Human Use and were in accordance with the tenets of the Declaration of Helsinki. Exclusion criteria included the following: individuals who had been diagnosed with dry eye or other ocular surface conditions or lid disease; laser-assisted in situ keratomilieusis (LASIK) and photorefractive keratectomy patients; and individuals who had worn rigid contact lenses within 60 days prior to study participation.

Eligible patients were extensively trained in tear collection, and they demonstrated that they were capable of providing small non-stimulated tear samples at a consistent rate not exceeding 0.33 μL per minute. Tears were collected using polished glass microcapillary tubes positioned at the lower lid margin. Contact with the ocular surface was avoided to prevent reflex lacrimation. If the flow rate exceeded 0.33 μL per minute during collection the sample was discarded and collection recommenced once the tear prism height matched that in the contralateral eye and flow rate was considered to have returned to baseline. If both eyes were reflex tearing, the patient was instructed to wait one to two minutes before continuing sample collection. Individuals who demonstrated an inability to provide a tear sample at the required rate were removed from the study.

Patients completed the Ocular Surface Disease Index (OSDI) questionnaire, and a series of clinical tests was performed to verify that there were no symptoms or signs of dry eye and to ensure that there was no intrinsic difference between the eyes of each individual. The clinical tests included slit lamp evaluation of the upper and lower eyelids and anterior segment, a measure of non-invasive break-up time (NIBUT), measure of tear meniscus height (TMH), sodium fluorescein and Lissamine green staining.

After clinical assessment, each individual was fitted, over-refracted and prescribed the study lenses for both eyes. Parameters for the study lenses are described in Table 5.

Table 5

Study Lens Parameters

Name	PureVision ®
Manufacturer	Bausch & Lomb
United States Adopted Name	balafilcon A
Material	silicone hydrogel
Replacement Schedule	30 days
Modulus	1.1
Water Content	36%
Spherical Power Range	+6.00 to -12.00 Diopter Sphere
Base Curves	8.3 mm and 8.6 mm
Diameter	14.0 mm
Oxygen Transmissibility	110 Dk/t
Center Thickness	0.05 mm to 0.50 mm

Subjects discontinued contact lens wear during a two-week wash-out period and then returned to the clinic to provide a 5.5 µL non-stimulated tear sample. This sample and all subsequent samples were diluted in 49.5 µl Beadlyte® Cytokine Assay Buffer (Millipore, Danvers, MA, USA), stored in 0.2 ml polymerase chain reaction (PCR) tubes and frozen at -80°C until the day of the assay.

One eye was randomly assigned to 30 days of continuous wear; the lens in the contralateral eye was worn daily for 14-17 hours. Subjects were instructed to soak the daily-wear lens for a minimum of six hours each night upon removal with Ciba Vision™ AOSEPT® Clear Care™ Disinfecting Solution (containing hydrogen peroxide, .85% NaCl stabilized with phosphonic acid and buffered with phosphates) (CIBA Vision, Duluth, GA).

At Days 3, 14 and 30, subjects returned to the clinic for a repeat slit lamp evaluation and tear sample collection from both eyes. At the end of the 30-day period, subjects were re-evaluated with the same clinical tests that were administered during the suitability assessment phase of the study. Figures 2 and 3 represent the timelines that each subject followed for Study I and Studies II and III.

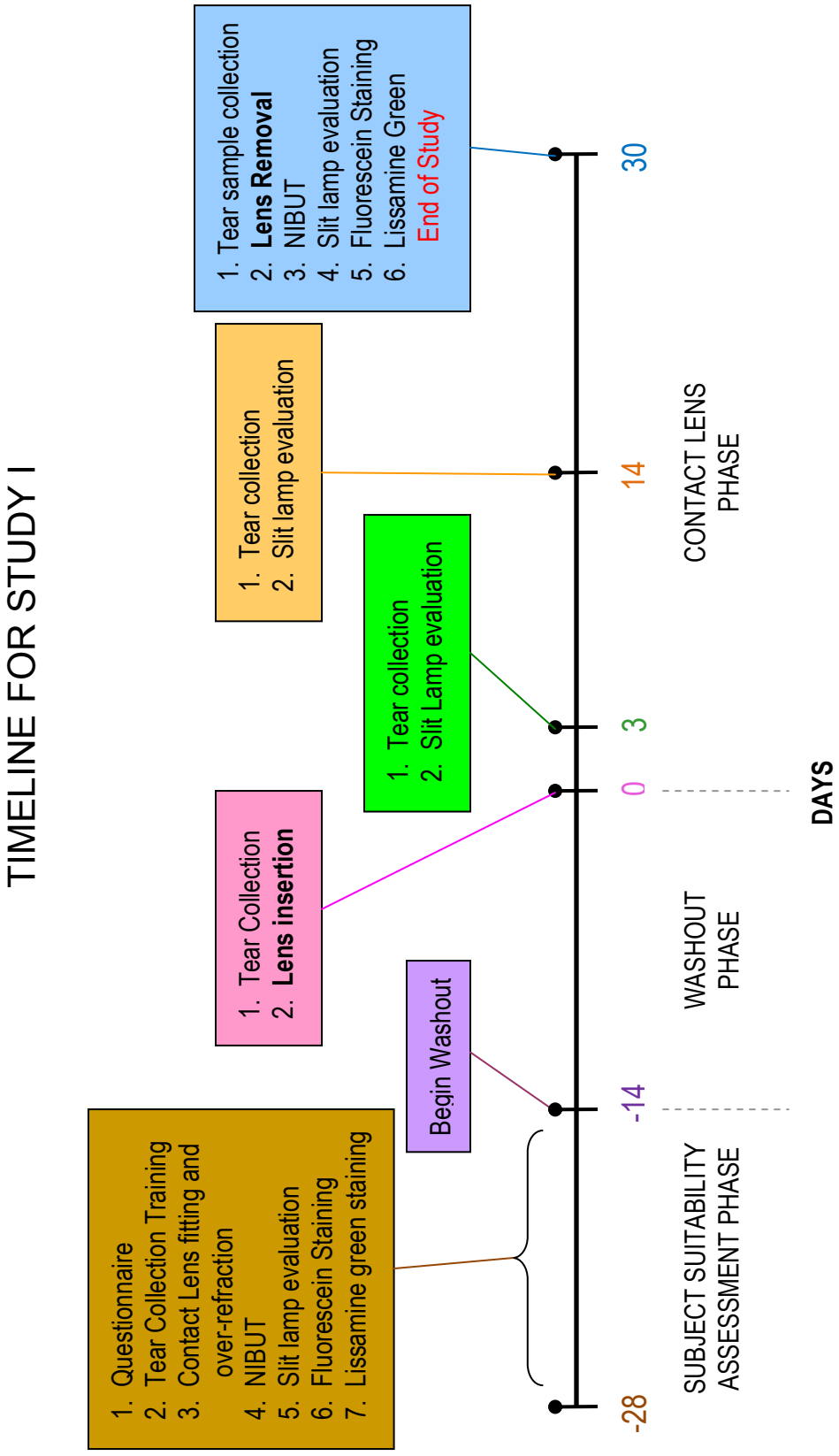

Figure 1. Study I Timeline

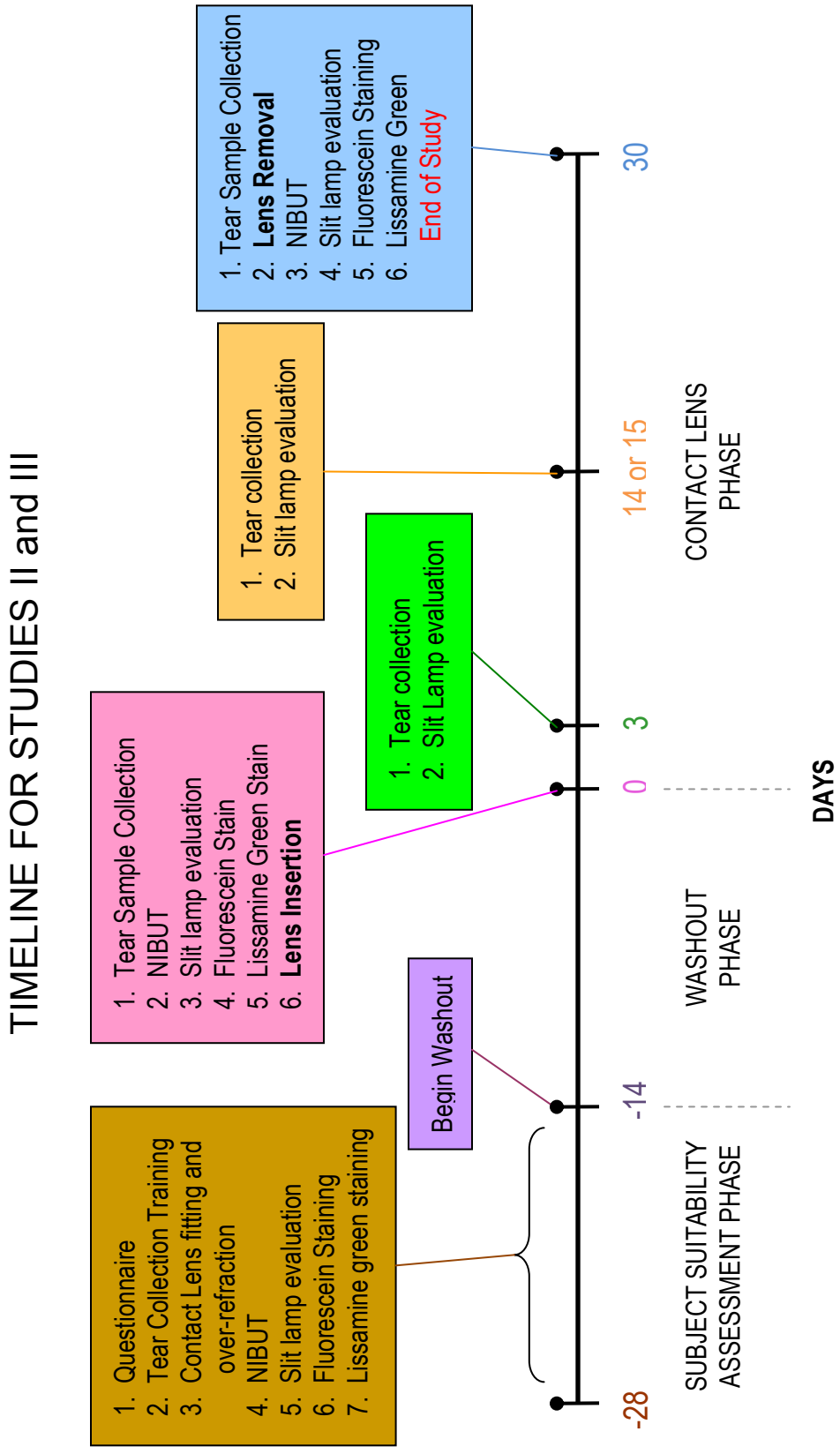

Figure 2. Timeline for Studies II and III

In Studies II and III, the clinical tests were repeated on Days 0 and 30 so that any clinical changes that occurred during the 30-day contact lens wearing period could be correlated with changes in cytokine levels.

In Study I, the Bio-Plex ® Human Cytokine 27-Plex Panel (Bio-Rad Laboratories, Inc., Hercules, CA) was used with the Luminex™ 200® flow cytometer system (Luminex Corp., Austin, TX) to quantify levels of cytokines in tear samples from both eyes of each subject. Results from Study I suggested that the use of a kit that produced more reliable data would reveal significance among trends that were seen in cytokine profiles in Study I. A comparison of two different formats of the 27-plex showed that better coefficients of variation were achieved with the Bio-Plex Pro™ Human Cytokine 27-Plex Panel, a magnetic bead format. Thus, this kit was used was used for the assay of tear samples in Study II. Results from Study II demonstrated that interference was increased with the Bio-Plex Pro™ assay leading to more inconclusive results. The Millipore™ High Sensitivity Human Cytokine 13-Plex kit targeting the following 13 cytokines; IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, GM-CSF, IFN-γ and TNF-α was used for Study III. This kit had previously performed better in spike-recovery experiments than the Bio-Rad™ 27-Plex kit used in Study I [60]. Protocols for Study I, the subsequent Assay Format Comparison, Study II and Study III are summarized in Table 6 and target analytes for the kits used in these studies are listed in Table 7.

Table 6

Summary of Study Protocols

Study	N	Kit	Format
I	17	Bio-Rad™ Human Cytokine 27-Plex	Polystyrene beads/ filter plates, manual vacuum manifold system
Assay Format Comparison	N/A	Bio-Rad™ Human Ctyokine 27-Plex vs. Bio-Rad™ Pro Human Cytokine 27-Plex	Magnetic beads/filter plates vs. polystyrene beads/flat-bottom plates, automated wash system
II	9	Bio-Rad™ Pro Human Cytokine 27-Plex	Magnetic beads/flat-bottom plates, automated wash system
III	16	Milliplex™ 13-Plex High Sensitivity Human Cytokine Assay Kit	Polystyrene beads/filter plates, manual vacuum manifold system

Table 7

Target Analytes in Bio-Rad™ 27-plex and Milliplex™ 13-plex Kits

Bio-Rad™ 27-Plex		Milliplex™13-Plex
IL-1β	Eotaxin	IL-1β
IL-2	FGF-b	IL-2
IL-4	G-CSF	IL-4
IL-5	IL-9	IL-5
IL-6	IL-15	IL-6
IL-7	IL-17	IL-7
IL-8	IP-10	IL-8
IL-10	MCP-1	IL-10
IL-12 (p70)	MIP-1α	IL-12 (p70)
IL-13	MIP-1β	IL-13
GM-CSF	PDGF-BB	GM-CSF
TNF-α	RANTES	TNF-α
IFN-γ	VEGF	IFN-γ
IL-1ra		

Statistical Approach

Statistical analyses were performed using SPSS® Statistics 17.0 software (Chicago, IL, USA). Repeated measure analysis of variance (ANOVA) was used to ascertain whether there was a significant difference in the mean concentration of any of the 27 cytokines in tears collected at each of the four visits (on days 0, 3, 14 (or 15) and 30) for daily wear and extended wear. Multiple Tukey's Honestly Significantly Different (HSD) post-hoc comparisons measuring the average change in each cytokine concentration were conducted for all combinations of visits. Mean cytokine concentrations in tears collected from eyes assigned to daily wear versus those assigned to extended wear were compared using paired sample *t*-tests for each of the four time points.

Gender effects on tear cytokine levels with daily and continuous wear were determined with ANOVA. Cytokine levels in samples collected at Visits 2, 3 and 4 were combined to compare overall differences between males and females to each treatment in Studies I – III. ANOVA was repeated to determine the significant differences between males and females at each visit for all cytokines. With Tukey's HSD, the significance of differences between visits within each group was established. The same comparisons were made to examine the effect of age on tear cytokine levels with daily and continuous wear between individuals 30 years and younger, and individuals over 30 in Studies I and II. Tear cytokines from individuals 26 and younger were compared to those who were over 26 in Study III.

Comparisons were also made for the results of the clinical tests done at the beginning and the end of the Studies II and III. Wilcoxon's Signed-Rank test was conducted to compare the average change in the fluorescein and Lissamine green staining grades, and

NIBUT between Visit 1 (day 0) and Visit 4 (day 30). Pearson's rho values and scatter-plots were generated to analyze the relationship between concentrations of cytokines that displayed parallel patterns and the corresponding clinical test results. Spearman's ρ values and scatter plots were generated to analyze the relationship between tear cytokine concentrations and clinical test results.

Coefficients of variation among replicate pooled tear samples were determined for comparison of the magnetic format used in Study II and filter plate formats used in Studies II and III. Paired sample *t*-tests were used to determine significance.

Details of Procedures

Assessment of tear film and ocular surface health was necessary to determine if a patient would be a successful extended contact lens wearer. For the purpose of this study, a contact lens-related dry eye questionnaire (OSDI), non-invasive break-up time (NIBUT), external slit lamp evaluation, and assessment of tear prism height, tear film debris, and staining with fluorescein and Lissamine green dyes were used at the beginning of the study as part of the Subject Suitability Assessment Phase. All of these tests were repeated at the end of the study to determine whether or not the different wearing patterns produced clinical changes in the tear film and ocular surface.

Questionnaire

The OSDI questionnaire was administered at the beginning of the study to screen prospective subjects as contact lens wearers without dry eye symptoms [61].

Non-invasive Tear Break-up Time

Break-up time is the time interval from a blink to the first dry spot formation on the surface of the eye. This assessment of tear film stability involves the use of an instrument that projects the image of a grid pattern onto the open eye, reflected by the tear film. Disruption of the reflected image indicates formation of a dry spot in the tear film. The NIBUT is measured as the time between the last complete blink and the first image disruption. Break-up time of at least 10 seconds is considered normal.

A reduced break-up time indicates tear film instability that may be attributed to a damaged and irregular epithelial surface [18] or altered lipid composition [62]. The destabilization of the tear film lipids has been attributed to phospholipase activity [63]. Phospholipase A_2 (PLA_2), an enzyme that degrades phospholipids, has been detected in tears of dry eye patients and was found in higher concentrations in tears of keratoconjunctivitis sicca (KCS) patients compared to normal individuals. Based on evidence that IL-1 and TNF-α have been found to stimulate production of PLA_2, it was expected that a reduced measure of NIBUT would correlate with increased levels of IL-1 and TNF-α [63]. Elevated IL-6 levels have also been significantly correlated with tear break-up time [64].

External Slit Lamp Evaluation

The tear film assessment of debris and prism height was incorporated into the external slit lamp evaluation at each of the four visits. Tear prism or tear meniscus height is an indicator of tear volume that can be measured under the high magnification of the slit lamp. A particularly low measure of the tear volume at the upper and lower menisci is

characteristic and indicative of dry eye disease. Debris in the tear film may also be detected in individuals with dry eye, in contact lens wearers or in response to inflammation. Tear film debris consists mainly of mucus threads from the mucin layer but may also contain desquamated epithelial cells. Increased concentrations of IL-1α, IL-1β [63], IL-6, and IL-8 have been linked to dry eye.

Sodium Fluorescein Staining

When sodium fluorescein is applied to the ocular surface, areas of epithelial cell loss will stain so that damaged areas of the corneal and conjunctival epithelium can be identified. A positive displacement pipette was used to instill 5 μL of Fluoresoft-0.35 %^{TM} high molecular weight sterile sodium fluorescein (Holles Laboratories, Inc., Cohasset, MA, US) into each inferior conjunctival sac. Illumination with the slit lamp was enhanced using a cobalt-blue exciter filter with 410-500 nm peak transmission over the light source and a Kodak Wratten 12 yellow absorption barrier filter transmitting above 495 nm over the slit lamp objective [65]. The staining pattern was compared to the Oxford grading scale to arrive at a staining score for each eye.

Exposure to 30 days of extended silicone hydrogel contact lens wear was expected to result in more intense staining compared to daily wear because of the micro-abrasion of post-lens debris against the corneal epithelium as well as the extended mechanical impact of the lens on the ocular surface [33]. The relationship between fluorescein staining and inflammation is yet to be established; however, it can be hypothesized that more intense staining will be linked to higher concentrations of cytokines that

are involved in wound healing and repair like FGF-b and GM-CSF [63] and/or lower concentrations of growth factors that inhibit epithelial cell growth (TGF-α and TGF-β).

Lissamine Green Staining

Staining of the cornea and conjunctiva with Lissamine green represents areas of the ocular surface that are devoid of the protective cover of mucin and glycocalyx, thus providing an assessment of the health and vitality of the surface cells. Lissamine green staining of the ocular surface was done during the Subject Suitability Assessment Phase and repeated on Day 30. Five microliter drops of 1.0% synthetic Lissamine green dye (Leiter's Pharmacy, San Jose, CA, US) were instilled into the lower conjunctival sacs of both eyes with a positive displacement pipette. Staining was viewed using the white light source from the slit lamp. Illumination was enhanced with a Kodak Wratten 92 red barrier filter over the objective as the dye absorbed wavelengths of 634-567 nm. The staining pattern was compared to an established grading scale to arrive at a staining score for each eye.

It was predicted that the lenses worn continuously for 30 days will induce more changes that caused prolonged disruption in the tear film and compromised the health of surface cells more than the lens worn daily, as described in the *Fluorescein Staining* section.

Cytometric Bead-Based Assay

The cytometric bead-based assay (CBA) was developed as a means of quantifying the concentrations of multiple analytes (proteins, DNA, RNA, cytokines, etc.) using the principles of ELISA and particle-based flow cytometry.

ELISA begins with an antibody adsorbed onto a solid medium (usually a microtiter plate). Antibody stabilized on a solid surface has high binding specificity for antigen contained in the test sample, thereby immobilizing the antigen once the sample is added to the well. An enzyme-linked secondary detection antibody is then added and allowed to bind to the sample antigen-stabilized antibody complex. When a substrate that will react with the enzyme on the detection antibody is added, a fluorescent signal of intensity relative to the concentration of the antibody is produced and measured with a spectrofluorometer. A standard curve of known sample concentrations is produced by plotting the concentration measured by the ELISA against the known concentration. This serves as a reference for the unknown sample assay to determine the concentration of the antigen in question.

In CBA assays, small polystyrene beads are pre-coated with a primary antibody with high affinity and binding specificity for the analytes of interest. The beads are color-coded with a ratio of red and infrared fluorophores, serving as the identifier for bead sets. The samples and beads are combined in wells of a 96-well plate where the analytes in the sample bind to the primary antibody onto the bead. A second detection antibody conjugated with fluorescent biotin is then added and allowed to bind the captured analyte. The antibody-analyte-antibody "sandwich" is then incubated with streptavidin-phycoerythrin, a substrate that activates the biotin on the secondary antibody and produc-

es a fluorescent signal. Once the assay is fully developed, the plate is placed in the Luminex® 200™ flow cytometry (Austin, TX, USA) instrument. In the Luminex® system, a flow column draws from each well sequentially. The beads with bound samples flow one-by-one through the flow column detection chamber, passing through a red and green laser beam. The red laser identifies the bead population based on the specific fluorophor ratio of red and infrared. The green reporter laser measures the fluorescent signal of the PE conjugated to the detection antibody bound to the sample, indicating the concentration of the sample based on fluorescent intensity

Study I Procedure

All buffers and solutions for the assay were warmed to room temperature prior to use. Stored samples were removed from -80°C storage and transferred to -30°C for one hour, then transferred to 4°C refrigeration for one hour to thaw. The lyophilized master standard was reconstituted with 500 µl of the same assay buffer that was used to dilute the tear samples. After a 30 minute incubation period, the master standard was serially diluted with a dilution factor of three to create nine standards for each cytokine (0.3 - 2000 pg/ml). Conjugated beads for the assay were prepared according to manufacturer instructions and applied to the 96-well filter plate that was pre-wet with assay buffer. Beads were then washed three times with wash buffer. The wash buffer and bead solution were removed using vacuum filtration. Thawed samples were removed from 4°C and transferred directly from the PCR tubes to the filter plate with washed beads according with an 8-channel electronic displacement pipet. Each plate included wells for blanks and standards in duplicates. The plate with each well containing beads with standards

and samples was covered with sealing tape, wrapped in aluminum foil to prevent exposure to light and incubated with agitation on a plate shaker overnight at 4°C.

After overnight incubation, the plate was removed from 4°C to allow contents to warm to room temperature. All fluid was removed from the wells with vacuum filtration. After three wash steps with assay buffer, 25 µl of detection antibody was added to each well followed by 30 minute incubation with agitation at room temperature. This was followed by 50 µl of streptavidin-phycoerythrin dispensed into each well and a 10-minute incubation at room temperature. The streptavidin-phycoerythrin was then removed with vacuum filtration and the samples were washed three times with wash buffer. The beads are finally re-suspended with 125 µL of assay buffer, agitated on a plate shaker for 30 seconds and placed into the Luminex® 2002™ for analysis and data acquisition.

Assay Format Comparison

The Bio-Plex™ Bio-Plex® CBA is available in two formats. One incorporates 5.6 µm polystyrene beads as the solid media for the primary capture antibodies. The polystyrene beads are incubated and washed in a 96-well filter plate using a vacuum manifold system. This format was compared with another that uses 6.5 µm polystyrene beads with a magnetic core. The magnetic format is performed with a flat-bottom plate and an automated plate washer. The two formats were compared using matched pooled samples that were divided into replicates on each plate format. An automated plate washer was used for all wash steps.

Study II Procedure

Participants were screened with the same clinical tests that were used in Study I. Clinical tests were conducted on the day of lens insertion (day 0). This step had not been included in Study I. Tests were repeated again on the day of lens removal (day 30) (see Figure 2). Subjects could return for tear collection for Visit 3 on Day 14 or 15 of contact lens wear.

Prior to the tear assay for Study II, the Luminex® instrument was validated and calibrated using the validation kit from BioRad™ (Hercules, CA, USA) to ensure the alignment of lasers and optics system, accuracy of bead population classification, and validation of the fluidics for proper laminar flow and reporter channel identification. All of the wash/vacuum steps were executed with the automated plate-washer using the magnetic bead buffer aspiration procedure. Magnets located on the platform of the automated washer drew the beads to the perimeter of each well while needles aspirated fluid from the center of the well, thus avoiding the beads. This automated system reduced the likelihood of variability by ensuring that equal volumes of all buffers were dispensed and aspirated with uniform pressure.

Unlike Study I, in which tear samples and cytokine standards were incubated overnight at 4° C, tear samples and cytokine standards were incubated with the magnetic beads for 1 hour at room temperature. The fully developed assays in each well of the assay plate were analyzed under the High Photomultiplier Tube (PMT) settings for better quantification of cytokines that are present at low concentrations in tears, in addition to the regular Low PMT setting analysis.

In addition to the repeat clinical tests on Day 0 and validation of the Luminex® instrument, samples serving as controls were included in the assays for Study II. Pooled stimulated tears from multiple donors (collected prior to the study) were diluted 1 in 10 in Millipore Assay Buffer, stored in 70 µL aliquots at -80°C until the day of the assay. Quadruplicates of these samples included in the assay of Study II provided measures of the intra-assay coefficient of variation.

Study III Procedure

The study timeline for Study III was the same for Study II (see Figure 2). High Sensitivity Human Cytokine Premixed 13-Plex kits (Millipore Corp., Billerica, MA) were used to assay the tear samples.

The 10× wash buffer and pre-mixed beads were prepared according to manufacturer's instructions. The Human Cytokine Standard mixture was reconstituted with assay buffer. A manual vacuum manifold was used for removal of fluid from the filter plate.

Two hundred µL of wash buffer were dispensed into each well of a 96-well filter plate. The plate was sealed and agitated on a plate shaker for 10 minutes at room temperature. After removal of the wash buffer, 25 µL of bead solution were dispensed into each well and the fluid removed by vacuum. Blanks, standards serially diluted from 0.008 to 2000 pg/ml and samples, were applied to the plate and incubated overnight as in Study I. After overnight incubation, the liquid in each well was removed from the plate, and well contents were washed twice with 200 µL of wash buffer. Fifty µL of detection antibody was then applied to each well of the plate. The plate was sealed, covered with foil, and incubated on a plate shaker at room temperature for one hour. This was followed by 50

µl of streptavidin-phycoerythrin dispensed into each well and a 30-minute incubation with agitation at room temperature. The liquid in each well was then removed by vacuum filtration and the samples were washed three times with 200 µL of wash buffer per well. The beads were finally re-suspended with 100 µL of sheath fluid, sealed, covered and agitated on a plate shaker for 5 minutes and placed into the Luminex® 2000™ for analysis and data acquisition.

RESULTS

Study I

Study I Cohort and Attrition

Forty individuals were screened for Study I; 29 females and 11 males. Eighteen individuals successfully completed the study. Patient demographics for Study I, including number enrolled, average age and gender distribution are summarized in Table 7. Six individuals were found to be clinically ineligible during the subject suitability assessment. One patient was excluded from Study because of inadequate NIBUT (less than 10 seconds). The other was excluded for corneal vascularization, endothelial opacity and stromal scarring. Several patients were either excluded from study participation or removed from Study I due to inability to collect tears (Table 8). The majority of patients excluded prior to study entry had great difficulty slowing the rate of tear collection to the required 0.3 μL/minute. Twenty-four percent of study entrants were unable to collect a sufficient volume of tears within the allotted time of 40 minutes.

Non-compliance with contact lens wearing schedules accounted for most of the study attrition during the 30-day contact lens wearing period. Six percent of subjects either fell asleep wearing the lens assigned to daily wear or removed the extended wear lens prior to the final visit. In all cases of early, unscheduled, extended wear lens removal, patients complained of lens discomfort (irritation, foreign body sensation, grittiness and stinging).

Table 8

Study I Cohort Demographics

Gender	Completed		Discontinued		Total	
	N (%)	Mean Age ± SD	N (%)	Mean Age ± SD	N (%)	Mean Age ± SD
Female	12 (30)	33.3 ± 10.4	17 (42.5)	34.8 ± 12.2	29 (72.5)	34.2 ± 11.3
Male	6 (15)	32.3 ± 7.0	5 (12.5)	30.8 ± 8.3	11 (27.5)	31.6 ± 72
Total	18 (45)	32.9 ± 9.2	22 (55.0)	33.9 ± 11.4	40 (100)	33.5 ± 10.3

Table 9

Study I Attrition

Reasons for Discontinuation	Frequency (%)
Clinical Ineligibility	4 (10)
Tear Collection	7 (17.5)
Missed Visit	4 (10)
Wear Schedule	7 (17.5)
Total	22 (55)

Study I Assay Results

Tear cytokine levels for the four study visits in Study I are shown in the following series of figures, divided for clarity according to mean cytokine concentration ranges: low (Figure 3a), medium (Figure 3b) and high (Figure 3c). Each figure is further subdivided into daily and extended wear data. FGF-b and MIP-1α were not detected in any samples and thus, are not included in the charts. Eotaxin was only detected on study Visits 3 and 4 for daily wear and exclusively on Visit 4 for extended wear (Figure 3a). However, in these cases, eotaxin was only detected in one individual. IL-9 was present at

the highest level at Visit 4 for daily wear and in particular extended wear (Figure 3a). However when compared to other study visits, this difference was not significant.

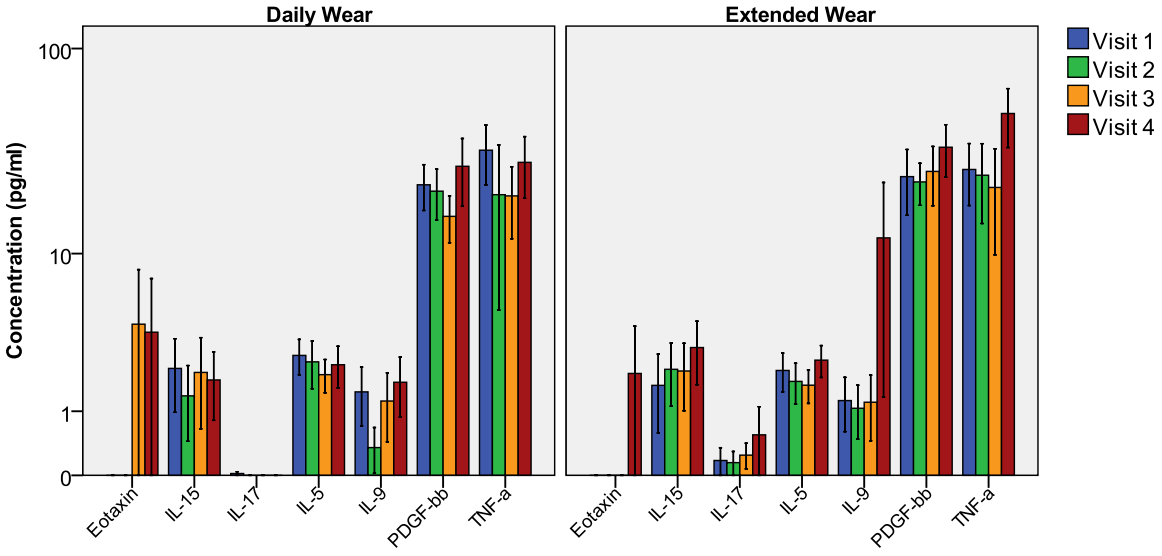

Figure 3a. Tear Cytokine Concentrations with Daily and Extended Wear in Study I. Mean concentrations of tear eotaxin, FGF-basic, IL-15, IL-17, IL-5 IL-9, MIP-1á, PDGF-bb and TNF-á at Visits 1, 2, 3 and 4 of daily and extended wear in Study I.

IL-1β levels were highest at Visit 4 for both daily and extended lens wear (Figure 3b). Again, these differences did not reach statistical significance. IL-1β was consistently observed in the vast majority of patients at all four study visits.

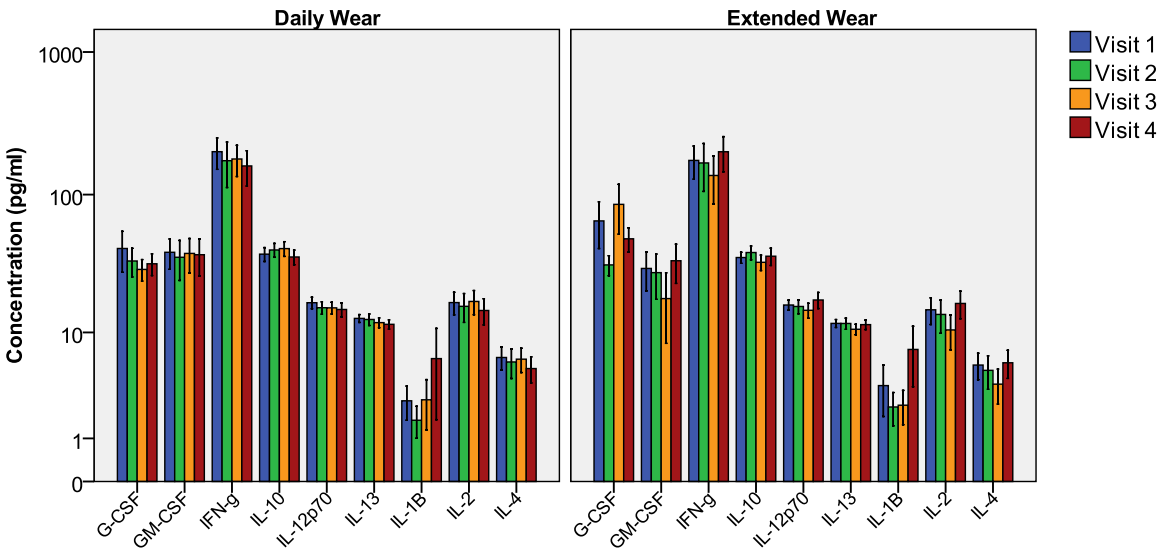

Figure 3b. Tear Cytokine Concentrations with Daily and Extended Wear in Study I. Mean concentrations of tear G-CSF, GM-CSF, IFN-ã, IL-10, IL-12p70, IL-13, IL-1â, IL-2 and IL-4 in samples collected at Visits 1, 2, 3 and 4 of daily and extended wear in Study I.

IL-1ra, which was detected consistently in all patient samples, was present at higher levels on Visit 4 than any of the other three visits for the daily wear eye (Figure 3c). However, the difference was not statistically significant. Conversely, the level of IL-1ra was significantly higher at Visit 4 for extended lens wear versus Visit 1 ($p < 0.05$) and Visit 2 ($p < 0.05$) (Figure 3c).

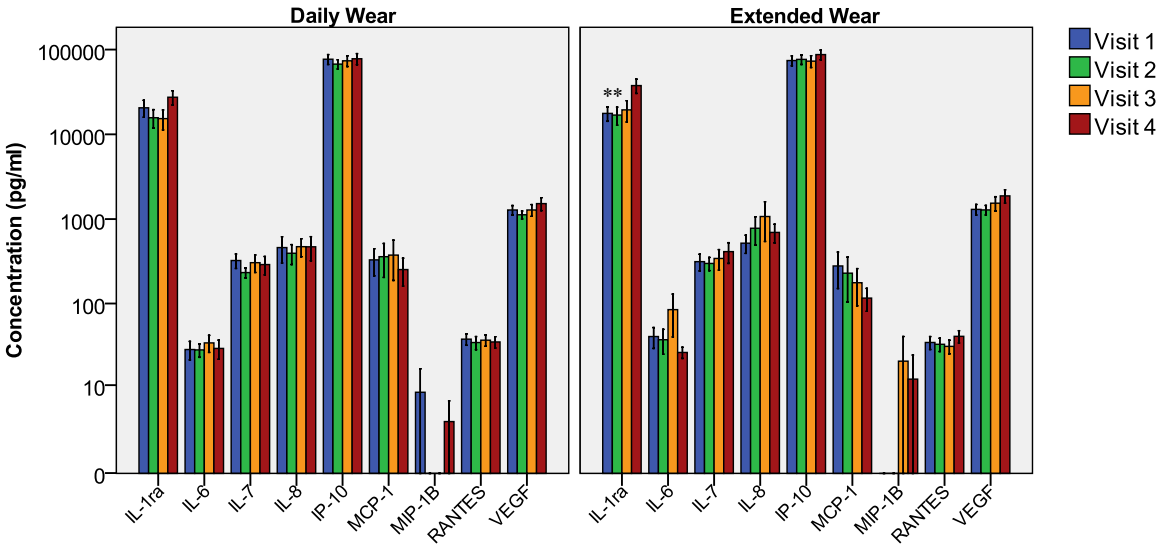

Figure 3c. Tear Cytokine Concentrations with Daily and Extended Wear in Study I. Mean concentrations of tear IL-1ra, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1â, RANTES and VEGF in samples collected at Visits 1, 2, 3 and 4 of daily and extended wear in Study I. *IL-1ra increased significantly from Visits 1 ($p < 0.05$) and 2 ($p < 0.05$) to Visit 4.

Figure 4 illustrates the variation in tear IL-1ra levels in both daily and extended lens wear across the four study visits and demonstrates the significant increase at Visit 4 versus 1 and 2 for extended wear. No significant differences were found for daily wear.

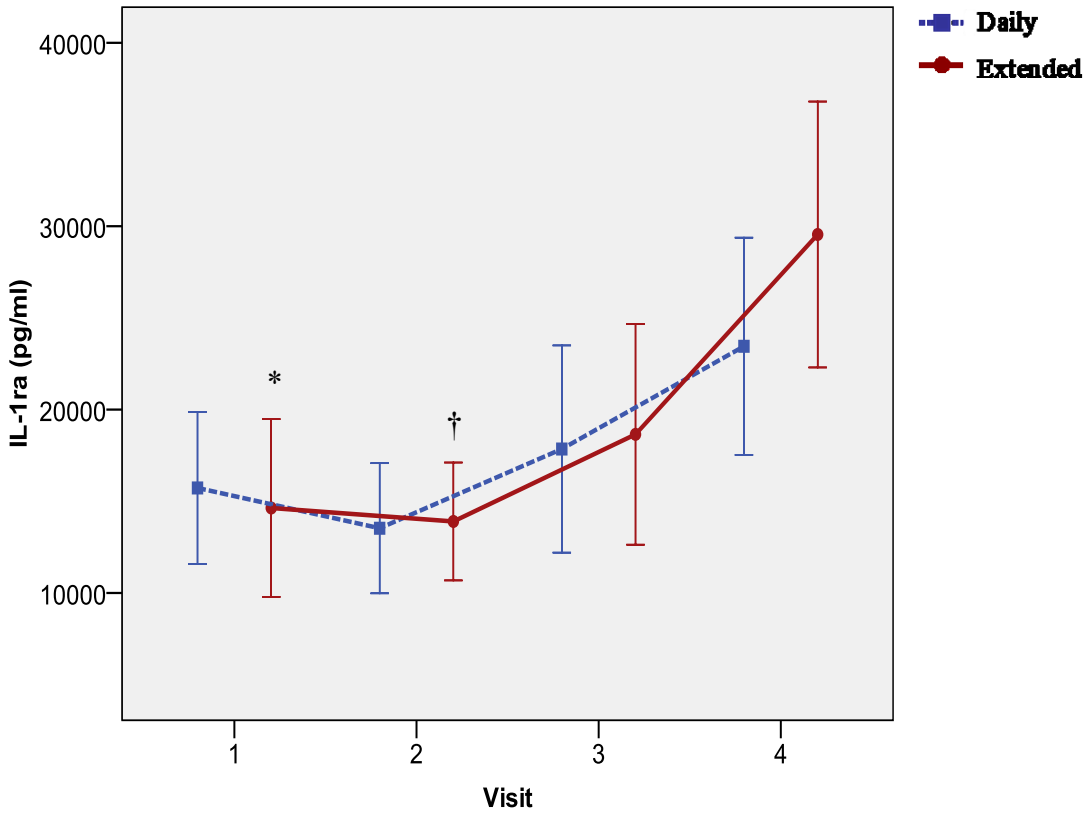

Figure 4. Tear IL-1 ra Levels with Daily and Extended Contact Lens Wear in Study I. Daily versus extended contact lens wear comparison of IL-1ra concentrations. Means and standard error of concentrations of tear IL-1ra collected at visits 1, 2, 3 and 4. Concentration at visit 4 of extended wear is significantly elevated from *Visit 1 ($p < 0.05$) and Visit 2 ($p < 0.05$).

Figures 5 and 6 illustrate the variation in tear IL-2 and -4 levels, respectively, in both daily and extended lens wear across the four study visits. There were no significant trends for either cytokine across the four study visits or between eyes at any given visit.

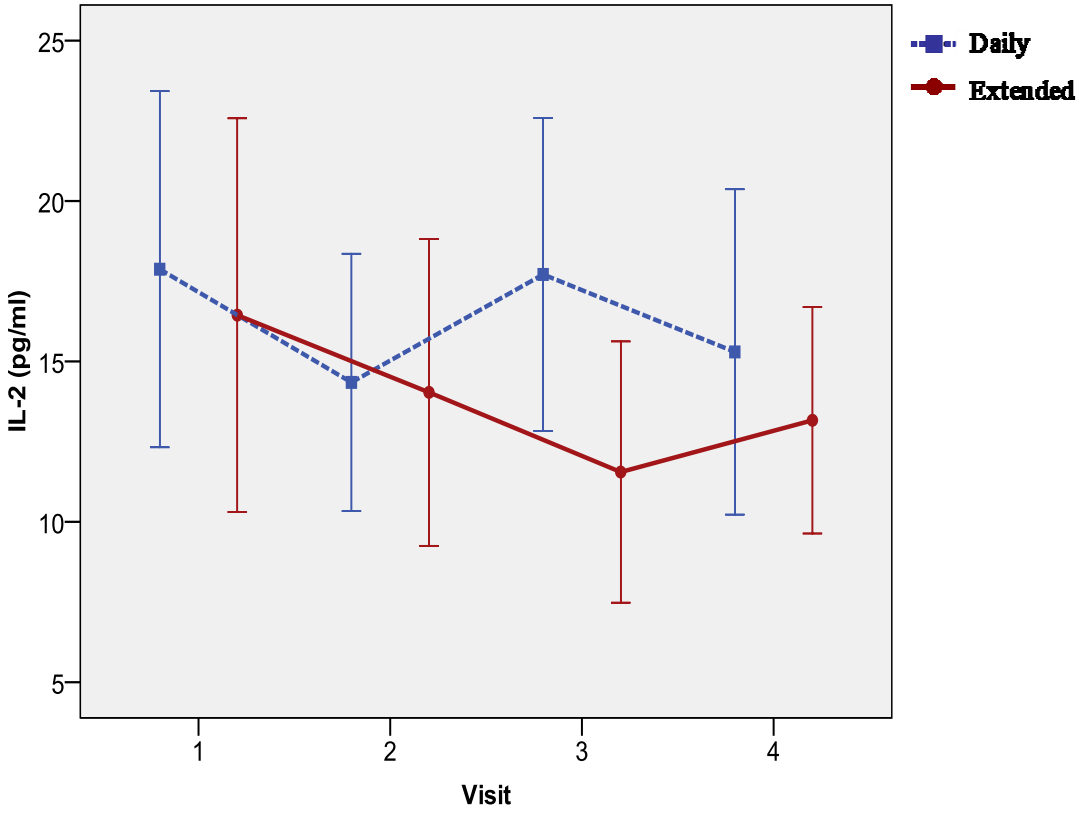

Figure 5. Tear IL-2 Levels with Daily and Extended Contact Lens Wear in Study I. Means and standard error of concentrations of tear IL-2 collected at visits 1, 2, 3 and 4. No significant differences in IL-2 levels were found between visits or between eyes.

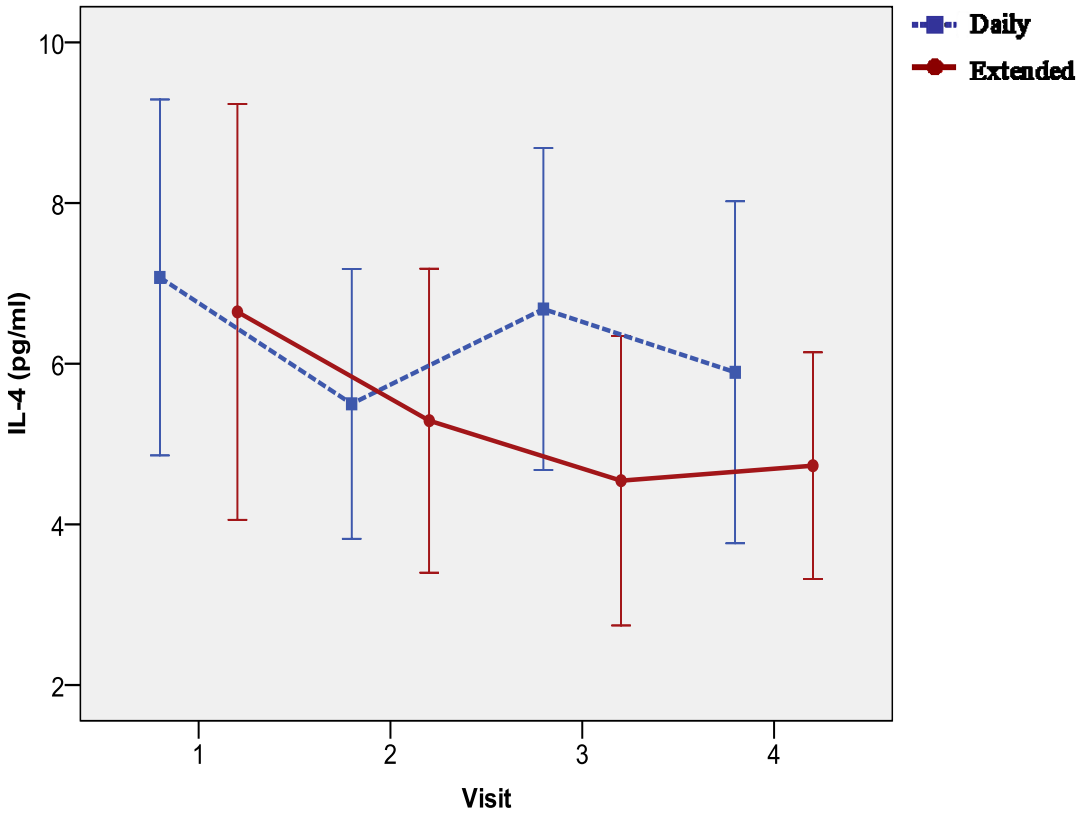

Figure 6. Tear IL-4 Levels with Daily and Extended Contact Lens Wear in Study I. Means and standard error of concentrations of tear IL-4 collected at visits 1, 2, 3 and 4. No significant differences in IL-4 levels were found between visits or between eyes.

IL-2 and IL-4 demonstrated notably similar patterns. To illustrate the potentially synergistic relationship between these two cytokines, the levels of tear IL-2 and IL-4 were correlated for all samples collected in Study I. A positive correlation is clearly evident for daily wear tear samples ($R^2 = 0.95$, Figure 7a) and for extended wear tear samples ($R^2 = 0.97$, Figure 7b). Conversely, to illustrate one of the many examples in which no such correlation was apparent, tear levels of IL-2 and IL-6 are plotted for both daily wear (Figure 8a) and continuous wear (Figure 8b).

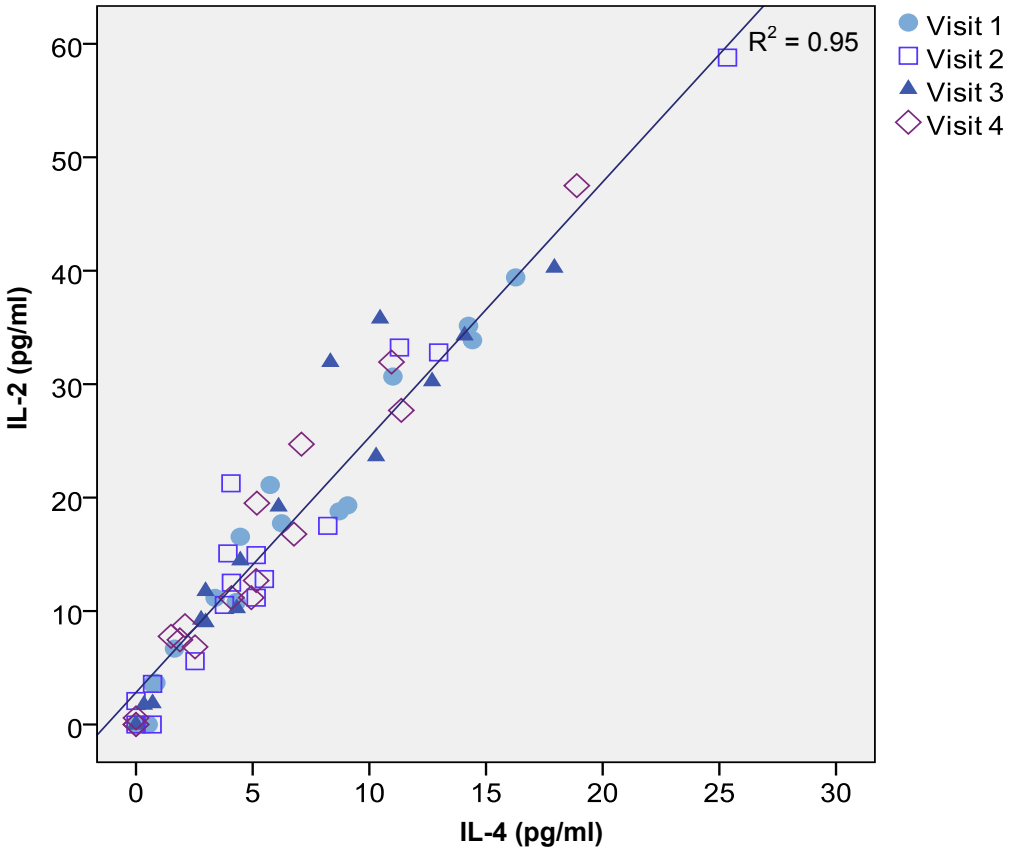

Figure 7a. Scatter Plot of Tear IL-2 and IL-4 with Daily Wear in Study I. Pearson's r = 0.984 ($p < 0.01$). Linear trendline equation: $y = 2.25x + 2.82$.

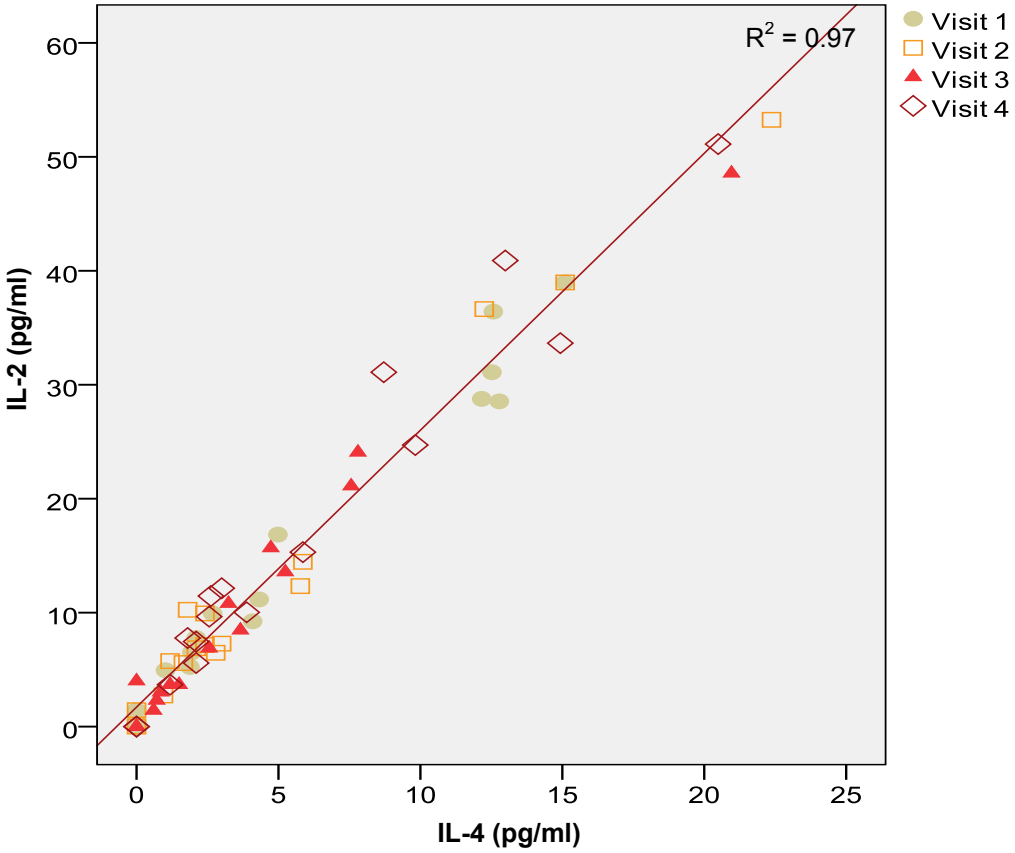

Figure 7b. Scatter Plot of Tear IL-2 and IL-4 with Extended Wear in Study I. Pearson's r =0.971 ($p < 0.01$). Linear trendline equation: $y = 2.43x + 1.71$.

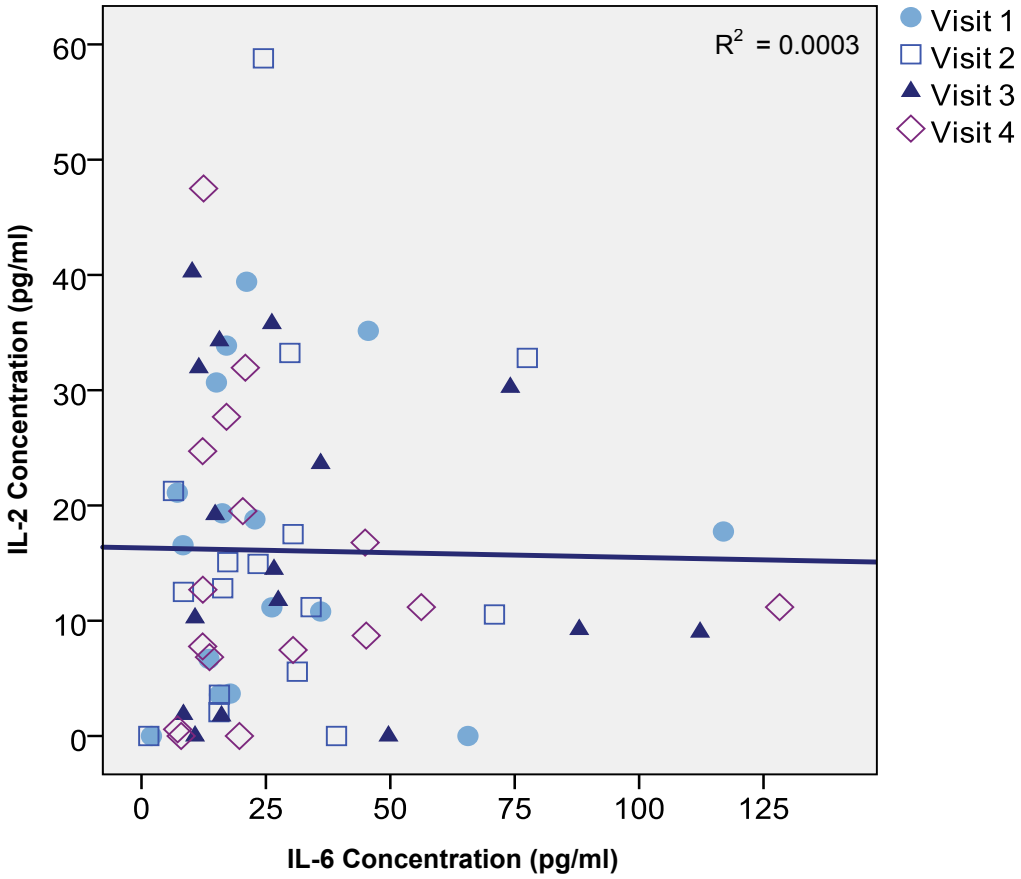

Figure 8a. Scatter Plot of Tear IL-2 and IL-6 with Daily Wear in Study I. Pearson's r = -0.02 ($p < 0.89$). Linear trendline equation: y = -0.083x+ 16.32.

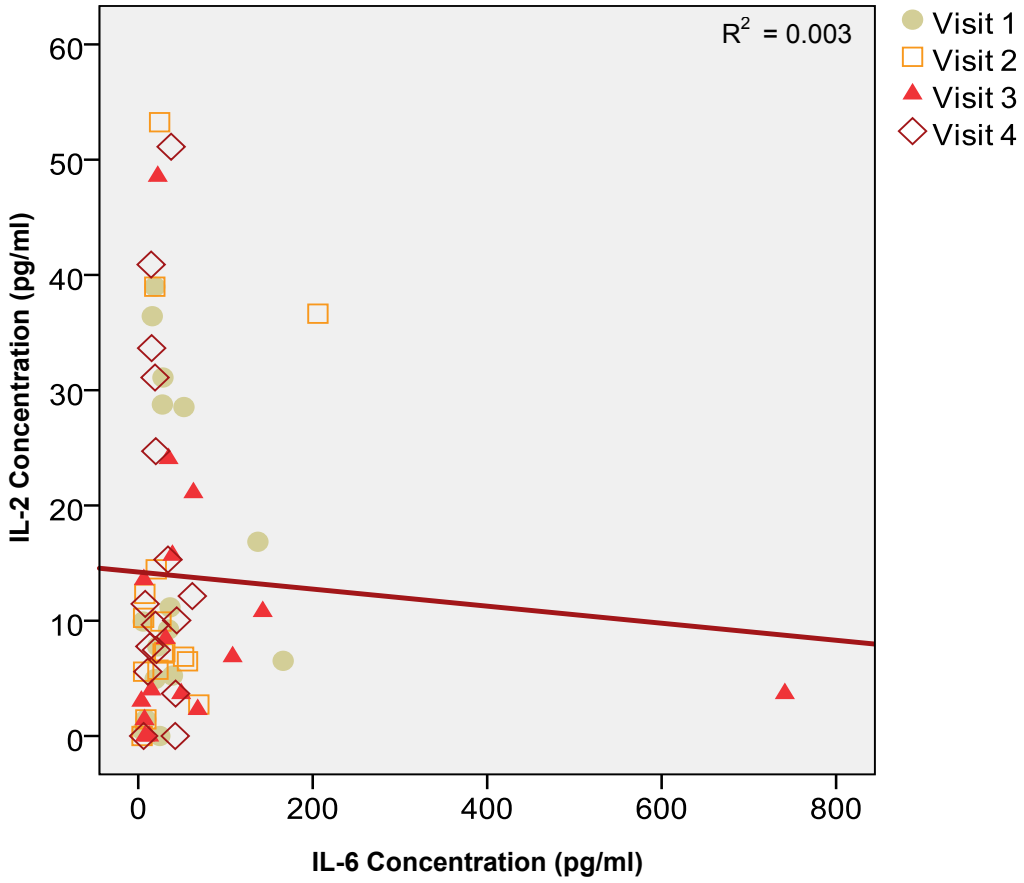

Figure 8b. Scatter Plot of Tear IL-2 and IL-6 with Extended Wear in Study I. Pearson's r = -0.05 ($p < 0.69$). Linear trendline equation: y = -0.007x + 14.22.

No statistically significant trends were found for tear IL-6 levels (Figure 9) or IL-8 levels (Figure 10) across the four study visits or between eyes at any given visit. There was a marked increase in levels of both cytokines at Visit 3 (day 14) of extended wear. However, these elevations did not reach significance.

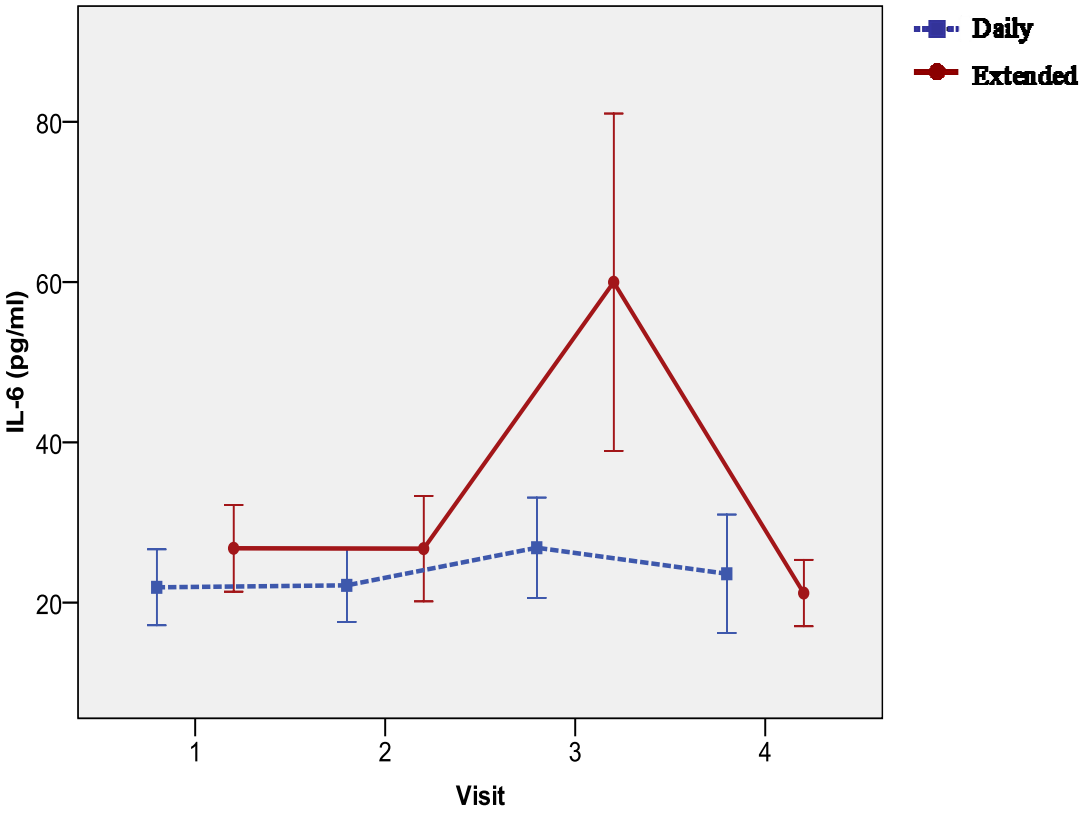

Figure 9. Tear IL-6 Levels with Daily and Extended Contact Lens Wear in Study I. Means and standard error of concentrations of tear IL-6 collected at visits 1, 2, 3 and 4. No significant differences in IL-6 levels were found between visits or between eyes.

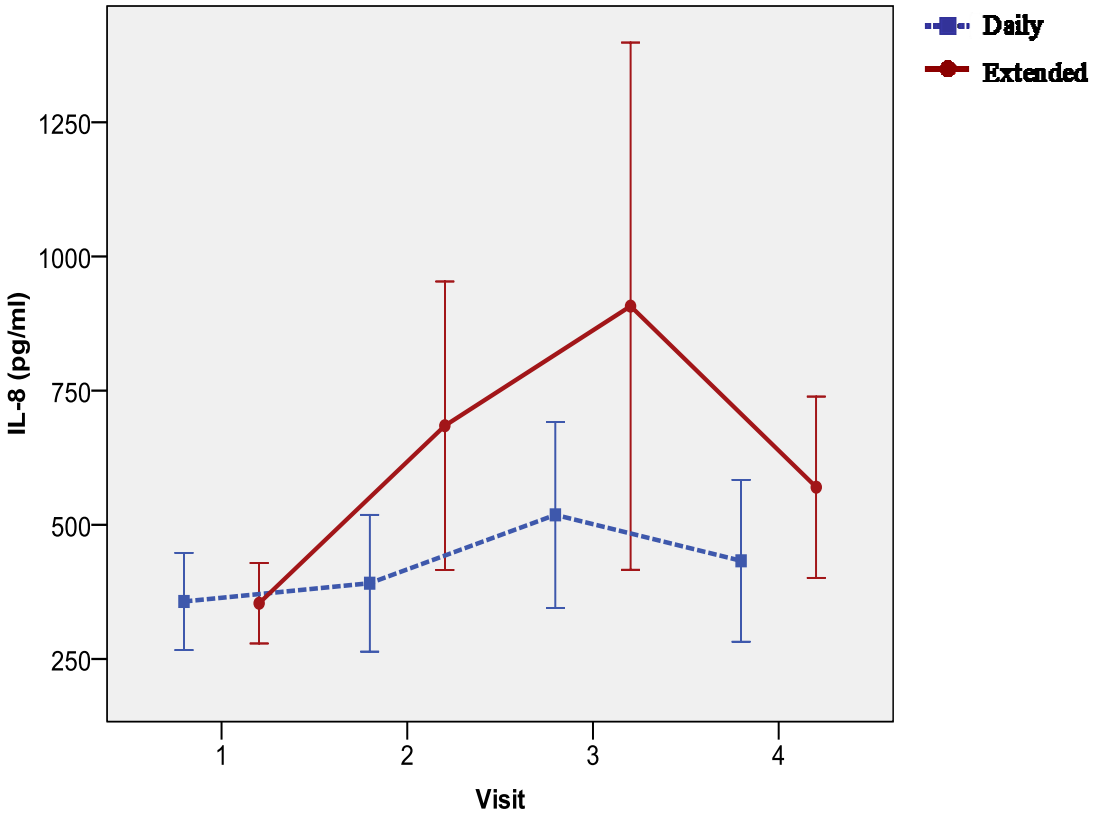

Figure 10. Tear IL-8 Levels with Daily and Extended Contact Lens Wear in Study I. Means and standard error of concentrations of tear IL-8 collected at visits 1, 2, 3 and 4. No significant differences in IL-8 levels were found between visits or between eyes.

No statistically significant trends were found for tear TNF-α levels across the four study visits or between eyes at any given visit (Figure 11).

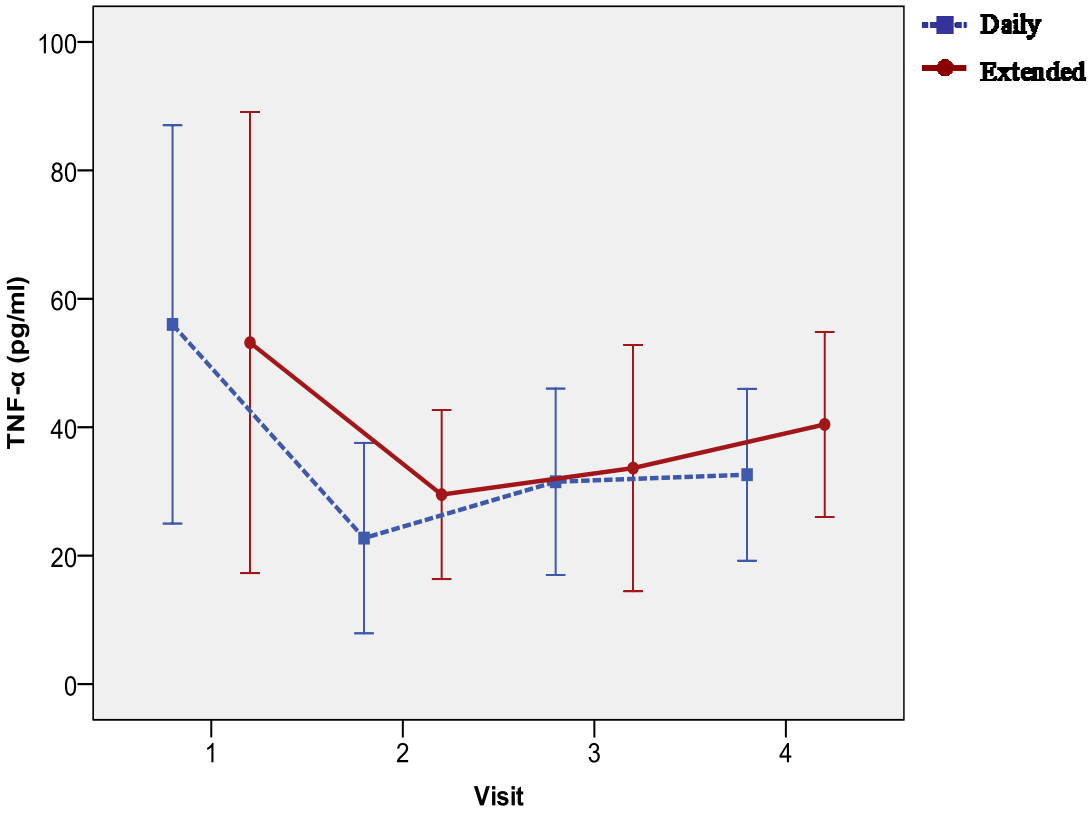

Figure 11. Tear TNF-α Levels with Daily and Extended Contact Lens Wear in Study I. Means and standard error of concentrations of tear TNF- α collected at visits 1, 2, 3 and 4. No significant differences in TNF-α levels were found between visits or between eyes.

Gender and Age Effects on Tear Cytokine Levels in Study I

Results from Study I were further analyzed for age and gender effects on tear cytokine levels. There were no significant differences in tear cytokine levels between males and females at Visit 1 (pre-lens wear), indicating baseline equality. Cytokine levels in samples collected at Visits 2, 3 and 4 were combined to investigate overall gender differences in response to contact lens wear. Analysis of variance revealed significant gender differences in levels of certain tear cytokines with daily and continuous contact lens wear.

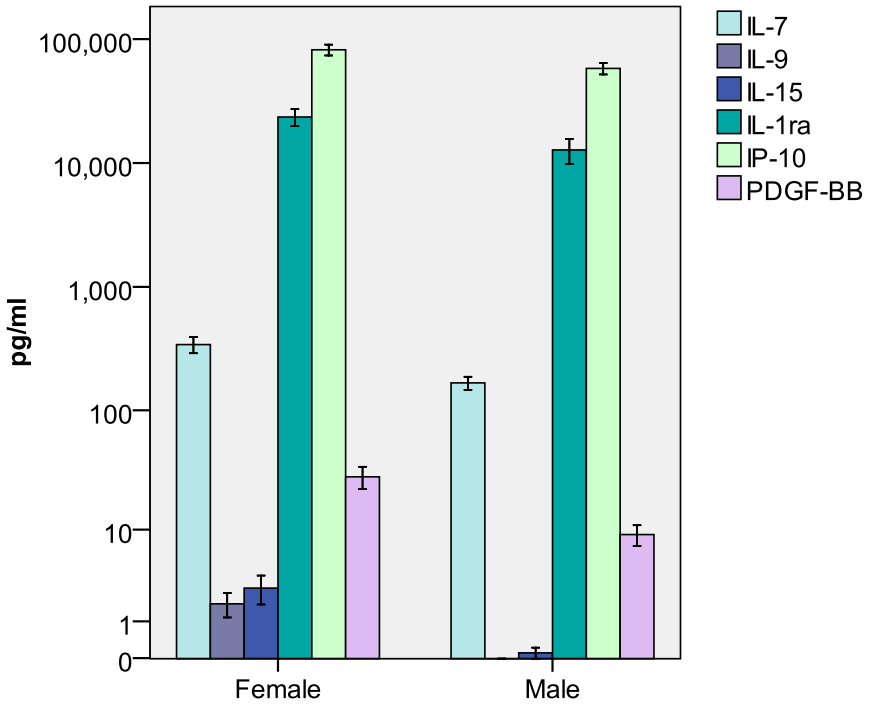

Figure 12a. Gender Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study I. Tear IL-7, IL-9, IL-15, IL-1ra, IP-10 and PDGF-BB levels in male and female daily wear samples in Study I. There were significantly higher levels of IL-7 ($p < 0.05$), IL-9 ($p < 0.05$), IL-15 ($p < 0.05$), IL-1ra ($p < 0.05$), IP-10 ($p < 0.05$), and PDGF-bb ($p < 0.05$) in female tear samples.

These differences were not seen with continuous wear (Figure 12b). Tear samples from males had a higher level of IP-10, but that did not reach significance.

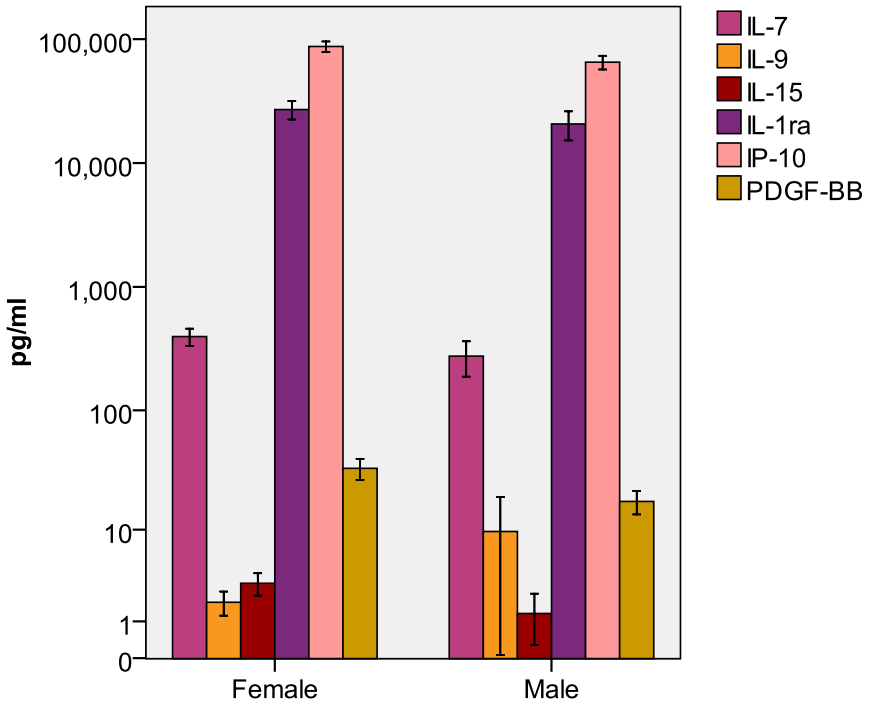

Figure 12b. Gender Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study I. Tear IL-7, IL-9, IL-15, IL-1ra, IP-10 and PDGF-BB levels in male and female continuous wear samples in Study I. There were significantly higher levels of IL-7 ($p < 0.05$), IL-9 ($p < 0.05$), IL-1ra ($p < 0.05$), IP-10 ($p < 0.05$), and PDGF-bb ($p < 0.05$) in female tear samples. There was no significant difference in IL-15 levels between males and female tear samples with continuous wear.

Although there were no significant gender differences in tear cytokine levels with continuous wear overall, comparisons at each visit revealed higher IL-1ra in female tear samples at Visit 2 ($p < 0.05$) of continuous wear. In addition, post-hoc comparisons of cytokine levels at each visit showed male tear IL-1ra at Visit 4 to be higher than IL-1ra at Visits 1 ($p < 0.05$), 2 ($p < 0.01$) and 3 ($p < 0.05$) with continuous wear.

Tear cytokine levels of individuals 30 years of age and younger were compared to those over 30. As with the gender comparison, there were no significant differences in tear cytokine levels between the two age groups at Visit 1 (pre-lens wear), indicating baseline equality. Cytokine levels in samples collected at Visits 2, 3 and 4 were combined for analysis. The following figures illustrate the comparison of tear cytokine levels with daily and continuous wear between the two groups in Study I. Age did not have a significant effect on tear cytokine levels in Study I with daily wear (Figure 14a). However, with continuous wear, IL-5, IL-7, IL-12p70, GM-CSF, IFN-ã, TNF-á and VEGF were significantly higher in the younger group ($p < 0.05$) (Figure 14b).

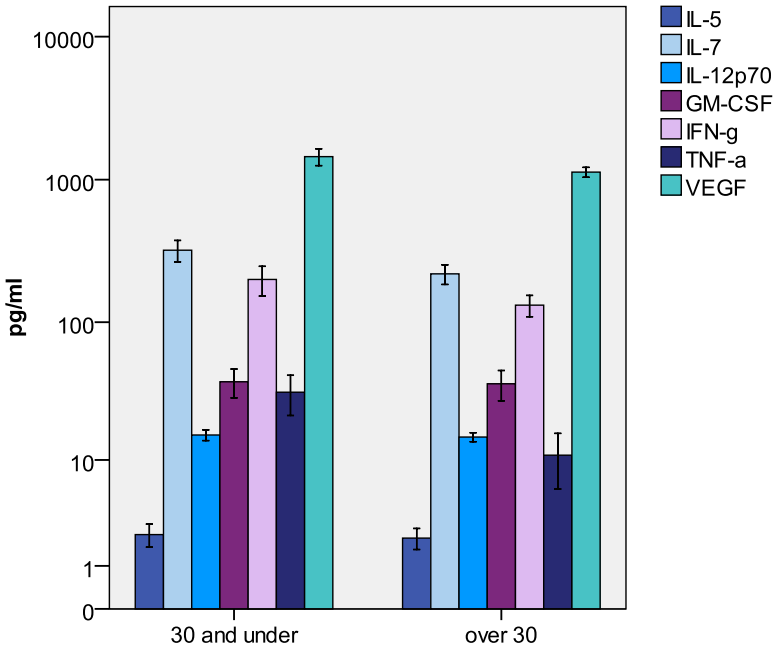

Figure 14a. Age Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study I. Tear IL-5, IL-7, IL-12p70, GM-CSF, IFN-ã, TNF-á and VEGF levels in samples from individuals 30 years old or younger compared to those over 30 with daily wear in Study I. There were no significant differences in tear cytokine levels between age groups with daily wear.

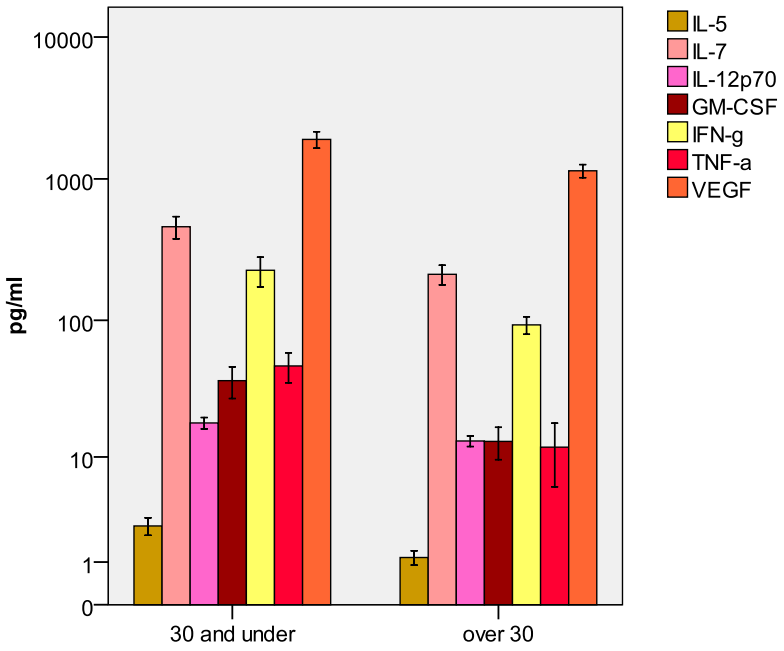

Figure 14b. Age Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study I. Tear IL-5, IL-7, IL-12p70, GM-CSF, IFN-ã, TNF-á and VEGF levels in samples from individuals 30 years old or younger compared to those over 30 with continuous wear in Study I. There were significantly higher levels of IL-5 ($p < 0.05$), IL-7 ($p < 0.05$), IL-12p70 ($p < 0.05$), GM-CSF ($p < 0.05$), IFN-ã ($p < 0.05$), TNF-á ($p < 0.05$), and VEGF ($p < 0.05$) in tear samples from the younger group.

ANOVA of cytokine levels at each visit between age groups in Study I revealed that at Visit 2 of continuous wear, the following cytokines were higher in the younger group: IL-7 ($p < 0.05$), IL-12p70 ($p < 0.05$), VEGF ($p < 0.05$), and PDGF-bb ($p < 0.05$).

Assay Format Comparison: Bridging Study between Study I and II

Due to the lack of significant overall trends in Study I and the announcement by Bio-Rad™ that they were converting to a magnetic bead format for all their cytokine assay kits, some developmental assays were conducted using the new 27-Plex magnetic bead kits (Bio-Plex Pro™). Matched tear samples were run using magnetic beads on a

filter plate (as employed with the Study I polystyrene bead kits) and a plastic-bottom (polystyrene), non-filter, plate. This second plate required the use of an automated plate washer with magnetic tray so that beads could be pulled to the bottom of the wells to allow aspiration of assay fluid during wash and reagent removal steps without bead loss. The plate washer also had an interchangeable vacuum wash tray to allow automated washing of the filter plate. Variability of results of the paired tear sample assays using the two different plate formats are shown in Table 10. Coefficients of variation (CV) for series of duplicate, triplicate and quadruplicate samples were compared in matched tear samples for all 27 cytokines. In virtually all cases, coefficients of variation were lower for the plastic plate and magnetic bead aspiration washing technique. Table 11 summarizes the overall mean coefficients of variation values across all tear samples. The finding of an approximate halving of the CV values with the magnetic plate format indicated that a change to magnetic beads with plastic plate should be adopted for Study II.

Table 10

Coefficients of Variation of Replicate Samples: Magnetic vs Vacuum Filtration Formats

	Duplicates		Triplicates		Quadruplicates	
Cytokine	MAG	VAC	MAG	VAC	MAG	VAC
Eotaxin	8.67	21.38	11.56	20.82	12.00	14.73
FGF-b	9.66	23.59	12.15	19.13	11.85	18.10
G-CSF	7.70	18.50	6.28	15.41	9.99	15.17
GM-CSF	11.98	20.23	14.56	17.13	13.33	18.26
IFN-γ	7.10	16.51	16.67	15.34	9.66	18.34
IL-10	9.04	18.97	12.85	18.86	14.20	21.50
IL-12(p70)	8.31	13.37	9.58	16.00	9.33	14.51
IL-13	7.46	16.27	13.28	14.22	8.04	16.28
IL-15	8.77	18.64	12.92	18.74	9.45	16.83
IL-17	6.91	17.37	9.74	14.58	11.31	18.73
IL-1β	8.66	18.75	9.91	12.95	8.75	9.69
IL-1ra	8.09	17.68	8.99	10.13	6.98	14.85
IL-2	5.90	17.46	18.53	13.71	10.92	15.67
IL-4	7.75	18.13	8.13	18.48	8.73	15.92
IL-5	11.40	19.99	13.02	11.79	7.25	8.62
IL-6	4.89	14.56	6.93	18.77	10.84	15.68
IL-7	5.69	12.47	7.84	16.24	9.00	13.93
IL-8	6.36	15.29	8.87	18.41	6.80	18.27
IL-9	10.08	16.69	10.98	16.38	12.11	18.91
IP-10	3.71	4.12	7.58	9.84	5.94	6.26
MCP-1	11.10	16.49	10.49	16.01	9.86	13.25
MIP-1α	11.75	19.67	11.90	19.48	10.27	20.02
MIP-1β	8.29	16.70	7.60	17.86	7.54	19.43
PDGF bb	9.15	18.70	10.16	24.36	10.75	13.88
RANTES	8.06	15.46	9.88	12.74	9.53	15.92
TNF-α	6.29	19.93	8.83	13.15	11.17	16.56
VEGF	5.29	21.55	8.06	17.99	9.64	21.44

Format abbreviations: MAG = magnetic format, VAC = vacuum filtration format, coefficients of variation expressed as a percentage of the mean.

Table 11

Mean Coefficients of Variation in Vacuum vs. Magnetic Formats

Vacuum CV			Magnetic CV		
Range	Mean	STE	Range	Mean	STE
0.03 -61.89	*16.92	0.62	0.06-32.35	*8.80	0.33

Range, mean and standard error (STE) of intra-assay coefficients of variation for magnetic and vacuum filtration formats. *Vacuum filtration format produced significantly higher coefficients of variation ($p < 0.05$).

Study II

Study II Cohort and Attrition

Nineteen individuals were screened for Study II; 14 females and 5 males. Nine individuals successfully completed the study. Patient demographics for Study II, including number enrolled, average age and gender distribution, are summarized in Table 12. Only one individual was found to be clinically ineligible during the subject suitability assessment. The reason for her exclusion from the study was an inadequate NIBUT (< 10 seconds). Inability to collect a sufficient volume of tears within the allocated time at a subsequent study visit accounted for most of the study attrition during the 30-day contact lens wearing period in Study II (Table 13). One subject removed the extended wear lens prior to the final visit, reporting grittiness and irritation from continuous wear.

Table 12

Study II Cohort Demographics

Gender	Completed		Discontinued		Total	
	N (%)	Mean Age ± SD	N (%)	Mean Age ± SD	N (%)	Mean Age ± SD
Female	7 (36.8)	23.3 ±11.4	7 (36.8)	26.7 ± 10.2	14 (73.7)	25.0 ± 10.6
Male	2 (10.5)	30.5 ± 3.5	3 (15.8)	33.0 ± 15.6	5 (26.3)	32.0 ± 11.2
Total	9 (47.4)	24.9 ± 10.5	10 (52.6)	28.6 ± 11.5	19 (100)	26.8 ±10.9

Table 13

Study II Attrition

Reasons for Discontinuation	Frequency (%)
Clinical Ineligibility	1 (5.3)
Missed Visit	3 (15.8)
Wear Schedule	1 (5.3)
Tear Collection	5 (26.3)
Total	10 (52.6)

Study II Assay Results

Tear cytokine levels for the four study visits in Study II are shown in a series of figures, divided for clarity according to mean cytokine concentration ranges that were seen in Study I: low (Figure 15a), medium (Figure 15b) and high (Figure 15c). It should be noted that unlike Study I, all tear cytokines levels in Figures 5a-c are within the same ranges of orders of magnitude in Study II. Cytokines like eotaxin and IL-9 that were only detected in a small number of patients in Study I were detected in all but one patient in

Study II. Differences between daily and extended wear did not reach statistical significance for any cytokines at any given visit.

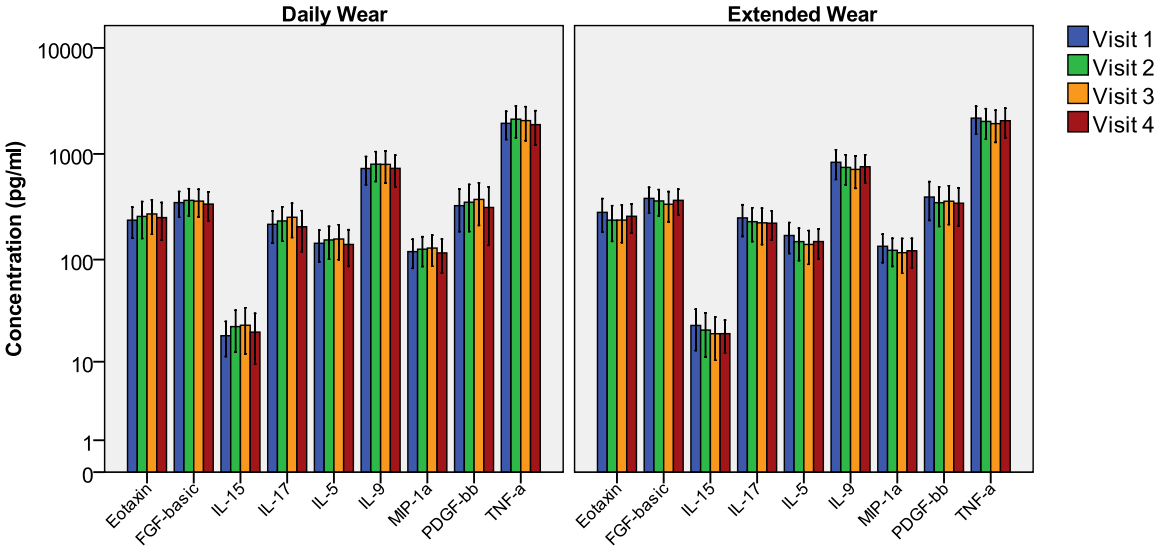

Figure 15a. Tear Cytokine Concentrations with Daily and Extended Wear in Study II. Mean concentrations of tear eotaxin, FGF-b, IL-15, IL-17, IL-5, IL-9, MIP-1á, PDGFbb and TNF-á in samples collected at Visits 1, 2, 3 and 4 of daily and extended wear in Study II.

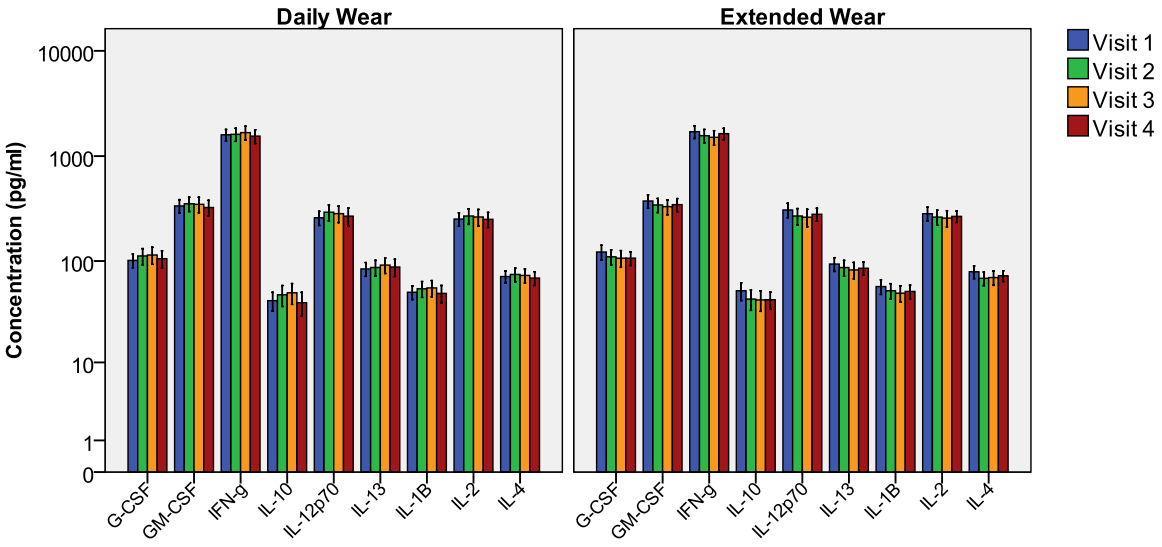

Figure 15b. Tear Cytokine Concentrations with Daily and Extended Wear in Study II. Mean concentrations of tear G-CSF, GM-CSF, IFN-ã, IL-10, IL-12p70, IL-13, IL-1â, IL-2 and IL-4 in samples collected at Visits 1, 2, 3 and 4 of daily and extended wear in Study II.

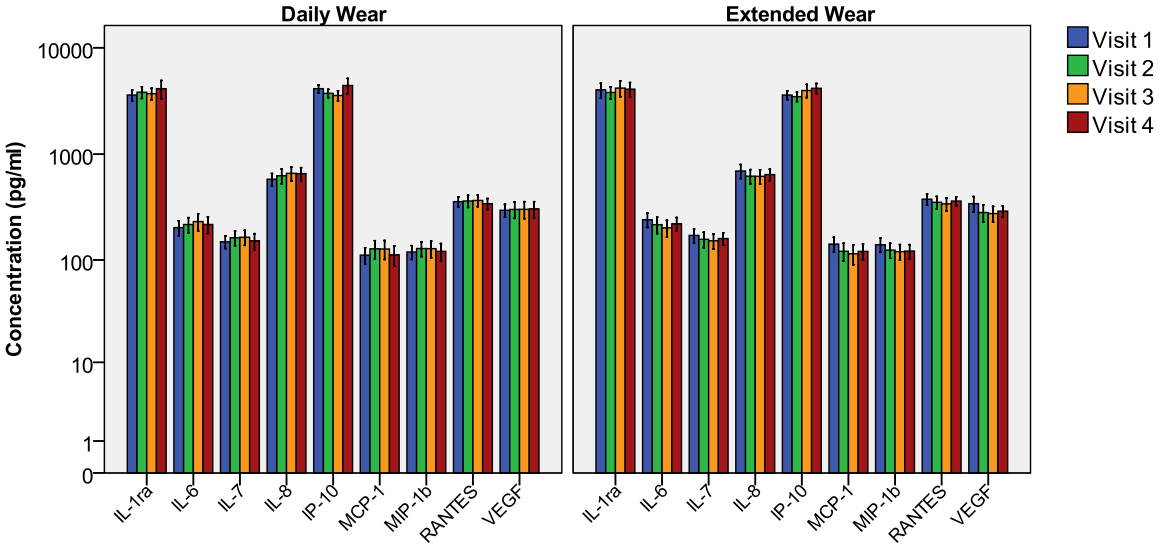

Figure 15c. Tear Cytokine Concentrations with Daily and Extended Wear in Study II. Mean concentrations of tear IL-1ra, IL-6, IL-7, IL-8, IP-10, MPC-1, MIP-1â, RANTES and VEGF in samples collected at Visits 1, 2, 3 and 4 of daily and extended wear in Study II.

Gender and Age Effects on Tear Ctyokine Levels in Study II

Results from Study II were further analyzed for age and gender effects on tear cytokine levels. There were no significant differences in tear cytokine levels between males and females at Visit 1 (pre-lens wear), indicating baseline equality. When cytokine levels in samples collected at Visits 2, 3 and 4 were combined for these analysis, the significant gender differences that were seen with daily wear in Study I were not found in Study II (Figure 16a). There were also no significant gender differences in tear cytokine levels with continuous wear (Figure 16b).

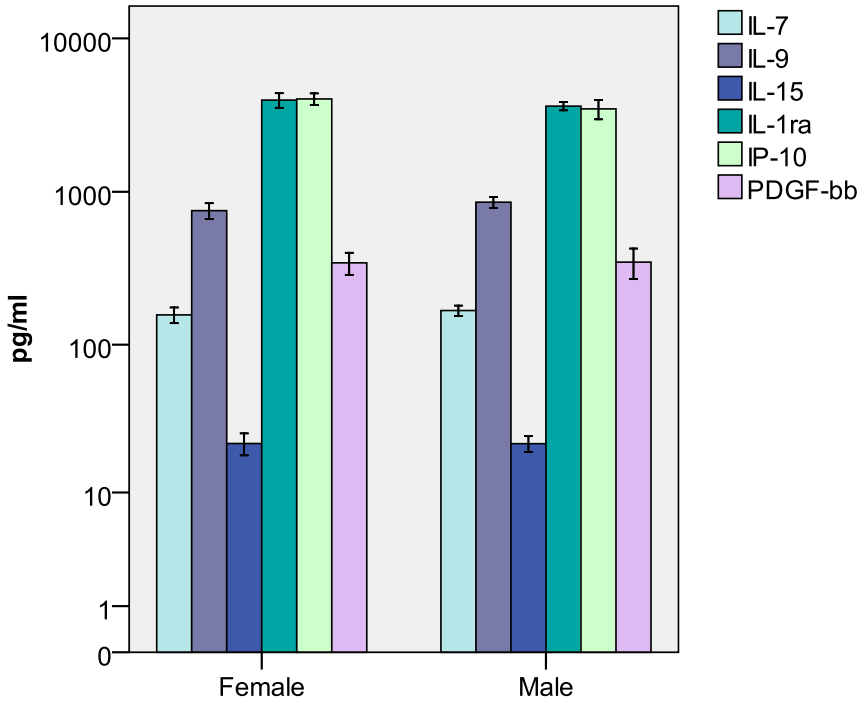

Figure 16a. Gender Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study II. No gender differences reached significance.

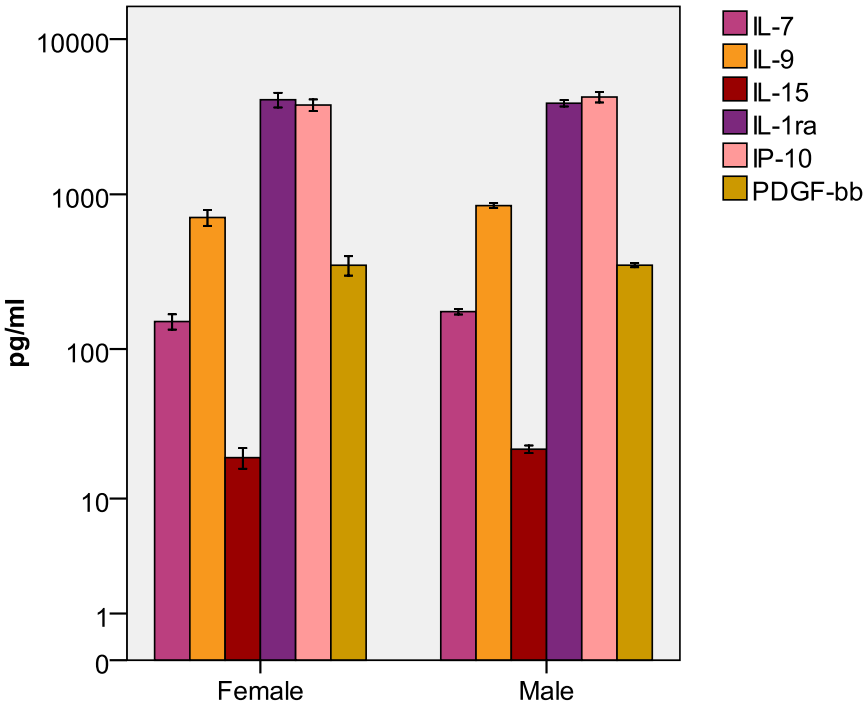

Figure 16b. Gender Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study II. No gender differences reached significance.

When further analyzed by visit, no differences were found between male and female cytokine levels. Moreover, no changes in cytokine levels over time within groups reached significance.

Results from Study II showed that there were significant differences in cytokine levels in tears between age groups. Tear cytokine levels of individuals 30 years of age and younger are compared to those over 30 in Study II. The following figures illustrate the comparison of tear cytokine levels with daily and continuous wear between the two groups. They are divided for clarity according to mean cytokine concentration ranges that were seen in Study I: low (Figures 17a and 17b), medium (Figures 17c and 17d) and

high (Figures 17e and 17f). Each cytokine was significantly higher in the younger group (30 and under) compared to those over 30 with daily wear ($p < 0.05$) (Figures 17a, 17c, and 17e) and with continuous wear, with the exception of IP-10 ($p < 0.05$) (Figures 17b, 17d, and 17f).

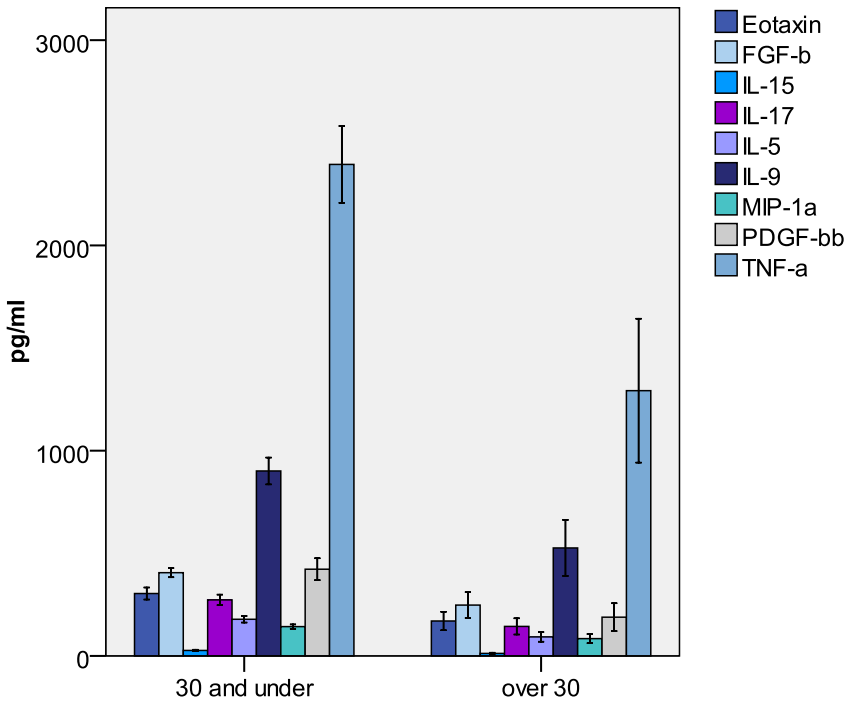

Figure 17a. Age Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study II. Tear eotaxin, FGF-b, IL-15, IL-17, IL-5, IL-9, MIP-1á, PDGF-bb and TNF-á levels in tear samples from individuals 30 years old or younger compared to those over 30 with daily wear in Study II. There were significantly higher levels of eotaxin ($p < 0.05$), FGF-b ($p < 0.01$), IL-15 ($p < 0.05$), IL-17 ($p < 0.05$), IL-5 ($p < 0.01$), IL-9 ($p < 0.01$), MIP-1á ($p < 0.05$), PDGF-bb ($p < 0.05$) and TNF-á ($p < 0.01$) in the younger group.

PDGF-bb, IL-1â, IL-2, IL-7, IL-9, eotaxin, and G-CSF were higher in tears from the younger group at the first Visit ($p < 0.05$) with continuous wear.

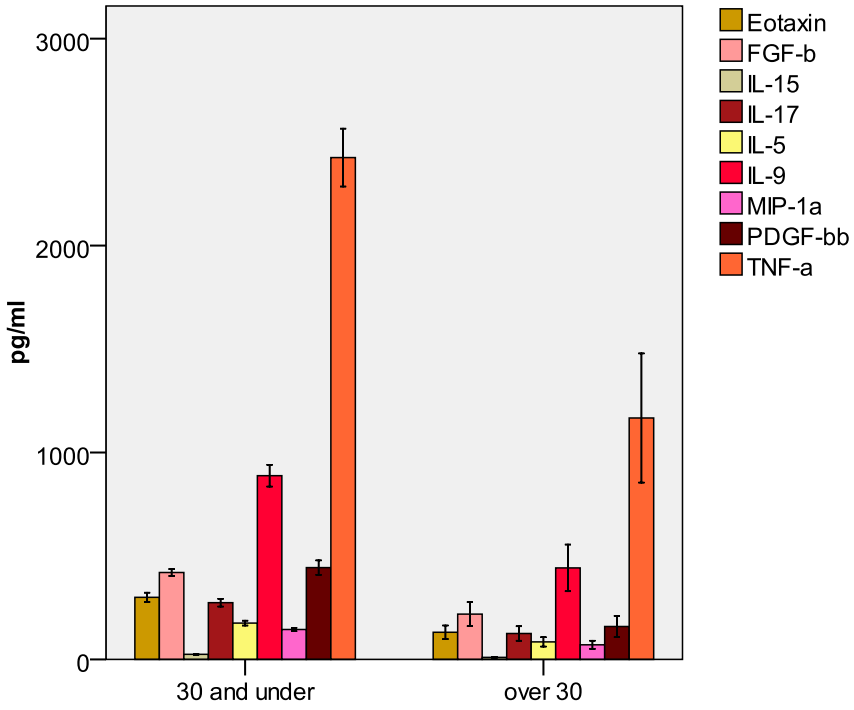

Figure 17b. Age Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study II. Tear eotaxin, FGF-b, IL-15, IL-17, IL-5, IL-9, MIP-1á, PDGF-bb and TNF-á levels in tear samples from individuals 30 years old or younger compared to those over 30 with continuous wear in Study II. There were significantly higher levels of eotaxin ($p < 0.01$), FGF-b ($p < 0.01$), IL-15 ($p < 0.01$), IL-17 ($p < 0.01$), IL-5 ($p < 0.01$), IL-9 ($p < 0.01$), MIP-1á ($p < 0.01$), PDGF-bb ($p < 0.01$) and TNF-á ($p < 0.01$) in the younger group.

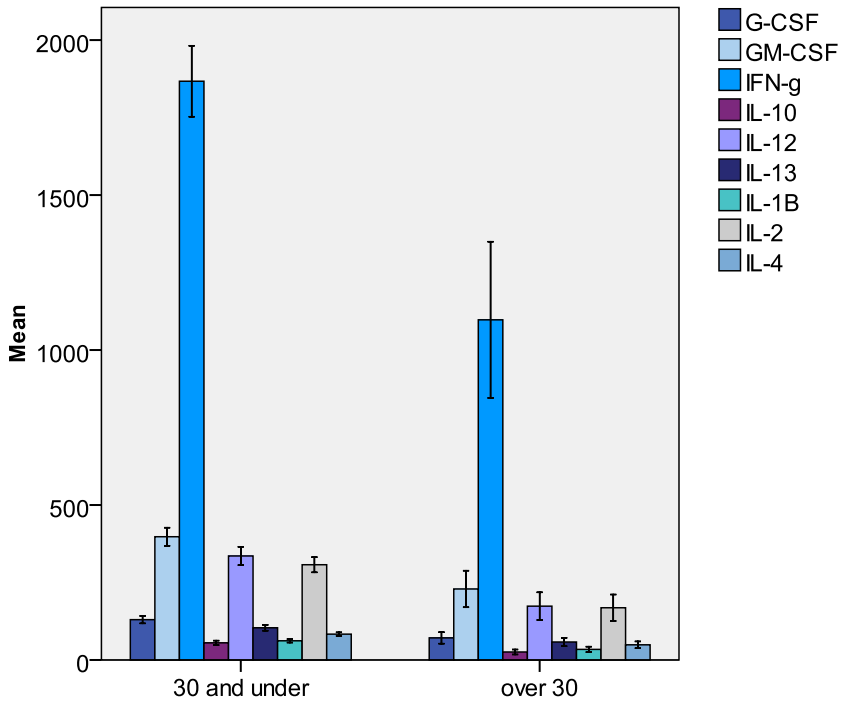

Figure 17c. Age Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study II. Tear G-CSF, GM-CSF, IFN-ã, IL-10, IL-12, IL-13, IL-1â, IL-2, and IL-4 levels in tear samples from individuals 30 years old or younger compared to those over 30 with daily wear in Study II. There were significantly higher levels of G-CSF ($p < 0.05$), GM-CSF ($p < 0.01$), IFN-ã ($p < 0.01$), IL-10 ($p < 0.05$), IL-12 ($p < 0.01$), IL-13 ($p < 0.01$), IL-1â ($p < 0.05$), IL-2 ($p < 0.01$), and IL-4 ($p < 0.01$) in the younger group.

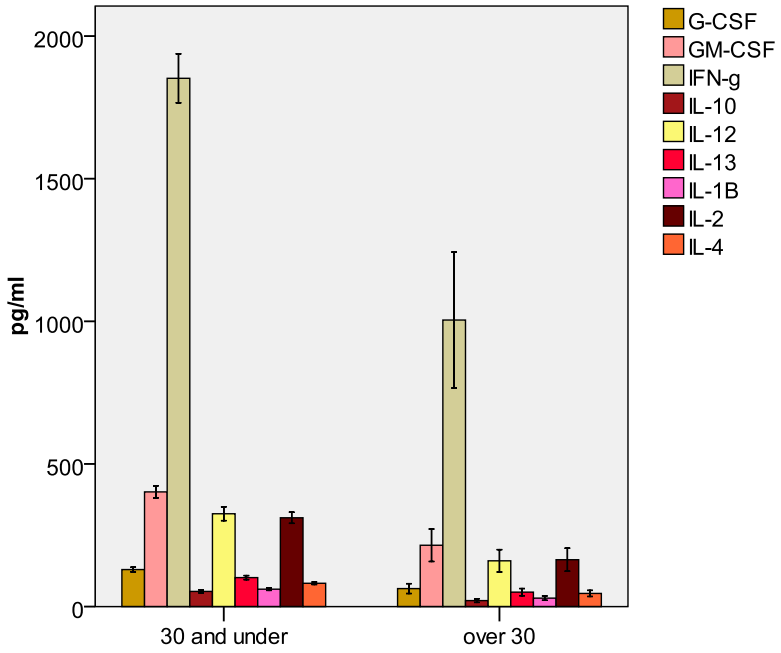

Figure 17d. Age Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study II. Tear G-CSF, GM-CSF, IFN-ã, IL-10, IL-12, IL-13, IL-1â, IL-2, and IL-4 levels in tear samples from individuals 30 years old or younger compared to those over 30 with continuous wear in Study II. There were significantly higher levels of G-CSF ($p < 0.01$), GM-CSF ($p < 0.01$), IFN-ã ($p < 0.01$), IL-10 ($p < 0.001$), IL-12 ($p < 0.01$), IL-13 ($p < 0.01$), IL-1â ($p < 0.01$), IL-2 ($p < 0.01$), and IL-4 ($p < 0.01$) in the younger group.

When analyzed by visit, IL-1ra was higher in tears from the younger group collected at Visit 1 ($p < 0.05$) with daily wear in Study II.

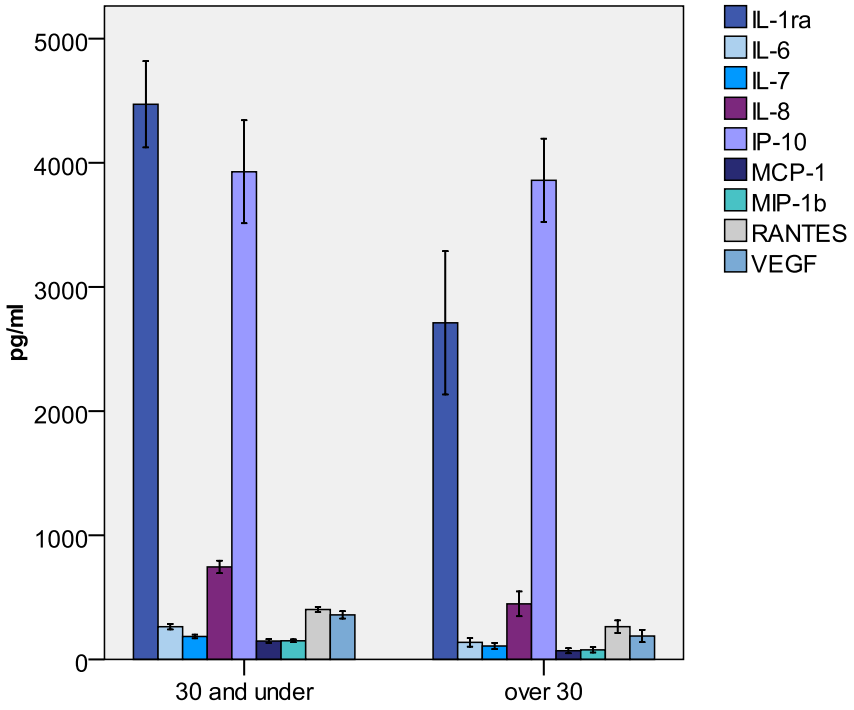

Figure 17e. Age Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study II. Tear IL-1ra, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1â, RANTES and VEGF levels in tear samples from individuals 30 years old or younger compared to those over 30 with daily wear in Study II. There were significantly higher levels of IL-1ra ($p < 0.05$, IL-6 ($p < 0.01$), IL-7 ($p < 0.01$), IL-8 ($p < 0.01$), MCP-1 ($p < 0.01$), MIP-1â ($p < 0.01$), RANTES ($p < 0.01$) and VEGF ($p < 0.01$) in the younger group. There was no significant difference between groups in levels of IP-10.

No changes in cytokine levels over time within groups reached significance. IL-12p70 ($p < 0.05$) and PDGF-bb ($p < 0.05$) were higher in the younger group at Visit 3.

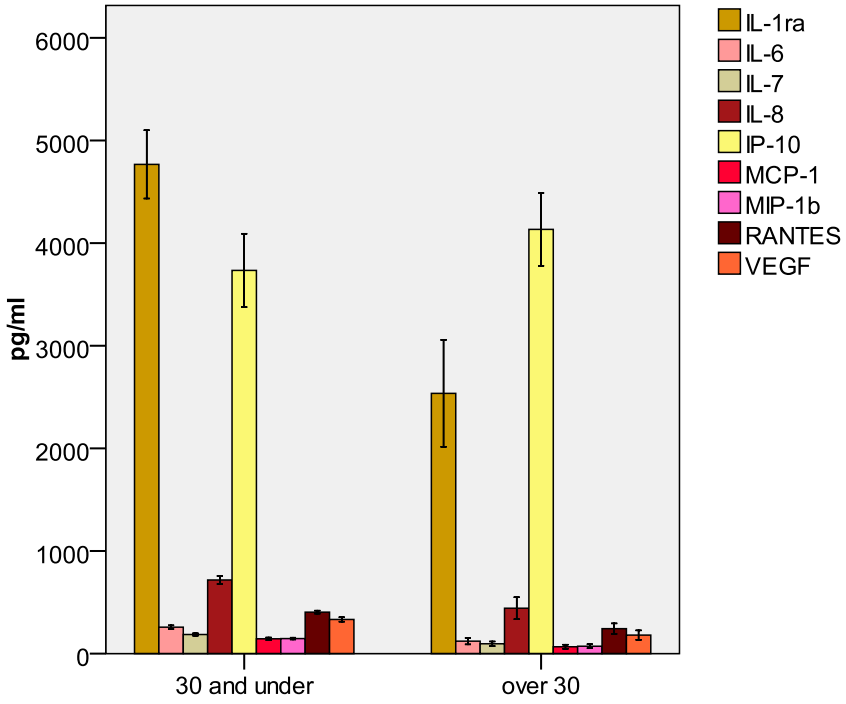

Figure 17f. Age Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study II. Tear IL-1ra, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1â, RANTES and VEGF levels in tear samples from individuals 30 years old or younger compared to those over 30 with continuous wear in Study II. There were significantly higher levels of IL-1ra ($p < 0.01$, IL-6 ($p < 0.01$), IL-7 ($p < 0.01$), IL-8 ($p < 0.01$), MCP-1 ($p < 0.02$), MIP-1â ($p < 0.01$), RANTES ($p < 0.01$) and VEGF ($p < 0.02$) in the younger group. There was no significant difference between groups in levels of IP-10.

IL-6, IL-17, G-CSF, GM-CSF, IFN-ã, MIP-1á, MIP-1â, TNF-á and VEGF were all higher in the younger group's tear samples at Visit 4 with continuous wear ($p < 0.05$). There were no significant changes between visits that reached significance for either group.

Study II Clinical Test Results

Clinical tests were conducted on the day of lens insertion and immediately after lens removal (day 30) on both eyes in Study II. Lissamine green staining score increased in both eyes and showed a greater increase after 30 days of daily wear than extended wear, although this change did not reach statistical significance (Figure 18). Similar results were found for fluorescein staining (Figure 19).

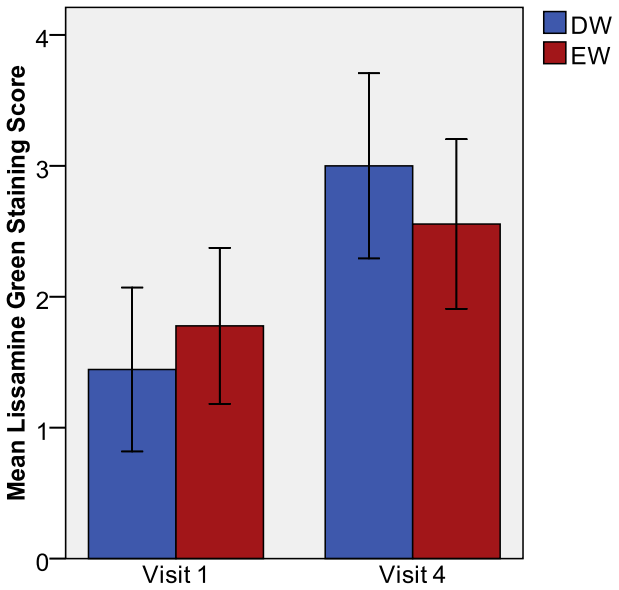

Figure 18. Study II Lissamine Green Staining Score with Daily and Extended Wear. Comparison of mean Lissamine green staining scores at Visit 1 (day 0) and 4 (day 30) of daily and extended contact lens wear.

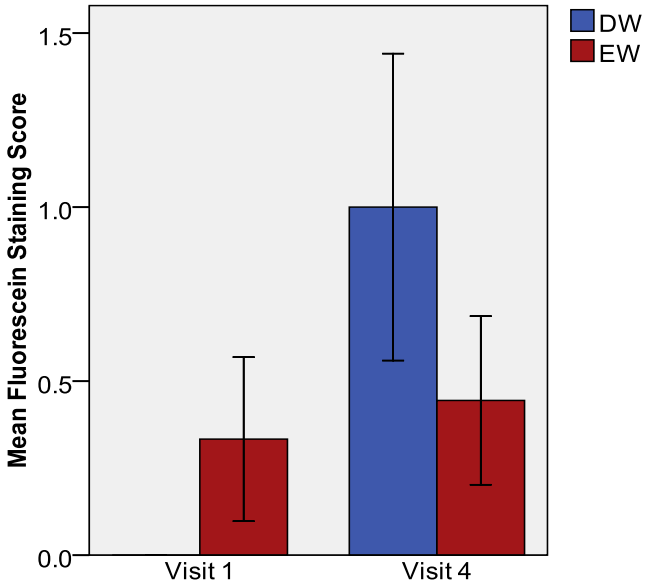

Figure 19. Study II Fluorescein Staining Score with Daily and Extended Wear. Comparison of mean fluorescein staining scores at Visits 1 (day 0) and 4 (day 30) of daily and extended contact lens wear.

There was no statistically significant difference between tear meniscus height after 30 days of daily wear versus 30 days of extended wear (Figure 20). Non-invasive break-up time did not change after 30 days of daily wear or extended wear (Figure 21).

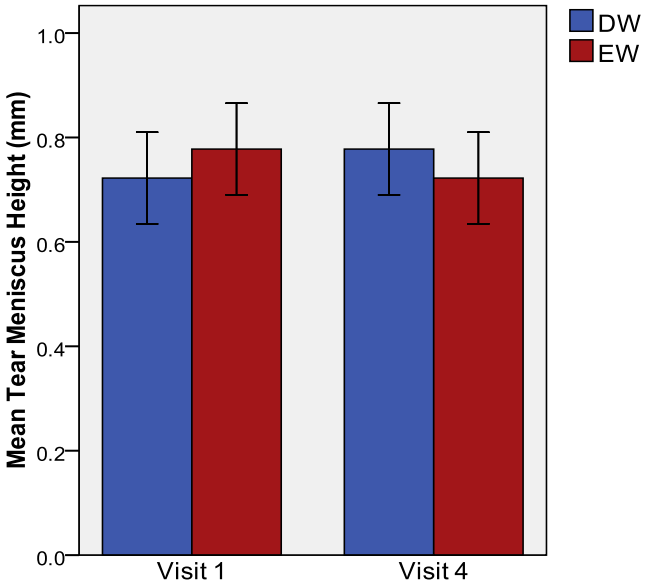

Figure 20. Study II Tear Meniscus Height with Daily and Extended Wear. Comparison of mean tear meniscus height at Visits 1 (day 0) and 4 (day 30) of daily and extended contact lens wear.

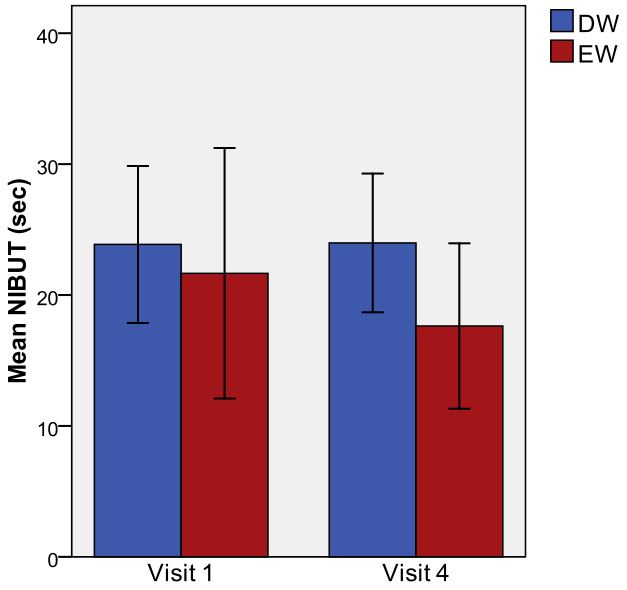

Figure 21. Study II Non-Invasive Break Up Time in Daily and Extended Wear. Comparison of mean non-invasive break up times at Visit 1 (day 0) and Visit 4 (day 30) of daily and extended contact lens wear.

Study III

Study III Cohort and Attrition

Twenty-six individuals were screened for Study III. Sixteen individuals successfully completed the study. Patient demographics for Study III, including number enrolled, average age and gender distribution are summarized in Table 14. Only one subject was clinically ineligible due to contact lens intolerance. Five individuals were either excluded from study participation or removed from Study III due to inability to collect tears (Table 15). Two subjects removed the extended wear lens prior to the final visit. These patients complained of lens discomfort (irritation and foreign body sensation).

Table 14

Study III Cohort Demographics

Gender	Completed		Discontinued		Total	
	N (%)	Mean Age	N (%)	Mean Age	N (%)	Mean Age
Female	13 (46.9)	26.6 ± 5.4	9 (36.6)	30.0 ± 11.9	22 (83.5)	28.0 ± 8.6
Male	3 (11.4)	28.0 ± 12.1	1 (5.1)	38.0	4 (16.5)	30.5 ± 11.1
Total	16 (58.3)	26.9 ± 6.6	10 (41.7)	30.8 ± 11.5	26 (100)	28.4 ± 8.8

Table 15

Study III Attrition

Reasons for Discontinuation	Frequency (%)
Clinical Ineligibility	1 (3.8)
Missed Visit	2 (7.7)
Wear Schedule	2 (7.7)
Tear Collection	5 (19.2)
Total	10 (38.4)

Study III Assay Results

Tear cytokine levels for the four study visits in Study III are shown in the following figures, divided for clarity according to mean cytokine concentration ranges: low (Figure 22a) and high (Figure 22b). Each figure is further subdivided into daily and extended wear data. GM-CSF and IL-12p70 were detected and at low levels in only one patient's samples (Figure 22a). There were no significant differences between daily and extended wear in any tear cytokine levels at any given visit.

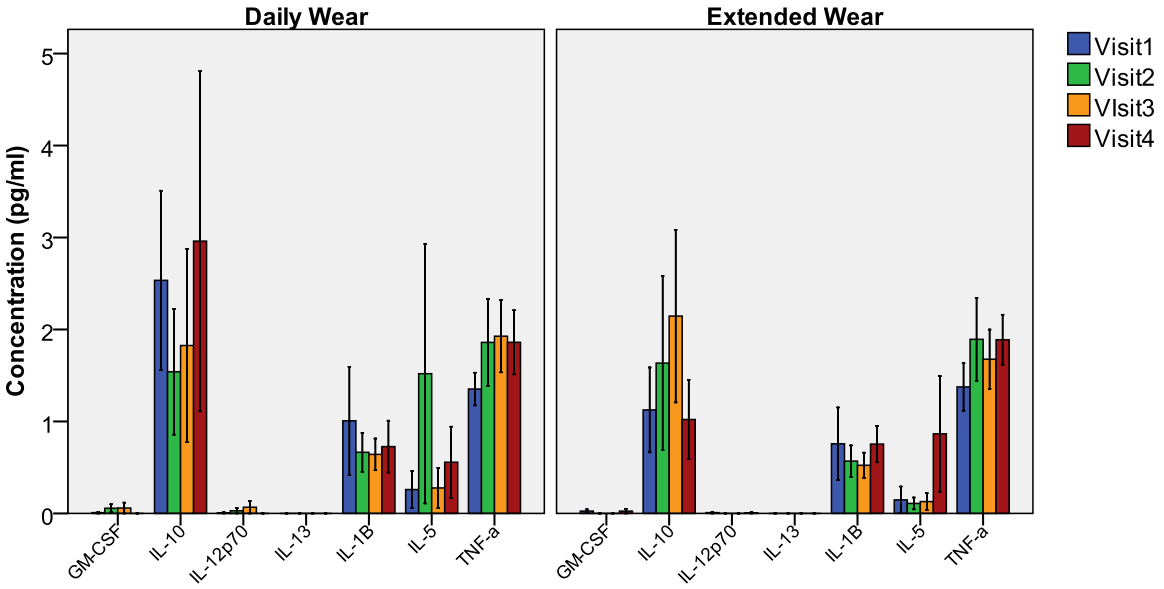

Figure 22a. Tear Cytokine Concentrations with Daily and Extended Wear in Study III. Mean concentrations of tear GM-CSF, IL-1â, IL-5, IL-10, IL-12p70, IL-13 and TNF-á in samples collected at Visits 1, 2, 3 and 4 of daily wear in Study III.

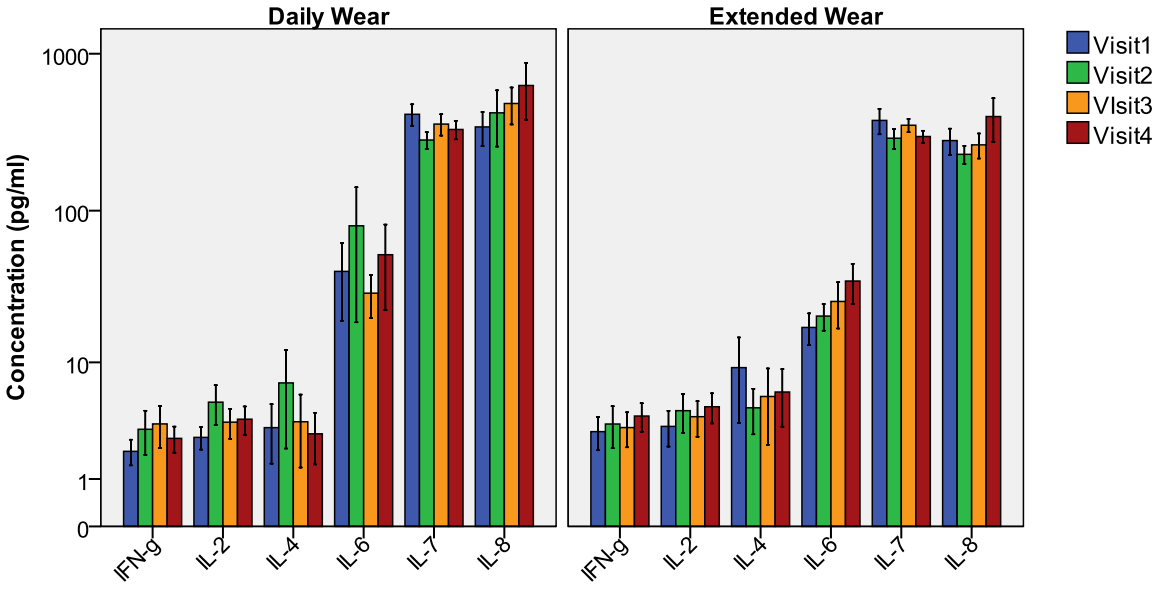

Figure 22b. Tear Cytokine Concentrations with Daily and Extended Wear in Study III. Mean concentrations of tear IFN-ã, IL-2, IL-4, IL-6, IL-7 and IL-8 in samples collected at Visits 1, 2, 3 and 4 of daily wear in Study III.

In contrast to findings in Study I, tear IL-2 (Figure 23) and IL-4 (Figure 24) do not display similar patterns to one another in daily and extended wear. No statistically significant trends were found for either one across the four study visits or between eyes at any given visit.

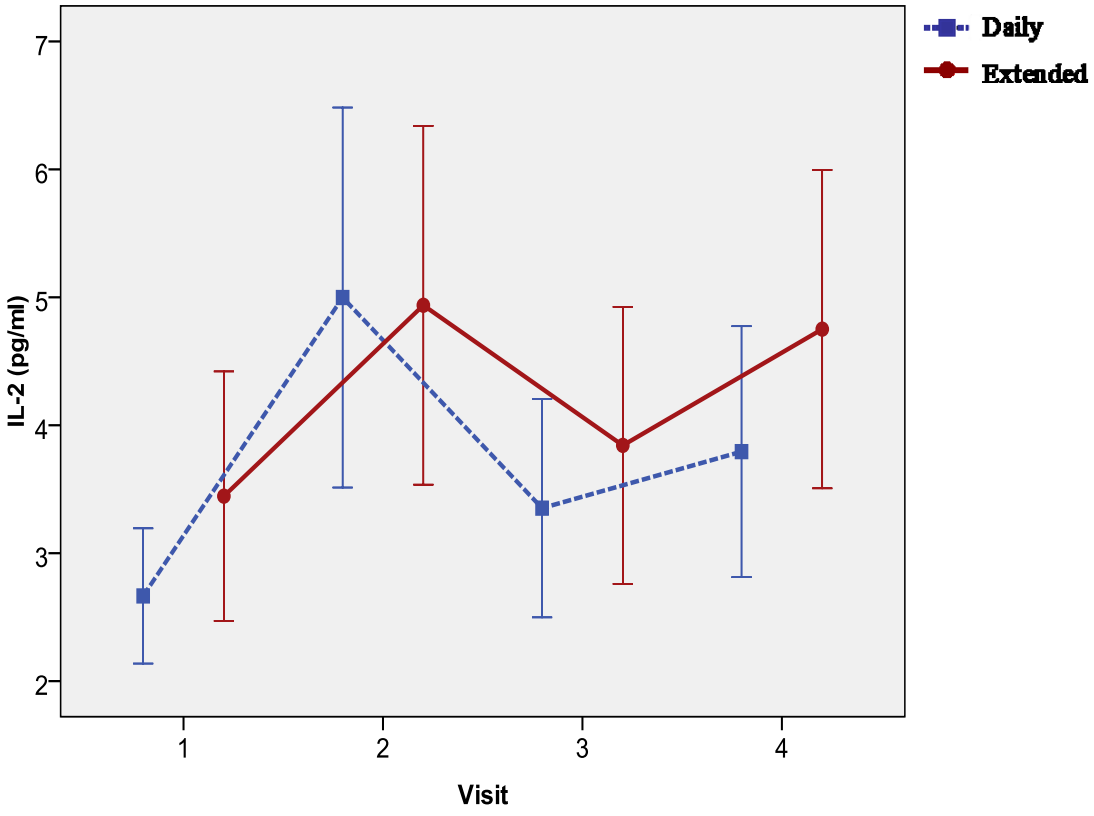

Figure 23. Tear IL-2 Levels with Daily and Extended Contact Lens Wear in Study III. Means and standard error of concentrations of tear IL-2 collected at Visits 1, 2, 3 and 4. No significant differences in IL-2 levels were found between visits or between eyes.

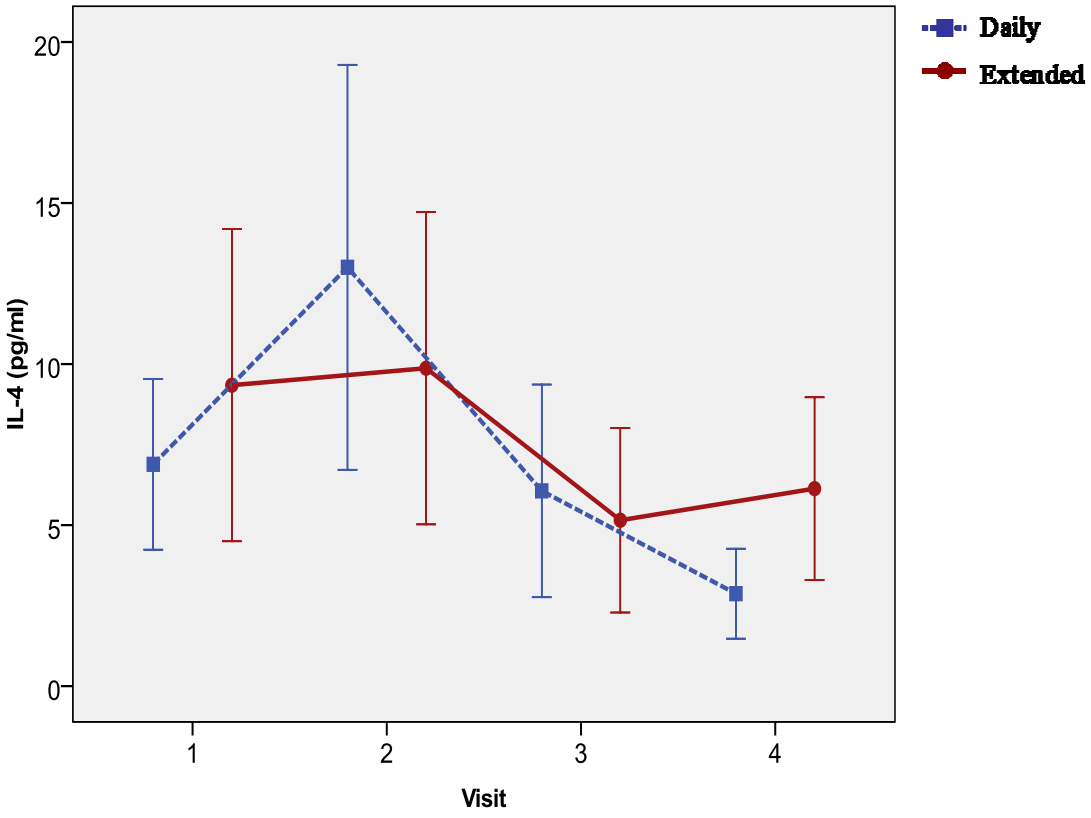

Figure 24. Tear IL-4 Levels with Daily and Extended Contact Lens Wear in Study III. Means and standard error of concentrations of tear IL-4 collected at visits 1, 2, 3 and 4. No significant differences in IL-4 levels were found between visits or between eyes.

There was a distinct elevation in tear IL-6 at Visit 2 of daily wear, although the change is not statistically significant (Figure 25). IL-6 with extended wear remained relatively level during the 30-day period.

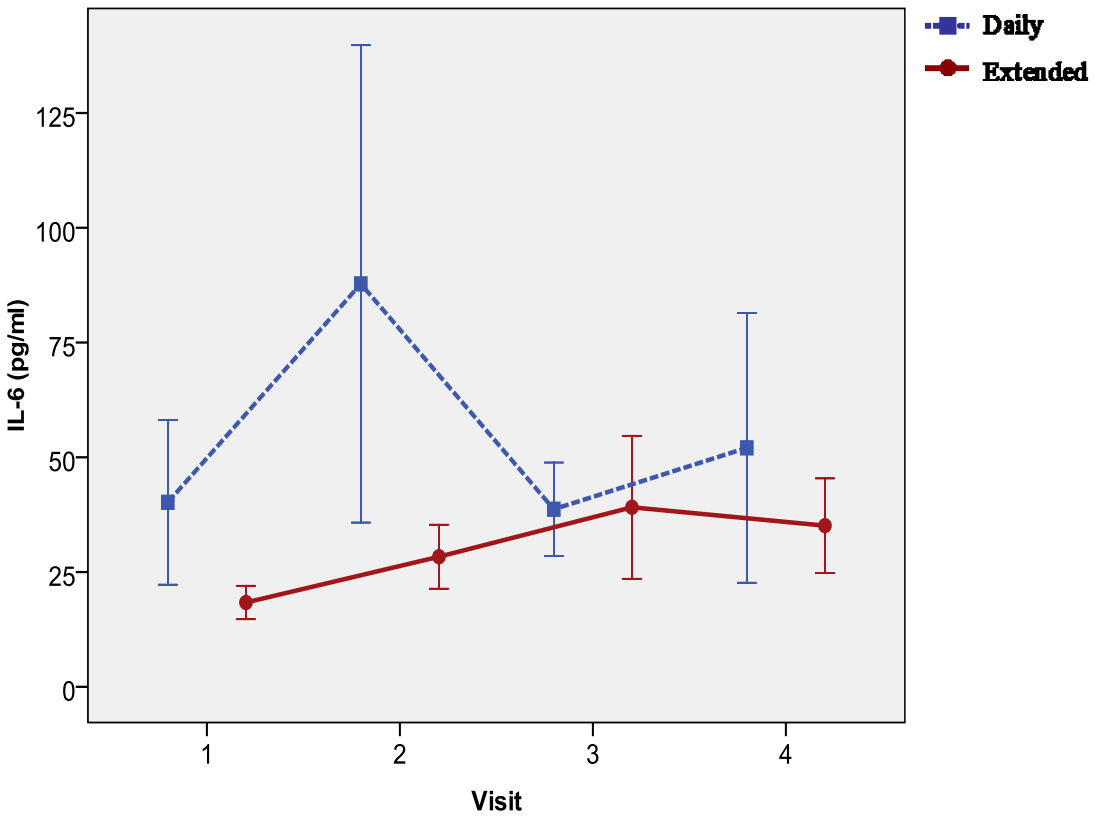

Figure 25. Tear IL-6 Levels with Daily and Extended Contact Lens Wear in Study III. Means and standard error of concentrations of tear IL-6 collected at visits 1, 2, 3 and 4. No significant differences in IL-6 levels were found between visits or between eyes.

Tear IL-8 appeared to increase steadily with 30 days of daily wear and remained at higher levels compared to extended wear across Visits 2 to Visit 4 (Figure 26). However, no changes or differences reached statistical significance. No statistically significant trends were found for TNF-á across the four study visits or between eyes at any given visit (Figure 27).

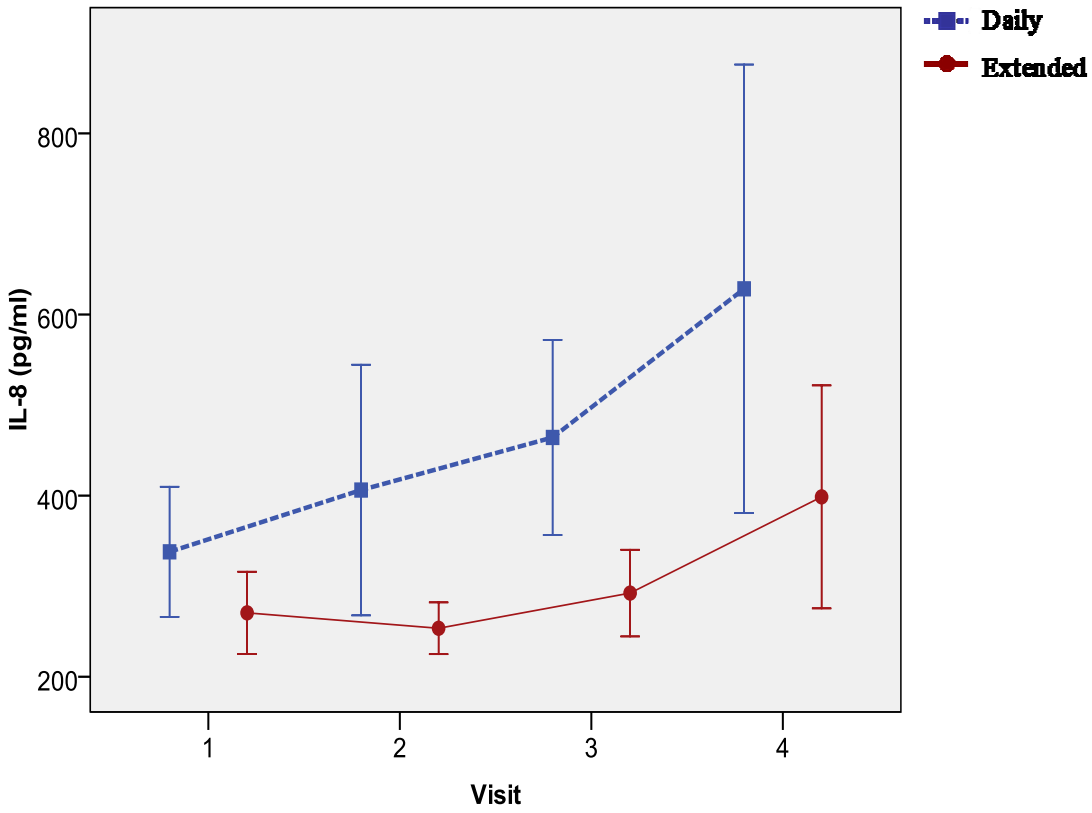

Figure 26. Tear IL-8 Levels with Daily and Extended Contact Lens Wear in Study III. Means and standard error of concentrations of tear IL-8 collected at visits 1, 2, 3 and 4. No significant differences in IL-8 levels were found between visits or between eyes.

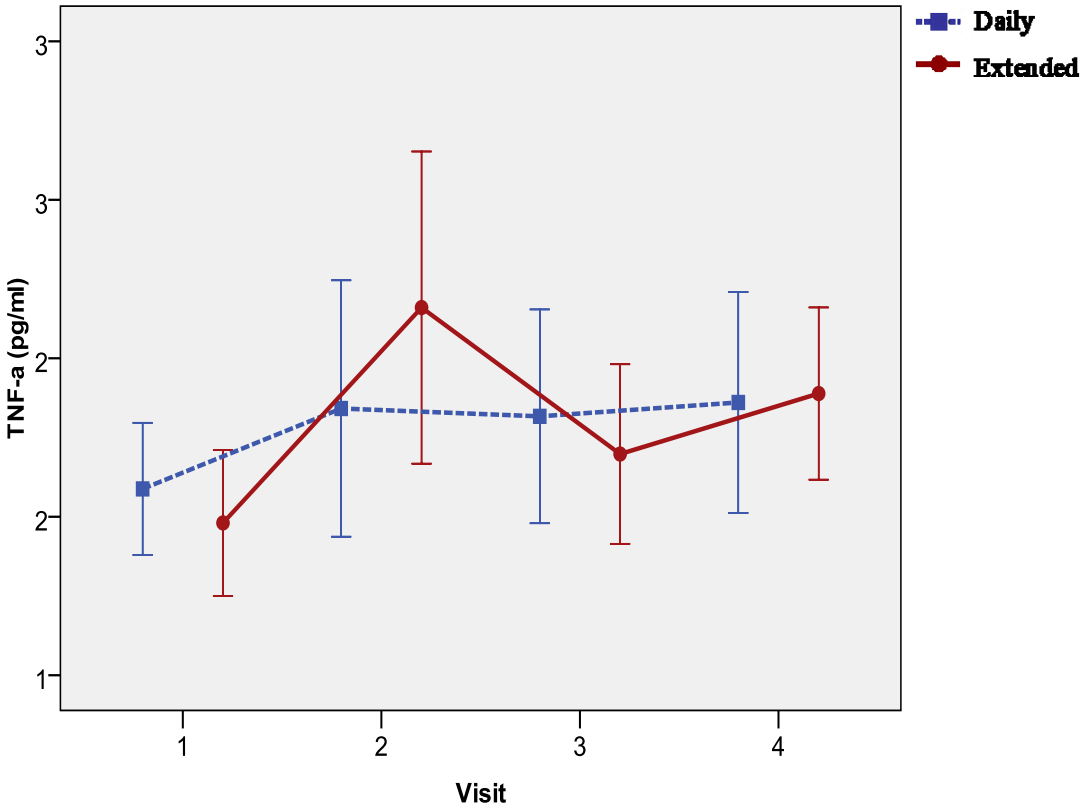

Figure 27. Tear TNF-α Levels with Daily and Extended Contact Lens Wear in Study III. Means and standard error of concentrations of tear TNF-α collected at visits 1, 2, 3 and 4. No significant differences in TNF-α levels were found between visits or between eyes.

Adverse events were experienced by two of the participants in Study III. The first case was reported 27 days into the 30-day wearing period. The patient reported that her left eye (the eye assigned to continuous wear) was irritated and that she eventually re-moved the lens as the discomfort increased. Shortly after lens removal, the patient noted what she described as "sharp pains" induced by light (photophobia). She returned to the clinic the following day, and it was noticed that her left eye was red and slightly swollen. She was also experiencing increased lacrimation. The patient was evaluated by slit lamp

examination and staining with sodium fluorescein. There was no staining present in either eye. However, infiltrates were observed in the left cornea. The patient was diagnosed with CLARE, prescribed Lotemax® (loteprednol etabonate ophthalmic suspension 0.5%) (Bausch & Lomb, Inc., Rochester, NY) and instructed to discontinue contact lens wear. She returned to the clinic four days later for a follow-up visit. The CLARE had completely resolved and the patient reported feeling no more discomfort and was no longer photophobic. Tear samples were collected at the follow-up visit and clinical tests were repeated for study purposes. The patient's individual tear IL-6, -8 and -10 profiles are shown in Figures 28-30, respectively.

The CLARE patient in Study III had a marked increase in tear IL-6 at Visit 3 (Day 14) of continuous wear with a subsequent decrease after treatment with Lotemax®. There were only minor fluctuations of IL-6 in daily-wear tear samples (Figure 28).

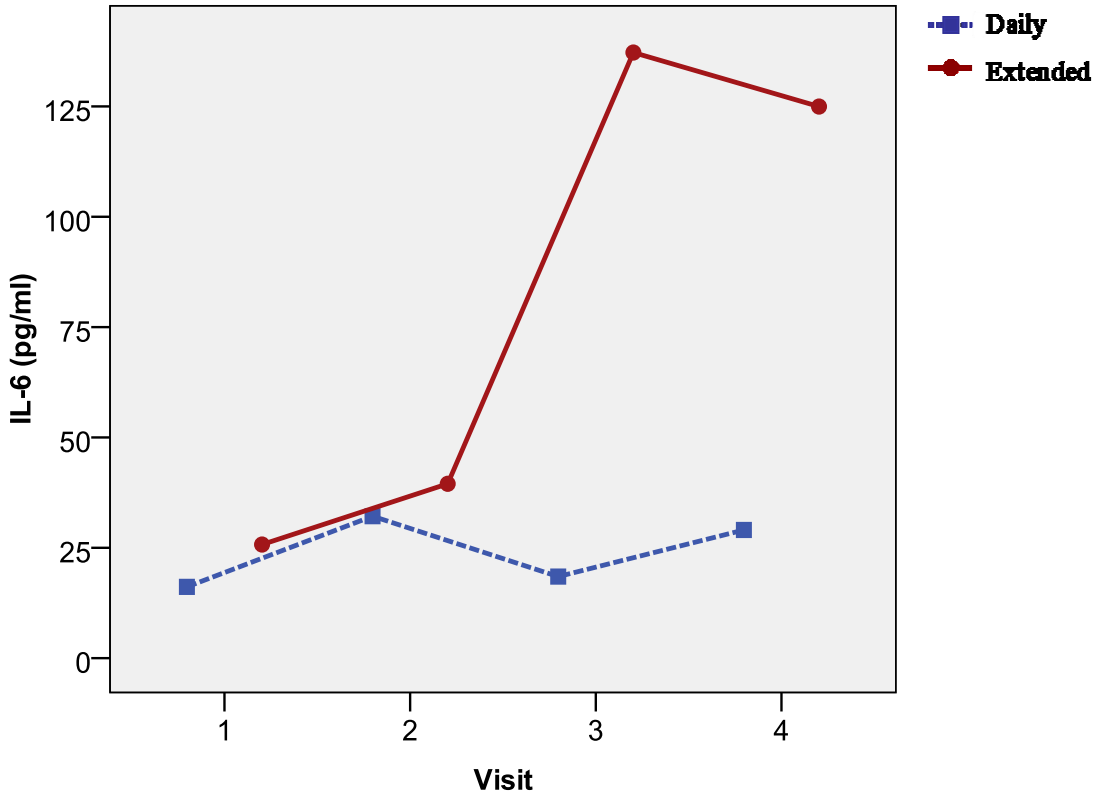

Figure 28. Tear IL-6 Profile of a Contact Lens Associated Red Eye Patient with 30 Days of Daily and Continuous Contact Lens Wear.

A sharp elevation was also seen in IL-8 (Figure 29) and IL-10 (Figure 30) with continuous wear. These occurred at Visit 4, after treatment with Lotemax®. Fluctuations were not as distinct with tear IL-8 or IL-10 in daily wear.

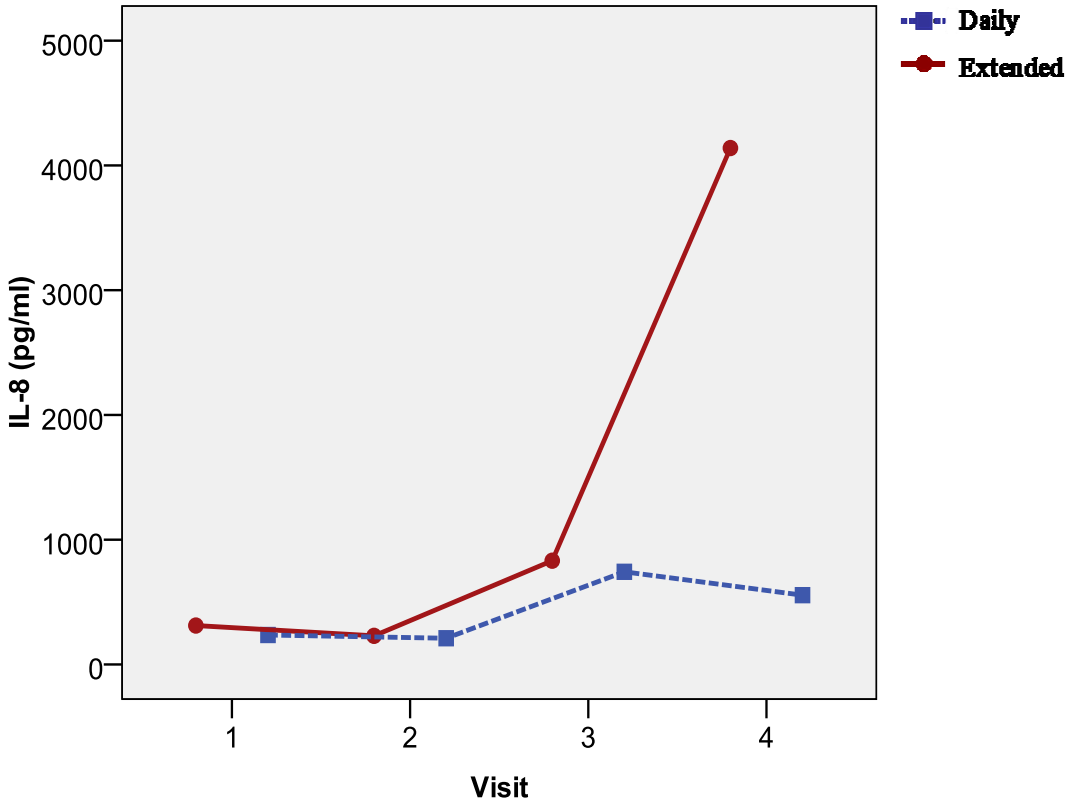

Figure 29. Tear IL-8 Profile of a Contact Lens Associated Red Eye Patient with 30 Days of Daily and Continuous Contact Lens Wear.

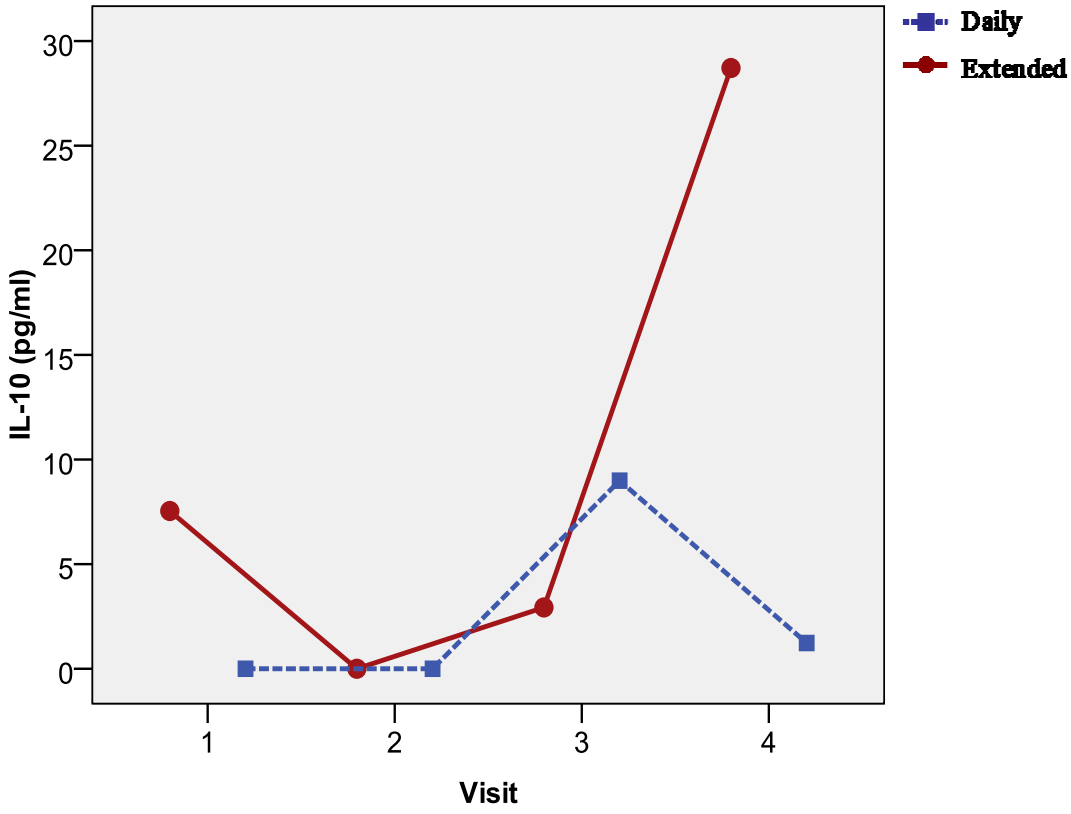

Figure 30. Tear IL-10 Profile of a Contact Lens Associated Red Eye Patient with 30 Days of Daily and Continuous Contact Lens Wear.

A second patient experienced an adverse event 23 days into the 30-day wearing period. This patient reported a foreign body sensation in her left eye (again, the eye assigned to extended wear). The patient first moved the lens around in the eye in an attempt to remove the foreign body from underneath the lens. Still feeling the sensation, she removed the lens and rinsed it OPTI-FREE® RepleniSH®. The lens was re-inserted but the eye began to feel irritated several hours later. The patient reported to the clinic the following day. The conjunctiva of the left eye was slightly red compared to the right eye. Slit lamp evaluation and corneal staining revealed infiltrates in the superior tempor-

al portion of the left cornea. The patient was prescribed the antibiotic Zymar® (gatifloxacin ophthalmic solution 0.3%) (Allergan, Inc., Irvine, CA) and instructed to discontinue contact lens wear. Tear samples from both eyes were collected (day 24) and clinical tests were repeated. When the patient returned to the clinic four days later for a follow-up visit, the issue had completely resolved. There were no remarkable trends in any cytokines in tear samples collected from this patient.

Gender and Age Effects on Tear Cytokine Levels in Study III

Results from Study III were further analyzed for age and gender effects on tear cytokine levels. There significant differences in tear cytokine levels between males and females at Visit 1 (pre-lens wear); males had higher IL-5 ($p < 0.05$), IL-6 ($p < 0.05$) and IL-8 ($p < 0.05$) at baseline with daily wear. When cytokine levels in samples collected at Visits 2, 3 and 4 were combined for analysis, males were found to have higher levels of tear IL-5 and IL-8; IFN-ã was higher in tear samples from females ($p < 0.05$) (Figure 31a). With continuous wear, males had significantly higher levels of IL-8 and again, females had higher IFN-ã ($p < 0.05$) but they did not differ in their levels of tear IL-5 (Figure 31b).

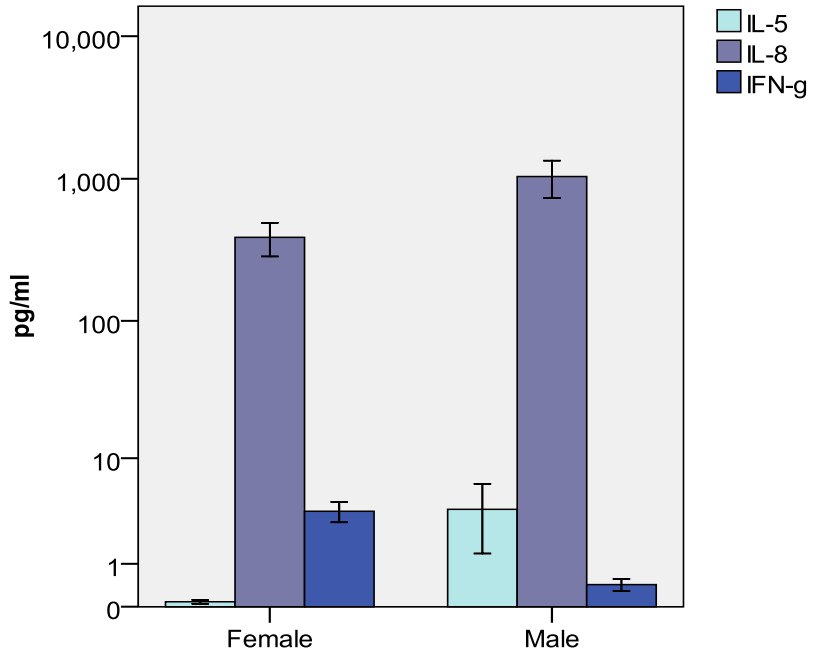

Figure 31a. Gender Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study III. Tear IL-5, IL-8 and IFN-ã levels in male and female daily wear samples in Study III. There were significantly higher levels of IL-5 ($p < 0.01$), IL-8 ($p < 0.05$), male tear samples. IFN-ã was significantly higher in tear samples from females ($p < 0.05$).

Comparisons made between groups at each visit revealed that males had higher levels of IL-5 and IL-8 than females at all four visits with daily wear in Study III. GM-CSF was also higher in tear samples from males at Visits 3 and 4 of continuous wear. Post-hoc analysis of changes in cytokine levels between visits for each group revealed that IFN-ã increased from Visit 1 to Visit 4 ($p < 0.05$) and from Visit 2 to Visit 4 ($p < 0.05$) in tear samples from females with both daily and continuous wear.

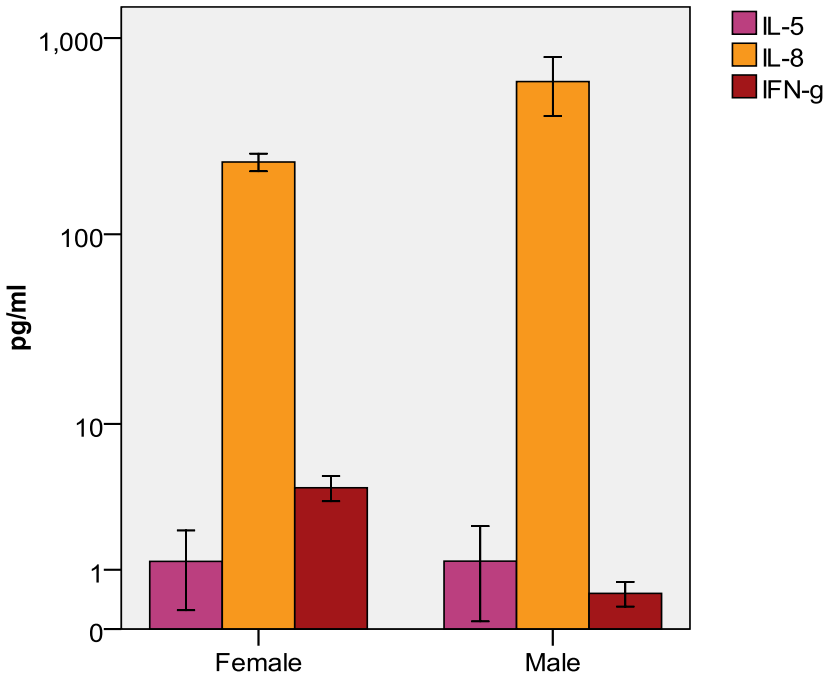

Figure 31b. Gender Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study III. Tear IL-5, IL-8 and IFN-ã levels in male and female daily wear samples in Study III. There was no significant difference in tear IL-5 between males and females. IL-8 was significantly higher in tear samples from males ($p < 0.01$); IFN-ã was significantly higher in samples from females ($p < 0.05$).

Tear cytokine levels of individuals 26 years of age and younger were compared to those over 26. The following figures illustrate the comparison of tear cytokine levels with daily and continuous wear between the two groups in Study III. Age did not have a significant effect on tear cytokine levels in Study III with either treatment. There were no significant differences in tear cytokine levels between age groups at Visit 1 (pre-lens wear), indicating baseline equality. The cytokines that were shown to have an age effect with continuous wear in Study I (IL-5, IL-7, IL-12p70, GM-CSF, IFN-ã and TNF-á) are shown for daily wear (Figure 32a) and continuous wear (Figure 32b) in Study III.

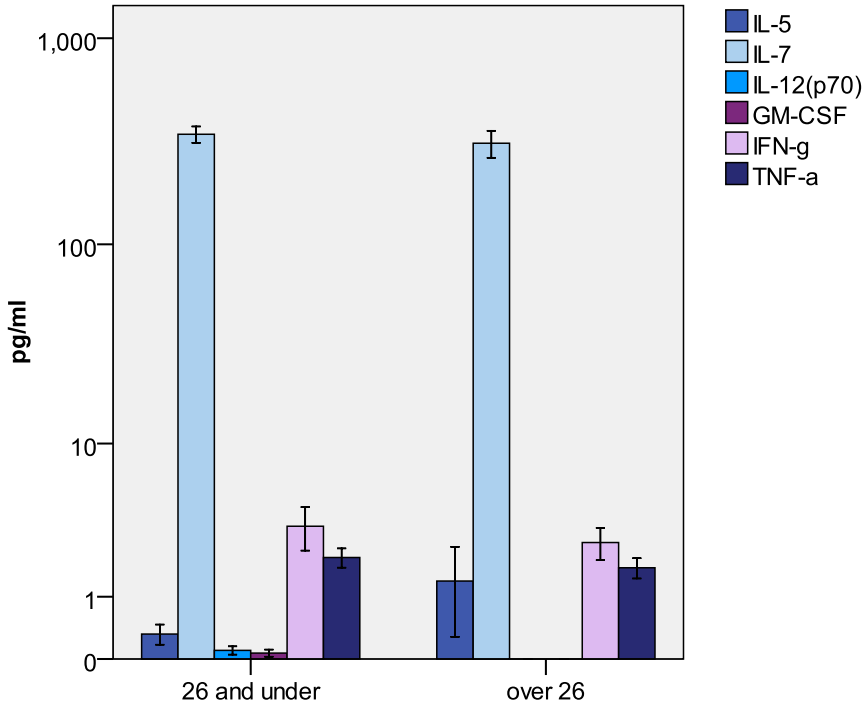

Figure 32a. Age Effect on Tear Cytokine Levels with Daily Contact Lens Wear in Study III. Tear IL-5, IL-7, IL-12p70, GM-CSF, IFN-ã, TNF-á and VEGF levels in samples from individuals 30 years old or younger compared to those over 30 with daily wear in Study III. There were no significant differences in tear cytokine levels between age groups with daily wear.

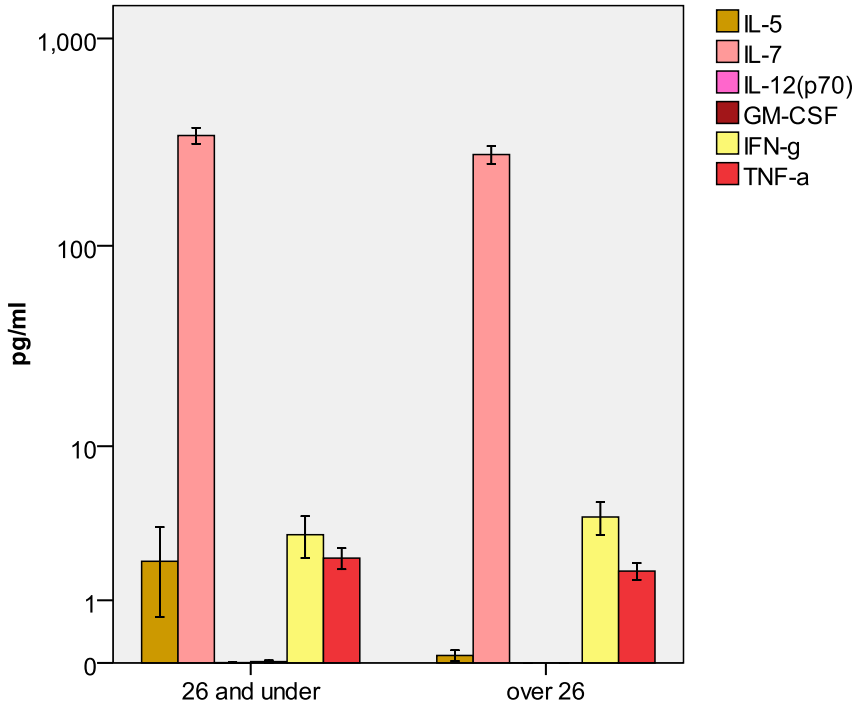

Figure 32b. Age Effect on Tear Cytokine Levels with Continuous Contact Lens Wear in Study III. Tear IL-5, IL-7, IL-12p70, GM-CSF, IFN-ã, TNF-á and VEGF levels in samples from individuals 30 years old or younger compared to those over 30 with continuous wear in Study III. There were no significant differences in tear cytokine levels between age groups with continuous wear.

There were no differences between age groups at any visit in Study III. No changes in cytokine levels over time reached significance in either group.

Study III Clinical Test Results

As in Study II, clinical tests were conducted on the day of lens insertion and immediately after lens removal (day 30) on both eyes in Study III. Lissamine green staining score increased in both eyes and showed a greater increase after 30 days of extended wear than daily wear, though this change did not reach statistical significance (Figure 33). Fluorescein staining score remained relatively unchanged after 30 days of daily wear and extended wear (Figure 34).

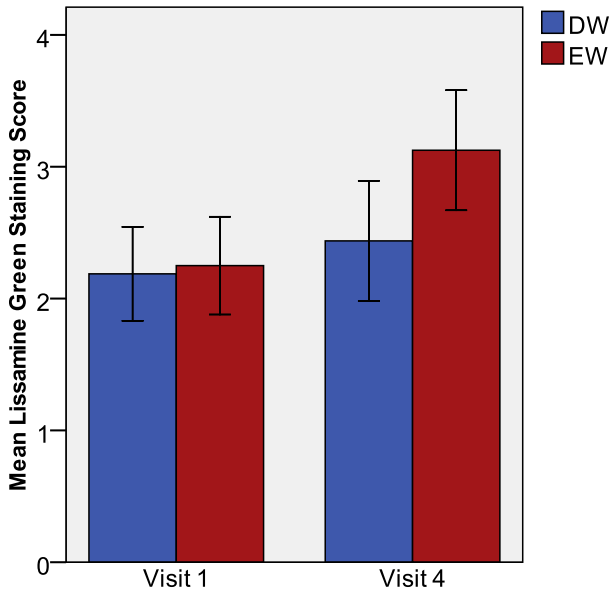

Figure 33. Study III Lissamine Green Staining Score with Daily and Extended Wear. Comparison of mean Lissamine green staining scores at Visits 1 (day 0) and 4 (day 3) of daily and extended contact lens wear.

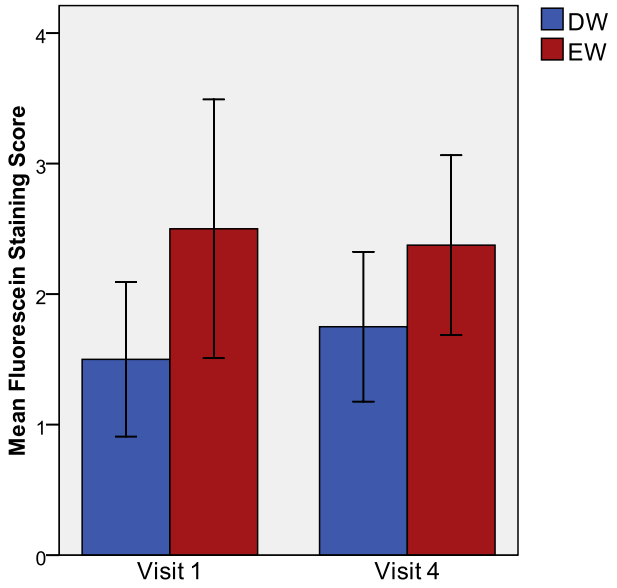

Figure 34. Study III Fluorescein Staining Score with Daily and Extended Wear. Comparison of mean fluorescein staining scores at Visits 1 (day 0) and 4 (day 30) of daily and extended contact lens wear.

Tear meniscus height and non-invasive break-up time decreased in both eyes after 30 days of daily and extended wear (Figures 35 and 36). However, this change did not reach significance.

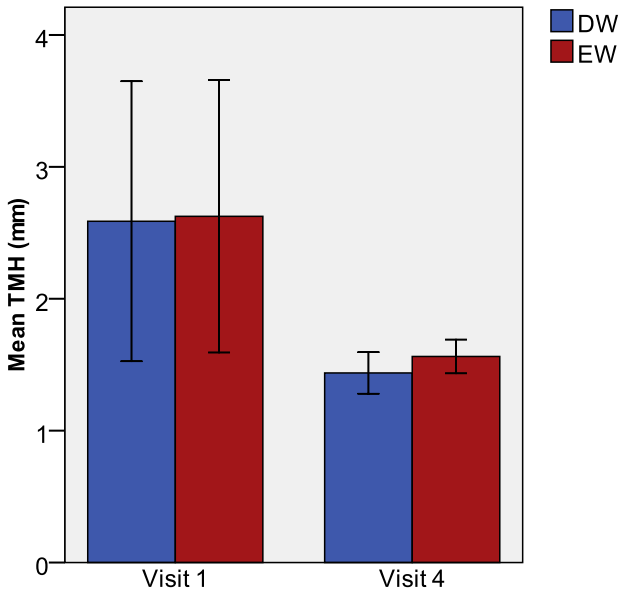

Figure 35. Study III Tear Meniscus Height with Daily and Extended Wear. Comparison of mean tear meniscus height at Visits 1 (day 0) and 4 (day 30) of daily and extended contact lens wear.

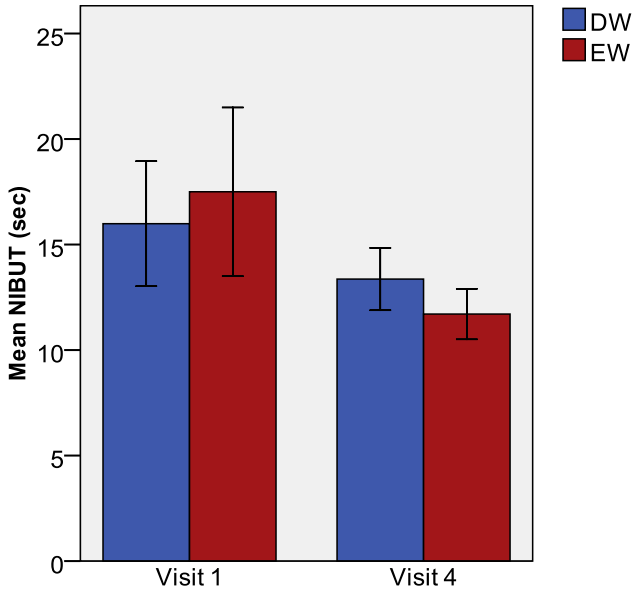

Figure 36. Study III Non-Invasive Break Up Time in Daily and Extended Wear Comparison of mean non-invasive break up times at Visit 1 (day 0) and Visit 4 (day 30) of daily and extended contact lens wear.

Although tear IL-8 and Lissamine green staining scores increased in both eyes after the 30-day contact lens wearing period, they did not correlate significantly with daily wear (Figure 37a) or extended wear (Figure 37b).

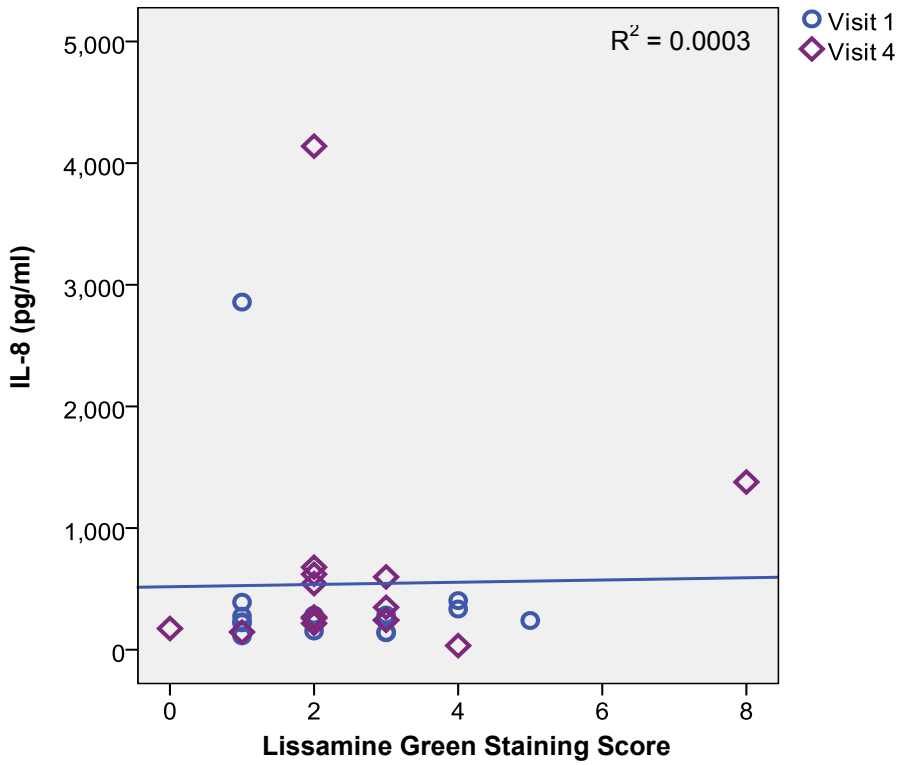

Figure 37a. Scatter Plot of Tear IL-8 and Lissamine green staining score with Daily Wear in Study III. Spearman's ñ = 0.98 (p = 0.61). Linear trendline equation: y = 9.21x + 517.74.

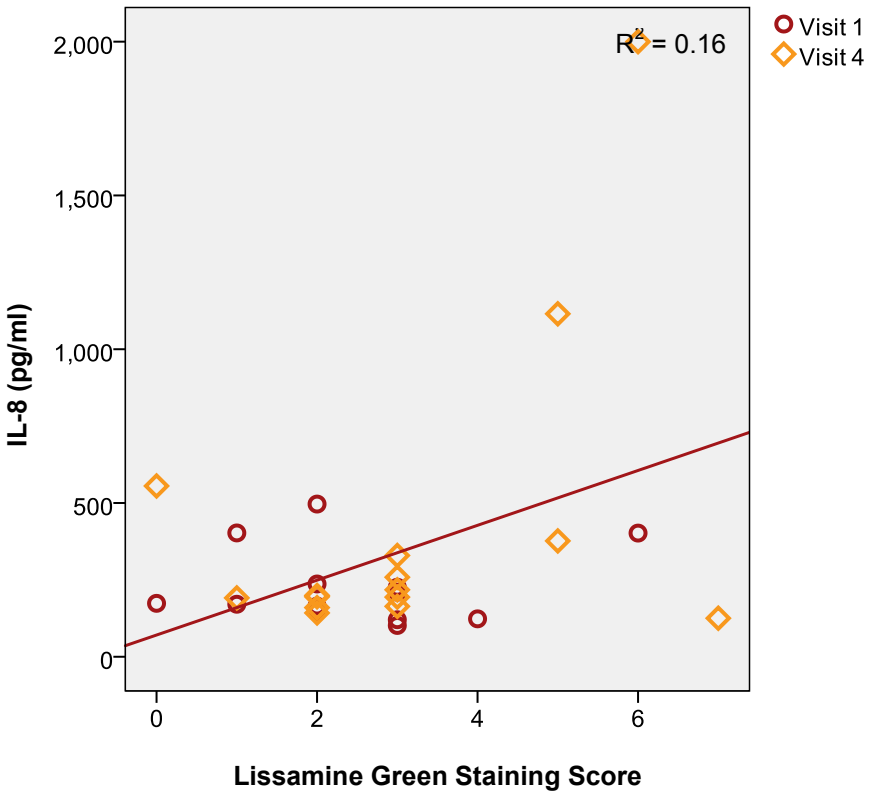

Figure 37b. Scatter Plot of Tear IL-8 and Lissamine green staining score with Extended Wear in Study III. Spearman's ñ = 0.75 (p = 0.70). Linear trendline equation: y = 89.18x + 70.66.

DISCUSSION

Findings in Study I were contrary to the hypothesis that 30 days of continuous contact lens wear would raise levels of pro-inflammatory cytokines and reduce levels of anti-inflammatory cytokines compared to 30 days of daily wear. There were no significant changes in pro-inflammatory cytokine levels with either treatment. However, IL-1ra (an anti-inflammatory cytokine) was elevated from Visit 1 ($p < 0.05$) and Visit 2 ($p < 0.05$) to Visit 4 of extended wear (See Figure 4).

With the exception of IL-1ra, results of Study I corresponded to findings in a similar study investigating the effects of punctual occlusion on tear cytokine levels [66]. Patients received a punctual plug in the superior and inferior puncta of one eye and no treatment in the contralateral eye. In that study, the same Bio-Plex™ Human Cytokine 27-Plex kits were used for CBA of tear samples collected from both eyes at four study visits during a four-week period. Differences in tear cytokine levels between the punctual plug treatment and control eyes did not reach significance.

In Study I, there were strong correlations between levels of tear IL-2 and IL-4 in both daily and extended wear during the 30-day period (Figures 7a, b). These two cytokines (along with IL-5, IFN-γ and others) are involved in regulating adaptive immunity. The parallel trends displayed by these two cytokines may demonstrate their synergistic activity in promoting the adaptive immune response. These significant correlations were rare among pairs of cytokines in Study I, as seen in Figures 8a and 8b where IL-2 and IL-6 levels do not correlate with either treatment. This lack of correlation may be explained by the roles of these two cytokines in different phases of the immune response; IL-2

plays a significant role in adaptive immunity and IL-6 is a key cytokine in the innate immune response.

More than 50% of tear samples in Study I had levels of eotaxin, IL-9, IL-17, MIP-1α, and MIP-1β that were below the limits of detection. FGF-b was below detection in all tear samples. The unexpected lack of detection for several cytokines may be explained by the unavoidable use of an older filter plate for the assay. On the day of the assay, there was some difficulty vacuuming fluid through the filter plate that was included in the kit due to several plugged wells. This was discovered during pre-wetting of the filter plate. Attempts to remove dispensed assay buffer from the plate using the vacuum manifold resulted in over-drying of several wells while excess fluid still remained in others. Rather than risk application and loss of tear samples on the defective plate, an older reserve plate was used for the assay. The reserve plate had been stored at room temperature in a cabinet for several months. It is not clear whether or not the storage conditions compromised the integrity of the plate and therefore may have caused some interference with the assay and detection of certain cytokines. One particular cytokine of interest, eotaxin, which has been detected in other studies in this lab [60], and even directly correlated with the severity of papillae grade [67], was undetectable in Study I samples (with the exception of tears from one individual). This prompted the investigation of modifications to the assay aimed at improving detection.

Although none were significant, noticeable trends in tear cytokine levels with daily and continuous contact lens wear suggested that improvements to the assay procedure may produce more reliable data. For example, the distinct elevations of tear IL-6 and IL-8 in Study I, Visit 3, of continuous wear approached, but did not reach, significance. To

explore alternatives for assay procedures to improve detection and sensitivity, several modifications were introduced. A newer Bio-Rad™ 27-Plex magnetic bead assay kit was investigated. An automated plate washer was also obtained to circumvent one potential source of variability: uneven washing and draining of assay wells using manual washing and vacuuming procedures.

Two formats of the CBA were then compared: the vacuum filtration format using filter plates and the magnetic format using flat-bottom plates. Results from the format comparison revealed that the mean intra-assay coefficients of variation with the magnetic washing format with flat-bottom plates were almost half that of the vacuum filtration format ($p < 0.05$) (Tables 10 and 11). This prompted the transition to the magnetic bead/flat-bottom plate format with the magnetic washing procedure for CBA in Study II.

Study II CBA results were expected to be similar to Study I, but enhanced detection of cytokines known to be present in tear samples were considered more likely to be seen. One reason for this expectation was based on Bio-Rad™ reports that magnetic and polystyrene bead-based assays had been extensively compared and found to perform equally. However, Bio-Rad™ tested only serum and cell lysate samples. Their data suggested enhanced sensitivity due to larger magnetic bead size and the ability to avoid vacuum filtration steps during the assay. Reduced variability between subjects was also expected. However, there were several, more prominent differences between results of tear cytokine levels in Study I and Study II.

None of the trends that were seen in Study I was observed in Study II. In fact, trends for daily and extended wear were remarkably similar across most cytokines over the 30-day period. Conversely, in Study I, cytokines seemed to have rather unique

trends. The similarity across cytokines in Study II was contradictory, not only to Study I, but also to what is known about cytokine function and concentrations in normal human tears. A possible explanation for the similarity among cytokines is the interaction of various components in tears with the material of the flat-bottom polystyrene plate that was used in the magnetic CBA format in Study II. Tear components have previously been shown to interfere with polystyrene plate ELISA of tears [68]. It is possible that these intrinsic factors interfere in a more systematic fashion with the analyte-antibody interactions when beads are drawn to the bottom of the plate by the magnet.

All 27 cytokines were detected in Study II, sometimes at unexpectedly high levels, particularly for normal individuals (see Figures 15a-c). For instance, eotaxin, IL-9, IL-17, MIP-1α, MIP-1β and FGF-b were detected in all Study II samples—these cytokines were only sporadically detected in Study I samples. It was also unusual that there were marked differences in tear cytokine concentrations between the two eyes immediately after the washout phase. The average difference between starting values (tear cytokine levels in samples collected prior to lens insertion) in Study I was much lower compared to Study II. Furthermore, there was an anomaly among the Study II samples: all of the tear samples from one particular patient had distinctly low levels of all 27 cytokines, all of which were detected in all other patient samples within the same cohort at substantially higher levels. Almost 40% of the cytokines quantified in this patient's sample set were below limits of detection.

The indeterminate assay results of Study II suggested, without considerable further experimentation, that the magnetic format was not the most suitable for the assay of proteins in tear fluid. Subsequently, the filter plate format with the Millipore™ High

Sensitivity 13-Plex kit was used for CBA of Study III samples. There were several reasons for choosing a different assay kit for Study III, rather than revert to the Study I kit. The 13-plex kit has demonstrated superior tear spike recovery to the Bio-Rad™ 27-Plex [60]. Improved spike recovery suggests a more reliable assay. The Millipore™ 13-Plex was not initially used in Study I because it did not include key cytokines that were of particular interest and thought to be pertinent to the impact of contact lens wear on the ocular surface, namely IL-1ra, eotaxin and VEGF.

Results of the CBA of tear samples in Study III were similar to Study I and confirmed that there were no significant differences in pro-inflammatory tear cytokine concentrations between eyes assigned to daily wear and eyes assigned to 30 days of continuous wear. No strong correlations were found between specific cytokines unlike the correlation of IL-2 (Figure 23) and IL-4 (Figure 24) in Study I. One possible interpretation of the strong IL-2 - IL-4 correlation in Study I and lack of such a correlation in Study III was that the Bio-Rad™ 27-Plex assay antibodies for either IL-2 or IL-4 may have been cross-reacting (that is, the IL-2 antibody may have been detecting both IL-2 and IL-4 or the IL-4 antibody may have been detecting both. This could be tested by spiking various amounts of IL-2 or IL-4 standards into tear samples and determining recovery with the Bio-Rad™ 27-Plex. Excess recovery of both on one bead coated with antibody raised against only one would indicate cross-reaction.

One patient in the study was diagnosed with CLARE in the eye assigned to continuous wear towards the end of the 30-day period in Study III. The cytokine profile of the CLARE patient was similar to the rest of the cohort in Study III. However, she had the highest levels of IL-6 (an acute phase cytokine), IL-8 (a potent PMN recruiter) and IL-10

(a modulator of the T_H2 response) at Visit 4 in the eye assigned to continuous wear. The tear sample collected at Visit 3 had a sudden increase in IL-6 compared to Visit 2 (Figure 28). This elevation occurred prior to the onset of CLARE symptoms. This supports the suggestion that increased tear IL-6 may be "a clinical marker in patients who are predisposed to contact lens-associated adverse responses" [53]. The increase in IL-6 from the eye that experienced the CLARE is consistent with findings from a previous study, where tear IL-6 was elevated in CLARE patients compared to normal individuals [54]. The increase at Visit 3 was followed by a slight decrease at Visit 4, after treatment with Lotemax® drops.

Elevations of IL-8 and IL-10 occurred at Visit 4 (see Figures 29 and 30), after the CLARE diagnosis and treatment. Therefore, it is not clear whether the elevated levels of IL-8 or IL-10 were a direct result of her condition or the ophthalmic steroid drops used to treat it. Lotemax® is an ophthalmic steroid drop that is used to suppress inflammation. As a result, it is probable that the increase in IL-10 at Visit 4 is associated with the treatment because IL-10 is an anti-inflammatory cytokine. In a study soon to be conducted in this laboratory, dry eye treatments, including Lotemax®, will be investigated both clinically and for their effects on tear cytokine profiles.

Further analysis of results in each of the studies revealed various gender and age effects on tear cytokines with contact lens wear. In Study I, there were no significant differences between males and females with respect to tear cytokine levels with continuous wear. However, comparisons of cytokine levels at each visit showed male tear IL-1ra to increase significantly from Visits 1, 2 and 3 to Visit 4 with continuous wear. With daily wear, females had significantly higher levels of IL-7, IL-9, IL-15, IL-1ra, IP-10 and

PDGF-BB (Figure 12a). In Study II, differences in cytokine levels between males and females did not reach significance (Figures 16a and 16b). On the other hand, In Study III, males had higher levels of IL-5 and IL-8 than females at all four visits with daily wear. Females had higher IFN-γ ($p < 0.05$) with both daily and continuous wear.

Hormonal distinctions between sexes may account for some of the differences in tear cytokines between men and women. Sex hormones are known to regulate cytokine release and lacrimal gland function [69]. A review article of the roles of sex hormones in allergic reactions discusses reports of disparities in the prevalence and severity of adverse reactions [70]. It highlights some of the many studies of gender-specific discrepancies in reactions to food allergies, drugs, and insect venom. The current study contributes to the understanding of the effect of gender on the ocular response to contact lens wear. To better address these differences, other gender-specific factors, like the menstrual cycle and menopause, would need to be considered in comparing tear cytokine levels in males and females.

It has been reported that reflex tear flow and tear protein composition vary with age [62, 71]. Tear cytokine levels of individuals 30 years of age and younger were compared to those over 30 in Studies I and II. In Study I, age did not have a significant effect on tear cytokine levels with daily wear. However, with continuous wear, several pro-inflammatory cytokines were significantly higher in the younger group's tear samples. In the overall comparison of cytokine levels with response to each treatment in Study II, the younger group had significantly higher levels of almost all cytokines with daily and continuous wear. Furthermore, PDGF-bb, IL-1â, IL-2, IL-7, IL-9, eotaxin, and G-CSF, were

significantly higher in the younger group's tear samples collected at the initial Visit, which may suggest that these tear cytokines display inherent age-specific differences.

Another account for the discrepancies in cytokine levels between age groups in Study II may be that each individual consistently fell within the same rank order for the concentrations of any given tear cytokine. For example, if someone had the highest IL-6, he or she consistently had the highest level of all other cytokines. Individuals that had consistently high levels of all cytokines also happened to fall into the younger age category. Thus, the younger group showed significantly higher levels of all cytokines with both daily and continuous wear, with the exception of IP-10 (Figures 17a-17f). This could be another manifestation of the interference effects on CBA.

It is possible that the same factors that interfere with the assay also increase in tears with age. One way to address the age discrepancy in cytokine levels that were seen using the protocol in Study II would be to analyze matching tear samples from a broad range of age groups on different formats of CBA. Results showing significant differences between age groups with only the magnetic bead format with the flat-bottom plate would provide further supporting evidence that this format is not appropriate for tear protein assays.

In the current study, any differences seen in age comparisons of cytokine levels, particularly in Study II, showed that levels were higher in the younger group. Age is in important element in immunity and disease profiles. Some ocular diseases are more prevalent in older individuals. VEGF and PDGF-bb are cytokines that are involved in angiogenesis. Evidence has shown that the expression of these cytokines changes with the onset of age-related macular degeneration (AMD), a posterior eye disease that is preva-

lent among older individuals. In the current study, VEGF and PDGF-bb are two cytokines that were consistently found at higher levels in tear samples from the younger group. Given what is known about these cytokines that promote angiogenesis, it is unclear why they would be higher in samples from younger individuals. It may be that their presence in the tear film is not indicative of their presence or function in the posterior eye.

There were drawbacks to the overall study design that did not allow insight into the clinical impact of 30 days of contact lens wear (daily and extended) in normal individuals as it relates to biomarkers in tears. In Studies II and III, clinical assessments (slit lamp examination of the ocular surface, NIBUT, TMH, sodium fluorescein and Lissamine green staining) were repeated immediately prior to lens insertion (on Day 0) and immediately after lens removal (Day 30). This was done to avoid any impact of intermittent removal and invasive tests on levels of tear cytokines.

One way to investigate the types of clinical changes that may be occurring concurrently with fluctuations in cytokine levels at different stages during the 30-day period would be to have a test group for each stage of evaluation. For example, one eye of each individual would be assigned to daily wear, the other to extended wear. They should be divided into three groups, each group assigned to a different wearing period: 3 days, 14 days and 30 days.

It has been suggested that shedding of the corneal epithelium decreases significantly early in a 30 day contact lens wearing period [45]. It has also been established that ocular surface cells release cytokines into the tear film. Further investigation exploring the impact of contact lens wear on cytokine levels with measures of exfoliation rate

would provide insight into the source and cause of the decreasing trend in certain tear cytokine levels that is seen with prolonged contact lens wear.

CBA is highly quantitative and sensitive and offers several benefits over older protein assay technologies. The assay does not require large sample volumes; which is particularly advantageous when the sample is difficult to produce, for instance, tear samples from dry eye patients who have difficulty producing tears. Additionally, many cytokines in one small sample may be captured and efficiently quantified concurrently. Because many cytokines function in concert with others, often their concentrations are regulated by the activity of others. Therefore, a great deal of useful information is gained when multiple cytokines can be analyzed simultaneously under various conditions [72]. It is also highly quantitative with high specificity and sensitivity. CBA also has advantages for studies of ocular health and disease. It provides the objectivity and reliability that is often lacking from common clinical tests. For example, grading of transient ocular surface stains like Lissamine and fluorescein is often subject to bias due to interpretation by the observer and variable grading scales. Also, the highly variable Schirmer strip test is unreliable because the strip is thought to induce reflex tearing, which does not provide a conclusive measure of basal tear production. CBA of tears, on the other hand, requires no interpretation and simply reports measures of many cytokines in tears.

There are, however, shortcomings inherent in the quantification of tear cytokines using CBA. Tears fluid has been reported to contain intrinsic components including proteases, anti-proteases, and auto-antibodies. The presence of these agents presents limitations to the CBA of tear film, potentially compromising results by interference, non-

specific binding of tear components between primary and secondary antibodies, producing a non-specific reaction, or other effects.

While the major vendors of CBA kits conduct extensive validation of the included antibody pairs and their performance in a multiplex assay environment, most of these quality control steps utilize serum or cell lysates - and thus do not address the interfering agents likely to be found in tears. Developmental CBA studies have been aimed at understanding the impact of known tear interfering agent effects on results of cytokine assays [60]. These studies included spike-recovery experiments in which known concentrations of human recombinant cytokine were spiked into tear samples to measure interference. Another study compared assay results of tear samples stored in buffer with and without protease inhibitor (unpublished data from this laboratory).

As an adjunct to tear CBA, complimentary assay techniques could be run in tandem. Measurement of the expression of cytokines and their receptors is one promising approach. Reverse transcriptase (RT) PCR could be used to validate the relative cytokine profiles determined by CBA. This would require collection of epithelial cells from the conjunctiva using impression cytology. Quantitative RT-PCR would provide a measure of the level of cytokine expression from the ocular surface in conjunction with the CBA measure of cytokines secreted from the lacrimal glands. This type of analysis would be particularly useful for the investigation of ocular allergy and disease states and response to drugs and therapy.

CONCLUSION

The first aim of this investigation was to determine if 30 days of continuous contact lens wear would produce a different ocular inflammatory response compared to daily wear.

In these investigations, the level of inflammatory response was determined through changes in the levels of cytokines released from the ocular surface and lacrimal glands into the tear film. The results of Study I, for which the Bio-Rad™ 27-Plex cytometric bead-based assay was used to assay tears, suggest that 30 days of continuous contact lens wear does not produce a more pro-inflammatory response compared to 30 days of daily wear. It did, however, show that IL-1ra increases significantly over one month of continuous contact lens wear. Further analysis revealed that levels of IL-1ra were significantly higher in females than males on the third day of continuous wear. The format comparison showed that the use of the flat-bottom plate format with magnetic-core polystyrene beads significantly reduced the coefficient of variation between samples when compared to the filter plate with vacuum filtration. This was the basis for the decision to use the Bio-Plex Pro™ assay kit, a magnetic bead-based format, for Study II. However, conclusions drawn from the results from the direct comparison of the vacuum format versus the magnetic format for these beads were misleading. Parallel trends and similar levels of cytokines in tear samples, large differences between starting values, and abnormally low values for all samples for one subject raised questions about the suitability of the flat-bottom polystyrene plate/magnetic bead format for tear assays. The magnetic format with plastic plates does not appear to be optimal for the assay of biomarkers in tears at this stage. The problem may lie with interaction of other intrinsic tear film com-

ponents with the material of the flat-bottom polystyrene plate causing interference with the detection of cytokines. Thus, the vacuum filtration format was used again in Study III with the Milliplex™ High Sensitivity 13-Plex kit, because it had demonstrated improved sensitivity and better detection of cytokines in past studies. Results of Study III corroborated the outcome of Study I; again, no changes in cytokine levels between 30 days of daily and extended wear reached significance.

The second aim of this project was to correlate changes in tear cytokine concentrations with changes in clinical test results. Studies II and III were designed to directly correlate any changes in cytokine levels with clinical profiles that were obtained on the day of lens insertion and lens removal. No significant correlations were present.

There were two adverse events experienced by the study participants during the 30-day wearing period; one case of CLARE and one of corneal infiltrates. The rest of the cohort experienced no adverse events. The lack of significant differences in cytokine concentrations between daily and extended contact lens wear may represent the absence of inflammatory or immune complications.

Given that the Bausch & Lomb PureVision lens is FDA approved for 30 days of continuous wear, the lack of a significantly more pro-inflammatory tear cytokine profile in the extended wear eye is not surprising. However, for studies in which a tear cytokine profile difference *is* expected, for example comparing dry eye patients with normals or looking at the anti-inflammatory effects of dry eye treatments, these CBA kits would be expected to elicit differences. Based on the outcome of the current study, it is suggested that the Bio-Rad™ 27-Plex polystyrene kit or the Milliplex™ 13-Plex polystyrene kit

may be useful. However, further investigation of the magnetic bead kit and restriction of its use to filter plates, would be required before it would be recommended.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and expires on October 26, 2010. The Assurance number is FWA00005960.

Principal Investigator:	KEHINDE, LUCY
Co-Investigator(s):	ELDER, KESHIA SIMS FULLARD, RODERICK J
Protocol Number:	**X080117002**
Protocol Title:	*Effects of Daily versus 30 Day Continuous Contact Lens Wear on Tear Cytokine Levels*

The IRB reviewed and approved the above named project on 12-02-08. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 12-02-08

Date IRB Approval Issued: 12-02-08

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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APPENDIX B

OCULAR SURFACE DISEASE INDEX

Ocular Surface Disease Index® (OSDI®)[2]

Ask your patient the following 12 questions, and circle the number in the box that best represents each answer. Then, fill in boxes A, B, C, D, and E according to the instructions beside each.

HAVE YOU EXPERIENCED ANY OF THE FOLLOWING *DURING THE LAST WEEK*:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time
1. Eyes that are sensitive to light?	4	3	2	1	0
2. Eyes that feel gritty?	4	3	2	1	0
3. Painful or sore eyes?	4	3	2	1	0
4. Blurred vision?	4	3	2	1	0
5. Poor vision?	4	3	2	1	0

Subtotal score for answers 1 to 5 (A)

HAVE PROBLEMS WITH YOUR EYES LIMITED YOU IN PERFORMING ANY OF THE FOLLOWING *DURING THE LAST WEEK*:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	
6. Reading?	4	3	2	1	0	N/A
7. Driving at night?	4	3	2	1	0	N/A
8. Working with a computer or bank machine (ATM)?	4	3	2	1	0	N/A
9. Watching TV?	4	3	2	1	0	N/A

Subtotal score for answers 6 to 9 (B)

HAVE YOUR EYES FELT UNCOMFORTABLE IN ANY OF THE FOLLOWING SITUATIONS *DURING THE LAST WEEK*:

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	
10. Windy conditions?	4	3	2	1	0	N/A
11. Places or areas with low humidity (very dry)?	4	3	2	1	0	N/A
12. Areas that are air conditioned?	4	3	2	1	0	N/A

Subtotal score for answers 10 to 12 (C)

ADD SUBTOTALS A, B, AND C TO OBTAIN D (D = SUM OF SCORES FOR ALL QUESTIONS ANSWERED) (D)

TOTAL NUMBER OF QUESTIONS ANSWERED (DO NOT INCLUDE QUESTIONS ANSWERED N/A) (E)

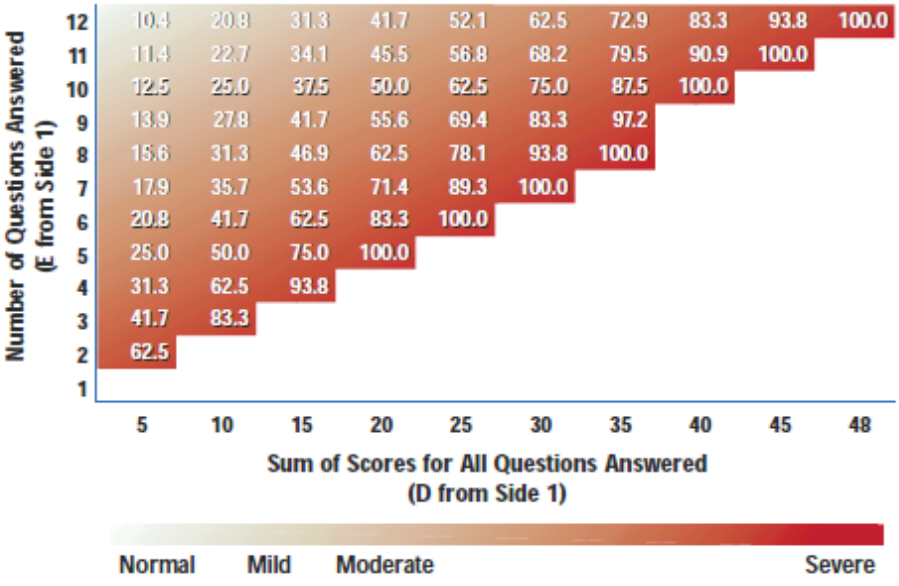
Please turn over the questionnaire to calculate the patient's final OSDI® score.

Evaluating the OSDI® Score[1]

The OSDI® is assessed on a scale of 0 to 100, with higher scores representing greater disability. The index demonstrates sensitivity and specificity in distinguishing between normal subjects and patients with dry eye disease. The OSDI® is a valid and reliable instrument for measuring dry eye disease severity (normal, mild to moderate, and severe) and effect on vision-related function.

Assessing Your Patient's Dry Eye Disease[1,2]

Use your answers D and E from Side 1 to compare the sum of scores for all questions answered (D) and the number of questions answered (E) with the chart below.*
Find where your patient's score would fall. Match the corresponding shade of red to the key below to determine whether your patient's score indicates normal, mild, moderate, or severe dry eye disease.

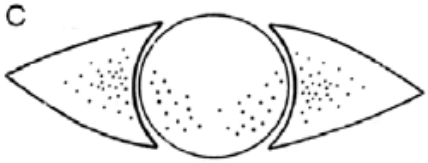
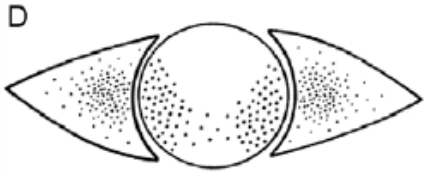
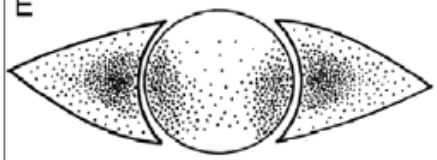


*Values to determine dry eye disease severity calculated using the OSDI® formula:

$$\text{OSDI}^{®} = \frac{(\text{sum of scores}) \times 25}{(\#\text{ of questions answered})}$$

APPENDIX C

OXFORD GRADING SCALE

PANEL	GRADE	VERBAL DESCRIPTOR
A 	0	Absent
B 	I	Minimal
C 	II	Mild
D 	III	Moderate
E 	IV	Marked
>E	V	Severe