Biomarkers of Dry Eye Disease

Rachel Kathryn Casemore

Doctor of Philosophy

Aston University

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Abstract

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The multifactorial nature of Dry Eye Disease (DED) and the poor correlation between signs and symptoms make diagnosis and management challenging, particularly in the early stages. An increasing prevalence, particularly in younger generations, has led to an increased workload for optometrists. Therefore, the search for validated biomarkers to accurately and objectively measure a disease whose pathogenesis is not yet fully understood, is vital.

The key focus of this thesis was to explore clinical signs, and potential biomarkers of evaporative DED from tears and meibum, in a young population.

Initially, UK primary care optometrists were surveyed to discover whether research, in particular use of an existing commercially available biomarker, is being translated into practice. The results unveiled current clinical practice patterns in the UK, and limited use of available biomarker tests, although an increase in evidence-based practice and steroid prescribing was identified.

A prospective, longitudinal study in young adults found significant overlap in clinical signs between subjects with and without DED. Conjunctival staining was the best single predictor, with female sex and contact lens wear the most significant risk factors; screen use and stress were key modifiable factors.

Findings implicating inflammation in the pathogenesis of early DED were found from tear protein and cytokine analysis, including the upregulation of albumin. Furthermore, zinc-α2-glycoprotein was downregulated and correlated with increasing signs of meibomian gland dysfunction (MGD). Along with the upregulation of certain pro-inflammatory cytokines (IL-1β and fractalkine), a corresponding upregulation of the anti-inflammatory cytokines IL-1Ra occurred, indicating a coordinated suppression of the immune response. Higher meibomian gland loss was significantly associated with up-regulation of polar phospholipids and down-regulation of (O-acyl)-ω-hydroxy fatty acids, which correlated with clinical signs of MGD.

Overall, this thesis has identified several key clinical findings and molecular biomarkers for the early detection of evaporative DED in a young cohort.

Key words

Dry Eye Disease, evidence-based practice, clinical practice, young adults, lifestyle factors, biomarkers, tears proteins, cytokines, meibum.

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> *"For now, we see in a mirror dimly, but then face to face. Now I know only in part; then I shall know fully, even as I have been fully known" 1 Corinthians 13 v.12.*

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List of Terminology and Abbreviations

ADDE - aqueous deficient dry eye

AMPs - antimicrobial proteins

BL - basal tears

BUT - breakup time

cDCD- corneal dendritic cell density

Cer - ceramide

ChE - cholesterol ester

CL - contact lens

DED - dry eye disease

DESOS - dry eye symptoms outweighing signs

DG - diglyceride

DTA - decision tree analysis

ECP - eye care practitioner

EDE - evaporative dry eye

EGF - epidermal growth factor

ELISA - enzyme-linked immunosorbent assay

FBUT - fluorescein breakup time

FL - flush tears

FML - fluorometholone

GCD - goblet cell density

GVHD - graft versus host disease

HPLC - high-performance liquid chromatography

IC - impression cytology

IFN-γ - interferon gamma

IgG - immunoglobulin G

IL - interleukin

IL-1Ra - interleukin 1 receptor antagonist

IP - independent prescriber

IPL - intense pulsed light therapy

IVCM - in vivo confocal microscopy

LC-MS/MS - liquid chromatography with tandem mass spectrometry

LLT - lipid layer thickness

LOC - local optical committee

LOCSU - local optical committee support unit

LWE - lid wiper epitheliopathy

MA - microarray

MAPK - mitogen-activated protein kinases

MC- microcapillary

MECS - minor eye conditions service

MG - meibomian gland

MGD - meibomian gland dysfunction

MHC - major histocompatibility complex

MMP-9 - matrix metalloproteinase-9

mRNA - messenger ribonucleic acid

MX - multiplex bead analysis

MS - mass spectrometry

NGF - nerve growth factor

NITBUT- non-invasive tear breakup time

NIKBUT - non-invasive keratograph breakup time

NK cells - natural killer cells

Non-SS - non-Sjögren's syndrome

NPSI-E - Neuropathic Pain Symptom Inventory for the Eye

OAHFA - (O-acyl)-ω-hydroxy-fatty acid

OSD - ocular surface disease

OSDI - ocular surface disease index

PEARS - primary eye care acute referral service

PCA - Principal Component Analysis

PIP - pipette

PIS - participant information sheet

PPI - patient and public involvement

pSS - primary Sjögren's syndrome

Px - patient

QOL - quality of life

RANTES - Regulated upon Activation, Normal T-cell Expressed and Presumably Secreted

RCT - randomised control trial

RX - reflex tears

SC - Schirmer test

SC1 - Schirmer test without anaesthetic (basal and reflex tears)

SC2 - Schirmer with anaesthetic (basal tears)

sIgA - secretory immunoglobulin A

SJS - Steven Johnson syndrome

SM - sphingomyelin

Sp - sponge

SS - Sjögren's syndrome

T2D - Type II diabetes

TAO - thyroid-associated orbitopathy

TBUT - tear breakup time

TCR - tear clearance rate

TG - triglyceride

TGF-β - transforming growth factor - beta

TFOS DEWS - Tear Film and Ocular Surface Society Dry Eye Workshop

TMH - tear meniscus height

TNF-α - tumour necrosis factor - alpha

TPC - total protein concentration

VEGF - vascular endothelial growth factor

WE - wax ester

w/o - without

WK- wick

ZAG - zinc-α2-glycoprotein

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Chapter 1- Introduction to Dry Eye Disease and biomarkers

1.1. Introduction

1.1.1. Definition and classification of Dry Eye Disease

Dry Eye Disease (DED) is characterised by a loss of homeostasis of the tear film, inflammation, and neurosensory changes; clinical signs include decreased tear production, decreased tear stability, epithelial and conjunctival damage with loss of conjunctival goblet cells (Craig et al., 2017a). Changes in corneal nerve density (reduced) and tortuosity (increased) (Labbé et al., 2013) and increased dendritic cell density (Aggarwal et al., 2021) have also been shown. The term dry eye disease (DED) covers a range of disease manifestations that can have numerous different and sometimes overlapping underlying causes: aqueous deficiency with or without underlying systemic disease, meibomin gland dysfunction, iatrogenic, hormonal, environmental, age-related (Willcox et al., 2017;Bron et al., 2017). In 2017 the TFOS DEWS II Definition and Classification subcommittee revised the definition of DED to:

> *Dry eye is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles (Craig et al., 2017b).*

They based their recommended classification on the two key pathophysiological mechanisms involved in DED i.e., aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE), acknowledging that these mechanisms may co-exist or overlap. Whether the patient is symptomatic, or asymptomatic, intrinsic, or extrinsic factors are at play, or whether there is underlying systemic inflammation or not, the aim of management is to restore homeostasis.

It has been proposed that DED could be thought of as a localised autoimmune process which occurs due to ocular surface immune tolerance disruption, probably initiated by tear hyperosmolarity and/or microbial stimuli (Stern et al., 2013). Dysfunction of this immune regulation has been shown to be at the core of several ocular surface diseases (Galletti et al., 2017). The ocular surface consists of the cornea, limbus, conjunctiva, eyelids, meibomian glands, lacrimal glands, and nerves. The ocular mucosal immune system is designed to keep the eye moist and protected from environmental threat. As with other mucosal surfaces in the body, the immune system of the ocular surface is equipped to defend itself against pathogens. A set of homeostatic or regulatory mechanisms also exist to prevent excessive inflammatory responses to foreign antigens which can lead to ocular surface damage and disease. To avoid unnecessary inflammation, the ocular surface's mucosal immune system needs to be able to discriminate between harmless and potentially dangerous antigens (Galletti and de Paiva, 2021).

1.1.2. Tear film composition

The tears are composed mainly of lipids, proteins, mucins, and electrolytes; the homeostasis of these components is essential for the maintenance of a healthy ocular surface. The tear film provides a mechanical/lubricating and antimicrobial barrier to the ocular surface and helps to ensure a smooth optical refractive surface. It is approximately three micrometres thick (King-Smith et al., 2000) and 8±3 µl in volume (Mishima et al., 1966). Its production by the lacrimal and meibomian glands is regulated by parasympathetic and sympathetic innervation. The aqueous/mucin layer makes up the bulk of the tears with the outer lipid layer having a mean thickness of 42nm (King-Smith et al., 2010).

The aqueous component of the mucoaqueous layer, secreted from the lacrimal glands, contains a specific balance of proteins, electrolytes, metabolites, oxygen, and water. It is responsible for providing oxygen and nutrients to the underlying avascular cornea and flushing away any cellular debris, foreign bodies, or toxins (Dartt and Willcox, 2013). The concentration of electrolytes in the mucoaqueous layer help to determine the osmolarity of the tear film, which can be increased in DED (Willcox et al., 2017). The antimicrobial peptides, proteins and soluble immunoglobulins that protect the ocular surface from infection are discussed in more detail in section 1.5.

Mucins are secreted by specialised goblet cells in the conjunctival epithelium and are thought to be under neurogenic control via the parasympathetic system (Dartt, 2002). They can be soluble, or membrane bound. Soluble mucins can also be gel-forming such as MUC5AC (Ohashi et al., 2006). Membrane-associated mucins or transmembrane mucins such as MUC1 and MUC16 are major components of the epithelial glycocalyx, a dense coating anchored to the epithelial cells responsible for maintaining surface integrity and tear film stabilisation (Argüeso, 2020).

The tear film lipid layer is produced mainly by the meibomian glands. These are embedded in the upper and lower tarsal plates in single rows. Numbers are reported to range from 30-40 in the upper lid and 20-25 in the lower lid (Blackie et al., 2010). Each gland is composed of multiple acini connected by a central duct. Lipids synthesized in the ducts are excreted through the gland orifices located at the mucocutaneous junction of the lid margin (McCulley and Shine, 2004). The glands are presumed to act in a coordinated fashion influenced by hormonal and neural regulation and by the mechanical forces of muscle contraction during a blink (Knop et al., 2011). Meibum can be expressed digitally from the glands. However, studies have shown that only a proportion of the glands are actively secreting at any one time; the nasal glands are the most active, followed by the central glands, and finally, the temporal glands (Tomlinson et al., 2011).

Meibum is a liquid at body temperature, with a melting range between 19 and 40 °C, when measured in vitro in bulk (Butovich et al., 2010). However, delivery to the ocular surface can be impeded if the ocular surface and eyelid temperature drop below 33 °C due to thickening of the meibum in the glands. A cause of meibomian gland dysfunction (MGD) is thought to be solidification of lipids due to an elevation in the melting point of meibum and an increase in proteinaceous particles in the secretions (Terada et al., 2004). The melting point for meibum appears to change due to variations in chemistry and viscosity, thus impacting the temperature at which the secretion becomes mostly liquid (Blackie et al., 2008). Secretions from severely obstructed glands tend to have higher melting points (up to 45°C) than the secretions from apparently normal, unobstructed glands (32°C) (Borchman, 2019).

Meibum contains non-polar lipids such as cholesterol and wax esters which make up the bulk of the lipid layer. Their most important role is to prevent evaporation of tears (Willcox et al., 2017). The polar phospholipids are thought to provide an interface between the hydrophilic aqueous-mucin layer and the bulk of the hydrophobic lipid layer by facilitating the spread of lipids over the aqueous to increase tear film stability, thus preventing evaporation (Campbell et al., 2011).

The cornea and lid margins are richly innervated with afferent trigeminal nerves. A reflex arc is responsible for maintaining tear secretion at a normal turn-over rate, replacing tears lost by evaporation or drainage (Dartt, 2009). Four different types of tears have been recognised: basal tears which are normally present on the ocular surface, reflex tears produced by stimulation, closed eye tears which bathe the eye during sleep, and emotional tears. Each type of tear has a different biochemical constitution (Willcox et al., 2017). The roles of these constituents will be discussed in more detail later.

1.1.3. Biomarker definition

Biomarkers are objective indications of medical signs that can be observed, measured, and reproduced accurately (Strimbu and Tavel, 2010). They aid in our understanding of the underlying mechanisms of disease and inflammatory processes and thus aid in diagnosis (diagnostic biomarkers). They may also assist in the monitoring (activity biomarkers) or predict the outcome (predictive/prognostic biomarkers) of therapeutic interventions (Enríquez-de-Salamanca et al., 2012).

In 2015, a working group convened by the FDA-NIH Joint Leadership Council, who developed the BEST (Biomarkers, Endpoints, and other Tools) Resource, defined a biomarker or biological marker as:

> *A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention,*

> *including therapeutic interventions. Biomarkers may include molecular, histologic, radiographic, or physiologic characteristics. A biomarker is not a measure of how an individual feels, functions, or survives (BEST, 2016) .*

They also defined a surrogate endpoint as:

> *An endpoint that is used in clinical trials as a substitute for a direct measure of how a patient feels, functions, or survives. A surrogate endpoint does not measure the clinical benefit of primary interest in and of itself but rather is expected to predict that clinical benefit or harm based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence (BEST, 2016).*

An ideal biomarker should be easily measured by a minimally invasive technique, allow for accurate diagnosis (high sensitivity and specificity) and classification of the disease in question, and predict the outcome and response to any treatment (Asbell, 2020). It needs to correlate with clinical signs and specific symptoms. Both genomic and proteomic studies have been applied to the search for new and specific biomarkers of diseases.

1.1.4. Established biomarkers used in medicine

1.1.4.1. Thyroid disease

Various thyroid function tests (TFT) can be used in the diagnosis of thyroid disease. The initial tests investigate the levels of thyroid-stimulating hormone (TSH), and free thyroxine (T4), biomarkers in the blood. High levels of TSH and low T4 in the blood may indicate hypothyroidism, whereas raised TSH but normal T4, may indicate a risk of developing the disease. Low levels of TSH and high T4 would indicate hyperthyroidism (Lab Tests Online, 2021). Further thyroid antibody tests may be indicted to rule out less common autoimmune thyroid conditions. Thyroid peroxidase antibodies (TPO-Ab) and TSH receptor antibody (TRAb) tests may be used to diagnose Hashimoto's thyroiditis and Graves' disease, respectively. Thyroglobulin (Tg) and calcitonin are important tumour biomarkers used to differentiate between thyroid carcinoma and medullary thyroid carcinoma, respectively (LabTestsOnline, 2017).

Around 25-50% of people with Graves's disease (autoimmune hyperthyroidism) develop thyroid-associated orbitopathy (TAO) or Grave's ophthalmopathy, an autoimmune disease of the orbit. Tear sampling has shown promise as a non-invasive method of measuring for subtle biomarkers in the tear contents of patients with TAO. A novel three-protein biomarker panel to enable discrimination between patients with and without Grave's ophthalmopathy has been established (Aass et al., 2017). DED frequently occurs in TAO and due to overlapping signs and symptoms differential diagnosis can prove difficult. Matheis et al. (2015) analysed the tears for specific biomarkers and reported a significantly different protein panel in TAO versus dry eye.

1.1.4.2. Diabetes

The prevalence and incidence of Type 2 diabetes (T2D) and its comorbidities are reaching epidemic proportions throughout the world. Early identification and intervention are, therefore, of great importance. T2D makes up more than 90% of diabetes cases and the two major mechanisms in its pathophysiology are insulin resistance, and defective insulin secretion from the pancreas (Laakso, 2019). The current recognised diagnostic biomarkers of T2D are random plasma glucose concentration ≥ 11.1 mmol/l, plasma glucose concentration greater than 11.0 mmol/L two hours after taking 75g of anhydrous glucose in an oral glucose tolerance test, or fasting plasma glucose concentration ≥ 7.0 mmol/l. HbA1c (haemoglobin A1c) evaluates the average amount of glucose in the blood over the previous 2 to 3 months.

HbA1c testing alone is not always appropriate and caution in interpreting the results in certain groups of people is required; for example, patients taking medications such as steroids or antipsychotics that may cause a rapid rise in glucose levels. T2D is understood to be a multifactorial disease and intense research is underway to identify new diagnostic markers. Large population-based studies and their meta-analyses have identified multiple potential genetic and metabolic biomarkers that could be used to identify pre-diabetes and T2D (Long et al., 2020). It is anticipated that biomarker information combined with consideration of lifestyle and environmental factors could pave the way for subgroup classification and precision medicine.

The tear protein profiles of diabetic patients have been found to vary from those of healthy individuals, with elevated levels of pro-inflammatory cytokines (von Thun Und Hohenstein-Blaul et al., 2013). Patients with proliferative diabetic retinopathy have been found to have higher tear levels of nerve growth factor (NGF) than non-diabetics or non-proliferative retinopathy patients. This has been proposed as a possible parameter by which to measure diabetic retinopathy status (Park et al., 2008). Substance P (SP) in the tears (a neurotransmitter involved in pain sensation) has also been suggested as a potential biomarker for diabetic peripheral neuropathy (Tummanapalli et al., 2019).

1.1.4.3. Sjögren’s Syndrome (SS)

Primary Sjögren’s Syndrome (pSS) is a chronic and progressive autoimmune rheumatic condition, found to affect approximately 0.6% of adult females in the UK (Bowman et al., 2004). However, variations in disease definitions and lack of a diagnostic gold standard make exact estimation of prevalence difficult. SS can be primary, if it occurs alone (pSS), or secondary (sSS) when it is associated with another autoimmune disease such as rheumatoid arthritis (Patel and Shahane, 2014). Women, particularly Caucasians, are more prone to develop pSS than men (Patel and Shahane, 2014). There

are two age peaks, with the first during the 20s to 30s and the second after menopause in the mid-50s (Fox, 2005).

SS is characterised by inflammation of the exocrine glands, particularly the salivary and lacrimal secretory glands, causing dry mouth and DED. Saliva from SS patients contains greater concentrations of Th1 cytokines than saliva from controls, including IL-1β, IL-6, TNF-α, and IFN-γ. Th2-derived cytokines, such as IL-10 and IL-4, were also found in greater quantities in salivary gland tissue from SS patients than in controls (Parisis et al., 2020). Elevated tear fluid levels of IL-1Ra, IL-2, IL-4, IL-8, IL-12p70, IL-17A, IFN-γ, IP-10, MIP-1β, and RANTES have been found in patients with pSS when compared to non-SS subjects and healthy controls (Chen et al., 2019b).

Primary SS may also manifest itself in a range of other systemic inflammatory conditions, from dry skin, joint pain and vaginal dryness to pancreatitis and lymphoma, making diagnosis difficult. Diagnosis involves both objective and subjective testing. The American-European consensus criteria for the diagnosis of SS includes blood tests to check for antibodies associated with Sjogren's, including anti-Ro (SS-A) and anti-La (SS-B) antibodies. However, the marker antibodies for SS are not always present; only 70% of Sjögren's patients test positive for SS-A and 40% positive for SS-B. These antibodies are also found in lupus patients. Furthermore, other autoantibodies which are also not unique to SS can be present, for example rheumatoid factor, complicating the presentation (Patel and Shahane, 2014).

Novel biomarkers for diagnosis of SS have been identified, including autoantibodies to salivary gland protein-1, parotid secretory protein, and carbonic anhydrase VI. These show greater sensitivity and specificity for early SS, with the latter two rarely observed in rheumatoid arthritis or normal controls (Shen et al., 2012). A commercially available diagnostic test kit, the Sjö® test (Bausch & Lomb, Rochester, NY, USA) incorporates these biomarkers along with the classic autoantibodies. This in-office finger prick test has been shown to identify SS patients who previously tested negative against traditional biomarkers only (Beckman et al., 2016).

Tear cathepsin S has also been suggested as a candidate biomarker for SS (Hamm-Alvarez et al., 2014). Its activity has been found to be similar in patients with primary and secondary SS but much higher in SS than in patients with non-specific dry eye disorder or normal controls. Tests such as this may improve diagnostic accuracy in suspected cases of SS and allow for disease identification at earlier stages.

1.1.4.4. Other diseases

Tear fluid has been analysed to propose biomarkers of several other ocular surface disorders, such as ocular rosacea (Barton et al., 1997), keratoconus, GVHD and pterygium (de Almeida Borges et al.,

2020) and ocular diseases such as glaucoma (Burgos-Blasco et al., 2020). Due to technological advances and the non-invasive method of sampling from patients, tear fluid analysis has also been used to identify biomarkers of systemic disease that do not have ocular complications. Tear fluid biomarkers for diseases such as Parkinson's, breast cancer and multiple sclerosis have been recognised (Hagan et al., 2016).

However, it can be seen from these diseases that established biomarkers may not always lead to accurate diagnosis, with no single test or biomarker having 100% specificity or sensitivity. Several elements, including the clinical picture of signs and symptoms, along with perhaps a panel of biomarkers may be necessary to achieve the correct diagnosis for every patient.

1.2. The Importance of biomarkers in DED

1.2.1. Increasing prevalence

In recent years there has been an increasing need to identify and validate biomarkers for the management of DED (Roy et al., 2017). Unfortunately, there is no single laboratory test with which to monitor dry eye status. A prevalence of 5-30% in the over 50s was reported by the first epidemiology subcommittee of the TFOS DEWS (2007). Ten years later the TFOS DEWS II Epidemiology Subcommittee performed a meta-analysis to investigate the impact of age and sex in varying severities of DED, with and without symptoms, and found a global prevalence range from 5-50%. They confirmed that prevalence increases with age and that women have a higher prevalence than men, although the difference becomes less significant with age (Stapleton et al., 2017). The increased prevalence with age is compounded by increased polypharmacy in older age groups (Zhang et al., 2020a), which has been hypothesised to cause or exacerbate DED due to decreased tear production, altered nerve input and changes in the tear film composition (Fraunfelder et al., 2012).

However, an increased prevalence of DED is also being noted in younger populations. Vehof et al. (2021) addressed the need for studies to be performed in populations under the age of 40, as identified by the 2017 TFOS DEWS II Epidemiology Report (Stapleton et al., 2017). They set up a large-scale cross-sectional study of 79,866 men and women across all adult age groups, using data obtained from a population- based cohort study in the Netherlands (Vehof et al., 2021). Subjects were classed as having dry eye via questionnaire only; the Women's Health Study dry eye questionnaire, which consists of two symptom- based questions and one concerning previous diagnosis of dry eye. The study found a relatively high prevalence of 20-to-30-year-olds with symptomatic dry eye. They also proposed an association with the use of hand-held screens amongst the younger adults in the cohort, as shown in other studies of paediatric populations (Moon et al., 2016). A recent study, using the TFOS

DEWS II criteria for diagnosis (Wolffsohn et al., 2017) and management (Jones et al., 2017), found dry eye which could be attributed to environmental factors such as digital screen use, in 33.33% of healthy children (Rojas-Carabali et al., 2020).

1.2.2. Economic burden

DED poses a significant economic burden, due to both direct healthcare costs and indirectly due to decreased productivity and loss of workdays (McDonald et al., 2016). Office workers/VDU users have lower self-reported work performance levels due to eye fatigue associated with dry eye symptoms and studies implicate low indoor air humidity as a factor (Wolkoff, 2020). An estimate of the financial burden of a disease is important when considering the value of any preventative and treatment strategies (Reddy et al., 2004). In 2006, Clegg et al. estimated the total annual healthcare cost of 1,000 dry eye patients managed by ophthalmologists in six European countries. Costs ranged from US $0.27 million in France to US $1.10 million in the UK. However, a large proportion of patients may be managed by their GP or self-treat, so the true cost may actually be higher (Clegg et al., 2006). In 2008, 7 to 10 million US citizens were estimated to spend an average of US $320 per year on over-the counter artificial tears (Gayton, 2009).

1.2.3. Impact on Quality of Life

DED can also have a considerable impact on a patient's quality of life (QOL) affecting crucial daily activities such as reading, computer use, professional work, driving, and watching TV (Miljanović et al., 2007). A survey including 500 dry eye patients across five countries reported that one in ten were unable to work because of the condition (Optometry Today, 2021).

To understand the impact of a health condition on a patient, QOL has been quantified using utility assessment scores (Schiffman et al., 2003). Scores range from 1.0 = perfect health to 0.0 = death. Mean utilities for self-reported mild-to-moderate dry eye groups have been scored at 0.72 ±0.23. Utility scores similar to those for severe angina, dialysis, and disabling hip fracture have been reported in severe dry eye (Buchholz et al., 2006). Additionally, a significant correlation has been found between the severity of DED and symptoms of depression and anxiety (Bitar et al., 2019). Patients were assessed for objective signs of DED, and subjective symptoms, by administering questionnaires related to anxiety and depression, before and after they were started on a treatment protocol suitable for their dry eye severity. The study concluded that effective DED treatment was positively correlated with improved symptoms of depression and could therefore have a positive impact (Bitar et al., 2019).

1.2.4. Poor correlation between signs and symptoms

Due to its multifactorial nature and the poor correlation between signs and symptoms accurate diagnosis and management of DED can prove challenging. Misdiagnosis can lead to substandard management and dissatisfied patients. While there are multiple clinical assessments available 'no single 'gold standard' sign or symptom that correlates perfectly with the DED state has been established' (Wolffsohn et al., 2017). A systematic review of 33 studies was unable to find any consistent, significant associations between specific measures of signs and symptoms in DED and noted low repeatability of some clinical tests (Bartlett et al., 2015). Additionally, treatment may lead to a reduction of patient symptoms without there being any improvement in clinical signs (Nichols et al., 2004b).

It has been suggested that in early/mild DED, corneal hyperalgesia may cause ocular symptoms prior to the evidence of any clinical signs. Later in the disease process, as corneal damage increases, the corneal sensation may decrease leading to fewer reported symptoms (Ong et al., 2018). A recent study to investigate inflammatory biomarkers in patients with mild dry eye characterised by symptoms outweighing signs (DESOS) was unable to conclude why there was a lack of correlation in this group and suggest clarification is still needed as to whether DESOS is only a characteristic in the progression of DED i.e., a pre-clinical phase, or a separate classification (Li et al., 2020a). There is also growing evidence that a subset of dry eye patients may show signs of dysfunctional somatosensory pathways, suggestive of neuropathic ocular pain (Goyal and Hamrah, 2016).

Patients' self-perceived health (female 82.7%) was found to have a strong association with the discordance between signs and symptoms of DED (Vehof et al., 2017). A composite score for signs was calculated from six clinical measurements: tear breakup time, tear osmolarity, Schirmer test, corneal and conjunctival staining and meibomian gland dysfunction. When looking for predictors of greater signs than symptoms, the presence of chronic pain syndrome (due to IBS, fibromyalgia, and chronic pelvic pain), allergy, antihistamine use, depression, osteoarthritis, and antidepressant use were all found to have a significant association.

Ong et al. (2018) re-examined these associations in a population with a high proportion of males (92%). The results of quantitative sensory testing found positive correlations with discordance of dry eye signs and symptoms. The results confirmed the findings of Vehof et al. (2017), that age negatively correlates with dry eye discordance and that comorbidities pose a greater likelihood for discordance.

1.2.5. Lack of effective curative or preventative strategies

Currently DED treatment is largely based around alleviation of symptoms with no curative or preventative strategies available (Inomata et al., 2020). Artificial tears, often the first line of treatment in DED, have been shown to give an improvement in clinical signs, however, large numbers of patients still report continuing symptoms. A questionnaire into the perceptions of dry eye management in clinical practice reported that 85.9% of practitioners who gave artificial tears as a first line therapy, identified a 20% failure rate (Williamson et al., 2014). A survey of US ophthalmologists' perceptions and treatment of patients with moderate to severe DED, found that the majority of participants felt treatment options lacked effectiveness and that a gap exists between artificial tears and effective treatments (Asbell and Spiegel, 2010). They also identified a need for additional options that provide continuous relief of DED symptoms which are acceptable to patients. Additionally, patients can often perceive that their DED is impossible to treat, leading to poor compliance or discontinuation of treatment (Aragona et al., 2020).

1.2.6. Need for evidence-based treatment strategies

The need to identify biomarkers in DED is also fuelled by a lack of strong scientific evidence for current treatments (Saldanha et al., 2017). The study highlighted the need for robust research-based evidence for the long-term benefits and harms that can be associated with treatments for this often, chronic condition. The TFOS DEWS II Management and Therapy Subcommittee (Jones et al., 2017) also identified this lack of evidence and introduced a staged management algorithm according to the two main disease types and severity. Although a very helpful stepwise approach, introduction of novel treatments will require further evidence.

1.2.7. Better inform outcomes of ocular surgery

A better understanding of the pathophysiology of DED is needed when considering the outcomes of ocular surgery. Surgical procedures, such as refractive surgery, may induce or exacerbate DED due to disruption of the corneal nerves. This and other risk factors for iatrogenic DED, such as postoperative topical medications or cataract surgery, can lead to patient dissatisfaction, visual disturbance, and poor surgical outcomes (Gomes et al., 2017). A prospective, multi-centre, observational study of 143 patients prior to cataract surgery, recommended careful monitoring of patients' ocular health pre- and post- surgery due to higher levels of OSD being noted in asymptomatic patients than anticipated (Trattler et al., 2017). The Corneal Clinical Committee of the American Society of Cataract and Refractive Surgery (ASCRS) constructed 'an algorithm for the preoperative diagnosis and treatment

of ocular surface disorders' in order to aid surgeons in diagnosing visually significant OSD, most commonly DED, and delay surgery until a treatment plan has been put in place and improvement found at a follow up visit (Starr et al., 2019).

1.2.8. Predictive, preventative, and personalised medicine

Analysis of tear fluid offers huge potential in the fields of predictive, preventative, and personalised medicine and could facilitate monitoring of a patient's response to therapeutic agents. Tear sampling provides a convenient, minimally invasive method of accessing a body fluid for investigation of biomarkers for both ocular and systemic disease (Hagan et al., 2016). As tear fluid is proximal to the site of interest, its specificity for OSD is increased (Zhou and Beuerman, 2017). Advances in technology allow for microfluidic protein analysis meaning tear samples as little as 4µl can be analysed (Wei et al., 2013). Studies suggest that a panel of biomarkers, rather than one single biomarker, are needed to define the aetiology of DED and distinguish between other ocular surface diseases with overlapping signs and symptoms (Versura et al., 2013). The results of a prospective case control study suggested that a panel of five protein biomarkers would allow for a clear discrimination between control subjects and patients with DED and those with DED due to MGD (Soria et al., 2013).

Inomata et al. propose the use of 'medical big data analyses' taking information from various biological analyses (multiomic studies) and mobile health applications (smartphones data collection) to identify biomarkers and pharmacological targets and so offer a more holistic approach to the management of DED, laying the foundation for personalised and precision medicine (Inomata et al., 2020). However, some resistance to this approach is likely unless patient confidentiality can be ensured.

1.2.9. The pathophysiology of DED: The vicious cycle of inflammation

There is a need for a better understanding of the mechanisms involved in the pathophysiology and chronicity of DED. Advances towards a more targeted approach to DED diagnosis and its management are being made, but a greater understanding of the 'vicious cycle of inflammation' (Craig et al., 2017b) and identification of objective markers of inflammation would allow for better management and therefore, more satisfied patients. The TFOS DEWS II definition highlights three key elements in the aetiology of DED: tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities (Craig et al., 2017b). Numerous extrinsic (e.g., environmental) and intrinsic (e.g., medications, aging) factors can contribute to initiation of the vicious cycle of inflammation. A simple explanation would lead us to understand that tear hyperosmolarity, either through excessive evaporation as in EDE or reduced lacrimal secretion as in ADDE, triggers a series of

acute immune-related signalling events within the ocular surface epithelial cells. This initiates a self-perpetuating cycle of ocular surface inflammation and damage (Bron et al., 2017). However, complex pathways and interactions are involved, which, with further research are gradually being understood.

Tear film instability, from any source, and hyperosmolarity can occur when any part of the lacrimal unit (the ocular surface, lacrimal gland, and their neural connections) is damaged. The production of tears is regulated via a neural reflex or feedback loop (Dartt, 2009). Stimulation of the ocular surface or the nasal mucosa by physical or microbial damage, or pain, sends impulses via the fifth cranial nerve to the lacrimal glands (Rhee and Mah, 2017). Symptoms may or may not develop in the early stages of inflammation and a compensatory reflex tearing may occur to maintain tear osmolarity. It has also been suggested that an increase in tear fluid production may compensate for loss of meibomian glands in individuals with MGD (Arita et al., 2015).

1.2.9.1. Epitheliopathy

Three immune-mediated changes are recognised in the pathogenesis of DED: epitheliopathy, lymphangiogenesis and neuropathy (Ganesalingam et al., 2019). The presence of epitheliopathy is the most easily recognised clinical feature of DED. Due to persistent irritation, the epithelial cells of the ocular surface react to the oxidative stress triggered by hyperosmolarity and to counter the threat, launch mechanisms for immune defence and tissue repair. It is proposed that mitogen-activated protein kinases (MAPKs) play a vital role in the cellular responses to stress (Luo et al., 2004). Activation of these signalling mediators within the epithelial cells, stimulate transcription factors such as nuclear factor (NF)-κB, and result in the increased expression of a variety of pro-inflammatory cytokines and chemokines, such as interleukin IL-1β, TNF-α and IL-8, and a number of matrix metalloproteinases (MMPs) (Li et al., 2006). The inflammatory mediators can activate immature corneal dendritic cells, triggering an upregulation of surface markers of the adaptive immune system, such as major histocompatibility complex (MHC) class II molecules and cell adhesion molecules such as ICAM-1 (Stevenson et al., 2012). These activated or mature ocular surface antigen presenting cells migrate through the afferent lymphatics to the draining lymphoid tissue where they are responsible for priming naïve T cells, leading to induction of helper T cells (Stern et al., 2013), essential components of the cell-mediated or adaptive immune system. The helper T cells then migrate through the efferent vessels to infiltrate the ocular surface, where they secrete additional pro-inflammatory cytokines (Stevenson et al., 2012). Large amounts of cytokines on the ocular surface are thought to cause epithelial cell loss and goblet cell loss (Pflugfelder et al., 2015) leading to reduced production of mucin and causing damage to the epithelial glycocalyx. This results in the familiar picture of punctate

epithelial staining and reduced tear breakup time, exacerbating the state of hyperosmolarity and leading to self-perpetuation of the disease (Bron et al., 2017).

There are three subtypes of T-helper cells, Th1, Th2, and Th17 each with its own set of directing cytokines that can activate a master transcriptional regulator to regulate the expression of a set of 'signature' effector cytokines (Meadows et al., 2016). Meadows et al. (2016) investigated the tears of different subtypes of DED to discover whether the signature cytokines, Th1 (IFN-γ, IL-2), Th2 (IL-4, -5, -13), and Th17 (IL-17) are present in all types of DED. They concluded that differential expression of the various T-cell mediated cytokines exists in different forms of DED (Meadows et al., 2016).

1.2.9.2. Lymphangiogenesis

DED has also been shown, both clinically and experimentally, to induce the expression of factors that promote lymphangiogenesis, the creation of afferent lymphatic vessels. In DED this occurs without haemangiogenesis, the formation of new blood vessels (Stevenson et al., 2012). The newly formed lymphatic vessels are an immune-mediated change that occurs as the need arises, to allow travel of antigens and antigen presenting cells between the ocular surface and lymph nodes. It has been proposed that blockage of lymphangiogenesis could be used therapeutically to prevent rejection of corneal transplants and may provide a further therapeutic strategy for immune-mediated corneal diseases such as DED management (Skobe and Dana, 2009).

1.2.9.3. Neuropathy

Neuropathy or abnormal corneal nerve morphology has been shown in DED, although there is conflicting data regarding changes in corneal sensation and sub-basal nerve density (Cruzat et al., 2017). A healthy cornea has approximately 7000 nerve endings per square millimetre, which are protected between layers of epithelial wing cells (Müller et al., 2003). When hyperosmolarity induces inflammation-mediated epitheliopathy, exposed nerve endings are subject to mechanical and inflammatory insult (Ganesalingam et al., 2019). Exposure to cytokines increases the production of neurotrophic factors that stimulate nerve growth, often resulting in nerves with reduced sensitivity and altered structure. Corneal nerve sprouts, tortuosity and thinning have been observed in DED (Benítez del Castillo et al., 2004), leading to further ocular surface damage and perpetuation of the vicious cycle. Increases in corneal dendritic cell density (antigen presenting cells) have also been found to correlate with dry eye severity (Aggarwal et al., 2020). Cox et al. (2021) found that compromised corneal sub-basal nerves changes were more pronounced in ADDE than EDE.

While the majority of studies support a loss of corneal nerve length in eyes with DED compared to healthy controls, not all studies agree. For example, while D'Souza et al. (2022) reported an increase

in total corneal dendritic cell density in subjects with DED, corneal nerve fibre length and main trunk density were slightly increased. A substantial amount of heterogeneity between studies has been observed with regard to the method of imaging, terminology and sample size (Chiang et al., 2023); standardisation of terminology and analysis method, along with confirmation of diagnostic accuracy is needed to improve clinical application.

Until recently little has been known about the involvement of Substance P in the pathogenesis of DED. However, an experimental dry eye study has demonstrated that it is derived from trigeminal ganglion neurones and released from sensory nerve endings at the ocular surface. Blockage of Substance P signalling has been shown to suppress antigen presenting cell acquisition of MHC class II, reduce Th17 cell activity and therefore reduce DED severity (Yu et al., 2020). This is an area for further investigation, with therapeutic potential.

Therefore, the interactions between inflammatory mediators are particularly complex, with some having been shown to upregulate each other, thus amplifying the inflammatory cascade (Pflugfelder and de Paiva, 2017). Several inflammatory mediators or biomarkers have been identified which will be discussed in more detail (see section 1.5).

1.3. Experimental and animal model studies

Experimental dry eye models have been used to determine the role of inflammation in the pathogenesis of DED. An early study inducing dry eye in mice by inhibiting aqueous production, using scopolamine, and causing desiccating stress by exposure to low humidity and air draft, demonstrated ocular surface epithelial changes mimicking those found in human DED (Dursun et al., 2002). A reduction in tear production and fluorescein clearance, altered corneal epithelial barrier function, reduced goblet cell density, and increased conjunctival epithelial proliferation were all noted. In a similar comparative study, the concentrations of IL-1β and MMP-9 from the tear fluid and IL-1β, and TNF-α from the corneal and conjunctival epithelia, were recorded in control mice and those with induced dry eye. A significant increase in the inflammatory cytokines and MMP-9 was found and activation of the MAPK pathways was demonstrated (Luo et al., 2004). Also using a murine model, Luo et al. (2005) demonstrated that hyperosmolar saline acts as a proinflammatory stress on the ocular surface and activates three MAPK intracellular signalling pathways. Further to this, human limbal epithelial cultures showed increased production of pro-inflammatory cytokines and chemokines when exposed to increasing hyperosmolarity (Li et al., 2006).

A study to determine the role of MMP-9 in corneal barrier disruption compared its concentration in the tear fluid of MMP-9 knock-out mice with wild-type counterparts; the MMP-9 knock-out mice were

resistant to corneal barrier disruption, thus proving MMP-9s role in the corneal epithelial barrier's function (Pflugfelder et al., 2005). Corneal epithelial disruption gives rise to the ocular irritation experienced in DED and it was suggested that this occurs due to increased loss of tight junctions (zonula occludins) in the differentiated apical corneal epithelial cells, with a resultant increase in permeability (Pflugfelder et al., 2005). De Paiva et al. (2006b) investigated the effects of two anti-inflammatory agents, the corticosteroid (methylprednisolone) and a tetracycline (doxycycline) on corneal epithelial disease. Utilising a similar murine experimental dry eye model, they demonstrated that both agents reduced the expression and activity of MMP-9, decreased the levels of inflammatory cytokines and reduced activation of MAPKs in the corneal epithelium.

Murine dry eye models have also been used to explore the role and significance of immune-mediated goblet cell loss in aqueous deficient DED. In addition to producing mucins to stabilise the tear film, goblet cells produce immunoregulatory factors, such as retinoic acid, which suppress cytokine production and maturation of phagocytic cells (immune defence cells) in the corneal stroma (Alam et al., 2020). Murine studies have shown that increased presence of cytokines, such as IFN-γ, are associated with secretory dysfunction and conjunctival goblet cell loss, and therapies to suppress these mediators can increase goblet cell density (Zhang et al., 2013). Pflugfelder et al. (2015) found IFN-γ expression in the conjunctiva was higher in aqueous deficiency and correlated with goblet cell loss and severity of conjunctival disease, supporting the findings of previous animal studies (Pflugfelder et al., 2015). The development of accessible clinical tests to evaluate these biomarkers (goblet cell numbers, retinoic acid metabolising enzymes in the conjunctiva or IFN-γ in the tears) would allow patients who would benefit from topical therapy to suppress IFN-γ production by the innate immune cells of the conjunctiva, to be identified (Alam et al., 2020).

1.4. Biomarkers used in the clinical diagnosis and monitoring of DED

When diagnosing DED, it is usual for a battery of tests, with the least invasive first, to be used in conjunction with self-reported symptom-based questionnaires such as the Ocular Surface Disease Index questionnaire (OSDI) (Wolffsohn et al., 2017). Significant advancements have been made in developing methods to assist eye care practitioners in the diagnosis, subclassification and monitoring of DED; early identification enabling prompt intervention and therefore better patient outcomes. When looking for signs of DED, whether it be ADDE, EDE or a combination of both, the clinical tests available can be broadly divided into those that evaluate the integrity of the ocular surface and those that consider tear production/quality. Several imaging and molecular biomarkers which evaluate these characteristics have been investigated for clinical use.

1.4.1. Imaging biomarkers (in vivo)

A variety of 'imaging biomarkers' giving automated, objective measurements, are becoming increasingly available, not only in research clinics and hospitals but also to high-street optometrists with a role in managing DED. It is important that any biomarker provides the specificity needed to differentiate between DED and other OSD, allows for analysis of the severity and be validated to ensure it is fit for purpose (Binotti et al., 2020).

Imaging biomarkers can be used to assess the tear films stability, quality, and volume, investigate the condition of the meibomian glands (infrared meibography) and explore the microanatomy of the cornea, thus helping in subclassification (Villani et al., 2020). Imaging devices which perform automated functions, such as NITBUT, TMH, and ocular redness scores, help to remove some of the subjective bias of these measurements.

1.4.1.1. In vivo confocal microscopy (IVCM)

IVCM allows for a minimally invasive, high-resolution assessment of the cornea and is currently used to provide information at a cellular level. IVCM allows qualitative and quantitative evaluation of the corneal nerves of the sub-basal plexus. Corneal IVCM has demonstrated differential changes in dendritic cell density, size and number between subtypes of DED, being more pronounced in ADDE (Cox et al., 2021), and particularly when underlying immune conditions were present (Kheirkhah et al., 2015). Central corneal sub-basal dendritic cells show potential as a marker of inflammation due to their role as antigen-presenting cells.

Dendritic cell density has been shown to correlate with the clinical severity of DED and morphological changes in dendritic cells have been observed in severe cases (Aggarwal et al., 2021). However, at present IVCM is an emerging technology and is currently only available in a research setting (see section1.2.9.3).

1.4.1.2. Lipid interferometry

Lipid interferometry is an established, non-invasive clinical examination to investigate the quality of the tear film, utilising lipid layer patterns and thickness, particularly useful in diagnosis of EDE. The Guillon grading scale (Guillon, 1998) is a useful tool in practice and describes the visibility and thickness of the lipid layer from a thin 'open meshwork' (grade 1) layer, through 'closed meshwork' (grade 2), 'wave pattern' (grade 3), 'amorphous' (grade 4), 'colour fringes' (grade 5) to a thick, irregular layer 'abnormal colour fringes'. Visualisation of the kinetics of the lipid/aqueous layers, although somewhat subjective, has been shown to facilitate assessment of tear film stability and therefore identification of the dry eye subgroup (Arita et al., 2016b). The Lipiview® Interferometer was the first automated

instrument available in practice and has been used to evaluate the correlation between lipid layer thickness (LLT) and MG function in patients with MGD. Recently, a prospective, cross-sectional study of three age groups, confirmed the findings of previous studies, that LLT is positively correlated with meibomian quality/expression and that LLT increases with age and should therefore be considered when analysing data (Li et al., 2020b).

1.4.1.3. Tear film breakup time (TBUT)

Tear film breakup time (TBUT) using sodium fluorescein to enhance visibility, has been a mainstay in the assessment of tear film stability for many years. In recent years there has been a move towards less invasive methods, which has seen the development of several commercially available automated, non-invasive (NI) systems. Utilising video-keratography to analyse for changes in the interblink appearance of reflected Placido rings, TBUT can be detected, mapped and data stored (Villani et al., 2020). However, some variation in measurements between devices have been reported for NITBUT and there has been some disagreement between studies comparing TBUT with NITBUT as to the diagnostic cut-off values (Binotti et al., 2020). The TFOS DEWS II diagnostic methodology committee have defined a cut off value of less than or equal to 10 seconds as an indicator for DED diagnosis, with 82% to 84% sensitivity and 76% to 94% specificity (Wolffsohn et al., 2017).

1.4.1.4. Tear-film orientated diagnosis (TFOD)

Interestingly, the Asia Dry Eye Society advocate the concept of tear-film orientated diagnosis (TFOD) and have proposed that the pattern of fluorescein tear breakup can be used to distinguish between three types of dry eye; aqueous deficient, decreased wettability, and increased evaporation, with ‘line’ or ‘area’ breaks, ‘spot’ or ‘dimple’ breaks, and ‘random’ breaks occurring respectively and corresponding to the aqueous, mucins and lipids (Tsubota et al., 2020). Although this provides a simple, practical method of evaluating the tear film, accessible to all, it remains prone to subjective bias and reiterates the need for exact precision in quantifying each layer of the tear film.

1.4.2. Imaging biomarkers (ex vivo)

1.4.2.1. Tear ferning

Tear ferning is a simple, visual method of observing the salt and protein patterns formed by crystallised tears. The tear fern pattern has been found to vary depending on the composition of the tear sample, with healthy tears producing a full dense fern pattern whilst a fragmented or absent fern pattern is noted in dry eye (Masmali et al., 2014). A prospective, randomised, controlled clinical study in healthy patients found agreement between the subjective analysis and a novel computer-based objective analysis of tear ferning grades (Dutta et al., 2019).

1.4.2.2. Extensional viscosity

A different approach to examine the integrity of the tear film was explored in a study to quantify the extensional viscosity of the tears using acoustically driven microfluidic extensional rheometry (McDonnell et al., 2019). The data suggested that changes in the tear film composition that can occur in DED, including changes in various lipids, proteins, and electrolytes, can impact its extensional properties. However, further investigation of its use in the different dry eye subclassifications, and consideration of variables such as diurnal variation and inter-day measurements will be needed before this method can be validated for clinical use.

1.4.3. Point-of-care tear film or molecular biomarkers

1.4.3.1. Tear Osmolarity

With an emphasis being placed on the loss of homeostasis of the tear film, many studies have investigated the potential of the tears themselves as biomarkers of DED. A biomarker which has received a significant amount of interest is tear osmolarity. Hyperosmolarity is mainly due to increased electrolyte concentration (Na+, K+, Ca2+) in the tears. Loss of homeostasis due to hyperosmolarity has been shown to be a causative mechanism in the pathogenesis of DED (Baudouin et al., 2013). Its measurement has gained popularity since the US Food and Drug administration (FDA) approved the TearLab® device in 2009, a commercially available point-of-care or "lab on a chip" test to diagnose dry eye. Measurement of osmolarity is attractive because it provides an objective numerical measurement which can be monitored. The TearLab® device enables an almost instant reading of osmolarity via collection of a small tear sample (50nL) from the temporal lower tear meniscus. Utilising electrical impedance, the tear fluid sample passes through a tiny channel in a single use chip which is embedded with gold electrodes.

The I-Pen osmolarity system (I-MED Pharma Inc, Dollard-des-Ormeaux, Quebec, Canada) is also considered a suitable method for clinical assessment of DED and available in the UK. The I-PEN®, used in conjunction with I-PEN® Single Use Sensors (SUS) measures tear osmolarity by measuring the electrical impedance of palpebral conjunctiva tissue rather than the tears themselves. In laboratory testing, measurements taken with the I-Pen were higher than those measured by with the TearLab® device (Rocha et al., 2017). However, when adopting a cut-off value of 318 mOsm/L, the I-Pen was able to differentiate 90.9% of DED patients (sensitivity) and 90.6% of normal subjects (specificity) (Park et al., 2021); tear osmolarity was negatively correlated with tear break-up time, and the Schirmer test, and was positively correlated with OSDI symptom score.

However, opinions have varied regarding the use of tear osmolarity as a marker to diagnose, grade and track therapeutic response in DED. Lemp et al. (2011) supported the opinion that tear osmolarity

measurement (measured with the TearLab® device) provides the best single metric to diagnose DED and allows for differentiation of mild or moderate dry eye from severe cases. The multicentre, prospective, observational study measured six objective tests for DED and found that tear osmolarity outperformed the others when sensitivity and specificity were used as the criteria. They concluded that a cut-off threshold greater than 308mOsm/L should be used for the most sensitive differentiation of normal to mild/moderate cases, and whilst other cut-off thresholds have been proposed, this has now been widely accepted (Willcox et al., 2017). A cut-off value of 316mOsm/L to differentiate moderate to severe DED, derived through meta-analysis, has also been accepted (Tomlinson et al., 2006).

A positive correlation between osmolarity and dry eye severity, associated with higher OSDI, discomfort sub-scores and corneal and conjunctival staining scores has been shown (Mathews et al., 2017) and agrees with the findings of other studies (Suzuki et al., 2010). Mathews et al. (2017) also reported that patients with more clinically significant dry eye showed more inter-eye variation than the patients with symptoms only or controls, thus confirming the greater than 8mOsm/L inter-eye difference considered significant by Sullivan (Sullivan, 2014).

Conversely, whilst acknowledging the importance hyperosmolarity plays in the development of DED, some criticism has been levelled at its repeatability and correlation with other clinical signs of DED. An early study of tear film osmolarity, measured with the TearLab® showed no correlations with six clinical signs of dry eye (Messmer et al., 2010). The test was not found to discriminate between patients with dry eye (308.9 ± 14.0 mosm/l) and the control group (307.1 ± 11.3 mosml/l) and further investigations were recommended before advocating it for daily clinical practice.

Despite these conflicting findings, use of tear film osmolarity is broadly supported in literature (Potvin et al., 2015). It is listed in the TFOS DEWS II Diagnostic Methodology report as a recommended appropriate test for diagnosis and assessment of dry eye. The report highlights the findings of Lemp et al. (2011), that variability in measurements should be treated as a possible indicator of DED (Wolffsohn et al., 2017). Osmolarity measurement also forms part of the American Academy of Ophthalmology Preferred Practice Patterns (Akpek et al., 2019) and is an 'essential' diagnostic test according to the American Society of Cataract and Refractive Surgery (ASCRS) Clinical Committee Algorithm for OSD (Starr et al., 2019).

1.4.3.2. Tear MMP-9

The InflammaDry® lateral flow device detects the inflammatory biomarker matrix metalloproteinase MMP-9 which is known to play a role in ocular surface inflammation and tissue breakdown that can occur in DED (Li et al., 2001). Its introduction was welcomed due to its rapid results and good sensitivity

and specificity. A sensitivity of 85%, specificity of 94%, negative predictive value of 73%, and positive predictive value of 97% have been demonstrated when compared with clinical assessments (Sambursky et al., 2013). MMP-9 activity has been shown to increase in proportion to dry eye severity and patients without DED had average levels of 8ng/mL (Chotikavanich et al., 2009). InflammaDry® only gives a positive result when MMP-9 levels reach 40ng/ml or more. This and the low positive test results found in studies of mild dry eye (Schargus et al., 2015) have led to the suggestion that it is better at detecting moderate to severe dry eye. Lanza et al. (2016) found no differences in the dry eye profiles of the 39% of their study participants who tested positive for MMP-9 with InflammaDry. Additionally, a positive InflammaDry® result does not distinguish between inflammation in DED or that caused by some other ocular surface disease, and so needs to be used in conjunction with other clinical tests.

InflammaDry® is suggested among the battery of tests recommended in the ASCRS Clinical Committee Algorithm, to identify patients who would benefit from anti-inflammatory therapy (Starr et al., 2019). An analysis of its use as a prognostic tool for predicting which patients would benefit from topical treatment with cyclosporine showed more favourable responses in those patients who had tested positive (Park et al., 2018). The signal intensity of a positive test result was also graded and shown to correlate well with the concentration of MMP-9 in the sample. Jun et al. (2020) used this to demonstrate a positive correlation between TMH and test band density, with denser test bands in the higher TMH groups than in the low and normal TMH groups. From this, they warn that InflammaDry® test results could be influenced by tear volume and could therefore result in false negatives in ADDE or false positives due to reflex tearing.

1.4.3.3. Lactoferrin

In 2020, the first meta-analysis of lactoferrin concentration in human tears was undertaken, which highlighted its potential role as a diagnostic biomarker for ocular diseases (Ponzini et al., 2020). Low levels of this lacrimal gland produced tear protein**,** which has anti-microbial and anti-inflammatory properties, have been understood to indicate ADDE since the 1990s (McCollum et al., 1994). A study of tear protein profiles in patients with dry eye, measured a mean lactoferrin concentration of 2.05 ± 1.12 mg/ml in control subjects, 0.69 ± 0.55 mg/ml in non-Sjögren's syndrome patients, 0.13 ± 0.22 mg/ml in patients with Sjögren's syndrome, and 0.26 ± 0.33mg/ml in Stevens-Johnson syndrome patients (Ohashi et al., 2003). Versura et al. (2013) also demonstrated, via electropherograms obtained using the Agilent 2100 Bioanalyzer, a significant reduction in the total tear protein content, including lactoferrin, in the tears of patients with early DED.

The TearScan® 300 Tear Analyzer (Advance Tear Diagnostics) is the only other currently available commercial point-of-care device for detecting tear biomarkers. The TearScan® Lactoferrin Diagnostic Test Kit uses a lateral assay which allows for differentiation between dry eye due to reduced tear secretion (ADDE) and that caused by rapid tear evaporation (EDE). An additional test is also available for IgE proteins, thus allowing for a differential diagnosis between dry eye and allergy. The system uses 0.5 µl of basal tears, collected by micropipette, which are diluted and loaded onto a test cassette. Any result below 0.9 mg/ml is indicative of tear-deficient dry eye. This is based on a study which found the average tear lactoferrin level to be 1.5 mg/ml (range: 0.9 to 2 mg/ml) (Zhou et al., 2012).

More recently, lower lactoferrin levels have been associated with greater severities of MGD, indicated by reduced eyelid meibomian gland expressibility and greater dry eye and allergy symptoms (Chao and Tong, 2018). It is proposed that the ocular surface inflammation associated with MGD may be sufficient to reduce tear lactoferrin production. Monitoring of tear lactoferrin would appear to be a useful strategy to employ in the early diagnosis and classification of DED.

1.5. Biomarkers from laboratory tear analysis

At present the number of commercially available biomarker devices is limited. However, studies show that there are numerous pro and anti-inflammatory biomarkers involved in the pathogenesis of DED, which can be identified and explored through tear sampling. Advances in metabolomic, proteomic, and lipidomic technology have allowed exploration of the tear fluid and broadened knowledge of its chemical composition.

Metabolomics has been used to extract molecular information i.e. the intermediate or end products of metabolism, from human tears (Benítez Del Castillo et al., 2021). Different metabolomic profiles have been found between individuals with and without dry eye and between different age groups (Jiang et al., 2020). Among the 51 metabolites investigated, 48 appeared to contribute to the incidence of dry eye. Analysis indicated that the main metabolic pathways included glucose, amino acid, and glutathione metabolism. It remains to be seen if information gained through metabolomics could prove useful in the accurate diagnosis of DED, prediction of prognosis, and development of personalized therapies.

The human tear proteome has been classified into the following groups (Zhou and Beuerman, 2012):

- Proteins secreted by the lacrimal gland, meibomian glands, goblet cells and accessory lacrimal glands i.e., indigenous proteins (Mann, 2007)
- Ocular cell/tissue proteins or serum proteins present in the tear fluid through leakage of normal secretions or due to cell damage or death

- Proteins released from diseased tissue which may be used as diagnostic biomarkers
- Foreign proteins e.g., protein released from an infectious organism

Tear proteins may also be classified according to their abundance; the bulk of the tear protein concentration is made up of a few high abundance lacrimal proteins (mg/ml to µg/ml): lysozyme, lipocalin, lactoferrin, secretory IgA, and plasma-derived albumin. These proteins make up around 80% of the total tear protein and have a molecular weight in the range of 14 to 147 kDa (Mann, 2007). Proteins such as lysozyme, lipocalin, lactoferrin are regulated proteins, having a level of production that varies according to tear flow rate. Secretory or constitutive proteins such as the immunoglobulins, and serum-derived proteins such as albumin, are more likely to vary with any subtle change in tear flow rate due to their constant rate of production (Ng et al., 2000).

Proteins secreted by the ocular surface tissue or cell signalling molecules tend to be of moderate abundance (µg/ml to ng/ml) and cytokines and growth factors in low abundance (ng/ml to pg/ml) (Zhou and Beuerman, 2012). These less abundant cytokines and chemokines have a wide variety of molecular weight ranges from approximately 6 to 70 kDa (Stenken and Poschenrieder, 2015).

Close to 1,800 proteins of various concentrations have been identified in the tears, using liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Zhou et al., 2012). However, by combining study data, 3,724 unique proteins (1% False Discovery Rate in each study) have been identified in tear fluid, with 1,397 (60%) unique proteins only identified in Schirmer's strip samples, and 60 (3%) in tear samples collected with a capillary tube (Ma et al., 2021) A reduction in the total tear protein content and a decrease in proteins with antibacterial and protective functions in early DED has been shown (Versura et al., 2013). A decrease in proteins with lipid-binding functions and an increase in some pro-inflammatory proteins was also found.

Several methods of tear collection are available for laboratory analysis; the methods used in this thesis and the pros and cons of different methods are discussed in section 3.2.4. Different methods of tear analysis are also reported in sections 3.2.6, 3.4.2 and 6.2.3. The tables of individual cytokine and chemokine studies described later in this section indicate the methods of tear collection and analysis used.

Tear proteins which have been studied in relation to different subtypes of DED are described in the following sections, starting with the more abundant lacrimal proteins.

1.5.1. High abundance tear proteins

The high abundance tear proteins can be described as 'indigenous' or 'lacrimal gland antimicrobial proteins' (AMPs). Studies have suggested a synergistic relationship between these antimicrobial molecules to provide protection to the ocular surface (McDermott, 2013).

1.5.1.1. Lysozyme and lactoferrin

Lysozyme is an antibacterial enzyme involved in innate immunity, which acts by destroying the cell walls of certain bacteria. It is one of the earliest proteins, produced in the lacrimal glands, to be studied in relation to DED. Its presence in the tears was identified by Alexander Fleming nearly 100 years ago (McDermott, 2013).

Also produced in the lacrimal gland, the concentration of lactoferrin provides a good index of its function; reduced levels (below 0.9mg/mL) have been used commercially as an indicator of ADDE (see section 1.4.3.3). A multifunctional iron-binding glycoprotein, it is also involved in innate immunity and plays a vital role in ocular health (McCollum et al., 1994). It can inhibit bacterial growth, is thought to prevent pro-inflammatory pathway activation, and have a significant impact on the development of adaptive immune responses (Siqueiros-Cendón et al., 2014). It has also been shown to inhibit biofilm formation and thus may play a role in protecting contact lens surfaces from colonization (Flanagan and Willcox, 2009).

Reduced levels of lysozyme and lactoferrin have been found in patients with ADDE, EDE and MDE and have been associated with higher infection rates (Di Zazzo et al., 2019). A literature review indicated that lysozyme, lactoferrin and lipocalin were all decreased in dry eye patients, although lysozyme was unchanged in some studies (Narayanan et al., 2013). This, and the fact that a single protein lysozyme test was found to be insufficient for diagnosis of DED (Mackie and Seal, 1986) may account for the lack of further interest in lysozyme as a biomarker for DED. A five-tear protein ELISA test to assay for five tear proteins, including lysozyme, lactoferrin, ceruloplasmin, IgA and IgG, developed by Mackie and Seal (1986), gave a more accurate assessment of a patient's dryness. Although lysozyme may allow for early detection of DED, it has not been found to be helpful in differentiation of DED.

1.5.1.2. Lipocalin

Lipocalin is also produced by the lacrimal gland and has a variety of functions including the binding and release of lipids, regulation of tear viscosity and anti-inflammatory activity (D'Souza and Tong, 2014). It has a low molecular weight of 18.2 kDa, in the same region as lysozyme (14.2 kDa) (Mann, 2007). A reduced concentration has been found in patients with obstructive and seborrheic MGD (Yamada et al., 2005). Its concentration correlated negatively with fluorescein staining scores and

positively with TBUT, and it has been suggested that its deficiency may be a predisposing factor in the development of MGD symptoms (Yamada et al., 2005). A positive correlation with tearing and heaviness of the lids has been shown in MGD patients when compared to controls (Tong et al., 2011). More recently, a prospective case-control study comparing stable SS patients with non- Sjögren's DED controls, found significantly lower levels of lipocalin in the SS-DED group (Kuo et al., 2019). The study also reported a significant correlation with clinical performance in the Sjögren's group. However, as this only appeared to be the case for NIKBUT, they suggested that more research was needed before lipocalin could be recommended as a biomarker for monitoring Sjögren's-DED.

1.5.1.3. Zinc-alpha-2- glycoprotein (ZAG)

Zinc-alpha-2- glycoprotein (ZAG) is found in several body fluids, secreted by the epithelia. It is known to stimulate the breakdown of fats by hydrolysis to release fatty acids (lipolysis) in adipocytes (fat cells) and its increase has been associated with the extreme weight loss that may occur with some forms of cancer (Hassan et al., 2008). It is thought to play a role in the expression of the immune response, however, its specific role in tears is not known (Versura et al., 2012). Its presence in the tears was validated by Versura et al. (2010). While no differences in its concentration were reported when comparing subjects with or without EDE, a later study reported reduced levels in DED subjects (Versura et al., 2013). Along with elevated levels of lysozyme, it has also been identified as a biomarker in thyroid eye disease (Aass et al., 2017) and is downregulated in keratoconus (Lema et al., 2010), intolerant CL wearers (Giannaccare et al., 2016), and fungal keratitis (Parthiban et al., 2019).

1.5.1.4. Secretory immunoglobulin A (sIgA)

Secretory immunoglobulin (sIgA) is a major AMP present in the tear film which prevents pathogen adhesion to the ocular mucosal surface epithelial cells and enhances phagocytosis (Willcox and Lan, 1999). It is a glycoprotein produced by B-lymphocytes as part of the adaptive immune system and is continuously secreted by the plasma cells of the lacrimal glands. It has a constant level of production and therefore its concentration decreases during increased tear production (Fullard and Tucker, 1991). Local production has been shown to occur in both the conjunctiva and the lacrimal gland (Knop et al., 2008). Its concentration has been shown to increase dramatically during prolonged eye closure, suggesting a possible protective role against the sub-clinical inflammation that can occur during sleep (Sack et al., 1992). It is thought to be reduced in the tear fluid of dry eye patients, but there has been no consistency in studies (Narayanan et al., 2013)

1.5.1.5. Proline-rich proteins

Proline-rich proteins also have anti-microbial properties and are thought to have a protective function on the ocular surface (Zhou and Beuerman, 2017). Lacrimal proline-rich protein 4 (LPRR4) was found to be reduced in ADDE and cases with combined ADDE and EDE, when compared to healthy controls (Boehm et al., 2013). A significant down regulation in all types of dry eye cases has been found to correlate with clinical measurements of disease severity and therefore it has been suggested as a biomarker of DED (Aluru et al., 2012).

1.5.1.6. Albumin

Although albumin is present in the tears due to vascular leakage from plasma serum via the blood-tear barrier (Aronson et al., 1971), it can be found in high abundance. As such, it can be used as a marker of loss of integrity of the blood-tear barrier and vascular leakage which have been shown to be increased in inflammatory ocular disorders, although it is uncertain whether it leaks passively due to ocular stress or is part of ocular homeostasis, being part of the adaptive immune system (Runström et al., 2013). It also plays a role in transport, stabilization of osmotic pressure and increases blood viscosity. Diurnal variation has been found in its concentration, which is highest on waking, stable during the day and increased in the evening (Runström et al., 2013). Albumin has been shown to suppress apoptosis of corneal cells in dry eye animal models and has therefore been shown to have potential therapeutic effect (Higuchi et al., 2007), (see section 1.7.6 biological tear substitutes).

1.5.1.7. Immunoglobulin G (IgG)

The immunoglobulin, IgG is also serum derived and present in the tear fluid in much smaller concentrations than sIgA. It is involved in immunoregulation in acquired immunity (Mahmoudi, 2009), but its exact role in the tears is unknown. Raised levels of IgG have been found whilst investigating the effect of contact lens wearer on the tear film (Mann, 2007). Low levels were present prior to the onset of redness and inflammation but increased during an inflammatory episode. IgG has therefore been suggested as a useful diagnostic marker of inflammation and increased vascular permeability (Mann, 2007). To date, there appears to be little research into its potential involvement in the vicious inflammatory cycle of DED.

1.5.1.8. Mucins

Mucins are high molecular-weight, hydrophilic glycoproteins produce by corneal and conjunctival epithelia and the lacrimal glands to maintain fluid on the eye’s surface. Of the twenty or more human mucin genes at least nine have been shown to be expressed on the ocular surface (Corrales et al., 2011). They are mostly categorised into three distinct families: gel forming mucins, soluble mucins

(both secretory mucins) and transmembrane mucins, according to their amino acid sequence (Gipson et al., 2004). Secreted mucins are thought to play a role in lipid organisation at the surface of the tear film, but further research is needed to prove this hypothesis (Willcox et al., 2017).

It is important that mucin production is tightly regulated as both too much and too little mucin can have a negative impact on tear film stability. Changes in the mucin layer may lead to increased evaporation, eventually contributing to hyperosmolarity and associated ocular surface inflammation. Tear ferning can be used as a non-invasive method of assessing mucous deficiency. However, it is not widely used due to the subjective nature of grading the patterns (Baudouin et al., 2019).

1.5.1.8.1. Gel-forming mucins

The gel forming mucins MUC2, MUC5AC, MUC5B, MUC6 and MUC19 are produced by specialised conjunctival goblet cells. MUC5AC, a large gel-forming mucin is the most abundantly expressed of these mucins and plays an important role in lubrication, hydration and barrier formation/protection (Zhang et al., 2013). An early study using tear samples obtained from Schirmer strips found that the mean MUC5AC concentration in tears was lower in the dry eye patients than in age and gender-matched healthy individuals (Zhao et al., 2001). This finding was confirmed by Argüeso et al. (2002) who also demonstrated a significant reduction of MUC5AC mRNA using conjunctival impression cytology. A study of office workers using visual display terminals reported a relationship between MUC5AC concentration in the tears and dry eye (Uchino et al., 2014). MUC5AC was lower in the workers with DED compared to those without, participants who worked longer hours, and those who had symptomatic eyestrain. A later study demonstrated significantly lower levels of MUC5AC and increased levels of IL-6 in a severe symptom group compared with a mild symptom group using subjective symptoms (OSDI score) (Zhang et al., 2013).

1.5.1.8.2. Soluble mucins

MUC7, MUC8 and MUC9 are soluble secretory mucins. MUC7 is expressed by the lacrimal gland acini and conjunctival epithelial squamous cells. Despite its mRNA having been demonstrated in these tissues, it has not been detected in human tears, possibly due to low concentrations, and its function in the tear film is not yet understood (Baudouin et al., 2019). Messenger RNA for other mucins has also been found in ocular surface epithelial cells but not yet identified in the tears.

1.5.1.8.3. Trans-membrane mucins

Membrane-associated mucins or trans-membrane mucins are expressed in the superficial cells of the cornea and conjunctival stratified epithelial cells and include MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, and MUC21 (Corrales et al., 2011). MUC1, MUC4 and

MUC16 are major components of the epithelial glycocalyx, a dense coating anchored to the epithelial cells forming a protective barrier responsible for maintaining surface integrity and tear film stabilisation (Argüeso, 2020). Several studies have noted changes in the concentration of these mucins in tears of dry eye patients, but some disagreement exists around the amount and direction of change.

Increased levels of MUC16 and MUC1 mRNA were found in the tears and conjunctiva of patients with SS when compared with other forms of ADDE and healthy controls (Caffery et al., 2008;Caffery et al., 2010). Increased soluble MUC1 expression was also found in non-SS DED compared to controls whereas no differences between these groups was found for MUC16. However, a nine- year prospective study of patients with moderate to severe non-SS DED found expressions of MUC1, MUC2, MUC4 and MUC5AC in the conjunctival epithelium to be lower than in normal subjects (Corrales et al., 2011).

Galectin-3 (a β-galactoside-binding lectin) is thought to be involved in the binding of transmembrane mucins on the apical glycocalyx of the ocular surface epithelial cells. It has been proposed as a diagnostic biomarker (Argüeso, 2020). Its concentration in tears has been reported to be significantly higher in patients with DED.

1.5.2. Moderate abundance proteins

Tear proteins secreted by the ocular surface tissue or cell signalling molecules are thought to be moderately abundant in the tears (µg/ml to ng/ml) (Zhou and Beuerman, 2012).

1.5.2.1. Matrix metalloproteinases (MMPs)

MMPs are a family of at least 23 proteases, or proteolytic enzymes, whose function is to breakdown or degrade the peptide bonds of extracellular matrix proteins (Klein and Bischoff, 2011). They are involved in angiogenesis, inflammation, wound repair, and tissue remodelling (Li et al., 2001). Several MMPs (1,3,9,13) have been found to be increased in the corneal epithelium in response to hyperosmolar stress (Li et al., 2004). Experimental dry eye models confirmed MMP-9's role in degeneration and loss of the corneal epithelial barrier as MMP-9 knock-out mice showed less response to desiccating stress than the control mice (Pflugfelder et al., 2005). MMP-9 is normally expressed at low levels in the ocular surface epithelia and is involved in tissue remodelling. However, at high levels it acts on proteins in the corneal epithelium basement membrane and tight junction proteins (occludins) leading to an irregular surface and cell apoptosis. MMP-9 (or gelatinase B) is believed to be an early marker of inflammation and has been shown to be elevated within 2 hours of an inflammatory stimulus (Kolaczkowska et al., 2008). When it is secreted in its proenzyme form it is bound to tissue inhibitors of metalloproteinases (TIMPs) and activated by other extracellular

proteinases (Klein and Bischoff, 2011). Concentrations of MMP-9 are usually negligible during the day and completely inhibited by TIMP-1. On waking MMP-9 shows a 200-fold increase in concentration, which suggests that other regulatory mechanisms are at play to prevent excess extracellular matrix degradation (Markoulli et al., 2012b).

Table 1.1. Association of MMP-9 with different subtypes of DED

Methods	SS-DED (ADDE)	Non-SS-DED (ADDE)	MGD (EDDE)	Combined ADDE/ EDDE	Controls	Correlations with signs/symptoms	Reference
BL, WK, ELISA	↑	↑	↑ (associated with rosacea)		Y		Solomon et al. (2001)
BL, Sp, ELISA		↑			Y		Acera et al. (2008)
BL, MC, ELISA				4 groups of increasing severity. MMP-9 ↑with ↑severity	Y	↓TBUT ↑Symptom scores ↑OS staining	Chotikavanich et al. (2009)
SC 1, MX					Controls only	↓SC 1 ↑Osmolarity	VanDerMeid et al. (2012)
Desiccating stress				↑	Y ↑	↑Corneal staining and TBUT in both groups	(López-Miguel et al., 2014)
SC 1, ELISA, InflammaDry®					20 Px with undiagnosed DED	↑Corneal staining No other correlations	Schargus et al. (2015)
InflammaDry®					Compared subjects with +ve versus -ve InflammaDry® results	No statistically significant correlations found	Lanza et al. (2016)
InflammaDry®				↑ in 40% of DE patients	Y	↑OSDI score ↓TBUT <5sec ↑OS staining ↓SC 1	Messmer et al. (2016)
FL, SC 1, ELISA			↑		Y	↑OS discomfort ↓TBUT ↓SC 1 ↑cDCD	Khamar et al. (2019)
FL, MP, ELISA	↑in stable SS-DED compared to non-SS				N	↑redness in SS group ↓ central TMH in SS group	Kuo et al. (2019)
InflammaDry® Osmolarity	63.5% of patients ↑				N	No correlations with DE indices	(Kook et al., 2020)

Types of tears: *BL=basal, RX=reflex, FL=flush.* ***Collection method***: *SC 1=Schirmer without anaesthetic (basal + reflex tears), SC 2= Schirmer with anaesthetic (basal tears) MC= microcapillary, MP=micropipette, WK=wick, Sp=sponge. OS=ocular surface*
Method of analysis: *MA= microarray, ELISA= enzyme-linked immunosorbent assay, MX= multiplex bead analysis, OSDI= ocular surface disease index.*
↑*= increase,* ↓*= decrease, Y= yes, N= no, cDCD= corneal dendritic cell density, TMH= tear meniscus height*

An early study comparing the MMP-9 concentration in the tears of dry eye patients with rosacea-associated MGD, SS-DED and controls found a significant elevation in the non-control groups (Solomon et al., 2001). Significantly elevated levels of MMP-9 have been shown in ocular allergy, conjunctivochalasis, blepharitis and dry eye (Acera et al., 2008). A study involving patients with newly diagnosed DED was able to show progressively higher levels of tear MMP-9 corresponding to increasing clinical levels of severity (Chotikavanich et al., 2009). As previously discussed, InflammaDry®, a point-of-care device, allows for detection of significant levels of inflammation in DED.

MMP-9 plays a crucial role in the disruption of the ocular surface epithelial barrier, a hallmark finding of DED, and its secretion is further promoted by IL-17 stemming from the adaptive immune response that later ensues (De Paiva et al., 2009). It has been the subject of extensive research in DED and correlations with some clinical measures have been noted (see ***Table 1.1***). The strongest of these would appear to be ocular surface staining, which is logical when considering the role of MMP-9 in the corneal epithelial barrier. However, Schargus et al. (2015) found no other significant correlations and a subsequent study found no statistically significant correlations when comparing subjects with and without a positive InflammaDry® result (Lanza et al., 2016).

1.5.3. Low abundance tear proteins: Cytokines and chemokines

Cytokines and chemokines play an essential role in the regulation and maintenance of inflammatory processes and their presence in the tears has been demonstrated in multiple studies of ocular surface disease. Basal, unstimulated secretion also occurs providing an 'immune tone' of cytokines and chemokines in normal tears (Carreño et al., 2010). Cytokines and chemokines can be difficult to detect due to their low levels in the circulation. However, Carreño et al. (2010) were able to report on the concentrations of 30 cytokines in the tears of healthy subjects using separate multiplex bead-based assays.

When considering studies of different cytokines/chemokines in DED, a review noted significant interstudy variation in biomarker concentrations in both DED and among non-DED patients (Roy et al., 2017). The review focussed on four cytokines which have been found to be consistently elevated in DED patients when compared to normal controls: TNF-α, IL-6, IL-17A and IL-8. Although some variations could be attributed to biological variations, it was suggested that variations in methodology, instrumentation and method of analysis all play a role, making direct comparisons between studies difficult (Roy et al., 2017).

Cytokines are a group of proteins, peptides or glycoproteins which are secreted by T cells as part of cell-mediated immunity. They are signalling molecules that mediate and regulate immunity and

inflammation by intercellular communication (Mahmoudi, 2009). The release of pro-inflammatory cytokines can be induced by osmotic, inflammatory, or mechanical damage, as often occurs in DED. Cytokines have been classified as interleukins, interferons, tumour necrosis factor, chemokines and colony stimulating factors (CSF). Cytokines that mediate chronic inflammatory processes or humoral responses include IL-4, IL-5, IL-6, IL-7, and IL-13, while those that regulate cellular responses include IL-1, IL-2, IL-3, IL-9, IL-10, IL-12, IFNs, TGF-β, and TNF-α and β (Hagan and Tomlinson, 2013). Cytokines have been classified according to their T-helper cell lineage: Th1 (IFN-γ), Th-2 (IL-4,5,13), Th17 (IL-17A) and Th-22 (TNF-α and IL-22) (Pflugfelder et al., 2013). They have also been considered in relation to systemic disease; IL-6,17,8 and TNF-α were found to be increased in DED without systemic disease while IL-1, 8 and 17 were increased in DED with systemic disease (Fong et al., 2019). IL-6 and 8 have been shown to correlate with pain in DED and increases in IL-6 and IL-1β are reported to be the earliest observable changes (Na et al., 2012). A meta-analysis of tear cytokines in DED indicted good evidence for higher tear levels of IL-1β, IL-6, IL-8, IL-10 IFN-γ and TNF-α in DED patients (Roda et al., 2020).

Interleukins are so called because they originate from leukocytes (white blood cells) and are used in communication between them. They are numbered based on the sequence of their discovery and play an important role in the adaptive immune response (Mahmoudi, 2009). Chemokines are small secreted proinflammatory cytokines which regulate the recruitment and movement of cells (leukocytes) from the blood to ocular surface cells by a process called chemotaxis. They are divided into four subgroups depending on their cysteine content and can compound the cycle of inflammation by attracting more inflammatory cells (Cytokine, 2003). IL-8 or CXCL8 is a chemokine that is consistently found in the tear film and conjunctiva of DE patients. It can mediate acute inflammation and allow leukocyte movement through the cornea.

1.5.3.1. Interleukin 1 (IL-1)

Several interleukins have been readily detected in the tears of DED patients and have therefore been the subject of numerous studies. Interleukin 1 (IL-1) is of particular interest as it can mediate both acute and chronic inflammation. The two inflammatory forms IL-1α and IL-1β, produce similar biological effects and are multifunctional (Acera et al., 2008). IL-1 is a potent inducer of other inflammatory cytokines such as IL-6, IL-8 and TNF-α (Solomon et al., 2001). It is known to alter the gene expression of these cytokines in corneal fibroblasts, epithelial cells, and endothelial cells (Narayanan et al., 2006). IL-1α has also been reported to stimulate the production of MMP-9 (Afonso et al., 1999). Using a murine dry eye model, a single injection of IL-1 was shown to cause a severe reversible inflammatory response in the lacrimal gland and a decrease in tear production (Zoukhri et al., 2007).

Several studies have explored the presence of IL-1 in human tear fluid and searched for any correlations with clinical signs and symptoms (see ***Table 1.2***). IL-1 would appear to be involved in the inflammatory cascade of all types of DED and can be seen to increase in the tear fluid in proportion to the severity. Correlations can be seen with OSDI score, ocular surface staining and Schirmer test.

Table 1.2. Association of IL-1 with different subtypes of DED

IL-1α	Methods	SS-DED (ADDE)	Non-SS-DED (ADDE)	MGD (EDE)	Combined ADDE/ EDE	Controls	Correlations with signs/ symptoms	Reference
	BL, WK, ELISA	↑		↑rosacea associated MGD)		Y	↑corneal staining	Solomon el. (2001)
	BL, MP, MX	↑SS & SJS		↑		Y	↑OS staining & ↓SC 1	Lam et al. (2009)
	SC 1, MX					Controls only	↓SC 1 ↑osmolarity	VanDerMeid et al. (2012)
	SC 1, ELISA			No change		Y		Khamar et al. (2019)
IL-1β	BL, WK, ELISA	↑ (precursor ↓)		↑rosacea associated MGD) (precursor ↓)		Y		Solomon et al. (2001)
	BL, SP, ELISA		No change			Y		Acera et al. (2008)
	BL, MC, MX				↑	Y	↑OSDI severity	Massingale et al. (2009)
	BL, MP, MX	↑SS & SJS		↑		Y	↑OS staining ↓SC 1	Lam et al. (2009)
	SC 1, MA		↑	No change	↑	Y		Boehm et al. (2011)
	SC 1, MX					Controls only	↓SC 1	VanDerMeid et al. (2012)
	Sp, MX				↑	Y	DEWS severity ↑	Na et al. (2012)
	SC 1, MX					Y (populat-ion-based study	Associated with eyelid crusts	(Tong et al., 2018)
	SC 1, ELISA			↑		Y	↑cDCD	Khamar et al. (2019)
	BL w anaestheti c, MC, MX			↑particularl y in DESOS group		Y	↑OSDI score ↑NPSI-E score	Li, B et al. (2020)
IL-1R α	BL, MC, MX			↑		Y	↓SC 1 ↓TBUT	Enriquez-de-Salamanca et al. (2010)
	MC (inferior fornix), MX				↑ with ↑severity (3 groups)	Y	↑OSDI ↑corneal staining	(Huang et al., 2012)

***Types of tears**: BL=basal, RX=reflex, FL=flush. **Collection method**: SC 1=Schirmer without anaesthetic (basal + reflex tears), SC 2= Schirmer with anaesthetic (basal tears) MC= microcapillary, MP=micropipette, WK=wick, Sp=sponge. **Method of analysis**: MA= microarray, ELISA= enzyme-linked immunosorbent assay, MX= multiplex bead analysis. ↑= increase, ↓= decrease, Y= yes, N= no, cDCD= corneal dendritic cell density, DEWS= dry eye workshop, DESOS= dry eye with symptoms outweighing signs, NPSI-E = Neuropathic Pain Symptom Inventory-Eye, OS=ocular surface, OSDI=ocular surface disease index, SJS= Stevens Johnson Syndrome.*

Interleukin-1 receptor antagonist (IL-Ra) is a naturally occurring anti-inflammatory or specific receptor antagonist of IL-1 activity. It has been found in abundance in normal corneal and conjunctival epithelial tissues. Higher levels in severe DED patients with ocular surface damage are thought to be a response to the release of inflammatory cytokines in inflamed tissues (Huang et al., 2012).

Although acknowledging the role of IL-β in mediating ocular surface inflammation in severe dry eye, its role in moderate DED has been questioned due to its absence in tear samples (Narayanan et al., 2006). However, the study population consisted of only 5 moderate dry eye patients. Later IL-1-receptor-1 knockout mice were used to demonstrate a significant increase in levels of IL-1α and IL-1β in response to EDE (Narayanan et al., 2008). As knockout mice would not be expected to show any changes in ocular surface cytokine expression if IL-1 activity were vital to EDE-associated changes, it was suggested that IL-1 is perhaps not the initiator of the inflammatory response but functions with other cytokines. Also using an experimental dry eye model, Chen et al. (2010b) suggested that the role of IL-1 in the squamous metaplasia of the ocular surface in SS could potentially be modulated by IL-1 receptor-1 targeted therapies.

A more recent study explored the correlation of cytokines and hypersensitivity in mild dry eye patients with MGD (Li et al., 2020a). Cytokines IL-1β, IL-6 and TNF-α are known to bind to the nociceptors of sensory nerve endings and indirectly induce the release of neuropeptides which have been implicated in sensory hypersensitivity (Lam et al., 2009). IL-1β, IL-2, IL-6 and TNF-α were all shown to correlate positively with OSDI and NPSI-E scores, suggesting they are involved in the hypersensitivity of these patients.

1.5.3.2. Interleukin 6 (IL-6)

IL-6 has been supported as the best indicator of disease and correlates well with DED severity. IL-6 and its mRNA levels have been found to be raised in DED in multiple studies when comparing controls to different patient groups (see ***Table 1.3***). It has multiple immune functions including activation of acute-phase proteins and MMPs, decrease of tear production and cell death (Fong et al., 2019). It also induces further release of inflammatory cytokines along with TGF-β by inducing the differentiation of naïve CD4+ T-cells into IL-17 producing Th17 cells, which are primarily responsible for the chronic nature of DED (Liu et al., 2017a).

A prospective, cross-sectional, clinical study based on tear osmolarity found a negative correlation between IL-6 with aqueous tear production (Jackson et al., 2016). Although IL-6 levels were unchanged between the two groups of patients, those with normal osmolarity and hyperosmolarity, it was suggested that lower tear production might be associated with higher levels of this proinflammatory cytokine, as can be seen in ***Table 1.3*** for ADDE.

Table 1.3. Association of IL-6 with different subtypes of DED

Methods	SS-DED (ADDE)	Non-SS-DED (ADDE)	MGD (EDE)	Combined ADDE/EDE	Controls	Correlations with signs/ symptoms	Reference
BL, PIP, ELISA	↑				Y		(Tishler et al., 1998)
BL, WK, IC, ELISA	↑				Y		Pflugfelder et al. (1999)
BL, MP, ELISA	↑	↑			Y	Disease severity Six OS parameters	(Yoon et al., 2007)
BL, Sp, ELISA		No change			Y		Acera et al. (2008)
BL, MC, MX				↑	Y	↑OSDI score	(Massingale et al., 2009)
BL, MP, MX	↑SS & SJS		↑		Y	↑OS staining ↓SC 1 ↑Irritation	Lam et al. (2009)
BL, MC, MX			No change		Y	Conjunctival stain ↑ ↑pain sensation scores ↑in older Px	Enriquez-de-Salamanca et al. (2010)
SC 1, MA		↑	No change	↑	Y		Boehm et al. (2011)
SC 1, MX					Controls only	↑osmolarity ↓SC 1	(VanDerMeid et al., 2012)
Sp, MX				↓	Y	↑DEWS severity	Na et al. (2012)
FL, MP, MX	↑	↑			Y	↓TBUT in non-SS ADDE	(Lee et al., 2013)
BL, MC, MX				No change	Y	↓SC 2	Jackson et al. (2016)
BL, MP, MX	↑	↑			Y	↑OSDI score ↓SC 1 ↓TBUT ↑corneal staining	Liu, R et al. (2017)
SC 1, MX					Y Population-based study	Associated with eyelid crusts.	(Tong et al., 2018)
BL, MC, ELISA & MX	↑	↑	↑		Y		Zhao et al. (2018)
BL with anaesthetic, MC, MX					Y	↑OSDI score N↑PSI-E score	Li, B et al. (2020)
MP,FL, MX			↑		Y	↓SC 1 ↑Meibography score	(Wu et al., 2020)
BL, MC, MX, & IC		↑			Y	Severity score↑	(Wei and Asbell, 2020)

***Types of tears**: BL=basal, RX=reflex, FL=flush. **Collection method**: SC 1=Schirmer without anaesthetic (basal + reflex tears), SC 2= Schirmer with anaesthetic (basal tears) MC= microcapillary, MP=micropipette, WK=wick, Sp=sponge. **Method of analysis**: MA= microarray, ELISA= enzyme-linked immunosorbent assay, MX= multiplex bead analysis.↑= increase, ↓= decrease, Y= yes, N= no, DEWS= dry eye workshop, IC= impression cytology, NPSI-E = Neuropathic Pain Symptom Inventory-Eye, OS=ocular surface, OSDI=ocular surface disease index, SJS= Stevens Johnson Syndrome.*

Raised IL-6 levels have been demonstrated in all the main types of DED. However, there does appear to be some disagreement. Acera et al. (2008) determined the concentration of IL-1β, IL-6 and MMP-9 in five patient groups: blepharitis, ocular allergic disease, dry eye (hyposecretory non-SS), conjunctivochalasis and controls. IL-1β and IL-6 were raised in patients with conjunctivochalasis but not in the dry eye patients when compared to controls. This would appear to disagree with earlier studies, however most of these were conducted with SS patients. Enriquez-de-Salamanca et al. (2010) detected IL-6 in only 65% of the tear samples collected from patients with EDE and did not find significantly different levels to controls. They reported a correlation of IL-6 and IL-8 with pain sensation scores, consistent with the proposal of Lam et al. (2009) that IL-6 in the tears of mild DED patients could be related to inflammation-induced hyperalgesia.

What would appear to be clear is the consistent increase in IL-6 levels in SS and non-SS patients. Some disagreement exists in the EDE group which could perhaps be related to the level of inflammation present in these patients. The variability in methods of tear collection, analysis, cohort size and selection criteria may all play a role in these discrepancies too.

1.5.3.3. Interleukin 17 (IL-17)

IL-17 is secreted mainly by Th-17 cells, a subset of T-cells, and consists of a family of six related proteins, IL-17A-F, with IL -17A being the 'signature' cytokine of the Th-17 pathway (Gu et al., 2013). IL-17 has been shown to indirectly exacerbate DED and play a role in ocular surface disease by mediating effects on stromal cells, resulting in production of inflammatory cytokines and promoting tissue infiltration with leukocytes, particularly neutrophils (Garbutcheon-Singh et al., 2019).

De Paiva et al. (2009) investigated IL-17s role in host defence and autoimmunity. Using human conjunctiva taken by IC they demonstrated increased gene expression of IL-23, IL-17A and IFN-γ, all Th-17 inducers. Also, using an experimental murine dry eye model they demonstrated that desiccating stress increased MMP-9, Th-17-associated genes (including IL-6, IL-23 and TGF-β1) and IFN-γ in the cornea and conjunctiva. Neutralisation of IL-17 in vivo in the experimental DE ameliorated desiccating stress-induced corneal epithelial barrier disruption and decreased the expression of MMP-9 and 3 mRNA, suggesting that IL-17 has a role in corneal barrier disruption in DED (De Paiva et al., 2009).

The majority of studies looking at IL-17 in human tears (see ***Table 1.4***) support a significant increase in DED but particularly those associated with a systemic disorder, with higher levels found in SS-DED than non-SS DED (Liu et al., 2017a;Tan et al., 2014). The only disagreement was found by Na et al. (2012) who found lower levels. However, they excluded patients from their study with DED severity level 4, due to insufficient tear volume. Investigation for the presence of IL-17 in various ocular

inflammatory diseases found the mean tear concentration to be elevated in all diseases when compared to controls (Kang et al., 2011). It also correlated with the clinical epitheliopathy scores, but only in those patients with a systemic inflammatory disease, providing a possible means of quantitatively evaluating the ocular surface inflammation severity in these patients (Kang et al., 2011).

Table 1.4. Association of IL-17 with different subtypes of DED

Methods	SS-DED (ADDE)	Non-SS DED (ADDE)	MGD (EDE)	Combined ADDE/EDE	Controls	Correlations with signs/symptoms	Reference
SC 1, ELISA	↑		↑	↑ (in simple dry eye)	Y	OS staining only in Px with systemic diseases	Kang et al. (2011)
Sp, MX	↑gene expression shown with IC	No change		↓ in tears (any dry eye type - graded on severity 1-4)	Y		Na et al. (2012)
FL, MP, MX	↑	↑			Y	↓TBUT & ↓SC 1 in SS Px	Lee et al. (2013)
BL, MC, ELISA	↑	↑			Y	↑Symptom score ↑keratopathy score ↓TBUT ↓SC 1	Tan et al. (2014)
SC 1, MA		↑	↑	↑	N (none in samples from healthy Px in a previous study)		Meadows et al. (2016)
BL, MP, MX	↑	↑			Y	↑OSDI score ↓SC 1 ↓TBUT ↑corneal staining	Liu, R et al. (2017)
SC 1, MX					Y (population-based study)	Associated with eyelid crusts in healthy Px	Tong et al. (2018)
SC 1, ELISA			↑		Y	↑OS discomfort, ↑ cDCD, ↓TBUT, ↓SC 1	Khamar et al. (2019)

***Types of tears**: BL=basal, RX=reflex, FL=flush.* ***Collection method**: SC 1=Schirmer without anaesthetic (basal + reflex tears), SC 2= Schirmer with anaesthetic (basal tears) MC= microcapillary, MP=micropipette, WK=wick, Sp=sponge.*
***Method of analysis**: MA= microarray, ELISA= enzyme-linked immunosorbent assay, MX= multiplex bead analysis. ↑= increase, ↓= decrease, Y= yes, N= no, cDCD= corneal dendritic cell density, IC=impression cytology, OS=ocular surface, OSDI= ocular surface disease index.*

1.5.3.4. Other interleukins

Several other interleukins have been studied, but to a lesser extent. Massingale et al. (2009) investigated the levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, and TNF-α in tears of patients with SS and non-SS DED and compared the results with normal controls. Although a significant

increase in all the cytokines analysed was found, the patient numbers were small, with only one patient in the SS category. IL-2 (Li et al., 2020a) and IL-2,4 and 5 (Massingale et al., 2009) have been shown to correlate with OSDI score. However, reduced levels of IL-2 and 4 have been found in patients with EDE (Khamar et al., 2019). Also, lower levels of IL-2 have been found in severe DED than in moderate DED patients (Pinto-Fraga et al., 2016). A decrease in IL-2 levels and an increase in signs and symptoms was found in patients subjected to controlled adverse environmental conditions who had been treated with polyvinyl alcohol compared to those who had been treated with FML. Those treated with FML maintained their IL-2 levels (Pinto-Fraga et al., 2016). This suggests that IL-2 may play a protective role in DED and has potential as a therapeutic biomarker.

IL-13, along with IL-4 and 5, is produced by Th-2cells, which primarily activate B cells in secondary lymphoid tissue i.e. humoral immunity (Hagan and Tomlinson, 2013). It has been detected in the tears of healthy patients (Carreño et al., 2010) and in the tears of all DED subtypes. Meadows et al. (2016) found its concentration levels as follows: ADDE>EDE>combined>non-specific group, suggesting no loss of IL-13 mediated homeostasis with increasing severity of DED, but rather a physiologic effort to restore it. It maintains homeostasis by regulating the mucous secreting conjunctival goblet cell density and differentiation (De Paiva et al., 2011). IL-13 signalling can be suppressed by IFN-γ leading to apoptosis and squamous metaplasia of the ocular surface, as has been seen in ADDE where IFN-γ expression is increased (Pflugfelder et al., 2013).

IL-10 and IL-12 are involved in cell-mediated immunity (Hagan and Tomlinson, 2013). IL-10 is an immunomodulatory cytokine which inhibits the activity of Th1 cells, natural killer cells and macrophages to reduce the production of inflammatory cytokines and antigen presenting cells (Couper et al., 2008).

IL-10 activation during chronic inflammation provides a negative feedback mechanism to initiate the establishment of a balance between activation and deactivation of cytokines at the site of inflammation (Aketa et al., 2017). Studies have found an increase of IL-10 levels in the aqueous humor of patients with ocular surface inflammation (Aketa et al., 2017) and in the tears of patients with SS (Lee et al., 2013;Zhao et al., 2018).

IL-12 has also been analysed less frequently than other interleukins. The active form IL-12p70 (IL-12p40 being the inactive form) is involved in T cell activation (Th-1) and progression of responses which in turn produce IFN-γ (Zhang and Wang, 2008). Increased levels of IL-12p70 have been shown in EDE, non-SS ADDE, but particularly in SS-DED, when compared to controls (Zhao et al., 2018). Lam et al. (2009) found significantly higher levels in DED patients with MGD than those without. However,

a more recent study did not find a significant difference when comparing to controls (Wu et al., 2020). More research is required before these two interleukins can be validated as biomarkers for DED.

1.5.3.5. Interferons (IFN): Interferon gamma

Interferons are involved in acute response intracellular signalling. They are cytokines produced by lymphocytes, dendritic cells, macrophages, natural killer cells, and T cells. IFN-γ is involved in activation of macrophages, expression of MHC class I and II, and increased antigen activation (Mahmoudi, 2009). In DED**,** IFN-γ is an immunoregulatory cytokine shown to play a role in Th-1 driven immune response at the ocular surface (Tamhane et al., 2019). IFN-γ has been shown to exacerbate goblet cell loss during desiccating stress, a key feature of DED (Zhang et al., 2011). Goblet cell loss has been modulated in a murine DE model by topical inhibition of IFN-γ (Zhang et al., 2014).

Table 1.5. Association of IFN-γ with different subtypes of DED

Method	SS-DED (ADDE)	Non-SS DED (ADDE)	MGD (EDE)	Combined ADDE/EDE	Controls	Correlations with signs/symptoms	Reference
BL, MP, MX				↑in DE & without MGD group	Y	↑OS staining	Lam et al. (2009)
BL, MC, MX				↑	Y	↑OSDI severity	Massingale et al. (2009)
SC 1, MA		↑	No change	↑	Y		Boehm et al. (2011)
Sp, MX				↓ with ↑severity	Y		Na et al. (2012)
FL, MP, MX	↑	↑			Y		Lee et al. (2013)
SC 1, MA		↑compared to EDE and combined	present	Present & in non-specific groups	N (none in samples from healthy Px in a previous study		Meadows et al. (2016)
BL, MC, MX			↑		Y	↑osmolarity ↑OS staining	Jackson et al. (2016)
SC 1, MX					Y (population-based study)	↑ocular discomfort & eyelid crusts	Tong et al. (2018)
BL, MC, ELISA, MX	↑	↑	↑		Y		Zhao et al. (2018)
SC 1, ELISA			↑		Y		Khamar et al. (2019)
FL, MP, FL, MX			↑		Y	↓TBUT	Wu et al. (2020)

Types of tears*: BL=basal, RX=reflex, FL=flush.* ***Collection method****: SC 1=Schirmer without anaesthetic (basal + reflex tears), SC 2= Schirmer with anaesthetic (basal tears) MC= microcapillary, MP=micropipette, WK=wick, Sp=sponge.*
Method of analysis*: MA= microarray, ELISA= enzyme-linked immunosorbent assay, MX= multiplex bead analysis, OS=ocular surface, OSDI= ocular surface disease index.↑= increase, ↓= decrease, Y= yes, N= no.*

In human studies, Pflugfelder et al. (2015) found higher levels of IFN-γ in the conjunctival tissue of patients with ADDE, in both SS and non-SS DED when compared to normal controls, supporting previous findings in murine studies. An increase in IFN-γ was shown to correlate with goblet cell loss, reduced mucin production and severity of conjunctival disruption (Pflugfelder et al., 2015). See ***Table 1.5*** for studies of IFN-γ in tear analysis and correlations with clinical findings.

Elevated levels have been demonstrated in all subtypes of DED and associated with tear hyperosmolarity (Jackson et al., 2016) increasing OSDI score (Massingale et al., 2009) and ocular surface staining (Lam et al., 2009;Jackson et al., 2016). Conversely Na et al. (2012) reported a decrease in concentration with increasing severity. However, the study did not specify DED subtypes, but graded according to clinical severity. As previously mentioned, some patients with severity level four were excluded due to insufficient tear volume, possibly skewing the results. IFN- γ has been suggested as one of the strongest biomarkers for disease severity (Pinto-Fraga et al., 2018). Therapies that suppress IFN- γ levels have potential for treating the ocular surface disease of dry eye.

1.5.3.6. Tumor necrosis factor alpha (TNF-α)

TNF-α is a potent pro-inflammatory cytokine that initiates a series of inflammatory responses on the ocular surface leading to necrosis or apoptosis, as part of the cell-mediated response (Idriss and Naismith, 2000). Raised levels have been found in DED and verified in experimental models (Li et al., 2006). TNF-α, along with IL-6 and IL-1β have been shown to decrease neurally-mediated lacrimal gland tear production in a murine DE model (Zoukhri et al., 2002). An early cytokine study in SS patients showed significantly elevated levels of the mRNA transcript for TNF-α in conjunctival epithelial specimens (Pflugfelder et al., 1999). Complex interactions occur between inflammatory mediators, with IL-1β shown to stimulate production of TNF-α and MMP-3; in turn, TNF-α stimulates MMP-9 production (Pflugfelder and de Paiva, 2017). TNF-α has also been shown to affect the expression of two major mucins (MUC1 and 16) found on the ocular surface, which may affect lubrication and protection (Albertsmeyer et al., 2010). Numerous studies have shown it to be consistently elevated in DED tears when compared to non-DED tears (Roy et al., 2017). See ***Table 1.6*** for the impact of TNF-α in DED.

TNF-α levels in the tears have been found to be increased in all subtypes of DED, although some disagreement exists in EDE where three studies show no statistically significant difference (Boehm et al., 2011;Wu et al., 2020;Khamar et al., 2019). Yoon et al. (2007) found elevated levels compared to normal controls, but no difference between SS and non-SS patients and no correlations with clinical findings. Other studies do show correlations with different clinical findings (Lam et al., 2009;Massingale et al., 2009;Lee et al., 2013;Tong et al., 2018;Oshida et al., 2004). However, TNF-α

has been found in the tears of healthy subjects (Carreño et al., 2010) and one study suggested that its presence is not a direct indication of inflammatory pathology (Puinhas et al., 2013).

Table 1.6. Association of TNF-α with different subtypes of DED

Method	SS-DED (ADDE)	Non-SS DED (ADDE)	MGD (EDE)	Combined ADDE/EDE	Controls	Correlations with signs/symptoms	Reference
ELISA	↑				Y	↑corneal staining	(Oshida et al., 2004)
BL, MP, ELISA	↑	↑			Y	None found	Yoon et al. (2007)
BL, MP, MX				↑ in all groups with & without MGD compared with controls	Y	None reported	Lam et al. (2009)
BL, MC, MX				↑	Y	↑OSDI severity	Massingale et al. (2009)
SC 1, MA		↑	No change	↑	Y		Boehm et al. (2011)
SC 1, MX					Controls only	↑osmolarity & ↓ Schirmer 1 No correlations with TBUT or OSDI	VanDerMeid et al. (2012)
FL, MP, MX	↑	↑			Y		Lee et al. (2013)
SC 1, MX					Y (population-based study)	Associated with eyelid crusts	Tong et al. (2018)
BL, MC, ELISA, MX	↑	↑	↑		Y		Zhao et al. (2018)
FL, SC 1, ELISA			No change		Y		Khamar et al. (2019)
BL w anaesthetic, MC, MX				↑in mild DE with symptoms outweighing signs compared to common DE & controls	Y	↑OSDI & NPSI-E	Li, B et al. (2020)
FL, MP, FL, MX			↑trend but not statistically significant		Y		Wu et al. (2020)

***Types of tears**: BL=basal, RX=reflex, FL=flush. **Collection method**: SC 1=Schirmer without anaesthetic (basal + reflex tears), SC 2= Schirmer with anaesthetic (basal tears) MC= microcapillary, MP=micropipette, WK=wick, Sp=sponge.*
***Method of analysis**: MA= microarray, ELISA= enzyme-linked immunosorbent assay, MX= multiplex bead analysis.*
↑= increase, ↓= decrease, Y= yes, N= no, NPSI-E = Neuropathic Pain Symptom Inventory-Eye, OS=ocular surface, OSDI= ocular surface disease index.

1.5.3.7. Chemokines

Chemokines are a family of proteins involved in leukocyte chemotaxis i.e., they regulate the directed migration of immune cells from blood to tissue. They are classified according to the receptor that they

mediate their biological effects through, with each receptor allocated to a specific chemokine subfamily name (C, CC, CXC, CX3C) (Cytokine, 2003). Prior to 2002 there was no clear system used for their classification. CXCL8 was previously known as IL-8 and is still often referred to as such. Several chemokines have been identified as playing a role in DED, such as CXCL8,9,10 and 11, CCL3 (or MIP-1α), CCL4 (or MIP-1β), CCL15 (or MIP-1δ), CCL2 (or MCP-1), CCL5 (or RANTES) and CX3CL1 (or Fractalkine). See ***Table 1.7.***

1.5.3.7.1. Interleukin 8 (IL-8/CXCL8)

IL-8 has been found to increase dramatically in DED in order to direct the migration of leukocytes during acute inflammation, particularly through avascular tissue such as the cornea. Its increase in the tear film is thought to be a potent signal to recruit T lymphocytes which infiltrate the ocular surface and in so doing, cause damage to it (Massingale et al., 2009). There appears to be evidence for IL-8 as a biomarker in DED and some correlations with clinical signs have been found (see ***Table 1.7***). Willems et al. (2020) reported no statistically significant change in IL-8 levels when comparing SS patients with controls. There appears to be some disagreement when investigating patients with EDE only. However, there is substantial evidence to conclude that IL-8 levels increase in non-SS and mixed DE groups (Massingale et al., 2009;Boehm et al., 2011;Huang et al., 2012;Dionne et al., 2016). IL-8 levels have been found to negatively correlate with Schirmer scores, and positively correlate with severity scores and ocular pain levels (Enríquez-de-Salamanca et al., 2010). Schirmer scores, however, are not included in the TFOS DEWS II diagnostic criteria, as the test has been shown to lack accuracy and reproducibility in less than severe cases of DED (Nichols et al., 2004a).

1.5.3.7.2. Other chemokines

Studies in dry eye patients have demonstrated elevated levels of other tear chemokines, such as the macrophage inflammatory protein 1 alpha/ chemokine (C-C motif) ligand 3 (MIP-1α/ CCL3), macrophage inflammatory protein 1 beta/chemokine (C-C motif) ligand 4 (MIP-1β/CCL4), RANTES/CCL5, Fractalkine/ chemokine (C-X3-C motif) ligand 1 (CX3CL1), chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11 and monocyte chemoattractant protein 1/ chemokine (C-C motif) ligand 2 (MCP-1/CCL2) (Tamhane et al., 2019). These chemokines bind to receptors (ligands) and are critical for the directed migration of monocytes and T-lymphocytes. Most have shown correlations to clinical parameters and disease severity (see ***Table 1.7***). The concentrations of these chemokines have been found to be higher in SS DED patients when compared with non-SS DED patients (Choi et al., 2012;Yoon et al., 2010).

Table 1.7. Association of various chemokines with different subtypes of DED

Chemokine	Method	SS-DED (ADDE)	Non-SS DED	MGD (EDE)	Combined ADDE/ EDE	Controls	Correlations with signs/ symptoms	Reference
IL-8 (CXCL8)	BL, MP, MX			↑ with & w/o MGD		Y	↑OS staining & ↓SC 1	Lam et al. (2009)
	BL, MC, MX				↑	Y	↑OSDI severity	Massingale et al. (2009)
	SC 1, MA		↑	No change	↑	Y		Boehm et al. (2011)
	SC 1, MX					Controls only	↓SC 1	VanDer-Meid (2012)
	Inf fornix by MC, MX				↑with DED severity (3 groups)	Y	↑OSDI & ↑corneal staining	Huang et al. (2012)
	SC 1, MX and MM				↑ in non-specific DE group	CL & non-CL wearers		Dionne et al. (2016)
	BL, MC, ELISA & MX	↑	↑	↑		Y		Zhao et al. (2018)
	SC 1, ELISA			No change		Y		Khamar et al. (2019)
	FL. MP, FL, ELISA			↑		Y	↓SC 1	Wu et al. (2020)
	BL, MC, MX and IC		↑			Y	↑severity scores	Wei & Asbell (2020)
CXCL9	BL, MC, ELISA	↑	No change			Y	none	Yoon et al. (2010)
CXCL10	BL, MC, ELISA	↑	No change			Y	↓BTS	Yoon et al. (2010)
	BL, MC, MX				↑	Y		Enriquez-de-Salamanca et al. (2010)
CXCL11	BL, MC, ELISA	↑	↑			Y	↓BTS, ↓TCR, ↑corneal stain, ↓GCD	Yoon et al. (2010)
CCL3 (MIP-1α)	BL, MP, MX					Y	↑OS staining & ↓SC 1	Lam et al (2009)
	BL, MC, ELISA	↑	↑			Y	↓TCR and ↓GCD	(Choi et al., 2012)
CCL4 (MIP-1β)	BL, MC, ELISA	↑	↑			Y	↓TCR and ↓GCD	(Choi et al., 2012)
CCL15 (MIP-1δ)	Sp, MX				↑	Y	↑DEWS severity	Na et al. (2012)
CCL2 (MCP-1)	Sp, MX				↑	Y		Na et al. (2012)
	SC 1, MX					Y (populat-ion-based study)	↓SC1	Tong et al. (2018)
	SC 1, ELISA			No change		Y		Khamar et al. (2019)

Chemokine	Method	SS-DED (ADDE)	Non-SS DED	MGD (EDE)	Combined ADDE/ EDE	Controls	Correlations with signs/ symptoms	Reference
CCL5 (RANTES)	BL, MP, MX			↑		Y		Lam et al. (2009)
	BL, MC, ELISA	↑	↑			Y	↑TBUT, ↑corneal sensitivity & ↓BTS ↓GCD	(Choi et al., 2012)
CXC3L1 (Fract-alkine)	BL, MC, MX			↑		Y	↓SC 1	Enriquez-de-Salamanca et al. (2010)
	Sp, MX				↑	Y	↑clinical severity	Na et al. (2012)

Types of tears: *BL=basal, RX=reflex, FL=flush.* ***Collection method***: *SC 1=Schirmer without anaesthetic (basal + reflex tears), SC 2= Schirmer with anaesthetic (basal tears), MC= microcapillary, MP=micropipette, WK=wick, Sp=sponge.*
Method of analysis: *MA= microarray, ELISA= enzyme-linked immunosorbent assay, MX= multiplex bead analysis. ↑= increase, ↓= decrease, Y= yes, N= no, BTS= basal tear secretion, GCD= goblet cell density, OS=ocular surface, OSDI= ocular surface disease index, TCR= tear clearance rate, w/o= without.*

CX3CL1 (fractalkine) differs from other chemokines as it can exist in soluble or membrane-bound form. Soluble fractalkine is a potent chemoattractant for the CX3CR1+ receptor on leukocytes (Enríquez-de-Salamanca et al., 2010). Using a murine SS model, a study showed that soluble fractalkine is separated from membrane-bound fractalkine by a cysteine protease, cathepsin (Fu et al., 2020b). Both cathepsin and fractalkine were found to be significantly elevated in the mouse tears. Previously, significantly elevated levels of fractalkine have been found in the tears of patients with DED when compared to normal controls and levels increased in proportion to the clinical severity grading (Na et al., 2012). Production appears to be stimulated by pro-inflammatory cytokines and decreased anti-inflammatory cytokines in the tears. A study investigating tear cytokine and chemokine levels specifically in EDE, found fractalkine in all the tear samples collected and noted a significant increase when compared to healthy controls (Enríquez-de-Salamanca et al., 2010). Its exact role in the inflammatory cascade in DED is yet to be explored.

1.5.3.8. Growth factors

Several growth factors are also found in the tears in low abundance, which have the potential to be used as biomarkers. They are produced by the lacrimal gland or other ocular surface glands and may provide trophic (growth) effects for the ocular surface epithelium (D'Souza and Tong, 2014).

1.5.3.8.1. Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a signal protein that promotes the growth of vascular endothelial cells, triggering vasodilation, and neovascularisation when over-expressed. Tear VEGF

levels have been found to be raised in EDE (Enríquez-de-Salamanca et al., 2010) and ADDE (Guyette et al., 2013) compared to normal controls. However, another study showed a significant decrease in DED compared to controls (Benitez-Del-Castillo Sánchez et al., 2017). This discrepancy may be explained in part by a study which investigated the tear film for nociception-associated factors (Khamar et al., 2019). VEGF is also known to modulate pain, with VEGF-B augmenting nociception and VEGF-A countering it. Higher levels of VEGF-B and lower levels of VEGF-A were observed in the tears of dry eye patients when compared to controls and both were found to correlate with increased ocular surface discomfort, increased corneal dendritic cell density (cDCD), decreased TBUT and decreased Schirmer scores (Khamar et al., 2019).

1.5.3.8.2. Epidermal growth factor (EGF)

EGF (epidermal growth factor) is produced by the lacrimal gland and plays an active role in wound healing and reflex tear production (Klenkler et al., 2007). Studies have shown decreased levels in DED, and therefore it can be used as an indicator of lacrimal gland function. Decreased tear concentrations of EGF have been correlated with increased severity levels of SS-DED and correlations with ocular surface staining and Schirmer test reported (Pflugfelder et al., 1999). These findings were confirmed in patients with DED without MGD (Lam et al., 2009). Conversely, a study of EDE patients reported an increase in the tear levels of EGF (Enríquez-de-Salamanca et al., 2010). However, they reported a borderline significant negative correlation with ocular surface staining, and correlations with pain, tear production and stability measures, which seem to confirm previous findings but contradict their report of increased EGF levels.

1.5.3.8.3. Transforming growth factor-beta-1 (TGF-β1)

TGF-β1 (transforming growth factor-beta-1) is a multifunctional cytokine with wide ranging biological functions. It is known to be involved in inflammatory and fibrotic pathways during wound healing and is thought to promote normal growth and differentiation of ocular surface epithelium and suppress inflammation (Klenkler et al., 2007). Tear TGF-β activity has been shown to increase in DED due to MGD, non-SS ADDE and SS ADDE, but particularly in SS (Zheng et al., 2010).

1.5.3.8.4. Nerve growth factor (NGF)

Evidence indicates that neurotrophic factors such as NGF play a role in ocular surface homeostasis by stimulating epithelial cell proliferation and innervation (Klenkler et al., 2007). Animal models have shown that corneal nerve impairment or injury induces an increase in local NGF levels. Injury can affect corneal function, leading to loss of epithelial metabolism and viability and possible ulceration (Lambiase et al., 2011). Significantly increased tear NGF levels were found in patients with DED (SS,

non-SS and ocular cicatricial pemphigoid) compared to normal controls and correlated with disease severity (Lambiase et al., 2011). A direct correlation with conjunctival hyperaemia and fluorescein staining was also found. Patients treated with prednisolone for 28 days were found to have a decrease in their NGF levels and a clinical improvement in their dry eye (Lee et al., 2006). However, further studies are required to determine why NGF levels are increased in DED. It is uncertain whether NGF is involved more with local tissue damage or with increased release from the lacrimal gland as part of the pathogenesis of DED (Lambiase et al., 2011).

1.5.3.9. Other neuromediators

Calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY), both found to be present in the lacrimal gland, have been shown to be reduced and significantly correlated with clinical factors in DED when compared to healthy subjects (Lambiase et al., 2011). Both neuromediators showed an inverse correlation with disease severity and ocular surface staining; CGRP showed a positive correlation with Schirmer test, while NPY correlated inversely with TBUT. NPY was decreased particularly in dry eye patients with underlying inflammation.

The neuropeptide, Substance P, has been recognised as a normal component of the tears (Yamada et al., 2003). It is involved in pain signalling and is reported to play a role in the maintenance of corneal homeostasis by promoting epithelial cell migration and proliferation (Markoulli et al., 2017).

When cytokines bind to their respective nociceptors on sensory nerve endings, igniting the action potential of the nerve, neuropeptides such as SP and CGRP are stimulated (Lambiase et al., 2011). These neuromediators promote local inflammation by inducing blood vessel dilatation, leukocyte leakage, immune cell activation, and synthesis and release of several cytokines, triggering reflex tearing and ocular discomfort. They have been implicated in sensory hypersensitivity (Li et al., 2020a). Further investigation is warranted into the potential use of these neuromediators as diagnostic biomarkers.

1.5.3.10. sPLA2-11a (secretory phospholipase A2)

sPLA2-11a is an inflammatory mediator/enzyme which hydrolyses phospholipids into fatty acids and other lipophilic substances. It is found in high concentration in the tears and is produced as part of the innate immune response to infection (Wei et al., 2011). Chen et al. (2009) showed that the activity of sPLA2-IIa was significantly increased in tears from patients with DED compared with normal subjects, particularly when the ocular surface was compromised; a role in chronic ocular surface inflammation was suggested. A recent study has confirmed that sPLA2-IIa activity is increased in DED and is not only associated with inflammatory changes but cooperates with TNF-α and IL-1β to induce inflammation

in conjunctival epithelial cells (Wei and Asbell, 2020). Further understanding of the role of this phospholipase may in time add to the understanding of DED pathogenesis.

1.5.4. Other proteins

Several other lesser studied tear proteins have been implicated in the pathogenesis of DED and suggested as potential biomarkers for the disease.

1.5.4.1. The S-100 proteins

The S-100 proteins are a family of at least 21 low molecular weight calcium-binding proteins which interact with other proteins to regulate cell proliferation, differentiation and migration, wound healing, and inflammation. S100 A8 (calgranulin A) and A9 (calgranulin B) are known to be pro-inflammatory and have been shown to be upregulated in DE patients (Soria et al., 2013). Zhou et al. (2009) also suggested that S100 A4 and S100 A11 may have functions related to osmotic stress and apoptosis, respectively. The same group were also able to show that S100 A8 and S100A9 correlated with MGD severity level and redness in patients with MGD related DE (Tong et al., 2011). However, they reported that the origin of these proteins remains uncertain, with suggestions being release from ocular surface cells due to epithelial desiccation, release from immune cells, or related to keratinisation of the MG ducts.

More recently, S100 A8 and S100 A6 (calcyclin) have been suggested as diagnostic biomarkers due to a strong correlation with DED clinical parameters. S100 A6 has also been found to be useful in severity grading (Benitez-Del-Castillo et al., 2021)

1.5.4.2. Aquaporins

Aquaporins are membrane proteins whose main function is to transport water across cell membranes in response to osmotic gradients to maintain tear volume and osmolarity of the ocular surface. Aquaporin 5 (AQP5) is thought to mediate rapid water transport in the apical membrane of the lacrimal gland (Ohashi et al., 2006). It was suggested that AQP5 protein may leak into the tears due to damage of the lacrimal acinar cells by lymphocyte infiltration (Ohashi et al., 2003). However, increased mRNA expression of AQP5 in mouse conjunctival epithelial cells has been shown to increase under increasingly hyperosmotic conditions (350 mOsm and 400 mOsm) (Bhattacharya et al., 2017).

1.5.4.3. Human leukocyte antigens

Human leukocyte antigens are a group of cell surface proteins that are encoded by the histocompatibility complex (MHC) gene complex and are responsible for the regulation of the immune system. HLA-DR (Antigen D-related) plays an important role in T-cell activation and is one of the most

commonly found biomarkers of inflammation in DED. It is detected in the conjunctiva by impression cytology and flow cytometric analysis (Fernandez et al., 2015). Its expression has been found to be highest in SS patients and to increase with clinical severity of DED. However, it's concentration has been noted to vary widely between studies, most likely due to different methodology and population samples (Epstein et al., 2013).

1.5.4.4. Cell adhesion molecules

Cell adhesion molecules are cell surface proteins that enhance cellular migration by binding components within the extracellular matrix. They promote the infiltration of immune cells such as lymphocytes onto the ocular surface in dry eye (Ganesalingam et al., 2019). Elevated levels of ICAM-1 (intercellular adhesion molecule-1) and vascular CAM-1 (vascular cellular adhesion molecule-1) have been identified in the conjunctiva and lacrimal glands in DED (Gao et al., 2004). Using an experimental DED model, a decrease in ocular surface inflammatory infiltrates resulted from treatment with monoclonal antibodies against murine ICAM-1 (Gao et al., 2004). A study comparing the levels of inflammatory molecules in tear samples between patients with MGD-related EDE and healthy subjects found significantly higher levels of ICAM-1 in the patient group; in addition, a positive correlation with meibography score was found for ICAM-1 and IL-6 (Wu et al., 2020). In recent years, lifitegrast, which appears to give improvement in both symptoms and signs of DED, has been approved by the US Food and Drug Administration (FDA) for the treatment of DED. Lifitegrast inhibits T cell-mediated inflammation by blocking the binding of the cell surface proteins, LFA-1 (lymphocyte function-associated antigen 1) located on T cells to ICAM-1, expressed on inflamed ocular surface cells, thus lessening overall inflammatory responses (Paton, 2016).

1.5.4.5. Leukotrienes and prostaglandins

Leukotrienes and prostaglandins are thought to act as lipid mediators in the tears and have a role in inflammation (Salmon and Higgs, 1987). During mechanical, chemical, or immunological challenge oxidative metabolism of arachidonic acid is increased in inflamed tissues with the production of eicosanoids such as prostaglandins (PGFs) and leukotrienes (LTs) (Salmon and Higgs, 1987). PGFs are potent vasodilators and hyperalgesic agents, contributing to erythema, oedema, and pain, while LTs are responsible for the accumulation of inflammatory leukocytes. Specific eicosanoids, such as leukotriene B4, have been identified as possible clinical indicators of MG obstruction (Ambaw et al., 2018). Upregulation of prostaglandin E2 has been consistently found in dry eye patients (Chistyakov et al., 2020).

1.5.5. Lipidomic analysis of tears and meibum

Lipidomics is an emerging field of biomedical research and involves the identification and quantification of lipid molecules. The tear lipid layer consists of an outer layer of non-polar hydrophobic lipids and an inner layer of amphiphilic polar lipids (see ***Figure 1.1***); these play a major role in the maintenance of tear film stability, delaying evaporation of the aqueous layer, reducing surface tension and acting as a first line of defence against the external environment (Bron et al., 2004). Its thickness is reported to range from 15 to 157nm, with a mean of 42nm (King-Smith et al., 2010). Current evidence suggests that the lipid components of meibum consist of ~30-45 mol%, WEs ~30-50 mol% cholesterol esters (ChEs), ~1.5 mol% triglycerides (TGs), and up to 4 mol % long-chain OAHFAs (Millar and Schuett, 2015). Other lipids include free cholesterol, which makes up < 0.5 mol% and phospholipids, which make up <0.01 mol% (Brown et al., 2013).

Asymptomatic, but at risk, and symptomatic patients can have altered tear lipidomes (Lam et al., 2014b); reduction in certain wax esters was noted in disease and significant correlations were found with the OSDI questionnaire, tear breakup time and Schirmer I.

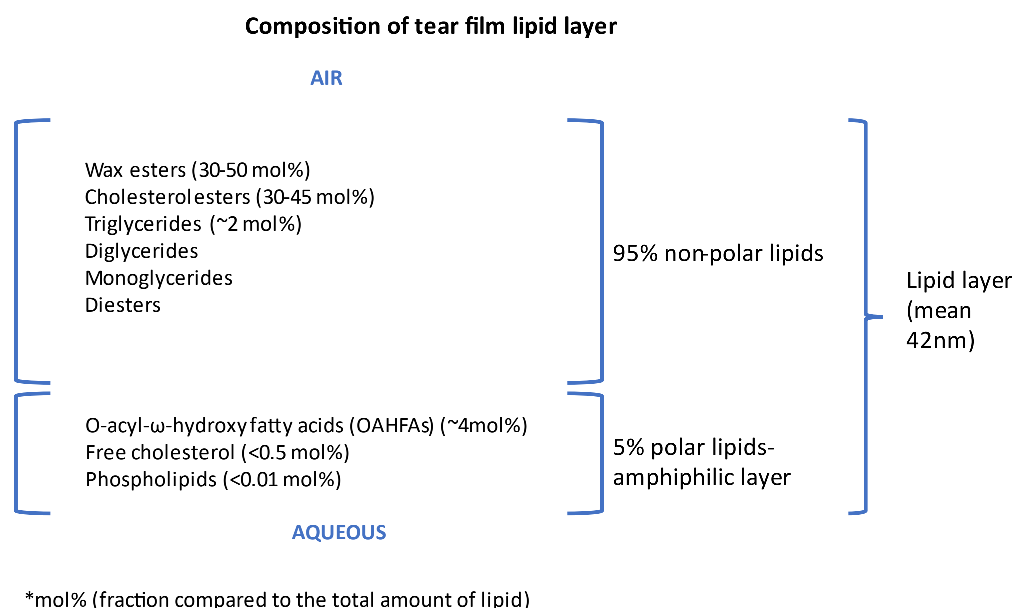

Figure 1.1. Composition of the tear lipid layer

The main source of lipids in the tear film is the meibomian gland secretion known as meibum. Meibum can be expressed from the meibomian orifices by pressing over the glands. The number of glands expressing, and the quality of the meibum expressed, can vary depending on the severity of disease; quality as well as quantity of lipids is critical (Ashraf et al., 2011).

The chemical composition of meibum is complex, being a mixture of diverse lipid classes rather than individual lipids, and despite advances in technology its evaluation and quantification is still a work in progress (Butovich, 2011). It is composed of approximately 95% nonpolar lipids which form the outer hydrophobic layer of the tears, and 5% amphiphilic lipids which form the inner polar layer (Willcox et al., 2017).

Non-polar lipids do not dissolve in water and are composed of wax esters (WE), cholesterol esters (ChE), diesters, free sterols, monoglycerides, diglycerides (DGs), triglycerides (TGs), free fatty acids, and hydrocarbons (Pucker and Nichols, 2012). Early studies report that wax and cholesteryl esters represent up to 60% of meibum lipids, with many individual members of these families having numerous isometric forms (Green-Church et al., 2011). Lam et al. (2011) found that the non-polar lipid classes of ChE, WE and TGs or triacylglycerides (TAGs) comprised approximately 90% of the total lipids present. Current evidence is that ChEs represent ~30-45 mol%, WEs ~30-50 mol%, and TAGs ~1.5 mol%, (Millar and Schuett, 2015). Other lipids include free cholesterol, which makes up <0.5 mol% and phospholipids, which make up <0.01 mol% (Brown et al., 2013). However, as in tear analysis, numerous different technologies have been used for collection and quantification, and therefore, absolute amounts are unknown. The range of mol% of total lipids also make any correlation analysis with dry eye parameters challenging, requiring a large change relative to normal (Willcox et al., 2017).

Polar lipids are relatively water soluble and consist of phospholipids (PL) and long-chain OAHFAs which are amphiphilic i.e., both hydrophilic and hydrophobic (Pucker and Haworth, 2015). OAHFAs belong to a family of lipids known as fatty acyl esters of hydroxy fatty acids (FAHFAs). The family includes (i) branched-chain FAHFAs, involved in the regulation of glucose metabolism and inflammation, with addition of an acyl group (acylation) to an internal branched-chain hydroxy-palmitic or -stearic acid; (ii) ω-FAHFAs or OAHFAs, which function as biosurfactants and are formed via acylation of the ω-hydroxyl group of very-long-chain fatty acids (30 to 34 carbons); and (iii) Ornithine-FAHFAs which are bacterial lipids (Wood, 2020). OAHFAs are structurally similar to wax esters, but carry an additional carboxyl group, which is negatively charged at neutral pH rendering them amphiphilic (Schuett and Millar, 2013).

OAHFAs constitute the majority of the polar lipid fraction making up approximately 3.5% of the total meibum lipids (Lam et al., 2011). They are thought to be responsible for stabilization of the tear film lipid layer by creating a surfactant interface between the non-polar lipids of meibum and the aqueous subphase beneath it, a role previously attributed to phospholipids (Butovich et al., 2009;Schuett and Millar, 2013). It has been speculated that a lipid polarity gradient is formed from the aqueous/lipid layer boundary to the external environment (from phospholipids, to OAHFAs, then OAHFA derivatives,

and ChEs/WEs), to ensure stability (Miyamoto et al., 2020). Variations in chain length and polar groups are thought to affect the phase behaviour of these lipids at the tear film surface (Bland et al., 2019). Several meibum-derived OAHFAs have shown significant associations with tear film thinning, whereas those derived from the tears did not, further implicating them in the underlying stabilisation and thinning of the tear film (Khanal et al., 2021). The concentration of OAHFA has been found to exceed that of phospholipids in meibum but the ratio is reversed in the tear film (Brown et al., 2013).

Phospholipids (sphingophospholipids ~30% and glycerophospholipids ~70%) have the lowest abundance of all the lipid classes in meibum, representing only 0.006 ± 0.003 mol% (Brown et al., 2013). In contrast, the phospholipids were shown to be highly abundant in tears at 12 ± 7 mol%; excluding phospholipids, the mole fractions of ChE and WE were found to be the same in tears and meibum, while OAHFAs and TAGs were 61% higher in the tears (Brown et al., 2013). A positive correlation between tear secretion and certain phospholipids has indicated a possible lacrimal rather than meibomian origin (Lam et al., 2014b). Both phospholipids and OAHFAs have been correlated with ocular surface disease, and it is likely that they both are important in maintaining homeostasis (Pucker and Haworth, 2015).

A summary of the key studies exploring the composition of the human meibum lipidome, are reported in ***Table 1.8***.

Table 1.8. Key findings of studies exploring the meibum lipidome

Study objective	Key findings	Reference
Analyse free fatty acid component in chronic blepharitis	• significant variation in proportions of lipid classes between individual samples • pooling misleading	(Tiffany, 1978)
Comparison of steer whole gland lipid and human excreted lipids.	• essentially the same lipid mixture • Coined the word 'meibum' to distinguish between lipid from the meibomian and sebaceous glands	(Nicolaides et al., 1981)
Determine polar lipid composition of human meibum	• polar phase composed of phospholipids and sphingolipids • proposed that free fatty acids and possibly small amounts of nonpolar lipids such as triglycerides also present in the polar phase	(Shine and McCulley, 2003)
Determine if aging associated with alterations in meibum	• Aging associated with significant sex-related changes in the polar and non-polar lipid profiles	(Sullivan et al., 2006)
Identification of FA and FFAs in human meibum	• positive-ion mode revealed a mass peak of m/z 282.3, oleamide, a predominant component • Myristic, palmitic, stearic, and oleic free fatty acids also identified • fatty acid amides (myristamide, palmitamide, stearamide, and erucamide).	(Nichols et al., 2007)
Comparison of meibum in MGD and ADDE	• ↑branched chain fatty acids (wax, cholesterol esters and triglycerides) in MGD • ↓saturated fatty acids palmitic (C16) and stearic (C18) acids in MGD	(Joffre et al., 2008)
Targeted analysis of meibum for presence of WE and related compounds	• very long chain OAHFAs discovered in meibum	(Butovich, 2009)

Study objective	Key findings	Reference
Determine major molecular components of the lipids in normal human meibum	• major peaks detected in positive-ion mode: nonpolar lipids, including WE, ChE, TAGs, and diesters. • negative mode polar lipids: free fatty acids and (O-acyl)-ω-hydroxy fatty acids.	(Chen et al., 2010a)
Analyse the composition of meibum in different age groups	• ↓ cholesterol esters in MGD similar to infants • differences in amount of ChE alone unlikely to be responsible for the greater stability of the infant tear film. Suggest specific features of ChE (chain length, branching, and degree of unsaturation) may change with age and disease.	(Shrestha et al., 2011)
Comparison of collection and extraction techniques	• total lipids showed significance for collection device but not for extraction technique or dry eye status • spatula collection & immediate extraction associated with highest average amount of cholesterol detected.	(Haworth et al., 2011)
Identification of phospholipids in meibum	• suggest tear phospholipids are derived from meibum	(Saville et al., 2011)
To explore the meibum lipid composition in Asians with DED	• several ChE species significantly higher in moderate than mild DED • No appreciable difference observed in overall lipid distribution between DED & controls • several unsaturated TAGS and PC species ↑ in DED. Needed larger cohort • ↓OAHFAs correlated with ↑DED severity but needed larger cohort.	(Lam et al., 2011)
Comparison of patient matched meibum and tear lipidomes	• meibum likely to supply majority of lipids in tear film lipid layer with exception of phospholipids • observed higher mole ratio of phospholipid in tears	(Brown et al., 2013)
Develop method for rapid identification of FA and OAHFAs	• retention time correlates with the number of double bonds and carbon chains	(Mori et al., 2014)
Explore inter-subject and inter-day variability in tear and meibum lipidomes	• Wax esters greatest inter- subject variation	(Brown et al., 2016)
To examine relation between changes in FFA composition of meibum and signs and symptoms of MGD	• elevated levels of linoleic acid associated with severity of plugging of gland orifices leading to disruption of lipid layer, and telangiectasia	(Arita et al., 2016a)
MS elucidation of OAHFAs	• used a standard Oleoyl stearate WE (WE 18:1/18:0) • confirmed the structure of the most abundant OAHFA found in human meibum, OAHFA 50:2	(Hancock et al., 2018)
Compare molar ratio of ChE/WE in MGD and controls	• ↓ cholesterol esters in MGD, but other factors, such as levels of saturation may be responsible for higher order MGD • No sex differences	(Borchman et al., 2019)
To investigate association between precorneal tear film (PCTF)– and meibum-derived OAHFAs & PCTF thinning in meibomian gland health & dysfunction.	• several human meibum-derived OAHFAs showed significant associations with PCTF thinning, suggesting that these OAHFAs could be implicated in the mechanism underlying the stabilization and thinning of the PCTF. • tear-film derived OAHFAs independent of rate of PCTF thinning. • suggest possible homeostatic balance among several OAHFAs in the TFLL	(Khanal et al., 2021)
To investigate alteration in meibum composition, signs & symptoms associated with aging & MGD	• MGD and aging affect ratio of major lipids • ↓in non-polar lipids (ChE) in MGD & elderly • and ↑polar lipids (free cholesterol, OAHFAs & free FA) • no differences in WE • vision-related symptom scores significantly negatively correlated with ratio of ChE, but significantly positively correlated with polar lipids Ch, OAHFA, and FA.	(Suzuki et al., 2022)

Analysis of meibum, and changes related to meibomian gland loss are discussed in more detail in Chapter 7 of this thesis.

1.5.6. Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are a heterogenous group of cell-secreted membranous structures broadly divided into two main categories, exosomes (small EVs of 30-150nm) and microvesicles (large EVs) dependent on their size (van Niel et al., 2018).They have been identified in several body fluids, including tears, and are secreted by many different cell types, including inflammatory cells. They were first reported in the 1970s and thought to be a mechanism for the elimination of unwanted substances from cells; they have since been shown to contain mRNAs and microRNAs (miRNAs) derived from secretory cells and are considered as an additional mechanism for intercellular communication, allowing cells to exchange proteins, lipids, and the genetic material (Hanayama, 2020). They may also act as signalling vehicles in normal cell homeostasis or occur as a consequence of pathology. Much is still unknown about their physiological and pathological origins and functions, and their possible use in clinical applications.

However, there is evidence that immunologically active exosomes can regulate both innate and adaptive immune responses and have been identified in several immune-mediated eye diseases, including SS DED (Li et al., 2019). They are considered to have potential as novel biomarkers and drug delivery vehicles.

A recent study using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to analyse the tear fingerprint from dry eye and normal tears, isolated EVs by means of a nanosized filter-based method (Zhang et al., 2020b). The EVs found in the DED patients were found to have a heavier molecular weight than those from normal controls. A significant amount of research is needed in this newly evolving area.

1.6. Treatment of Dry Eye Disease

Following an evidence-based review of current dry eye therapies and management options, the TFOS DEWS II Management and Therapy Subcommittee devised a staged management plan for DED, with a stepwise approach to implementation, dependent on disease severity. They reported a lack of Level 1 evidence (meta-analysis, systematic review or RCTs) to support many of the recommended managements for DED, often due to poor study design (Jones et al., 2017).

Selection of the most appropriate management strategy must be based on an initial differentiation between ADDE and EDE, although often there can be overlap. Initial or step 1 management involves patient education and modification of the local environment (Jones et al., 2017). A thorough history can elucidate modifiable risk factors which can be mitigated against, such as screen time use, air-conditioning and hydration and contact lens use. Controlled adverse environment chambers are being used to study the effects of different environmental conditions, such as temperature, humidity and airflow on DED and its treatments (Calonge et al., 2018). They can also be used to study patients in controlled normal healthy environments. Several studies have identified an increased concentration of inflammatory markers in the tears of patients with DED; one study found that tear levels of IL-6 and MMP-9 significantly increased and EGF significantly decreased after exposure to simulated air cabin conditions (Tesón et al., 2013).

1.6.1. Dietary modifications and nutritional supplements

The recent TFOS Lifestyle Workshop, which focused on the direct or indirect consequences of lifestyle choices on the ocular surface and adnexa, addressed the impact of nutrition, a key modifiable lifestyle factor (Markoulli et al., 2023). Dietary modifications and nutritional supplements have received considerable interest and extensive research. Nutritional supplements with anti-inflammatory and anti-oxidative properties are used with the aim of interrupting the viscous cycle of inflammation in DED. Omega-3 and omega-6 are important macronutrient lipids that have been the source of significant investigation with respect to their role in ocular surface health. The human body is incapable of synthesizing them de novo, and therefore they must be consumed in the diet. Diets dominated by omega-6 fatty acids are primarily pro-inflammatory, while diets dominated by omega-3 fatty acids tend to be anti-inflammatory (Simopoulos, 2002).

While a possible role for long-chain omega-3 supplementation in managing DED has been suggested, the evidence remains uncertain and inconsistent. Further research is needed to gain a full understanding of the role of omega-3 and 6 supplements and provide information about how supplement characteristics, such as dose, form, and composition affect clinical outcomes (Downie et al., 2019). Current studies have included participants from 13 countries with dry eye of variable severity and aetiology. Variations in the length of follow-up, lack of published protocols and high risk of bias in one or more domains of previously published studies also confound their reliability.

Deficiency in micronutrients such as vitamins A, B12, C and D are also thought to play a role in the health of the ocular surface (Jones et al., 2017). Vitamin A is naturally present in the tear film of healthy eyes and is known to be involved in ocular surface health. Deficiency causes goblet cell loss, mucin

deficiency, and keratinization, which lead tear film instability (Tseng, 1986). Both topical and systemic vitamin A have been shown to play a role in the treatment of DED. Short-term oral supplementation has shown an improvement in the quality, but not quantity, of tears (Alanazi et al., 2019). Further studies of longer duration with larger cohorts are needed in this area.

Vitamin B12 deficiency has been implicated in neurosensory abnormalities, while vitamin C plays a role in antioxidant defence and cornel wound healing (Pellegrini et al., 2020). Vitamin D also plays a role, by modulating the expression of inflammatory cytokines, and protecting the corneal barrier function by reducing the expression and activation of MMPs (Shetty et al., 2016).

1.6.1.1. Omega-3

Omega-3 polyunsaturated fatty acids are essential structural components of cell membranes, regulate lipid metabolism and have anti-inflammatory properties. They must be obtained from the diet and can exist as both short and long chain subtypes (Jones et al., 2017). They can inhibit the formation of omega-6 fatty acid derived pro-inflammatory eicosanoids (signalling molecules) or form potent anti-inflammatory lipid mediators (e.g. resolvins and protectins) (Serhan and Petasis, 2011). They have been shown to block the gene transcription of the pro-inflammatory cytokines (TNF-α, IL-1α and IL-1β) that block neurotransmitter release in the lacrimal gland (Kang and Weylandt, 2008). Their biological activity is dependent on their ratio with Omega-6 obtained through the diet and is ideally 4:1 (Pellegrini et al., 2020).

The majority of the many studies (RCTs and meta-analyses) exploring the role of long chain Omega-3 supplementation in DED have shown a significant improvement in several clinical features (ocular surface staining, TBUT, Schirmer test, osmolarity, MMP-9 positivity) as well as symptom scores (OSDI) (Pellegrini et al., 2020). The efficacy of two forms of Omega-3 supplementation , a phospholipid (krill oil) and a triacyl glyceride (fish oil) in treating DED have been assessed in a randomized, double-masked, placebo-controlled clinical trial (Deinema et al., 2017). Both resulted in reduced tear osmolarity and increased tear stability. Also, the effect of using Omega-3 supplements for 12 months would appear to endure after they are discontinued, with outcomes not significantly different in those who continued their use (Hussain et al., 2020).

Topical use of low concentrations of linoleic acid (a plant derived short chain fatty acid) has been shown to improve the stability and spreading of the tear lipid layer by increasing its elasticity and compressibility (Mudgil, 2020). Flaxseed oil has also been shown to improve the efficacy of a tear substitute in ameliorating the signs and symptoms of DED (Downie et al., 2020). Further work is needed to confirm optimum dosages of both methods of administration.

1.6.1.2. Complimentary medicines

Complementary medicines, including herbal/natural products and acupuncture have also been explored. Curcumin has been applied to treating inflammatory diseases such as chronic anterior uveitis, as it is a natural polyphenol or micronutrient shown to have antioxidant properties (Chen et al., 2010b). Chen et al. (2010b) were able to show that curcumin completely abolished the increased production of the pro-inflammatory IL-1β which occurred in a hyperosmolar-induced culture of human corneal epithelial cells. They therefore suggested that curcumin may have therapeutic potential as an anti-inflammatory agent in the treatment of DED.

1.6.2. Modification of topical and systemic medications

Several topical and systemic medications have been implicated in DED. Numerous systemic drugs, particularly those with antimuscarinic effects, such as antidepressants, antihistamines and antihypertensive drugs have been shown to contribute or aggravate DED (Jones et al., 2017). Several topical treatments, particularly those used to treat glaucoma, such as the prostaglandins, may also induce or worsen DED (Jones et al., 2017). A reduction in tear film stability and an increase in ocular surface inflammation, including an increase in the inflammatory marker IL-1β, has been documented in patients using preserved timolol 0.5% eyedrops when compared to non-preserved drops (Manni et al., 2005). Therefore, preservative free options are often recommended for long term use.

1.6.3. Lid hygiene and warm compresses

Lid hygiene and warm compresses have been found to be beneficial in lid conditions associated with DED. A wide variety of lid cleansing products, including scrubs, foams, solutions, and wipes are available to reduce the bacterial load and toxins which can build up on the lids in anterior and posterior blepharitis. Eyelid cleanser has been shown to reduce MMP-9 expression, confirmed by reduced clinical signs of inflammation (Sung et al., 2018).

Identification and treatment of demodex blepharitis is mentioned in Step 2 of the TFOS DEWS II management plan (Jones et al., 2017). Infestation is a causative factor in blepharitis and is often associated with DED (Zhao et al., 2012). Tea tree oil (TTO), a natural essential oil with antimicrobial, anti-inflammatory, antifungal, and antiviral properties, has been shown to be toxic to Demodex, and the eye if used in its pure form. However, the active component 4-Terpineol is commercially available in pre-formulated wipes that are equivalent to a quarter of the concentration in pure TTO (Tighe et al., 2013b).

Warm compresses have been proven to be effective in management of MGD (or posterior blepharitis) in several clinical studies. The optimum method, time, and temperature at which to heat the lids and soften or liquefy the meibum secretions, and the effect on the tear fluid lipidome has been investigated (Lam et al., 2014a). A reduction in phospholipase activity, an excess of which can be detrimental to tear film stability through the excessive breakdown of phospholipids, correlated with a reduction in tear evaporation rate and improved ocular symptoms. In addition, treatment also lead to an improvement in OAHFAs (Lam et al., 2014a).

1.6.4. Over-the-counter ocular lubricants

Prescribing of over- the- counter ocular lubricants is often the first line of treatment in DED. Artificial tears can be used to increase tear volume, improve lubrication, nourish the ocular surface, improve tear film stability, and dilute the concentration of inflammatory cytokines. A wide variety of compositions and choices are available with relatively few RCTs comparing products for superiority (Jones et al., 2017). This can make the decision of which lubricant to prescribe difficult and often untargeted (see discussion in Chapter 2, Dry Eye in Clinical Practice Survey).

Step 2 of the TFOS DEWS II management plan recommends preservative free lubricants to minimize preservative-induced toxicity (Jones et al., 2017). Due to the chronic and progressive nature of DED, topical products may be administered several times a day, over a period of many years and therefore preservative free options may be preferred. Considerable evidence has shown that the most commonly employed and effective preservative, benzalkonium chloride, can cause adverse effects on the ocular surface and an increase in inflammatory cytokines and so its use should be avoided in treating DED (Walsh and Jones, 2019).

Treatment with preservative-free versus preserved 0.1% sodium hyaluronate and 0.1% FML eye drops combined with 0.05% cyclosporine, showed no initial significant difference in the mean concentrations of IL-1β, IL-6, IL-12, and TNF-α in tears (Jee et al., 2014). However, in the preservative-free group, there was a statistically significant decrease at 1, 2, and 3 months in all markers compared to initial values. Carboxymethycellulose glycerine castor oil has also been shown to reduce up to six tear inflammatory markers by more than 25% reduction in 40% of participants (Martin et al., 2018). The same reduction (> 25%) was seen in only 10% of participants treated with carboxymethycellulose and in none with hydroxypropyl guar.

1.6.4.1. Osmoprotectants

Protection of the corneal epithelium is a key feature in DED therapy, to break the vicious cycle that is sustained by po-inflammatory cytokines produced during epithelial damage. Trehalose, a naturally

occurring sugar, has been recognised as an osmoprotectant with potential as a therapeutic tool due to its ability to confer resistance to desiccation and control inflammation (Aragona et al., 2020). Hyaluronic acid is another naturally occurring ingredient of several artificial tear formulations, used due to its ability to increase viscosity, improve retention time, and improve ocular surface hydration and lubrication (Jones et al., 2017). It is naturally distributed in connective, neural, and epithelial tissue and has been shown to have the ability to bind ocular surface cells and promote wound healing (Inoue and Katakami, 1993).

Eyedrops containing trehalose/hyaluronate can reduce tear levels of IL-1β, IL-6, and IL-8 indicating possible reduction in ocular surface damage (Fariselli et al., 2018). This is thought to be due to the synergistic action of hyaluronic acid and trehalose in targeting different aspects of the dry eye inflammatory cycle (Fariselli et al., 2018). Tear substitute formulations which are designed not only to alleviate symptoms but also to target the cause are known as Multiple Action Tear Substitutes (MATS).

1.6.5. Tear conservation

Further treatment options included in step 2 of the TFOS DEWS II include tear conservation, overnight treatments (ointment or moisture chamber devices) and in-office treatments (Jones et al., 2017). Conservation of tears can be achieved through spectacles or goggles designed to reduce evaporation by reducing airflow across the ocular surface or by providing a moist environment. A randomised controlled trial of moisture chamber spectacles demonstrated an improvement in ocular comfort and tear film stability (Shen et al., 2016).

1.6.5.1. Punctal plugs

Conservation of tears in ADDE can also be achieved by occluding the puncta with punctal plugs, either temporary absorbable plugs, non-absorbable plugs, or permanent surgical occlusion. Punctal occlusion is reported to be supported by numerous studies which all indicate improved symptoms or tear function (Jones et al., 2017). Non-absorbable punctal plugs can reduce inflammatory proteins and stimulate lacrimal proteins, leading to an improvement in DED in some patients (Tong et al., 2017).

1.6.6. In-office treatments

Physical treatments for MGD include forceful expression of the glands, debridement of the lid margins, intraductal probing and heating devices such as LipiFlow® and intense pulsed light (IPL) therapy. A study investigating the efficacy of four in-office forceful gland expressions over a 6- month period found an improvement in the number of expressible glands, quality of secretion and lipid layer

thickness and improved comfort and decreased symptoms, when used in conjunction with daily warm compresses (Korb and Greiner, 1994).

1.6.6.1. LipiFlow®

A single 12- minute treatment with LipiFlow® thermal pulsation has been shown to be effective and superior to traditional warm compresses, with the effect sustainable for up to a year (Blackie et al., 2016). A study to investigate the effect of thermal pulsation treatment on MMP-9 levels and osmolarity of the tear film demonstrated significant improvements in both subjective and objective measures of DED (Kim et al., 2017). Treatment with the LipiFlow® device led to improvements in TBUT, OSDI score, osmolarity and MMP-9 (50% positive rate pre-treatment compared to 26% positive rate post- treatment), in patients with moderate or severe DED.

1.6.6.2. Intense pulsed light (IPL) therapy

IPL uses intense pulses of noncoherent polychromatic light in the wavelength range of 500 to 1200 nm which is converted to heat in the tissues to treat various skin conditions (Raulin et al., 2003). When a concurrent improvement in ocular surface conditions was observed in patients treated for rosacea, its potential for the treatment of MGD was realised. Treatment in the periocular region has been shown to improve tear film quality, decrease ocular surface inflammation and reduce dry eye symptoms in MGD (Craig et al., 2015). A randomized, double-masked, controlled study analysed tear samples at baseline and following three sessions of IPL or placebo treatment. The inflammatory markers, IL-17A and IL-6 showed statistically significant decreases compared to placebo treatment and correlated well with ocular surface parameters of the lower eyelid (Liu et al., 2017b). A further study has shown that tear levels of IL-4, IL-6, IL-10, IL-17A, and TNF-α have been shown to decrease after IPL treatment; IL-6 and TNF-α were also found to correlate with the improvement in meibum quality and expressibility (Choi et al., 2019).

Other non-pharmaceutical options for the treatment of DED include use of therapeutic contact lenses, amniotic membrane grafts and other surgical approaches, such as tarsorrhaphy (Jones et al., 2017).

1.7. Pharmaceutical treatments and biomarker studies

There are several pharmaceutical agents which can be used for the treatment of DED. Information in ***Table 1.9*** has been based around the recommended stepwise management approach of the TFOS DEWS II Management and Therapy Report (Jones et al., 2017). Information has also been collated from The College of Optometrists' Formulary and a research paper reporting on diagnosis and treatment of dry eye (Kojima et al., 2020).

Table 1.9. Pharmaceutical drugs used for the management of DED

	Drug type	Drug name	Drug action/use
Step 2 of the TFOS DEWS II management plan			
	Topical antibiotic and/or antibiotic/steroid combination	• Fusidic acid 1% • Chloramphenicol 0.5%	Reduce bacterial load on lid margins in DED associated with blepharitis
	Topical corticosteroid for limited duration	• 0.5% loteprednol etabonate (Lotemax®) • 0.5% prednisolone sodium phosphate • 1% prednisolone acetate (Pred Forte®) • 0.1% fluorometholone (FML®) • 0.1% dexamethasone sodium phosphate	Anti-inflammatory action to break the vicious cycle. Caution in use due to possible adverse effects, such as glaucoma, cataract, increased risk of infection and delayed wound healing.
	Topical secretagogues	• Diquasol sodium 3% (unlicensed in UK)	Promotes secretion of tear fluid via an ↑in intracellular Ca+ concentration & ↑ mucin secretion
		• Rebamipide 2% (unlicensed in UK)	Mucin secretagogue promotes production of mucin-like glycoproteins
	Topical non-glucocorticoid immunomodulators	• Ciclosporin 0.05% (Ikervis®) • Restasis® (CsA 0.05%) (unlicensed in the UK)	Immunomodulatory with anti-inflammatory properties. ↓inflammation by inhibiting T cell recruitment and activation & ↓expression of HLA-DR
	Topical LFA-1 antagonist	• Lifitegrast ophthalmic solution 5% (Xiidra®) (unlicensed in the UK)	Lymphocyte function-associated antigen 1 (LFA-1) antagonist
	Oral macrolide antibiotic		Anti-infective and anti-inflammatory properties
	Oral tetracycline antibiotic		Broad spectrum antibiotics with anti-inflammatory properties. Inhibit protein synthesis
	Topical macrolide	• 1.5% azithromycin dihydrate (Azyter®)	Anti-infective and anti-inflammatory for lid margin
	Topical immunosuppressant	• Tacrolimus 0.03%	Immunosuppressant: blocks T-cell activity. Lid margin disease.
Step 3 of the TFOS DEWS II management plan			
	Oral secretagogues	• Pilocarpine • Cevimeline	Cholinergic agonists to counteract autoantibodies that bind to muscarinic acetylcholine receptors

	Drug type	Drug name	Drug action/use
			in SS. Stimulate tear production
	Autologous or allogeneic serum	• See text	Fluid component of blood with similar biochemical characteristics to tears. i.e. growth factors and proteins. Inhibit release inflammatory cytokines, ↑goblet cell and mucin, enhance wound healing and nerve regeneration
Step 4 of the TFOS DEWS II management plan			
	Topical steroid for longer duration	As above	As above
Other potential pharmaceutical therapies			
	Biologics	Recombinant human lubricin (no commercially available products)	Lubricating, mucin-like glycoprotein
		Regener-Eyes (Licensed in USA, not available in the UK)	Uses naturally occurring anti-inflammatory cytokines, growth factors and chemokines to ↓inflammation & stimulate healing and repair of OS.
		IL-1 receptor antagonist (Anakinra 2.5%; Kineret®) ↓corneal staining in MGD associated dry eye study (Amparo et al., 2013)	Inhibits the pro-inflammatory action of IL-1α and IL-1β by competitively blocking their binding to the IL-1 cell receptor 1
		TNF-α blocker (systemic infliximab and etanercept and topical infliximab) (research only)	Block the co-stimulatory anti-inflammatory effects of TNF-α
		Anti-IL-17 (animal studies only)	Block the disruption of the corneal epithelium caused by IL-17
	Tear-based biotherapeutics		
		Lactoferrin oral supplement (270mg/day). Clinical trial in SS patients (Dogru et al., 2007)	Multifunctional tear glycoprotein with antibacterial, anti-inflammatory and anti-angiogenic properties
		Topical lacritin. Clinical trial in ADDE (Vijmasi et al., 2014)	Glycoprotein which promotes basal tearing (Samudre et al., 2011)
		Recombinant tear lipocalin (Karnati et al., 2013)	Main lipid binding protein in tears

Items in green are not listed as part of the current TFOS DEWS II management plan. ↑= increased ↓= decreased

As well as providing information to aid in the diagnosis of DED, tear film biomarkers have been investigated for their usefulness as predictive and therapeutic biomarkers of DED treatments. Studies

of biomarkers taken at baseline, and then during or after treatment, can indicate whether a certain biomarker is affected by the intervention and therefore if the treatment is beneficial. Building a cytokine profile prior to instigating a therapy, particularly immune based therapies, could allow for differentiation between expected responders and non-responders to the treatment and therefore better management. Patients with similar cytokine profiles could receive similar interventions allowing for a more targeted management approach. Some therapeutic agents have been specifically studied to investigate for changes in inflammatory biomarkers.

1.7.1. Topical steroids

Glucocorticoids are used successfully to treat a wide range of inflammatory conditions, including DED. They inhibit expression of many genes involved in inflammatory and immune responses, including inflammatory mediators such as cytokines, chemokines, cell surface receptors, adhesion molecules, tissue growth factor and degradative proteinases (Barnes, 2006). The TFOS DEWS II staged management and treatment guidelines suggest that repeated short-term pulse therapy of corticosteroids can be an alternative approach (step 2) for patients with moderate-to severe DED not controlled with other therapies (Jones et al., 2017). Studies exploring the use of loteprednol etabonate (Aragona et al., 2015), prednisolone (Lee et al., 2006) and fluorometholone (Li et al., 2020a) have shown a beneficial reduction in inflammatory cytokines.

1.7.2. Topical secretagogues

Diquasol tetrasodium 3% ophthalmic solution is an aqueous/mucous secretagogue approved for use in dry eye treatment in several Asian countries. It acts on P2Y2 receptors to stimulate tear fluid secretion from corneal and conjunctival epithelial cells and promotes mucin secretion from conjunctival goblet cells and thus improves tear film stability (Kojima et al., 2020). When compared both diquafosol 3% and sodium hyaluronate 0.1% ophthalmic solutions exhibited similar efficacy in improving fluorescein staining scores in dry eye patients (Takamura et al., 2012). However, diquafosol was shown to exhibit superior efficacy in improving rose bengal staining scores. Several other studies have shown its use to be effective and beneficial in various dry eye disorders, but as yet it is not approved for use in the UK and failed to achieve FDA approval in the USA (Jones et al., 2017).

Similarly, 2% rebamipide ophthalmic suspension, a mucin secretagogue, which increases numbers of goblet cells in the bulbar conjunctiva and the lid wiper area of palpebral conjunctiva (Hori, 2018) has not been approved for treatment of DED in the UK.

1.7.3. Topical non-glucocorticoid immunomodulators: Cyclosporine A

Topical cyclosporine A (also known as ciclosporin A) (CsA), an immunomodulatory drug with immunosuppressive properties, is prescribed to target inflammation by blocking the transcription of cytokine genes in activated T-cells (Matsuda and Koyasu, 2000), and is used systemically in autoimmune diseases and to prevent organ transplant rejection. There is a strong body of level 1 evidence to support its topical use in the treatment of DED, although its therapeutic effect can take several months (Jones et al., 2017). A Cochrane Database Systematic Review reported an increase in goblet cell density as a major attribute of its use (de Paiva et al., 2019). However, evidence did not support that the increase translated to improved symptoms or clinical signs.

1.7.4. Topical LFA-1 antagonist: Lifitegrast

Lifitegrast was approved by the US Food and Drug Administration for use in patients with DED, in 2016. It has been designed to reduce inflammation by preventing the interaction of lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) thus reducing the release of cytokines from T cells (Paton, 2016). Four clinical trials have shown that Lifitegrast is well tolerated and effective in improving signs and symptoms of DED over a 12- week period; a fifth trial showed long-term safety over 1 year (Chan and Lisa Prokopich, 2019). As yet Lifitegrast remains unlicensed in the UK.

1.7.5. Oral antibiotics

1.7.5.1. Tetracycline antibiotics

Although oral antibiotics are used commonly in the management of OSD, until recently relatively few studies have assessed their efficacy in a clinically meaningful way. Tetracycline and its analogues, minocycline and doxycycline, are broad spectrum antibiotics used to treat dry eye associated with disorders such as MGD, blepharitis and acne rosacea (Jones et al., 2017). They have both anti-inflammatory and antibacterial properties. They have been shown to decrease the activity of collagenase, phospholipaseA2, several MMPs and production of inflammatory mediators such as IL-1 and TNF-α (De Paiva et al., 2006a). Their antibacterial role has been hypothesized to cause a decrease in bacteria-producing lipolytic enzymes and inhibition of lipase production resulting in a decrease in meibomian lipid breakdown products (Dougherty et al., 1991).

1.7.5.2. Macrolide antibiotics

Azithromycin is a macrolide antibiotic, also with antibacterial and anti-inflammatory properties but with fewer side effects than the tetracyclines, namely photosensitivity and gastrointestinal symptoms.

It is available in both topical and systemic forms and is thought to assist in bacterial flora control, lid inflammation and inhibiting production of inflammatory cytokines (Rubin, 2004). Both doxycycline and azithromycin have been shown to suppress production of MMP-9 and inflammatory cytokines and doxycycline to preserve corneal barrier function in the murine dry eye models (De Paiva et al., 2006a;De Paiva et al., 2006b).

Topical application of azithromycin has been shown to temporarily suppress the expression of proinflammatory mediators IL-1β, IL-8, and MMP-9 mRNA and restore transforming growth factor β1 (TGF-β1) levels (Zhang et al., 2015). Similar results were noted with topical administration of azithromycin ophthalmic solution when compared to systemic administration; some superiority was noted with topical application, with more persistent results likely due to the higher ocular tissue concentration with topical administration (Yildiz et al., 2018). Oral administration of both azithromycin and doxycycline are effective for treating patients with persistent MGD, although azithromycin was shown to be superior, with a reduced dose and a shorter course of therapy required (5 days vs. 4 weeks) (De Benedetti and Vaiano, 2019).

1.7.6. Biological tear substitutes

Several blood derivatives have been proposed for the treatment of various severe OSDs that are refractory to conventional therapy. The source of the blood can vary from the patient's own peripheral blood (autologous) to donor blood, mainly allogeneic peripheral blood or umbilical cord blood. Serum is the fluid component that remains after blood has clotted**.** The advantage of autologous serum is that many of its biochemical characteristics are similar to that of human tears, such as albumin and growth factors EGF and NGF (Villatoro et al., 2017)). In vitro and in vivo studies have shown that serum and other blood derivatives can enhance corneal epithelial wound healing, inhibit the release of inflammatory cytokines, and increase the number of goblet cells and mucin expression in the conjunctiva (Jones et al., 2017).

1.7.6.1. Autologous serum

Despite the legal and logistical requirements around the use of autologous serum for the treatment of severe OSD, it is gaining more widespread acceptance. A large cohort study to assess patient satisfaction with autologous serum tears for the treatment of dry DED concluded that the treatments significantly reduced symptoms in severe cases, with high patient satisfaction scores (Kreimei et al., 2019).

1.7.6.2. Allogenic serum

Allogeneic serum can be used as an alternative for patients who have active systemic inflammation as it can be prepared from previously stored blood. However, there is then a theoretical risk of an immune response to foreign antigens. As yet, there is limited clinical data regarding its use in DED.

1.7.6.3. Cord blood serum

There appears to be more research into the use of umbilical cord serum possibly because it is easier to collect in large amounts. Umbilical cord serum has the additional benefit over other serum as a source of stem cells, which are being used in the field of regenerative medicine because they can differentiate into various mature cells (Giannaccare et al., 2020).

1.8. Summary of biomarker research

The presence of clinically significant inflammation has been confirmed in the tears of 40%–65% of patients with symptoms of dry eye (Sambursky, 2016). Significant interest exists in the inflammatory biomarkers involved in the underlying pathophysiology of DED, and development of biomarkers to aid in diagnosis, classification, treatment efficacy and clinical trial endpoints. Review of the literature points to several biomarker candidates for which there has been a large amount of research, whereas understanding of others such as phospholipase (PL)A2-IIa is more limited. While the presence of certain pro-inflammatory cytokines, such as IL-1β and IL-6 have been implicated in ocular surface stress and damage (Lam et al., 2009;Enríquez-de-Salamanca et al., 2012), proteins and growth factors have been used to assess lacrimal gland function. Cytokines that are known to mediate chronic inflammatory processes include IL-4, IL-5, IL-6, IL-7, and IL-13, while those that regulate cellular responses include IL-1, IL-2, IL-3, IL-9, IL-10, IL-12, IFNs, TGF-β, and TNF-α and β (Hagan and Tomlinson, 2013).

Potential biomarkers have been considered in relation to systemic disease; an increase in IL-6, IL-17, IL-8 and TNF-α has been reported in DED without systemic disease, while IL-1, IL-8 and IL-17 are found to be increased in DED with systemic disease (Fong et al., 2019). Potential tear film biomarkers have been explored in relation to DED with an underlying inflammatory disease such as SS, SJS and GVHD. Primary SS patients have shown elevated tear fluid levels of IL-1Ra, IL-2, IL-4, IL-8, IL-12p70, IL-17A, IFN-γ, IP-10, MIP-1β, and RANTES when compared to non-SS subjects and healthy controls (Chen et al., 2019b).

Na et al. (2012) reported IL-6 and Il-1β to be the earliest detectable biomarker indicators of DED, while IL-6, IL-1β, IL-8 and TNF-α have all been implicated in pain sensation ((Lam et al., 2009;Na et al., 2012;Li

et al., 2020a). A review by Roy et al. (2017) reported that IL-6, IL-17a, IL-8 and TNF-α appear to be consistently elevated in the tears of patients with DED when compared to controls, while a later meta-analysis indicated IL-1β, IL-6, IL-8, IL-10, IFN-γ and TNF-α to be key players in DED (Roda et al., 2020).

However, considerable interstudy variation in concentrations of these biomarkers exists. Confounding influences include methodology for tear collection, time of collection and analysis method, differences in statistical analysis, patient selection, and criteria for defining severity and subtype of DED. Some cytokine concentrations have been shown to vary significantly depending on time of collection (IL-10 and IL-1β), whilst others could be measured reproducibly (Benito et al., 2014). Variations in the tear protein profile have also been reported when comparing open and closed eye tears, with the balance of cytokines moved to pro-inflammatory status in closed eyes (Uchino et al., 2006).

Additionally, some proteins such as S100A8 and S100A9, which have been found to be associated with the severity of MGD (Tong et al., 2011) have also been reported in patients with glaucoma, indicating that these inflammatory proteins are not expressed uniquely in patients with dry eye (Wong et al., 2011).

To overcome the variabilities and confounding factors mentioned, Wei et al. (2013) established and validated standard operating procedures for human tear inflammatory cytokine assessment. They demonstrated that a panel of tear inflammatory cytokines, IL-1β, IL-6, IFN-γ, and TNF-α could be used to monitor inflammatory diseases of the ocular surface such as DED. Use of a panel of tear proteins in conjunction with clinical signs and symptoms has been proposed to support clinicians in the diagnosis of mild or early stage DED, as individual biomarkers can lack the required sensitivity and specificity (Versura et al., 2013). The panel proposed included lipocalin-1, exudated serum albumin and total protein content, with a sensitivity of 91% and specificity of 99%. Further longitudinal clinical studies have also been recommended to strengthen the validity of potential biomarkers (D'Souza and Tong, 2014).

Despite extensive research and advances in our understanding of the underlying pathological processes involved in DED, many questions around therapeutic management remain. Fewer studies have explored the effect of treatments on inflammatory biomarkers. Novel disease targets and innovative targeted rather than palliative therapeutic strategies are being developed offering promising new treatment options for this complex, multifactorial disease. However, despite supportive preclinical evidence many therapeutic candidates for DED fail in clinical trials with clinical trial length, environmental factors, heterogeneity in study populations (e.g., age, gender, comorbidities), and the heterogeneity of DED itself being potential confounders (Chao et al., 2016).

Therefore, although a significant amount of progress has already been made, the search continues for the ideal biomarkers to enable accurate and timely diagnosis, classification, monitoring and even prediction of DED, in order to alleviate the burden it presents.

1.9. Thesis overview

Around 32% of the UK population is estimated to have DED (Vidal-Rohr et al., 2023). This not only has a potential negative effect on the quality of life of our patients but also poses a significant economic burden. While advances in evidence-based treatment options exist, diagnosis of a disease where signs and symptoms do not always corelate can prove challenging.

Just a small part of the considerable body of research into biomarkers for DED has been reported in this chapter. While numerous studies have focused on ADDE, with a known systemic inflammatory component, fewer researchers have explored inflammatory activity in EDE, particularly in younger populations; although the prevalence of DED is known to increase with age, several reports indicating an increasing prevalence in younger cohorts have emerged over recent years. Therefore, the main aim of this thesis was to explore the tears and meibum of a young adult cohort for biomarkers of evaporative DED.

Furthermore, it is relevant to know if existing research results are actually being translated into evidence-based practice. Therefore, a further objective of this thesis, addressed in Chapter 2, is to gather information on the clinical practice patterns in DED management of UK optometrists. To this end, an online survey aimed to address questions such as: the awareness of optometrists working in grass roots optometry of the evidence behind the clinical procedures they use, awareness of the existing validated point-of-care devices available which utilise tear film biomarkers, perceptions of DED, and satisfaction with currently available treatment options. Also addressed is the question of whether gaining independent prescriber status has impacted DED management in the UK. The results of the survey are discussed in chapter two.

Chapter 3 reports on a pilot study to confirm the repeatability of a method of micro-fluidics based electrophoresis to explore the tear protein profile and total protein content of human tears, prior to its application in the larger study reported in chapters 4 to 7. Although it is possible to generate considerable amounts of information regarding hundreds of tear proteins using methods such as high-performance liquid chromatography (HPLC), and mass spectrometry, this method aimed to simplify the data into a tear profile of the main lacrimal proteins. The hypothesis that inter-visit tear protein profiles measured using the Agilent 2100 Bioanalyzer, are repeatable, is therefore explored in order

to provide a reliable method for investigating tear proteins. Details of the methodology for tear collection, storage and subsequent analysis using this method are also addressed.

Chapters 4 to 7 report the findings of a prospective, longitudinal study to explore evaporative DED in a young adult cohort. Chapter 4 describes the methodology used in the study and the clinical results. It also explores risk factors for evaporative DED, including modifiable lifestyle factors and forms the basis for the following three chapters. Chapter 5 details the results of the tear protein analysis of samples collected at both visits of the longitudinal study and is based on application of the method reported in Chapter 3. Further investigation for potential tear protein biomarkers was performed on tear samples collected with Schirmer strips, the details of which are reported in Chapter 6. Chapter 7 describes investigation of the composition of meibum samples collected during the young adult study, in order to identify any lipid biomarkers for evaporative DED.

1.9.1. Hypotheses

The main hypothesis of this thesis is that clinical, tear, and meibum findings will differ between young adults with and without evaporative DED, allowing for the identification of potential biomarkers.

Further hypotheses specific to each chapter are:

- Inter-visit measurements of tear protein profiles are repeatable using the quantitative microfluidic system of the Agilent 2100 Bioanalyzer (chapter 3)
- There is a direct correlation between specific dry eye clinical measures and specific lifestyle factors (chapter 3)
- Tear protein biomarkers are different in healthy patients compared to EDE participants (chapter 4)
- Specific tear inflammatory protein and cytokine concentrations directly correlate with signs of EDE (chapters 5 and 6)
- The composition of meibum is different in healthy compared to EDE participants (chapter 7)

1.9.2. Thesis aims

- **Objective 1**. The main objective of this thesis is to explore biomarkers of evaporative dry eye disease, specifically in a young population.

In order to explore this main objective, other objectives of this thesis include:

- **Objective 2**. Determination of the repeatability of tear protein profiles using the quantitative microfluidic system of the Agilent 2100 Bioanalyzer
- **Objective 3**. Exploration of the clinical and tear film characteristics of young adults with and without DED
- **Objective 4**. Exploration of the meibum composition and identify any potential lipid biomarkers based on percentage of meibomian gland loss
- **Objective 5**. Determination of any progression in any disease characteristic or biomarker in young adults over one year.

To achieve objectives 2 to 5, data was collected during a longitudinal research project conducted at Aston University.

A further objective of this thesis is to discover whether biomarker research findings are being translated into evidence-based practice, by evaluating the clinical DED practice patterns of UK optometrists, and specifically the use of existing tear biomarker point-of-care devices.

Chapter 2 - Dry Eye in Clinical Practice Survey

This chapter has been published as –

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This chapter was presented as -

- Casemore, R.K. (2023). Clinical Practice Patterns for Dry Eye Disease Management in the UK. Free paper presentation at BCLA Conference, Cutting Edge Track, 9th June 2023, Manchester, UK.

2.1. Introduction

The increasing prevalence of DED poses a significant economic burden, both in time and resources (see section 1.2.1). Due to its multifactorial nature and the poor correlation between signs and symptoms, accurate diagnosis and management of DED can prove challenging (Bartlett et al., 2015). Considerable research has already been undertaken in search of tear biomarkers which permit accurate diagnosis and subclassification of DED, as well as improving our understanding of the disease pathogenesis, in order to provide more targeted treatment options. Therefore, it is worthwhile exploring whether dry eye research is actually being translated into practice; in particular how aware practitioners are of existing validated point-of-care devices which utilise tear film biomarkers.

In recent years, the role of optometrists in the UK has extended, with many pursuing further qualifications to develop their interest in specialist areas of practice, such as independent prescribing and DED management. In the primary care sector, DED may be managed during a routine eye examination, fall into the remit of an enhanced commissioned service, or may be managed during a specialist dry eye appointment.

Of the approximately 17,000 UK optometrists registered with the General Optical Council, around 1441 were registered as Independent Prescribers, as of February 2023; an increase of approximately 400 over the last two years (General Optical Council, 2023). The specialty-registration of independent prescribing (IP) was first introduced for optometrists in 2008. This permitted qualified optometrists to clinically assess a patient, establish a diagnosis, determine the clinical management needed and prescribe where necessary. Following on from this, extended General Ophthalmic Services (GOS) were implemented, initially by the Scottish Government in 2010, with the aim of reducing the burden on general practitioners and the hospital eye service. This shifted the contact point for primary eye care toward community optometrists, who became the designated first port of call for primary and supplementary or unplanned eye care provision. The clinical decision-making ability of experienced and trained IP optometrists working in acute ophthalmic services has been shown to be concordant with consultant ophthalmologists (Todd et al., 2020).

Many optometrists are also becoming involved in Clinical Commissioning Group (CCGs) minor eye condition service (MECS) provision. The Local Optical Committee Support Unit (LOCSU) reported that in April 2020, 84 out of 135 CCGs had a MEC-type service commissioned for delivery by optical practices (LOCSU, 2020). Between the start of the COVID-19 pandemic and November 2020, there was a reported increase of 24% in the number of CCGs commissioning urgent and MEC-type services.

A recent literature review found 12 articles published in the previous two decades containing the results of surveys investigating trends in diagnosis and treatment of DED by community eye care practitioners (ECPs) (Hantera, 2021). The first qualitative study of UK practitioners attitudes towards DED in 2005 reported an overall poor satisfaction with diagnostic and therapeutic options for DED management (Turner et al., 2005). Recently, a survey of Australasian ophthalmologists (7.6%) and optometrists (92.4%) reported similar attitudes towards DED diagnostic tests and therapies. However higher rates of satisfaction with available tests and therapeutic options were found (Li et al., 2022). In 2021, TFOS reported the results of an international survey examining the management patterns of eye care professionals in the context of severity and subtype of (Wolffsohn et al., 2021a). The survey included ophthalmologists (37%) and optometrists (58%) from 51 countries and found that management at each severity level and between DED subtypes differed across continents and countries. Although the survey included 93 UK optometrists (Wolffsohn, 2023), (142 eye care practitioners in total), their specific responses were not reported.

The only other survey to include UK optometrists was in 2016, which compared self-reported optometric DED practices between Australian and UK optometrists (Downie et al., 2016). However, this survey was conducted in 2015 or before, and was only administered to practitioners who were members of a contact lens association, which may not reflect the true wider picture of optometric practice. Since that time considerable evidence-based guidance regarding diagnosis and management of DED, in the form of the TFOS DEWS II reports (Wolffsohn et al., 2017;Jones et al., 2017), has been widely promoted to ECPs in the UK.

Therefore, in view of the increased roles of UK optometrists working in the primary care sector, the aim of this study was to gain a current insight into their perceptions of dry eye as a disease, knowledge and confidence in diagnosis and management, and satisfaction with currently available first-line treatment options. Furthermore, the study aimed to gain an insight into whether dry eye research is being translated into practice, particularly with regard to awareness of existing validated point-of-care devices which utilise tear film biomarkers.

2.2. Methods

2.2.1. Participants

The survey was aimed at UK optometrists who diagnose and manage DED specifically in a primary care setting. Those who worked only in a hospital or secondary care setting were requested not to complete the survey. Personal information that could enable participants to be identified was not collected unless they entered the prize draw, in which case an email address was required. Data was

stored on a secure cloud storage device and any emails were deleted once the prize draw had taken place on completion of the study. Participants were informed that the results would form part of a PhD research project and could be published. They were also given the researchers email address should they wish to receive a summary of the results.

2.2.2. Survey design

Initially a pilot study was undertaken in February/March 2021. Six optometrists from Aston University, who work in a primary care setting, were asked to complete a paper version of the questionnaire, and give feedback on:

- Length of time to complete the questionnaire
- Any errors/disagreements
- Clarity of questions
- Design

Suggestions included clarity over answering questions according to current practice, rather than what the respondent thinks should be done, and explaining the abbreviation DED. A finalised anonymous, web-based questionnaire was designed, containing a variety of question types including multiple choice (single or multiple answer), Likert-type scale questions, grid questions, selection list questions and single-line free text questions. This was made available through Aston University's preferred survey platform www.onlinesurveys.ac.uk. The survey was estimated to take approximately 15 minutes to complete. It was made available, via the links in **Appendix 1**, between October 2021 and June 2022. A paper version was used for administering in face to face conferences.

The landing page for the online survey included participant information and links to the full Participant Information Sheet (PIS) (**Appendix 2**). Following this, completion of a required consent form enabled the respondent to proceed to the survey. Printed copies of the participant information and consent form were also used at in-person conferences. In order to get a true picture of optometrists' interest and level of involvement, all respondents were encouraged to complete the questionnaire regardless of their level of engagement in DED management.

The survey consisted of 16 questions (including several sub-questions) that collected information primarily on the following areas:

- Practice patterns and practitioner's demographics including experience, practice location, and current knowledge and understanding of DED
- Preferred diagnostic techniques used for DED

- Preferred treatment and patterns of intervention based on severity of DED
- Therapeutic qualification and MECS involvement

Questions were presented sequentially, with participants able to go back and review or alter previous questions. This feature enabled practitioners ample time and flexibility to reflect their true practice patterns. Participants were also given the space to add their own responses.

Question-1, with 10 sub-sections and question-7 with 4 sub-sections, were designed to gain an understanding of optometrists' opinions about dry eye as a disease and whether they felt sufficiently knowledgeable and equipped to diagnose (Q1) and manage (Q7) the condition. Respondents were asked to indicate on a scale of 1-10, where 1=strongly disagree and 10=strongly agree, their level of agreement with several statements.

Question-2 had 19 sub-sections which aimed to identify preferred diagnostic procedures for DED at three different appointment types: a routine eye examination with no dryness symptoms reported, a routine eye examination with dryness symptoms reported, or a specific appointment for a dry eye assessment (including MECS).

Question-3 required participants to indicate from a list of 19 options up to five clinical procedures that they commonly employ for diagnosis of DED.

Question-4 sought to examine what management decisions were made based on the severity (mild, moderate, and severe) of the disease from a pool of 19 options.

From a list of 16 options, question-5 asked for the three most commonly employed clinical procedures used at a follow up appointment (if performed) that may confirm a successful outcome of a treatment.

Questions-6 explored the rationale behind prescribing artificial tears; participants were required to indicate which options (more than one answer could be selected) they based their prescribing decision on when recommending an artificial tear to their patients.

Question-8 and 9 were optional questions aimed at optometrists performing specific appointments for DED, including exploring the use of a lateral flow test using a small tear sample.

The remaining questions 10-16 aimed to gather information on optometrists' practice patterns such as number of patients seen in a typical month, years of experience, involvement in a MECS or Primary Eye Care Acute Referral scheme (PEARS), therapeutic qualification, and area of practice.

Although the survey was anonymous, respondents were given the opportunity to be included in an optional £100 prize draw. Email addresses were collected for the purpose of contacting the winner only and deleted on completion of the study.

2.2.3. Inclusion and exclusion criteria

The survey was administered to UK optometrists only. Given that practice patterns of hospital-based optometrists are significantly different, and the majority of UK optometrists are employed in a high-street practice setting, hospital-based optometrists were excluded in this survey.

2.2.4. Distribution

A web link and QR code were made available on the research pages of the LOCSU and the Optometry Today websites. Links were also distributed through colleagues and via several Local Optical Committees by email to their databases. In an attempt to cover all regions of the UK, the links were also distributed via Optometry Scotland, Optometry Wales and the Northern Ireland Optometric Committee. Links were also included at a couple seminars on DED at optometric conferences (Optometry Tomorrow, Telford 2022, and 100% Optical, London 2022).

2.2.5. Data Analysis

Data analysis was performed using Microsoft® Office Excel®, GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA, and IBM SPSS Statistics for Windows, Version 26 for Decision Tree Analysis. As the questionnaire data cannot be considered to be continuous, the Mann-Whitney non-parametric test was used to compare practitioner opinions between groups. Descriptive statistics such as median, mode and interquartile range, were used to describe the practitioner demographics. Practitioners' opinions were reported as a mean rank and standard deviation. Fisher's exact test was used to compare categorical data for the therapeutic approach of practitioners to different DED severities. Where any data was missing, responses were calculated on the total number of responses to the question. For all tests an alpha value of 0.05 was adopted for statistical significance.

2.2.6. Ethics Approval

The study followed the tenets of the Declaration of Helsinki and was approved by Aston University's College of Health and Life Sciences Ethics Committee (#1795) (see **Appendix 3**). Data was collected anonymously and accessible only by the researchers. Prior to completing the questionnaire, consent was received, and participants were informed about the length of the survey.

2.3. Results

2.3.1. Practitioner demographics

In total, 131 optometrists responded to the survey. Response rates to some of the demographic questions varied (from 123 to 129) and therefore percentages of the responses are also given. The practitioner demographics are summarised in ***Table 2.1***.

Table 2.1. Summary of practitioner demographics

Characteristic	Respondents, n (%)		
Years of practice			
Less than 5 years	23 (18.5)		
6-10 years	20 (16.1)		
11-15 years	18 (14.5)		
16-25 years	35 (28.2)		
More than 25 years	28 (22.6)		
Area of UK in which practice			
England	82 (63.6)		
Wales	17 (13.2)		
England and Wales	3 (2.3)		
Scotland	22 (17.1)		
Northern Ireland	3 (2.3)		
Preferred not to say	2 (1.6)		
Type of practice where dry eye patients examined			
High Street Multiple	52 (41.9)		
Small chain	3 (2.4)		
Independent	57 (46.0)		
Academic institution	3 (2.4)		
Other (not specified)	9 (7.3)		
IP qualified			
No	100 (81.3)		
Yes	23 (18.7)	England	10 (43.5)
		Wales	5 (21.7)
		Scotland	6 (26.0)
		Northern Ireland	1(4.4)
		Not specified	1 (4.4)
Involved in a MECs-type scheme			
No	65 (52.4)		
Yes	59 (47.6)	England	35 (59.3)
		Wales	16 (27.1)
		England and Wales	3 (5.1)
		Scotland	3 (5.1)
		Northern Ireland	2 (3.4)

Responses were gathered from all four constituent countries of the UK, the majority from England (63.6%). Eighty-eight percent of responses were obtained from practitioners working in either a

multiple or independent practice primary care setting. Although the highest number of responses came from optometrists with 16-25 years of experience, a good range of experience was represented. Of the optometrists who had been qualified for less than 5 years, 71.4% of them reported working for a multiple practice.

Twenty-three respondents (18.7% of the responses) were identified as holding independent prescribing status and just under a half reported involvement in minor eye condition service provision. The UK figure for IP status is approximately 8.4% of optometrists based on the data in section 2.1. However, regional differences exist; as of March 2019, there were 278 IP optometrists in Scotland i.e. 23.4% (El-Abiary et al., 2021). Six of the 22 respondents (22%) from Scotland had IP status.

Practitioners' estimation of the percentage of their adult patients who they believed to have DED varied widely; a mean of 47.1 ± 21.2 percent was found, with median and mode values of 50 and 60 percent, respectively, and an IQR of 35. Optometrists reported seeing an average of 33.3 ± 31.0 patients with DED per month. The median and mode values were 20 with an IQR of 27.5.

2.3.2. Practitioner's opinions about DED

Figures 2.1. **to** ***2.14*** shows optometrists' opinions regarding DED, their knowledge, confidence, and satisfaction with DED diagnosis. Overall, optometrists were aware of the importance of DED (rank 8.43 ± 2.07) and felt they have adequate knowledge and equipment to diagnose DED. They regularly included questions related to DED in their work up and agreed that signs and symptoms in DED may not always correlate. Optometrists generally agreed that ocular surface inflammation causes DED, however there was a stronger perception that DED is likely to induce ocular surface disease. While optometrists are generally confident in diagnosis of DED there was no specific pattern found in responses in terms of requirement of more specific diagnostic tests. Optometrists may not have adequate time to manage dry eye patients during a routine eye examination but were still likely to treat a patient with signs, but no symptoms of dryness.

Most optometrists believed they are confident and have adequate knowledge in managing DED. However, IP optometrists were significantly more confident in managing patients with DED (p= 0.03) and felt more knowledgeable about available treatment options (p= 0.049) when compared to their non-IP colleagues. Mixed responses were received regarding confidence in patient specific targeted treatment, although IP optometrists were again significantly more confident (p=0.08).

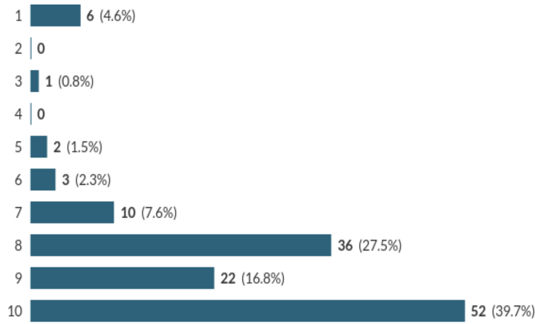


Figure 2.1. DED is an important eye disease

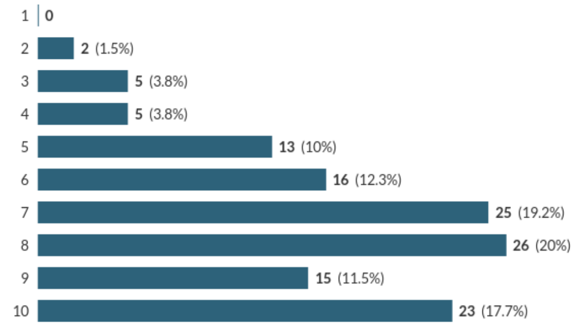


Figure 2.5. I have sufficient knowledge to diagnose DED subtype

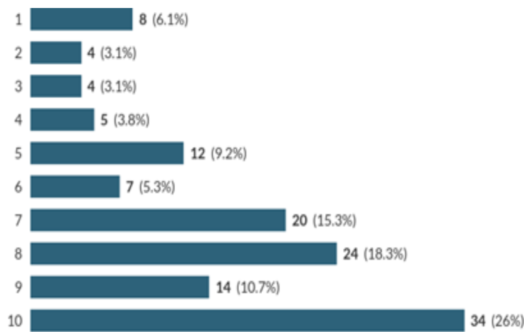


Figure 2.2. I routinely ask my patients whether they have any symptoms of DED

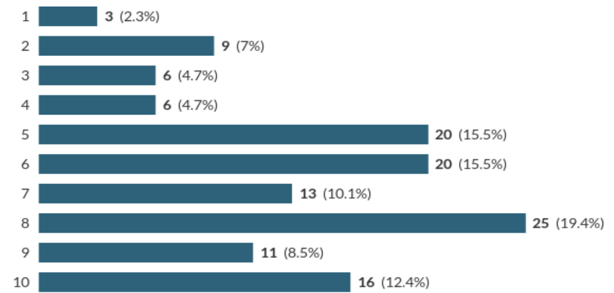


Figure 2.6. More definitive diagnostic tests are needed

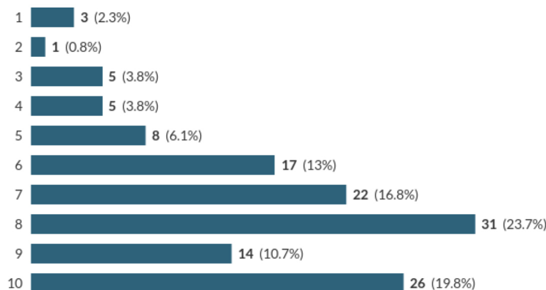


Figure 2.3. In my experience, patients can often have dryness symptoms but few ocular surface signs

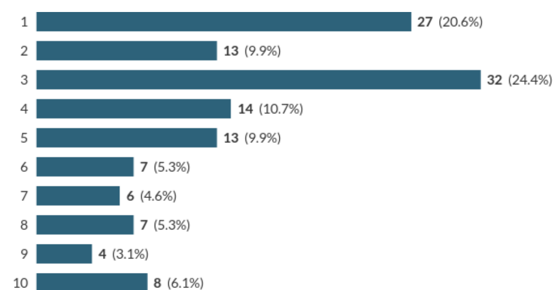


Figure 2.7. If a patient has ocular surface signs but no symptoms, I do not manage them

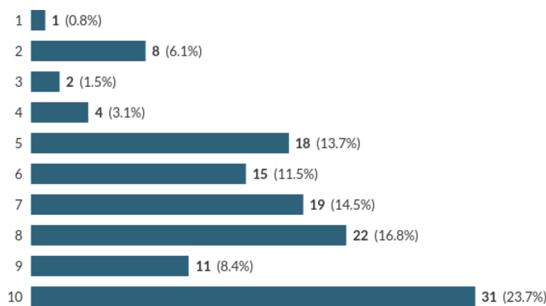


Figure 2.4. I have sufficient equipment available to me in order to diagnose DED

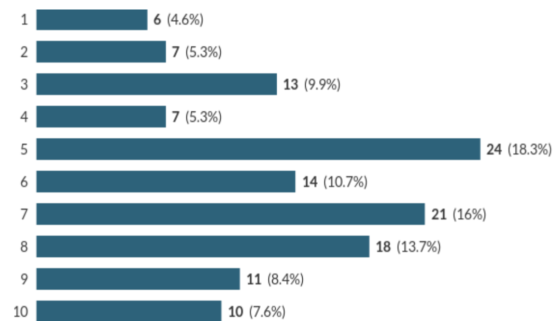


Figure 2.8. Patient symptoms drive my decision as to the severity of their DED, rather than signs

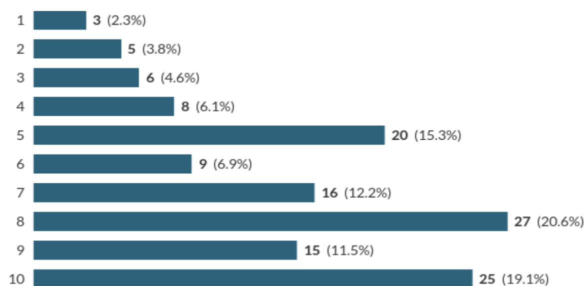


Figure 2.9. Ocular surface inflammation causes DED

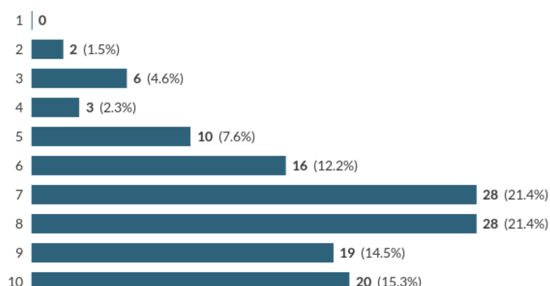


Figure 2.12. I am confident in managing patients with DED

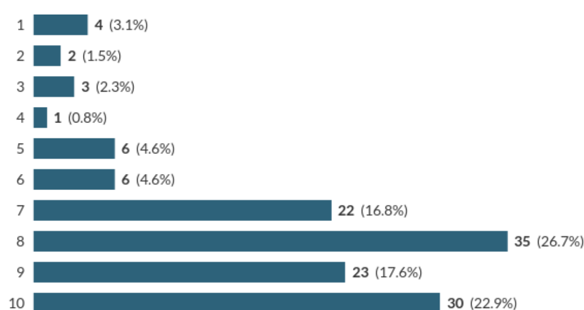


Figure 2.10. Dry eye causes ocular surface inflammation

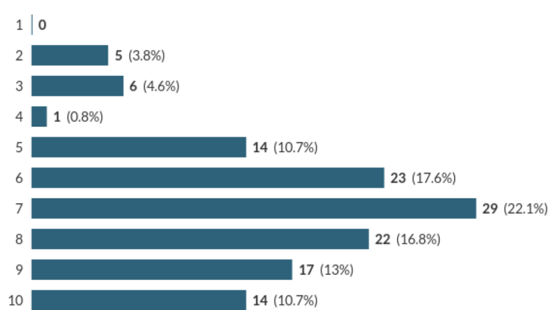


Figure 2.13. I have sufficient knowledge about DED treatments

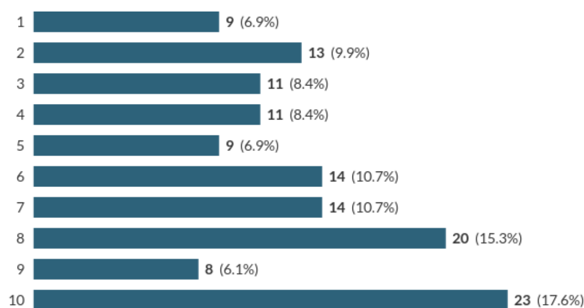


Figure 2.11. During a routine eye examination, I do not have enough time for dry eye management

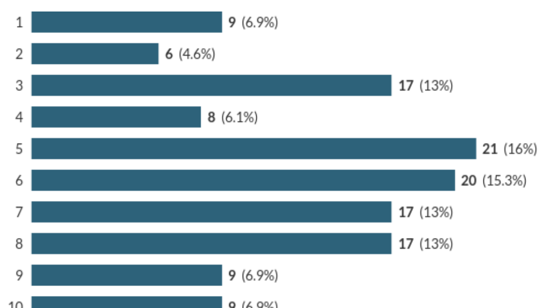


Figure 2.14. I am not always confident that I have prescribed the most effective treatment

The mean rank, variance, standard deviation, and lower and upper quartiles for each question's responses are displayed in ***Table 2.2.*** These results are also illustrated as Box Whisker plots in ***Figures 2.15*** *and* ***2.16,*** where a score of 1= strongly disagree and 10= strongly agree*.* ***Figure 2.15*** corresponds with ***Figures 2.1-2.10*** while ***Figure 2.16*** corresponds with ***Figures 2.11-2.14***. The upper and lower whiskers represent the highest and lowest scores for a given statement, while the line in the centre of the box represents the median score. The ends of the boxes represent the lower (25%) and upper (75%) quartiles, and the 'x' indicates the mean score.

The optometrist's location, practice type and experience did not have any significance when considering the responses to these questions.

Table 2.2. Optometrists' opinions regarding DED

	Mean rank ± SD	Variance	Lower quartile limit	Upper quartile limit
Dry Eye Disease is an important eye disease.	8.43 ± 2.07 (7.96 ± 1.83)	4.28 (3.35)	8.0 (7.0)	10.0 (9.5)
I routinely ask my patients whether they have any symptoms of DED.	7.18 ± 2.66 (6.96 ± 2.15)	7.07 (4.62)	5.75 (5.75)	10.0 (8.25)
In my experience, patients can often have dryness symptoms but few ocular surface signs.	7.35 ± 2.15 (7.35 ± 1.83)	4.64 (3.36)	6.0 (6.0)	9.0 (8.0)
I have sufficient equipment available to me in order to diagnose DED.	7.15 ± 2.37 (8.22 ± 1.74)	5.60 (3.04)	5.5 (7.0)	9.0 (10.0)
I have sufficient knowledge to diagnose DED subtype	7.29 ± 2.01 (8.04 ± 1.76)	4.04 (3.09)	6.0 (7.0)	9.0 (10.0)
More definitive diagnostic tests are needed.	6.46 ± 2.42 (6.32 ± 2.46)	5.88 (6.08)	5.0 (5.0)	8.0 (8.0)
If a patient has ocular surface signs but no symptoms, I do not manage them.	4.02 ± 2.65 (3.7 ± 2.40)	7.04 (5.78)	2.0 (2.0)	5.0 (4.0)
Patient symptoms drive my decision as to the severity of their DED, rather than signs	5.96 ± 2.44 (5.09 ± 2.57)	5.98 (6.6)	4.5 (3.0)	8.0 (8.0)
Ocular surface inflammation causes dry eye.	6.94 ± 2.45 (7.04 ± 2.37)	5.98 (5.61)	5.0 (5.0)	9.0 (8.5)
Dry eye causes ocular surface inflammation.	7.79 ± 2.12 (8.22 ± 1.74)	4.52 (3.04)	7.0 (8.0)	9.0 (10.0)
During a routine eye examination, I do not have enough time for dry eye management	6.07 ± 2.91 (6.30 ± 2.94)	8.47 (8.65)	3.75 (4.0)	8.0 (8.0)
I am confident in managing patients withDED.	7.36 ± 1.94 (8.25 ± 1.48)	3.76 (2.19)	6.0 (7.0)	9.0 (9.25)
I have sufficient knowledge about DED available treatments	6.96 ± 2.0 (7.78 ± 1.53)	4.01 (2.34)	6.0 (6.5)	8.0 (9.0)
I am not always confident that I have prescribed the most effective treatment for a given patient and would like to be able to target treatment more precisely	5.68 ± 2.48 (4.88 ± 2.42)	6.19 (5.86)	4.0 (2.75)	8.0 (7.0)

(1=strongly disagree and 10=strongly agree). Numbers in brackets denote the results obtained from the optometrists with IP qualification only.

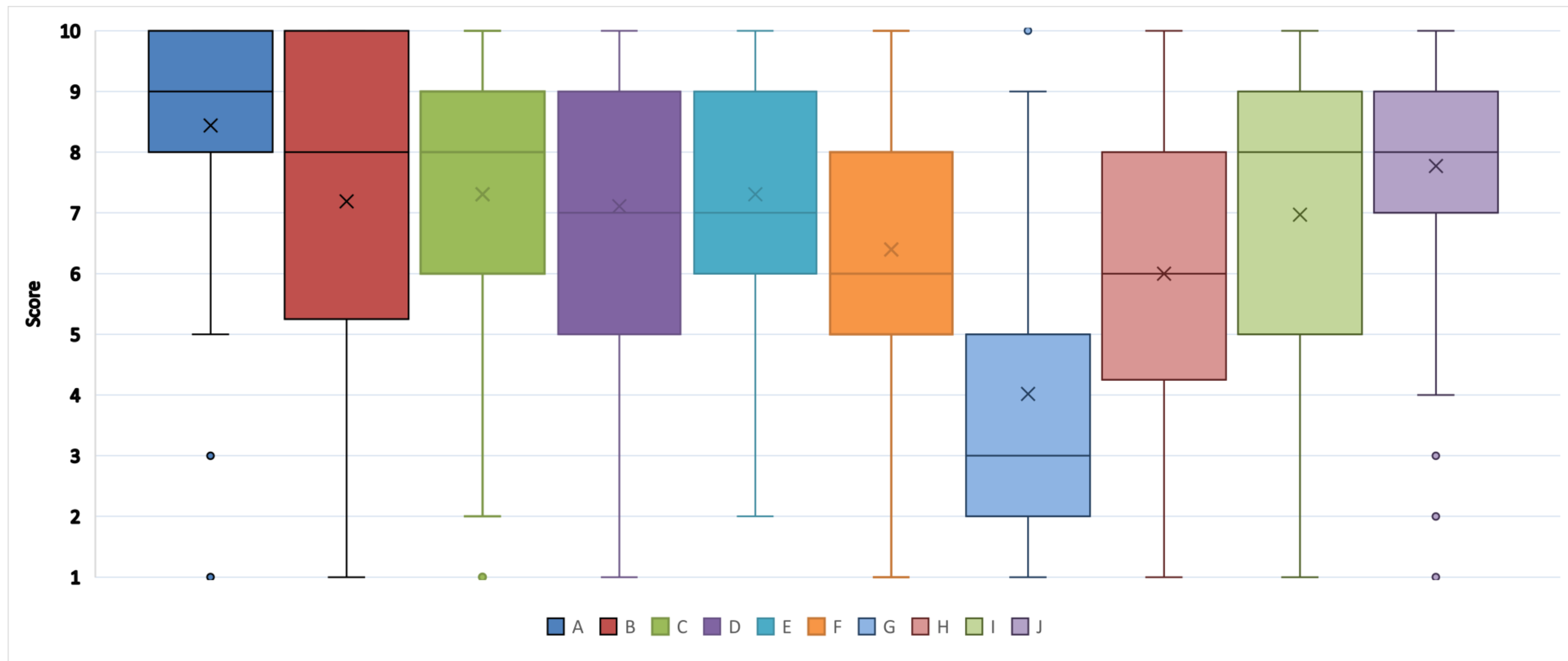


Figure 2.15. Box whisker plot results for question 1

A-J correspond with Figures 2.1-2.10.The minimum, lower quartile, median, mean (represented by a x), upper quartile and maximum scores, where a score of 1= strongly disagree to 10=strongly agree are illustrated. Outliers are represented by a dot (identified as 1.5 multiplied by the IQR value of the data).

A= Dry Eye Disease is an important eye disease

B= I routinely ask my patients whether they have any symptoms of DED

C= In my experience, patients can often have dryness symptoms but few ocular surface signs

D= I have sufficient equipment available to me in order to diagnose DED

E= I have sufficient knowledge to diagnose DED subtype

F= More definitive diagnostic tests are needed

G= If a patient has ocular surface signs but no symptoms, I do not manage them

H= Patient symptoms drive my decision as to the severity of their DED rather than signs

I= Ocular surface inflammation causes dry eye

J= Dry eye causes ocular surface inflammation

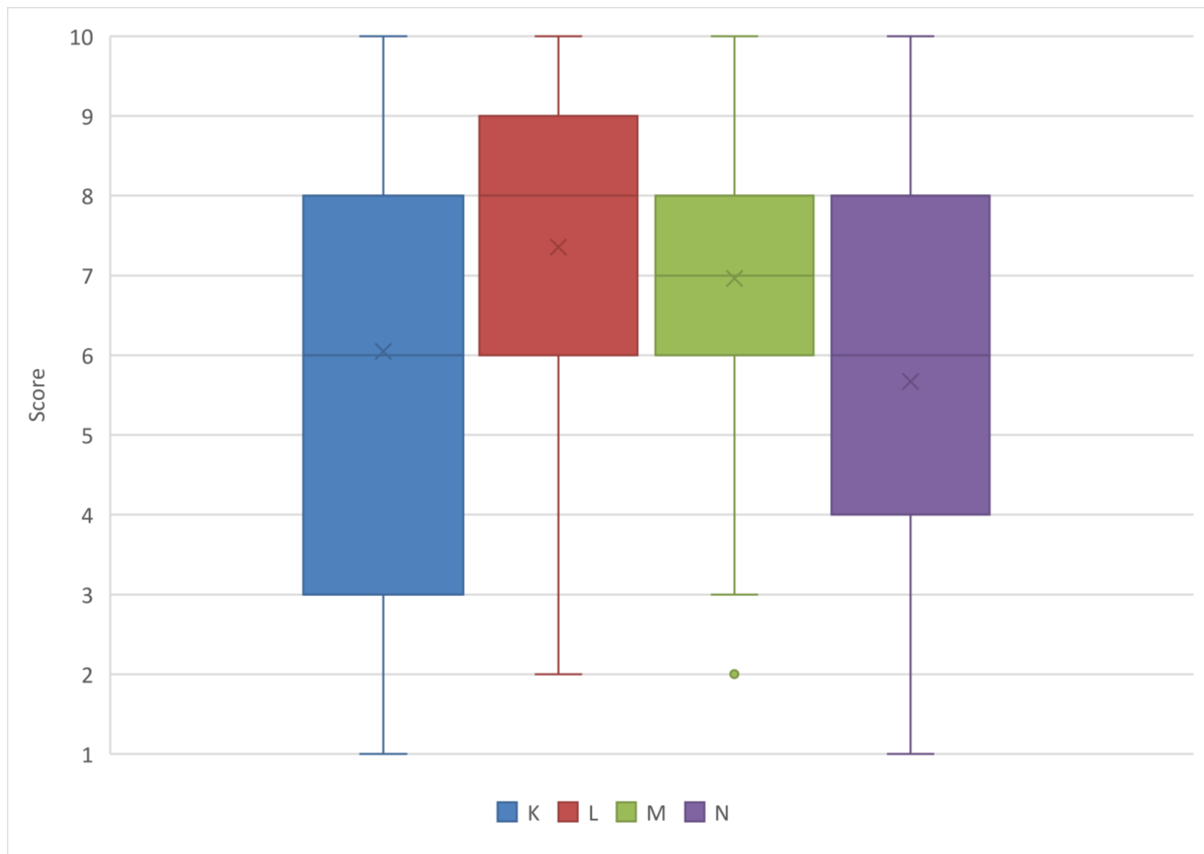


Figure 2.16. Box whisker plot results for question 7
K-N correspond with Figures 2.11. – 2.14.
The minimum, lower quartile, median, mean (represented by a x), upper quartile and maximum scores, where a score of 1= strongly disagree to 10=strongly agree are illustrated. Outliers are represented by a dot (identified as 1.5 multiplied by the IQR value of the data).
K= During a routine eye examination I do not have enough time for dry eye management
L= I am confident in managing patients with DED
M= I have sufficient knowledge about available treatments
N= I am not always confident that I have prescribed the most effective treatment for a given patient and would like to be able to target treatment more precisely

2.3.3. Diagnostic clinical procedures recorded or performed

Figure 2.17. details the preferred diagnostic clinical procedures performed by optometrists for: (1) a routine eye examination with no dry eye symptoms, (2) a routine eye examination with dryness symptoms reported, (3) a specific appointment for a dry eye assessment (including a MECS-type appointment). Respondents were asked only to complete the survey for the type of appointments that they provide for patients. Fewer responses were obtained from practitioners seeing patients for a specific dry eye appointment (n=66), which may reflect the fact that less than half of the total respondents (n=59) reported involvement in MECS-type provision.

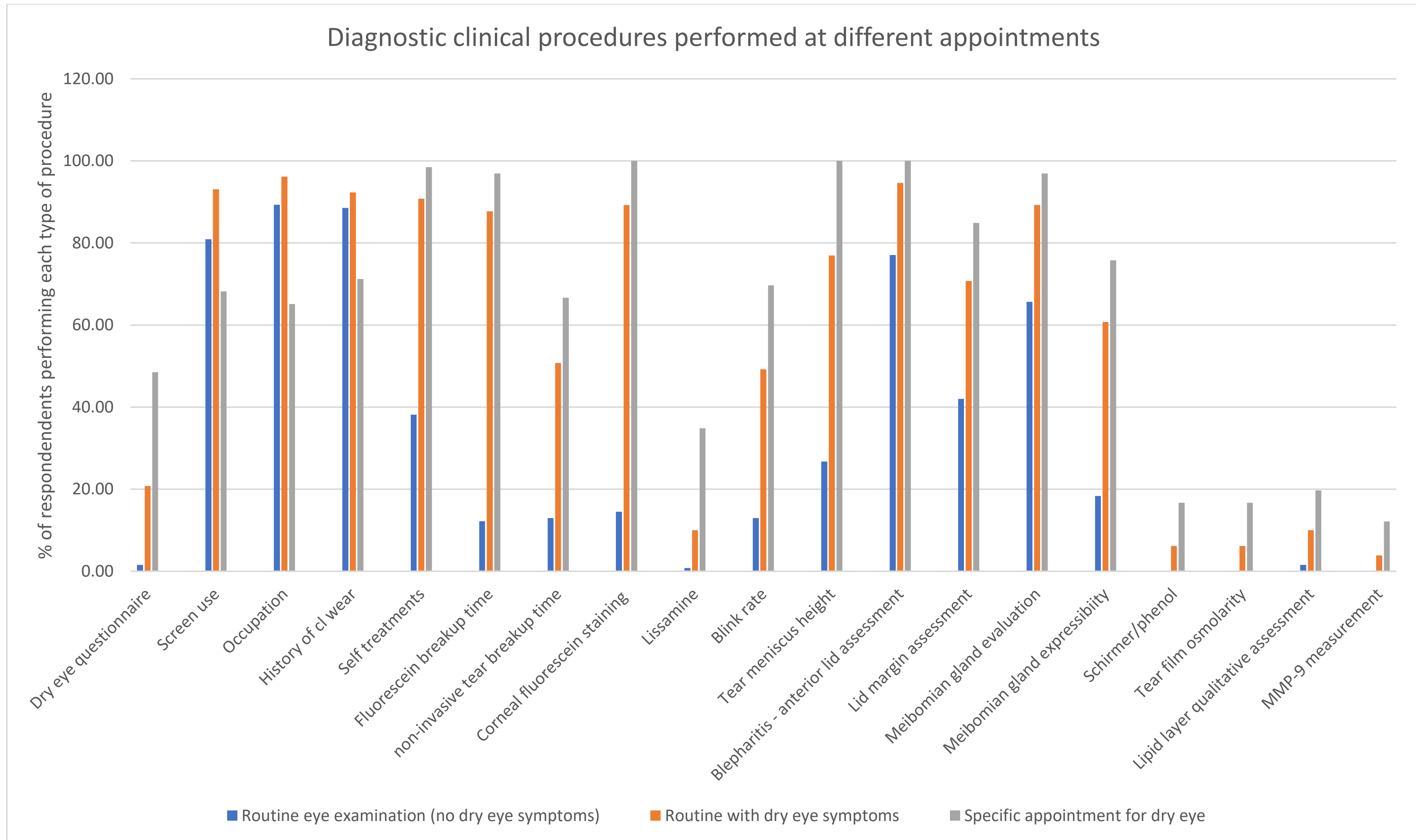


Figure 2.17. The five most used clinical procedures for diagnosis of DED

Assessment of screen use (81%), history of occupation (89%), contact lens wear (89%), and blepharitis (77%) were popular tests during routine eye examination. Sixty-six percent of optometrists reported also evaluating the meibomian glands during a routine examination. Interestingly, none of the tear film assessments such as NIBUT, FBUT or TMH were performed during routine eye examination by many practitioners. MMP-9, Schirmer's/Phenol, and tear osmolarity tests are not performed at this stage, and only one optometrist would perform a Lissamine green test.

The responses changed substantially when patients report dry eye symptoms. Approximately 90% of the practitioners would ask about self-treatments and perform fluorescein corneal staining and break-up time, and meibomian gland evaluation, in addition to the tests performed for routine non-dry eye symptoms. Additional attention would be given to tear film parameters, lid margin, and meibomian gland expressibility when patients report dry eye symptoms. When patients are booked specifically for dry eye management (including a MECS-type appointment), all optometrists would perform assessment for blepharitis, TMH, corneal fluorescein staining, and meibomian gland expression. Tear film osmolarity, lipid layer qualitative assessment and MMP-9 measurements were performed relatively rarely; approximately 1 in 5 at specific dry eye appointments.

Respondents were also asked to specify any procedures they use for diagnosis which were not already listed for this question. Incomplete blinking, conjunctival redness grading, and tear film assessment were individually listed by four respondents.

2.3.4. Five most commonly used clinical methods for DED diagnosis

Twenty-nine responses were excluded from the analysis due to more than five options being selected. Fluorescein breakup time was the most commonly used clinical procedure, favoured by more than 72% of the respondents (see ***Figure 2.18***). This was followed by corneal fluorescein staining (>70%), assessment of blepharitis (65%), and meibomian gland evaluation (64%) which were the other three popular choices. Approximately one-third of the respondents preferred assessing TMH and daily screen use. Approximately a quarter of the participants favoured evaluating non-invasive breakup time and history of contact lens wear to diagnose DED. None of the participants preferred Schirmer's or Phenol red test for this purpose.

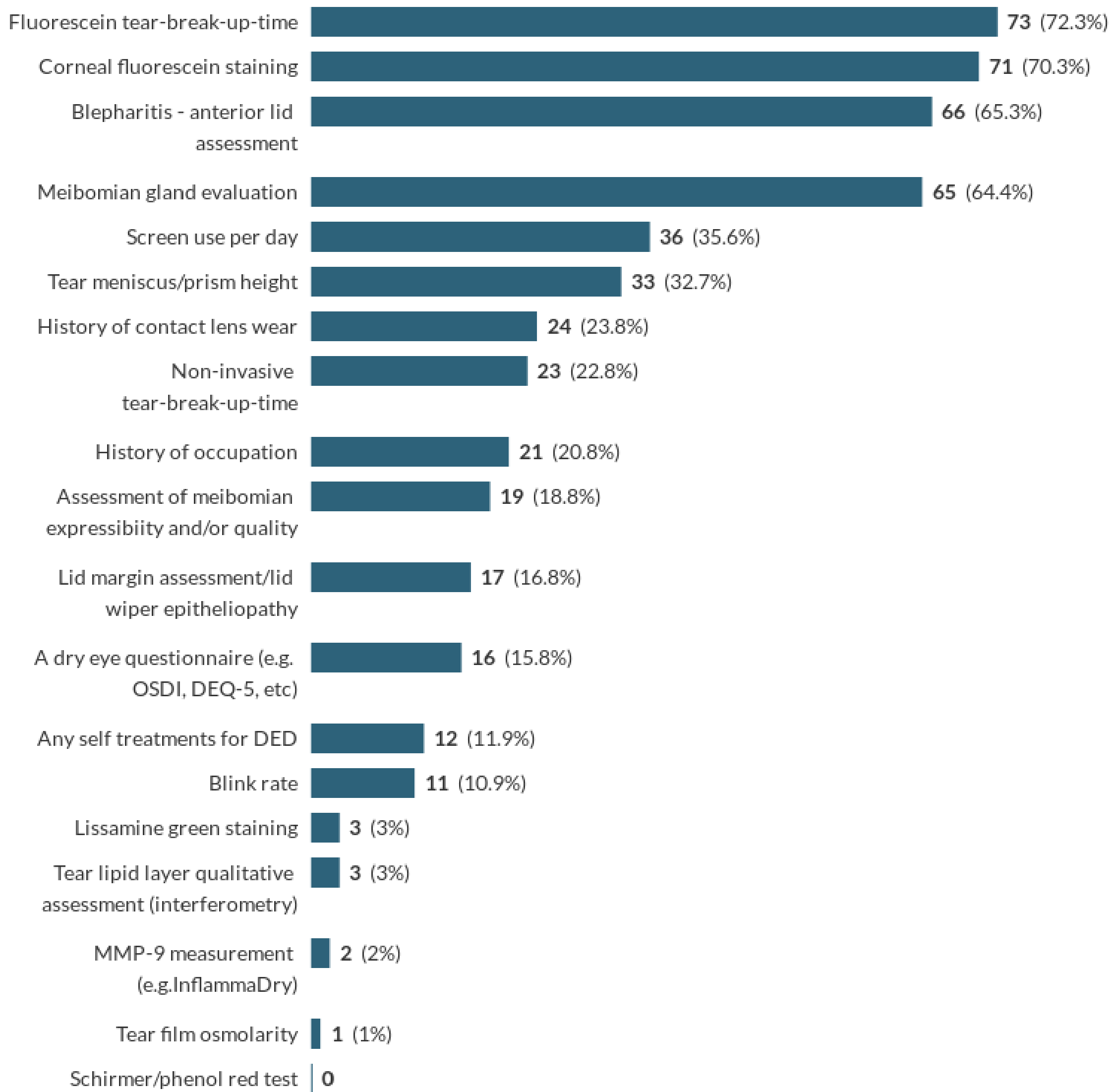


Figure 2.18. The five most commonly used procedures preferred for diagnosis of DED

2.3.5. Preferred management options for mild, moderate, and severe DED

Table 2.3. details the number of responses to the questions regarding management options for mild, moderate, and severe DED. Response rates to some questions varied and therefore percentages of the responses are also given. Modification of local environment such as drying conditions and digital device use, lid hygiene and hot compresses, and prescribing non-preserved tear supplements were the preferred treatments for managing mild DED. In addition, dietary advice, topical ointment at bedtime and advanced therapies such as IPL or Lipiview were preferred for moderate DED management. Preserved tear supplements were largely avoided for moderate and severe DED cases.

Table 2.3. Number of responses to the question regarding the management options for mild, moderate, and severe DED

Treatment modality	**Severity of dry eye disease (n)**		
	Mild **n** (%)	Moderate **n** (%)	Severe **n** (%)
Modification of local environment - drying conditions and environmental pollutants	89 (71.8)	117* (82.3)	121 (81.5)
Modification of local environment - managing use of digital devices	93 (75.0)	123* (81.5)	122 (83.1)
Dietary advice - diet rich in Omega 3 essential fatty acids	58 (48.3)	103* (96.0)	120* (100)
Lid hygiene and hot compresses	85 (65.9)	124* (92.2)	128 (88.4)
Preserved tear supplements	59 (79.7)	38* (59.4)	25 (39.1)
Liposomal sprays	35 (57.4)	38* (62.3)	40 (67.2)
Non-preserved tear supplements	76 (62.3)	115* (93.4)	116 (89.3)
Topical ointment at bedtime	6 (4.9)	87* (64.2)	116* (93.5)
Punctal plugs	2 (2.7)	15*(19.2)	73* (98.6)
Topical corticosteroids	1 (2.4)	9* (21.4)	41* (100)
Systemic tetracycline e.g., doxycycline	0	8* (14.8)	54* (100)
Topical macrolide e.g., azyter	0	3 (12.5)	23* (100)
Systemic macrolide e.g., azithromycin	0	6* (18.8)	32* (100)
Cyclosporin	0	2 (7.4)	27* (100)
Advanced therapies such as thermal pulsation (Lipiview) or intense pulsed light (IPL)	4 (10.3)	14* (35.9)	38* (97.4)
Protection with therapeutic contact lenses	0	7* (14.3)	49* (100)
Scleral contact lenses	0	3 (11.1)	27* (100)
Refer to a colleague	0	11* (19.3)	57* (100)
Refer to an ophthalmologist	0	9* (8.2)	110* (99.1)

**Indicates a statistically significant difference from the previous severity category where p<0.05, using Fishers Exact test.*
Step 1 of the TFOS DEWS II staged management and treatment recommendations for dry eye disease are indicated in the green boxes, Step 2 in yellow and Step 3 in orange. The % given is the percentage of responses to the question.

For severe DED, practitioners preferred to add almost all remaining additional measures that ranged from punctal plugs, systemic and topical medications, to therapeutic and scleral lenses. All respondents would like to manage mild DED cases by themselves, whereas a small number of optometrists may refer moderate cases to a colleague or ophthalmologist. The majority of respondents would consider referring severe cases that they did not feel they could manage satisfactorily themselves. Interestingly one respondent also suggested that patients could monitor urine colour as an indicator of dehydration.

Table 2.4. details the responses, as a percentage, from optometrists who examined patients under a MECS/PEARS or non-MECS/PEARS appointment. These show similar trends, with no significant differences between management preferences between the two appointment types.

Table 2.5. shows the percentage of IP and non-IP responses that indicated preferences in DED treatment modalities for mild, moderate, and severe cases. When treating a patient with mild DED, a significantly ($p<0.05$) higher percentage of IP-qualified optometrists preferred to modify the local environment, such as protection against drying conditions and environmental pollutants. When considering moderate DED, a significantly higher percentage of IP-qualified optometrists preferred to use punctal plugs, topical corticosteroids, systemic tetracycline, topical and systemic macrolides, cyclosporin and other advanced therapies. A similar trend was observed when treating patients with severe DED, however, a significantly higher percentage of non-IP qualified optometrists would prefer to refer to a colleague ($P<0.05$).

Around a third (34.1%) of all respondents reported recommending a prescription of topical steroids, while 19.4% and 41.9% reported recommending cyclosporine or systemic tetracycline, respectively for severe DED. Practice location, practice type and years of experience did not have any bearing on these recommendations.

Table 2.4. Treatment modality responses based on involvement in an extended service

Treatment Modality	Severity of dry eye disease (%)					
	Mild		Moderate		Severe	
	MECS/PEARS (n=59)	Non-MECS/PEARS (n=65)	MECS/PEARS (n=59)	Non-MECS/PEARS (n=65)	MECS/PEARS (n=59)	Non-MECS/PEARS (n=65)
Modification of local environment - drying conditions and environmental pollutants	64.4	72.3	93.2	87.7	94.9	90.8
Modification of local environment - managing use of digital devices	71.2	72.3	89.8	86.2	98.3	89.2
Dietary advice - diet rich in Omega 3 essential fatty acids	44.1	44.6	74.6	81.5	91.5	89.2
Lid hygiene and hot compresses	67.8	63.1	94.9	95.4	96.6	96.9
Preserved tear supplements	47.5	43.1	28.8	32.3	11.9	26.2
Liposomal sprays	30.5	26.2	40.7	43.1	50.8	46.2
Non-preserved tear supplements	59.3	60.0	89.8	90.8	94.9	92.3
Topical ointment at bedtime	1.7	6.2	59.3	64.7	93.2	95.4
Punctal plugs	3.4	0	6.8	15.4	57.6	56.9
Topical corticosteroids	1.7	0	3.4	9.2	32.2	32.3
Systemic tetracycline e.g., doxycycline	0	0	5.1	7.7	42.4	41.5
Topical macrolide e.g., azyter	0	0	1.7	1.5	15.3	21.5
Systemic macrolide e.g., azithromycin	0	0	3.4	4.6	22.0	27.7
Cyclosporin	0	0	1.7	1.5	20.3	21.5
Advanced therapies such as thermal pulsation (Lipiview) or IPL	3.4	3.1	8.5	13.8	28.8	30.8
Protection with therapeutic contact lenses	0	0	3.4	6.2	40.7	35.4
Scleral contact lenses	0	0	0	3.1	20.3	20.0
Refer to a colleague	0	0	3.4	9.2	40.0	46.2
Refer to an ophthalmologist	0	0	1.7	7.7	84.7	81.5

Step 1 of the TFOS DEWS II staged management and treatment recommendations for dry eye disease are indicated in the green boxes, Step 2 in yellow and Step 3 in orange.

Table 2.5. Percentage of IP and non-IP respondents that indicated recommending each dry eye treatment modality for managing mild, moderate, and severe DED

Treatment Modality	Severity of dry eye disease (%)					
	Mild		Moderate		Severe	
	IP (n=23)	Non-IP (n=98)	IP (n=23)	Non-IP (n=98)	IP (n=23)	Non-IP (n=98)
Modification of local environment - drying conditions and environmental pollutants	91.3*	65.3	100	87.8	100	93.9
Modification of local environment - managing use of digital devices	82.6	71.4	95.7	88.8	91.3	93.9
Dietary advice - diet rich in Omega 3 essential fatty acids	39.1	45.9	82.6	78.6	87.0	92.9
Lid hygiene and hot compresses	73.9	65.3	100	100	100	99.0
Preserved tear supplements	43.5	39.8	39.1	28.6	21.7	19.4
Liposomal sprays	21.7	29.6	34.8	41.8	42.9	50.0
Non-preserved tear supplements	47.8	62.2	95.7	91.8	100	94.9
Topical ointment at bedtime	0	5.1	60.9	60.2	91.3	94.9
Punctal plugs	4.35	0.0	26.1*	7.1	65.2	55.1
Topical corticosteroids	4.35	0.0	26.1*	2.0	65.2*	24.5
Systemic tetracycline e.g., doxycycline	0	0.0	26.1*	2.0	78.2*	33.7
Topical macrolide e.g., azyter	0	0.0	8.70*	0.0	30.4*	15.3
Systemic macrolide e.g., azithromycin	0	0.0	21.7*	0.0	56.2*	17.3
Cyclosporin	0	0.0	8.70*	0.0	34.9	17.3
Advanced therapies such as thermal pulsation (Lipiview) or intense pulsed light (IPL)	4.35	3.1	26.1*	7.1	39.1	26.5
Protection with therapeutic contact lenses	0	0.0	13.0	3.1	52.2	34.7
Scleral contact lenses	0	0.0	0	2.0	26.1	18.4
Refer to a colleague	0	0.0	0	8.2	17.4	51.0*
Refer to an ophthalmologist	0	0.0	0	5.1	82.6	85.7

**Indicates statistically significant differences between the percentages of the two groups (p<0.05), using Fishers exact test. Step 1 of the TFOS DEWS II staged management and treatment recommendations for dry eye disease are indicated in the green boxes, Step 2 in yellow and Step 3 in orange.*

2.3.6. Three most commonly used clinical procedures used to help decide if treatment successful

Eleven participants did not provide any responses to this question, possibly due to not performing follow up appointments. Eighteen responses were excluded due to the respondent selecting more than three options. Therefore, there were 102 valid responses to this question (see ***Figure 2.19***). Exclusion of the incorrect responses did not affect the order of the top six procedures. Corneal fluorescein staining and fluorescein tear breakup were the most commonly used tests to determine success of ongoing treatment. This was followed by assessment of blepharitis, meibomian gland evaluation and use of DED questionnaires. Schirmer's test, MMP-9 measurement, tear lipid layer quantification and blink rates were the least popular tests to characterise treatment outcome.

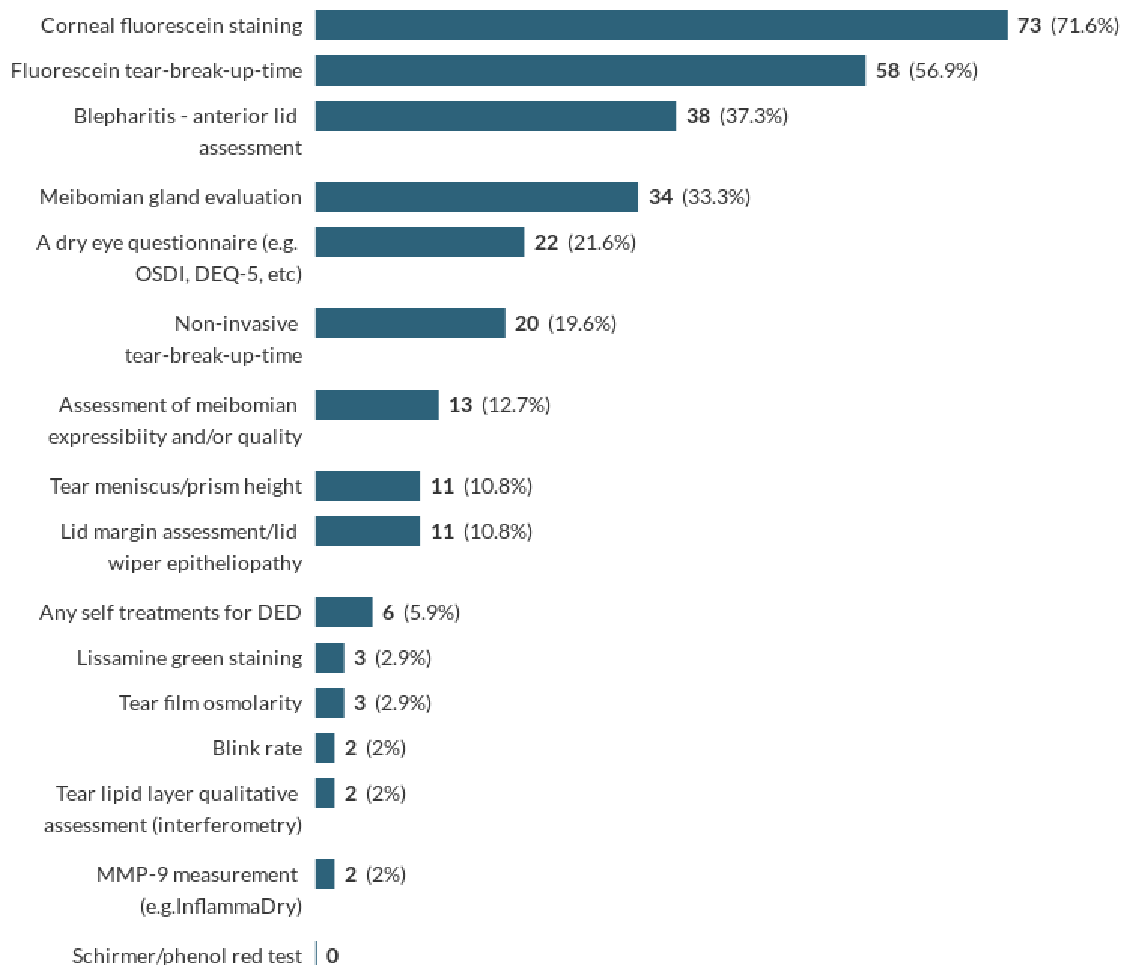


Figure 2.19. The three most commonly used clinical procedures preferred for determination of successful treatment

Numbers in bold represent the number of responses.

Other responses included six suggestions that the patient's subjective response as to whether their symptoms had improved be used, rather than a standardised written questionnaire. One respondent also suggested the use of photographs where applicable.

2.3.7. Decision on artificial tears

The majority of respondents would prefer to prescribe preservative free artificial tears. Almost half of their prescriptions would depend on work-place availability and/or drop ingredients. Other considerations practitioners reported when choosing which artificial tears to prescribe included type of dry eye, cost, personal experience of the product, viscosity, lipid content, ease of use/manipulation, and regional formulary prescribing guidelines. Sixty percent (± 14.1%; IQR 40) of optometrists felt all their patients are satisfied with and could be managed with artificial tears alone. However, the median and mode values were 50%, which indicate that almost half of the optometrists would manage DED patients with additional treatment and management. See ***Figure 2.20***.

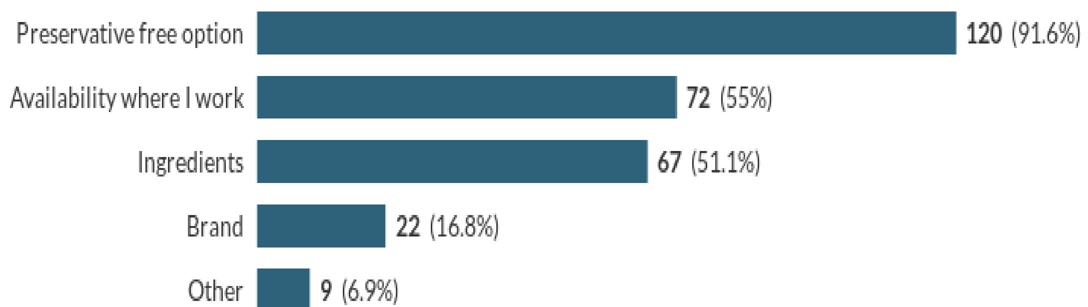
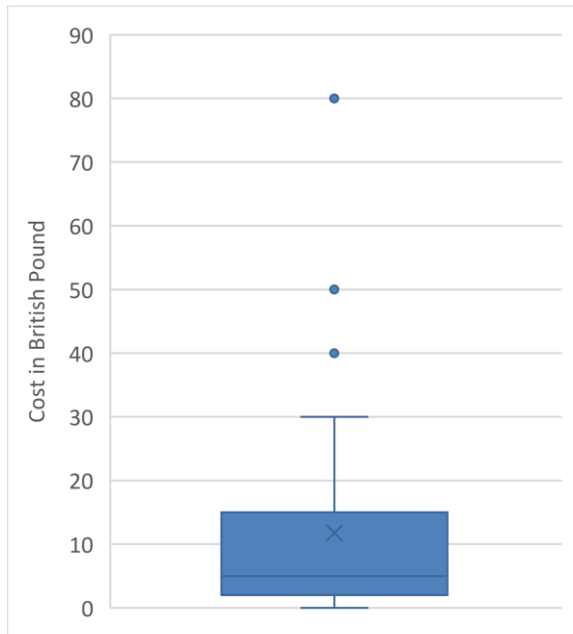


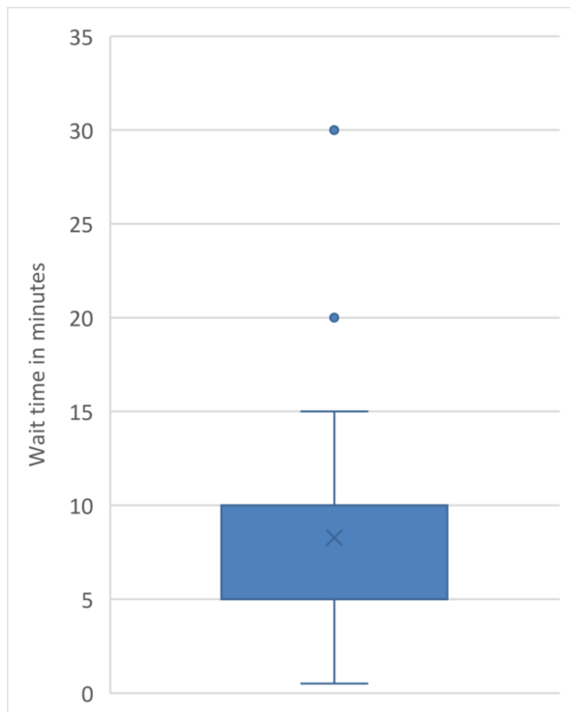
Figure 2.20. Considerations behind prescribing of artificial tears
Numbers in bold represent the number of responses.

2.3.8. Lateral flow device to diagnose DED



Fifty-five participants responded to the optional question regarding the cost of a potential lateral flow device for DED diagnosis (in pounds) that would be viable to include in their clinic. The average suggested cost was £12.00 ± £14.80, with a median value of £5. There were two significant outliers of £500 and £1000, which were removed from the analysis. Other outliers at £40, £50, and £80 can be seen in ***Figure 2.21.***

Figure 2.21. Box whisker plot showing the results for the optional question regarding the viable cost of a lateral flow device



Sixty-five participants responded to the optional question regarding the preferred length of time for a lateral flow device result. The mean time was 8.26 ± 7.01 minutes, following removal of a significant outlier of 120 minutes. The median and mode were both 5 minutes, with an IQR of 5 minutes (see ***Figure 2.22***). One respondent indicated that they would be happy to send the lateral flow device away.

Figure 2.22. Box whisker plot showing the time practitioners would be willing to wait for the result of a lateral flow device

2.4. Discussion

This survey has demonstrated a wide range of attitudes towards DED amongst UK optometrists. While it is possible that those who completed the survey in response to links distributed at CPD events may have altered their opinions through what was presented, the questions related to current practice and so the responses were unlikely to have been influenced. It was also made clear that the opinions of optometrists with any level of interest in DED were being sought.

Most practitioners expressed positive views regarding the importance of DED, and their knowledge and confidence in managing it. Previously, a smaller UK based survey found an overall poor satisfaction with diagnostic and therapeutic options (Turner et al., 2005). While ECPs have recently expressed thoughts of 'frustration' and 'dread' in the context of a disease with a perceived 'time-consuming' nature with 'limited therapeutic options' (Li et al., 2022), there does appear to be an increased awareness of evidence based practice, with engagement in training for extended roles and further qualifications a likely reason.

Practitioners did not show any significant agreement or disagreement to the statement 'I am not always confident that I have prescribed the most effective treatment for a given patient and would like to be able to target treatment more precisely'. A similar result was found when isolating the responses of those who reported recommending a topical steroid, suggesting there is still some uncertainty. Years of experience did not appear to affect the response either.

Practitioners estimated that 47% of their adult patients have DED. Global prevalence has been reported to range from 5-50% (Stapleton et al., 2017), with the large variation presumed to be the result of several factors including geographical location, study population variations and lack of consistent diagnostic criteria. The most recent UK based study, which diagnosed DED according to the TFOS DEWS II criteria, suggested that just over one third of the UK adult population has DED (Vidal-Rohr et al., 2023).

Practitioners reported examining around 33 patients a month with DED, but with a range of 1 to 130. If nearly half of the practitioners' adult patients are estimated to have DED, then this may suggest that some patients are not having their dry eye managed. Further information regarding the total number of patients examined per month would be needed to verify this, including its reason.

After patient history or clinical judgement, a combination of fluorescein BUT, corneal staining and meibomian gland evaluation have previously been rated as the predominant objective clinical procedures used for dry eye diagnosis (Hantera, 2021) (see Table 2.6).

Table 2.6. Previous surveys reporting the most commonly used objective clinical procedures used to diagnose DED

Study	Country	Respondents	Rank		
			1st	2nd	3rd
(Turner et al., 2005)	UK	Ophthalmologists (n=32) Optometrists (n=6)	FBUT	Fluorescein staining	MG evaluation
(Downie et al., 2013)	Australia	Optometrists (n=144)	FBUT	MG evaluation	Corneal fluorescein staining
(Williamson et al., 2014)	USA	Optometrists, ophthalmologists & corneal specialists (n= 38)	Fluorescein staining	TBUT	Lissamine staining
(van Tilborg et al., 2015)	Netherlands	Optometrists (n=138) GPs (n=93)	TBUT	Lissamine staining	Fluorescein staining
(Downie et al., 2016)	Australia and UK	Optometrists (n=317, UK =173)	FBUT	MG evaluation	Corneal fluorescein staining
(Asiedu et al., 2016)	Ghana	Optometrists (n=142) Ophthalmologists (n=20)	TBUT	Patient history	Schirmer I
(Xue et al., 2017)	New Zealand	Optometrists (n=174) Ophthalmologists (n=29)	Meibomian gland evaluation	FBUT	Conjunctival fluorescein stain
(Li et al., 2022)	Australasia	Optometrists (n=133) Ophthalmologists (n=11)	Fluorescein staining	MG evaluation	FBUT

FBUT= fluorescein breakup time, TBUT=tear breakup time, MG=meibomian gland

The results of this UK survey are in agreement with a retrospective study in 2000, which looked at the records of 467 patients with a previous diagnosis of DED. That study found the most commonly performed objective tests after symptom assessment (82.8%), were fluorescein staining (55.5%), TBUT (40.7%), and tear assessment (22.2%) (Nichols et al., 2000).

Only 15.8% of the UK optometrists who responded said they incorporate a dry eye questionnaire as one of their five most used procedures for diagnosis; of those who ranked it first, all were IP qualified. However, DED is defined as the presence of both clinical signs and patient reported symptoms using one of the two validated questionnaires (Wolffsohn et al., 2017). The relatively low usage of these questionnaires would imply an unfounded confidence in DED diagnosis, with most practitioners not using the well-established global consensus established by the Tear Film and Ocular Surface Society (TFOS) Dry Eye Workshop (DEWS) II (Wolffsohn et al., 2017).

Just over a fifth of respondents ranked a dry eye questionnaire as one of their three most commonly used clinical procedures for determination of successful treatment. These percentages are lower than the 31% (for diagnostic use) reported by Downie (2016). However, this study had a greater diversity of respondents, being directed towards general optometry, rather than those with a specialist interest in the anterior eye or contact lenses. Furthermore, just under one half (48.5%) of the UK practitioners who responded to this survey, who perform specific dry eye appointments said they would use a questionnaire.

It is possible that practitioners prefer a verbal assessment of symptoms, as could be inferred by the higher ratings for questions regarding risk factors for DED (screen use, history of CL wear, occupation) over a validated questionnaire. Further research questions exploring the use of questionnaires and any barriers to their uptake, such as ease of use and time constraints, could address this question.

The survey questions exploring management of different severities of DED has provided evidence that practitioners are incorporating a stepwise treatment approach, consistent with the TFOS DEWS II management recommendations, into their practice (Jones et al., 2017). Patient education regarding local environment, digital device-use, diet, artificial tears, and warm compress recommendations (step 1 interventions) were found to be a common approach among practitioners. Statistically significant increases were found for all treatment options falling into Step 2 recommendations when comparing mild to moderate disease. Statistically significant increases were also found for all treatment options when comparing moderate to severe disease, except for preserved tear supplements which declined in recommendation as severity increased. Provision of an extended service did not alter the management options employed.

A previous survey including community optometrists, ophthalmologists, and corneal specialists reported that 80.8% listed artificial tears as their first-line recommended treatment, although failure rates were high; almost 40% of respondents reported that 20-40% of their patients failed treatment, while a further third of respondents reported a 40-60% failure (Williamson et al., 2014)

In this survey, sixty percent of respondents reported that they believed all their patients are satisfied/managed with artificial tears alone. This is consistent with the more recent findings of Craig et al. (2021) who found that one in three of their participants did not respond with improved signs or symptoms to either of the newer, more targeted artificial tear therapies evaluated.

As pointed out by one respondent, success with artificial tears depends largely on patient compliance. Therefore, a further question based around patient compliance would be helpful in determining perceived success of artificial tear prescribing, as would the criteria practitioners use to determine

success, whether improvement in signs, symptoms, or both. This question also assumes that patients are being seen for a follow up appointment; further information regarding follow-up rates would also help determine success.

While practitioners prescribe artificial tears across all severity levels of DED, they are more than twice (2.24 times) as likely to prescribe preservative free supplements over preserved options; more than that found in a recent global survey including ophthalmologists (1.27 times) (Wolffsohn et al., 2021a). This study also indicates that unpreserved options, as with lubricating ointment at bedtime, are more likely to be prescribed in the UK as the severity of the disease increases.

The IP practitioners were significantly more likely to manage a patient with a pharmaceutical agent. Of the total respondents with IP training, 87% agreed that the qualification had widened their ability to diagnose and treat DED. This widened ability was reflected in the IP optometrist's higher likelihood of recommending prescription medications for the treatment of moderate and severe DED. IP optometrists were more than twice as likely to recommend a topical steroid to treat the inflammation associated with moderate and severe DED compared to non-IP optometrists. Previously a significant difference in the prevalence of topical steroid prescribing for moderate (1% and 14%) and severe DED (8% and 52%) has been reported between the UK and Australia respectively (Downie et al., 2016). The low rate of steroid prescribing by UK practitioners when the survey took place in 2016, was attributed to the low number of prescribing optometrists at the time; approximately 2% of the workforce. Therefore, this study provides evidence of an increase in steroid prescribing for moderate and severe DED in the UK.

Interestingly, 82.6% of IP optometrists, a similar percentage to non-IP optometrists (85.7%), would still refer severe DED to an ophthalmologist. A previously reported reason for over 50% of UK referrals was for prescription of topical medications (Downie et al., 2016). This survey did not address the current reasons for referral. However, although more optometrists are now able to prescribe, regional differences in Clinical Commissioning Group prescribing formularies are known to exist. A third of the IP optometrists who responded in this survey would recommend, but not necessarily be able to prescribe, cyclosporin for severe DED. This survey did not identify any regional differences in prescribing.

Regarding clinical procedures used to determine successful treatment, reduction in corneal fluorescein staining was the primary indicator reported, followed by FBUT and anterior lid assessment. A symptomology questionnaire was ranked fifth. Previously an international survey, including dry eye researchers and corneal specialists, reported corneal fluorescein staining as the top sign to indicate treatment response (Sy et al., 2015) despite its reported poor repeatability (Nichols et al., 2004a).

Another study reported patient history as the primary gauge of therapeutic effect (Asiedu et al., 2016). Regardless of whether clinical signs or patient reported symptoms are used, the low associations between them and the low repeatability of clinical tests can confound DED assessment and monitoring of response to treatment (Bartlett et al., 2015). Hence the need for further research and identification of biomarkers that give a more reliable metric of treatment response.

For that reason, one of the aims of this survey was to ascertain whether dry eye research is being translated into practice, specifically the use of tear film biomarkers to give a more quantitative measurement. Very few practitioners reported using tear film osmolarity or MMP-9 measurement to assist in DED diagnosis, even though there is a good evidence base for them (Lemp et al., 2011;Sambursky et al., 2014). Tear osmolarity has been reported to be the best single metric both to diagnose and classify dry eye disease and has been shown to correlate with dry eye severity (Jacobi et al., 2011).

Quantitative and objective measurement of the tear inflammatory biomarker matrix metalloproteinase MMP-9 is possible with the point of care lateral flow device InflammaDry® (Positive Impact, East Sussex, UK). Costing £15 per test it can provide a positive or negative reading in 10 minutes (PositiveImpact, 2023). Even though there is a good evidence base for its use (see section 1.4.3.2) it was assumed that some practitioners may not be aware of it, and therefore a question regarding the feasibility of incorporating a similar device into their practice was included. While less than ten percent of respondents (7.6%) reported using InflammaDry at a specific dry eye appointment, practitioners appeared willing to incorporate a similar device at a very similar cost (mean £12). Practitioners would also be willing to wait a similar length of time (1.7 minutes less) for the results of such an in-office device.

Therefore, further research exploring practitioner’s awareness and knowledge of these products and any constraints to their use would be appropriate.

2.5. Limitations

This survey was completed by 131 participants. Limitations of the study include the unknown response rate and whether the study is truly representative of all experience levels i.e., a sufficient sample size. A priori sample size calculation for a t-test with medium effect size (Cohen’s 0.5) and 80% power was 128, with 64 in each group (see ***Figure 2.23***). For comparisons of the respondents involved in MECS provision with those who are not, post hoc data analysis showed the study was slightly underpowered for a medium effect size (power = 0.78) but exceeded the desired power (0.99) for a large effect size.

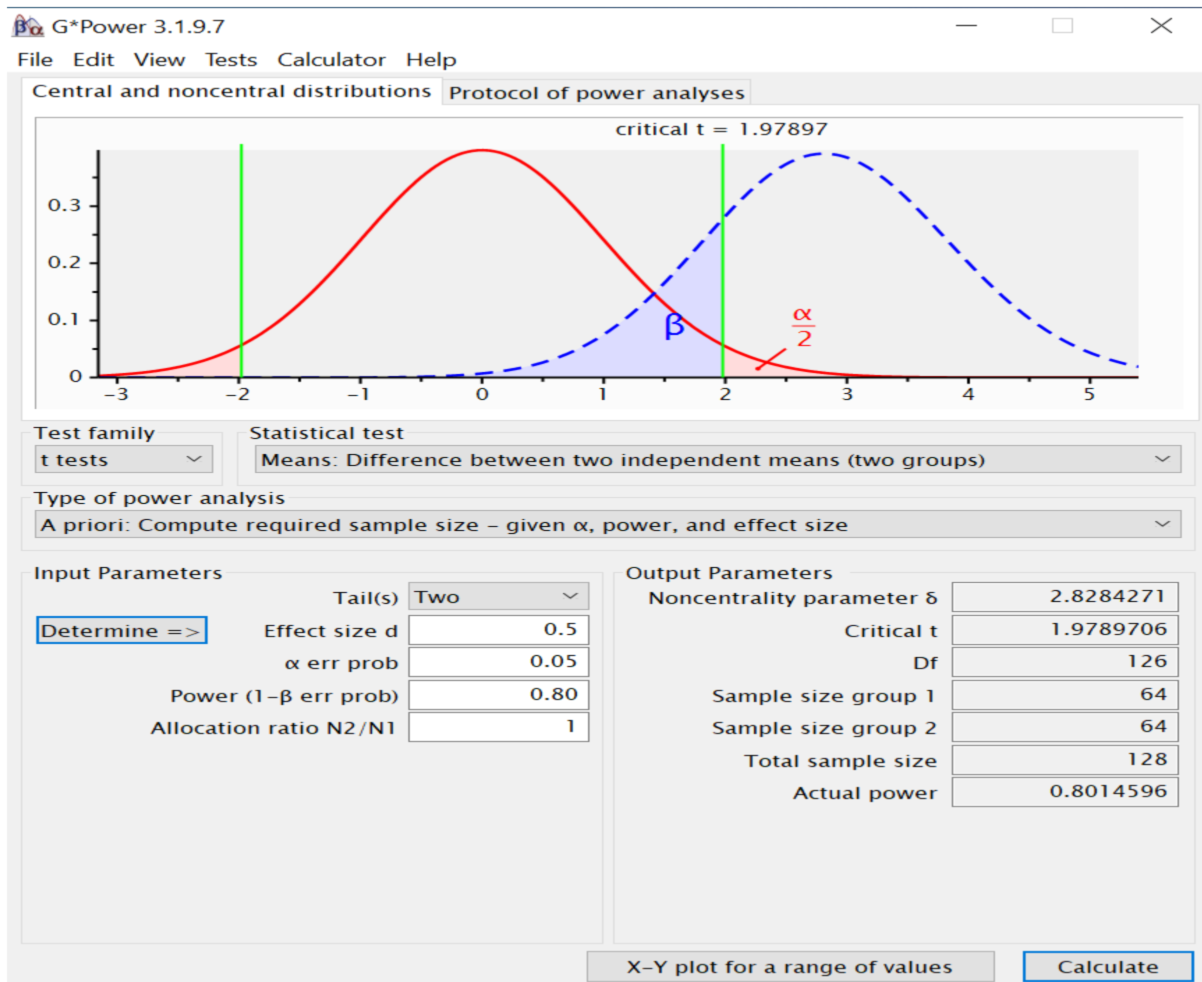


Figure 2.23. A priori sample size calculation for a t-test

Similarly, when considering IP versus non-IP respondents a power of 0.93 could be achieved with a large effect size (0.8), but the study was underpowered (0.57) with a medium effect size. Therefore, a larger sample size would be desirable for any future survey. The study sample size was sufficient to perform x^2 tests for goodness-of-fit tests for the more commonly used treatment options, (A priori sample size of 108 for a medium effect size and 80% power), but not for the lesser prescribed treatment options.

The study potentially carried some bias towards people interested in DED. To avoid this a statement was include on the landing page of the online survey encouraging participation regardless of level of interest or engagement in DED management. Although the survey asked about IP specialism and involvement in extended service provision a further question to identify those with a specialist interest in DED could be incorporated.

The study did not seek to differentiate between the diagnosis and management of aqueous versus evaporative DED, although participants were asked how confident they are in their differential diagnosis. Any future survey could further develop questions around the use of techniques such as

TMH, lipid layer assessment and meibography for subclassification purposes and optimization of management decisions. A clearer distinction between verbal symptomatology and a standardised written questionnaire would also be helpful when considering the success of any management strategies.

This study confirmed practitioner's awareness of the importance of meibomian gland assessment when considering DED of an evaporative aetiology, with approximately two thirds of respondents employing it as one of their top five clinical procedures for diagnosis. However, the survey did not address the specific method used for gland evaluation. Therefore, it is unclear whether the respondents' evaluation consists of a visual inspection of the lid margin or infrared meibography imaging. Meibography was not reported by any respondents in 'any other procedures' but would be expected to be limited to more specialist clinics. Therefore, any further survey should make specific reference to the method employed.

2.6. Summary

In summary, this chapter has provided a current view of UK optometrists opinions and clinical practice patterns with regard to DED diagnosis and management. It has identified an increase in therapeutic management and shown that a stepwise approach to management is being employed. Although an increase in evidence-based practice can be seen, the limited adoption of tear film biomarkers for diagnosis and management highlights the potential to further improve the translation of dry eye research evidence into clinical practice.

A repeatable method for analysing tear film biomarkers may not only enable differentiation of the dry eye versus non-dry eye tear protein profile but may also be used to inform and monitor treatment strategies. Therefore, the following chapter will explore the methodology and repeatability of a microfluidics-based platform for the simultaneous detection and quantification of potential tear protein biomarkers, which is then used to assess the tear protein profiles of the participants in the study reported in chapters 4 to 7.

Chapter 3 - Methodology and repeatability of tear film protein assessment using the Agilent 2100 Bioanalyzer

This chapter has been published as -

- Casemore, R. K., Wolffsohn, J. S., Dutta, D. Human Tear Protein Analysis Using a Quantitative Microfluidic System: A Pilot Study. Eye and Contact Lens. 49(11):498-504, November 2023.

This chapter was presented as -

- Casemore, R. K., Wolffsohn, J. S., Dutta, D. Human Tear Protein Analysis Using a Quantitative Microfluidic System: A Pilot Study. Association for Research in Vision and Ophthalmology (ARVO) Conference, 23-27th April 2023, New Orleans, LA, USA. Invest. Ophthalmol. Vis. Sci. 2023;64(8):3951.

3.1. Introduction

The Agilent 2100 Bioanalyzer is a microfluidics-based platform for the simultaneous detection of proteins, DNA/RNA or cells, by miniaturised capillary gel electrophoresis in conjunction with an appropriate LabChip kit. Three protein kit assays are available: Protein 80 kit, Protein 230 kit, High Sensitivity protein kit, for sizing and quantifying protein samples from 5 to 80kDa, 14 to 230kDa and 5 to 250kDa, respectively. The studies reported in this thesis utilised the Protein 230 LabChip® kit, which had previously been identified as the best kit to separate proteins of interest in the tears (Versura et al., 2012). Each chip contains an interconnected set of microchannels that sieve proteins by size as they are driven through it by means of electrophoresis. During gel electrophoresis, the proteins to be separated are pushed by an electrical field through the gel and detected with laser-induced fluorescence.

The bioanalyzer software allows the data for each individual sample to be displayed as a gel-like image in bands, as in SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), or as electropherogram peaks. Peaks in the protein profile can be identified based on their molecular weight/mass (kDa) identity. Peak height, area, relative concentration, and percentage of the overall protein content can also be investigated. Comparisons between tear samples taken at different times are possible by overlaying the electropherograms, allowing for any changes in the profile to be identified.

This chapter details the method of preparation and analysis of tear samples using the Agilent 2100 Bioanalyzer (Agilent Technologies, UK, Ltd). The results of a pilot study to ascertain the repeatability of the method are reported. The techniques used for tear collection and storage for this study and subsequent chapters are described.

3.1.1. Study hypothesis and aims

This pilot study tests the hypothesis that the tear protein profiles measured by electrophoresis using the Agilent 2100 Bioanalyzer are repeatable. It seeks to validate the bioanalyzer as a reliable method for investigating tear proteins, in order to compare the protein profiles of dry eye and non-dry eye subjects reported in chapter 5.

3.2. Methods

This was a prospective, controlled pilot study to investigate the repeatability of the tear protein profile found by electrophoresis utilising the Agilent 2100 Bioanalyzer.

3.2.1. Study protocol

Participant suitability for the study was assessed at visit 1.The inclusion criteria were as follows:

- Aged over 18 years
- Best-corrected vision at least 6/12 or better in each eye
- Willing to attend for two visits, with a seven-day interval in between
- Able to provide written consent in English

The exclusion criteria were:

- Any current or previous use (within three months) of any topical or systemic medications which could alter the tear film composition
- Any history of any major systemic or ocular conditions (within last three months)
- Previous ocular surgery
- Any active inflammation or infection
- Pregnancy

Contact lens wearers were allowed to participate in the study and were permitted to wear their lenses between visits but asked not to wear them on an appointment day. Written consent was obtained at the first appointment and participants informed that they could withdraw from the study at any time.

Null hypothesis:

- Inter-visit tear protein profiles are not repeatable.

3.2.2. Ethics approval

The study received ethics approval from the University Research Integrity and Ethics Committee (URIEC) (ref# AUREC 1375 (21-04)) and followed the tenets of the declaration of Helsinki, see Appendix 3.

3.2.3. Participant enrolment and clinical procedures

Ten participants with no known systemic or ocular disease (other than possible DED) were recruited from the student population. All investigations were conducted 1 week ± 2 days apart. A thorough ocular and systemic history were taken from each participant. The 12-item Ocular Surface Disease

Index (OSDI) questionnaire was administered to all participants at visit 1, to assess any symptoms of dry eye disease during the previous week. This included 3 ocular symptom questions, 6 visual function-related questions and 3 environmental trigger questions. Scores from 0 (none of the time) to 4 (all the time) were used to calculate a final score. A score of ≥13 combined with one or more signs of a loss of homeostasis of the tear film; non-invasive tear breakup time (NIBUT) < 10s or ocular surface staining (> 5 fluorescein corneal spots, > 9 lissamine green spots or lid wiper staining ≥ 2 mm length & ≥ 25% width) were used to diagnose dry eye disease (DED) (Wolffsohn et al., 2017).

Clinical evaluations were made at both visits using the Keratograph 5M (Oculus, Wetzlar, Germany) in a non-invasive to invasive sequence. These included tear meniscus height (average of three measurements taken at the lower limbus), non-invasive breakup time (NIBUT) (average of three readings), ocular redness, then tear collection, followed by corneal and conjunctival staining with fluorescein (Bio Fluoro 1mg fluorescein sodium ophthalmic strips, Biotech Visioncare, India) and lissamine green (Green Glo Ophthalmic Strips 1.5mg lissamine green, Contacare Ophthalmics and Diagnostics, India), respectively. Corneal and conjunctival staining were graded according to the Oxford Grading Scale (Bron et al., 2003). Ocular redness was graded using the automated Keratograph 5M JENVIS grading scale of 0 to 4 in 0.1 steps, in both the bulbar and limbal areas nasally and temporally i.e., four measurements.

3.2.4. Methods of tear collection

Glass microcapillary tubes were used to collect tears in this study. Various methods exist, including Schirmer strips with or without anaesthesia (Boehm et al., 2011;Jackson et al., 2016), mini sponges (Acera et al., 2008), micropipettes (Yoon et al., 2007), and glass or plastic microcapillary tubes (Esmaeelpour et al., 2008). Studies using these methods have been reported in ***Tables 1*.*1*** to ***1.7*** of chapter 1, each having their own advantages and disadvantages that can affect tear analysis results (Zhou and Beuerman, 2012).

Although Schirmer strips are used routinely for the quantitative assessment of tear production and can be easily applied to tear collection for sample extraction and analysis, the test is known to cause some irritation of the conjunctiva, stimulation of reflex tearing and possible contamination of the sample by cellular proteins from the conjunctival epithelial cells (Posa et al., 2013). Use of an anaesthetic in conjunction with the Schirmer strip (Schirmer II test) to prevent this, may allow for collection of basal tears only, but may cause contamination of the sample. A significant increase in tear serum proteins has been shown with Schirmer strip collection compared to collected without mechanical stimulation of the conjunctiva (Stuchell et al., 1984). The properties of commercially

available Schirmer strips have also been shown to vary as there is no standardisation between them (Garcia-Porta et al., 2018).

Green-Church et al (2008) found some proteins were predominantly observed in tears collected by capillary, whereas a higher number were only identified in those collected by Schirmer strips, with some overlap between the two methods. They proposed that the difference arose through the Schirmer strip's interaction with the epithelium of the ocular surface.

An advantage of capillary tubes is their simple post-collection handling, as the complete volume of undiluted conjunctival cell-free tear fluid can be collected following a quick centrifugation step, while Schirmer strips require the tear fluid to be extracted. The choice of solvent, time, temperature, and volume may all influence the extraction efficiency (Bachhuber et al., 2021).

While both methods have been reported to be safe and well tolerated, the capillary tube method was considered more comfortable and caused less frequent and shorter foreign body sensations (Bachhuber et al., 2021). Higher total protein concentrations were found in samples collected with capillary tubes as well as more repeatability in sequential sample collections.

3.2.4.1. Microcapillary basal tear collection method

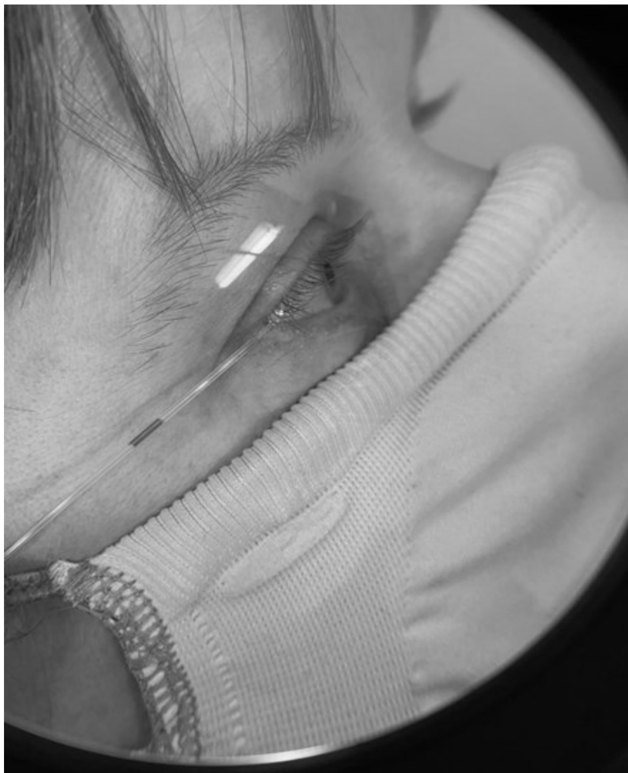


Figure 3.1. Glass microcapillary tear collection

Glass microcapillary tubes (10 µl, Sigma-Aldrich, USA) were used for the collection of non-stimulated tears from participants in the repeatability study, in accordance with Aston University's Standard Operating Procedure (SOP) for tear collection (see **Appendix 4**) and the protocol of the Optometry and Vision Sciences Research group (**Appendix 5**). Due to possible diurnal variations in secreted proteins (Puinhas et al., 2013), all the tear samples were collected at the same time of day (between 2 and 4pm) except for two, where the visits had to be scheduled in the late morning. Tear collection rate was not tracked.

Tear collection was performed as follows:

- Sterile latex/vinyl gloves were worn to prevent contamination from the skin.
- Participants were asked to tilt their head towards the side of the collection and look up and away, while up to 10 µL of basal tears were collected from one eye.
- The microcapillary tube was placed carefully at the lateral tear meniscus (see **Figure 3.1**), with minimal touch to the bulbar conjunctiva or lid margin to avoid stimulation of reflex tearing.
- Participants were able to blink during the procedure and the collection was paused if any reflex tearing was suspected.
- A five minute time limit was set for tear collection. Tear flow rate was monitored, and collection paused if reflex tearing occurred.
- Collected samples were expelled from the microcapillary tubes with a plunger into 0.5ml microcentrifuge tubes Eppendorf Tubes® (Eppendorf, Germany), labelled, and kept on ice temporarily before storage.

3.2.4.2. Microcapillary flush tear collection method

The flush method was used only when insufficient basal tears could be collected after around 5 minutes. The procedure employed was as follows:

- Non-preserved unit dose saline was warmed to 35˚C to avoid any reflex tearing due to cold stimulus to the ocular surface.
- Flush tears were collected by instilling a single 20 µL drop of non-preserved unit dose saline into the inferior palpebral fold using an Eppendorf pipette. Care was taken not to touch the eye with the sharp pipette tip.
- Participants were asked to gently close their eyes and rotate them twice prior to tear collection with a 20 µL microcapillary tube. The ocular fluid was collected within a minute of instillation to avoid reflex tear as described before. This procedure was then repeated (see discussion section 3.4.1).

3.2.5. Tear storage

Collected samples were kept on ice and transferred to storage on the same day. Prior to storage, samples were centrifuged at 5000g for 10 minutes at 4˚C to remove any cellular debris (Masoudi et al., 2017). The aqueous supernatant was collected and aliquoted to a fresh microcentrifuge tube for storage at -80°C, for later analysis. The effect of storage on the total tear protein and major protein concentrations of tear samples collected by capillary tube and analysed with SDS-PAGE has previously been determined (Sitaramamma et al., 1998). The study indicated that tears should be stored

preferably at a low temperature (-70 ˚C), as higher temperatures were shown to cause a reduction in protein concentration in a shorter period of time.

3.2.6. Tear Analysis with the Agilent 2100 bioanalyzer

3.2.6.1. Sample preparation

The tear samples were stored for up to 2 months before analysis was performed. Prior to analysis the samples (analytes) were pre-treated according to the protocols provided with the Protein 230 LabChip® kit. This involved the addition of 2 µl of denaturing solution to 4 µl of the analyte. The denaturing solution was prepared by combining 200 µl of sample buffer with 7µl of the reducing agent β-mercaptoethanol. The sample buffer contained upper and lower markers of known molecular weight, identical to those in the standard ladder and were therefore incorporated into each analyte allowing direct comparison against it.

The analytes and ladder (6µl) were denatured by placing the tubes in a hot water bath (95-100°C) for 5 min, cooled immediately and centrifuged for 15s. Eighty-four microlitres of deionized water was then added to each analyte and the ladder. As deionized water contains no charge, only denatured proteins, which have a net negative charge, conduct electricity.

3.2.6.2. Loading of the protein chip

A 6 µl aliquot of each analyte (into wells 1 to 10) and the ladder (into well L) were loaded onto the chip, which was initially filled with 12 µl of a gel/dye mix into wells marked **G** and a de-staining solution into well **DS** (see ***Fig 3.2***). See **Appendix 5** for the conditions of storage. All reagents were kept covered as they contained dye which decomposes when exposed to light.

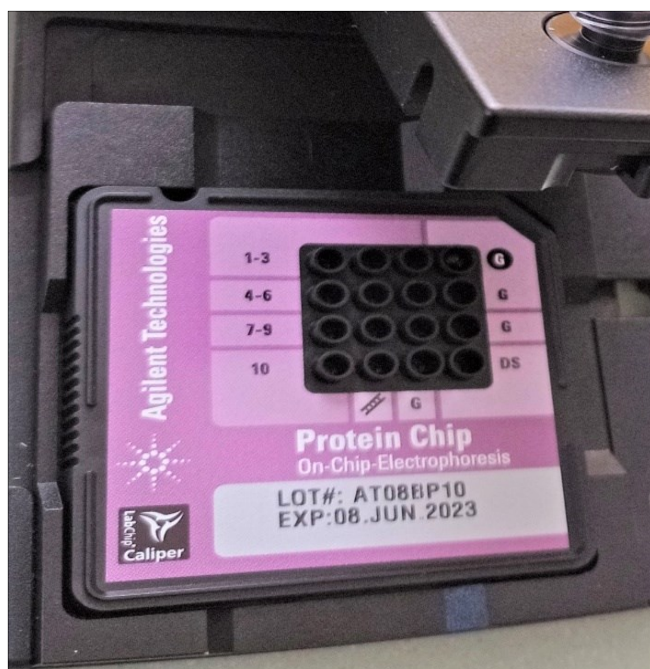
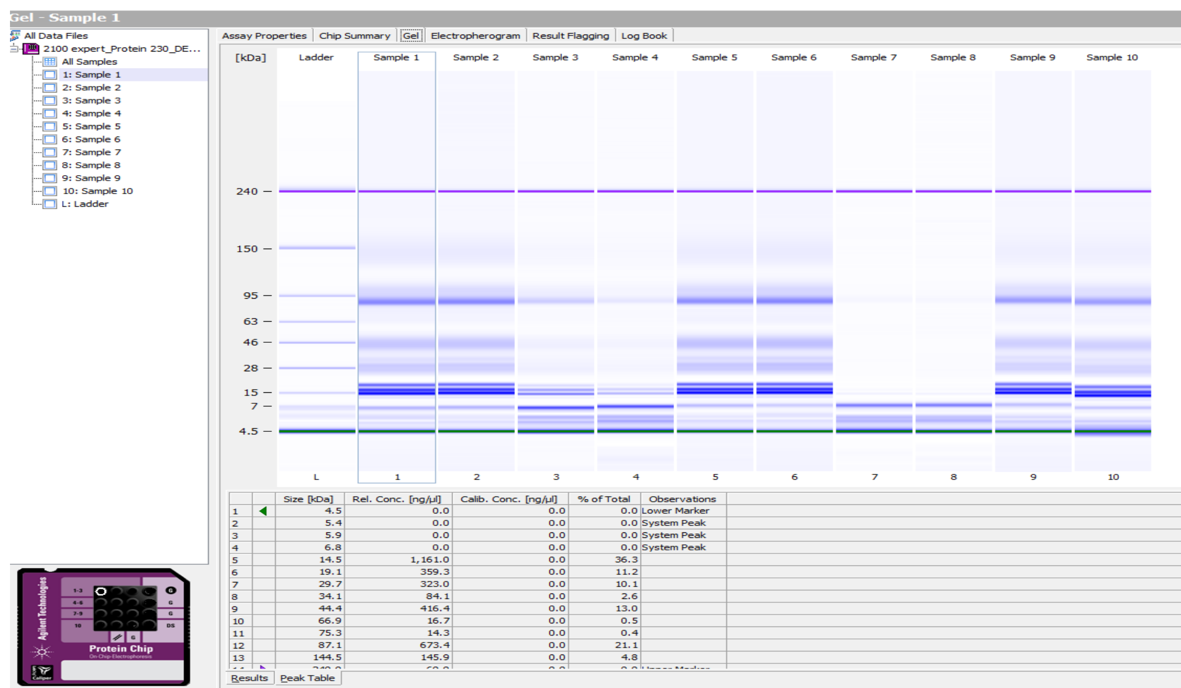


Figure 3.2. Protein 230 LabChip (Agilent technologies)

3.2.6.3. Analysis of tear samples

Once loaded the chip was placed carefully into the bioanalyzer. Each chip run took 30 to 40 minutes, during which time the ladder and then each sample was processed in turn. Results could be displayed as gel-like image bands (see ***Fig 3.3***) or as electropherogram peaks (see ***Fig 3.4***) for each analyte.



		Size [kDa]	Rel. Conc. [ng/µl]	Calib. Conc. [ng/µl]	% of Total	Observations
1	◀	4.5	0.0	0.0	0.0	Lower Marker
2		5.4	0.0	0.0	0.0	System Peak
3		5.9	0.0	0.0	0.0	System Peak
4		6.8	0.0	0.0	0.0	System Peak
5		14.5	1,161.0	0.0	36.3	
6		19.1	359.3	0.0	11.2	
7		29.7	323.0	0.0	10.1	
8		34.1	84.1	0.0	2.6	
9		44.4	416.4	0.0	13.0	
10		66.9	16.7	0.0	0.5	
11		75.3	14.3	0.0	0.4	
12		87.1	673.4	0.0	21.1	
13		144.5	145.9	0.0	4.8	

Figure 3.3. Image of gel-like protein profiles for tear samples from an initial trial run.
Image shows the molecular weight marker ladder (L) on the left-hand side and analytes (lanes 1-10).

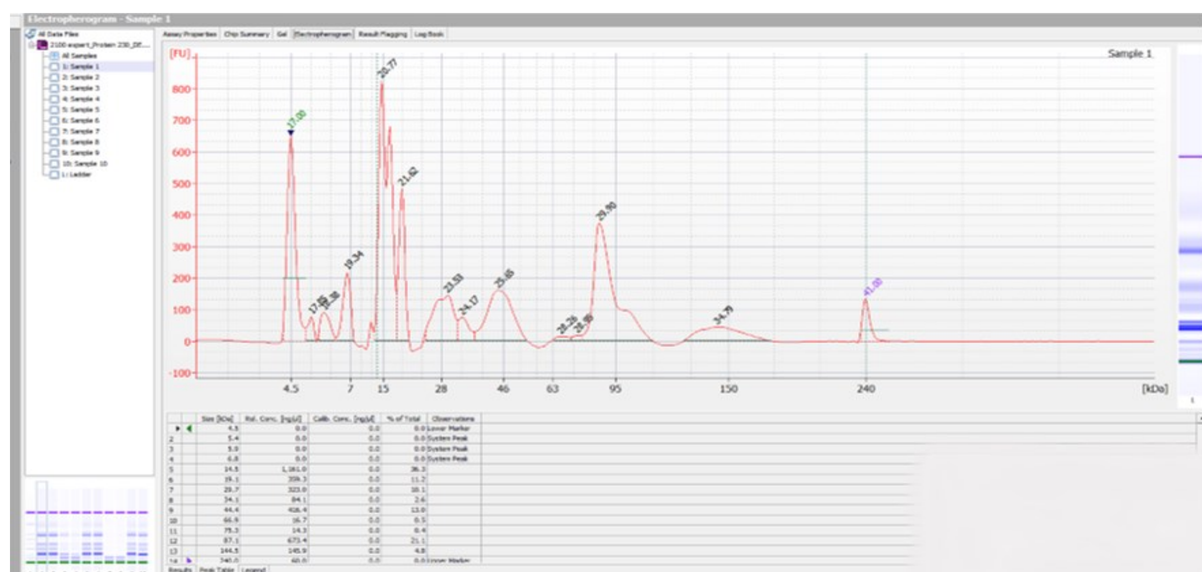


Figure 3.4. Electropherogram peaks for the samples in Lane 1 of Figure 3.3.
The y-axis indicates protein concentration in fluorescence, the x-axis identifies the molecular weight (kDa).

Samples from Visit 1 and 2 for participants 1,2,3 and 5 were analysed on one chip while the 6,7,8 and 9 were loaded on a separate chip on a different day. Samples for participants 4 and 10 were analysed on a separate chip. The addition of sample buffer (containing the denaturing solution), temperature and duration of incubation were controlled tightly, according to the manufacturer's instructions. Peaks in the protein profile were identified based on their molecular weight/mass (kDa) and the findings from previous studies (Kuizenga et al., 1991;Molloy et al., 1997;Mann, 2007;Versura et al., 2012;Versura et al., 2013;Mann et al., 2020).

3.2.7. Sample size calculation

There was limited data available on which to base the repeatability of tear protein analysis using the bioanalyzer instrument. Given the fact that tear protein concentrations and their percentage of the total protein concentration may vary substantially based on the employed analytical method, it was important to assess this method at the start of this doctoral research. Hence, this pilot study sought to include ten participants in order to inform sample size calculations for subsequent chapters (see section 4.2.3).

3.2.8. Statistical Analysis

Data analysis was performed using Microsoft® Office Excel® and GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA. A comparison of the main proteins identified and the protein concentrations at each visit was made for each participant. Quantitative data are expressed as mean ± standard deviation (SD). Frequency and percentage were used for the categorical data and clinical parameters. The coefficient of repeatability (1.96 x standard deviation of the mean of the differences) was calculated for the percentage of the total concentration for each major protein identified at both visit 1 and 2. The Kolmogorov-Smirnov test was used to test for normality. As the data was not normally distributed the non-parametric Wilcoxon matched pairs t-test (Wilcoxon signed ranks test) was used where proteins were present in both visit samples. The strength of any correlation between the two visits (Spearman's correlation coefficient r) was estimated according to the following coefficient magnitudes: −1 (perfect negative correlation) through 0 (no correlation) to +1 (perfect positive correlation) (Mukaka, 2012). A high correlation was considered to be 0.5-1.0, moderate 0.5-0.3, and 0.3 to 0.1 a small if any correlation. A p value of <0.05 was considered statistically significant.

3.3. Results

Ten participants with no ocular or systemic disease were recruited from the student population and attended for two visits one week apart. Participants consisted of 9 females and 1 male with a mean age of 20.8 ± 1.6 years. Three participants fulfilled the TFOS DEWS II diagnostic criteria for DED (RS01, RS06 and RS09). Four participants wore soft contact lens, two of whom had dry eye signs and symptoms. Four participants reported occasional use of rewetting or lubricating drops, but not immediately prior to the visit. See ***Table 3.1*** below for the demographic, ocular surface, and tear film characteristics of the study participants.

Table 3.1. Demographic and clinical measurement data for the study participants.

Characteristic	Scale/ range	Values	Values for non-DED participants n=7	Values for DED participants n=3	p-value
Demographics					
Age	years	20.8 ± 1.62			
Female sex	n/a	9 (90%)			
TFOS Dews II dry eye criteria met	n/a	3 (30%)			
Clinical measures					
OSDI score**	0-100		4.52 ± 4.36	28.10 ± 22.48	**0.02***
Tear meniscus height	mm		0.32 ± 0.07	0.34 ± 0.08	0.62
Non-invasive tear breakup time	sec		9.30 ± 2.24	7.88 ± 2.51	0.14
Bulbar redness (composite score)	0 to 4 (Jenvis)		1.26 ± 0.56	0.98 ± 0.32	0.38
Limbal redness (composite score)	0 to 4 (Jenvis)		0.85 ± 0.64	0.52 ± 0.18	0.38
Corneal staining score	0 to 4 (Oxford)		0.14 ± 0.36	0.50 ± 0.55	0.11
Conjunctival staining score	0 to 4 (Oxford)		0.36 ± 0.80	1.17 ± 041	0.26

*Data are represented as mean ± SD, or percentages. *p value of <0.05 considered significant. ** All data was combined for visits 1 and 2 except for OSDI which was not repeated at visit 2.*

3.3.1. Total protein content and protein concentrations

In this study the total protein concentration collected by microcapillary tubes was 6.72 ± 3.56 mg/ml. See ***Figure 3.5*** for the total protein concentrations per visit for each participant.

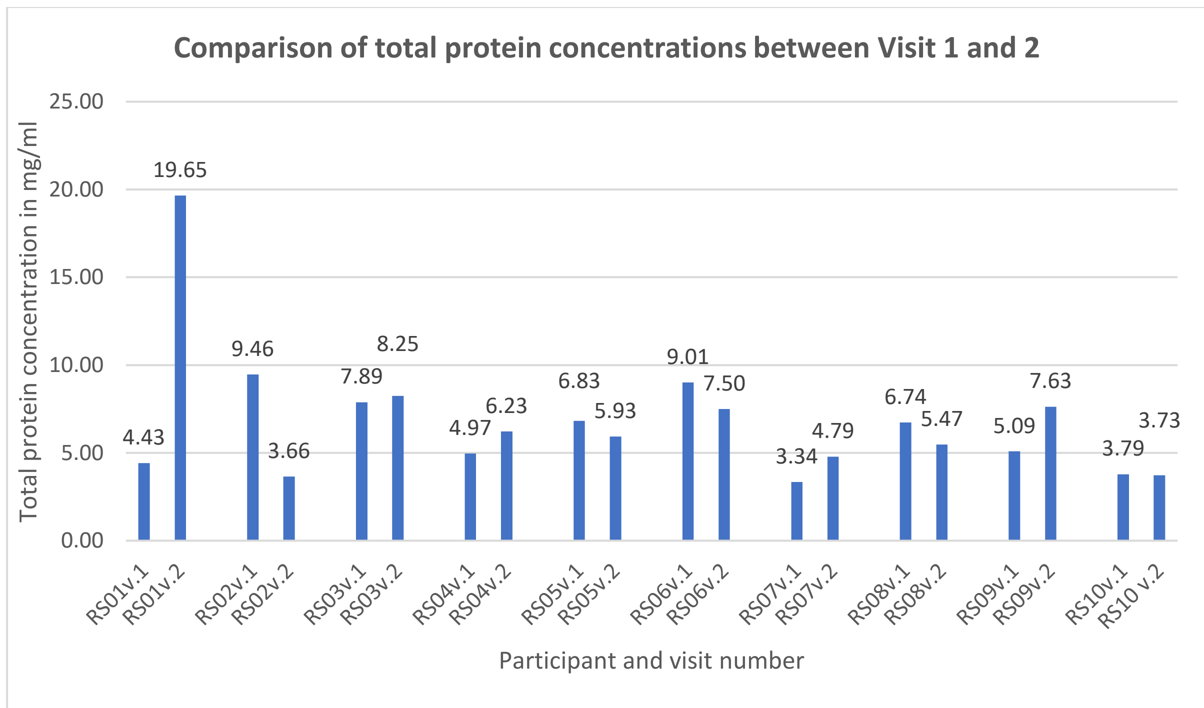


Figure 3.5. Total protein concentrations at each visit for each participant

Participant 1 (RS01) Visit 2 had a substantially higher total tear protein concentration of 19.64mg/ml, which appeared as an outlier above the upper limit (upper quartile plus IQR x 1.5) of 12.20 mg/ml (see ***Figure 3.6***). The upper and lower whiskers of the box plots denote the total protein concentration maximum and minimum values, while the boxes show the interquartile ranges.

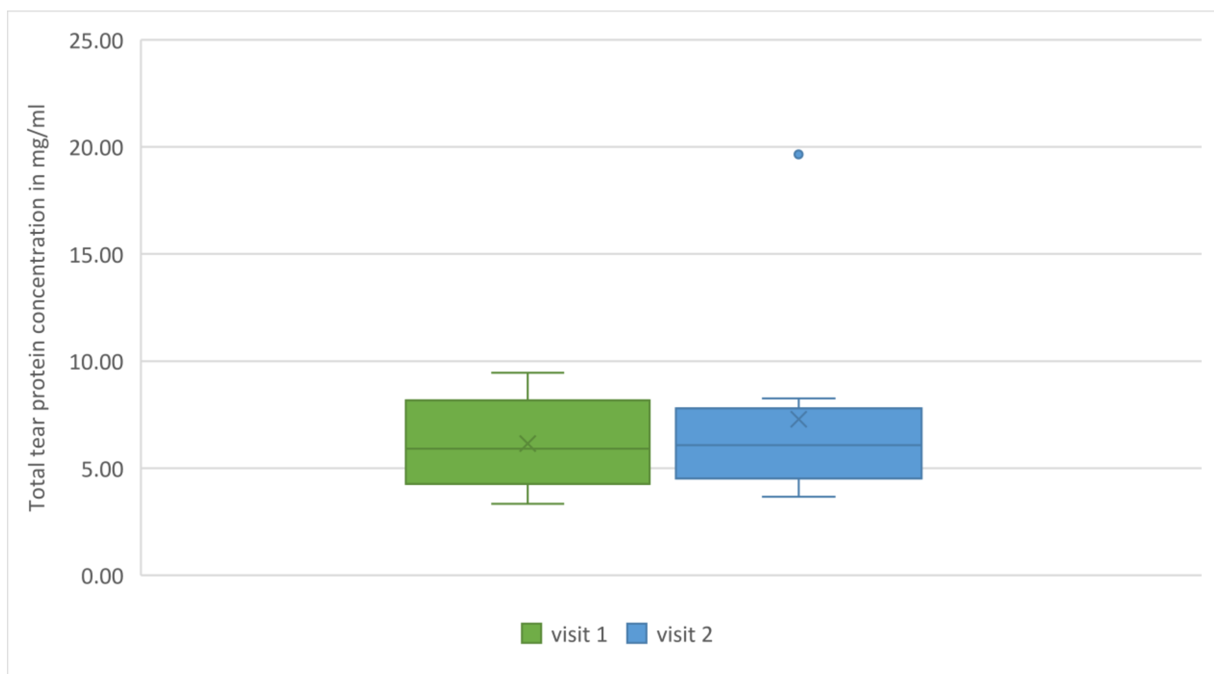


Figure 3.6. Box plot showing the distribution of total protein concentrations at Visit 1 and 2
The total protein concentration found for one participant at Visit 2 was higher and indicated as an outlier.

Median and mean protein concentrations during Visit 1 were 5.91 mg/µl and 6.15 ± 2.16 mg/µl and during Visit 2 were 6.08 mg/µl and 7.28 ± 4.62mg/µl respectively. After removing the outlier data of Participant 1 Visit 2, the mean total protein concentration for visit 1 and 2 combined was 6.04 ± 1.90 mg/ml. There was no significant difference ($P<0.05$) in the total protein concentrations found between visits.

3.3.2. Protein analysis and interpretation

To check that the samples had run correctly, the ladder well results were viewed in the electropherogram display. A successful ladder featured 7 well resolved ladder peaks, a flat baseline and readings of at least 20 fluorescence units higher than the baseline readings; these features were found. A lower and upper marker, 4.5 and 240 kDa respectively, was present in each ladder and each analyte run. The standard protein ladder contained proteins of known concentrations and molecular weights allowing for semiquantitative analysis.

The results for each of the chip runs also displayed the main features of a successful protein sample run; sample peaks were between the lower and upper marker peaks, two marker (system) peaks were present at 4.5 kDa and 240 kDa, which were well resolved from the sample peaks.

A range of 6 to 12 protein peaks were found in the twenty analytes, the mean being 9.65 ± 1.70 proteins identified. Based on literature review and previous studies (Molloy et al., 1997;Markoulli et al., 2012a;Versura et al., 2012;Mann and Tighe, 2013;Versura et al., 2013), six of the most abundant proteins were identified. ***Table 3.2*** shows data relating to these proteins, their theoretical size, the study size range, mean concentrations, and confidence intervals. ***Figure 3.7*** shows the gel-image protein bands for each participant, with Visit 1 and Visit 2 alongside each other. The darker bands correspond to the electropherogram peaks. The upper and lower markers of known molecular weight contained in the sample buffer appear at 240 and 4.5 kDa respectively, for each sample. These allow direct comparison to the ladder.

Table 3.2. Tear proteins recognised by the Agilent 2100 Bioanalyzer in this study, their theoretical molecular weight (kDa) and the range found with the 230 protein chip

Protein name	Theoretical size (kDa)	Study size range (kDa)	Mean concentration ± SD (mg/ml)	Confidence interval (mg/ml)	Function (identified by previous studies)
Lysozyme	14.0	14.0-15.4	2.58 ± 1.47	1.73 - 3.12	Antimicrobial enzyme
Lipocalin-1	17.0	18.1-19.8	0.46 ± 0.29	0.31 - 0.63	Bind & transport hydrophobic molecules
sIgA* (light chain)	26.0	26.7-30.5	1.07 ± 0.81	0.75 - 1.47	Antibody
Zinc-α2-glycoprotein (ZAG)	34.2	32.0-34.1	0.31 ± 0.15	0.23 - 0.39	Lipid breakdown
~47 kDa unidentified		43.1-47.9	0.63 ± 0.49	0.26 - 0.85	
Albumin	68.0	60.6-68.2	0.01 ± 0.12	0.03 - 0.16	Stabilize osmotic pressure
Lactoferrin	82.0	83.2-88.4	1.51 ± 1.13	0.97 - 2.22	Inhibits bacterial growth
~100 kDa unidentified		98.4-103.6	0.41 ± 0.30	0.36 - 0.59	

**Secretory immunoglobulin A*

An overview of all the proteins present at each visit for each participant can be seen in ***Figure 3.8*** below. Not all proteins were present in the same concentrations at each visit. ***Figure 3.9*** displays each protein concentration as a percentage of the total concentration.

It was possible to overlay and view the electropherogram peaks from both visits for each participant, see ***Figures 3.10-20.*** It is worth noting that the fluorescence scale on the y-axis is not the same for all the participant electropherograms. However, by overlaying the results, it can be seen that the protein peaks are broadly consistent between each visit for each participant.

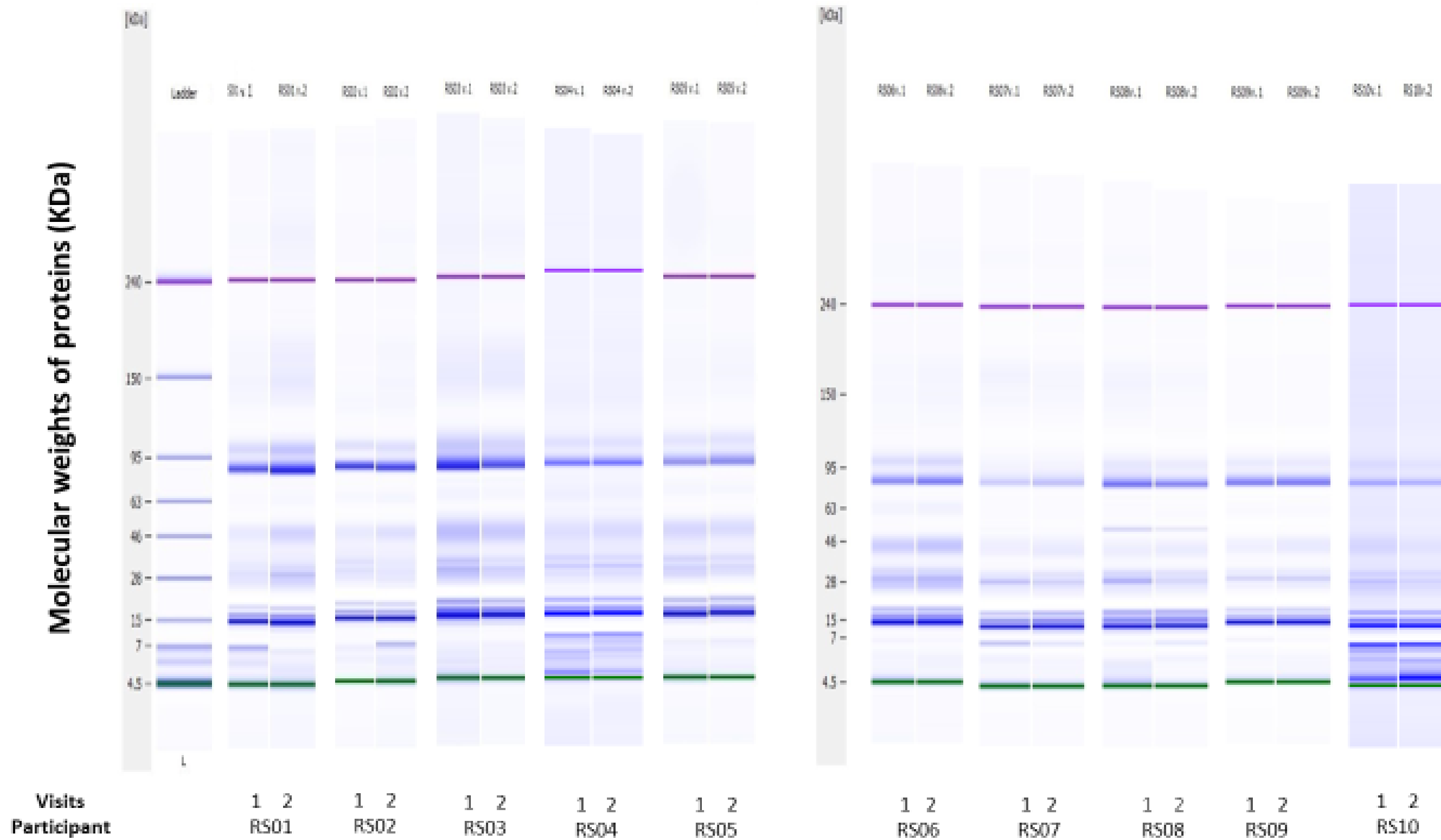


Figure 3.7. Gel images showing the protein bands for each participant
Data for visit 1 and 2 for each participant are alongside each other. Chip run 1 contains data for participants 1-5. Chip run 2 contains data for participants 6-10.

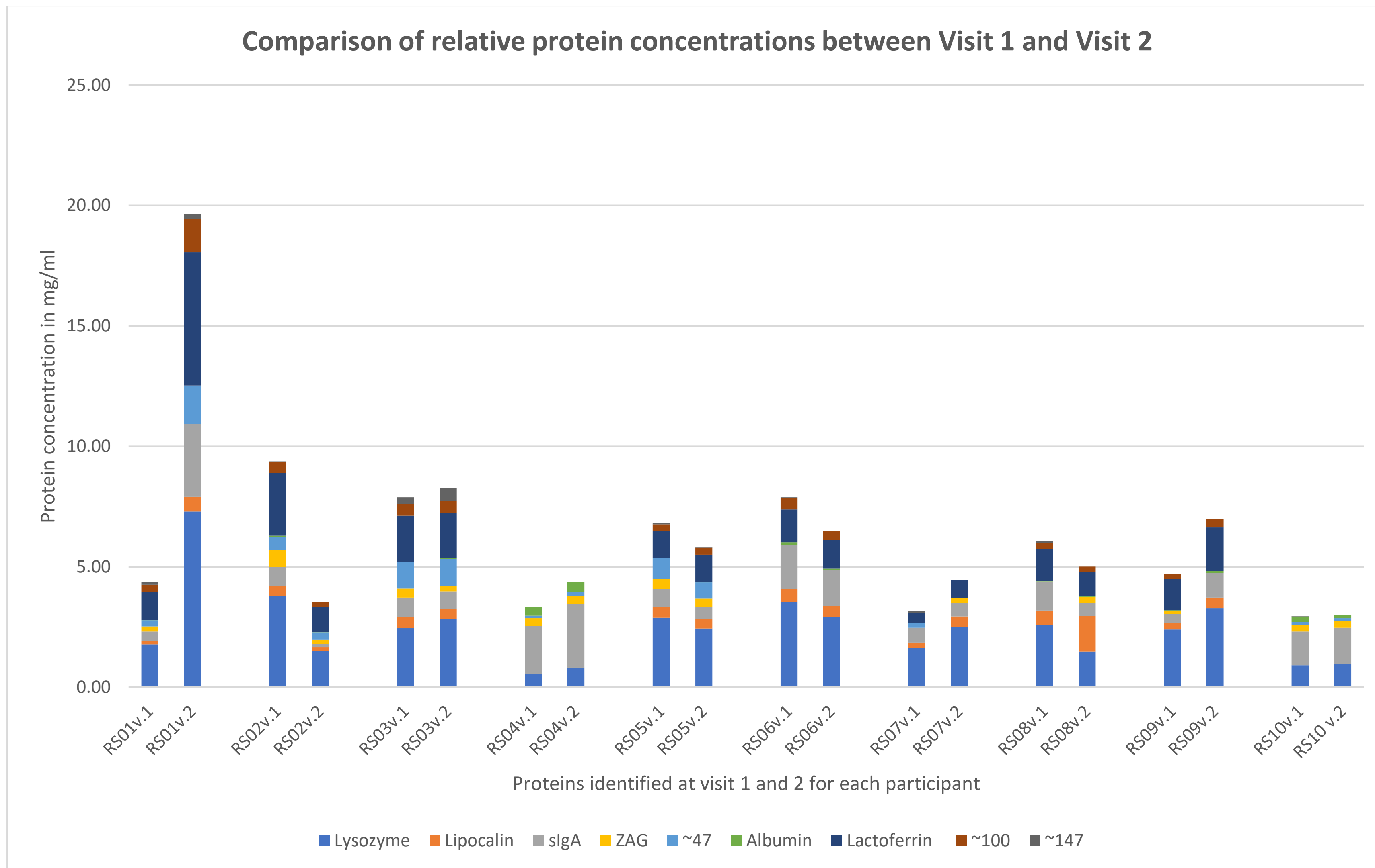


Figure 3.8. The main protein concentrations present at each visit for each participant
SIgA indicates secretory immunoglobulin-A. ZAG indicates Zinc-α2-glycoprotein.

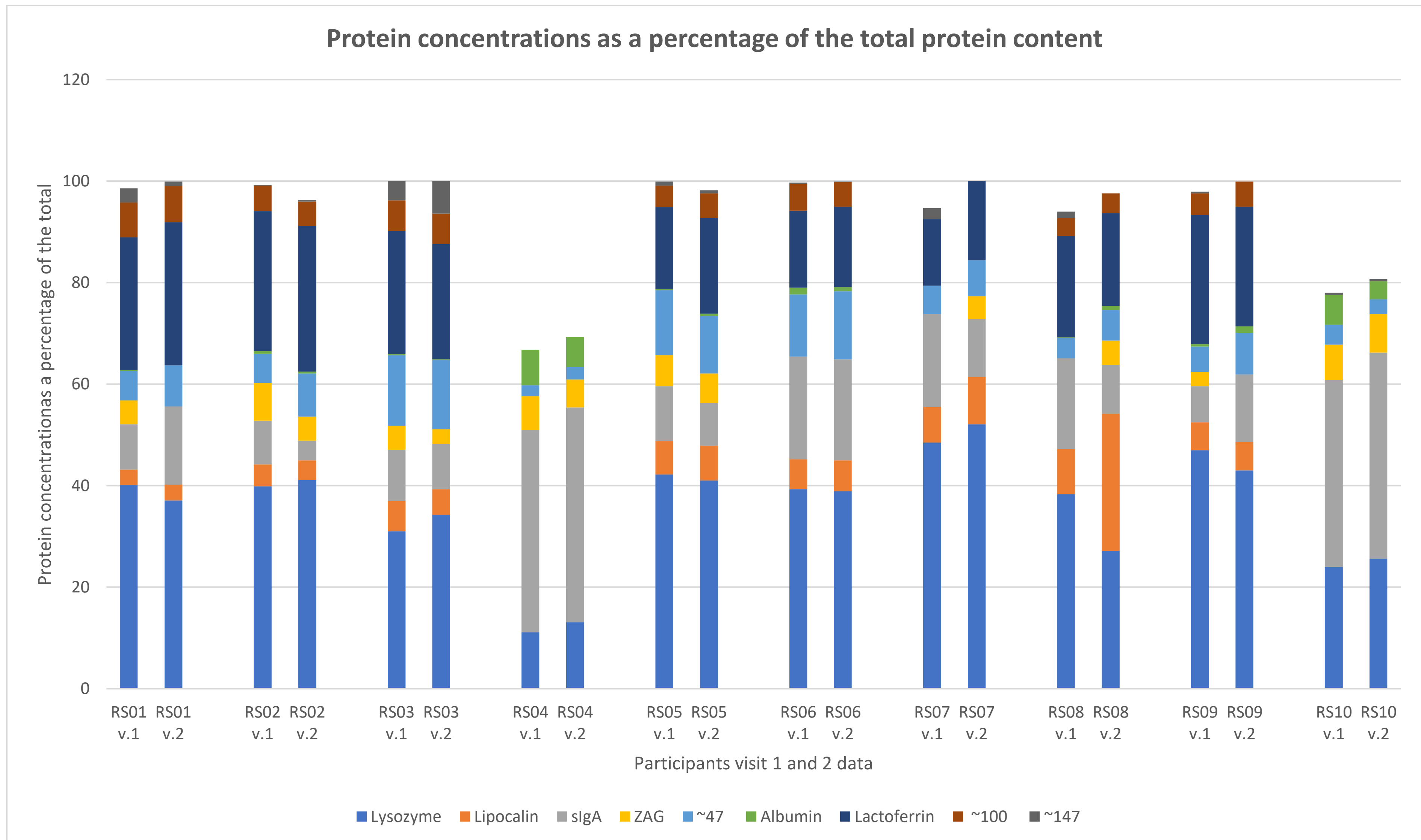


Figure 3.9. Protein concentrations displayed as percentages of the total protein concentration for each participant at each visit

V1 indicates Visit 1, V2 Visit 2. SIgA indicates secretory immunoglobulin-A. ZAG indicates Zinc-α2- glycoprotein.

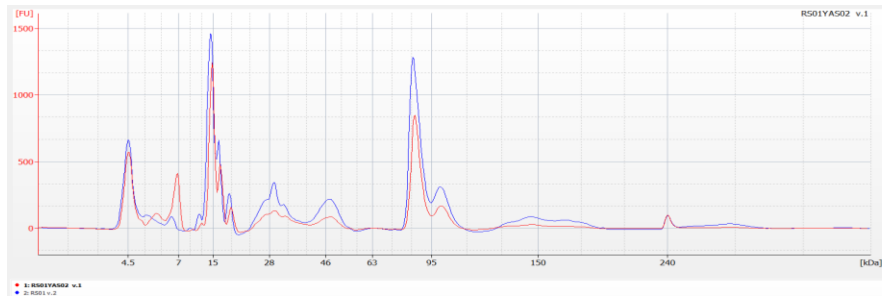


Figure 3.10. Overlay electropherogram image for participant 1 (RS01)
The red line denotes Visit 1; the blue line Visit 2.

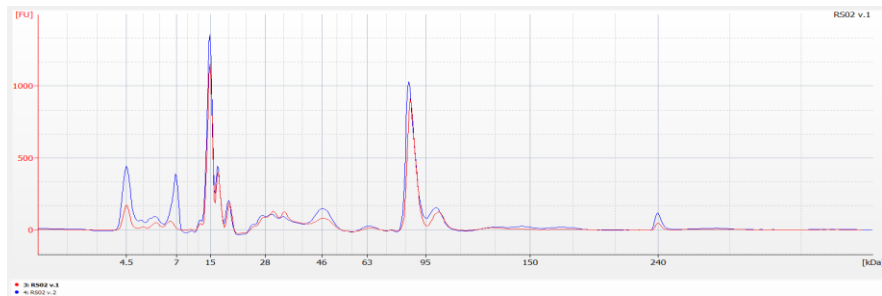


Figure 3.11. Overlay electropherogram image for participant 2 (RS02)
The red line denotes Visit 1; the blue line Visit 2.

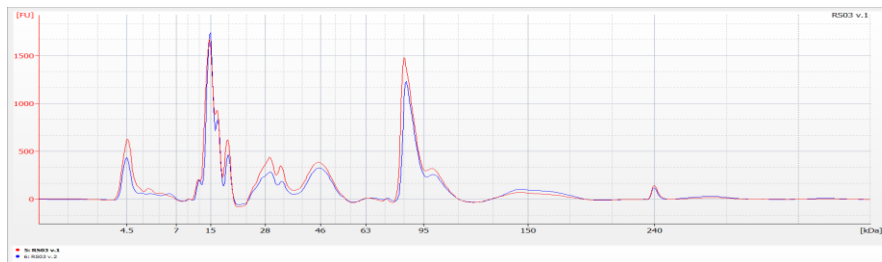


Figure 3.12. Overlay electropherogram image for participant 3 (RS03)
The red line denotes Visit 1; the blue line Visit 2.

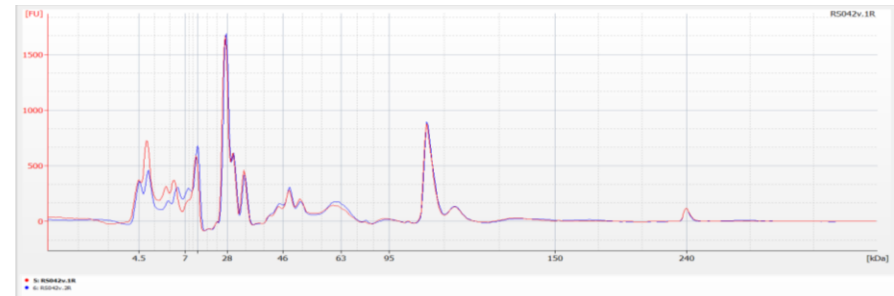


Figure 3.13. Overlay electropherogram image for participant 4 (RS04)
The red line denotes Visit 1; the blue line Visit 2.

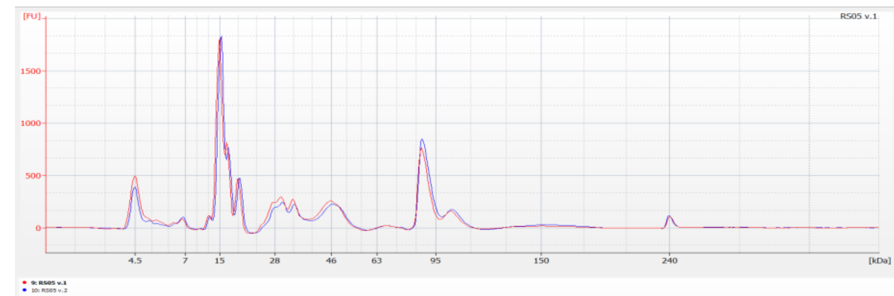


Figure 3.14. Overlay electropherogram image for participant 5 (RS05)
The red line denotes Visit 1; the blue line Visit 2.

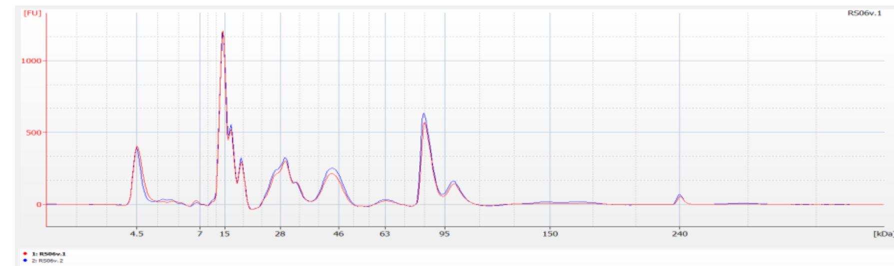


Figure 3.15. Overlay electropherogram image for participant 6 (RS06)
The red line denotes Visit 1; the blue line Visit 2.

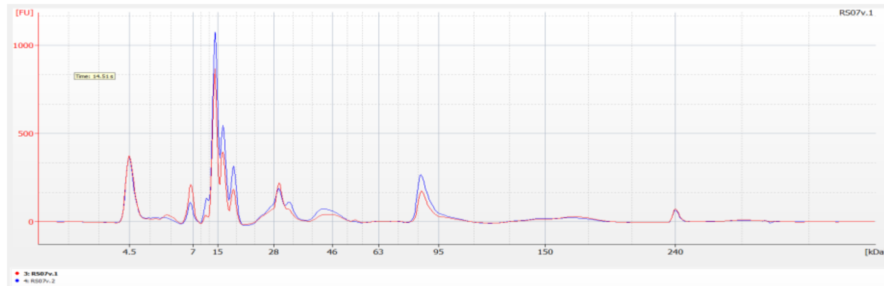


Figure 3.16. Overlay electropherogram image for participant 7 (RS07)
The red line denotes Visit 1; the blue line Visit 2.

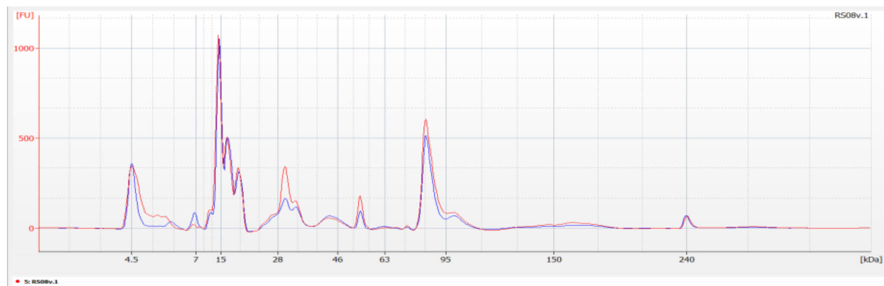


Figure 3.17. Overlay electropherogram image for participant 8 (RS08)
The red line denotes visit 1, the blue line Visit 2.

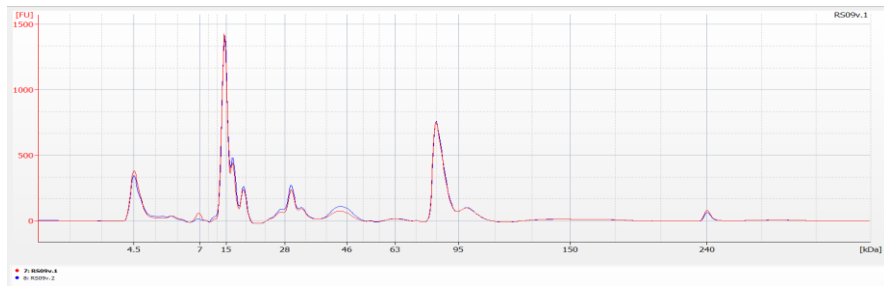


Figure 3.18. Overlay electropherogram image for participant 9 (RS09)
The red line denotes visit 1, the blue line Visit 2.

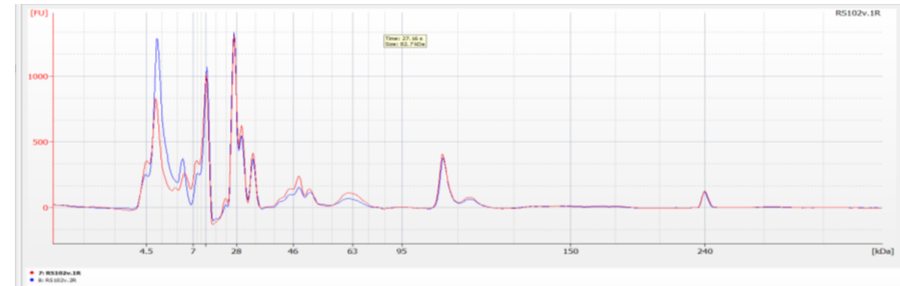


Figure 3.19. Overlay electropherogram image for participant 10 (RS10)
The red line denotes visit 1, the blue line Visit 2.

3.3.3. Statistical analysis of the protein peaks

Table 3.3 includes data for the seven proteins that were present at both visits for seven or more participants. Considering each protein as a percentage of the total protein concentration (TPC), the coefficient of repeatability between visits, limits of agreement, and the Wilcoxon non-parametric matched pairs test for each protein are reported.

Table 3.3. Data for the main proteins present at both visits based on the percentage of the total concentration for each protein.

Protein	Coefficient of repeatability (% of the total protein concentration)	Limits of agreement (% of the total protein concentration)	Wilcoxon t-test (between two visits)	Spearman's correlation coefficient (between two visits)
Lysozyme	8.38	-6.62 to 10.16	p=0.43	r=0.93
Lipocalin	11.83	-14.01 to 9.65	p=0.47	r=0.93
sIgA (light chain)	10.21	-9.72 to 10.70	p=0.70	r=0.63
~47 kDa unidentified	4.96	-5.50 to 4.42	p=0.28	r=0.91
Albumin	1.77	-1.52 to 2.02	p=0.69	r=0.71
Lactoferrin	3.80	-4.30 to 3.30	p=0.46	r=0.98
~100 kDa unidentified	0.85	-1.02 to 0.68	p=0.31	r=0.69

r=non-parametric Spearman correlation coefficient. p=two-tailed p value

The values returned for the coefficient of repeatability for each protein, ranged from 0.85 to 11.83 and were high for the majority of proteins (see ***Table 3.3***). Limits of agreement estimate the interval between which two repeat measurements will be less than, in 95% of cases; the smallest intervals were found for albumin and the ~100kDa protein. Data for the Wilcoxon paired or matched t-test, and Spearman's correlation coefficients are also displayed. The Wilcoxon t-test indicated that no significant differences were found for the percentages of the total concentrations between the two visits, for any of the proteins listed. The Spearman's correlation coefficients for the percentages of the total concentration between the two visits were all between 0.5-1.0, considered to be high.

ZAG was found at both visits in only 5 of the participants with a coefficient of repeatability of 2.51, limit of agreement -1.45 to 3.57, and r=0.30 (p=0.19). The percentage of the total concentration ranged from 2.8 to 7.4%.

3.4. Discussion

This study showed that tear proteins assessed by electrophoresis with the Agilent 2100 Bioanalyzer are repeatable. It can determine a panel of key proteins such as lysozyme, lactoferrin, lipocalin and sIgA. This laboratory-on-chip approach is particularly suitable for ocular surface and dry eye research, where collection of large tear volumes is challenging. The technique could be used to provide a relatively quick screening or monitoring of disease to allow a better understanding of underlying pathophysiology in various ocular surface diseases.

3.4.1. Tear collection

Given the nature of DED, collection of sufficient tears can be difficult, particularly if the patient has an element of aqueous deficiency. The flush method, while allowing faster collection of tears, has been shown to procure significantly less concentrated tears than basal tears, but essentially with the same spectrum of proteins in similar proportions (Markoulli et al., 2011). Collection of basal tears was preferrable in this study, as less abundant proteins can be harder to locate by gel electrophoresis and may theoretically be lost with the flush technique.

It was, however, necessary to use the flush method for one participant (RS07) at both visits of this study. The tear collection protocol (**Appendix 5**) recommends a single 40 µL drop of non-preserved unit dose saline be instilled into the inferior palpebral fold using a micropipette (Guyette et al., 2013). However, whilst using this technique during a different study, it was evident that most of this amount of saline was either blinked away, or if collected caused a significant dilution of the tear constituents. The outcome of a previous study, by Markoulli et al. (2017), has also indicated that a smaller volume of saline (20 µL) may be more appropriate for a repeatable result. Therefore, the technique was altered to two separate applications of 20 µL for this study. Tears were collected within one minute of each application to avoid reflex tearing which can influence the spread of proteins in the sample; the main regulated proteins of the lacrimal gland, such as lysozyme, lactoferrin, and tear lipocalin should remain constant if reflexing is induced, while others such as immunoglobulin A and G and albumin have been shown to change dramatically (Fullard and Tucker, 1991).

Therefore, when comparing results, it was better to consider the percentage of the total concentration rather than the TPC or relative concentration of each protein, which could be affected by the volume of saline used during the flush technique. Equally, while every effort was made to avoid collection of reflex tears during basal collection, the percentage of the TPC was considered a more accurate measure.

3.4.2. Tear analysis

The Agilent 2100 Bioanalyzer was used to find the total protein concentration, the relative concentration of each protein peak, and the percentage of the total tear protein concentration. The absence of proteins could also be observed. In comparison with the Western blot or ELISA (enzyme-linked immunosorbent assay) methods, which can only measure one or a handful of analytes at a time and detect one or two proteins, the Bioanalyzer can detect a panel of proteins. Other customizable multiplex arrays which allow quantification of multiple proteins, require a well-validated antibody pair, and involve multiple steps. Therefore, the Bioanalyzer provided a relatively quick analysis of ten samples, requiring only 4 µL of each, in comparison to the typical 25–50 µL sample volume required to test for multiple targets, dependent on the recommended dilution factors.

3.4.3. Total protein concentration (TPC)

The TPC collected by microcapillary tubes in this study was 6.72 ± 3.56 mg/ml. This was calculated from the basal tears of nine participants, and flush tears from one (RS07). The TPC and individual protein concentrations of the flush tears was similar to other participants at Visit 2, but lower at Visit 1, which may have reduced the study's mean TPC for Visit 1. When the outlier for participant 1 (RS01) Visit 2 result was removed, the standard deviation reduced and the TPC was 6.04 ± 1.90 mg/ml, which agrees with other studies. The TPC of human tears varies according to collection method but has been reported to range from 3.5 to 9.5 mg/ml in basal tears and reflex tears, and is increased in closed eye conditions (6 to 18 mg/ml) (Sack et al., 2003).

The pilot nature of this study meant that only three participants were identified as having DED and no correlation between TPC and dry eye status was found. Proteins with antibacterial and protective functions (indigenous proteins) reduce in concentration in DED, whilst pro-inflammatory proteins increase (Zhou et al., 2012). Previously, a TPC of 9.89 ± 2.28 mg/ml has been reported in healthy subjects and 6.44 ± 2.1 mg/ml in dry eye subjects (Versura et al., 2013). The TPC found in this study was nearer to that of the dry eye participants, but within the normal range. Variations have also been reported based on collection method, with capillary tubes the highest, followed by Schirmer strips, flushing, and cellulose micro sponges (Krajčíková et al., 2022).

Distinct diurnal variations in the total protein concentration of tear fluid have also been shown; from 6.0 in reflex tears, to 9.0 in open eye tears, and 18.0 mg/ml in closed eye tears (Sack et al., 1992). Closed eye tears were not collected in this study, but the higher TPC at Visit 2 for participant 1 could potentially be explained by the removal of a daily disposable contact lens just prior to tear collection.

3.4.4. Peak interpretation

Between six to twelve protein peaks were found in the twenty analytes in this study. The identity of some of the less frequently presented peaks or those with small concentrations are uncertain. Reproducible inter-visit detection of seven of the most abundant protein peaks was demonstratable. However, assigning a specific protein identity to all of the peaks detected was challenging due to the experimentally derived molecular weights not correlating precisely with the known theoretical weights. This could be attributed to glycosylation of proteins leading to heavier molecular weights or the presence of low molecular weight fragments occurring due to disulphide bond reduction as the protein was denatured through heating (Mann, 2007). Therefore, the protein peaks detected were listed under a protein size range rather than a specific molecular weight.

Lysozyme, lactoferrin, ZAG and serum albumin could be identified as they had previously been validated (Versura et al., 2012). A protein peak between 144.7-149.2 kDa was present at both visits in five of the participants and at a single visit in three participants. Mann (2007) also found a protein peak between 146.2-148.5 kDa which they suggested to be secretory immunoglobulin A whole molecule, present due to dimerization.

The total size of sIgA is 385 kDa, composed of a light-chain and a heavy-chain, which are linked by a J-Chain (15 kDa), and a glycoprotein called secretory component (Kijlstra, 1998). The secretory component (~85 kDa) has been reported to migrate with lactoferrin (Kuizenga et al., 1991). Mann (2007) reported fragments of sIgA at ~26 kDa (light-chain) and ~73 kDa (heavy chain). Other studies have reported similar findings; light chain 28 kDa and heavy chain 64 kDa (Kuizenga et al., 1991;Sack et al., 1992). Therefore, the peak present in this study between 26.7 and 30.5 kDa could be identified as light chain sIgA. Previously, tear samples separated on 12.5% SDS-PAGE under reducing conditions, indicated a significant increase in the gel band corresponding to this molecular weight in closed eye tears, when compared to open or reflex tears, suggesting its correct identification (Sack et al., 1992).

Further evidence for the correct identification of this peak can be found when considering the results from a study which explored the top 20 proteins by percentage of the total (Perumal et al., 2016). Secretory IgA was found to be among the top three most prevalent proteins, depending on dry eye type and status. In this study the protein peak between 28.1-30.5 kDa was found to be the third most concentrated protein, which correlates with the findings in the study's non-dry eye subjects.

The broad unidentified peak present between 43.9-47.9 kDa, which was found in all participants at both visits, has previously been reported as a common finding (Mann, 2007). It was suggested that this could potentially be a lipocalin dimer. The process of dimerization occurs when two molecules of

similar chemical composition join together leading to a protein of approximately twice the weight. However, due to its broad nature, this peak has been suggested to indicate the presence of several potentially undetermined proteins (Mann, 2007). A later study using SDS-gel mono-dimensional electrophoresis in parallel to the Bioanalyzer, confirmed that IgA-heavy chain could be observed in tears around 52-69 kDa (Giannaccare et al., 2016). The protein ~47 kDa could possibly be assigned the identity of IgA-heavy chain. However, its concentration does not correlate with that of IgA-light chain ($r=0.144$, $p=0.639$) which would be expected.

A protein peak ~100 kDa appeared consistently in 7 of the 10 repeated samples; it did not appear in the protein profiles of participants RS04, RS07 and RS10. Previous studies have reported the protein peak for lactoferrin in the regions of 95.4 - 98.1 (Mann, 2007) and 93.7 - 99.3 kDa (Versura et al., 2013), with a shoulder peak also reported between 105.2 - 110 kDa (Versura et al., 2013). As lactoferrin is known to constitute up to 25% of the protein in human tears (Kijlstra et al., 1983) and a high concentration peak was found between 83.2-88.4 kDa (theoretical mass 82 kDa), it would appear logical to identify this peak as lactoferrin. Therefore, it could be speculated that the peak ~100 kDa could be a shoulder peak of lactoferrin.

Participants RS04 and RS10 did not have peaks between 83.2-88.4 kDa but did show repeatable smaller peaks between 105.2 - 110 kDa. As the identity of these peaks was not certain they were not included in the data for ***Figure 3.9***, which accounts for the apparent lower total concentration of tears for these participants. When visit 1 and 2 electropherograms were overlaid the greatest repeatability of peaks was found for participants RS05, RS06 and RS09.

3.4.5. Individual concentrations and percentages of the total protein concentration

The mean concentrations of the four most readily identified proteins in this study, lysozyme, lipocalin, lactoferrin and light chain sIgA were 2.58 ± 1.47, 0.46 ± 0.29, 1.51 ± 1.13, and 1.07 ± 0.81mg/ml respectively. These principal indigenous proteins make up around 80% of the total tear protein (Mann, 2007). Several studies have quantified the concentrations of these major tear proteins. Using selection monitoring mass spectrometry, concentrations of 2.11 ± 1.50, 1.75 ± 0.99, and 1.20 ± 0.77 mg/ml for lysozyme, lipocalin, and lactoferrin, respectively, have been reported (Masoudi et al., 2014). This is consistent with previous ELISA studies (Fullard and Tucker, 1991). The concentrations found in this study are lower for lipocalin, but within a similar range for lysozyme and lactoferrin.

The concentration levels of these three proteins can be assumed to be relatively constant from day to day as they are regulated proteins. However, the concentration of constitutive or secretory proteins (those that have a constant level of production) such as immunoglobulins, and serum-derived proteins

such as albumin, are more likely to vary with any subtle change in tear flow rate (Ng et al., 2000). For example, serum albumin has been shown to increase from 0.02 to 0.06 to 1.10 mg/ml in reflex, open eye and closed eye tears, respectively, while the major tear proteins remained essentially static (Sack et al., 1992).

When considering the percentage of the total concentration for the major proteins identified at each visit, participants RS02, RS03, RS05, and RS06 showed good repeatability (***Figure 3.8***). The values returned for the coefficient of repeatability, which represents the difference between which two repeat measurements should fall with 95% probability, were high for most proteins (***Table 3.3***). This may be due in part to the large measurement range and the relatively small number of repeated measurements in this study. As the concentration of albumin is more likely to be variable than the main indigenous proteins, it is contrary to expectation that this should have the smallest coefficient of repeatability.

The Wilcoxon t-test (***Table 3.3***) showed no significant differences ($p>0.05$) for the percentages of the total concentrations between the two visits, for any of the proteins listed, indicating good repeatability. The Spearman's correlation coefficients for each protein as a percentage of the TPC between the two visits, also showed good repeatability for all the main proteins present. The coefficients were all higher than 0.5, with between 0.5-1.0 considered to be high, and 1 indicating a perfect positive correlation. The main indigenous proteins lysozyme, lipocalin and lactoferrin showed excellent repeatability with coefficients of 0.93, 0.93 and 0.98 respectively. Repeatability of the percentages was not affected by DED status either. However, due to the pilot nature of the study only three participants were diagnosed as having DED.

When considering lysozyme concentration as a percentage of the TPC, a retrospective sample size calculation of 77 participants was found. This calculation was performed with G-Power (version 3.1.9.7) using the difference between two dependent means (matched pairs) t-test, with a two-tailed α-level of 5%, an 80% power level and a calculated effect size of 0.62. Previous work with the bioanalyzer to explore tear protein profiles has shown lysozyme to be the most important protein for predicting adverse events and corneal infiltrates during extended contact lens wear (Lakkis et al., 2019). However, when specifically considering DED, lactoferrin has shown the strongest evidence as a potential clinical marker (D'Souza and Tong, 2014). A similar retrospective sample size calculation, using the data obtained from this pilot study for lactoferrin, gives a total sample size of 46. The data for lipocalin provides a similar total sample size of 52.

3.4.6. Limitations of the Agilent 2100 Bioanalyzer for tear protein analysis

A limitation of tear analysis with the Bioanalyzer is its inability to detect smaller or less abundant proteins, such as the many cytokines and chemokines which are altered during inflammation in DED (ng/ml to pg/ml concentrations) (Zhou and Beuerman, 2012). A small study to analyse peptides and proteins in the 1-20 kDa range, by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) coupled with C18 magnetic beads, found no significant variability in healthy individuals over a seven-day period (González et al., 2012). However, interstudy variability in results for tear analysis from studies utilising mass spectrometry have occurred due to variations in methodology. Despite technical advances, the large dynamic range of multiple tear proteins still represents a challenge in the search for individual biomarkers (Ponzini et al., 2021). The current trend is to focus on protein panels rather than on single proteins.

A further limitation of the Bioanalyzer is its inability to identify the protein from the peak without prior knowledge of the theoretical molecular weight of the suspected protein. Proteins separated by traditional gel electrophoresis can only be identified by removal and analysis with mass spectrometry for identification of the peptide sequences. Databases such as UniProt (Boutet et al., 2007) are then required to identify the proteins from their peptide sequences. Therefore, definitive identification of the proteins identified at ~47, 100 and 147 kDa would require either a specific separate analysis such as ELISA or multiplex, or mass spectrometry, thus requiring a larger tear sample.

3.5. Conclusion

The results obtained from this pilot study support the hypothesis that the inter-visit tear protein profiles measured by electrophoresis with the Agilent 2100 Bioanalyzer are repeatable, thus indicating it as a reliable method for investigating tear proteins. The study highlighted the need for tight control of conditions when analysing the samples, with particular care to analyse the samples to be compared on the same day, using the same batch of reagents.

The advantages of electrophoresis with the Agilent 2100 Bioanalyzer are the relative quantification of several proteins in a single analysis requiring only a small quantity of tears (4µL), and the short time to obtain the results. Decreased levels of lactoferrin, lysozyme and lipocalin as well as increased levels of albumin, in conjunction with clinical signs may allow for differential diagnosis of DED. As the method reported here was found to be repeatable, it was employed to investigate the tear protein profiles of a young adult cohort (chapter 5), in order to identify any potential biomarkers of evaporative DED.

The following chapters (4-7) describe the methodology and findings of a prospective, longitudinal study to explore early evaporative DED in young adults.

Chapter 4 - Prospective, longitudinal study assessing dry eye among young adults - clinical outcomes

Preliminary data from this chapter was presented at:

- Casemore, R.K. Understanding dry eye in young adults: a translational approach. Aston University's 4th Interdisciplinary Postgraduate Research Conference, 3rd October 2022, Birmingham, UK.

- A Casemore, R. K., Wolffsohn, J. S., Dutta, D. Characterisation of Dry Eye in Young Adults. Poster presentation at the British Congress of Optometry and Vision Science Conference (BCOV), 13th September 2022, Birmingham, UK.

An abstract of this chapter was submitted (Dec, 2023) to the Association for Research in Vision and Ophthalmology (ARVO) Conference, 2024 and accepted as a poster presentation.

- Casemore, R. K., Wolffsohn, J. S., Dutta, D. A prospective, longitudinal study to characterise progression of dry eye disease in young adults. Association for Research in Vision and Ophthalmology (ARVO) Conference, 5th to 9th May 2024, Seattle, USA.

4.1. Introduction

This chapter introduces a prospective, longitudinal study to explore DED in young adults, and reports on clinical and lifestyle findings, while subsequent chapters investigate the tears and meibum for potential biomarkers of evaporative DED in the same cohort. The prevalence and severity of Dry Eye Disease (DED) is known to increase with age (Stapleton et al., 2017); previous longitudinal and cross-sectional DED studies have largely focused on older age groups, with very few investigating the characteristics and associated risk factors specifically for evaporative DED in young adults.

Although DED is a common condition, affecting around a third of the UK population (Vidal-Rohr et al., 2023), substantial symptomatic DED exists among younger people. Several recent studies have reported a high prevalence in younger populations (Vehof et al., 2021), with a reported worldwide prevalence of around 20% (Papas, 2021). Increased use of video display terminals, particularly during the COVID-19 pandemic, has been associated with symptomatic DED in 70.8% of university students; other associated risk factors were higher perceived stress scale scores, prolonged contact lenses wear, and female sex (Tangmonkongvoragul et al., 2022).

Data obtained from a large population-based cohort study in the Netherlands, found a particularly high prevalence of dry eye symptoms in 20-to-30-year-olds, with the highest prevalence for men of any age being in this age range (Vehof et al., 2021). Up to 5% of women and 3% of men aged 20–30 years had either ‘often’ or ‘constant’ symptoms of dry eye (data extracted from presented bar chart). An association with the use of hand-held screens amongst the younger adults in the cohort was proposed. A similar trend was found in a large paediatric population of 7–12-year-olds, where 6.6% were diagnosed with DED; smartphone use was strongly associated with diagnosis whereas outdoor activity was protective to its development (Moon et al., 2016).

The prevalence rates of DED appear to vary between studies, depending on the diagnostic clinical test, type of questionnaire, or cut-off values employed. In 2016, a cross-sectional study of Ghanaian undergraduates reported symptomatic dry eye in 44.3% of the students (Asiedu et al., 2017), while in 2018, a lower prevalence of clinically diagnosed DED (10%) was reported among 901 university students from Shanghai (Li et al., 2018).

A large cross-sectional study of 2140 Brazilian university students reported a higher prevalence of dry eye symptoms in the students (23.5%) in comparison to the general population of over 40-year-olds (Yang et al., 2021). As in the Netherlands population-based study, dry eye was defined by the Women’s Health Study (WHS) questionnaire; either a clinical diagnosis of dry eye and/or symptoms of both dryness and irritation of the eyes ‘often’ or ‘constantly (Vehof et al., 2021). In addition to known risk

factors, sleeping less than six hours and use of a visual display terminal for more than six hours a day were found to be associated with DED. While comparing between ethnicities, a higher prevalence of DED has been reported in Asians than Caucasians (Stapleton et al., 2017).

Evaporative DED forms the largest subgroup of DED, accounting for around 85% of cases (Lemp et al., 2012); clinic and population-based studies have indicated Meibomian Gland Dysfunction (MGD) as the leading cause (Craig et al., 2017c). Use of a digital device, which is rampant among young adults, has been linked to meibomian gland loss and reduced or incomplete blink rates (Wolffsohn et al., 2021b), known risk factors for evaporative DED (Portello et al., 2013).

The apparent paucity of research studies exploring the characteristics and associated risk factors specifically for evaporative DED in young adults, diagnosed according to the TFOS DEWS II diagnostic methodology (Wolffsohn et al., 2017), led to the development of the prospective, longitudinal study reported here.

4.1.1. Study objectives

This study aimed to provide an insight into the development and progression of evaporative DED in its early stages, by exploring the clinical parameters of the eye over one year in a young cohort. Analysis of tears and meibomian gland lipids for biomarkers of evaporative DED collected over the period of the study are reported in Chapters 5, 6 and 7.

This longitudinal study also endeavoured to expand on existing knowledge of the association of dry eye with potentially modifiable lifestyle risk factors, such as digital screen use, to better inform clinical management including early preventative advice for patients. Early identification of those who are likely to develop dry eye signs and symptoms due to evaporative dry eye disease could not only impact their quality of life, but also be of economic significance. Therefore, participants were followed up after one year to determine whether any progression had occurred in that time interval. The clinical results are reported here in Chapter 4. ***Table 4.1***. shows the study objectives.

Null hypotheses:

- There are no correlations between specific dry eye clinical measures and specific lifestyle factors for young adults.
- There would be no progression of the disease over one year of the study period.

4.2. Methods

This was a prospective, longitudinal, observational, single centre study where participants were followed up over one year, to characterise the signs and symptoms of evaporative DED. The study took place at Aston University between September 2021 and March 2023.

Table 4.1. Study objectives

	Objectives	**Outcome Measures**
Primary	1. to characterise the anterior eye, ocular surface, tear and meibum parameters in evaporative dry eye in young adults and compare to controls 2. to explore associations with digital screen use and other lifestyle factors 3. to determine any progression of the disease after one year	-Medical history, lifestyle, and dry eye questionnaire (OSDI) -Blink rate and incomplete blinks -Tear meniscus height (TMH) -Non-invasive keratometric break- up-time (NIKBUT) -Conjunctival hyperaemia -Tear lipid layer assessment -Tear (microcapillary and Schirmer 1) and meibum for biomarker analysis (reported in chapters 5 and 6) -Tear film MMP-9 (InflammaDry®) with band imaging for semiquantitative assessment. -Corneal, conjunctival and lid wiper staining with sodium fluorescein and lissamine green -Meibomian glands secretion and meibum quality. -Infrared Meibography
Secondary	to determine any progression of the disease stratified by severity.	-Repeated measurements as above in both control and patient groups. Report on any changes in ocular surface and tear film parameters, meibography scores, symptom severity and any associated lifestyle factors

4.2.1. Ethics approval

The study received ethics approval from Aston University Research Ethics Committee (ref# AUREC 1756, see **Appendix 6**) and followed the tenets of the declaration of Helsinki. All participants received a participant information sheet and subsequently signed a consent form to participate in the study (see **Appendix 8** and **9**, respectively).

4.2.2. Participant enrolment and selection

Participants (young adults, aged 18-25) were recruited from within the general student population and the School of Optometry at Aston University, via newsletters and email correspondence from various year and subject leads, see **Appendix 7** for the advertisement. Recruitment took place between September 2021 and May 2022. Potential participants were asked to read the Participant Information Sheet and Consent Form (**Appendix 8** and **9**), which were sent via email, prior to the appointment. As one of the objectives of the study was to explore associations between dry eye and digital screen use, a minimum of one hour reported screen use was a requirement for participation.

Contact lens wearers were allowed to participate in the study and were permitted to wear their lenses between visits but not on an appointment day. Each appointment was expected to take approximately 45 minutes. Participants received a £10 gift card for each visit in acknowledgement of their time. Written consent was obtained at the appointment and participants were informed that they could withdraw from the study at any time. Participants were not required to have DED in order to participate but were subsequently placed into one of the following groups:

- Patient group: 18-25-year olds diagnosed with evaporative DED as defined by the TFOS DEWS II Diagnostic Methodology report (Wolffsohn et al., 2017)
- Control group: Healthy, age-matched 18-25-year olds

Inclusion Criteria:

- Aged 18-25 and spend at least 1 hour a day on a digital device
- Vision at least 6/12 (LogMAR 0.3) or better in each eye
- Able to attend follow up visit in 1 year ± 2 months
- Do not anticipate any major ocular changes over 1 year
- Able to provide written consent in English

Exclusion Criteria:

- Aqueous deficient dry eye (ADDE) (defined by a TMH less than 0.20mm or symptoms suggestive of ADDE such as dry mouth or swollen glands)
- Use of systemic or topical medications known to affect the eye in the previous 3 months
- Previous ocular surgery
- Allergic conjunctivitis with the use of antihistamine drugs
- History of major systemic or ophthalmic conditions
- Any active infection or inflammation
- Currently enrolled on another clinical trial

Participants with known ADDE, which is more likely to be associated with inflammation, or a history of an inflammatory condition were excluded from the study in order to remove any potential confounding factors when analysing the tear and meibum for biomarkers of DED (see chapters 5, 6 and 7). Lipid layer thickness, MGD and TMH were considered when subclassifying participants as evaporative DED (Wolffsohn et al., 2017).

4.2.3. Clinical methods and grading scales

Once eligibility for the study was confirmed, participants were asked to complete a lifestyle questionnaire (see **Appendix 10**) which incorporated questions about ethnicity, sex, smoking, contact lens wear, screen use and exercise (hours). The questionnaire was designed based on known risk factors for DED (Inomata et al., 2019;Wang et al., 2021b;Wolffsohn et al., 2021b). Participants were also asked to rate their diet quality, health quality and stress levels on a Likert scale (1-4) as follows: diet quality 1=poor, 2=fair, 3=good,4=excellent; health quality 1=poor, 2=fair, 3=good, 4=excellent, and stress levels 1=high, 2=medium, 3=mild, 4=low.

The OSDI validated 12-item dry eye questionnaire (described in 3.2.5) was used to provide a quick assessment of any symptoms of ocular discomfort consistent with dry eye, symptom severity, and consequence on vision-related functioning over the preceding week. Dry eye symptoms were categorised as follows: normal (scores 0–12), mild (13–22), moderate (23–32), and severe (33–100) (Schiffman et al., 2000). A score of ≥13 combined with one or more signs of a loss of homeostasis of the tear film were used to diagnose DED in the current study (Wolffsohn et al., 2017).

A thorough history and symptoms, including questions regarding general health, medications (both topical and systemic), supplements, dry eye treatments and allergies was performed prior to any clinical assessments. ***Table 4.2***. details the sequence of the clinical and analytical procedures performed at each visit, including their grading scales. Measurements were performed in a least to most invasive order, to cause minimal disruption to the tear film (Wolffsohn et al., 2017).

All clinical measures were performed on the right eye only, using the Keratograph 5M (Oculus, Wetzlar, Germany) and recorded on a Clinical Record Form (CRF) designed for the study (see **Appendix 11**). A slit lamp was used to assist assessment of meibomian gland expressibility and meibum collection. Corneal (> 5 spots) and conjunctival (> 9 spots) staining were graded with the Oxford Scale (Bron et al., 2003) and lid wiper staining with the Korb Scale (Korb et al., 2010). Staining was evaluated with fluorescein and blue light with a yellow observation filter (Bio Fluoro 1mg fluorescein sodium ophthalmic strips, Biotech Visioncare, India) and lissamine green (Green Glo

Ophthalmic Strips 1.5mg lissamine green, Contacare Ophthalmics and Diagnostics, India). The Guillon scale (see ***Figure 4.1***) was used to subjectively grade lipid layer thickness (Guillon, 1998).

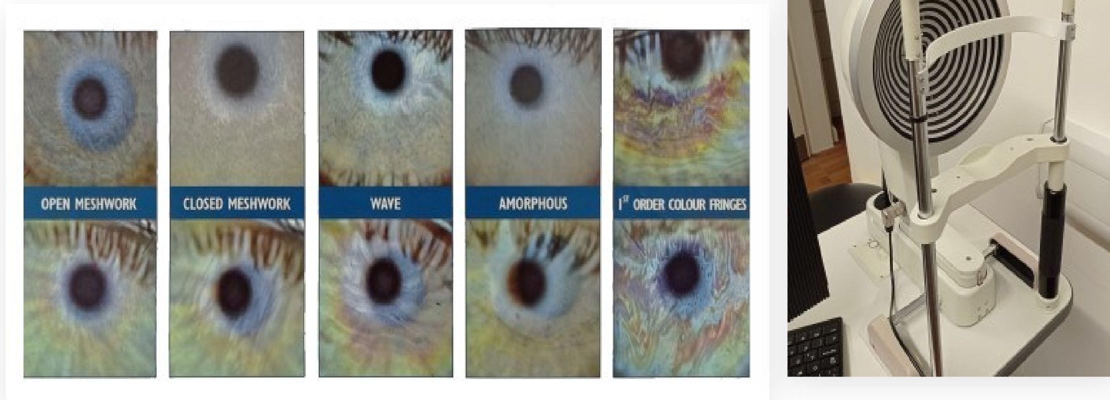


Figure 4.1. The Guillon lipid layer grading scale (left) measured with the Oculus Keratograph 5M (right)

Table 4.2. The sequence of clinical and analytical procedures performed at each visit

Procedures	Grading scale/unit	Visit 1	Visit 2
Check with inclusion and exclusion criteria		✓	✓
Written consent		✓	
OSDI questionnaire	(0-100)	✓	✓
General medical and ocular history		✓	✓
Lifestyle and digital device use questions	Screen use in hours	✓	✓
Visual acuity assessment	LogMAR	✓	✓
Blink rate and incomplete blinks (K5 instrument)	number in 30 seconds	✓	✓
Tear meniscus height (K5 instrument)	mm	✓	✓
Non-invasive keratographic breakup time (NIKBUT; K5 instrument)	sec (average of 3 measurements)	✓	✓
Conjunctival hyperaemia (K5 instrument)	Jenvis scale: 0= no findings, 1= single injections, 2= mild diffuse injections, 3= severe local injections and 4= severe diffuse injections. Measured in nasal and temporal bulbar, and nasal and temporal limbal	✓	✓
Lipid layer grading (K5 instrument)	Guillon-Keeler scale 1= Open meshwork pattern: very thin lipid layer with no detail visible, 2= Closed meshwork: even well-mixed lipid layer with minimal detail visible, 3= Wave pattern: grey streaks visible with flow pattern during the blink process, 4= Amorphous pattern: flow pattern with some colour fringes, 5= Colour fringe pattern: interference colours (1st order) visible in part of the field, 6= Colour fringe pattern: interference colours (2nd order) covering the majority of the field	✓	✓
Tear collection with glass capillary tubes	µl	✓	✓
Tear collection using Schirmer's paper	mm	✓	✓
MMP-9 (InflammaDry)	Negative, trace positive, weak positive, positive, strong positive	✓	✓
Expressibility of meibomian glands and quality of meibum (using digital MGE)	Glands secreting: 0=>75% (almost all), 1=50-75%, 2= 25-50%, 3= <25%, 4= 0%. Meibum quality: 0=clear, 1=cloudy, 2= cloudy with debris, 3= thick, toothpaste- like, 4= waxy, inexpressible	✓	✓
Meibum collection using a sterile spatula		✓	✓
Sodium fluorescein and lissamine green staining of cornea and conjunctiva	Oxford grading scale: 0=absent, 1=minimal (at least 5 corneal spots or 9 conjunctival spots), 2=mild, 3=moderate, 4=marked, 5=severe		
Lissamine green staining - lid wiper epitheliopathy (LWE)	Korb scale Sagittal width: 0=< 25%, 1= 26-50%, 2= 51-75%, 3= >75%. Horizontal length: 0=<2mm, 1= 2-4mm, 2= 5-9mm, 3=>10mm	✓	✓
Infrared Meibography (K5 instrument))	Pult meiboscale: 0=0%, 1= < 25%, 2= 26-50%, 3= 51-75%, 4= >75% area of loss	✓	✓

4.2.3.1. Meibography

Infrared meibography images of the upper and lower lids were taken with the Keratograph 5M (Oculus, Wetzlar, Germany) and subjectively graded according to the 5-point meiboscale (Pult and Riede-Pult, 2013), see ***Table 4.2***. The upper lid gland images were also graded semi-objectively by a masked observer using an Advanced Ophthalmic Systems (AOS) software application provided by SPARCA (Croyden, UK). The glands were detected within the area of interest selected for each uploaded image (***Figure 4.2***) using the same threshold (0.4) for all images, and with artifacts removed when necessary. The results were presented as a percentage loss and scored on a scale based on the Pult meiboscale (Pult and Riede-Pult, 2013). However, the scale differed for gland loss less than 25%, where 0= < 10% and 1= 10-25%, in comparison to 0= no loss and 1= <25% for the subjective grading scale. To allow for comparison of the two methods, participants with meibography grades 0 and 1 from the automated analysis were classed as grade 1; no participants had zero gland loss when graded this way.

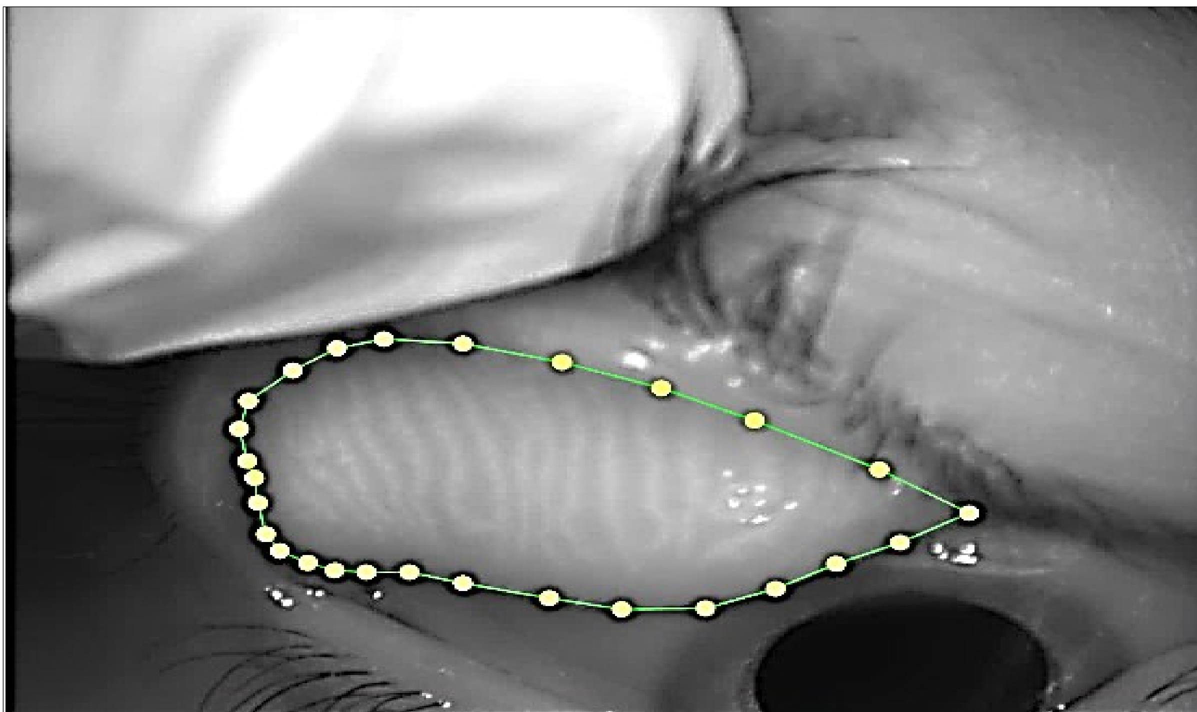


Figure 4.2. Meibography - Area of interest selected using AOS software

4.2.3.2. InflammaDry® test

The InflammaDry® test (Quidel Corporation, San Diego, USA) was used to check for the presence of the inflammatory marker MMP-9 (see section 1.4.3.2). The test was performed according to the manufacturer's guidelines. In short, the eyelid was gently lowered to expose the palpebral conjunctiva and the sampling fleece was dabbed 8 to 10 times in multiple locations until saturated. The test

cassette was assembled, and the absorbent tip was immersed into buffer solution for 20 seconds. It was then laid flat on a horizontal surface for 10 minutes. The test was read and rated positive when a second even faint line appeared in the result zone. Semi-quantitative assessment was made for any positive results (above 40 ng/ml threshold) and the bands imaged, according to a previously designed scale (Khamar et al., 2019;Park et al., 2018;Jun et al., 2020;Kim et al., 2021), see ***Figure 4.3.***

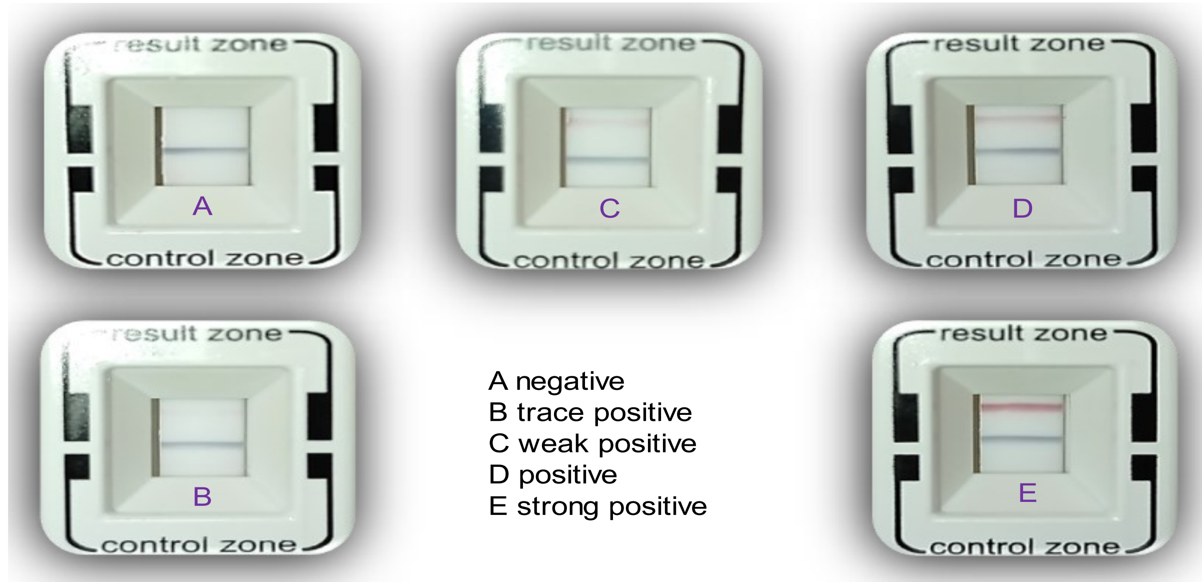


Figure 4.3. Semi-quantitative grading of the InflammaDry® test

4.2.3.3. Tear and meibum collection

The protocol and collection methods for tear and meibum are reported in chapters 5 and 6 respectively. These were performed prior to the instillation of fluorescein or lissamine to prevent contamination.

4.2.4. Sample size calculation

One of the main aims of this research study was to explore any differences in tear proteins between the DED and non-DED participants. Therefore, data for lactoferrin obtained from the pilot study reported in Chapter 3 could be used to calculate a retrospective total sample size of 46 (23 in each group); lactoferrin has previously been shown to have the strongest potential as a clinical biomarker in DED (D'Souza and Tong, 2014). The sample size calculation was performed with G-Power (Version 3.1.9.7) (Faul et al., 2007), using a dependent samples t-test with a two-tailed α-level of 5%, an 80% power level and a calculated effect size of 0.42.

As this calculation assumes matched pairs and is calculated from ten sets of data to assess repeatability of the bioanalyzer, further sample size calculations were made using data for lactoferrin

from a previous study which reported protein concentrations in early DED, using the Agilent 2100 Bioanalyzer (Versura et al., 2013). Using an independent samples t-test with a two-tailed α-level of 5%, an 80% power level and a calculated effect size of 0.95, a total sample size of 42 (21 in each group), would be required to demonstrate statistically different lactoferrin concentrations between the two groups, see ***Figure 4.4***. The sample size was increased by 10% to compensate for any deviation from a normal distribution. The same calculation applied to data for lysozyme and lipocalin produced total sample size calculations of 40 (effect size 0.97, actual power 0.80) and 20 (effect size 1.44, actual power 0.82), respectively.

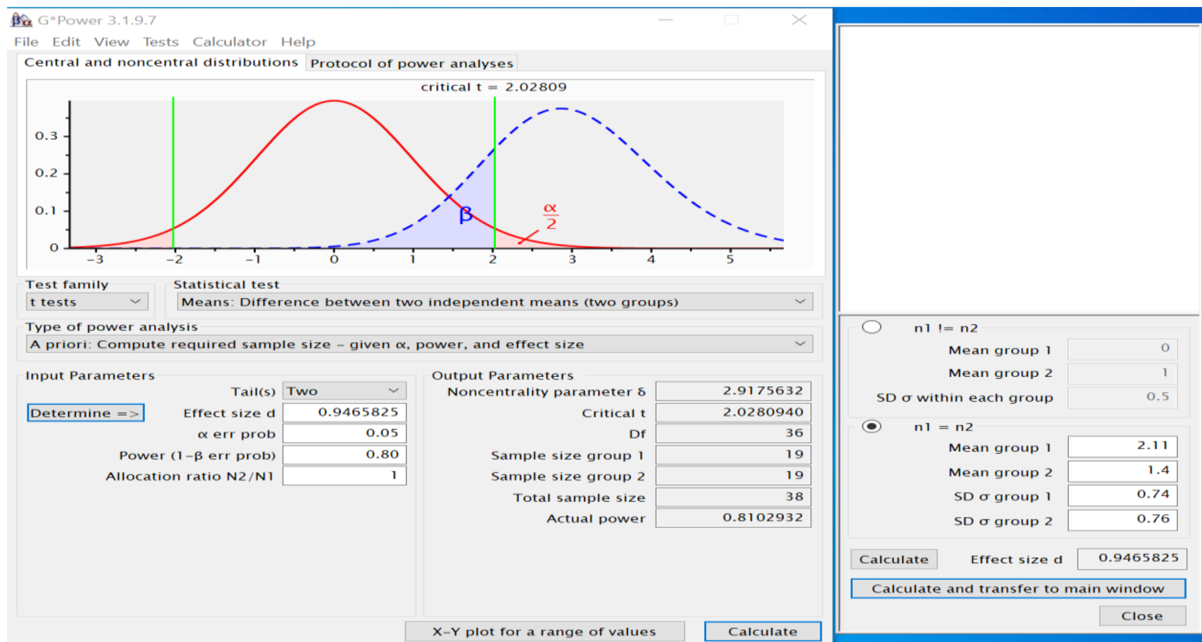


Figure 4.4. A priori sample size calculation for lactoferrin using G-Power and an independent means t-test

As tear samples were also to be analysed for inflammatory cytokines, a further sample size calculation was based on data from a study comparing cytokines in MGD related dry eye and control participants (Wu et al., 2020). Using an independent samples t-test with a two-tailed α-level of 5%, an 80% power level and a calculated effect size of 0.98, a total sample size of 40 (compensated by 10%) would be required to demonstrate statistically different IL-8 concentrations between the two groups.

Furthermore, this study sought to identify any correlations between cytokine concentrations and clinical measures in the controls and dry eye subjects. IL-6 has been shown to have significant correlation with the symptoms and severity of DED (Lam et al., 2009). Using the data for correlations between IL-6 concentration and corneal staining (r=0.41, p<0.01) a total corrected sample size of 48 was calculated, see ***Figure 4.5***. This final figure, which was adopted for the study, also supports the recommended minimum sample sizes per group reported in the TFOS DEWS II Diagnostic

methodology report (Wolffsohn et al., 2017) for clinical measures including NIKBUT and TMH performed with the Oculus Keratograph 5M.

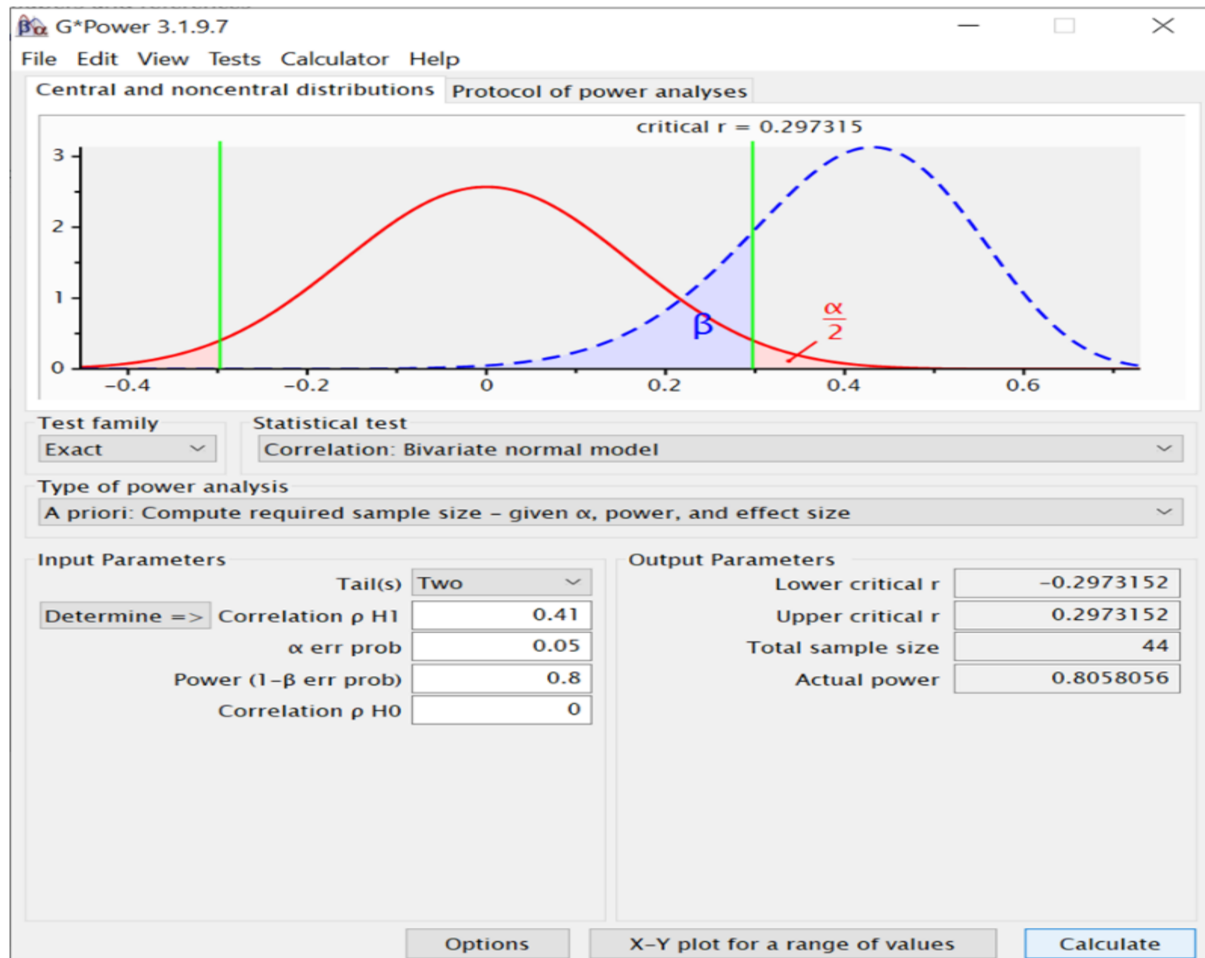


Figure 4.5. A priori sample size calculation for IL-6 using G-Power and a correlation test

4.2.5. Data analysis

Data analysis was performed using Microsoft® Office Excel®, GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA and IBM SPSS Statistics for Windows, Version 26. Descriptive statistics such as mean and standard deviation were used to present participant demographics. Odds ratios and their 95% confidence intervals were used to report the strength of association between risk factors and DED.

The Kolmogorov-Smirnov test was used to check for normality. Data was not found to be sampled from Gaussian distributions for the majority of measurements. Therefore, the non-parametric Mann-Whitney U test was used for comparisons of the DED and non-DED participants. Where the K-S normality test was passed, but standard deviations were different, the Welch test was used.

Decision Tree Analysis (DTA) was performed using IBM SPSS Statistics for Windows, Version 26; CHAID (chi-squared automatic interaction detection) tree growing algorithm was used to determine the influence of several independent variables on either a scale or ordinal dependent variable. Continuous data were transformed into ordinal predictors by SPSS before analysis. Tree growth was limited to a minimum of 6 parent nodes, 3 child nodes (5% of the total cohort size) and a maximum tree depth dependent on the number of independent variables analysed. This allowed identification of the independent variable with the strongest association with the selected dependent variable, created by the first branch of the tree.

To quantify any correlations between the clinical parameters, Spearman ranked correlation was used. The strength of any correlation between the data (Spearman's correlation coefficient r) was estimated according to the following coefficient magnitudes: −1 (perfect negative correlation) through 0 (no correlation) to +1 (perfect positive correlation) (Mukaka, 2012). A high correlation was 0.5-1.0, moderate 0.5-0.3, and 0.3 to 0.1 a small (weak) or if any correlation. To measure the association between nominal and numerical data, such as lifestyle questions and dry eye status, Univariate and Multivariate Analysis of Variance was used to calculate partial eta-squared where $\eta^2 = 0.01$ indicates a small effect, $\eta^2 = 0.06$ a medium effect and $\eta^2 = 0.14$ indicates a large effect (Watson, 2021). Fisher's exact test was used to calculate odds ratios.

For all tests an alpha value of 0.05 was adopted for statistical significance. Bonferroni correction was not applied to the data and therefore the possibility of false significance is acknowledged (Armstrong, 2014).

4.3. Results

4.3.1. Participant demographics

The study included 50 participants, 72% of which were females (n=36). The average age was 19.9 ± 1.6 years. The OSDI questionnaire scores ranged from 0 to 66.7, with a mean of 23.5 ± 17.6. The participant demographics for Visit 1 are presented in ***Table 4.3.***

Of the 50 participants at Visit 1, 22 (44%) were classed as the healthy controls or non-DED, according to the TFOS DEWS II diagnostic criteria and 28 were diagnosed with DED. Of the remaining 42 participants at Visit 2, 25 were categorised as having DED. However, at Visit 2, four participants had moved from the non-dry eye to the dry eye group, while two had changed from dry eye to non-dry eye categories based on the TFOS DEWS II diagnostic criteria (see ***Table 4.4***).

Table 4.3. Participant demographics for Visit 1

Characteristic	Participants n (%)		
	Total cohort (n=50)		
Age (years; range)	19.9 ± 1.6 (18.3-25.1)		
Dry eye status		non-DED	DED
		22 (44%)	**28** (56%)
Sex (female)	36 (72%)	12 (55%)	24 (86%)
Sex (male)	14 (28%)	10 (45%)	4 (14%)
Smokers	3 (4 in the past)	1 (4.5%)	2 (7%)
Systemic conditions	8*	5 (23%)	3 (11%)
Contact lens wearers	16 (32%)	2 (9.5%)	14 (50%)
Screen hours	8.0 ± 2.7 (range 4 to 18)	7.9 ± 3.2 (range 4 to 18)	8.1 ± 2.3 (range 4 to 14)
Exercise (hours/week)	4.6 ± 3.5 (range 0 to 16)	3.8 ± 2.5 (range 0 to 9)	5.2 ± 4.1 (range 0 to 16)
Outdoor activity (hours/week)	3.8 ± 5.0 (range 0 to 28)	2.4 ± 2.3 (range 0 to 9)	4.9 ± 6.2 (range 0 to 28)
Allergies	13** (26%)	4 (18 %)	9 (32%)
Medications	4 (8%)	2 (9%)	2 (7%)
Nutritional supplements/vitamins	15 (30%)	6 (27%)	9 (32%)
Dry eye treatments	18 (36%)	2 (9%)	16 (57%)

**Gilbert's syndrome (n=1), mild asthma(n=1), vitiligo (n=1), mild eczema (n=2), congenital heart murmur (n=1), bursitis in hips (n=1) ** Hayfever (n=11), peanuts (n=1), jackfruit (n=1).*

Table 4.4. Diagnostic details for participants who changed dry eye status

Participant ID	OSDI score		Average NIKBUT (seconds)		Ocular surface staining	
Non-dry eye to dry eye	Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2
YAS 15	11.4	20.8*	6.12	10.13	LWE	LWE & grade 2 conjunctival & corneal stain
YAS 37	41.7	25.0	16.2	20.3	None	LWE*
YAS 51	12.5	31.8*	6.6	5.2	LWE & grade 3 conjunctival stain	LWE & grade 3 conjunctival stain
YAS 21	10.42	18.75*	3.55	4.71	grade 1 conjunctival staining	no staining
Dry eye to non-dry eye						
YAS 28	20.2	0*	9.2	3.5	Grade 1 corneal & conjunctival stain	LWE & grade 1 conjunctival stain
YAS 34	38.6	41.7	9.1	12.8*	None	LWE*

**Measurements responsible for the change in dry eye status*

The dropout rate was 16% after 1 year ± 2 months (n=8). The primary reasons were: one drop out due to inflamed chalazia at the time of the appointment, one withdrawal due to limited available time, two participants having left the university and four were unreachable. Five of these eight drop out

participants had been diagnosed with DED according to the TFOS DEWS II diagnostic criteria at the first visit. Therefore, the study included 42 participants at Visit 2, 74% of which were females (n=31). The average age was 20.7 ± 1.5 years and the OSDI questionnaire scores ranged from 0 to 84.1, with a mean of 23.7 ± 20.3.

The female participants were five times more likely to have DED than the males at Visit 1 (OR 5.0; CI 1.30 to 19.3, p=0.025); the odds were higher at Visit 2 (OR 6.82; CI 1.47 to 31.6, p=0.014).

Systemic conditions reported to be present at the time of the study included: Gilbert's syndrome, Coeliac disease, congenital heart murmur, asthma, and bursitis in the hips. Two participants reported asthma; one required the use of the preventative inhaler, Seretide, while the other only reported use of the reliever inhaler, Salbutamol at Visit 1 only. The participant with bursitis reported previous localised steroid injection, more than three weeks prior to the study appointment. Two other participants reported the use of a systemic contraceptive pill at both visits.

Thirteen of the Visit 1 participants reported allergy; this included hayfever (n=11), peanuts (n=1) and jackfruit (n=1). Thirty-one percent of those that reported an allergy were non-dry eye participants, while 69% (n=9) were diagnosed with DED (odds ratio 1.80; 95%CI 0.46-7.01; p=0.512). None of the participants with hayfever were taking antihistamines at the time of the study visits, which occurred between the months of September and March.

At Visit 2, the number of allergy sufferers had reduced to 11, including eight hayfever suffers, one of whom reported hayfever and dust allergy. Thirty-six percent of those that reported allergy were non-dry eye participants (n=4) and 64% had a DED diagnosis (odds ratio 1.26; 95% CI 0.31 to 5.23; p=1.000). Again, due to the time of year, none of the participants were taking antihistamines for hayfever at the second visit. Two new previously unreported allergies were noted, peanuts (n=1) and gluten (n=1), but these were dealt with by avoidance. The participant demographics for Visit 2 are presented in ***Table 4.5***.

No associations between allergy and any clinical parameter were found at Visit 1. However, at Visit 2 associations were found with TMH and horizontal LWE (η^2 =0.144, p=0.013 and η^2= 0.095, p=0.046, respectively).

At Visit 1, 12 participants reported taking a vitamin or supplement. These included multivitamins (n=8), vitamin D (n=4), iron (n=3), cod liver oil (n=1), omega 3 fish oil (n=2) and Vitamin B12 + folic acid. An increased number of participants (n=15) reported taking a vitamin or supplement, either regularly or occasionally at Visit 2. Although four participants reported to have stopped taking their supplements, ten other participants had started taking one or more by Visit 2. These included an

increase in participants taking vitamin D (n=7), omega 3 fish oil (n=3) and two participants reporting taking herbal supplements such as turmeric and ginger.

Table 4.5. Participant demographics for Visit 2

Characteristic	Participants n (%)		
	Total cohort (n=42)		
Age (years; range)	20.7 ± 1.5 (19.2-26.1)		
Dry eye status		**non-DED** **17** (40%)	**DED** **25** (60%)
Sex (female)	31 (74%)	9 (53%)	23 (92%)
Sex (male)	11 (26%)	8 (47%)	3(12%)
Smokers	3 (4 in the past)	1 (4.5%)	2 (7%)
Systemic conditions	8*	5 (29%)	3 (12%)
Contact lens wear	17 (40%)	4 (24%)	13 (52%)
Average screen hours	7.3 ± 2.5 (range 3 to 16)	7.1 ± 2.1 (range 4 to 12)	7.3 ± 2.7 (range 3 to 16)
Exercise (hours/week)	4.8 ± 4.1 (range 0 to 20)	3.7 ± 2.9 (range 0 to 8)	5.2 ± 4.6 (range 0 to 20)
Outdoor activity (hours/week)	3.6 ± 3.3 (range 0 to 14)	2.8 ± 2.1 (range 0 to 7)	4.2 ± 3.9 (range 0 to 14)
Allergies	11** (26%)	4 (23%)	7 (28%)
Medications	4 (9.5%)	1 (5.9%)	3(12%)
Nutritional supplements/vitamins	19 (45%)	9 (53%)	10 (40%)
Dry eye treatments	14 (33%)	3 (18%)	11 (44%)

**Gilbert's syndrome (n=1), mild asthma(n=1), vitiligo (n=1), mild eczema (n=2), congenital heart murmur (n=1), bursitis in hips (n=1), coeliac (n=1) ** Hayfever (n=8), hayfever and dust (n=1), peanuts (n=1), gluten (n=1).*

Dry eye treatments included drops containing hypromellose, hyaluronic acid, carmellose sodium, and glycerol. One participant reported using calendula & hamamelis. Drops were used on an 'as required' basis in over half of the participants. Participants were asked not to use any drops on the day of the appointment. At Visit 1, eighteen participants (36%) reported using an ocular lubricant; half of them were also contact lenses wearers. At Visit 2, a third of the participants (n=14) reported using some form of lubricant, with 79% (n=11) of them being contact lens wearers. Two participants who didn't report use of a lubricant at Visit 1 had started doing so at Visit 2; one a contact lens wearer on an 'as required' basis and the other a DED participant. One other participant changed brand of hyaluronic acid based lubricant, while another changed to this from a glycerol based solution.

Most of the participants were of southern and eastern Asian ethnicity (66% (n=33) at Visit 1 and 69% (n=29) at Visit 2), including Indian, Pakistani, Bangladeshi, Sri Lankan, and Afghan participants. There were also two Chinese (eastern Asian) and one Vietnamese (south-eastern Asian) participants; therefore, 72% (Visit 1) and 74% (Visit 2) of the cohort were of Asian ethnicity. There were eight white

(English, Irish, Romanian, and Irish) at both visits and four and three Black (Black British and Black Caribbean) participants at visits 1 and 2, respectively . See ***Figure 4.6***.

The odds of having DED were more than twice as high in the Asian participants: OR 2.10; 95% CI 0.74 to 7.33; p=0.344 at Visit 1 and OR 2.18; 95% CI 0.54 to 8.81; p=0.305 at Visit 2.

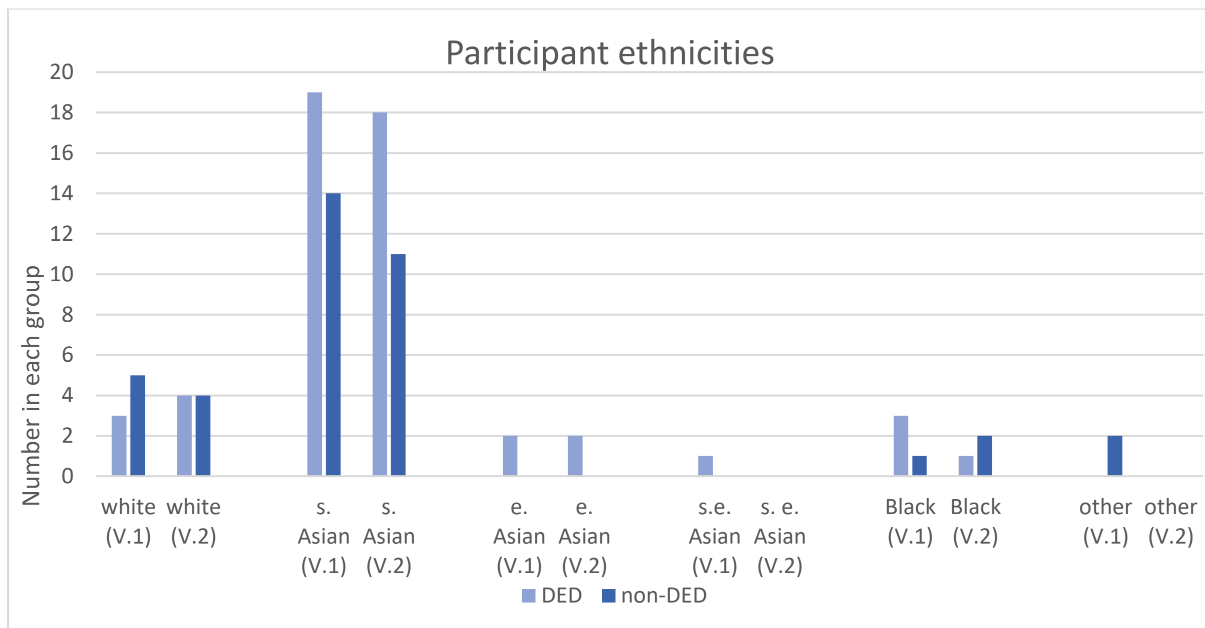


Figure 4.6. Participant ethnicities at each visit separated by dry eye status
V.1 = Visit 1, V.2 = Visit 2. s.Asian = southern Asian, e. Asian = eastern Asian, s.e. Asian = south-eastern Asian

4.3.2. Participant lifestyle factors

Only three participants were current smokers, with four reporting having smoked in the past. Sixteen (32%) of the 50 participants at Visit 1 wore contact lenses (see ***Table 4.3***). Fifty percent (n=14) of the DED participants wore contact lens, compared to only 9.5% (n=2) of the non-DED participants. Of the 16 contact lens wearers, 87.5% (n=14) had DED, (odds ratio 10; 95% CI 1.96-51.11; p=0.002). At Visit 2, 40.5% (n=17) of the total 42 participants wore contact lenses (see ***Table 4.4***). Around half of the DED participants (n=13, 52%) wore contact lens, whereas 24% (n=4) of the non-DED participants did. Of the 17 CL wearers, 76.5% (n=13) were diagnosed with DED according to the TFOS DEWS II diagnostic criteria (odds ratio 3.52; 95% CI 0.90-13.83; p=0.109). The mean OSDI score was 28.5 ± 14.6 and 30.6 ± 21.4 in the CL wearers at visits 1 and 2 respectively and 21.1 ± 18.6 and 19.7 ± 18.7 in the non-CL wearers.

The average screen use of the DED and non-DED participants at Visit 1 was 8.1 ± 2.3 and 7.9 ± 3.2 hours respectively; the difference was not statistically significant different (p=0.401). Similarly, for

Visit 2 there was no significant difference (p=0.636) when comparing the average screen use hours between DED (8.1 ± 2.3) and non-DED participants (7.3 ± 2.7), see ***Figure 4.7***. No correlation between screen hours and OSDI score was found at either visit.

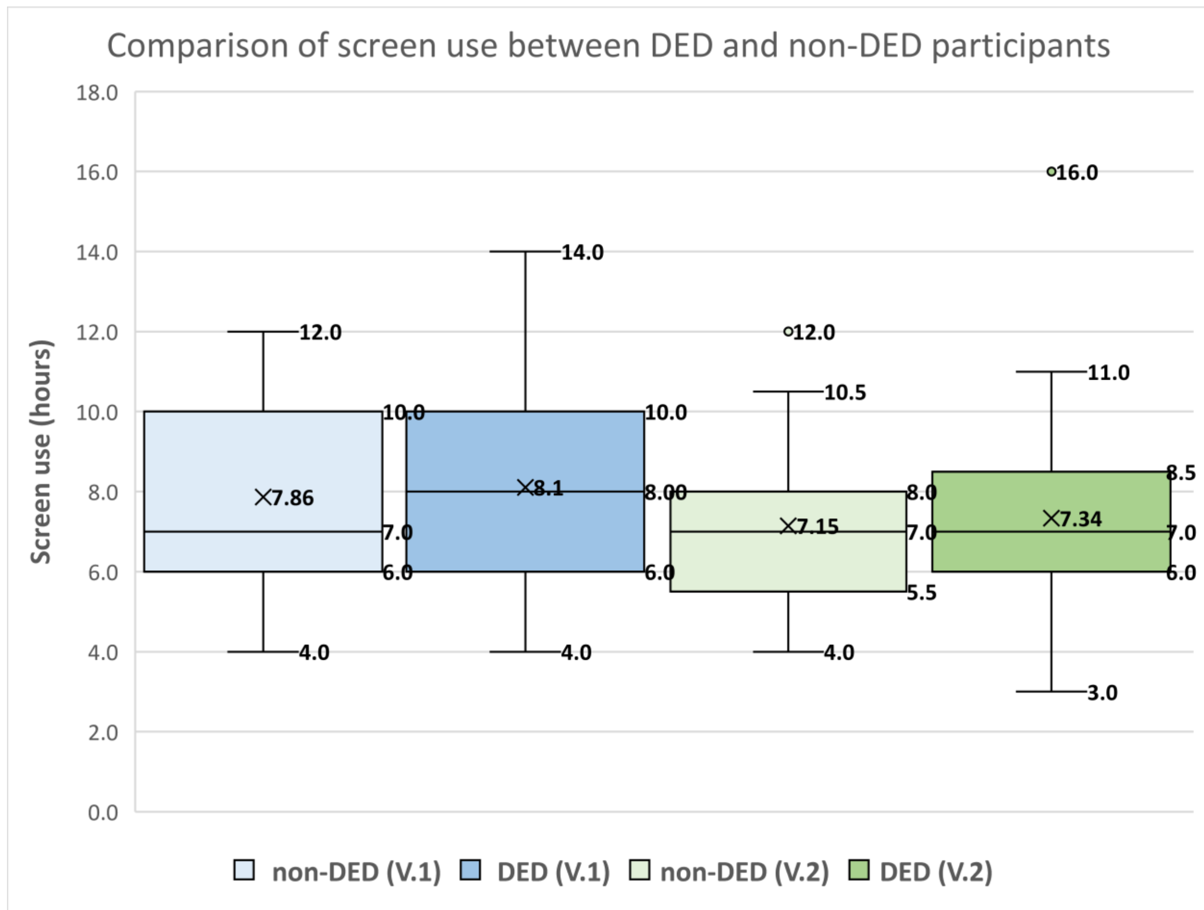


Figure 4.7. Box whisker plots showing the screen use hours for each group
The minimum, lower quartile, median, mean (represented by 'x'), upper quartile and maximum hours are illustrated. Outliers are represented by a dot (identified as 1.5 multiplied by the IQR value of the data).

When exploring whether any lifestyle factors were predictive of signs of DED, screen use was the only variable to be associated with an ocular surface sign in the Visit 1 data. ***Figure 4.8***. illustrates the relationship found between screen hours and sagittal LWE, with a moderate but significant correlation coefficient of r=0.430 (p=0.002).

Significant but weak correlations for screen time with horizontal LWE (r=0.289, p=0.042), lower meibography score (r=-0.308, p=0.030) and upper meibography score (r=-0.319, p=0.024) were also found. A moderate correlation with upper meibography score was the only correlation with screen time hours at Visit 2.

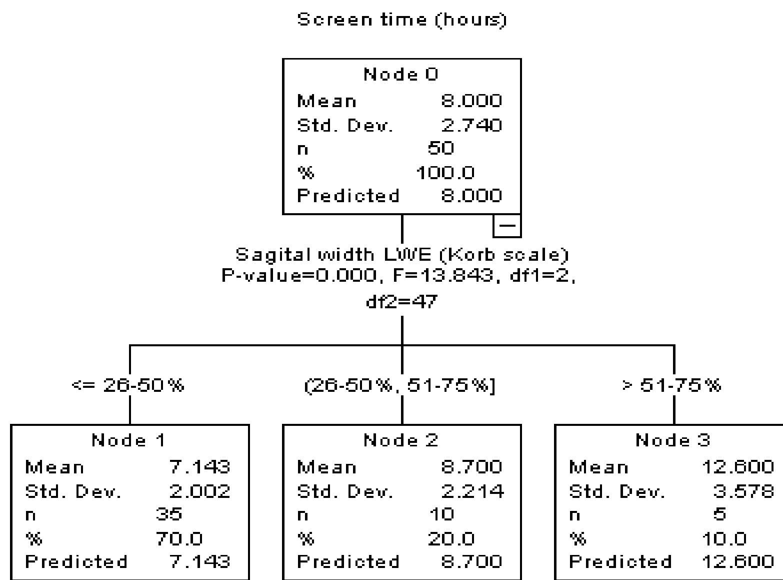


Figure 4.8. DTA showing relationship between screen use and sagittal LWE (Visit 1)

The presence of incomplete blinks was significantly associated (p=0.009) with longer screen use (> 8 hours) at Visit 1. At Visit 2, the presence of incomplete blinks was associated with a lower mean screen time (6.52 ± 1.78 hours). However, the participants (Visit 2) with incomplete blinks and a blink rate of > 16 per minute spent significantly (p=0.01) longer using a screen (7.21 ± 1.51 hours) than those who blinked less than 16 times per minute (4.86 ± 1.22 hours), see ***Figure 4.9***.

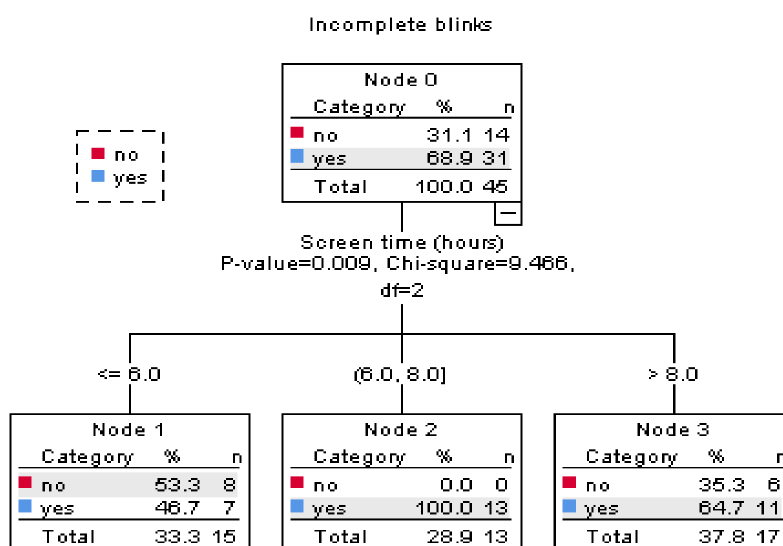


Figure 4.9. DTA showing the relationship between incomplete blinking and screen use (Visit 1)

No other significant correlations were found for LWE (sagittal or horizontal) at Visit 2. Higher blink rates were associated with CL wear at Visit 2 (p=0.042) with a medium partial eta-squared of η^2 =0.067 (p=0.098), but the association was not significant at Visit 1.

The reported hours of exercise and outdoor activity were similar when comparing Visit 1 and 2 (see ***Tables 4.4*** and ***4.5***). Although the DED participants reported spending more time exercising and outdoors compared to the non-DED participants, the differences did not reach statistical significance at either visit.

The results of the lifestyle questions around self-assessed diet quality, health quality and stress levels are shown in ***Figure 4.10-4.12***.

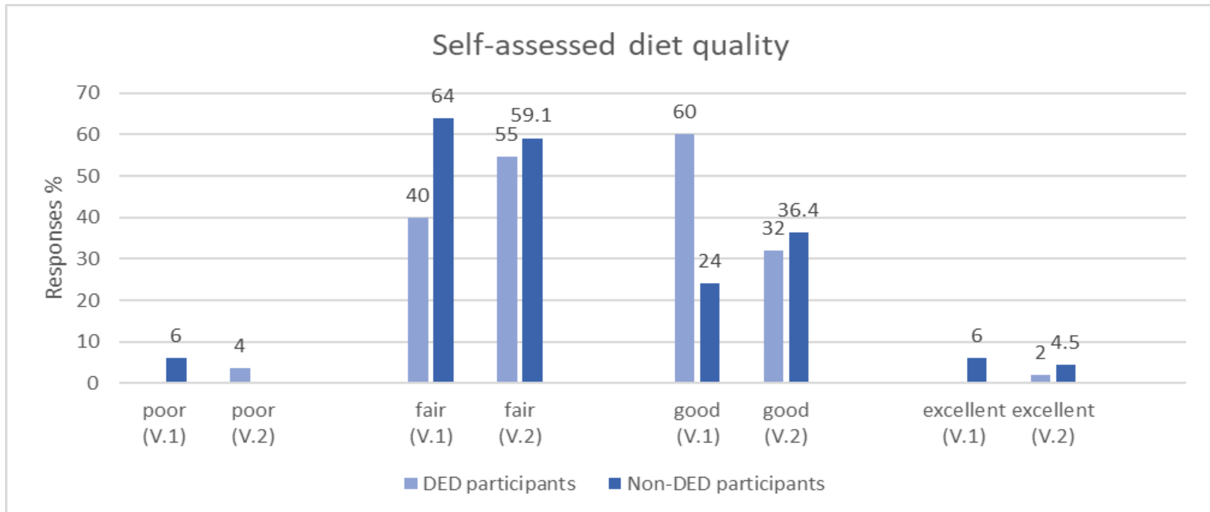


Figure 4.10. Self-assessed diet quality

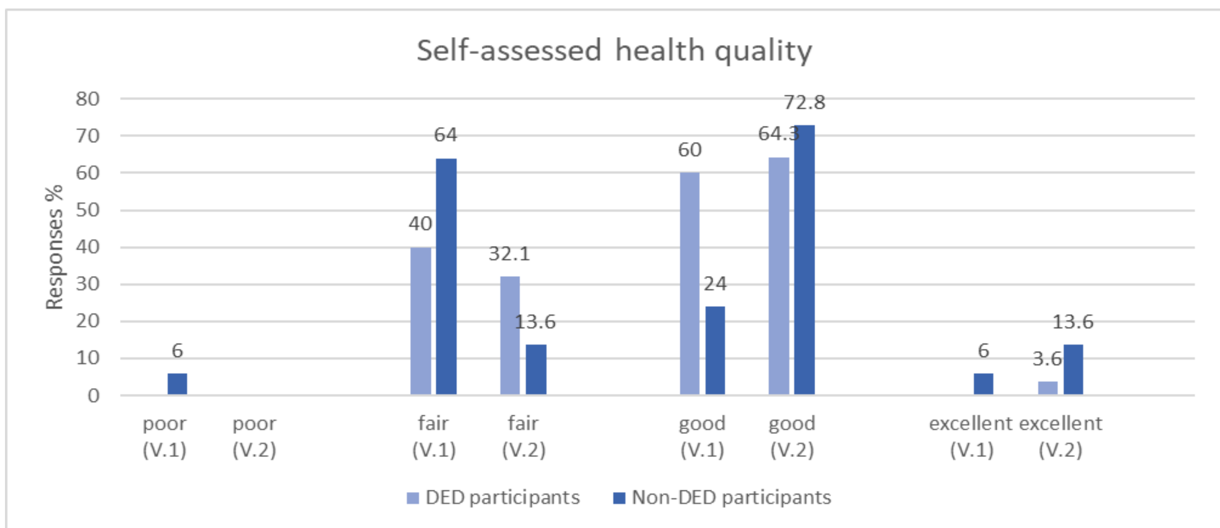


Figure 4.11. Self-assessed health quality

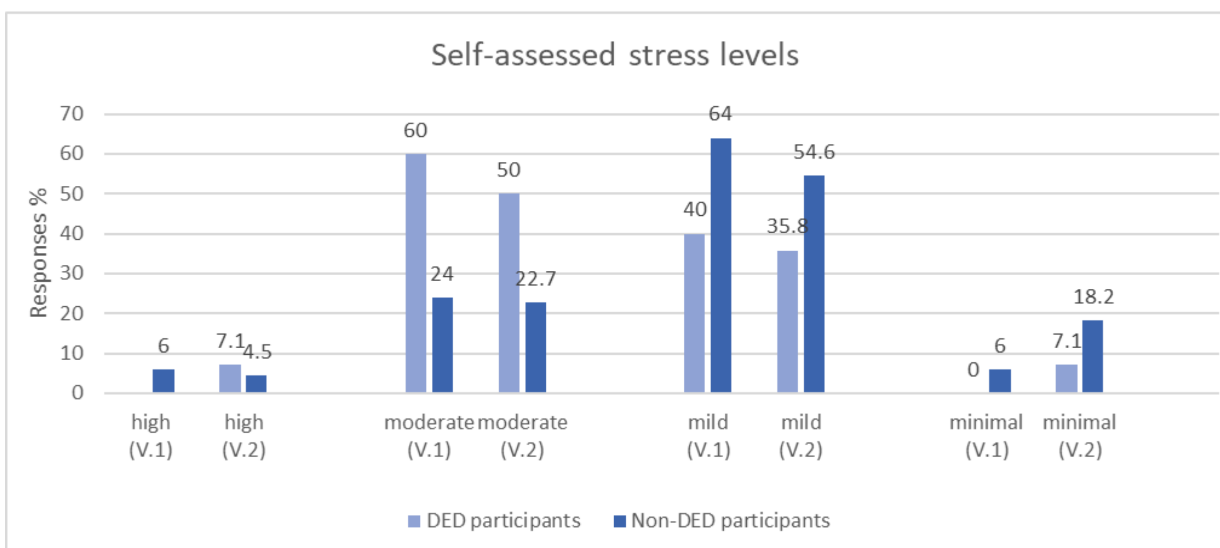


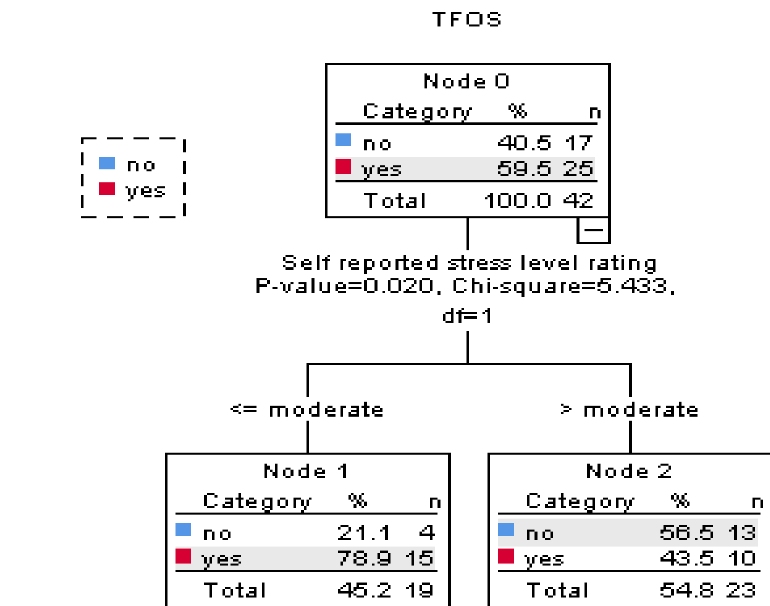
Figure 4.12. Self-assessed stress levels

The mean scores for self-reported diet, stress and health are reported in ***Table 4.6***. Significantly higher stress levels were found in the DED participants at Visit 1, where '1'=high stress and '4'= minimal (medium effect size η^2=0.078, p=0.049).

Table 4.6. Lifestyle questionnaire scores

	Dry eye status	**Score (mean ± SD)**	
		Visit 1	**Visit 2**
Self-reported diet rating	DED	2.46 ± 0.69	2.48 ± 0.65
	non-DED	2.45 ± 0.60	2.41 ± 0.62
Self-reported stress levels	DED	2.43 ± 0.74	2.36 ± 0.81
	non-DED	**2.86 ± 0.77**	**3.00 ± 0.71**
Self-reported health status	DED	2.71 ± 0.54	2.80 ± 0.41
	non-DED	3.00 ± 0.53	2.81 ± 0.51

Similarly at Visit 2, stress levels were significantly higher in the DED participants (large effect size η^2=0.149, p=0.012). The odds of having moderate to high stress and DED was OR 3.36; CI 1.07 to 11.81; p=0.047 at Visit 1; the OR of 1.58 was not statistically significant at Visit 2. There was no association with diet rating at either visit, and a medium, not quite significant association with lower self-perceived health status was found in the DED participants at Visit 1 only. ***Figure 4.13.*** illustrates the significant association between DED and moderate and above self-assessed stress levels (grade 2 or 1).



stress (Visit 2)

4.3.3. Participant clinical results

Table 4.7. presents the results for all the clinical measurements performed with the Oculus Keratograph 5M and compares the difference in means between each group from Visit 1 (in black) and Visit 2 (in blue).

Participants were categorised as either DED or non-DED, according to the TFOS DEWS II diagnostic criteria.

Table 4.7. Comparison of the DED and non-DED clinical results at each visit

	Non-DED participants (n)		**DED participants (n)**		**Mann-Whitney U test**	
Clinical assessment	**Visit 1** (mean & SD)	**Visit 2** (mean & SD)	**Visit 1** (mean & SD)	**Visit 2** (mean & SD)	**Visit 1** (p-value)	**Visit 2** (p-value)
Blink rate (no. in 30 seconds)	8.00 ± 5.32	10.4 ± 7.36	10.2 ± 5.93	11.4 ± 5.40	p=0.184	p=0.633
NIKBUT (sec)	8.17 ± 4.04	6.88 ± 3.33	8.63 ± 5.66	7.00 ± 4.31	p=0.748	P=0.921
TMH (mm)	0.32 ± 0.09	0.29 ± 0.05	0.29 ± 0.10	0.28 ± 0.08	p=0.233	p=0.540
LLT (Guillon-Keeler scale)	2.73 ± 0.70	2.65 ± 1.00	2.96 ± 1.14	2.64 ± 1.04	p=0.396	p=0.983
Bulbar redness# (Jenvis scale)	1.25 ± 0.72	1.78 ± 0.75	1.30 ± 0.71	1.36 ± 0.53	p=0.777	**p=0.038***
Limbal redness# (Jenvis scale)	0.77 ± 0.48	1.21 ± 0.59	0.89 ± 0.52	0.96 ± 0.50	p=0.430	p=0.149
Corneal stain (Oxford Scale)	0.23 ± 0.53	0.06 ± 0.24	0.29 ± 0.46	0.36 ± 0.70	P=0.678	p=0.097
Conjunctival stain (Oxford scale)	0.64 ± 0.90	0.82 ± 0.81	1.04 ± 0.69	1.04 ± 0.89	p=0.083*	p=0.427
LWE horizontal (Korb scale)	1.59 ± 1.37	2.06 ± 1.03	1.82 ± 1.25	2.16 ± 1.11	p=0.537	p=0.766
LWE sagittal (Korb scale)	0.91 ± 1.02	1.71 ± 1.11	1.07 ± 1.02	1.40 ± 1.00	P=0.578	P=0.357
Meibography upper (Pult scale)	1.55 ± 1.10	1.41 ± 0.94	1.50 ± 1.04	1.36 ± 1.00	p=0.882	p=0.866
Meibography lower (Pult scale)	1.14 ± 0.94	1.41 ± 0.94	1.25 ± 0.89	1.36 ± 0.91	p=0.663	P=0.859

** P-values <0.05 were considered significant and are shown in bold. #Nasal and temporal scores combined. * near significance*

TMH was lower in the DED participants (mean 0.29 ± 0.05mm) than in the non-DED participants (mean 0.32 ± 0.09mm) at Visit 1 but was not significant (p=0.233). Interestingly, the non-DED participants had a greater mean bulbar redness (1.78 ± 0.75) than the DED participants (1.36 ± 0.53), at Visit 2 (p=0.038). An increase in conjunctival staining was found between the non-DED (0.73 ± 1.03) and DED (1.04 ± 0.69) participants at Visit 1 only, but this did not reach statistical significance (p=0.083). No

other significant differences were found between the two groups for the data for either visit of the study.

Table 4.8. Comparison of the clinical results between visits, based on the TFOS DEWS II criteria for DED diagnosis

	Non-DED participants (n)		DED participants (n)		Mann-Whitney U test	
	Visit 1	**Visit 2**	**Visit 1**	**Visit 2**	**Visit 1**	**Visit 2**
Participants	22 (44%)	17 (40%)	28 (56%)	25 (60%)		
OSDI >13	2 (4%)	1 (2.4%)	28 (56%)	25 (60%)		
OSDI (0-100) ± SD (range)	8.2 ± 9.2 (range 0 to 41.7)	6.5 ± 9.6 (range 0 to 41.7)	35.5 ± 12.5 (range 14.6 to 66.7)	35.3 ± 17.2 (range 16.8 to 84.1)	**p<0.001***	**p<0.001***
NIKBUT <10 sec	16 (73%)	12 (71%)	18 (64%)	20 (80%)	p=0.157	p=0.651#
Corneal staining (>5 spots)	4 (18%)	1 (6%)	8 (29%)	6 (24%)	too few values	too few values
Conjunctival staining (>9 spots)	10 (45%)	10 (59%)	24 (86%)	17 (68%)	p=0.156	p=0.666
Lower LWE (≥2mm length & ≥25% width)	14 (64%)	16 (94%)	20 (71%)	22 (88%)	p= 0.888	p=0.359
>25% upper meibomian gland loss	10 (45%)	8 (47%)	11 (44%)	10 (40%)	p= 0.966	p=0.485
>25% lower meibomian gland loss	4 (18%)	5 (29%)	6 (21%)	3 (12%)	too few values	too few values

** P-values <0.05 were considered significant and are shown in bold. # parametric test performed as passed the K-S normality test. Numbers in brackets present the percentage of the total participants.*

Table 4.8 reports the clinical assessment results of the parameters used to diagnose DED, for example corneal staining of > 5 spots, according to the TFOS DEWS II diagnostic criteria. Other than OSDI (as would be expected) no significant differences were found in these diagnostic clinical measurements between the DED and non-DED participants for Visit 1 (data in black) or Visit 2 (data in blue). Correlations between OSDI score and corneal and conjunctival staining (r=0.319, p=0.024 and r=0.293, p=0.039, respectively) were found at Visit 1 only.

Incomplete blinking was observed in 70% of the participants at Visit 1 and 57% at Visit 2, see ***Figure 4.14***. When comparing the Visit 1 data for presence of incomplete blinks with absence of incomplete blinks there was no significant association with dry eye status (odds ratio 1.17; 95% CI: 0.3459 to 3.935,

p=1.00). Similarly, for Visit 2 data, there was no significant association (odds ratio 0.89; 95% CI: 0.26 to 3.16, p=1.00). When comparing the positive incomplete blink rates between Visit 1 and 2 there was no statistically significant difference (p=0.790).

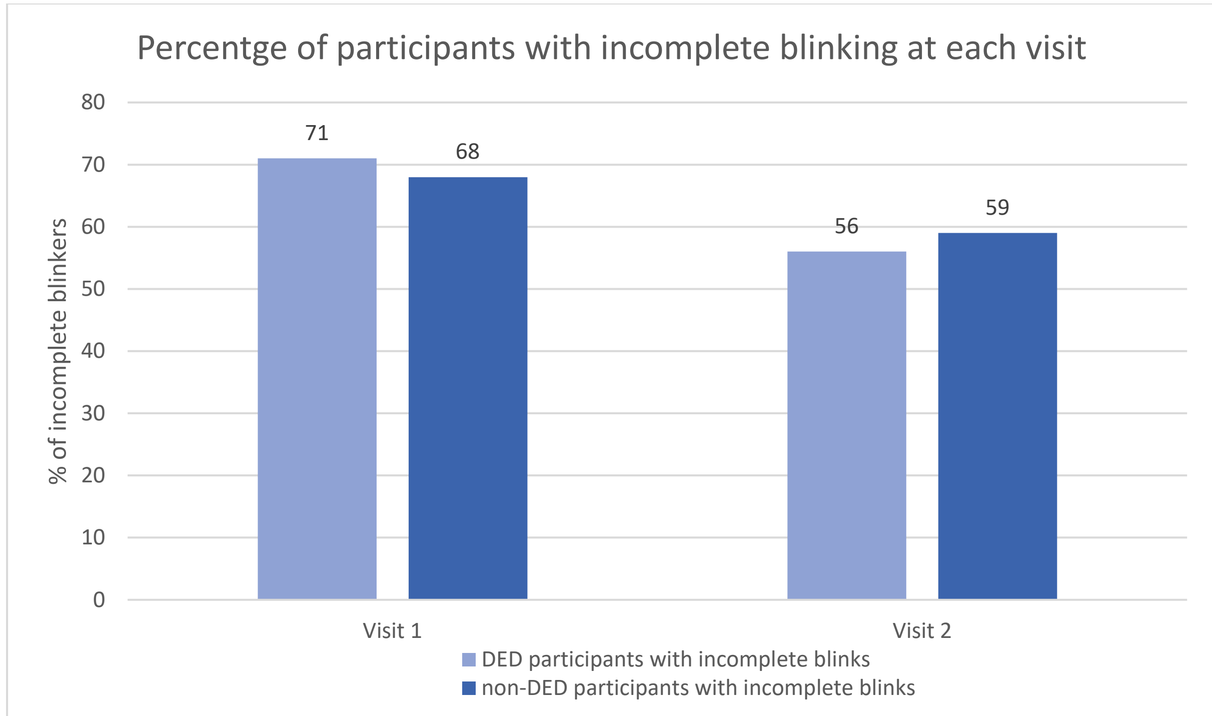


Figure 4.14. A comparison of incomplete blinking between DED and non-DED participants at both visits

4.3.4. Meibography results

Forty-eight percent of the participants from both visits (n=24 at Visit 1, and n=20 at Visit 2) were found to have at least 25% meibomian gland loss in either lid despite their dry eye status.

The subjective and semi-automated upper gland loss grades can be seen in ***Figure 4.15***. which compares the percentages for each grade between visits. Eight participants (16%) were given a subjective grade of 0 (no gland loss) for upper gland loss (Visit 1). However, no participants had no gland loss at all when graded with the software and only one participant was found to have less than 10% gland loss.

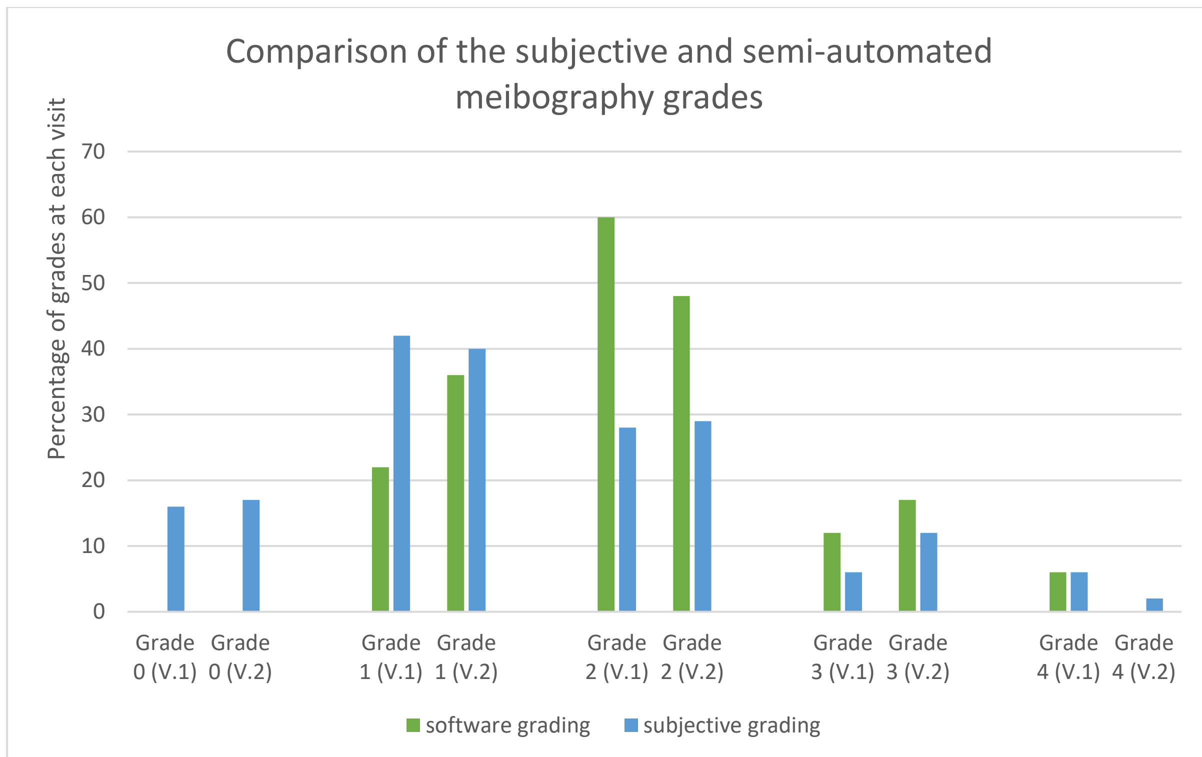


Figure 4.15. Comparison of the subjective and semi-automated meibography grades at both visits

The participants with a semi-automated grade of '0' or '1' were classed as grade '1' for the purpose of analysis. At Visit 2, six participants were graded subjectively with no gland loss; two of these had less than 10% gland loss when graded by the software.

Agreement was found between the subjective and semi-automated upper meibography grading in 48% (n=24) of the participants (Visit 1). This increased to 76% if the eight gradings where the subjective grade was '0' were excluded. Where the grades differed, all were due to the semi-automated grade being one grade higher than the subjective grade. At Visit 2, excluding the six results where a subjective grade of '0' was found, 58% (n=21) of the grades agreed; four grades were lower and eleven higher when calculated by the software.

The mean percentage gland loss for all participants calculated by the software for Visit 1 was 37.2 ± 17.9 %. When comparing the percentage gland loss between the DED (38.5 ± 15.6%) and non-DED (35.5 ± 20.7%) participants no significant difference (p=0.572) was found.

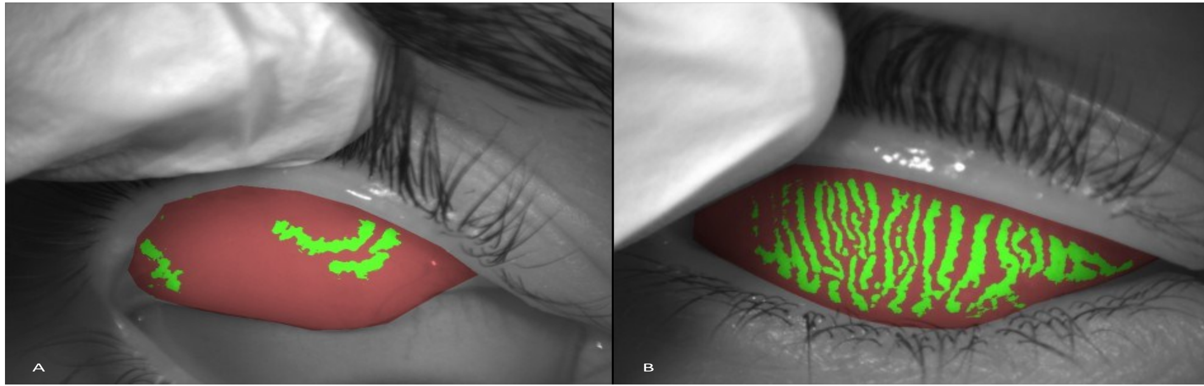


Figure 4.16. Gland detection with the AOS software illustrating Grade 4 gland loss (A) and Grade 0 (B)

At Visit 2, the mean total percentage of upper gland loss was 31.9 ± 16.4%. Similarly, no significant difference (p=0.708) was found between the two groups: DED 33.7 ± 18.8% and non-DED 31.5 ± 16.3%. This agrees with the subjective upper meibomian gland loss grades reported in ***Table 4.6***, where no significant differences were found between the two groups at either visit. No progression was found between the visits with either method of grading either (see ***Table 4.8*** for mean subjective grades between visits).

Also, no significant differences were found between the DED and non-DED participants at Visit 1 (p=1.00) or Visit 2 (p=1.00) for meibomian gland expression. ***Figure 4.17*** shows a comparison of the grades given for the number of glands expressing between the two participant groups.

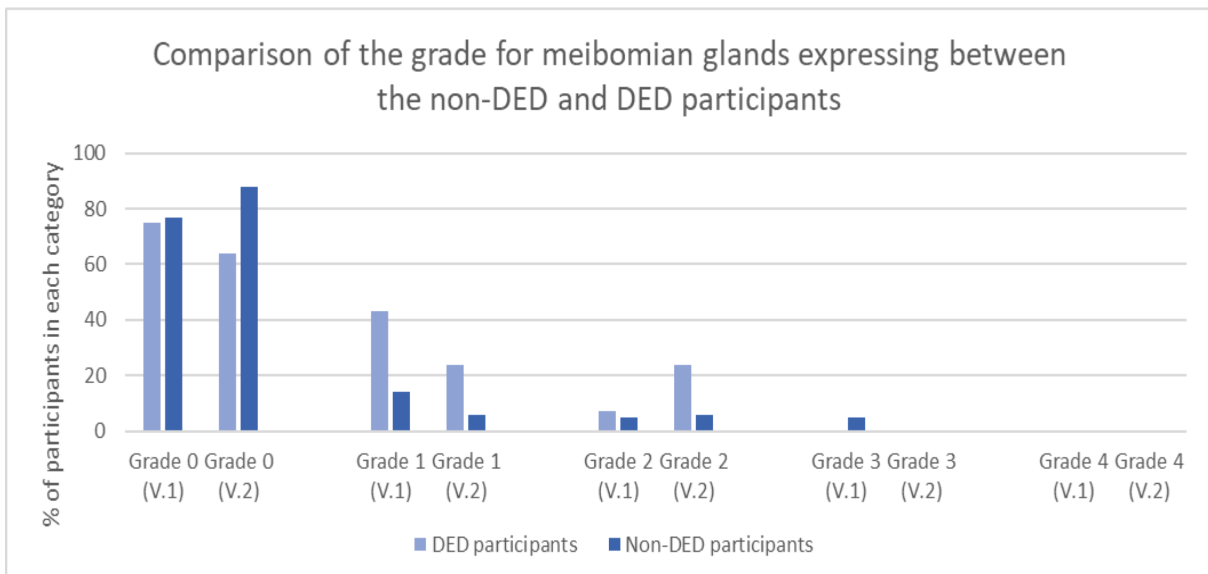


Figure 4.17. Comparison between visits of the grades for meibomian glands expressing, based on dry eye status.
Meibum expressibility grades: 0=>75%, grade 1=50-75%, grade 2= 25-50%, grade 3= <25%, grade 4= 0%

The quality of the meibum expressed digitally also showed no statistically significant differences dependent on dry eye status in Visit 1 (p= 0.386) or Visit 2 (p=0.618). Eighty-six percent of the Visit 1

and Visit 2 participants had clear meibum, while no participants were found to have grade 2 (cloudy with debris) or above, see ***Figure 4.18***.

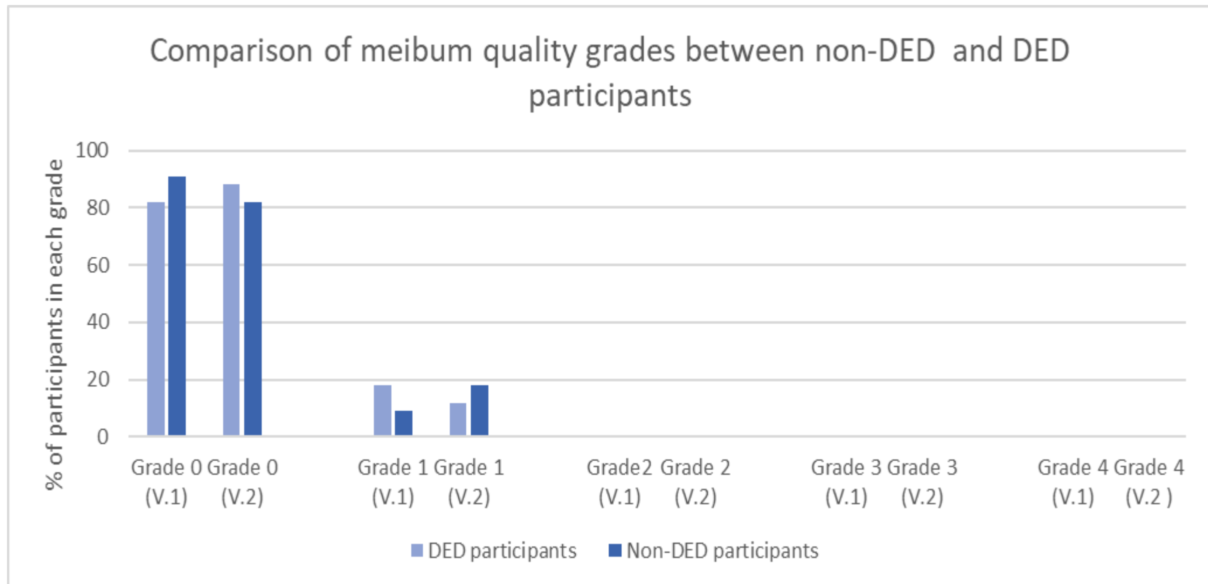


Figure 4.18. Comparison between visits of the meibum quality grade based on dry eye status
Meibum quality grades: 0=clear, 1=cloudy, 2= cloudy with debris, 3= thick, toothpaste- like, 4= waxy.

4.3.5. MMP-9 results

Sixty percent of participants at Visit 1 (n=30) had a positively graded result for MMP-9; of these 47% were non-DED (n=14) and 53% (n=16) DED participants, (odds ratio 0.76; 95% CI 0.24-2.40, p=0.773). At Visit 2, 74% of the participants (n=31) had a positively graded result; of these 39% were non-DED participants (n=12) and 61% DED participants (n=19), (odds ratio 1.32; 95% CI 0.33-5.30, p=0.733). There were no statistically significant differences between the two groups for Visit 1 (p=0.682) or Visit 2 (p=0.693). The semi-quantitative breakdown of these measurements is shown in ***Figure 4.19***.

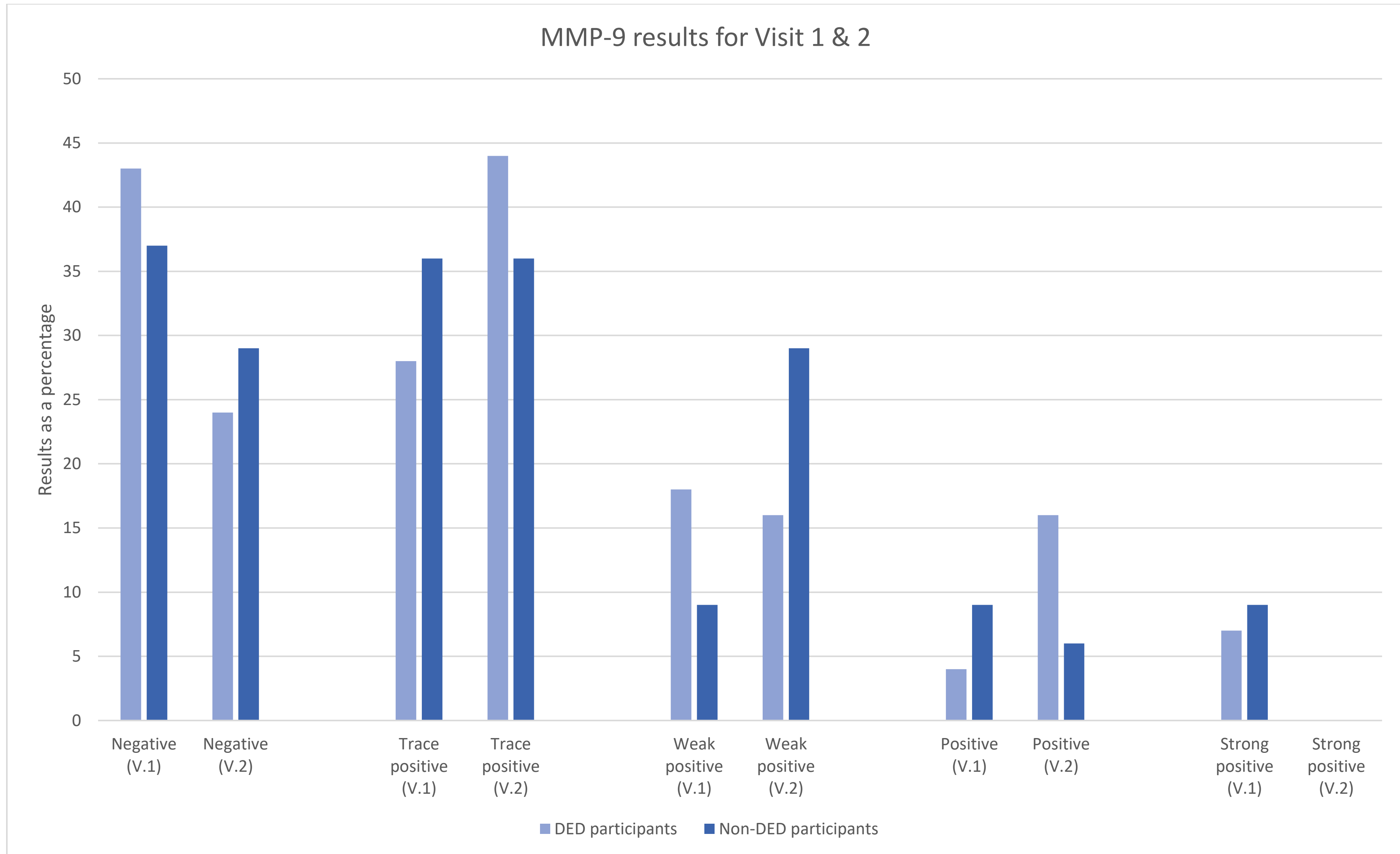
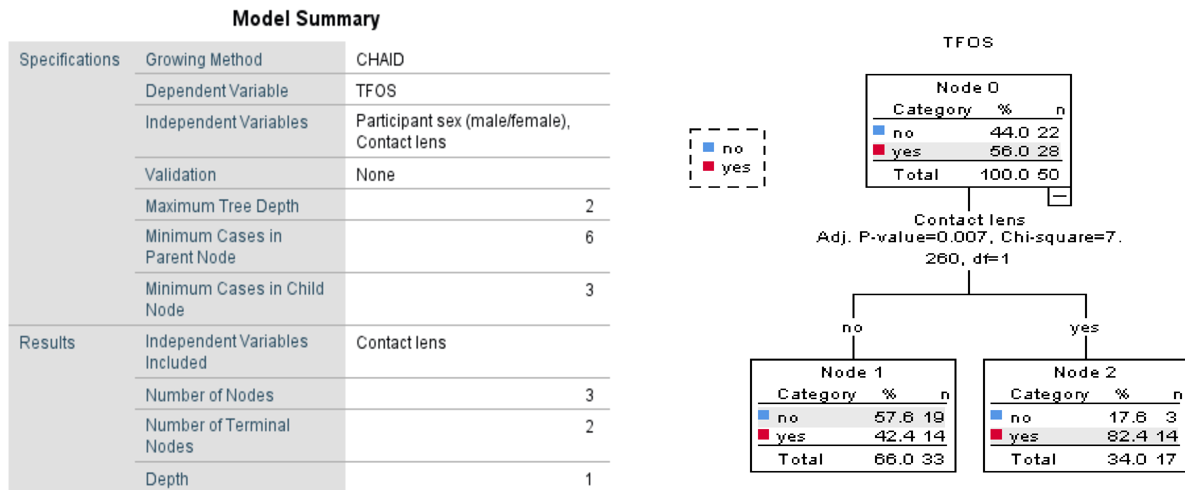


Figure 4.19. Results of the semi-quantitative assessment of MMP-9 for all participants at both visits

4.3.6. Predictors for DED and correlations within the clinical data from Visit 1

Decision Tree Analysis (DTA) was used to identify the hierarchy of the most significant predictors for DED and identify specific relationships within the data for further correlation analysis.

The Visit 1 data shown in ***Figure 4.20***. indicates that contact lens wear was a more significant predictor (p=0.007) for DED (TFOS criteria), than sex.

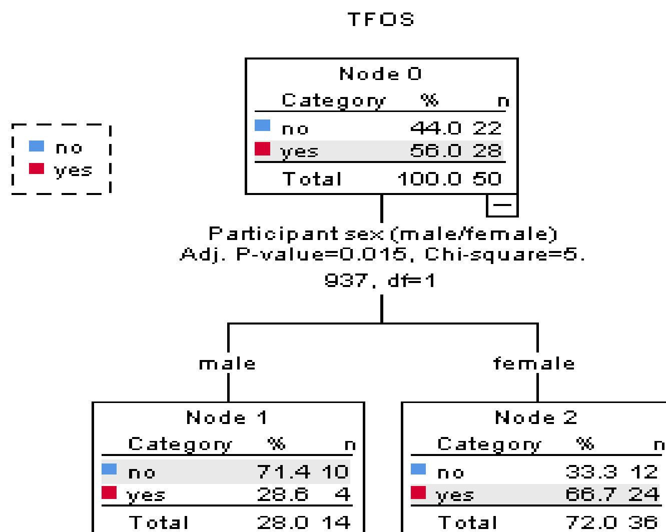


Model Summary

Specifications	Growing Method	CHAID
	Dependent Variable	TFOS
	Independent Variables	Participant sex (male/female), Contact lens
	Validation	None
	Maximum Tree Depth	2
	Minimum Cases in Parent Node	6
	Minimum Cases in Child Node	3
Results	Independent Variables Included	Contact lens
	Number of Nodes	3
	Number of Terminal Nodes	2
	Depth	1

Figure 4.20. DTA for TFOS positive/negative status and contact lens wear (Visit 1)

However, participant sex alone shows that the males were significantly less likely to have DED than the females (p=0.015), see ***Figure 4.21***.



The presence of conjunctival staining (grade > 0) at Visit 1, was also a good predictor for DED (TFOS criteria), where p=0.006 (see ***Figure 4.22***). However, the association between severity of conjunctival staining and dry eye status was not quite significant (p=0.08).

Figure 4.21. DTA for dry eye status and sex (Visit 1)

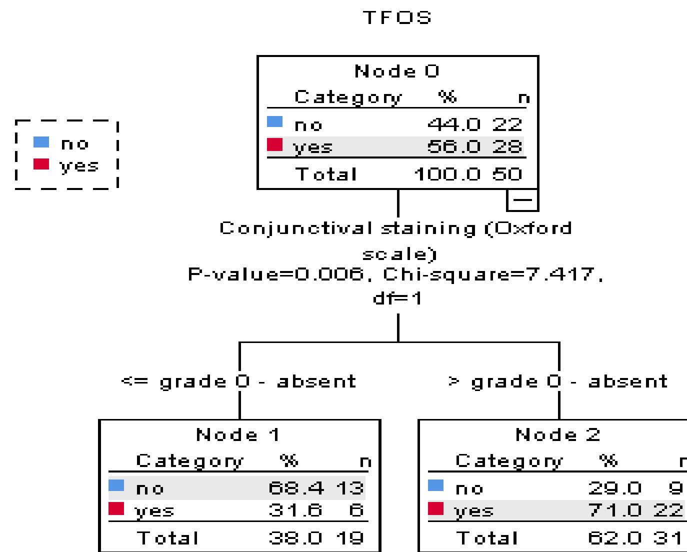


Figure 4.22. DTA indicating conjunctival staining as a predictor for DED (Visit 1)

There was a moderate correlation between conjunctival and corneal staining (r=0.319, p=0.024), but only a weak, not significant correlation was found between conjunctival staining grade and OSDI score (r=0.27 p=0.06). The non-CL wearers were significantly more likely not to have conjunctival staining (p=0.06).

Increased blink rate was found to be a significant predictor for lower NIKBUT (p< 0.001) (see *Figure 4.23*). Correlation analysis also confirmed a reduction in NIKBUT as the blink rate increased, with a high negative correlation coefficient of r=-0.52, p<0.001 (illustrated in ***Figure 4.24***).

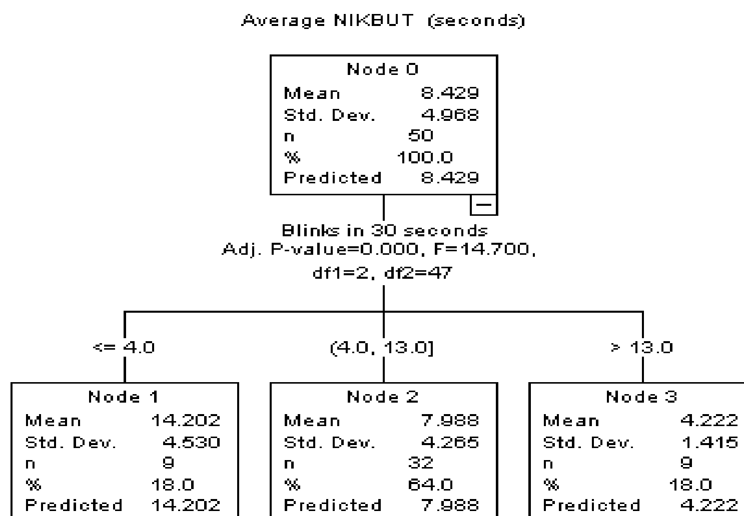


Figure 4.23. DTA for average NIKBUT and blink rate (Visit 1)

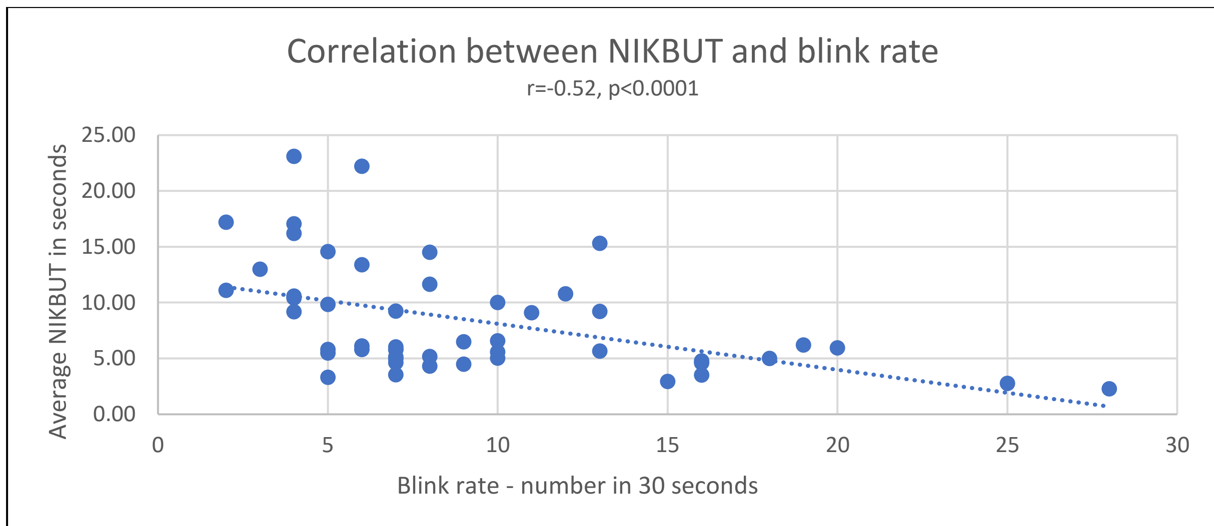


Figure 4.24. Scatter plot showing Spearman correlations for average NIKBUT and blink rate (Visit 1)

Tear meniscus height (TMH) showed a significant negative correlation with sagittal LWE (r=-0.42, p=0.002). However, only a weak, not quite significant correlation was found between TMH and horizontal LWE grade (r=-0.27, p=0.062).

Upper and lower lid meibography scores were found to correlate moderately with each other (r=0.40, p=0.004), and both correlated with meibum quality (upper lid, r=0.341, p=0.015 and lower lid, r=0.40, p=0.004), but not with the number of glands secreting. The quality of the meibum secretions also correlated with the number of glands expressing (r=0.308, p=0.29); as the quality of the meibum reduced the expression also reduced.

Lipid layer thickness (LLT) grading was not found to differ between the DED and non-DED participants and no significant correlations were found with any other clinical parameters at either visit. A small but not statistically significant correlation was found with sagittal and horizontal LWE, at Visit 2 only (r=-0.280, p=0.07 and r=-0.286, p=0.07).

4.3.7. Predictors for DED and correlations within the clinical data from Visit 2

The Visit 2 data also indicated contact lens wear and female sex to be significant predictors of DED (p=0.024 and p=0.001, respectively), see ***Figures 4.25*** and ***4.26***. Females had a significantly higher mean OSDI score (p=0.03), (27.8 ± 20.8) than the males (12.9 ± 13.9).

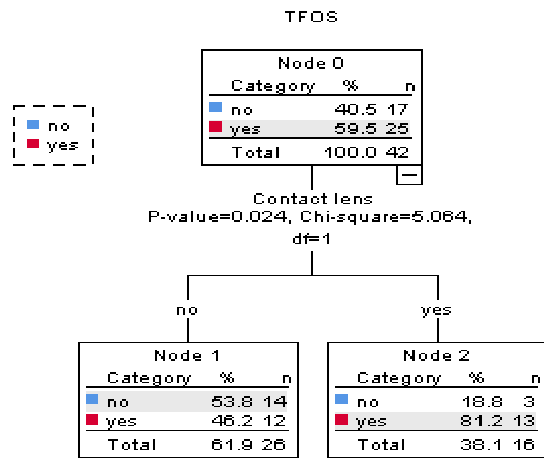


Figure 4.25. DTA for contact lens wear and dry eye status (Visit 2)

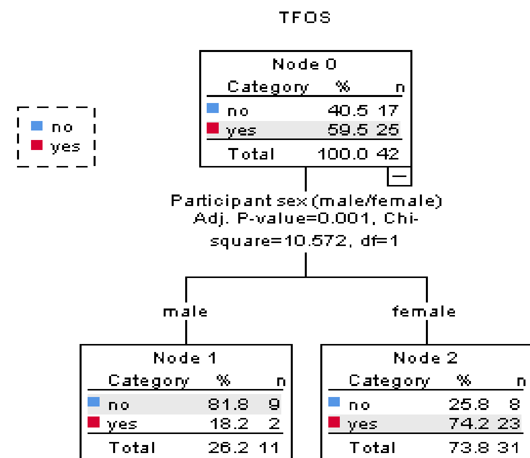


Figure 4.26. DTA for sex and dry eye status (Visit 2)

The highest proportion of MMP-9 results were in the trace positive category (40.5%); of these 76.5% (n=14) had horizontal LWE of >2-4mm. A negative MMP-9 test was found to be significantly (p=0.001) associated with the absence of or < 2mm horizontal LWE (see ***Figure 4.27***). A moderate but significant correlation (r=0.44, p=0.004) was found.

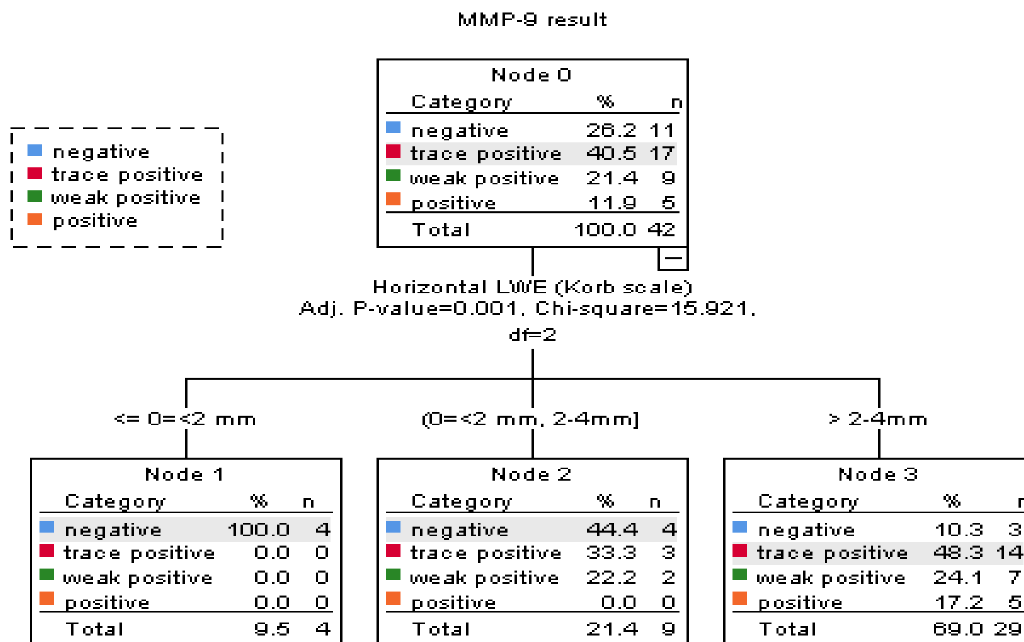


Figure 4.27. DTA results for MM-9 and horizontal LWE (Visit 2)

Sagittal LWE was associated with contact lens wear, with a moderate effect size of $\eta^2 = 0.092$, p=0.050, but no other clinical parameters at Visit 2. As would be expected, horizontal and sagittal LWE were highly correlated (r=0.747, p<0.001 at visit 1 and r=0.519, p<0.01 at Visit 2). The average TMH was lower in the participants who reported allergy (0.24 ± 0.05) compared to those who didn't (0.30 ± 0.07), p=0.013.

There was a moderate but significant positive correlation, (r=0.37, p=0.02) between upper and lower lid subjective meibography scores. This is also illustrated in the DTA in ***Figure 4.28***. No significant correlations were found between the meibography grade for either lid and the quality of meibum or expression from the glands. The presence of incomplete blinking was significantly more likely with greater upper lid gland loss (p=0.037), but this did not follow for the lower lid.

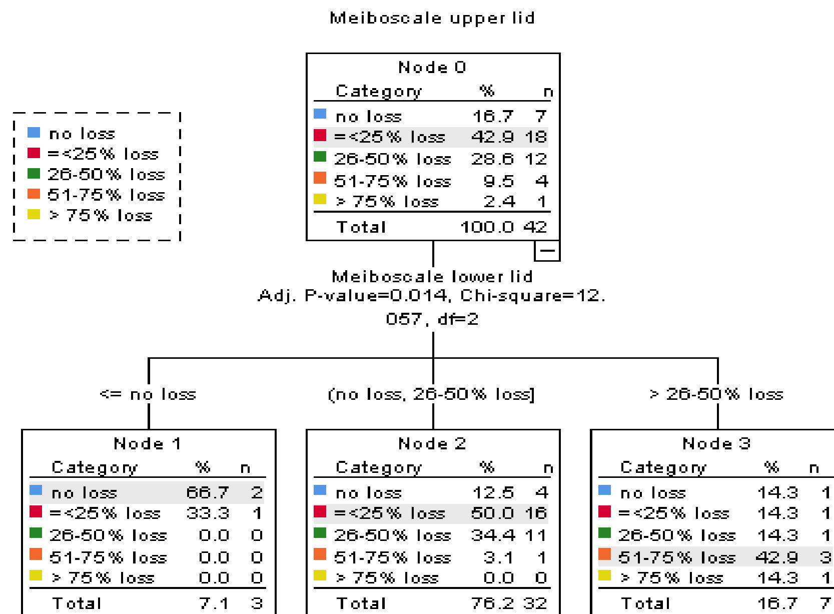


Figure 4.28. DTA results for upper and lower meibography (Visit 2)

As would be expected fewer glands secreting in the lower lid was associated with greater lower lid gland loss, (shown in ***Figure 4.29***). Lipid layer thickness (LLT) grading was not found to differ between the DED and non-DED participants and no significant correlations were found with any other clinical parameters at either visit. A small, but not statistically significant correlation was found with sagittal and horizontal LWE, at Visit 2 only (r=-0.280, p=0.07 and r=-0.286, p=0.07).

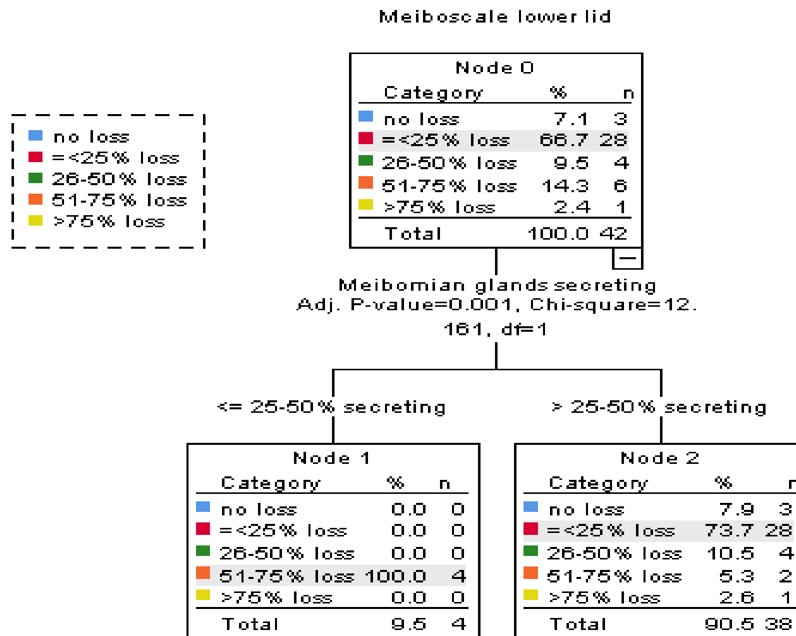


Figure 4.29. DTA results for lower meibography score and glands secreting (Visit 2)

4.3.8. Disease progression

At Visit 1, 56% (n=28) of the participants were diagnosed as having DED as defined by the TFOS DEWS II diagnostic criteria. Similarly, at Visit 2, 60% (n=25) had both signs and symptoms of DED. ***Figure 4.30***. presents a comparison of the distribution of the OSDI symptom questionnaire scores for each visit. A lower percentage of the participants had severe symptoms at Visit 2, but the mild and moderate categories increased.

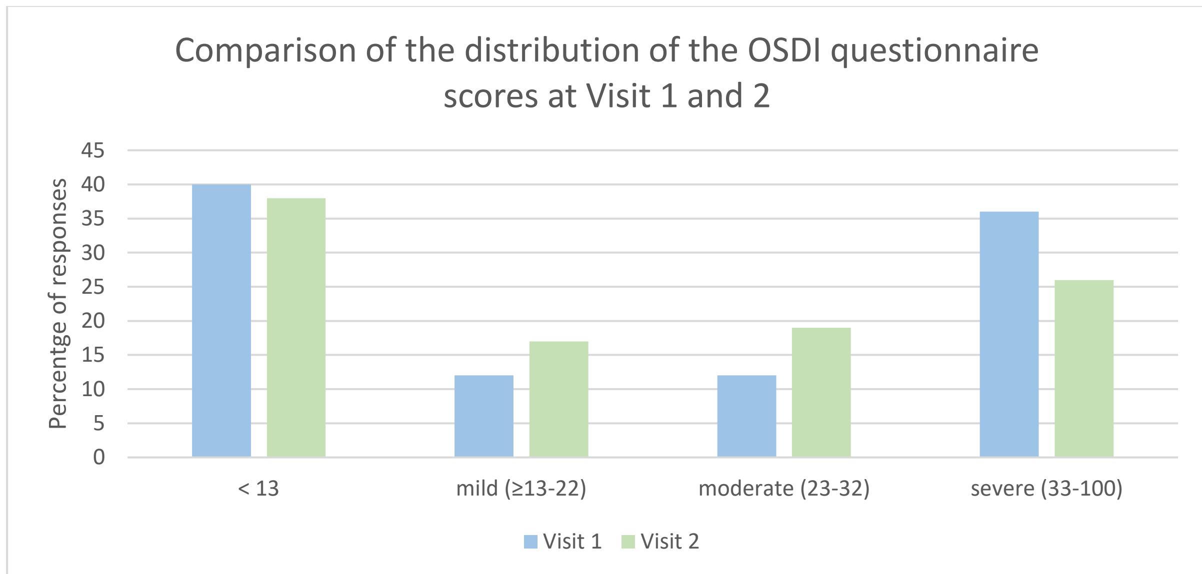


Figure 4.30. Comparison of the distribution of OSDI questionnaire scores between visits

When comparing the clinical measurements obtained from all the Visit 1 and 2 participants (see ***Table 4.9***), statistically significant increases in blink rate, bulbar and limbal redness, and sagittal LWE were found (p<0.05). Nearly significant differences were also found for lower NIKBUT and increased horizontal LWE at Visit 2. When only the participants that were present at both visits were compared, the statistically significant differences were stronger, and the horizontal LWE had also increased significantly. ***Table 4.10*** shows all the results for the pairwise comparisons of the clinical findings between visits. Both the subjective and semi-automated upper meibography grades were lower at Visit 2.

Table 4.9. Comparison of the clinical measurements between all Visit 1 and 2 participants

	Visit 1 (n=50)	Visit 2 (n=42)	Mann-Whitney U Test
Blink rate (no. in 30 seconds)	9.22 ± 5.72	10.98 ± 6.21	**p=0.049***
NIKBUT (sec)	8.43 ± 4.97	6.95 ± 3.90	p=0.097
TMH (mm)	0.30 ± 0.09	0.29 ± 0.07	p=0.185#
LLT	2.86 ± 0.97	2.64 ± 1.00	p=0.247
Bulbar redness (Jenvis scale)	1.28 ± 0.71	1.53 ± 0.65	**p=0.019***
Limbal redness (Jenvis scale)	0.84 ± 0.50	1.06 ± 0.55	**p=0.035***
Corneal stain (Oxford Scale)	0.26 ± 0.49	0.24 ± 0.58	p=0.490
Conjunctival stain (Oxford scale)	0.86 ± 0.81	0.95 ± 0.85	p=0.614
LWE horizontal (Korb scale)	1.72 ± 1.30	2.12 ± 1.06	p=0.128
LWE sagittal (Korb scale)	1.00 ± 1.01	1.52 ± 1.04	**p=0.017***
Meibography upper (Pult scale) - subjective grading	1.52 ± 1.05	1.38 ± 0.96	p=0.599
Meibography upper - semi-automated grading	2.00 ± 0.85	1.74 ± 0.89	p=0.194
Meibography lower (Pult scale) - subjective grading	1.20 ± 0.90	1.38 ± 0.91	p=0.236

** P values <0.05 were considered statistically significant. # parametric test performed as passed the K-S normality test*

Table 4.10. Pairwise comparison of the clinical measurements between participants

	Visit 1 (n=42)	Visit 2 (n=42)	Paired t-test
OSDI questionnaire score	22.9 ± 17.6	23.6 ± 20.3	p=0.615
Blink rate (no. in 30 seconds)	8.98 ± 5.18	10.98 ± 6.21	**p=0.009***
NIKBUT (sec)	8.50 ± 4.99	6.95 ± 3.90	p=0.081
TMH (mm)	0.31 ± 0.09	0.29 ± 0.07	p=0.140#
LLT	2.74 ± 0.91	2.64 ± 1.00	p=0.486
Bulbar redness (Jenvis scale)	1.29 ± 0.73	1.53 ± 0.65	**p=0.007***
Limbal redness (Jenvis scale)	0.87 ± 0.51	1.06 ± 0.55	**p=0.003***
Corneal stain (Oxford Scale)	0.21 ± 0.47	0.24 ± 0.58	p=0.812
Conjunctival stain (Oxford scale)	0.79 ± 0.72	0.95 ± 0.85	p=0.255
LWE horizontal (Korb scale)	1.55 ± 1.33	2.12 ± 1.06	**p=0.028***
LWE sagittal (Korb scale)	0.88 ± 0.99	1.52 ± 1.04	**p=0.001***
Meibography upper (Pult scale) - subjective grading	1.50 ± 1.06	1.38 ± 0.96	p=0.303
Meibography upper - semi-automated grading	2.00 ± 0.79	1.83 ± 0.76	p=0.322
Meibography lower (Pult scale) - subjective grading	1.24 ± 0.91	1.38 ± 0.91	p=0.279

** P values <0.05 were considered statistically significant. # parametric test performed as passed the K-S normality test*

Changes within each group (DED versus non-DED) between visits are shown in ***Table 4.11***. There was a significant difference in the limbal and bulbar redness in the non-DED participants. This was due to both the limbal and bulbar redness being higher in the non-DED group than the DED group at the second visit.

Table 4.11. Results of the Mann-Whitney U test comparing the data between visits for the DED and non-DED participants

	Non-DED participants			**DED participants**		
	Visit 1	**Visit 2**	**p-value**	**Visit 1**	**Visit 2**	**p-value**
Blink rate (no. in 30 seconds)	8.00 ± 5.32	10.5 ± 7.38	p=0.159#	10.2 ± 5.93	11.4 ± 5.40	p=0.171
NIKBUT (sec)	8.17 ± 4.04	7.09 ± 3.59	p=0.388	8.63 ± 5.66	7.03 ± 4.29	P=0.314
TMH (mm)	0.32 ± 0.09	0.30 ± 0.06	p=0.439#	0.29 ± 0.10	0.27 ± 0.07	p=0.872
LLT (Guillon-Keeler scale)	2.72 ± 0.70	2.65 ± 0.99	p=0.593	2.73 ± 1.14	2.64 ± 1.00	p=0.553
Bulbar redness (Jenvis scale)	1.25 ± 0.72	1.78 ± 0.75	**p=0.026***	1.30 ± 0.71	1.36 ± 0.53	p=0.303
Limbal redness (Jenvis scale)	0.77 ± 0.48	1.21 ± 0.59	**p=0.016*#**	0.89 ± 0.52	0.96 ± 0.50	p=0.549#
Corneal stain (Oxford Scale)	0.23 ± 0.53	0.06 ± 0.24	p=0.262	0.29 ± 0.46	0.40 ± 0.70	p=0.830
Conjunctival stain (Oxford scale)	0.73 ± 1.03	0.82 ± 0.81	p=0.497	1.04 ± 0.69	1.04 ± 0.89	p=0.932
LWE horizontal (Korb scale)	1.59 ± 1.37	2.06 ± 1.03	p=0.282	1.82 ± 1.25	2.16 ± 1.11	p=0.264
LWE sagittal (Korb scale)	0.91 ± 1.02	1.71 ± 1.11	**p=0.029***	1.14 ± 1.08	1.40 ± 1.00	p=0.341
Meibography upper (Pult scale)	1.45 ± 1.14	1.41 ± 0.94	p=0.894	1.39 ± 1.03	1.44 ± 1.04	p=0.888
Meibography lower (Pult scale)	1.14 ± 0.94	1.35 ± 0.93	p=0.399	1.39 ± 0.92	1.20 ± 0.71	p=0.448

** P values <0.05 were considered statistically significant. # parametric test performed as passed the K-S normality test*

4.4. Discussion

The results of this study show substantial overlap of signs and symptoms, as suggested by TFOS DEWS II for DED diagnosis, in both the evaporative and non-evaporative young adult participants. The longitudinal nature of the study was able to capture DED progression in young adults, which was characterised by increased blinking, bulbar and limbal redness, and horizontal and sagittal LWE. Although not significant, there was a clear trend toward reduced NIKBUT, which further supports disease progression in the young adult participants.

Eighty-two percent of the non-dry eye participants had one or more positive signs of ocular surface disease (88% at Visit 2). The poor correlation between objective clinical tests and severity of symptoms is well known (Nichols et al., 2004a). A study of sixty healthy children between the ages of 7 and 17,

reported that all participants had at least one abnormal ocular surface characteristic and a third had a dry eye diagnosis according to the TFOS DEWS II diagnostic criteria (Rojas-Carabali et al., 2020).

The female participants outnumbered the male participants, and were significantly more likely to have DED; this aligns with the body of evidence from large epidemiological studies (Stapleton et al., 2017). At Visit 1, contact lens wear was a more significant predictor or risk factor for DED than sex; a third of the participants wore contact lenses and composed half of the total DED participants. Again, at Visit 2, half of the DED participants were CL wearers. The odds of the CL wearers having DED were higher in this study (OR of 10 and 3.52, at visits 1 and 2 respectively) compared to the findings of other larger cohort studies (ORs of 1.35 and 1.42) (Wang et al., 2021b;Wolffsohn et al., 2021b).

Higher mean OSDI scores were found in the CL wearers at both visits of this study. Significant associations with conjunctival staining at Visit 1 and both higher blink rate and sagittal LWE were found at Visit 2. Several studies have reported an association between CL use and DED or symptoms of it (Tangmonkongvoragul et al., 2022;Vehof et al., 2021;Wolffsohn et al., 2021b;Inomata et al., 2019). While no association between CL wear and meibomian gland loss was found in this study, alterations of the MG morphology prior to clinically apparent alterations in MG function (meibum quality and expressibility), have been suggested to play a potential role in CL discomfort; once discomfort appears, MG loss may play a role in the degree of CL-related symptoms (Blanco-Vázquez et al., 2022).

The highest proportion of participants at both visits were of Asian ethnicity. Although the odds of having DED were over two times higher in the Asian participants compared to the other participants at both visits, the findings were not statistically significant. Previous reports have implicated Asian ethnicity as one of the predisposing risk factors for DED (Craig et al., 2016;Wolffsohn et al., 2021b;Stapleton et al., 2017). When comparing South Asian and White ethnicities, Wolffsohn et al. (2021b) reported a significantly higher odds of DED in the South Asian participants (OR 1.42; CI 0.98–2.05; p= 0.07). No significant associations were found between ethnicity and any lifestyle factors or clinical parameters at either visit in this study, although higher degrees of meibomian gland dropout and incomplete blinking have been reported in adult Asians compared with Caucasians (Craig et al., 2016).

When considering allergy, the odds of a participant having DED were higher at Visit 1 than at Visit 2 (OR 2.13 and 1.26, respectively). No associations were found between allergy and any clinical parameter at Visit 1, while at Visit 2 it was associated with a lower TMH (p=0.013). The study data collection took place between September to March, outside of hayfever season, and therefore it can be assumed that the clinical signs found were not confounded by signs of allergy. Patients with allergy and atopic disorders have previously been reported to have greater signs and symptoms of DED (Vehof

et al., 2017). One suggestion was that the sensitivity of the corneal nerves could be heightened due to inflammatory processes leading to symptoms of dry eye even when homeostasis is minimally compromised.

Considering lifestyle factors, the reported hours of exercise and outdoor activity were similar when comparing Visit 1 and 2. Although the DED participants reported spending more time exercising and outdoors compared to the non-DED participants, the differences did not reach statistical significance at either visit. Previously, the odds of having DED has been shown not to increase significantly with more outdoor activity (Wolffsohn et al., 2021b).

In this study no significant differences were found between the non-DED and DED participants for the number of hours spent using any form of digital device during a day at Visit 1. Although the reported time was greater in the DED participants at Visit 2, this did not reach significance. Increased digital device screen exposure has previously been reported as a modifiable risk factor for DED (Wang et al., 2021b).

Nevertheless, screen time was found to correlate with several ocular surface signs including sagittal LWE, horizontal LWE and lower and upper lid meibography score. Other studies have reported an association between extended screen time, reduced tear film stability, and symptoms of DED (Muntz et al., 2022;Yang et al., 2021).

Significantly higher self-reported stress levels were found in the DED participants at both visits of this study; this is in agreement with other studies (Yilmaz et al., 2015;Bitar et al., 2019;Wolffsohn et al., 2021b;Wang et al., 2021a). The odds of having moderate to high self-reported stress levels and DED were higher in this study at Visit 1 (OR 3.36; CI 1.07 to 11.81; p=0.047) compared to those previously reported by Yilmaz (2015) (OR 2.33; CI, 1.48-3.67) and Wolffsohn (2021b) (OR 1.29; CI, 1.11–1.51) p=0.001).

The OSDI questionnaire was used in this study to assess symptoms of DED. The range of scores in the non-DED participants did not vary from Visit 1 to Visit 2 (0 to 41.7%). No significant change was found in the OSDI score between visits for the DED participants either. When comparing the OSDI scores between visits, more participants reported mild and moderate symptoms at Visit 2, but this was not significant.

While no statistically significant differences in clinical signs were found between the DED and non-DED participants, correlations were found amongst them at each visit. Conjunctival staining was significantly more likely to be present in the DED participants (p=0.006) at Visit 1. However, the mean grade did not reach significance when comparing the DED and non-DED participants (p=0.083).

Corneal staining showed no association with dry eye status. These findings align with the current understanding that corneal and conjunctival staining are later features of DED (Craig et al., 2021). Significantly more conjunctival staining, but not corneal, was found in the CL wearers (all soft) in this study ($p=0.021$), but this did not correlate with symptoms.

Lid wiper epitheliopathy (LWE) was recorded in the lower lid only in this study. The horizontal LWE grade was higher in the DED participants at both visits but did not reach significance. Similarly sagittal LWE was greater in the DED participants but at Visit 1 only. When considering the TFOS DEWS II diagnostic criteria for LWE, more than half the non-DED participants at Visit 1 (55%) and 82% at Visit 2 had LWE ≥2mm length & ≥25% width. Although LWE occurs more frequently in patients with DED than without (Korb et al., 2010), it is known to be a frequent finding in the absence of routine clinical dry eye findings (Korb et al., 2005). No association was found between LWE and OSDI questionnaire symptom score at either visit; the severity of LWE has previously been reported to correlate with dry eye-related symptoms (Korb et al., 2005;Pult et al., 2011).

At Visit 2, there was a significant association between sagittal LWE and CL wear, with a medium but significant effect size $\eta^2 = 0.092$, p-=0.05. Liu et al. (2020) found that moderate to severe LWE was associated with greater symptoms in CL wearers. Interestingly, while examining both lids, Shiraishi et al. (2014) found a higher prevalence of LWE in the lower lids, regardless of CL wear, which suggested that this may be due to the continuous friction of the lower eyelid on the same region of the cornea during blinking.

At Visit 1, a significant correlation was found between sagittal LWE and TMH ($r=-0.42$, $p=0.002$); a weak, but non-significant correlation was also found with horizontal LWE grade ($r=-0.27$, $p=0.062$). This agrees with previous studies in DED participants, which also found correlations with tear breakup time (Liu et al., 2020;Kato et al., 2022).

Horizontal LWE was also found to be associated with a positive result for MMP-9. Seventy-six percent (n=14) of the participants with a trace positive result had more than 2-4mm of horizontal LWE. A moderate positive correlation was found with MMP-9 result ($r=0.44$, $p=0.004$) and a negative MMP-9 result was significantly associated with the absence of horizontal LWE ($p=0.001$).

In this study, the ability to distinguish between DED and non-DED was inconclusive; almost half of the non-DED participants at Visit 1 had a positive (trace, weak, positive, or strong positive) MMP-9 result. The odds of having DED with a positive MMP-9 result were slightly higher at Visit 2, but not significant. This result is in contrast with previous reports which showed 85% sensitivity, 94% specificity, negative 73% predictive value, and 97% positive predictive value (Sambursky et al., 2013).

No correlations with any clinical parameter indicative of ocular surface inflammation or tissue breakdown, other than horizontal LWE, were found in this study. No correlation was found with OSDI score, although MMP-9 activity has previously been shown to increase in proportion to dry eye severity (Chotikavanich et al., 2009).

Due to the current study criteria precluding the possibility of any other ocular surface inflammatory conditions, the results reported here would indicate a low level of inflammation was present in many of the DED and non-DED participants, particularly those with trace positive results. Previously, the low number of positive results in studies of mild DED led to the suggestion that MMP-9 measurement with InflammaDry® is better at detecting moderate to severe dry eye (Schargus et al., 2015).

In the current study, two other methods of tear collection were performed prior to administration of the InflammaDry® test. Due to possible contamination of the tear samples, it was performed after microcapillary and Schirmer tear collection. However, MMP-9 is believed to be an early marker of inflammation and has shown elevated levels within 2 hours of an inflammatory stimuli (Kolaczkowska et al., 2008). One of the manufacturers recommendations is that the InflammaDry® test should not be used within 20 minutes of performing a Schirmer test, due to the possible stimulation of MMM-P degranulation leading to a false positive result. Therefore, it is possible that some of the positive results found in this study could be attributed to that. However, care was taken during the procedure not to rub the InflammaDry® fleece along the tarsal conjunctiva, but rather a dab, hold, and lift technique was employed. Therefore, the positive correlation with horizontal LWE and any relationship with extended screen use may still be valid and warrant further investigation in this previously under-studied age group.

No association between incomplete blinking and DED status or ocular surface staining was found in this study. However, the presence of incomplete blinks was significantly associated with longer screen time at Visit 1 ($p=0.009$). This is in agreement with previous studies; the percentage of incomplete blinks has been shown to increase 6.5 times during a concentrated task (Bilkhu et al., 2020).

The presence of incomplete blinks was associated with higher blink rates at both visits of this study. Visit 2 participants, who exhibited incomplete blinking and a blink rate of > 16 per minute, also spent significantly longer using a screen than those who blinked less than 16 times per minute.

Incomplete blinking has also been associated with a lower TBUT (Jie et al., 2019), but this was not found at either visit of this study. The association found between incomplete blinking and meibomian gland loss in this study, similar to previous studies, has been proposed as a potential mechanism in the development of MGD (Wan et al., 2016).

The blink rate was higher in the DED participants at both visits, but not significantly. It was significantly higher at Visit 2 when comparing all the participants. Blink rate is known to decrease during concentrated tasks such as electronic device use (Bilkhu et al., 2020) and has been suggested as a primary mechanism in the worsening of DED (Portello et al., 2013). Worryingly, these changes are also being reported in paediatric populations, with extended screen use being the suggested cause of atrophy and changes in morphology of the meibomian gland (Cremers et al., 2021;Kocamiş et al., 2021).

Increased blink rate was also found to be a significant predictor for lower NIKBUT in this study, which agrees with previous findings (Nosch et al., 2016). It is suggested as a compensatory mechanism to alleviate the effects of reduced tear film stability.

This is understood to be the first report in a longitudinal study to document significant increases in bulbar and limbal redness in a young cohort, which aligns with DED progression. The change was observed when comparing the 42 participants who were present for both visits ($p<0.001$ for each). This was due to significantly higher levels of redness in the non-DED compared to the DED participants at Visit 2. As these measurements were objectively measured with the Keratograph 5M (Oculus, Wetzlar, Germany), they could not be attributed to observer bias. This result supports the understanding that progression of DED is gradual.

Tear film lipid layer thickness (LLT), number of glands expressing, quality of expressed meibum and upper and lower lid meibography were all considered to be diagnostic markers specific to evaporative DED in this study. The subjective meibography grading of both lids did not show any differences between the DED and non-DED participants at either visit, with almost half of the participants having at least 25% gland loss in either lid. Similarly, no difference in the mean percentage gland loss of the upper lids was found between the two groups by semi-automated gland detection software performed by a masked observer. No differences were found in LLT, or meibum expressibility and quality from the lower lids.

In this study the upper and lower lid meibography scores correlated at both visits. There was a non-significant increase in lower lid gland loss at Visit 2. Upper lid gland loss was lower at Visit 2, both for all the participants and when a pairwise analysis was made (n=42). This could be attributed the subjective nature of the measurements if this finding was exclusive to the subjective grading. However, the semi-automated grading was done by a masked observer and a similar reduction was reported. A possible reason for this could be that the lid eversion and imaging was better at Visit 2.

The morphology of the upper and lower meibomian glands have previously been shown to correlate significantly for all aspects except thickness, which was greater in the lower lids (Pult et al., 2012). Shortening of the glands in the lower rather than upper lid was found to be significantly more frequent in patients with EDE than healthy subjects (Crespo-Treviño et al., 2021).

The only other significant correlations between meibomian gland related features were between lower meibography score and meibum quality, and the percentage of glands secreting and meibum quality, at Visit 1. It is reported that at the 'preclinical stage' altered quality of expressed secretions and/or decreased or absent expression may be present, but with progression, patients are likely to become symptomatic, with additional lid margin signs, such as telangiectasis (Tomlinson et al., 2011). A recent study found that meibum expressibility, meibum quality, lid margin changes, and MG dropout were unable to distinguish between asymptomatic MGD, symptomatic MGD, and MGD-related EDE; LLT (the quantity of meibum) was the only factor to be associated with MGD with DED (Chiou et al., 2023).

This study employed the five-point Pult grading scale to subjectively identify the area of gland loss in each lid (Pult and Riede-Pult, 2013). When comparing just the upper and lower meibography scores which showed > 25% gland loss, more gland loss was found in the upper lids in both the DED and non-DED participants at both visits. The mean upper gland loss for all participants calculated semi-objectively with the software was 37.0 ± 17.8 % at Visit 1, and 31.9 ± 16.4% at Visit 2. These figures are considerably higher than the objective threshold values for predicting DED recommended by Pult: a minimal gland loss of 16.9% for the upper eyelid and 28.7% for the lower eyelid (Pult and Riede-Pult, 2013). However, the mean age of the 20 participants in that study was 44.5 years. This would suggest that although the young participants in this study had more gland loss, they were relatively less symptomatic.

In this study no associations were found between any meibomian gland related parameter and sex, ethnicity or contact lens wear. Although gland loss was a common finding in both lids, no participant was found to have meibum quality worse than grade 1 (cloudy), although poor expressibility of the glands (less than 50% expressing) was found in both DED and non-DED participants. The potential predisposition of Asians to the development of DED appears to be related to greater levels of meibomian gland drop out and incomplete blinking (Craig et al., 2016).

Although no differences were found in this study, there has been a longstanding clinical impression that contact lens wear increases the risk of MGD, although the evidence for this is inconclusive (Schaumberg et al., 2011). Recently further studies have explored this relationship by examining the MG structure and function, but still with conflicting findings (Rueff et al., 2022). Von Ahrentschildt et

al. (2022) were unable to find any MG morphology metrics with a clinically meaningful predictive value for detecting DED in a group of current and previous CL wearers. Reviewing current studies, Rueff et al. (2022) reported a lack of standardized clinical MG assessments and longitudinal studies and recommended that all CL wearers should undergo a full MG evaluation until more definitive answers are found.

While no overall progression in gland loss was detected over the one-year period of this study, an interesting finding was observed in an individual participant. Due to the participant having a meibomian cyst in the interval between the visits, a significant area of gland drop out was observed (see Figure 4.31.). Clinically there was a small nodule remaining in the lid, which could be felt digitally, but the patient was asymptomatic at the time of the visit. This finding would reinforce the need for a thorough history and symptoms when examining patients at follow up visits.

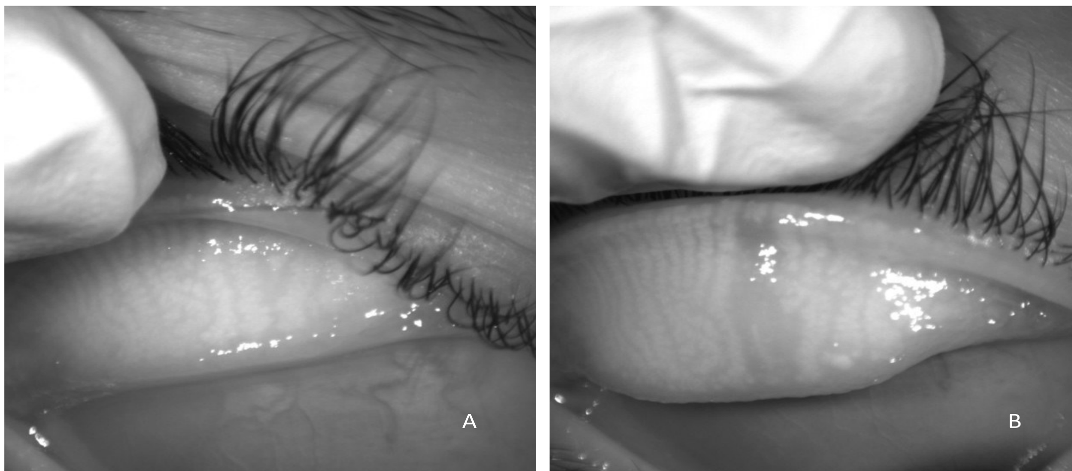


Figure 4.31. Comparison of upper lid meibography at Visit 1 (A) and Visit 2 (B)

As yet there does not appear to be any published data on longitudinal assessment of the meibomian glands. Neither does there appear to be any assessment of potential subtle changes and/or fluctuations over shorter periods of time. While there has been considerable research into various meibography techniques, morphological changes in the glands and their diagnostic relevance, much is still unknown.

It was evident from this study that particular attention should be placed on good eyelid eversion and lighting, as these were critical for the good image quality required for objective processing, allowing an accurate comparison of images at subsequent visits. Any future investigation of MGD would also benefit from including observation of gland length and lid margin abnormalities, such as gland capping and telangiectasia. Furthermore, investigation of the lipid composition of expressed meibum could

shed more light on the physiological changes occurring in the meibomian glands. Comparison of the meibum in participants with and without gland loss could help to distinguish participants likely to go on to develop DED. To this end, meibum samples were collected as part of the study reported here; the result of their analysis is reported in Chapter 7.

4.5. Conclusion

This longitudinal study in young adults found a significant overlap in ocular surface signs and symptoms in both evaporative DED and non-DED participants. Certainly, the overlap of signs and symptoms and the variation found between visits in this study serves to reinforce the requirement to screen for both in order to diagnose DED, with no one sign alone adequate to demonstrate a loss of tear film homeostasis.

This study has, for the first time, observed a young population in a longitudinal study and has shown that a significant number of young adults have ocular surface signs of DED, but as yet no symptoms; the term 'pre-clinical' DED may be better applied to these subjects. In particular, upper meibomian gland loss and reduced NIKBUT were particularly prevalent in the non-DED participants, but individually would not be good clinical indicators of DED. Considering all the clinical parameters measured, conjunctival staining appeared to be the best single clinical predictor of DED, while several correlations were found with LWE, incomplete blinks, increased blink rate and reduced NIKBUT amongst all participants.

Contact lens wear and screen use were found to be key modifiable risk factors for DED in young adults and correlated well with signs of ocular surface inflammation. The association between LWE and digital screen use hours lend weight to the evidence that screen use is a risk factor for DED. Therefore, the use of digital devices requires careful monitoring for early DED diagnosis and prevention of severe DED. This study highlights the importance of examining the ocular surface and lids of all young adults, not just symptomatic patients, or CL wearers. Therefore, it is important that young people in particular are counselled for the key modifiable risk factors of DED regardless of their dry eye status and before the onset of symptoms.

The finding that four participants moved to the DED group after one year, with three of these due to an increase in symptoms accompanying diagnostic signs, indicates possible progression of the disease. Furthermore, increases in ocular redness and LWE also support a gradual progression of the disease over the period of the study. The measurable changes in signs and symptoms are possibly just the 'tip of the iceberg', where a larger longitudinal study may be able to uncover the magnitude of the disease progression.

The findings reported here also serve to reinforce the need for objective biomarkers to aid in the early detection and differentiation of those patients who are likely to progress to symptomatic DED. The following two chapters, therefore, describe the results of tear analysis for potential biomarkers of evaporative DED in the tear proteome of these young adults.

Chapter 5 - Prospective, longitudinal study assessing dry eye among young adults - tear protein assessment

An abstract of this chapter was submitted (Dec, 2023) to the Association for Research in Vision and Ophthalmology (ARVO) Conference, 2024 and accepted as a poster presentation.

- Dutta, D., Casemore, R. K., Wolffsohn, J. S. A prospective, longitudinal study to characterize tear proteome in young adults during progression of dry eye disease. Association for Research in Vision and Ophthalmology (ARVO) Conference, 5th to 9th May 2024, Seattle, USA.

5.1. Introduction

Proteomic analysis of the tears has played a major role in our understanding of the pathogenesis and management of DED. Over the past two decades tears have emerged as a reliable biomarker for the identification of several ocular and systemic conditions (Enríquez-de-Salamanca et al., 2012;Cocho et al., 2016;Hamm-Alvarez et al., 2014;Aass et al., 2017;Tummanapalli et al., 2019;Zhan et al., 2021); the high concentration of proteins and the minimally invasive collection techniques available provide an attractive means of assessing changes in the biochemical properties of the tear film (Hagan et al., 2016). Various methods of tear collection are available, including Schirmer strips with or without anaesthesia (Boehm et al., 2011;Jackson et al., 2016), mini sponges (Acera et al., 2008), micropipettes (Yoon et al., 2007), and glass or plastic microcapillary tubes (Esmaeelpour et al., 2008). Increasing numbers of proteins (up to 3724 unique proteins) have been identified as techniques for analysis have developed, with some proteins being unique to the tear collection method used (Ma et al., 2021).

Opinion varies regarding the best method of tear collection (see discussion Chapter 3). A comparison of tear proteins collected by Schirmer strips and capillary tubes, the two methods employed in the study reported in this thesis, have shown significant increases in serum proteins with Schirmer strip collection (Stuchell et al., 1984). However, numerous studies have explored the tear proteome using both methods in search of ideal biomarkers to optimise diagnosis, subclassification, therapeutic management and monitoring of DED. Ma et al (2021) propose that 435 commonly reported proteins can be identified regardless of the tear fluid collection method and are the higher abundant, core proteins in the composition of tear fluid.

Collection of tear samples by both glass microcapillary tube and Schirmer I test was performed as part of the prospective, longitudinal, observational, single centre study reported in the previous chapter. The tear protein profiles of the tears collected by glass microcapillary in the young adult cohort, whose clinical findings have been reported in Chapter 4, are reported here. The results for the tear cytokine analysis with tears collected by Schirmer strips is reported in Chapter 6.

Recent research has identified a high prevalence of DED or symptoms of DED in young adults (Asiedu et al., 2017;Li et al., 2018;Vehof et al., 2021). A weak correlation between subjective symptoms and objective signs is particularly apparent in the early stages of DED, when clinical signs may be mild or absent all together (Nichols et al., 2004b).The tear proteome has been evaluated with various analytical methods (see Chapter 1), but with particular reference to changes that reflect the state of the ocular surface in more advanced DED. As yet few researchers have explored the tear proteome in early DED or in young adults with DED. Therefore, detection of any tear biomarkers that could broaden

knowledge surrounding the pathogenesis of DED and enable its early detection and differentiation could be beneficial.

5.1.1. Study objectives

The study reported here, which was conducted as part of the study reported in Chapter 4, aimed to investigate the tear protein profiles in an 18-25 year old cohort for biomarkers of evaporative DED. Furthermore, the study aimed to identify any correlations between any tear proteins and the clinical parameters previously reported.

Null hypotheses:

- There are no differences in tear proteins when comparing young DED to non-DED participants.
- There are no associations between specific tear proteins and severity of evaporative DED in a young population.
- There would be no change or progression in the protein profiles over the one year study period.

5.2. Methods

Samples collected with glass microcapillary tubes were analysed with the Agilent 2100 Bioanalyzer in order to detect the presence of the main tear proteins, their concentrations and concentration as a percentage of the total tear protein concentration (TPC), and the TPC. The tear samples collected with Schirmer strips underwent cytokine analysis by Luminex Assay (see Chapter 6).

5.2.1. Tear collection with microcapillary tubes and method of analysis

The basal tear samples collected by glass microcapillary tubes (10 µl, Sigma-Aldrich, USA), were done so in accordance with Aston University's Standard Operating Procedure (SOP) for tear collection (see **Appendix 4**) and the protocol of the Ophthalmic Vision Science Research Group (OVSRG) (**Appendix 5**), as previously described in Chapter 3 section 3.2.4.1.

The tear protein profiles of the samples collected by this method were investigated using the Agilent 2100 Bioanalyzer (Agilent Technologies, UK, Ltd). The sample preparation, chip loading and method of analysis for this technique are described in the pilot study reported in Chapter 3 of this thesis. Only basal samples and those with six or more protein peaks were included in the analysis.

5.2.2. Data analysis and sample size

Data analysis was performed using Microsoft® Office Excel®, GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA and IBM SPSS Statistics for Windows, Version 26. Descriptive statistics such as mean and standard deviation were used to report biomarker concentrations. Fischer's exact test was used to compare the frequency of presentation of each protein between visits for the DED and non-DED participants. The non-parametric Mann-Whitney U test was used for comparisons of TPC, relative protein concentration and percentage of the total concentration between the DED and non-DED participants.

To quantify correlations between clinical parameters and tear biomarker levels, Spearman ranked correlation was used. The strength of any correlation between the two visits (Spearman's correlation coefficient r) was estimated according to the following coefficient magnitudes: 0.5-1.0 represents a high correlation, 0.5-0.3 moderate, and 0.3 to 0.1 weak. Pearson's correlation coefficient was calculated where the data passed the Kolmogorov-Smirnov test for normality.

To measure the association between nominal and numerical data, such as contact lens wear and protein values, Multivariate Analysis of Variance was used to calculate partial eta-squared where $\eta 2 = 0.01$ indicates a small effect, $\eta 2 = 0.06$ a medium effect and $\eta 2 = 0.14$ indicates a large effect (Watson, 2021). For all tests an alpha value of 0.05 was adopted for statistical significance. Bonferroni correction was not applied to the data and therefore the possibility of false significance is acknowledged (Armstrong, 2014).

The sample size calculation for tear protein analysis has been reported in Chapter 4. A total sample size of 42 (21 in each group) with a two-tailed α-level of 5%, an 80% power level and a calculated effect size of 0.95, was required to demonstrate statistically different lactoferrin concentrations between two groups.

5.3. Results

Tear samples collected by microcapillary tubes were analysed using the Agilent 2100 Bioanalyzer in conjunction with the Protein 230 LabChip Kit. In total, tear protein profile results were obtained for 45 participants at Visit 1, and 39 from Visit 2. At Visit 1 tear collection was not possible from one participant. Other results were excluded due to missing or erroneous data; where less than 6 protein peaks were present, or peaks did not appear at the expected molecular weights, the samples were excluded from the analysis. The protein peaks were identified according to their molecular weight and knowledge from previous validation studies reported in Chapter 3.

Lysozyme, lipocalin and lactoferrin were observed in the tear protein profiles of all the participants at Visit 1 and 2, except for one participant (Visit 2) where a peak was observed at 17.2 kDa. This was not included in the analysis due to it not falling within the previously reported range for lipocalin of 18.1-19.8 kDa (see Chapter 3). The percentage frequency of presentation of each protein for all the participants at each visit, separated according to dry eye status, can be seen in ***Table 5.1***.

Table 5.1. Percentage frequency of presentation for the main tear proteins identified

Protein	**All participants**		**non-DED participants**		**DED participants**	
	Visit 1 (n=45)	**Visit 2 (n=39)**	**Visit 1 (n=20)**	**Visit 2 (n=14)**	**Visit 1 (n=25)**	**Visit 2 (n=25)**
Lysozyme	100	100	100	100	100	100
Lipocalin	100	97	100	100	100	96
SIgA**	100	95	100	86	100	100
ZAG	51	67	70	71	36*	64
~47kDa	91	95	90	100	92	92
Albumin	62	67	45	57	80	72
Lactoferrin	100	100	100	100	100	100
~100 kDa	76	69	65	79	84	64
~147 kDa	53	41	55	36	52	44
~167 kDa	60	36	65	43	56	32

** indicates a statistically significant difference in the percentage frequency between visits (p=0.048), Fischer's exact test. ** secretory immunoglobulin A (light-chain)*

5.3.1. Visit 1 tear protein results.

The total protein concentration (TPC) at Visit 1 was 4.50 ± 2.45 mg/ml (CI 3.76 – 5.24) and 4.93 ± 2.70 mg/ml (CI 4.05 – 5.80) at Visit 2 (p=0.50). A comparison of the mean and standard deviations of the TPC, the number of peaks and the relative concentrations for each main protein identified according to dry eye status at Visit 1 are reported in ***Table 5.2***. Protein profiles were obtained for 25 DED and 20 non-DED participants.

DED participants had a significantly higher concentration (p<0.001) and percentage (p=0.003) of albumin compared to the non-DED participants. The percentage of lactoferrin was found to be significantly lower (p=0.01) in the DED participants.

Table 5.2. Tear protein data for Visit 1

Visit 1	**non-DED participants**		**DED participants**		**Mann-Whitney U test p value**	
Participants	n=20		n=25			
Number of peaks	9.65 ± 1.66		9.84 ± 2.15		p=0.747#	
Total protein concentration (mg/ml)	4.12 ± 2.32		4.85 ± 2.49		p=0.182	
Protein	**Relative conc. mean ± SD (range) (mg/ml)**	**% of total concentration mean % ± SD (range)**	**Relative conc. mean ± SD (range) (mg/ml)**	**% of total concentration mean % ± SD (range)**	**p value for relative conc.**	**p value for % of total conc.**
Lysozyme	1.69 ± 1.12 (0.69 – 5.08)	39.5 ± 6.8 (30.6 – 52.3)	1.90 ± 1.05 (0.42 – 4.55)	38.7 ± 7.0 (23.0 – 50.8)	p=0.213	p=0.964
Lipocalin	0.27 ± 0.18 (0.05 – 0.68)	6.2 ± 2.1 (2.7 – 8.8)	0.28 ± 0.15 (0.09 -0.34)	6.2 ± 2.3 (0.90 – 10.2)	p=0.937#	p=0.992#
SIgA	0.43 ± 0.29 (0.10 – 1.10)	9.4 ± 3.0 (3.8 – 16.8)	0.61 ± 0.49 (0.13 – 2.38)	12.1 ± 4.9 (5.0 – 21.7)	p=0.113	p=0.112
ZAG	0.14 ± 0.16 (0.00 – 0.51)	2.9 ± 2.3 (0.0 – 6.1)	0.09 ± 0.18 (0.00 – 0.84)	1.8 ± 2.7 (0.0 – 5.5)	p=0.082*#	p=0.060*#
~47kDa	0.39 ± 0.29 (0.00 – 1.09)	8.9 ± 1.4 (0.0 – 13.8)	0.50 ± 0.34 (0.00 – 1.5)	10.5 ± 4.9 (0.0 – 22.9)	p=0.174	p=0.232#
Albumin	0.006 ± 0.011 (0.00 – 0.04)	0.2 ± 0.3 (0.0 – 1.4)	0.024 ± 0.020 (0.00 – 0.06)	0.5 ± 0.5 (0.0 – 1.8)	**p<0.001***	**p=0.003***
Lactoferrin	0.98 ± 0.49 (0.44 – 2.55)	24.3 ± 4.8 (14.6 – 34.7)	1.03 ± 0.63 (0.31 – 3.18)	20.7 ± 4.0 (14.3 – 29.2)	p=0.771#	**p=0.009*#**
~100 kDa	0.11 ± 0.10 (0.00 – 0.31)	2.9 ± 2.6 (0.0 – 6.5)	0.20 ± 0.17 (0.00 – 0.64)	3.7 ± 2.2 (0.0 – 7.6)	p=0.057*	p=0.407
~147 kDa	0.03 ± 0.05 (0.00 – 0.17)	1.0 ± 1.6 (0.0 – 6.4)	0.05 ± 0.07 (0.00- 0.23)	1.2 ± 1.7 (0.0 – 5.4)	p=0.876	p=0.771
~167 kDa	0.05 ± 0.12 (0.00 – 0.52)	1.0 ± 1.4 (0.0 – 5.7)	0.05 ± 0.09 (0.00 – 0.36)	1.0 ± 1.4 (0.0 – 4.0)	p=0.723	p=0.601

*#indicates data that passed the KS normality test – parametric unpaired t-test used. *P-values <0.05 were considered significant and are shown in bold. * near significance.*

The concentration of ZAG was lower in the DED participants, but not quite to significance (p=0.08), while a higher concentration of the protein with a mass of ~100kDa showed borderline significance (p=0.06).

The results of the correlation analysis between the total and relative tear protein concentrations and the clinical parameters used to classify participants by dry eye status are reported in ***Table 5.3***.

Table 5.3. Spearman correlation coefficients for the total and relative tear protein concentrations and clinical parameters used to classify dry eye status (Visit 1)

Variables Visit 1		OSDI	TMH	Average NIKBUT	corneal staining	conjunctival staining	LWE horizontal	LWE sagittal
TPC	r	0.127	0.152	-0.027	0.003	0.004	-0.155	-0.137
	p-value	0.405	0.318	0.861	0.985	0.978	0.310	0.371
Lysozyme	r	0.100	0.156	-0.012	-0.037	-0.037	-0.147	-0.106
	p-value	0.513	0.305	0.938	0.810	0.810	0.337	0.489
Lipocalin	r	0.087	-0.089#	-0.018	-0.287	-0.007	0.140	0.129
	p-value	0.570	0.562	0.905	0.056*	0.964	0.359	0.400
SIgA	r	0.244	0.094	-0.019	0.173	0.203	-0.104	-0.142
	p-value	0.106	0.540	0.902	0.257	0.181	0.496	0.352
ZAG	r	0.316	-0.181#	-0.065	-0.319	-0.162	0.209	0.066
	p-value	0.142	0.257	0.671	**0.032***	0.288	0.168	0.668
~47 kDa	r	0.180	0.157	0.029	-0.060	0.071	0.008	-0.081
	p-value	0.237	0.304	0.849	0.695	0.643	0.961	0.598
Albumin	r	0.265	0.166	-0.024	0.010	0.141	-0.182	-0.107
	p-value	0.095*	0.299	0.878	0.948	0.354	0.233	0.485
Lactoferrin	r	-0.044	0.197	-0.084	-0.019	-0.064	-0.167	-0.210
	p-value	0.774	0.195	0.581	0.899	0.674	0.272	0.167
~100 kDa	r	0.124	0.086	-0.045	0.082	0.142	-0.033	-0.005
	p-value	0.416	0.576	0.771	0.592	0.351	0.832	0.974
~147 kDa	r	0.141	-0.202	0.174	0.000	-0.077	0.179	0.244
	p-value	0.355	0.184	0.253	1.000	0.615	0.240	0.106
~167 kDa	r	0.072	-0.085	0.127	-0.066	-0.032	0.255	0.115
	p-value	0.637	0.578	0.407	0.668	0.836	0.091*	0.451

*# Pearson's correlation coefficient used as data found to have a normal distribution. * P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

The correlation coefficients for the percentage tear protein concentration and clinical parameters used to classify participants by dry eye status at Visit 1 are reported in ***Table 5.4***.

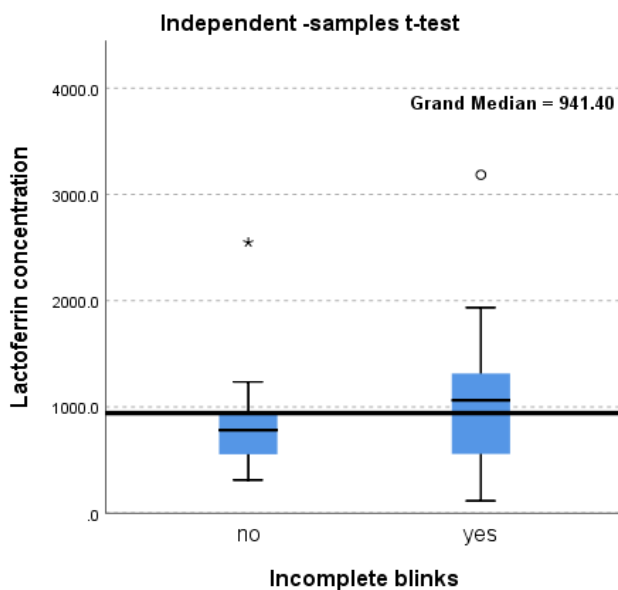
A significant moderate negative correlation was found between the percentage (r=-0.341, p=0.022) of lipocalin with corneal fluorescein staining. A weak negative correlation was also found for the relative concentration (r=-0.287, p=0.056) but this did not reach significance. A positive correlation with horizontal LWE was also found with the percentage of lipocalin (r=0.300, p=0.045). A statistically significant moderate negative correlation between ZAG concentration (r=-0.319, p=0.032) and percentage (r=-0.360, p=0.016), and corneal staining was found.

The percentage of lactoferrin was noted to be moderately negatively correlated with OSDI score (r=-0.296, p=0.048). A near significant correlation was also noted between lactoferrin concentration and TMH (r=0.266, p=0.077). TMH and corneal staining were also found to have small but nearly significant positive correlations with the percentage of the protein with a mass of ~167 kDa.

Table 5.4. Spearman correlation coefficients for the percentage of tear proteins and clinical parameters used to classify dry eye status (Visit 1)

Variables Visit 1		OSDI	TMH	Average NIKBUT	corneal staining	conjunctival staining	LWE horizontal	LWE sagittal
Lysozyme	r	0.097	0.047	0.039	-0.114	0.039	-0.154	-0.030
	p-value	0.524	0.759	0.800	0.454	0.800	0.313	0.843
Lipocalin	r	-0.039	-0.232#	0.113	-0.341	-0.037	0.300	0.289
	p-value	0.801	0.126	0.458	**0.022***	0.811	**0.045***	0.054*
SIgA	r	-0.145	-0.015	0.054	0.147	0.179	-0.032	-0.091
	p-value	0.373	0.921	0.727	0.335	0.241	0.836	0.550
ZAG	r	0.051	-0.201#	-0.066	-0.360	-0.188	0.246	0.144
	p-value	0.817	0.214	0.670	**0.016***	0.221	0.108	0.350
~47 kDa	r	0.071	0.043#	0.034	0.067	0.065	0.112	0.082
	p-value	0.651	0.785	0.826	0.662	0.671	0.462	0.594
Albumin	r	0.232	0.148	0.007	-0.082	0.104	-0.202	-0.121
	p-value	0.144	0.355	0.964	0.590	0.495	0.184	0.430
Lactoferrin	r	-0.296	0.220	-0.029	-0.057	-0.176	-0.211	-0.243
	p-value	**0.048***	0.146	0.848	0.709	0.249	0.164	0.108
~100 kDa	r	0.046	-0.011	-0.021	-0.021	0.139	0.169	0.095
	p-value	0.775	0.945	0.892	0.893	0.362	0.268	0.536
~147 kDa	r	0.046	0.073	-0.021	-0.021	0.139	0.169	0.095
	p-value	0.764	0.634	0.892	0.893	0.362	0.268	0.536
~167 kDa	r value	-0.208	0.253	0.109	0.263	0.047	-0.055	0.057
	p-value	0.169	0.093*	0.475	0.081*	0.759	0.718	0.709

 # Pearson's correlation coefficient used as data found to have a normal distribution. P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*



The presence of incomplete blinks showed a significant association with the mean concentration of lactoferrin (p=0.031) only, see ***Figure 5.1***. Blink rate and ocular redness (bulbar and limbal) showed no significant correlation with any of the protein concentrations or percentages, other than a moderate negative correlation between limbal redness and the concentration of ~167 kDa (r=-0344, p=0.021).

Figure 5.1. In complete blinks and lactoferrin concentration (Visit 1)

The results for subjective meibography parameters are reported in ***Table 5.5***.

Table 5.5. Spearman correlation coefficients for meibomian gland parameters and tear proteins (Visit 1)

Protein			Meibum quality	Meibum expression	Lipid layer thickness	Upper meibography score	Lower meibography score
Total protein concentration		r	-0.198	0.026	0.017	-0.094	-0.177
		p-value	0.192	0.866	0.913	0.540	0.245
Lysozyme	Concentration (mg/ml)	r	-0.208	0.019	0.033	-0.133	-0.157
		p-value	0.171	0.904	0.829	0.384	0.304
	Percentage	r	-0.099	0.202	0.052	-0.121	-0.049
		p-value	0.517	0.183	0.735	0.428	0.748
Lipocalin	Concentration (mg/ml)	r	-0.208	-0.074	-0.117	-0.066	-0.140
		p-value	0.171	0.628	0.446	0.668	0.358
	Percentage	r	-0.149	-0.158	-0.083	-0.141	-0.136
		p-value	0.329	0.299	0.589	0.356	0.372
SIgA	Concentration (mg/ml)	r	-0.076	0.148	0.009	0.162	-0.029
		p-value	0.622	0.332	0.955	0.289	0.852
	Percentage	r	0.052	0.081	0.027	0.203	0.127
		p-value	0.735	0.595	0.861	0.180	0.404
ZAG	Concentration (mg/ml)	r	-0.337	-0.155	-0.008	-0.306	-0.276
		p-value	**0.024***	0.309	0.961	**0.041***	0.066*
	Percentage	r	-0.334	-0.173	-0.014	-0.296	-0.297
		p-value	**0.025***	0.255	0.929	**0.049***	**0.047***
~47 kDa	Concentration (mg/ml)	r	-0.03	0.020	-0.099	-0.003	-0.157
		p-value	0.829	0.889	0.519	0.984	0.304
	Percentage	r	0.229	0.011	-0.078	0.050	-0.016
		p-value	0.130	0.943	0.609	0.745	0.915
Albumin	Concentration (mg/ml)	r	0.043	-0.057	0.163	-0.164	-0.025
		p-value	0.777	0.711	0.286	0.282	0.870
	Percentage	r	0.049	-0.053	0.129	-0.169	0.014
		p-value	0.751	0.730	0.399	0.267	0.930
Lactoferrin	Concentration (mg/ml)	r	-0.212	-0.060	0.008	-0.116	-0.210
		p-value	0.161	0.693	0.957	0.448	0.166
	Percentage	r	-0.146	-0.126	0.051	-0.219	-0.098
		p-value	0.337	0.408	0.739	0.148	0.522
~100 kDa	Concentration (mg/ml)	r	-0.124	-0.030	0.070	-0.036	-0.155
		p-value	0.418	0.842	0.649	0.814	0.311
	Percentage	r	-0.203	-0.094	0.145	-0.016	0.055
		p-value	0.182	0.539	0.343	0.919	0.720
~147 kDa	Concentration (mg/ml)	r	0.184	-0.018	-0.056	-0.018	0.121
		p-value	0.226	0.905	0.713	0.904	0.427
	Percentage	r	0.246	-0.058	0-.035	-0.055	0.138
		p-value	0.103	0.706	0.820	0.721	0.366
~167 kDa	Concentration (mg/ml)	r	0.032	0.040	0.093	-0.017	-0.195
		p-value	0.836	0.793	0.543	0.913	0.200
	Percentage	r	0.059	0.087	0.123	-0.052	-0.191
		p-value	0.699	0.568	0.422	0.735	0.208

**P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

Interestingly, the concentration and percentage of ZAG correlated with meibum quality, upper and lower meibography scores.

Other ordinal data such as that obtained for the MMP-9 results are shown in ***Table 5.6***.

Table 5.6. Spearman correlation coefficients for protein parameters and MMP-9 (Visit 1)

Visit 1		**MMP-9 subjective semi-quantitative grade**	
		correlation coefficient ®	**p-value**
Total protein concentration		0.278	0.066*
Lysozyme	concentration	0.265	0.079*
	percentage	-0.078	0.610
Lipocalin	concentration	0.221	0.146
	percentage	0.002	0.133
SIgA	concentration	0.287	0.056*
	percentage	0.170	0.265
ZAG	concentration	-0.096	0.531
	percentage	-0.093	0.543
~47 kDa	concentration	0.263	0.081*
	percentage	0.151	0.321
Albumin	concentration	0.026	0.866
	percentage	-0.044	0.247
Lactoferrin	concentration	0.244	0.106
	percentage	0.003	0.985
~100 kDa	concentration	0.093	0.543
	percentage	-0.055	0.719
~147 kDa	concentration	-0.131	0.392
	percentage	-0.157	0.304
~167 kDa	concentration	0.145	0.341
	percentage	0.127	0.406

**P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance*

No statistically significant correlations were found between any tear protein and MMP-9 grade. Small positive correlation coefficients were found with TPC, and the concentration of lysozyme, sIgA and ~47kDa; however, they were not quite statistically significant.

When considering differences based on sex (34 females, 11 males), no statistically significant associations were found with the TPC, relative concentration or percentage of the total concentration. Increases were found in the concentration and percentage of sIgA and albumin in the contact lens wearers (30 non-CL, 15 CL wearers), but only reached significance with a medium effect size for sIgA percentage (η^2=0.091, p=0.045). Decreases in ZAG and ~167 kDa were found but the small effect sizes were not significant. The mean percentage and distribution of lipocalin was found to be higher in the participants who reported allergy (n=12) than in those who did not but did not reach statistical significance (p=0.076 and p=0.062, respectively).

Furthermore, no significant associations or correlations were found with participant ethnicity or for time reported using a screen each day. However, correlations between lifestyle factors and certain proteins were found at Visit 1. These included increases in the percentage of lipocalin and ZAG with improved self-perceived health status (r=0.300, p=0.045 and r=0.320, p=0.032, respectively), and higher levels of stress (where 1=high and 4=minimal) with a higher percentage of sIgA (r=-0.298, p=0.047).

5.3.2. Visit 2 tear protein results.

A comparison of the mean and standard deviations of the total protein concentration, the number of peaks and the relative concentrations for each main protein identified according to dry eye status at Visit 2 are reported in ***Table 5.7***.

Table 5.7. Tear protein data from Visit 2

Visit 2	**non-DED participants**		**DED participants**		**Mann-Whitney U test p value**	
Participants	n= 14		n= 25			
Number of peaks	9.57 ± 2.59		9.36 ± 1.68		p= 0.759#	
TPC (mg/ml)	4.76 ± 2.56		5.02 ± 2.82		p=0.774#	
Protein	**Relative conc. mean ± SD (range) (mg/ml)**	**% of total concentration mean % ± SD (range)**	**Relative conc. mean ± SD (range) (mg/ml)**	**% of total concentration mean % ± SD (range)**	**p value for relative conc.**	**p value for % of total conc.**
Lysozyme	2.13 ± 1.33 (0.81 – 4.98)	43.5 ± 6.1 (30.6 – 52.2)	2.00 ± 1.25 (0.74 – 5.90)	40.7 ± 7.5 (24.5 – 53.5)	p=0.849	p=0.237#
Lipocalin	0.25 ± 0.18 (0.02 – 0.72)	5.7 ± 1.9 (2.9 – 8.3)	0.32 ± 0.20 (0.10 – 1.14)	6.8 ± 2.3 (0.0 – 10.2)	p=0.261#	p=0.104
SIgA	0.39 ± 0.41 (0.00 – 1.50)	7.6 ± 5.1 (0.0 – 18.0)	0.54 ± 0.35 (0.07 – 1.27)	10.9 ± 5.0 (4.0 – 22.4)	p=0.237#	p=0.056#*
ZAG	0.17 ± 0.22 (0.00 – 0.67)	3.6 ± 3.7 (0.0 – 11.8)	0.14 ± 0.17 (0.00 – 0.74)	2.6 ± 2.1 (0.0 – 6.1)	p=0.823	p=0.654
~47kDa	0.40 ± 0.28 (0.18 – 1.10)	8.6 ± 2.9 (4.3 – 13.4)	0.44 ± 0.37 (0.00 – 1.59)	8.2 ± 4.4 (0.0 – 14.6)	p=0.683	p=0.762#
Albumin	0.015 ± 0.017 (0.00 – 0.047)	0.4 ± 0.5 (0.0 – 1.6)	0.017 ± 0.020 (0.00 – 0.069)	0.5 ± 0.4 (0.0 – 1.4)	p=0.602	p=0.832
Lactoferrin	1.08 ± 0.47 (0.30 – 2.12)	23.7 ± 5.3 (16.5 – 24.3)	1.03 ± 0.55 (0.43 – 2.84)	21.7 ± 4.0 913.6 – 28.2)	p=0.752#	p=0.197#
~100 kDa	0.16 ± 0.12 (0.00 – 0.41)	3.4 ± 2.1 (0.0 – 5.8)	0.13 ± 0.14 (0.00 – 0.52)	2.8 ± 2.5 (0.0 – 8.4)	p=0.357	p=0.373
~147 kDa	0.02 ± 0.04 (0.00 – 0.12)	0.6 ± 1.0 (0.0 – 2.7)	0.04 ± 0.06 (0.00 – 0.23)	1.0 ± 1.5 (0.0 – 5.2)	p=0.706	p=0.511
~167 kDa	0.01 ± 0.03 (0.00 – 0.11)	0.3 ± 0.3 (0.0 – 0.3)	0.06 ± 0.15 (0.00 – 0.71)	1.0 ± 2.2 (0.0 – 1.0)	p=0.905	p=0.753

#indicates data that passed the KS normality test – parametric unpaired t-test used. near significance*

Fewer protein profiles for the non-DED participants were due to participants either being lost to follow up (n=4), tear analysis results being excluded (n=1) or changes in dry eye status (n=1).

No significant differences were found in the relative concentration or the percentage of the total concentration for any of the proteins between the DED and non-DED participants at Visit 2. A near statistically significant higher percentage of sIgA (p=0.056) was found in the DED participants, but this was not found at Visit 1.

The results of the correlation analysis between the total and relative tear protein concentrations and the clinical parameters used to classify participants by dry eye status at Visit 2 are reported in ***Table 5.8.***

Table 5.8. Spearman correlation coefficients for the total and relative tear protein concentrations and clinical parameters used to classify dry eye status (Visit 2)

Variables Visit 2		OSDI	TMH	Average NIKBUT	corneal staining	conjunctival staining	LWE horizontal	LWE sagittal
Total protein concentration	r p-value	-0.187 0.255	-0.044 0.790	-0.076 0.646	-0.037 0.825	0.001 0.993	-0.023 0.887	0.105 0.524
Lysozyme	r p-value	-0.268 0.099	0.015 0.927	-0.029 0.859	-0.093 0.573	-0.068 0.682	-0.062 0.710	0.146 0.374
Lipocalin	r p-value	-0.038 0.820	0.074 0.653	-0.217 0.185	0.084 0.610	0.132 0.423	-0.021 0.901	0.293 0.070*
SIgA	r p-value	0.000 0.996	-0.073 0.657	0.044 0.790	-0.001 0.997	0.098 0.552	0.179 0.274	0.269 0.098
ZAG	r p-value	-0.147 0.372	0.073 0.660	-0.396 **0.013***	0.061 0.713	0.000 0.998	-0.246 0.131	0.053 0.750
~47 kDa	r p-value	-0.083 0.615	-0.042 0.799	-0.184 0.262	0.224 0.171	0.323 **0.045***	-0.060 0.716	0.160 0.332
Albumin	r p-value	-0.061 0.712	-0.053 0.748	0.046 0.781	0.135 0.412	0.139 0.399	-0.061 0.711	-0.117 0.480
Lactoferrin	r p-value	-0.197 0.229	0.029 0.862	-0.131 0.425	0.026 0.877	-0.043 0.796	-0.129 0.434	0.050 0.762
~100 kDa	r p-value	-0.123 0.457	0.168 0.306	0.120 0.467	-0.288 0.075*	-0.095 0.566	0.039 0.812	0.002 0.992
~147 kDa	r p-value	0.269 0.098	-0.281 0.083*	-0.256 0.115	0.294 0.070*	0.437 **0.005***	0.442 **0.005***	0.085 0.608
~167 kDa	r p-value	-0.061 0.712	0.056 0.736	0.138 0.403	-0.046 0.782	0.073 0.661	0.140 0.395	0.226 0.167

**P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

Significant moderate correlations with protein concentrations were found. ZAG negatively correlated with average NIKBUT (r=-0.396 p=0.013). Conjunctival staining was found to positively correlate with the protein with a mass of~47 kDa (r=0.323, p=0.045) and ~147 kDa (r=0.437, p=0.005). This latter protein also correlated with horizontal LWE (r=0.442, p=0.005). Lipocalin showed a nearly significant correlation with sagittal LWE (r=0.293, p=0.070) as in the Visit 1 results (r=0.289, p=0.054). Other weak

correlations which did not quite reach significance can be seen in ***Tables 5.8***, marked with a red Asterix.

When considering the percentages of the proteins, several statistically significant moderate correlations were noted at Visit 2. These are reported in ***Table 5.9***.

Table 5.9. Spearman correlation coefficients for the percentage of tear proteins and clinical parameters used to classify dry eye status (Visit 2)

Variables Visit 2		OSDI	TMH	Average NIKBUT	corneal staining	conjunctival staining	LWE horizontal	LWE sagittal
Lysozyme	r p-value	-0.312# 0.053*	0.002 0.990	0.104 0.527	-0.299 0.064*	-0.318 **0.049***	0.034 0.839	0.100 0.543
Lipocalin	r p-value	0.145# 0.378	0.133 0.418	-0.377 **0.018***	0.046 0.781	0.231 0.158	0.128 0.439	0.271 0.095*
SIgA	r p-value	0.235# 0.150	0.014 0.931	0.235 0.149	-0.047 0.777	0.143 0.387	0.238 0.144	0.205 0.210
ZAG	r p-value	-0.085 0.608	0.136 0.410	-0.351 **0.029***	0.044 0.792	0.015 0.928	-0.229 0.162	0.003 0.987
~47 kDa	r p-value	-0.032# 0.847	-0.097 0.559	-0.158 0.337	0.349 **0.030***	0.428 **0.007***	-0.014 0.933	0.154 0.348
Albumin	r p-value	0.022 0.893	0.089 0.590	0.009 0.957	0.176 0.285	0.193 0.240	0.014 0.934	-0.100 0.544
Lactoferrin	r p-value	0.073# 0.659	0.065 0.695	-0.196 0.231	0.184 0.262	0.002 0.988	-0.094 0.570	-0.234 0.152
~100 kDa	r p-value	0.042 0.798	0.263 0.106	0.269 0.098	-0.312 0.053*	-0.107 0.516	0.028 0.868	-0.060 0.718
~147 kDa	r p-value	0.246 0.131	-0.243 0.136	-0.122 0.459	0.214 0.191	0.355 **0.027***	0.384 **0.016***	0.003 0.984
~167 kDa	r value p-value	-0.094 0.568	0.009 0.956	0.121 0.465	-0.029 0.860	0.113 0.493	0.133 0.421	0.213 0.193

*# Pearson's correlation coefficient used as data found to have a normal distribution. *P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

Average NIKBUT was found to significantly negatively correlate with the percentage of lipocalin (r=-0.377, p=0.018) and ZAG (r=-0.351, p=0.029). A negative correlation was also found between lysozyme and conjunctival staining (r=-0.318, p=0.049). The protein with a mass of ~47 kDa was noted to positively correlate with corneal and conjunctival staining, r=0.349, p=0.030 and r=0.428, p=0.007, respectively. Conjunctival staining was also found to correlate with the protein with a mass of ~167 kDa (r=0.355,p=0.027) as was horizontal LWE (r=0.384, p=0.016).

The concentration and percentage of the protein with a mass of ~100 kDa had a moderate negative correlation with corneal staining, but this did not quite reach significance for either, r=-0.288, p=0.075 and r=-0.312, p=0.053, respectively.

The results for subjective meibography parameters are reported in ***Table 5.10***.

Table 5.10. Spearman correlation coefficients for meibomian gland parameters and tear proteins (Visit 2)

Protein			Meibum quality	Meibum expression	Upper meibography score	Lower meibography score
Total protein		r	-0.101	-0.263	-0.282	0.019
		p-value	0.541	0.106	0.082*	0.911
Lysozyme	Concentration (mg/ml)	r	-0.177	-0.211	-0.233	0.105
		p-value	0.282	0.198	0.154	0.525
	Percentage	r	-0.047	0.032	-0.132	0.338
		p-value	0.775	0.845	0.424	**0.035***
Lipocalin	Concentration (mg/ml)	r	-0.183	-0.079	-0.202	-0.096
		p-value	0.264	0.632	0.218	0.561
	Percentage	r	0.009	0.239	0.049	-0.165
		p-value	0.954	0.143	0.766	0.316
SIgA	Concentration (mg/ml)	r	0.032	-0.061	-0.221	-0.062
		p-value	0.849	0.710	0.176	0.708
	Percentage	r	0.088	0.161	-0.032	-0.010
		p-value	0.592	0.328	0.849	0.952
ZAG	Concentration (mg/ml)	r	-0.039	-0.143	-0.037	0.083
		p-value	0.816	0.384	0.822	0.617
	Percentage	r	-0.026	-0.022	0.091	0.009
		p-value	0.876	0.894	0.581	0.954
~47 kDa	Concentration (mg/ml)	r	-0.152	-0.006	-0.119	-0.031
		p-value	0.357	0.970	0.471	0.853
	Percentage	r	-0.133	0.170	0.039	-0.067
		p-value	0.421	0.302	0.815	0.686
Albumin	Concentration (mg/ml)	r	0.167	0.047	-0.007	-0.061
		p-value	0.309	0.777	0.965	0.713
	Percentage	r	0.053	0.191	-0.022	-0.040
		p-value	0.749	0.244	0.895	0.811
Lactoferrin	Concentration (mg/ml)	r	-0.126	-0.389	-0.266	-0.045
		p-value	0.444	**0.014***	0.102	0.788
	Percentage	r	-0.054	-0.308	0.040	-0.044
		p-value	0.745	0.056*	0.807	0.792
~100 kDa	Concentration (mg/ml)	r	0.042	-0.142	-0.286	-.015
		p-value	0.801	0.388	0.077*	0.929
	Percentage	r	0.045	0.052	-0.095	0.014
		p-value	0.786	0.753	0.566	0.933
~147 kDa	Concentration (mg/ml)	r	0.212	0.194	-0.028	-0.239
		p-value	0.194	0.237	0.863	0.142
	Percentage	r	0.198	0.194	-0.119	-0.258
		p-value	0.226	0.238	0.471	0.112
~167 kDa	Concentration (mg/ml)	r	0.118	0.088	0.216	0.132
		p-value	0.475	0.594	0.186	0.425
	Percentage	r	0.135	-0.004	-0.265	0.062
		p-value	0.411	0.981	0.103	0.710

** P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

Moderate positive correlations were found between lower meibography score and the percentage of lysozyme (r=-0.338,p=0.035) and lactoferrin concentration, and meibomian gland expressibility (r=0.389, p=0.014). However, these were not the same correlations as those found at Visit 1.

The presence of incomplete blinks showed a significant association with the mean rank of the concentration of lactoferrin (p=0.029) and ~47kDa (p=0.042), being higher when incomplete blinks were present (***Figure 5.2***). As in Visit 1, the mean concentration of lactoferrin was higher when incomplete blinks were present but not statistically significant. Blink rate showed a positive moderate correlation with the percentage of ZAG (r=0.371, p=0.020), but with no other proteins. Ocular redness (bulbar and limbal) showed no significant correlation with any of the protein concentrations or percentages.

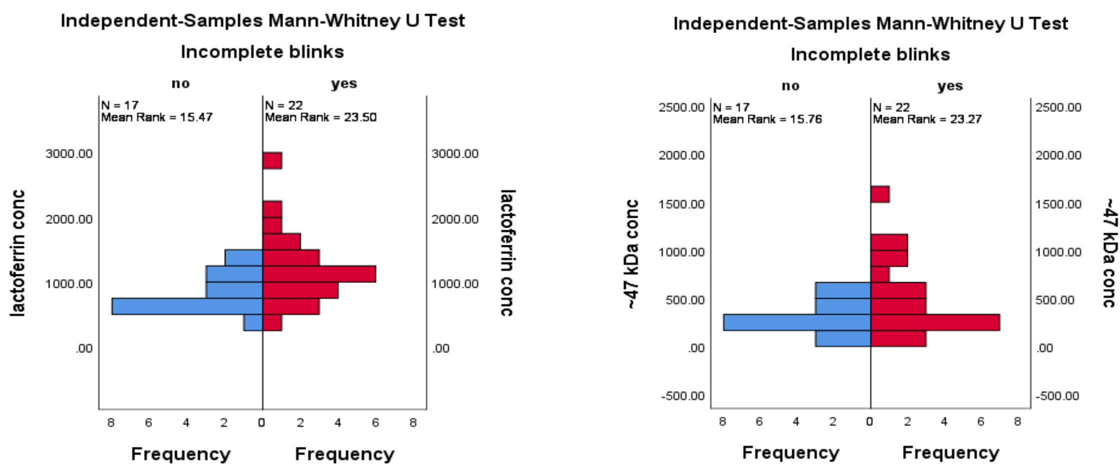


Figure 5.2. Mean ranks of lactoferrin (left) and protein ~47 kDa (right) concentrations

No correlations were found at Visit 2 between MMP-9 semi-quantitative grading and any of the tear proteins. See ***Table 5.11.***

Table 5.11. Spearman correlation coefficients for protein parameters and MMP-9 (Visit 2)

Visit 2		MMP-9 subjective semi-quantitative grade	
		correlation coefficient ®	p-value
Total protein concentration		0.055	0.740
Lysozyme	concentration	0.063	0.703
	percentage	0.113	0.492
Lipocalin	concentration	-0.035	0.831.
	percentage	-0.019	0.909
SIgA	concentration	0.071	0.666
	percentage	0.120	0.467
ZAG	concentration	-0.065	0.694
	percentage	-0.122	0.459
~47 kDa	concentration	-0.016	0.923
	percentage	-0.037	0.821
Albumin	concentration	0.026	0.875
	percentage	0.016	0.923
Lactoferrin	concentration	-0.014	0.931
	percentage	-0.093	0.574
~100 kDa	concentration	0.061	0.711
	percentage	-0.074	0.652
~147 kDa	concentration	0.149	0.365
	percentage	0.089	0.591
~167 kDa	concentration	0.039	0.814
	percentage	0.057	0.730

When considering differences based on contact lens wear (23 non-CL, 16 CL wearers), sex (29 females, 10 males), and between those who reported allergy (n=9) and those who didn't (n=30), no statistically significant associations were found with any of the tear protein parameters. As in Visit 1, increases in sIgA and albumin were found in CL wearers, but the small effect sizes found were not significant. Small but not significant decreases in ZAG and ~167 kDa in the CL wearers were also repeated but not significant. The distribution of the percentages of lipocalin and ~47kDa were greater in females but did not reach statistical significance (p=0.079 and p=0.059, respectively). The mean concentration of the protein with ~100 kDa mass was also higher in females but did not reach significance (p=0.054). The only association found between ethnicity and any protein parameter was for the percentage of lipocalin being above 5.5% in significantly more southern Asian participants (p=0.002).

As at Visit 1 no significant correlation with any of the tear protein parameters and screen time hours were found. However, the correlations with self-perceived health quality found at Visit 1 were repeated at Visit 2. These included increases in the percentage of lipocalin and ZAG (r=0.321, p=0.046 and r=0.349, p=0.029, respectively), but also with the concentration of ZAG (r=0.322, p=0.046), confirming the significance further.

5.3.3. Visit 1 and 2 protein comparison.

A comparison of the relative concentrations of the main proteins identified, and their percentages of the total protein concentration, between visits, and for all participants is shown in ***Table 5.12***. ***Table 5.13*** provides a similar comparison, but only for the participants where matched pairs of data were obtained.

Table 5.12. Comparison of tear protein parameters for all participants between visits

	Visit 1		Visit 2		Mann-Whitney U test p value	
Participants	n=45		n=39			
Total protein concentration (µg/µl)	4.53 ± 2.42		4.93 ± 2.70		p=0.518	
Protein	**Relative conc. (µg/µl) (range)**	**% of total concentration (range)**	**Relative conc. (µg/µl) (range)**	**% of total concentration (range)**	**p value for relative conc.**	**p value for % of total conc.**
Lysozyme	1.81 ± 1.07	39.1 ± 6.9	2.05 ± 1.26	41.7 ± 7.1	p=0.425	p=0.088#*
Lipocalin	0.28 ± 0.16	6.2 ± 2.1	0.32 ± 0.23	6.4 ± 2.2	p=0.414	p=0.720#
SIgA	0.53 ± 0.42	10.9 ± 4.3	0.49 ± 0.37	9.7 ± 5.2	p=0.660	p=0.243#
ZAG	0.12 ± 0.17	2.3 ± 2.6	0.15 ± 0.19	2.9 ± 2.8	p=0.237	p=0.299
~47kDa	0.45 ± 0.32	9.8 ± 4.6	0.42 ± 0.34	8.3 ± 3.9	p=0.459	p=0.096*
Albumin	0.02 ± 0.02	0.4 ± 0.3	0.02 ± 0.02	0.4 ± 0.5	p=0.812	p=0.900
Lactoferrin	1.01 ± 0.57	23.3 ± 4.7	1.05 ± 0.51	22.4 ± 4.5	p=0.493	p=0.898#
~100 kDa	0.16 ± 0.15	3.3 ± 2.4	0.15 ± 0.13	3.0 ± 2.4	p=0.849	p=0.625
~147 kDa	0.04 ± 0.06	1.1 ± 1.6	0.03 ± 0.06	0.9 ± 1.3	p=0.362	p=0.393
~167 kDa	0.05 ± 0.10	1.0 ± 1.4	0.04 ± 0.12	0.7 ± 1.8	**p=0.049***	**p=0.044***

*# indicates data that passed the KS normality test – parametric unpaired t-test used. * P-values <0.05 were considered significant and are shown in bold. * near significance.*

The overall percentage of lysozyme was higher at Visit 2, and significantly so when paired data was assessed (p=0.019). The coefficient of repeatability was 11.0, higher than that found in the pilot study (coefficient of repeatability 8.38, limits of agreement -6.62 to 10.2) reported in chapter 3. A decrease

in the percentage of ~47kDa was found, which again was significant only when comparing matched data (p=0.020); the coefficient of repeatability was 5.9 (4.96 in the pilot study, with limits of agreement -5.50 to 4.42). A significant decrease in the concentration and percentage of the tear protein with a mass of ~167 kDa was found at Visit 2 (p=0.049 and p=0.044, respectively) when all the data was considered. However, this was not repeatable when matched pairs were used.

Table 5.13. Pairwise comparison of the tear protein parameters for participants at both visits

	Visit 1		**Visit 2**		**Wilcoxon paired t-test p value**	
Participants	n=35		n=35			
Total protein concentration (µg/µl)	4.77 ± 2.37		4.78 ± 2.68		p=0.967	
Protein	**Relative conc. (µg/µl) (range)**	**% of total concentration (range)**	**Relative conc. (µg/µl) (range)**	**% of total concentration (range)**	**p value for relative conc.**	**p value for % of total conc.**
Lysozyme	1.90 ± 1.00	39.2 ± 6.7	1.96 ± 1.22	41.6 ± 7.0	p=0.812	**p=0.019*#**
Lipocalin	0.29 ± 0.16	6.3 ± 2.2	0.30 ± 0.19	6.6 ± 2.2	p=0.708	p=0.296
SIgA	0.56 ± 0.44	11.0 ± 4.3	0.46 ± 0.33	9.5 ± 4.9	p=0.116	p=0.070* #
ZAG	0.12 ± 0.18	2.4 ± 2.6	0.14 ± 0.17	2.8 ± 2.4	p=0.491	p=0.339
~47kDa	0.46 ± 0.32	9.5 ± 4.1	0.40 ± 0.32	8.5 ± 3.8	p=0.098	**p=0.020***
Albumin	0.02 ± 0.02	0.38 ± 0.40	0.02 ± 0.02	0.35 ± 0.43	p=0.837	p=0.768
Lactoferrin	1.09 ± 0.59	22.7 ± 4.7	1.03 ± 0.53	22.7 ± 4.6	p=0.362	p=0.927#
~100 kDa	0.17 ± 0.16	3.1 ± 2.5	0.13 ± 0.14	2.9 ± 2.4	p=0.303	p=0.468
~147 kDa	0.04 ± 0.06	1.1 ± 1.6	0.03 ± 0.06	0.73 ± 1.19	p=0.683	p=0.243
~167 kDa	0.06 ± 0.11	1.0 ± 1.5	0.05 ± 0.13	0.8 ± 1.9	p=0.375	p=0.482

*# indicates data that passed the KS normality test – parametric paired t-test used. * P-values <0.05 were considered significant and are shown in bold. * near significance.*

5.4. Discussion

The electrophoretic assessment of tears employed in this study has permitted an objective assessment of the relationship between certain ocular surface signs and tear protein profiles in an attempt to determine DED biomarkers in a young adult cohort.

The results identify that certain tear proteins can be used as potential DED biomarkers, such as lactoferrin and ZAG which significantly decreases, whereas albumin increases with increasing signs of DED. ZAG, a protein thought to be involved in the breakdown of lipids, is identified as a prime candidate with several significant negative correlations with key DED signs such as NIKBUT, corneal staining, meibomian gland dropouts and meibum quality. Lactoferrin, a previously known candidate also showed negative correlation with OSDI scores and meibum expression. The results underpin the importance of monitoring the kinetics of five key tear proteins: ZAG, lipocalin, lactoferrin, albumin and lysozyme, which could be used as a combined biomarker for diagnosis and prognosis of DED.

Ten proteins were identified in the tear protein profiles of this young adult cohort. The identity of six (lysozyme, lipocalin, sIgA, ZAG, albumin and lactoferrin) have previously been reported (see Chapter 3); the identity of four remain uncertain and were therefore reported according to their molecular weight (~47, ~100, ~147 and ~167 kDa). While the percentage frequency of the main lacrimal proteins (lysozyme, lipocalin and lactoferrin) was high, the unidentified proteins ~100, ~147 and ~167 kDa were present less frequently.

The total protein concentration of basal tears, as collected here, has been reported to range from 3.5 to 9.5mg/mL, dependent on the methods used (Craig et al., 2013). In this study, the TPC found at Visit 1 was 4.53 ± 2.42 mg/mL, and 4.93 ± 2.70 mg/mL at Visit 2; no significant differences were found based on dry eye status. These TPCs are lower than that reported in chapter 3 of this thesis (6.72 ± 3.56 mg/mL), which included 10 participants, only three of which had DED. Previously, lower total protein concentrations have been reported in subjects with early DED (Versura et al., 2013). However, the mean age of the participants was higher than in this study (47.6±16.1 in the females and 49.9±15.2 in the males) and the type of DED was not specified. Also, some of the earliest collected samples from Visit 1 had been stored at -80° C for up to 18 months before analysis, whereas all the samples from the pilot study were analysed within two months of collection. Previously, when investigating the most abundant tear proteins (lactoferrin, sIgA light and heavy chains, albumin, lipocalin and lysozyme), long-term storage (>4 months at -70 °C) has been shown to reduce the TPC (Sitaramamma et al., 1998).

No significant differences in the concentration or percentage of the TPC were found between the two visits for any of the individual proteins. Although more than half of the participants in this cohort were

classified as having DED, it was observed that there was at least one ocular surface sign in 85% of the non-DED participants at Visit 1 and 86% at Visit 2; this overlap of DED signs may account for there being no correlations between the TPC and any of the clinical parameters.

When considering only the 35 protein profiles that were repeatable between visits, the percentage of the TPC for lysozyme and ~47 kDa protein were significantly different between visits. However, the coefficient of repeatability for the two proteins were greater than those found in the pilot study; lysozyme percentage was also outside the limits of agreement. The findings in the pilot study were based on a sample size of 10, and therefore comparison of the results from this study should be treated with caution.

ZAG was reduced in the DED participants in this study. It was absent or below the level of detection in more DED participants than non-DED participants at Visit 1 (p=0.009); this was not found at Visit 2. The relative concentration and percentage were also lower in the DED participants at Visit 1 and 2, not quite reaching significance at Visit 1 (p=0.082 and p=0.060, respectively). Previously recognised and validated by Versura et al. (2010), no significant differences were found in its concentration when comparing subjects with or without EDE. However, the lower levels in DED found here do agree with their later findings (Versura et al., 2013).

At Visit 1 several significant correlations were found; in agreement with the 2013 findings (Versura et al., 2013) the concentration and percentage of ZAG correlated negatively with corneal staining (r=-0.319, p=0.032, and r=-0.360, p=0.016, respectively). Correlations were also found with lower meibum quality (r=-0.337, p=0.024 and r=-0.334, p=0.025) and greater meibography loss (r=- 0.306, p=0.041 and r=-0.296, p=0.049) with lower concentrations and percentages of ZAG. Lower lid gland loss also correlated with ZAG percentage (r=-0.297, p=0.047) but not significantly with the concentration (r=-0.276, p=0.066). Although none of these correlations were repeated at Visit 2, it is interesting that this protein was found to correlate with several signs of MGD, which may support a previous suggestion that ZAG may play a role in managing the lipids in tear secretions (Versura et al., 2013). It is also interesting that a repeatable positive correlation was found between the concentration and percentage of ZAG and self-perceived health quality at both visits.

At Visit 2 a significant negative correlation between the mean concentration and percentage of ZAG with the average NIKBUT was found (r=-0.396, p=0.013 and r=-0.351, p=0.029 respectively); as the tear breakup time worsened the concentration and percentage of ZAG increased. However, this is contrary to expectation and previous findings (Versura et al., 2013). There was also a positive correlation with blink rate at Visit 2 only (r=0.371, p=0.020). As increased blink rate and low TBUT are

known to correlate with each other (Su et al., 2018), corresponding changes in the ZAG parameters might be expected, as were found here.

A significantly higher composite meibography score (upper and lower combined), indicating greater gland loss, was also found in the participants where ZAG was absent or below the level of detection (p=0.006) at Visit 1. The mean composite meibography score was 2.04 ± 1.36 (n=22) when ZAG was present and 3.45 ± 1.85 (n=22) when ZAG was absent; again, this was not found at Visit 2, where ZAG was found in 26 participants, and absent in 13. The specific role of ZAG is still uncertain but given its suggested role in lipid breakdown in fat storage cells (Versura et al., 2012) these findings could implicate ZAG as a potential biomarker for EDE. However, further research with a larger cohort would be required to confirm this finding.

The concentrations of the main lacrimal proteins have previously been reported to range between 0.7 - 3.0 mg/mL (lysozyme), 0.5 - 3.5 mg/ml (lipocalin) and 0.7 - 4.0 mg/mL (lactoferrin) dependent on tear collection method (Craig et al., 2013;Fullard and Tucker, 1991;Sack et al., 1992). In this study the concentrations fall within these ranges, except for lipocalin which was lower at both visits (0.28 ± 0.16 mg/mL at Visit 1 and 0.32 ± 0.23mg/mL at Visit 2).

The concentration and percentage of lipocalin was very similar between the DED and non-DED participants at Visit 1 and were higher in the DED participants at Visit 2, but not significantly. Previously the percentage of lipocalin was found to be reduced in ADDE, EDE and mixed DED (Perumal et al., 2016). Lower mean concentrations of lipocalin have also been reported in subjects with obstructive and seborrheic MGD when compared to normal controls (Yamada et al., 2005), and in EDE (Versura et al., 2010).

However, ocular surface signs consistent with a DED diagnosis were found to correlate with lipocalin. At Visit 1 negative correlations were found between the mean concentration and percentage of lipocalin with corneal staining (r=-0.287, p=0.056 and r=-0.341, p=0.022, respectively). A positive correlation between percentage of lipocalin and horizontal LWE (r=0.300, p=0.045) was also found at Visit 1. At Visit 2 a significant negative correlation for lipocalin percentage with NIKBUT was noted (r=-0.377, p=0.018). This appears to be contrary to the findings of previous studies where a positive correlation between tear film breakup time and lipocalin concentration were reported (Yamada et al., 2005;Versura et al., 2013). Yamada et al. (2005) suggested that deficiency of lipocalin, one of whose functions is the binding and release of lipids, may be a predisposing factor for the expression of symptoms in MGD. The negative correlation with corneal fluorescein staining scores found at Visit 1, however, agrees with both studies.

The association between higher mean percentage and distribution of lipocalin and allergy, found at Visit 1, was interesting and may have been statistically significant had there been a larger sample size. Previously there does not appear to be a study which has linked them, and so further investigation may be warranted given its anti-inflammatory role.

Lactoferrin accounted for 23.3 ± 4.7% of the TPC at Visit 1 and 22.4 ± 4.5% at Visit 2. The mean concentrations were 1.01 ± 0.57 mg/mL (Visit 1) and 1.05 ± 0.51mg/mL (Visit 2). Its percentage of the TPC was found to be lower in the DED participants at both visits, but significantly so at Visit 1 (p=0.009). This agrees with a previous study which reported lower levels of lactoferrin (and lipocalin) in subjects with evaporative DED (Versura et al., 2010). This was not statistically significant at Visit 2 or when the data for all participants between the two visits was compared. The percentage of lactoferrin was found to correlate negatively with the OSDI questionnaire score at Visit 1 only (r=-0.296, p=0.048, also in agreement with a previous study (Versura et al., 2013).

At both visits the mean concentration of lactoferrin was higher when incomplete blinking was present and reached significance at Visit 1 (p=0.031). The concentration of lactoferrin had a moderate, negative correlation (r=-0.389, p=0.014) with the percentage of glands secreting meibum from the lower lid, at Visit 2, but not the quality; the concentration was lower when fewer glands were expressing. Indeed, lower tear lactoferrin concentration, along with greater signs of DED and symptoms of allergy, have previously been associated with the inflammation and discomfort found in patients with MGD (Chao and Tong, 2018). It was suggested that lower meibomian gland expressibility, but not gland loss, potentially damages the adjacent palpebral structures, which may induce inflammation in accessory lacrimal glands and reduce lactoferrin secretion.

The relative concentration and percentage of lysozyme did not differ significantly between DED and non-DED participants at either visit of this study; a small but not significantly higher percentage (p=0.088) was found at Visit 2, which was significant when matched pairs were considered (p=0.019). While some studies have found a decrease in lysozyme levels in subjects with ADDE, EDE and MGD (Di Zazzo et al., 2019), others who aimed to specifically investigate EDE, as in this study, have not (Versura et al., 2010).

Conjunctival staining was noted to correlate with contact lens wear (r=0.349, p=0.019) at Visit 1 (see Chapter 4). A moderate, negative correlation between lysozyme percentage and conjunctival staining was found at Visit 2 (r=-0.318, p=0.049), with a near but not significant correlation with corneal staining also (r=-0.299, p=0.064). This agrees with the findings of Versura et al. (2013) for conjunctival staining (r=-0.16, p=0.01) and corneal staining (r=-0.28 p=0.0001). A further significant correlation

(r=0.338, p=0.035) between lysozyme percentage and lower meibography score was noted, but only at Visit 2.

Albumin was found significantly less frequently in the non-DED participants at Visit 1. Significantly higher levels were noted in the DED participants at Visit 1 (p<0.001 for relative concentration and p=0.003 for percentage), in agreement with previous studies (Versura et al., 2010;Runström et al., 2013;Versura et al., 2013). Smaller, non-significant differences were found at Visit 2.

In this study tear samples were collected between the hours of 10.30am and 5.30pm. Diurnal variations in the concentration of albumin have previously been observed, with the lowest but most consistent levels 2-6 hours post waking (Runström et al., 2013). However, this was not specifically controlled for in the study reported here. No association with contact lens wear was noted, although increased levels have previously been reported in CL-related problems (Nichols and Green-Church, 2009). Furthermore, no correlations with clinical parameters were found in this study.

Secretory immunoglobulin A was found in all the participants at Visit 1 and 95% (n=37) at Visit 2. Concentrations of 0.53 ± 0.42mg/mL and 0.49 ± 0.37 mg/mL were found at Visit 1 and 2 respectively. These are lower than the concentrations of 0.9 ± 0.1 and 6.7 ± 1.2 mg/mL which have been reported in previous studies by Willcox et al. (2019) although the methods of collection and analysis were not reported. Higher percentages of sIgA were found in the non-DED participants in this study than in a previous study including healthy young adults (<27 years) which used the same method of collection and analysis as reported here (Mann et al., 2020).

Higher levels of sIgA were found in the DED participants at both visits but did not reach statistical significance; Visit 2, p=0.056. Although the levels of sIgA are thought to be increased in DED, no consistency has been found among studies (Narayanan et al., 2013).

A higher percentage of sIgA was also found in those with higher self-reported stress levels (p=0.047) at Visit 1. As previously reported in Chapter 4, the DED participants were significantly more likely to have high or moderate stress levels (p=0.02). A positive correlation (r=0.287, p=0.056) between sIgA concentration and the inflammatory biomarker MMP-9 was also found at Visit 1, although it did not reach significance. Given its suggested protective role in inflammation (Sack et al., 1992) a correlation would seem logical; however, this was not repeated at Visit 2.

An unidentified protein with a mass of ~47 kDa was found repeatedly in an average of 93% of participants, with no statistically significant differences in its concentration or percentage between the visits or dry eye status. Previously there has been speculation that this could be a lipocalin dimer (Mann, 2007). It has also been suggested as heavy chain IgA (Giannaccare et al., 2016); however, its

presence does not correlate with SIgA light chain, and therefore this is unlikely, with the first suggestion more probable.

At Visit 2, where the DED participants (n=25) outnumbered the non-DED participants (n=14) significant positive correlations were found with conjunctival and corneal staining. The presence of incomplete blinks showed a significant association with the mean rank of the concentration of the ~47kDa protein (p=0.042) but not the mean concentration, at Visit 2. Further research into the exact identity and role of this protein is warranted.

A protein with a molecular weight of ~100kDa (study range-98.4-103.6 kDa) was identified in 76% of the participants at Visit 1 and 69% at Visit 2. Although the concentrations and percentages were higher in the DED participants at both visits, they were not statistically significant, and no correlations were found with clinical parameters. The identity of this protein is not certain, but based on previous findings it could be a shoulder peak of lactoferrin, occurring during the electrophoretic separation of the proteins (Versura et al., 2013).

Two further protein peaks, ~147 kDa and ~167 kDa, were identified with less frequency in both the DED and non-DED participants at both visits. The protein peak found between 144.7-149.2 kDa has previously been reported by (Mann, 2007), who suggested it could be a dimer of sIgA. However, they also suggested that IgG, whose molecular weight is in the same region (theoretical mass 150kDa) may be masked by the higher concentration of the sIgA dimer. Mean concentrations of 0.03 ± 0.05 mg/mL and 0.02 ± 0.04 mg/mL were found in the in the non-DED participants at Visit 1 and 2 of this study. These concentrations are similar to the median concentration of IgG (0.03mg/mL (IQR 6.6–76.1)), reported in the tears of healthy subjects, collected with capillary tubes (Bachhuber et al., 2021).

Both the mean concentration and percentage of the ~147 kDa protein found at Visit 2 showed a significant positive correlation with conjunctival staining and horizontal LWE, both indicators of inflammation. No significant findings were found in relation to the protein peak found at ~167 kDa and any clinical parameter.

Significant progression of four clinical parameters (blink rate, limbal and bulbar redness and sagittal LWE) were found at Visit 2 and reported in Chapter 4. However, no correlations were identified with any of the tear proteins.

Nevertheless, several interesting correlations have been found between the tear proteins identified and the clinical parameters measured. As shown in the previous chapter, a significant amount of overlap of clinical features, such as meibomian gland loss, was found between the DED and non-DED participants. At Visit 2, a higher proportion of the tear sample results were from DED participants

(64%) than at Visit 1 (56%), meaning the study was underpowered for comparing the difference between the two groups at Visit 2. A larger sample size would improve the statistical power of the study, particularly for the less abundant proteins. Also, the number of tear protein profiles obtained was reduced compared to the number of participants initially enrolled in the study. This was due in part to difficulty obtaining tear samples from a couple of participants, but also due to a reduction in the participant numbers at Visit 2.

A further limitation of the study was the unknown identity of four of the proteins. In order for identification and validation, the proteins of interest would need to be located by gel electrophoresis, excised, and extracted for analysis by mass spectrometry. The proteins could then be identified based on their amino acid sequence. This work was beyond the scope of this current study but could prove to be of great interest, particularly for determining the identity of the protein ~147 kDa.

Going forward, a larger cohort study to determine the repeatability of these findings would add weight to their validity in detecting EDE in the early stages. Although this young cohort was followed over a one year period, a longitudinal study over a longer 5-10 year period would enable monitoring of the clinical parameters and tear protein profiles for change, should more severe signs and symptoms develop.

5.5. Conclusion

In summary, this chapter has shown several interesting differences between the tear protein profiles of young adults with and without DED. Given its role in the binding and release of lipids, the unchanged levels of lipocalin in the early EDE participants warrants further investigation, with more advanced cases of evaporative DED included. The repeatable up-regulation of albumin, in agreement with previous studies, adds to the evidence implicating inflammation in the early pathogenesis of DED. Although the downregulation of lactoferrin has been used as a biomarker for ADDE in older patients, this appears to be the first study documenting downregulation in early EDE, and its association with the number of meibomian glands expressing in early disease warrants further investigation. Furthermore, the downregulation of ZAG and its remarkable correlation with signs of evaporative DED during this longitudinal study, suggest it as a potential biomarker in early disease.

The following chapter reports on cytokine and chemokine analysis of tears collected by Schirmer strips, which also took place during the prospective, longitudinal study of evaporative DED in the young adult cohort, whose clinical findings are reported in chapter 4.

Chapter 6 - Prospective, longitudinal study assessing dry eye among young adults - tear cytokine results

6.1. Introduction

The majority of the tear protein concentration is made up of a few high abundance proteins (mg/ml to µg/ml), as investigated in the previous chapter. Cytokines and chemokines secreted by ocular surface tissue or cell signalling molecules, however, tend to be less abundant (ng/ml to pg/ml) (Zhou and Beuerman, 2012). While some have molecular weights within the detectable range of the Agilent 2100 Bioanalyzer when used in conjunction with the Protein 230 LabChip® kit, other proteins such as fractalkine, IL-8 and EGF are lower and out of range. Irrespective of molecular weight, cytokines and chemokines were not present in high enough quantities to be detected with the Bioanalyzer and therefore it was necessary to employ a different method of analysis to detect them.

Tears collected by Schirmer strips during the study reported in the preceding two chapters, were analysed for cytokines and chemokines, potential biomarkers of inflammation. Inflammation is known to play a key role in the pathogenesis of the ocular surface disease that develops in dry eye (Bron et al., 2017); tear hyperosmolarity stimulates a cascade of events in the ocular surface epithelial cells due to activation of the innate inflammatory pathways. This leads to the up-regulation of inflammatory cytokines produced by the infiltrating T-helper cells, ocular surface epithelial pathology and the ensuing vicious cycle of inflammation.

The normal tear film contains several pro- and anti-inflammatory cytokines which play an integral role in the coordination and maintenance of inflammatory processes (Carreño et al., 2010). Cytokines are a group of small, soluble proteins or peptides that are produced by a variety of cells such as T cells. Cytokines participate in the immune response and are important mediators in the complex communication network of the immune system (Khalifian et al., 2015). Chemokines, or chemotactic cytokines, such as IL-8/CXCL8 and fractalkine/CX3CL1, regulate the recruitment and movement of cells (leukocytes) from the blood to ocular surface cells by a process called chemotaxis (Thakur and Willcox, 1998).

Ten proteins, eight cytokines and two chemokines, were selected for analysis based on their previously reported roles in the inflammatory response in DED (see Chapter 1). IL-10 and IL-1Ra were selected due to their anti-inflammatory roles, while IL-1β, IL-6, IL-12p70, EGF, TNF-α, VEGF, IL-8/CXCL-8 and fractalkine/ CXC3L1 were selected due to their pro-inflammatory actions (Roda et al., 2020). IL-17 and IFN-γ were not selected based on low detection levels (13% and 30%, respectively) in a previous study investigating evaporative DED (Enríquez-de-Salamanca et al., 2010).

As yet few studies have reported on the tear proteome in early evaporative DED or in young adults with DED. Neither does there appear to be any report of longitudinal data pertaining to cytokine levels

in the tears. Therefore, this study explores whether tear cytokine analysis could be more sensitive as a disease marker than traditional ocular signs and symptoms in early DED. The results of the tear cytokine and chemokine analysis performed during the prospective, longitudinal, observational, single centre study described in Chapter 4 are reported here.

6.1.1. Study objectives

The study reported here, which forms part of the study reported in Chapter 4, aimed to investigate tear cytokines and chemokines in an 18-25 year old cohort. Furthermore, the study aimed to identify any correlations with the clinical parameters previously reported in Chapter 4.

Null hypotheses:

- There are no differences in tear cytokines or chemokines when comparing young evaporative DED to non-DED participants.
- There are no associations between specific tear cytokines or chemokines and severity of evaporative DED in a young population.
- There would be no change or progression in the tear cytokines or chemokines over the one year study period.

6.2. Methods

Tear samples were collected with Schirmer strips and analysed by Luminex Assay following the technique detailed below. This was done in collaboration with colleagues at the Department of Ophthalmology, Oslo University Hospital, Norway.

6.2.1. Tear collection with Schirmer strips

Tear samples were collected in accordance with Aston University's Ophthalmic Vision Science Research Group (OVSRG) Standard Operating Procedure (A07) for Schirmer-1 test (without anaesthesia) (see **Appendix 12**). The protocol provided by Tor Utheim (Department of Ophthalmology, Oslo University Hospital, Norway) was also followed to ensure appropriate collection and storage (see **Appendix 13**) prior to cytokine analysis by multiplex immunoassay (Landsend et al., 2018). Schirmer strips were used for the purpose of collecting tears only, rather than diagnosis of ADDE. Schirmer scores are not included in the TFOS DEWS II diagnostic criteria, as the test is considered unnecessarily invasive and has been shown to lack accuracy and reproducibility in less than severe cases of DED (Nichols et al., 2004a).

Briefly, tears were collected using ink free Schirmer strips (Haag-Streit, Essex, UK) from the right eye only. Schirmer strips were placed over the lid margin in the lateral third of the lower lid and kept in place for 5 min while the participants closed their eyes. The strips were then removed and transferred to 1.5ml Eppendorf® tubes (Eppendorf, Germany) containing 250µl PBS. Schirmer wetting values were recorded on the tubes in millimetres and the samples were transferred to storage at -80°C. As with capillary tear collection, gloves and tweezers were used to avoid contamination.

6.2.2. Analysis of tear cytokines

The tear samples were prepared and analysed in accordance with the manufacturer's protocol using a customized 10-plex Luminex Human Discovery Assay (LXSAHM-10) (R&D Systems, Biotechne, Abington, UK) at the Department of Ophthalmology, Oslo University Hospital, Norway. The cytokines included were IL-1β, IL-1Ra, IL-6, IL-10, IL-12p70, EGF, TNF-α, VEGF, IL-8/CXCL8 and fractalkine/CX3CL1.

The Luminex system allows for the rapid, simultaneous detection of several analytes in a single 96-well plate, using small volumes of tears (VanDerMeid et al., 2011). The multi-analyte assay system technology is based on the principles of flow cytometry; the passage of cells in single file in front of a laser so they can be detected and quantified (VanDerMeid et al., 2011). Luminex assays enable the measurements of cytokines by utilizing hundreds of specially prepared magnetic microbeads or microspheres, which are internally dyed with a graded mixture of red or infrared fluorescent dyes giving them individual fluorescent profiles (Khalifian et al., 2015). Capture/detection antibodies specific for each cytokine are present on different bead sets in each well and are also conjugated with a reporter dye, which provides the microsphere with an additional distinct fluorescent emission signature when it binds the cytokine of interest (Tighe et al., 2013a). The amount of reporter dye bound to the microsphere is dependent on the concentration of the particular cytokine in the tear sample and is excited by a green laser. Therefore, the light emitted from the dyes can be measured and used for quantitative analysis.

This method is considered to offer the benefits of enzyme-linked immunosorbent assay (ELISA), but with the advantages of high throughput, high sensitivity, smaller sample volumes, and lower cost, which are suitable for multiple samples analysis (Zhao et al., 2018). While ELISA and multiplex are based on a similar concept of capturing proteins of interest with the use of relevant antibodies, the large surface area offered by microarray beads allows for more potential binding sites of analytes (Hagan and Tomlinson, 2013). The method has been shown to correlate well with individual ELISA for several of the cytokines reported in this study (IL-6, IL-8, IL-12P70, and TNF-γ) (Zhao et al., 2018). A

good correlation between ELISA and multiplex for IL-10 and TNF-α was also demonstrated in small volumes of tear samples in childhood and asthma studies (Bomert et al., 2011).

6.2.2.1. Sample preparation and assay procedure

The samples were allowed to thaw and left on ice during preparation. Each sample was vortexed and spun down at 16000g for 10 minutes at 4°C. To obtain a dilution factor of two, 70 µl of each sample was added to a tube containing 70 µl of a calibrator diluent (RD6-52; (R&D Systems, Bio-techne, Abington, UK) containing carrier protein. Each individual sample was analyzed in the same plate in duplicate**.** In total three plates were needed to analyze all the samples, with samples from Visit 1 and 2 for the same participant analyzed on the same plate. Internal in-house spiked controls were included and used to determine the intra- and inter coefficient of variation expressed as a percentage (% Coefficient of Variation). Spiked samples contain a known quantity of analyte in order to validate and determine the recovery of the analytes (Denisin et al., 2012).

Four standards of different concentrations (see ***Table 6.1***) were prepared from the standards supplied, which contained each of the ten analytes. A colour-coded microparticle cocktail of pre-coated analyte-specific antibodies was also supplied in the kit.

Table 6.1. Details of the standard cocktail concentrations supplied

Analyte	Bead Region	Standard pg/ml	Sample dilution	Cocktail µL
Il-1β	28	4,430	1:1	P333154 Standard A 225µl P327999 Standard B 250µl P356687 Standard C 225µl P303242 Standard L 250µl
IL-1Ra	30	5,620		
IL-6	13	370		
IL-8/CXCL8	18	1030		
IL-10	22	460		
IL-12p70	56	37,580		
Fractalkine /CX3CL1	46	162,560		
EGF	25	2,020		
TNF-α	12	1,800		
VEGF	26	1,990		

This was added to each well of the micro-assay plate, after addition of a sample or standard. The plate was then covered to protect the microparticles from light and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12” orbit) set at 800 ± 50 rpm. After incubation, each well was washed three times with 100 µl of wash buffer using an automated plate-washer incorporating a magnetic device to ensure no loss of the microparticles. The next step involved the addition of 50 µl of a cytokine specific labelling Biotin-Antibody cocktail to each well. This was covered and incubated for 30 minutes at room temperature on the shaker set at 800 ± 50 rpm.

Following three further washes, 50 µL of diluted Streptavidin-PE (used to aid in detection) was added to each well and the previous incubation step repeated. Prior to reading of the plate, the wash step was repeated, and the microparticles resuspend in 100 µL of Wash Buffer and incubated for 2 minutes on the shaker set at 800 ± 50 rpm.

The mean fluorescent intensity (MFI) for each tear cytokine was measured with a Luminex IS 200 (Bio-Rad laboratories, Inc., Hercules, CA, USA). Standard curves were generated from the standard cytokine gradient concentrations allowing the concentrations of each cytokine in the tear samples to be calculated. Cytokine levels were adjusted for total protein concentration and expressed as pg of cytokine/mg of total protein.

The total protein concentration was calculated based on absorbance on a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). The formula 'Protein conc (mg/mL) = (1.55 x Abs.280) – (0.76 x Abs.260)' was used to adjust for presence of nucleic acids in the sample (Dara et al., 2022).

6.2.3. Data analysis and sample size

Data analysis was performed using Microsoft® Office Excel®, GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA and IBM SPSS Statistics for Windows, Version 26. Descriptive statistics such as mean and standard deviation were used to report biomarker concentrations. Where the cytokine data did not pass the Kolmogorov-Smirnov normality test the non-parametric Mann Whitney U-test was used for comparisons of cytokine concentrations between the DED and non-DED participants.

To quantify correlations between clinical parameters and cytokine concentrations, Spearman ranked correlation was used. The strength of any correlation between the two visits (Spearman's correlation coefficient r) was estimated according to the following coefficient magnitudes: 0.5-1.0 represents a high correlation, 0.5-0.3 moderate, and 0.3 to 0.1 weak. Pearson's correlation coefficient was used where data was found to have a normal distribution.

To measure the association between nominal and numerical data, such as contact lens wear and cytokine concentrations, Univariate or Multivariate Analysis of Variance was used to calculate partial eta-squared, where $\eta^2 = 0.01$ indicates a small effect, $\eta^2 = 0.06$ a medium effect and $\eta^2 = 0.14$ indicates a large effect (Watson, 2021).

For all tests an alpha value of 0.05 was adopted for statistical significance. Bonferroni correction was not applied to the data and therefore the possibility of false significance is acknowledged (Armstrong, 2014).

The sample size calculation for the tear cytokine analysis has been reported in Chapter 4. A total sample size of 40 (20 in each group) with a two-tailed α-level of 5%, an 80% power level and a calculated effect size of 0.98, was required to demonstrate statistically different IL-8 concentrations between two groups.

6.3. Results

All ten cytokines were present in the tear samples collected with the Schirmer strips. Their concentrations are presented as 'pg cytokine/mg total protein', as this was considered the most accurate way of presenting the results. The concentrations have also been calculated based on the length of the wetted Schirmer strip, which was recorded at the time of tear collection. However, at the time of cytokine assay preparation, three samples were noted to have less than 250 µl PBS, when inspected visually. The uncertainty of how much PBS was adsorbed by the strip itself, also made the amount of PBS hard to estimate.

Successful standard curves were obtained for each cytokine allowing the concentrations of individual cytokines in each tear samples to be calculated. The intra and inter coefficient of variation, expressed as a percentage, was calculated for each cytokine in all three plates. The lowest inter coefficient of variation between plates was 1.5% CV (IL-1β), while the highest was 15.5% (IL-10). For plates 1, 2 and 3 the lowest intra coefficient of variations were 2.3% (TNF-α), 2.0% (VEGF) and 2.1% (IL-1Ra), respectively. The highest intra coefficient of variations were all found for IL-10; 13.4%, 17.4% and 11.1%, respectively. Therefore, IL-10 showed the greatest variation from the mean concentration.

Schirmer strips were collected and analysed for all participants at Visit 1 (n=50). Forty samples were analysed at Visit 2; 41 samples were collected but one was excluded due to contamination with makeup. ***Table 6.2*** details the percentage of participants where detectable levels of each cytokine were found.

Table 6.2. Percentage frequency of presentation for each cytokine

Cytokine	All participants	All participants	non-DED participants	non-DED participants	DED participants	DED participants
	Visit 1 (n=50)	Visit 2 (n=40)	Visit 1 (n=22)	Visit 2 (n=17)	Visit 1 (n=28)	Visit 2 (n=23)
IL-1Ra	100	100	100	100	100	100
IL-10	94	93	100	94	94	91
IL-1β	100*	100*	100*	100*	100*	100*
IL-6	100	100	100	100	100	100
IL-8	100	100	100	100	100	100
IL-12p70	94*	95*	95*	88*	93*	100*
Fractalkine	100	100	100	100	100	100
TNF-α	100*	100	100*	100	100*	100
VEGF	100	100	100	100	100	100
EGF	100	100	100	100	100	100

**indicates where some of the data has been extrapolated*

The results obtained for IL-1Ra concentration were all above the range of the highest standard and therefore the final concentrations obtained were predicted by extrapolation of the results. Conversely, almost all (94.4%) the data for IL-12p70 was below the range of the least concentrated standard, and so these were also extrapolated. The concentration of IL-12p70 was below the limit of detection in five samples. Four percent (n=2) of the results for TNF-α were extrapolated due to being below the range of the standards; 14% (n=7) of the results for IL-1 β at Visit 1, and 17.5% (n= 7) at Visit 2 were also extrapolated.

The Schirmer wetting length was recorded for 49 of the 50 samples at Visit 1 and 40 strips at Visit 2; the mean wetting lengths were 27.1 ± 12.64 mm and 30.45 ± 11.8 mm, respectively. The wetting lengths were highly correlated with the total tear protein concentration at both visits: Spearman correlation r= 0.744 ($p<0.001$) at Visit 1 and r=0.720 ($p<0.001$) at Visit 2. There was no significant difference between the means of the wetting length between visits (p=0.192). Moderate or strong negative correlations with Schirmer wetting length were found for most of the individual cytokines at both visits, see ***Table 6.3***.

Table 6.3. Spearman correlation coefficients for Schirmer wetting length and cytokine concentrations (pg/mg/mL)

Cytokine	Visit	Correlation coefficient ®	p-value
IL-1Ra (pg/mg of TPC)	1	**-0.801**	**p<0.001***
	2	**-0.798**	**p<0.001***
IL-10 (pg/mg of TPC)	1	**-0.474**	**p=0.001***
	2#	-0.190	p=0.239
IL-1β (pg/mg of TPC)	1	**-0.407**	**p=0.004***
	2	**-0.572**	**p<0.001***
IL-6 (pg/mg of TPC)	1	**-0.519**	**p<0.001***
	2	**-0.496**	**p=0.001***
IL-8 (pg/mg of TPC)	1	**-0.362**	**p=0.0118**
	2	-0.124	p=0.446
IL-12p70 (pg/mg of TPC)	1	**-0.525**	**p<0.001***
	2	**-0.498**	**p=0.001***
Fractalkine (pg/mg of TPC)	1	**-0.581**	**p<0.001***
	2#	**-0.559**	**p<0.001***
TNF-α (pg/mg of TPC)	1	**-0.560**	**p<0.001***
	2	**-0.408**	**p=0.009***
VEGF (pg/mg of TPC)	1	-0.092	p=0.529
	2#	0.229	p=0.156
EGF (pg/mg of TPC)	1	**-0.300**	**p=0.036***
	2	-0.247	p=0.124

*# Pearson's correlation coefficient used as data found to have a normal distribution. * P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded.*

6.3.1. Cytokine analysis results - Visit 1

A comparison of the cytokine concentrations (pg per mg of the total concentration) found in the DED and non-DED participants are shown in ***Table 6.3***. No statistically significant differences were found between the two groups when multivariate analysis was performed. The concentration of vascular endothelial growth factor (VEGF) was higher in the DED participants with borderline significance (p=0.062).

When the data between the DED and non-DED participants was analysed according to the Schirmer wetting length, rather than as pg per mg of the total concentration, no significant differences (p>0.05) were found between the DED and non-DED participants.

Table 6.3. Tear cytokine data from Visit 1

Visit 1	non-DED participants	DED participants	Multivariate analysis	
			η^2	p value
Participants	n=22	n=28		
Total protein concentration (mg/ml)	1.19 ± 0.50	1.13 ± 0.45	η^2=0.004	p=0.671
Cytokine	**pg per mg of the total concentration ± SD (range)**	**pg per mg of the total concentration ± SD (range)**		
IL-1Ra	6551 ± 3261 (2568 – 14665)	6531 ± 3136 (2799 – 15062)	η^2=0.000	p=0.982
IL-10	6.6 ± 3.1 (0.0 – 15)	7.1 ± 4.0 (0.0 – 16)	η^2=0.005	p=0.625
IL-1β	6.5 ± 3.6 (1.6 – 14)	8.4 ± 5.9 (1.6 – 26)	η^2=0.034	p=0.197
IL-6	2.7 ± 1.8 (0.7 – 7.4)	3.8 ± 5.6 (0.3 – 30)	η^2=0.016	p=0.377
IL-8	66 ± 36 (18 – 149)	81 ± 74 (18 – 343)	η^2=0.016	p=0.378
IL-12p70	17 ± 13 (0.0 – 49)	17 ± 15 (0.0 – 62)	η^2=0.001	p=0.858
Fractalkine	1491 ± 771 (256 – 3734)	1603 ± 1035 (374 – 6104)	η^2=0.004	p=0.675
TNF-α	2.9 ± 1.6 (0.7 – 8.2)	2.9 ± 1.6 (1.5 – 5.8)	η^2=0.000	p=0.986
VEGF	121 ± 29 (74 – 196)	106 ± 25 (59 – 166)	η^2=0.071	p=0.062*
EGF	120 ± 39 (67 – 183)	121 ± 62 (30 – 260)	η^2=0.000	p=0.949

*#partial eta-squared where η^2 = 0.01 indicates a small effect, η^2 = 0.06 a medium effect and η^2 = 0.14 indicates a large effect. *P-values <0.05 were considered significant. *indicates p values near significance.*

The results of the correlation analysis between the cytokine concentrations and the clinical parameters used to classify participants by dry eye status, detailed in Chapter 4, are reported in ***Table 6.4***. The TPC reduced with increasing signs and symptoms of DED. However, this was only significant with corneal staining (r=-0.298, p=0.036) and average NIKBUT (r=0.367, p=0.009). As the average NIKBUT decreased or worsened, the concentration of all the cytokines increased with six showing statistical significance. This included the pro- and anti-inflammatory cytokines (IL-1Ra and IL-10).

Table 6.4. Spearman correlation coefficients for the TPC and each cytokine with clinical parameters used to classify dry eye status (Visit 1)

Variables Visit 1		OSDI	TMH	Average NIKBUT	corneal staining	conjunctival staining	LWE horizontal	LWE sagittal
TPC	r	-0.022	-0.138#	**0.367**	**-0.298**	-0.204	-0.084	-0.130
(mg/mL)	p-value	0.878	0.339	**0.009***	**0.036***	0.155	0.563	0.369
IL-1Ra	r	0.056	0.032	**-0.393**	0.196	0.212	0.181	0.195
(pg/mg of TPC)	p-value	0.702	0.828	**0.005***	0.172	0.139	0.209	0.175
IL-10	r	0.089	-0.017#	**-0.326**	0.133	0.211	**0.315**	0.150
(pg/mg of TPC)	p-value	0.539	0.905	**0.021***	0.357	0.141	**0.026***	0.297
IL-1β	r	0.023	0.159	**-0.340**	0.266	0.061	-0.134	-0.185
(pg/mg of TPC)	p-value	0.873	0.269	**0.016***	0.062*	0.675	0.354	0.198
IL-6	r	0.017	0.170	**-0.415**	**0.283**	0.146	-0.036	-0.067
(pg/mg of TPC)	p-value	0.908	0.238	**0.003***	**0.046***	0.312	0.806	0.645
IL-8	r	0.022	0.191	-0.221	**0.329**	-0.035	-0.022	-0.054
(pg/mg of TPC)	p-value	0.880	0.184	0.123	**0.020***	0.810	0.878	0.709
IL-12p70	r	0.100	-0.025	-0.257	0.000	0.237	**0.291**	0.273
(pg/mg of TPC)	p-value	0.491	0.862	0.072*	1.000	0.098*	**0.040***	0.055*
Fractalkine	r	0.111	0.139	**-0.367**	0.213	**0.285**	0.206	0.120
(pg/mg of TPC)	p-value	0.442	0.336	**0.009***	0.138	**0.045***	0.152	0.405
TNF-α	r	-0.027	0.086	**-0.343**	0.214	**0.288**	0.026	-0.030
(pg/mg of TPC)	p-value	0.853	0.553	**0.015***	0.135	**0.042***	0.856	0.833
VEGF	r	**-0.299**	-0.040#	-0.137	-0.064	-0.144	-0.113	0.025
(pg/mg of TPC)	p-value	**0.035***	0.784	0.343	0.660	0.319	0.435	0.865
EGF	r	-0.024	-0.065	-0.046	0.256	**0.298**	0.194	0.099
(pg/mg of TPC)	p-value	0.868	0.653	0.749	0.072*	**0.036***	0.176	0.495

*# Pearson's correlation coefficient used as data found to have a normal distribution. * P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

When considering ocular surface staining, the majority of cytokines (except VEGF) showed an increase in concentration with worsening signs. The significant correlations between corneal staining, and IL-6 and IL-8, were not repeated for conjunctival staining. On the other hand, correlations between fractalkine, TNF-α and EGF were found with conjunctival, but not corneal staining, while IL-10 and IL-12p70 significantly correlated with horizontal LWE, see ***Table 6.4***. No correlations were found between any cytokine and corneal and conjunctival staining when the analysis was performed with the cytokine concentrations adjusted for Schirmer wetting length. However, the significant findings for horizontal LWE were repeated when Schirmer wetting length was considered; IL-10 (r=0.380, p=0.008) and IL-12p70 (r=0.358, p=0.013), and also IL-1Ra (r=0.296, p=0.041), fractalkine (r=0.329, p=0.022) and EGF (r=0.326, p=0.024).

VEGF showed a negative, but not statistically significant correlation with all clinical signs used to diagnose DED except sagittal lid wiper epitheliopathy, indicating lower concentrations as signs of DED increase. A statistically significant correlation with the OSDI symptom score was also found (r=-0.299 p=0.035).

Spearman correlation analysis for each cytokine, adjusted for Schirmer wetting length, with the clinical parameters used to classify dry eye status at Visit 1, found correlations with NIKBUT and LWE only. The results for NIKBUT are as follows: IL-1Ra (r=-0.392, p=0.006), IL-10 (r=-0.321, p=0.026), IL-1β (r=-0.415,p=0.03), IL-6 (r=-0.395, p=0.005), IL-12p70 (r=-0.380, p=0.007), fractalkine (r=-0.408, p=0.004), an TNF-α (r=-0.356, p=0.013). These confirm the findings reported in ***Table 6.4***.

Considering other clinical parameters, no correlations were found between the TPC or any of the cytokines with incomplete blinking or blink rate at Visit 1. IL-8 was found to significantly, positively correlate with both bulbar (r=0.318, p=0.024) and limbal (r=0.288, p=0.040) ocular redness. IL-12p70 also correlated with bulbar redness (r=0.288 p=0.043). VEGF showed a negative but not statistically significant correlation with limbal redness (r=-0.243 p=0.09), while a positive correlation between IL-1β and bulbar redness was also not significant (r=0.251 p=0.078).

The results for subjective meibography parameters are reported in ***Table 6.5***. Interestingly, the concentration of IL-1β positively correlated with meibum quality (r=0.328, p=0.020) and upper meibography score (r=0.426, p=0.002); the concentration increased with higher meibum gland loss and poorer quality of meibum.

Table 6.5. Spearman correlation coefficients for meibomian gland parameters and cytokines (Visit 1)

Variables Visit 1		**Meibum quality**	**Meibum expression**	**Lipid layer thickness**	**Upper meibography score**	**Lower meibography score**
TPC (mg/mL)	r p-value	-0.128 0.376	-0.158 0.273	-0.085 0.559	-0.172 0.232	0.172 0.231
IL-1Ra (pg/mg of TPC)	r p-value	0.022 0.880	0.037 0.800	0.109 0.449	0.092 0.526	-0.145 0.314
IL-10 (pg/mg of TPC)	r p-value	-0.014 0.923	-0.076 0.598	0.226 0.115	-0.093 0.522	-0.140 0.332
IL-1β (pg/mg of TPC)	r p-value	**0.328** **0.020***	**0.298** **0.036***	0.001 0.997	**0.426** **0.002***	0.030 0.835
IL-6 (pg/mg of TPC)	r p-value	0.126 0.384	0.142 0.324	-0.028 0.849	0.258 0.070*	-0.107 0.458
IL-8 (pg/mg of TPC)	r p-value	0.182 0.207	0.120 0.407	0.016 0.914	0.136 0.348	-0.142 0.325
IL-12p70 (pg/mg of TPC)	r p-value	-0.048 0.741	-0.002 0.988	-0.033 0.819	0.015 0.916	-0.173 0.231
Fractalkine (pg/mg of TPC)	r p-value	0.122 0.399	0.020 0.888	0.046 0.753	0.005 0.972	-0.137 0.343
TNF-α (pg/mg of TPC)	r p-value	0.200 0.164	0.123 0.394	0.229 0.110	0.108 0.456	-0.141 0.330
VEGF (pg/mg of TPC)	r p-value	-0.102 0.482	0.045 0.758	-0.010 0.944	0.188 0.192	-0.055 0.705
EGF (pg/mg of TPC)	r p-value	0.174 0.228	-0.038 0.794	0.191 0.184	0.059 0.686	0.167 0.246

** P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

The significant correlation between IL-1β and upper meibography score was also found when considering concentration adjusted for Schirmer wetting length (r=0.334, p=0.020). Meibum expression was significantly positively correlated with the mean concentration of IL-1β (pg per mg of TPC) (r=0.298, p=0.036) i.e., poorer expression was correlated with lower levels of IL-1β. No other cytokines showed a significant correlation with any of the meibomian gland parameters, with either method.

Table 6.6 reports the results of the cytokine correlation analysis with the subjective semi-quantitative gradings for MMP-9. A statistically significant moderate positive correlation was found between IL-8 (r=0.334, p=0.018) and MMP-9 grade. Small not significant correlations were also found for IL-6 and IL-1β. No correlations were found when the concentration was adjustment for Schirmer length.

Table 6.6. Spearman correlation coefficients for cytokines and MMP-9 (Visit 1)

Visit 1	MMP-9 subjective semi-quantitative grade	
	correlation coefficient ®	p-value
Total protein concentration (TPC) (mg/mL)	-0.175	0.224
IL-1Ra (pg/mg of TPC)	0.132	0.361
IL-10 (pg/mg of TPC)	0.141	0.328
IL-1β (pg/mg of TPC)	0.240	0.093*
IL-6 (pg/mg of TPC)	0.258	0.071*
IL-8 (pg/mg of TPC)	**0.334**	**0.018***
IL-12p70 (pg/mg of TPC)	0.036	0.805
Fractalkine (pg/mg of TPC)	0.161	0.264
TNF-α (pg/mg of TPC)	0.013	0.930
VEGF (pg/mg of TPC)	0.225	0.116
EGF (pg/mg of TPC)	-0.089	0.538

** P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance*

When considering any differences based on sex (36 females, 14 males), no statistically significant associations were found with the total protein concentration or any of the cytokines. Sixty-six percent of the participants (n=33) were non-CL wearers; 36% were current wearers. Twenty-eight percent of the participants reported a history of allergy (n=14). No statistically significant associations were found between contact lens wear or allergy with any cytokine. Furthermore, no significant associations or correlations were found with participant ethnicity, time reported using a screen each day, outdoor activity or exercise.

When considering other lifestyle factors, the only significant correlation found was between self-perceived stress levels and TPC ($r=-0.290$, $p=0.041$); higher levels of stress (where 1=high and 4=minimal) correlated with higher TPCs.

6.3.2. Cytokine analysis results - Visit 2

A comparison of the cytokine concentrations (pg per mg of the total concentration) found in the DED and non-DED participants at Visit 2 are shown in ***Table 6.7***.

Multivariate analysis showed no statistically significant differences between the DED and non-DED participants for the TPC, as in Visit 1. However, significantly higher levels of IL-1Ra and fractalkine were observed at Visit 2, with a medium effect size ($p=0.047$ and=0.025, respectively). This was also found when using the cytokine concentration based on Schirmer wetting length; IL-1Ra ($\eta^2=0.138$, $p=0.018$) and fractalkine ($\eta^2=0.159$, $p=0.011$). As at Visit 1, higher mean pg per mg of the total concentrations were found for IL-10, IL-1β, and Il-8, but were not significant. However, when considering Schirmer wetting length, the differences were significant for IL-10 and IL-12p70; $\eta^2=0.088$, $p=0.049$, and $\eta^2=0.110$, $p=0.037$, respectively. A lower concentration of VEGF was found at Visit 2, as in Visit 1, but the difference was not significant.

Table 6.7. Tear cytokine data from Visit 2

Visit 2	non-DED participants	DED participants	Multivariate analysis	
			η^2	**p value**
Participants	n=17	n=23		
Total protein concentration (mg/ml)	1.62 ± 0.52	1.35 ± 0.51	η^2=0.066	p=0.110
Cytokine	**pg per mg of the total concentration ± SD (range)**	**pg per mg of the total concentration ± SD (range)**		
IL-1Ra	4192 ± 1794 (1155 – 8150)	5599 ± 2355 (2668 – 11788)	η^2=0.100	**p=0.047***
IL-10	4.4 ± 3.0 (0.0 – 13)	6.2 ± 3.1 (0.0 – 13)	η^2=0.075	p=0.087*
IL-1β	5.8 ± 3.9 (1.3 – 13)	6.7 ± 6.2 (1.5 – 32)	η^2=0.006	p=0.629
IL-6	3.4 ± 7.8 (0.3 – 34)	2.0 ± 0.8 (0.6 – 4.1)	η^2=0.020	p=0.387
IL-8	48 ± 30 (13 – 120)	88 ± 101 (16 – 425)	η^2=0.060	p=0.128
IL-12p70	10 ± 7.7 (0.0 – 32)	15 ± 8.9 (1.7 – 41)	η^2=0.085	p=0.067*
Fractalkine	1006 ± 422 (396 – 2118)	1330 ± 441 (707 – 2319)	η^2=0.126	**p=0.025***
TNF-α	2.9 ± 1.8 (0.9 – 8.8)	3.1 ± 1.6 (1.2 – 8.4)	η^2=0.002	p=0.794
VEGF	120 ± 27 (68 – 185)	112 ± 31 (72 – 191)	η^2=0.023	p=0.352
EGF	99 ± 49 (43 – 249)	104 ± 49 (48 – 256)	η^2=0.003	p=0.751

*#partial eta-squared where η^2 = 0.01 indicates a small effect, η^2 = 0.06 a medium effect and η^2 = 0.14 indicates a large effect. *P-values <0.05 were considered significant and are shown in bold. *indicates p values near significance.*

The results of the correlation analysis between the cytokine concentrations and the clinical parameters used to classify participants by dry eye status at Visit 2, detailed in Chapter 4, are reported in ***Table 6.8***. Again, the TPC could be seen to reduce with increasing signs and symptoms of DED. This was again significant with corneal staining (r=-0.431, p=0.006), but not average NIKBUT.

When considering cytokine correlations with ocular surface staining at Visit 2, there was more variation; none of the correlations found at Visit 1 were repeated. However, several statistically significant positive correlations were found between corneal staining and individual cytokines; IL-1Ra (r=0.444, p=0.004), IL-10 (r=0.368, p=0.019), IL-12p70 (r=0.474, p=0.002), and fractalkine (r=0.380,

p=0.015). EGF did not correlate with corneal staining at Visit 1 (r=0.256, p=0.072), but did at Visit 2 (r=0.331, p=0.037).

Table 6.8. Spearman correlation coefficients for the total protein concentration (TPC) and each cytokine with clinical parameters used to classify dry eye status (Visit 2)

Variables Visit 2		OSDI	TMH	Average NIKBUT	corneal staining	conjunctival staining	LWE horizontal	LWE sagittal
TPC (mg/mL)	r p-value	-0.206 0.202	0.163# 0.314	0.149 0.360	**-0.431** **0.006***	-0.241 0.134	-0.102 0.529	-0.003 0.988
IL-1Ra (pg/mg of TPC)	r p-value	0.210 0.192	-0.287 0.073*	-0.180 0.267	**0.444** **0.004***	0.264 0.100	0.152 0.348	0.195 0.550
IL-10 (pg/mg of TPC)	r p-value	0.295 0.064*	-0.244# 0.128	-0.156 0.366	**0.368** **0.019***	0.107 0.512	-0.072 0.657	-0.201 0.214
IL-1β (pg/mg of TPC)	r p-value	0.008 0.962	0.027 0.870	-0.140 0.390	0.116 0.478	0.158 0.330	0.171 0.290	0.013 0.935
IL-6 (pg/mg of TPC)	r p-value	0.259 0.107	-0.154 0.344	-0.024 0.885	0.149 0.360	-0.011 0.948	0.102 0.531	0.232 0.149
IL-8 (pg/mg of TPC)	r p-value	0.175 0.280	0.032 0.843	-0.005 0.975	-0.075 0.644	0.116 0.478	**0.379** **0.016***	0.243 0.132
IL-12p70 (pg/mg of TPC)	r p-value	0.310 0.052*	-0.248 0.123	-0.224 0.165	**0.474** **0.002***	**0.355** **0.025***	0.040 0.840	0.039 0.810
Fractalkine (pg/mg of TPC)	r p-value	**0.342** **0.031***	-0.156# 0.338	0.028 0.864	**0.380** **0.015***	0.305 0.056*	0.143 0.377	0.095 0.558
TNF-α (pg/mg of TPC)	r p-value	0.147 0.365	-0.206 0.202	0.006 0.970	0.211 0.191	0.199 0.219	-0.060 0.715	-0.060 0.711
VEGF (pg/mg of TPC)	r p-value	-0.174 0.283	0.154# 0.344	0.146 0.369	**-0.322** **0.043***	-0.165 0.308	-0.053 0.746	0.072 0.657
EGF (pg/mg of TPC)	r p-value	0.040 0.805	-0.013 0.935	0.215 0.182	**0.331** **0.037***	0.170 0.294	-0.086 0.598	-0.009 0.958

 # Pearson's correlation coefficient used as data found to have a normal distribution. P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

The correlations between corneal staining and IL-1Ra, IL-1β and fractalkine were confirmed when the cytokine concentration was calculated based on Schirmer wetting length; r=0.560, p<0.0001, r=0.408, p=0.009, and r=0.348, p=0.028, respectively. A significant negative correlation in the concentration of VEGF (pg per mg of TPC) was found (r=-0.322, p=0.043) in agreement with the reductions found at Visit 1.

The non-statistically significant positive correlation found between IL-12p70 and conjunctival staining at Visit 1, became significant at Visit 2 (r=0.474, p=0.002), and confirmed by Schirmer wetting length concentration (r=0.317, p=0.046). The statistically significant positive correlation with fractalkine at Visit 1, did not reach significance at Visit 2 (r= 0.305, p=0.056). At Visit 2, fractalkine was the only cytokine to correlate with OSDI score (r=0.342, p=0.031).

Considering other clinical parameters, again no correlations were found between the TPC or any of the cytokines with incomplete blinking or blink rate at Visit 2. IL-6, rather than IL-8 as at Visit 1, showed

a strong positive correlation with both bulbar (r=0.419, p=0.007) and limbal (r=0.472, p=0.002) ocular redness.

The Visit 2 results for subjective meibography parameters are reported in ***Table 6.9***. Interestingly, the concentration of IL-1β again positively correlated with meibum expression (r=0.317, p=0.046); as the expression of the glands worsened its concentration reduced. No correlation with upper gland loss and poorer meibum quality was found at Visit 2. Other correlations found at Visit 2 included significant negative correlations between IL-6 and lipid layer thickness (r=-0.343, p=0.030), and IL-12p70 with lower lid meibography score (r=-0.388, p=0.013). This last correlation was also found when the concentration was calculated based on Schirmer wetting length (r=-0.394, p=0.012).

Table 6.9. Spearman correlation coefficients for meibomian gland parameters and cytokines (Visit 2)

Variables Visit 2		Meibum quality	Meibum expression	Lipid layer thickness	Upper meibography score	Lower meibography score
TPC (mg/mL)	r p-value	0.069 0.673	-0.268 0.094*	0.000 0.998	-0.032 0.843	0.006 0.973
IL-1Ra (pg/mg of TPC)	r p-value	-0.102 0.533	0.301 0.059*	-0.075 0.647	0.080 0.626	-0.028 0.866
IL-10 (pg/mg of TPC)	r p-value	-0.154 0.343	0.077 0.639	-0.129 0.428	0.151 0.352	-0.147 0.366
IL-1β (pg/mg of TPC)	r p-value	0.082 0.616	**0.317** **0.046***	-0.240 0.137	-0.233 0.148	-0.059 0.718
IL-6 (pg/mg of TPC)	r p-value	-0.141 0.386	0.237 0.140	**-0.343** **0.030***	-0.007 0.965	-0.049 0.765
IL-8 (pg/mg of TPC)	r p-value	-0.003 0.984	0.253 0.115	-0.262 0.103	0.066 0.684	0.015 0.927
IL-12p70 (pg/mg of TPC)	r p-value	-0.033 0.841	0.153 0.346	-0.119 0.464	-0.164 0.312	**-0.388** **0.013***
Fractalkine (pg/mg of TPC)	r p-value	-0.141 0.386	0.163 0.314	-0.262 0.103	0.016 0.923	-0.120 0.460
TNF-α (pg/mg of TPC)	r p-value	0.278 0.082*	0.255 0.113	0.036 0.826	-0.035 0.828	-0.197 0.223
VEGF (pg/mg of TPC)	r p-value	-0.075 0.644	-0.232 0.150	-0.117 0.474	-0.032 0.843	-0.032 0.844
EGF (pg/mg of TPC)	r p-value	-0.311 0.051*	-0.006 0.972	-0.190 0.240	0.065 0.688	-0.170 0.296

** P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

Table 6.10 reports the results of the cytokine correlation analysis with the subjective semi-quantitative gradings for MMP-9 at Visit 2. In agreement with Visit 1, a strong positive correlation was found between IL-8 and MMP-9 subjective semi-quantitative grading (r=0.575, p<0.001). At Visit 2 a moderate positive correlation was also found with IL-6 (r=0.395, p=0.012). Both IL-6 and IL-8 were found to correlate with the MMP-9 grading, when the concentrations were based on Schirmer wetting length; r=0.491, p=0.001, and r=0.596, p<0.001.

Table 6.10. Spearman correlation coefficients for cytokines and MMP-9 (Visit 2)

Visit 2	MMP-9 subjective semi-quantitative grade	
	correlation coefficient ®	p-value
Total protein concentration (TPC) (mg/mL)	0.013	0.937
IL-1R (pg/mg of TPC)	0.026	0.875
IL-10 (pg/mg of TPC)	-0.111	0.495
IL-1β (pg/mg of TPC)	0.093	0.570
IL-6 (pg/mg of TPC)	**0.395**	**0.012***
IL-8 (pg/mg of TPC)	**0.575**	**<0.001***
IL-12p70 (pg/mg of TPC)	-0.069	0.674
Fractalkine (pg/mg of TPC)	0.074	0.648
TNF-α (pg/mg of TPC)	-0.044	0.788
VEGF (pg/mg of TPC)	-0.028	0.862
EGF (pg/mg of TPC)	-0.156	0.337

** P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance.*

When considering differences based on sex at Visit 2 (29 females, 11 males), unlike at Visit 1, associations were found with the cytokines. Significantly higher levels of fractalkine, IL-Ra, and IL-12p70 were found in the females (η^2 =0.122, p=0.027, η^2=0.181, p=0.006, and η^2=0.177, p=0.031, respectively), while lower levels of the TPC were found (η^2=0.185, p=0.006). Lower levels of Il-6 were found in the females too but were not significant.

Twenty-five of the participants (37.5%) were contact lens wearers. A significantly lower mean concentration of EGF was found in these participants (η^2=0.101, p=0.046). Twenty-five percent of the participants reported a history of allergy (n=10); no statistically significant associations were found with any cytokine. Furthermore, no significant associations or correlations were found with participant ethnicity or for time reported using a screen each day.

When considering other lifestyle factors, no associations were found with exercise or outdoor activity. Significant correlations were found for self-perceived stress levels and health quality with certain cytokines; higher stress levels and poorer self-perceived health status correlated with higher cytokine

concentrations. IL-8 was moderately negatively correlated with stress (r=-0.346, p=0.029), but was not significant for health status (r=-0.269, p=0.093), while IL-6 correlated with health (r=-0.413, p=0.08), but not stress (r=-0.273, p=0.089). Fractalkine concentration was associated, but not significantly, with both stress (r=-0.288, p=0.071) and health (r=-0.299, p=0.061). Poorer self-perceived health quality was also significantly negatively correlated with higher levels of VEGF (r=-0.341, p=0.031). No correlations were found with self-reported diet quality.

6.3.3. Visit 1 and 2 cytokine comparison

Where data was obtained for the same participant at each visit, the mean concentrations (pg per mg of the TPC) are compared in ***Table 6.11***. The TPC was significantly higher at Visit 2 (p=0.012). A significantly lower concentration of IL-Ra (p=0.006) was found at Visit 2; fractalkine concentration was also lower but did not reach significance (p=0.057). IL-10, Il-1β, Il-6 and EGF were also lower at Visit 2 but not significantly.

Table 6.11. Comparison of the cytokines between visits

	Visit 1 (n=40)	**Visit 2 (n=40)**	**Wilcoxon paired t-test p value**
Total protein concentration (mg/mL)	1.24 ± 0.48# (0.43 – 2.52)	1.46 ± 0.52# (0.60 – 2.66)	**p=0.012**
Cytokine	**pg per mg of the total concentration ± SD (range)**	**pg per mg of the total concentration ± SD (range)**	
IL-1Ra	6030 ± 2924 (2568 – 14665)	4991 ± 2230 (1155 – 11788)	**p=0.006**
IL-10	6.1 ± 3.4# (0.0 – 15)	5.4 ± 3.2# (0.0 – 13)	p=0.204
IL-1β	7.1 ± 4.4 (1.6 – 21)	6.3 ± 5.3 (1.3 – 32)	p=0.186
IL-6	3.3 ± 4.8 (0.3 – 30)	2.6 ± 5.1 (0.3 – 34)	p=0.207
IL-8	72 ± 64 (18 – 343)	70 ± 81 (13 – 425)	p=0.614
IL-12p70	15 ± 13 (0.0 – 49)	13 ± 8.9 (0.0 – 41)	p=0.368
Fractalkine	1475 ± 987 (256 – 6104)	1196 ± 454 (396 – 2319)	p=0.057*
TNF-α	2.7 ± 1.3 (0.7 – 8.2)	2.9 ± 1.6 (0.9 – 8.8)	p=0.497
VEGF	113 ± 27# (64 – 196)	115 ± 29# (68 – 191)	p=0.518
EGF	113 ± 47 (30 – 237)	101 ± 49 (43 – 256)	p=0.121

*# indicates data that passed the K-S normality test - parametric unpaired t-test used. *P-values <0.05 were considered significant and are shown in bold. * near significance.*

The cytokines were also compared between visits based on a calculation of the mean concentration calculated using Schirmer wetting length, see ***Table 6.12***. When calculated this way, no significant difference in the TPC or any cytokine concentration was found between visits.

Table 6.12. Comparison of the cytokines between visits, based on the Schirmer wetting length

	Visit 1 (n=36)	**Visit 2 (n=36)**	**Wilcoxon paired t-test p value**
Total protein concentration (mg/mL)/mm of Schirmer strip	0.05 ± 0.02 (0.02 – 0.13) 0.003	0.05 ± 0.01 (0.03 – 0.09) 0.002	p=0.5367
Cytokine	**pg per mm of Schirmer strip ± SD (range) SEM**	**pg per mm of Schirmer strip ± SD (range) SEM**	
IL-1Ra	300 ± 217 (112 – 1051) 36	254 ± 148 (75 – 708) 25	p=0.111
IL-10	0.31 ± 0.23 (0.00 – 0.93) 0.04	0.27 ± 0.19 (0.00 – 0.77) 0.03	p=0.372
IL-1β	0.34 ± 0.23# (0.05 – 0.87) 0.04	0.32 ± 0.26# (0.05 – 1.30) 0.04	p=0.772
IL-6	0.19 ± 0.34 (0.01 – 1.88) 0.06	0.15 ± 0.37 (0.02 – 2.29) 0.06	p=0.471
IL-8	3.7 ± 4.5 (0.65 – 21 0.75	3.6 ± 4.2 (0.41 – 22) 0.70	p=0.689
IL-12p70	0.71 ± 0.69 (0.0 – 3.1) 0.12	0.69 ± 0.56 (0.0 – 2.2) 0.09	p=0.902
Fractalkine	66 ± 38# (7.4 – 164) 6.6	59 ± 30# (20 – 130) 5.0	p=0.209
TNF-α	0.13 ± 0.09 (0.02 – 0.4) 0.02	0.15 ± 0.12 (0.04 – 0.61) 0.02	p=0.282
VEGF	5.6 ± 3.0# (1.87 – 18) 0.50	5.7 ± 2.0# (3.20 – 13) 0.33	p=0.991
EGF	5.8 ± 4.1 (1.0 – 17) 0.69	5.0 ± 2.8 (2.0 – 15) 0.46	p=0.906

 # parametric paired t test used.

The TPC and cytokine concentrations for the DED and non-DED were also compared between visits, see ***Table 6.13***.

Table 6.13. Comparison of the DED and non-DED participants between visits

	Participants classed as non-DED (at Visit 1)		Wilcoxon paired t-test p value	Participants classed as DED (at Visit 1)		Wilcoxon paired t-test p value
Participants	19			21		
Cytokine (pg per mg of the total conc. ± SD (range) SEM	**Visit 1**	**Visit 2**		**Visit 1**	**Visit 2**	
TPC (mg/mL)	1.25 ± 0.50 (0.43 – 2.52) 0.1	1.63 ± 0.59 (0.60 – 2.66) 0.1	**p=0.007**	1.23 ± 0.47# (0.56 – 2.22) 0.1	1.31 ± 0.41# (0.80 – 2.18) 0.1	p=0.402
IL-1Ra	6146 ± 3135 (2568 – 14665) 719	4463 ± 2497 (1155 – 11788) 573	**p=0.001**	5926 ± 2795 (2800 – 2682) 610	5470 ± 1894 (2988 – 8265) 413	p=0.452
IL-10	6.2 ± 3.1 (0.0 – 15) 0.7	5.5 ± 3.5 (1.7 – 13) 0.8	p=0.196	6.1 ± 3.7# (0.0 – 12) 0.8	5.3 ± 2.9# (0.0 – 11) 0.6	p=0.459
IL-1β	6.2 ± 3.4 (1.6 – 14) 0.8	7.0 ± 7.1 (1.3 – 32) 1.6	p=0.891	7.8 ± 5.1# (1.6 – 21) 1.1	5.6 ± 3.1# (1.5 – 13) 0.7	p=0.094
IL-6	2.6 ± 1.8 (0.7 – 7.4) 0.4	3.3 ± 7.4 (0.3 – 34) 1.7	p=0.312	3.9 ± 6.4 (0.3 – 30) 1.4	2.0 ± 0.9 (0.6 – 4.1) 0.2	p=0.475
IL-8	64 ± 37 (18 – 149) 8.6	49 ± 29 (13 – 120) 6.6	p=0.156	80 ± 82 (18 – 343) 18	90 ± 106 (16 – 425) 23	p=0.609
IL-12p70	16 ± 14 (0.0 – 49) 3.1	11 ± 7.7 (0.0 – 32) 1.8	p=0.134	14 ± 12 (0.0 – 49) 2.6	15 ± 9.7 (1.7 – 41) 2.1	p=0.919
Fractalkine	1429 ± 801# (256 – 3734) 184	1085 ± 466# (396 – 2118) 107	**p=0.028**	1517 ± 1149 (374 – 6104) 251	1295 ± 430 (707 – 2318) 94	p=0.683
TNF-α	2.8 ± 1.7 (0.7 – 8.2) 0.4	2.6 ± 1.7 (0.9 – 8.8) 0.4	p=0.441	2.7 ± 1.0# (1.5 – 4.2) 0.2	3.2 ± 1.5# (1.7 – 8.4) 0.3	p=0.129
VEGF	116 ± 29 (74 – 196) 6.6	118 ± 27 (68 – 185) 6.3	p>0.999	109 ± 26# (64 – 166) 5.6	114 ± 31# (72 – 191) 6.9	p=0.395
EGF	119 ± 39 (58 -183) 8.9	112 ± 58 (43 – 256) 14	p=0.490	107 ± 54 (30 – 237) 12	92 ± 35 (43 – 182) 7.6	p=0.137

 # parametric paired t test used

The significant difference in the total protein concentration between visits can mostly be attributed to an increase in the non-DED participants at Visit 2. The lower concentrations of IL-1Ra and fractalkine at Visit 2, shown in ***Table 6.11***, were observed to be due to significant changes in the non-DED participants at Visit 2.

6.4. Discussion

The results of this study confirm the presence of an inflammatory component even in the early stages of the pathogenesis of MGD-related evaporative DED. While significant differences in individual cytokine levels, such as fractalkine, were found between the two study groups, several significant correlations between cytokines and clinical features of DED, particularly corneal staining, were also apparent.

The values obtained for the TPC in the samples at each visit of this study, for all participants (1.16 ± 0.47 mg/mL at Visit 1, and 1.46 ± 0.52 mg/mL at Visit 2), were lower than those obtained by capillary tubes (4.50 ± 2.45 and 4.93 ± 2.70 mg/mL) reported in Chapter 5. These findings are in close agreement with a previous study comparing the effect of collection and analysis method on the TPC found spectrophotometrically, where a TPC of 4.34 ± 1.91 mg/mL was found for tears collected by capillary tubes, and 0.97 ± 0.12 mg/mL for Schirmer collection (Krajčíková et al., 2022). It was suggested that these differences could originate from the use of a larger amount of extraction reagent, not required for extraction from capillary tubes, and retention of proteins by strips. In this study Schirmer strips were stored in 250 µl of PBS before 70 µl was removed and diluted with a further 70 µl of calibrator diluent prior to analysis. Whereas 4 µl of the tears collected by capillary tubes (reported in chapter 5) were diluted to 90 µl before analysis.

Although Schirmer strips provide a simple method to collect tears for sample extraction and analysis, irritation of the conjunctiva and stimulation of reflex tearing can potentially cause contamination of the sample by cellular proteins from the conjunctival epithelial cells (Posa et al., 2013). Significantly higher serum proteins have been reported in tears collected by Schirmer strips compared to those collected without mechanical stimulation of the conjunctiva, as in capillary collection (Stuchell et al., 1984). However, Schirmer strips have also been shown to retain tear proteins to varying extents dependent on their molecular weight (Denisin et al., 2012). Significant differences have also been observed in the tear flow rate between sequential tear collections with Schirmer strips but not with capillary tubes (Bachhuber et al., 2021). Further comparison of the collection amount between visits for each collection method could prove interesting but is beyond the remit of this chapter.

Reflex tearing has also been shown to significantly reduce the concentration of many tear proteins (Fullard and Tucker, 1991). It was evident that some reflex tearing was present in a few of the participants at both visits, potentially diluting the cytokine concentration in these subjects. However, reflex tearing does not explain the higher TPC found at Visit 2, particularly as the mean Schirmer wetting lengths were also higher at Visit 2. Where tear samples were present from both visits, all were

analysed on the same microplate, thereby eliminating the possibility of a batch effect. The ink-free Schirmer strip samples were also stored 'wet'. Recently, this has been reported to be preferrable to 'dry' storage; the addition of PBS so the Schirmer strip is submerged appears to be beneficial due to the water-based salt solution providing the ideal environment for protein dynamics, stability and structure (Gijs et al., 2023).

However, some of the earliest collected samples from Visit 1 had been stored at -80° C for up to 18 months before analysis. Previously, when investigating the most abundant tear proteins (lactoferrin, sIgA light and heavy chains, albumin, lipocalin and lysozyme), long-term storage (>4 months at -70 °C) has been shown to reduce the TPC (Sitaramamma et al., 1998). De Jager et al. (2009), on the other hand, showed that cytokines are stable for a period of up to 2 years when stored at -80°C, but multiple freeze thaw cycles can alter their concentrations. While samples were added to the freezer storage boxes as quickly as possible to prevent thawing, it is difficult to be certain whether either of these factors contributed to any differential reduction in individual tear cytokine or lacrimal protein, and therefore the lower TPC at Visit 1. This would suggest that in any future longitudinal studies, it may be preferrable to analyse the tear samples following each visit. Nevertheless, when the Schirmer values for the 36 samples obtained at both visits were used to calculate the TPC, no significant difference was found.

When comparing the TPC between DED and non-DED, lower, but not statistically significant, concentrations were found in the DED participants at both visits. This has been found previously (Versura et al., 2013). While lacrimal gland proteins, which make up the bulk of the TPC can be reduced in DED, pro-inflammatory proteins are often upregulated (Zhou et al., 2012). The TPC was also found to positively correlate with average NIKBUT (r=0.367, p=0.009 at Visit 1); as the TPC reduced the NIKBUT also reduced, as would be found in DED. All other diagnostic criteria for DED diagnosis increased with lower TPC, but not significantly, except for TMH which decreased with decreased TPC, as expected, but only at Visit 2.

All the cytokines were detected in at least 88% of participants. IL-10, IL-1β, Il-8, and fractalkine were all increased in the DED participants at both visits, but only fractalkine reached statistical significance at Visit 2 (p=0.025). TNF-α and EGF showed no significant variation associated with dry eye status at either visit, while IL-1Ra appeared unchanged at Visit 1, but significantly higher in the DED participants at Visit 2 (p=0.047). The concentration of IL-12p70 was unchanged at Visit 1, but higher in the DED participants at Visit 2 (p=0.067). Conversely, VEGF concentrations were reduced in the DED participants at both visits but did not reach significance. However, it can be seen from the data in

Tables 6.3 and 6.6, that large variations in the mean cytokine concentrations were present in both DED and non-DED participants at both visits.

Although not statistically significant, the concentrations of IL-6 appeared to alter between visits, with higher concentrations in the DED participants at Visit 1, but in the non-DED participants at Visit 2. The finding at Visit 1 is in agreement with previous studies of different subtypes of DED (Yoon et al., 2007;Massingale et al., 2009;Wu et al., 2020). However, the higher concentration in the non-DED participants at Visit 2 could be attributed to a single significant outlier. When removed, the mean pg concentration per mg of the total protein reduced to 1.5 ± 0.97 pg per mg, lower than the DED mean (2.0 ± 0.84 pg per mg of the TPC) but not significantly. A significant outlier for IL-6 was also found at Visit 1 for one of the DED participants. When this was removed, the mean concentration reduced from 3.8 ± 5.6 to 2.8 ± 2.2 pg per mg of TPC, still higher than the non-DED participant mean (2.7 ± 1.8). Therefore, with the outliers removed, IL-6 did not differ significantly dependent on dry eye status as found in a previous study comparing EDE to healthy eyes (Enríquez-de-Salamanca et al., 2010). The reason for the outliers is unclear, as there were no other outliers among the corresponding cytokines in the samples.

Along with IL-6, the chemokine IL-8 has been reported to display the earliest observable changes in DED (Roda et al., 2020). The increased levels of IL-8 in the DED participants found in this study, although not statistically significant, is in agreement with previous studies (Lam et al., 2009;Zhao et al., 2018;Wu et al., 2020). However, other studies have not reported any statistically significant increase in EDE (Boehm et al., 2011;Khamar et al., 2019). Indeed, the similarity found between the tear cytokine profiles of healthy and EDE subjects lead Boehm et al. (2011) to assume a pathological mechanism other than inflammation to be responsible in EDE.

IL-6 and IL-8 were found to correlate with corneal and conjunctival staining at Visit 1 of this study. Previously Lam et al. (2009) reported an increase in both cytokines correlated with ocular surface staining when comparing subjects with EDE to controls. Increased conjunctival staining (Enríquez-de-Salamanca et al., 2010) and corneal staining (Liu et al., 2017a) have previously been reported to correlate with IL-6. A correlation between IL-8 and corneal staining has also been reported in a study comparing DED of different severities (Huang et al., 2012). However, the findings in this study were not consistent between visits or when the Schirmer wetting length was used to calculate the cytokine concentrations.

Significant positive correlations between IL-8 and both limbal and bulbar ocular redness were found at Visit 1. However, at Visit 2, IL-6, rather than IL-8, showed a strong positive correlation with both

bulbar and limbal ocular redness. These ocular surface indicators of inflammation do not appear to have been reported elsewhere in DED research.

The increase in the mean concentration of fractalkine/CXC3L1 found in this study also aligns with the findings of previous studies (Enríquez-de-Salamanca et al., 2010;Na et al., 2012). Carreño et al. (2010) were the first to detect fractalkine in healthy human tears. The same research group then showed significantly higher levels in subjects with EDE compared to controls (Enríquez-de-Salamanca et al., 2010). Elevated levels of fractalkine have been demonstrated in the tears of a Sjögren's syndrome murine model, indicating a role in inflammation (Fu et al., 2020a).

At Visit 2, a significant positive correlation was found between fractalkine concentration and OSDI questionnaire score (r= 0.342, p=0.031). A positive correlation with OSDI score was also found with IL-10, but was not significant (r=0.295, p=0.064). Previously Enriquez et al (2010) found no correlation between any cytokines (including EGF, fractalkine, IL-Ra, IL-8, VEGF, IL-6, IL-1β and IL-10) and DED symptom scores.

VEGF (Vascular Endothelial Growth Factor) was detected in all the tear samples in this study. The standard cocktails used to prepare the Luminex plates contained VEGF-A (commonly known as VEGF). Lower concentrations were found in the DED participants at both visits but did not reach significance at either (p=0.062 at Visit 1 and p=0.352). Negative correlations with all the clinical parameters were also found, but only corneal staining was significant at Visit 2 (r=-0.322, p=0.043).

Previous studies have reported conflicting findings with regard to VEGF (Kumar et al., 2023). Enriquez et al. (2010) reported significantly increased levels of VEGF in mild-to-moderate EDE. However, a larger study comparing EDE patients with controls reported significantly lower levels of VEGF in the dry eye patients, as found in this study (Benitez-Del-Castillo Sánchez et al., 2017); higher levels of VEGF-B and lower levels of VEGF-A were observed in the tears of DED patients compared to the controls in that study.

VEGF was the only cytokine to correlate with the OSDI questionnaire (r=-0.299, p=0.035) at Visit 1 only. Several previous studies have reported positive correlations between other cytokines and OSDI score, including IL-1β (Li et al., 2020a), IL-1Ra and IL-6 (Huang et al., 2012), and, IL-8 and TNF-α (Massingale et al., 2009).

The other growth factor, EGF (Epidermal Growth Factor) was also detected in all the tear samples in this study. Its mean concentration did not differ between the DED and non-DED participants at either visit. A positive correlation with conjunctival staining was found at Visit 1 only (r=0.298, p=0.036) and corneal staining at Visit 2 only (r=0.331, p=0.037).

Previous studies have shown EGF to be reduced in Sjögren's syndrome and ADDE (Pflugfelder et al., 1999;Ohashi et al., 2003), but increased in EDE due to MGD (Enríquez-de-Salamanca et al., 2010). Enriquez et al. (2010) also found a significant negative correlation with conjunctival staining; as conjunctival staining increased the concentration of EGF reduced. This correlation, however, would seem more likely if EGF concentration reduced in DED. Therefore, the role of EGF in EDE still remains unclear.

IL-1β was also present in all the tear samples analysed. Previously its modulatory role in DED has been questioned due to its very low levels in tear samples, with no statistically significant differences found between dry eye and control subjects (Narayanan et al., 2006). The mean concentration, in pg per mg of the TPC, was higher in the DED participants, but not statistically significant. The two inflammatory forms of IL-1, IL-1β and IL-1α, are both multifunctional and produce similar biological effects (Acera et al., 2008). Higher levels of IL-1β in DED have been reported in previous studies comparing EDE to control subjects (Solomon et al., 2001;Lam et al., 2009;Khamar et al., 2019;Li et al., 2020a). Murine models have also been used to demonstrate elevated levels of IL-1β in EDE, although it was suggested that IL-1 is not the initiator of the inflammatory response but rather functions with other cytokines (Narayanan et al., 2008). Interestingly, the concentration of IL-1β correlated significantly with increasing signs of MGD: meibum quality, expressibility of the glands, and upper meibography score. This trend was confirmed when Schirmer wetting length was used to calculate the concentration, and by the repeated finding of a significant positive correlation with meibum expression at Visit 2.

The anti-inflammatory antagonist of IL-1 activity, Interleukin-1 receptor antagonist (IL-1Ra), was found in high concentrations in all participants at both visits. The results obtained were all above the range of the highest standard, so the final concentrations obtained were predicted by extrapolation of the results and should therefore be interpreted with some caution. It has been suggested that higher levels of the anti-inflammatory IL-1Ra than the pro-inflammatory forms may be a natural homeostatic mechanism for preventing inappropriate activation of IL-1–mediated inflammatory events on the ocular surface (Solomon et al., 2001).

While no difference was found in the concentrations of IL-1Ra between the DED and non-DED groups at Visit 1, levels were significantly higher in the DED participants at Visit 2 ($p=0.047$). This is in agreement with previous studies (Enríquez-de-Salamanca et al., 2010;Huang et al., 2012). The current study also found a negative correlation of IL-1Ra concentration with average NIKBUT ($r=-0.393$, $p=0.005$) at Visit 1, which is similar to findings by Enriquez et al. (2010). IL-1Ra was also one of the six cytokines found to correlate with corneal staining ($r=0.444$, $p=0.004$) at Visit 2 (see Table 6.7). This

finding has also been reported previously, and further confirmed by the significant correlations with Schirmer wetting length (Huang et al., 2012).

The other anti-inflammatory cytokine investigated in this study, IL-10, was found in 91% or more of the participants at each visit. The concentrations found were higher than those reported in a previous study using Luminex Assays with tears collected by microcapillary; 5.3 ± 2.4 and 3.92 ± 1.3 pg/mg/mL in slight/moderate DED subjects and controls, respectively (Benitez-Del-Castillo Sánchez et al., 2017).

Higher mean concentrations were found in the DED participants at both visits of this study, but only reached significance when the concentration based on Schirmer wetting length was used (p=0.049 at Visit 2). Higher levels have previously been reported in subjects with Sjögren's syndrome (Lee et al., 2013;Zhao et al., 2018) and in non- Sjögren's DED (Massingale et al., 2009;Benitez-Del-Castillo Sánchez et al., 2017). However, conflicting findings have been reported regarding the association of IL-10 and MGD. Whereas Zhao et al. (2018) did not report any significant changes, Wu et al. (2020) reported higher levels in the DED subjects.

In this study, IL-10 was among the six cytokines (IL-1Ra, IL-10, IL-1β, IL-6, fractalkine, and TNF-α) showing significant correlations with average NIKBUT (r=-0.326, p=0.021), although none of these were repeated at Visit 2. In agreement with the increased levels of IL-10 in the DED participants, the concentration of IL-10 increased as the NIKBUT worsened. Significant positive correlations with horizontal LWE at Visit 1 (r=0.315, p=0.026) and corneal staining at Visit 2 (r=0.368, p=0.019) were also found. These results agree with a population-based study which found that IL-10 was increased in subjects with increased frequency of ocular discomfort, lower Schirmer scores and eyelid crusting (Tong et al., 2018). Other than this, there appears to be a paucity of studies reporting any correlations between IL-10 and any clinical features of EDE. Given its known role in providing a negative feedback mechanism, balancing the activation and deactivation of cytokines during chronic inflammation (Aketa et al., 2017), further study is warranted.

IL-12p70 was present in 88% or more of the DED and non-DED participants in this study. However, almost all (94.4%) of the relative concentrations fell below the range of least concentrated standard, with the remainder below the limit of detection, and so the data was extrapolated. The concentration (pg/mg/mL) was unchanged between the DED and non-DED participants at Visit 1, and slightly increased, at Visit 2 (p=0.037) when based on Schirmer wetting length. IL-12p70 is known to be critical to the initiation and progression of T cell activation (Th-1) in the immune response (Zhang and Wang, 2008), but only a few studies have investigated it, with conflicting findings (Lam et al., 2009;Na et al., 2012;Wu et al., 2020).

The IL-12p70 concentration correlated significantly with bulbar redness and horizontal LWE at Visit 1 of this study (r=0.288 p=0.043 and r=0.291, p=0.040, respectively). At Visit 2, positive correlations with corneal and conjunctival staining were found (r=0.474, p=0.002 and r=0.355, p=0.025, respectively). It is interesting that this cytokine was found to correlate with ocular surface staining, all-be-it differently at each visit. A previous comparison of MGD-related EDE with controls reported no correlation of this cytokine with TMH, Schirmer I test, TBUT, bulbar redness, meibography score or OSDI (Wu et al., 2020).

TNF-α, a potent pro-inflammatory cytokine, was found in all the tear samples at both visits of this study. Very similar concentrations were found in the DED and non-DED participants. Previously, it has been found to be upregulated in Sjögren's syndrome and ADDE (Oshida et al., 2004;Yoon et al., 2007;Lee et al., 2013). Studies involving subjects with EDE have found no significant differences when comparing to controls (Boehm et al., 2013;Khamar et al., 2019). However, a study comparing Sjögren's syndrome DED, non-SS ADDE, and MGD reported increased levels of TNF-α in all dry eye subtypes compared to controls (Zhao et al., 2018).

Although correlations were noted between NIKBUT at Visit 1 (r=-0.343, p=0.015) and conjunctival staining at Visit 2 (r=0.288, p=0.042), no correlations were found with the inflammatory marker MMP-9, which might be expected if TNF-α plays a role in an early inflammatory response in MGD-related DED.

No associations between sex or ethnicity and any cytokines were found at Visit 1. At Visit 2, significantly higher levels of fractalkine, IL-Ra, and IL-12p70 were found in the females (η^2 =0.122, p=0.027, η^2=0.181, p=0.006, and η^2=0.177, p=0.031, respectively), while lower levels of the total protein concentration were found (η^2=0.185, p=0.006). Female sex was shown to be a significant predictor of DED at both visits of this study (reported in Chapter 4). Therefore, higher levels of certain cytokines in the females would be logical; the reason for the elevated levels in the particular cytokines reported here is unclear though. Higher levels of fractalkine have previously been reported in non-DED men, but in a wider age range (18-88 years) than reported in this study (18-25 years) (Fernández et al., 2021).

No statistically significant associations were found between allergy with any cytokine at either visit. T-cell mediated inflammation has previously been implicated in the pathophysiology of chronic allergic eye disease (Metz et al., 1997). Higher levels of IL-1β and IL-6 have been reported in allergic eye disease (Acera et al., 2008). As the participants in this study were screened for any active ocular surface disease or allergy, the negative findings here give some reassurance that the results found were not compounded by any ocular surface disease other than DED.

When considering contact lens wear, there was no association with any cytokine at Visit 1. At Visit 2, a significantly lower mean concentration of EGF was found in the contact lens wearers (η^2=0.101, p=0.046). This finding is contrary to a previous report (González-Pérez et al., 2012). A recent review reported up-regulation of several inflammatory molecules in tears of contact lens wearers, including EGF, IL-1β, IL-6, IL-8, IL-17, leukotriene B4, and MMP-9 (Insua Pereira et al., 2022). While an increase in inflammatory markers may occur, particularly in CL-related disorders, these can also be adsorbed by the contact lens, thus potentially reducing their presence (Mann and Tighe, 2013). However, the participants in this study did not wear their lenses on the day of attendance, and all wore soft monthly or daily disposable lenses.

At Visit 2, two participants who did not report use of an ocular lubricant at Visit 1 had started using doing so at Visit 2; one a contact lens wearer on an 'as required' basis and the other a DED participant. Another participant changed brand of hyaluronic acid based lubricant, while one other changed to this from a glycerol based solution. Artificial tears are known to protect the eye from hyperosmolarity and thus reduce inflammation by disrupting the processes that promote the recruitment of cytokines and (Yagci and Gurdal, 2014). While few human studies have explored the effect of hyaluronic acid alone on the level of tear cytokines, a reduction of inflammatory cytokines such as TNF-α have been demonstrated in vitro (Chatard-Baptiste et al., 2018). A larger cohort would be needed to further explore the effect of ocular lubricants in reducing the levels of inflammatory cytokines in a young cohort, particularly where contact lens wear is a confounding factor.

When considering other lifestyle factors, no associations were found between any cytokine at either visit with screen use, exercise, or outdoor activity. Higher levels of stress correlated with higher total protein concentrations at Visit 1 (r=-0.290, p=0.041). At Visit 2, correlations were found between higher stress levels and poorer self-perceived health status with higher cytokine concentrations: IL-8 was moderately negatively correlated with stress (r=-0.346, p=0.029) and IL-6 correlated with health (r=-0.413, p=0.08). Poorer self-perceived health quality was also significantly negatively correlated with higher levels of VEGF (r=-0.341, p=0.031). Poorer self-perceived health quality and higher stress levels have previously been shown to correlate with DED, and therefore the changes in cytokine levels found in this study are concordant.

The number of cytokines investigated in this study was limited to ten. Due to the correlations between individual cytokines and the clinical parameters found in this young cohort, further investigation including other inflammatory cytokines such as IFN-γ would be of interest. The smaller sample size at Visit 2 led to the study being underpowered for correlation analysis and comparisons between groups; a larger sample size would improve the statistical power of the study.

A further limitation of this study included the need to extrapolate data for IL-1Ra and IL-12p70. The large volumes of tears (12-25µL) required for the method described in this study, which required further dilution prior to analysis, may have contributed to a loss of sensitivity, whereby the cytokine level fell below the lower limit of detection. During Luminex the microbeads themselves may settle out of any solution, and the magnetic field can become disrupted during the washing and decanting steps leading to a loss of material (Dionne et al., 2016). Furthermore, the signal-to-noise ratio can be disrupted by blooming effects, matrix effects from other components in the sample, and cross-talk between capture and probe antibodies, thus reducing the reliability of the results (Li et al., 2008). However, tears eluted from Schirmer strips, as in this study, have been found to exhibit lower noise than those collected by microcapillary tubes, thereby reducing tear-specific matrix effects, and improving sensitivity.

A further study involving a larger cohort would help to determine the repeatability of the findings reported here, in order to validate them for detecting EDE in the early stages. The incorporation of another validated symptom questionnaire such as the Neuropathic Pain Inventory modified for Eye (NPSI-E) would also provide more precise information regarding the symptoms that this young cohort present with. Although this study was novel in following young participants over a one-year period, a longer study would also enable monitoring of the clinical parameters and cytokine levels for change, should there be disease progression. Furthermore, this would also aid in detecting a panel of the most relevant biomarkers for early detection of EDE.

6.5. Conclusion

The findings of the cytokine analysis in this study suggest that inflammation plays an important role in early DED and not just in severe cases or older cohorts. Differences in the tear cytokine levels were found in young adults with and without DED, in agreement with previous studies of EDE in older cohorts. A lower total protein concentration, as expected in DED, was significantly correlated at both visits with increased corneal staining. Several cytokines were upregulated in the DED participants, more notably at Visit 2, including IL-1Ra, IL-10, both with anti-inflammatory actions, and the pro-inflammatory cytokines, IL-12p70 and fractalkine. The concentration of several cytokines significantly correlated with the average NIKBUT at Visit 1, while at Visit 2 significant correlations with corneal staining were observed. These findings were further validated when cytokine concentrations were based on Schirmer wetting length. Correlations with ocular surface signs, such as horizontal LWE (IL-10, IL-12p70, IL-Ra, fractalkine and EGF) and ocular redness (IL-8 and IL-12p70) were also observed. The repeatable correlation between the up-regulation of IL-6 and IL-8 with MMP-9 lends further

support to the role of inflammation in the early pathogenesis of DED. Significant associations between IL-1β and several meibomian gland parameters indicated its role in evaporative DED.

The next chapter explores the composition of meibomian gland secretions collected during the study reported here, for further potential biomarkers of evaporative DED.

Chapter 7 - Prospective, longitudinal study assessing dry eye among young adults - meibum lipidome analysis

7.1. Introduction

Meibum or meibomian lipid is the secretory product of the meibomian glands; modified sebaceous, holocrine glands present in the upper and lower lids. Meibum is spread onto the tear film, a thin layer approximately 2-5.5µm thick, with each blink (Willcox et al., 2017). The tear film lipid layer (TFLL), supplied principally by the meibomian glands, has been found to vary between 15-200nm, with a mean thickness of approximately 42nm (King-Smith et al., 2010). It creates a thin hydrophobic film which provides a smooth optical surface for the cornea and protects against evaporation of aqueous tears from the eye (Brown et al., 2016). It also forms a hydrophobic barrier along the margins of the eyelids to contain the tear film and prevent spillage (Bron et al., 2004). Alterations in its composition can alter the physical properties of the tear film, and in turn may affect evaporation. Although a normal tear film should resist evaporation, Millar and Schuett (2015) argue that it is perhaps too simplistic to think of it as merely an evaporative blanket, the main role of the TFLL being to allow the spread of the tears and prevent its collapse onto the ocular surface. Numerous studies have shown, however, that the increased instability of the tear film of dry eye patients is linked to deficiencies in meibum production and/or alterations in the lipid (Joffre et al., 2008;Lam et al., 2014b;Borchman et al., 2019;Suzuki et al., 2022)

7.1.1. Meibum as a candidate biomarker of meibomian gland dysfunction

Meibomian gland dysfunction (MGD) is defined as 'a chronic diffuse abnormality of the meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative and quantitative changes in glandular secretions' (Nelson et al., 2011). It has been suggested that the lipid composition in meibum plays a critical role in the pathogenesis of MGD. The biochemical differences in the composition or distribution of lipids could act as biomarkers; a single biomarker may not be sufficient and therefore, as with tears, a panel of multiple biomarkers has been suggested (Asiedu, 2022). However, few studies have compared the lipid profiles of MGD subjects to controls, and those that have found alterations appear to report conflicting results. Therefore, a comparison of the meibum composition between participants with and without MGD could broaden the knowledge surrounding the pathogenesis of evaporative DED due to MGD in early disease.

7.1.2. Study objectives

This study aimed to compare the composition of polar and non-polar lipids in the meibum of a young adult cohort. Meibum was collected as part of the study reported in Chapter 4 of this thesis, exploring clinical and molecular biomarkers of DED in a young adult cohort. Meibum analysis aimed to provide

an insight into the development of evaporative DED due to MGD, in its early stages. Due to the symptomatic element of a DED diagnosis, and the significant overlap in signs of MGD between DED and non-DED participants previously reported in this thesis (chapter 4), meibum samples were compared based on the extent of clinical signs of MGD rather than a DED diagnosis.

Null hypothesis

- There are no differences in meibum composition when comparing participants with more than grade 2 composite meibography score to those with less than or equal to grade 2 composite score
- There are no correlations with any clinical parameters used to identify MGD
- There would be no change or progression in the meibomian lipids over the one year study period

7.2. Methods

7.2.1. Meibum collection

Meibum was collected in accordance with the Aston University Optometry and Vision Sciences Research Group (OVSRG) Optometry Translational Research Protocol for Meibum Collection (see **Appendix 14**). The spatula method was used to collect meibum in this study, as it has been shown to have the most balanced benefits, particularly where yield is concerned (Haworth et al., 2011).

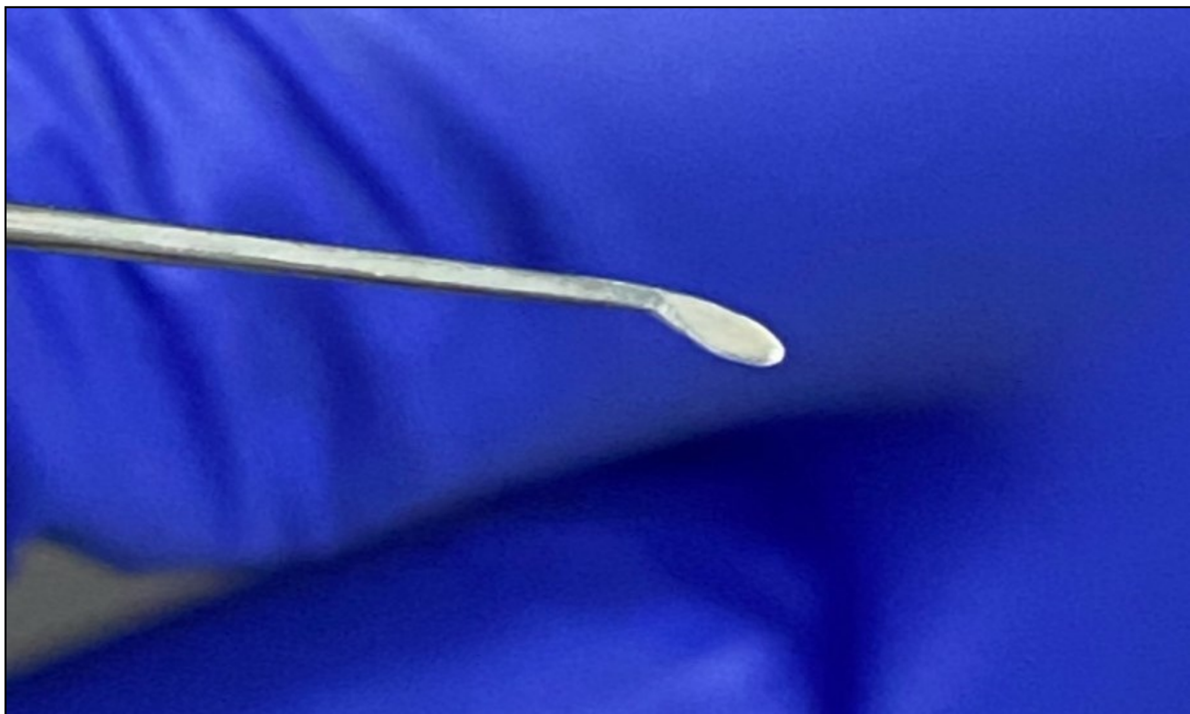


Figure 7.1. Fresh meibum sample collected from the lower lid margin

As with tear collection, sterile latex/vinyl gloves were worn to prevent contamination with skin lipids. The eyelid margin was cleaned prior to collection by gently swabbing with a cotton bud soaked in sterile saline to reduce possible contamination of the sample with tears and to avoid collection of epithelial cells. Gentle digital pressure was applied to the lower lid to express the meibomian glands and any expressed meibum was collected using the long side of the angled portion and the rounded tip of a sterile metal golf club spud spatula (Malosa Medical, UK), see ***Figure 7.1***. During collection, the lid margin was rolled away from the globe, where possible, to minimise contamination with tears and conjunctival cells.

Samples were immediately transferred from the spatula to an amber glass vial containing 270µl of HPLC grade chloroform, to prevent contamination through the use of plastic containers (Butovich et al., 2008). Samples were then dried using exposure to nitrogen gas prior to storage at -80°C, avoiding exposure to moisture and light, and capped to prevent possible lipid breakdown.

7.2.2. Meibum analysis

The meibum lipidome was analysed for the main lipid species from the major lipid classes, including non-polar cholesteryl ester (ChE), wax ester (WE), triglycerides (TG), and the polar or amphiphilic lipids including OAHFA, glycerophospholipids (phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidic acid (PA); phosphatidylserine (PS); phosphatidylinositol (PI); phosphatidylglycerol (PG)) and sphingophospholipids (sphingomyelin (SM); ceramide (Cer)). Samples were analysed by LC-MS/MS; high-performance liquid-chromatography coupled with mass spectrometry (HPLC/MS), and tandem mass spectrometry (MS/MS) for qualitative and quantitative analysis.

7.2.2.1. Meibum preparation

Meibum samples were prepared and analysed by Professor Corinne Spickett, Aston University, UK. Each meibum sample, of between 1-2 µg, was redissolved in 100 µL of Optima grade isopropanol (IPA) (Fischer Scientific, UK). Initially, four test samples were used, and diluted to either 10+40 µL IPA (1/5) or 10+90 µL IPA (1/10). However, the sample signals were too weak with these dilutions, so subsequently the initial concentration of meibum solution (100 µL) was used for analysis.

7.2.2.2. Mass spectrometry

Mass spectrometry (MS) is a sensitive method of analysis which permits both detection and identification of small samples (Green-Church et al., 2011). Mass spectrometers operate by converting the analyte molecules to a charged (ionised) state, with subsequent analysis of the ions and any

fragment ions that are produced during the ionisation process, on the basis of their mass to charge ratio (*m/z*) (Pitt, 2009).

While several different technologies are available for infusion, ionization, separation, and detection, this study used untargeted LC-MS/MS to explore the lipid composition within each sample individually. Aliquots (2 µL) were analysed by liquid chromatography-MS^e on a Waters I-class UPLC connected to a Waters SELECT Series Cyclic IMS. 5 µL of the diluted lipid extract was injected onto an Accucore™ C30 reverse phase column, 150 x 2.1 mm (length x internal diameter), 2.6 µm particle size (ThermoScientific, UK) at a flow rate of 200 µL/min and temp of 50 ^{o}C using a 30 minute gradient of mobile phases. The m/z range for both ionization modes was 100-2000 Da with a scan time of 0.5s. Separate runs for positive and negative ion modes were made.

A single OAHFA standard (16:0; 18:1) (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) was analysed and its retention time used to optimise the system methodology in order to identify other OAHFAs.

7.2.3. Lipid data analysis

Following pre-selection of possible adducts likely to be found in the samples, the LC-MS/MS data for each sample run was imported into the Progenesis QI discovery analysis software (Version 2, Nonlinear Dynamics, UK). This was performed by Professor Andrew Pitt, Manchester University, UK. Adduct selection for positive ion mode was M + H, M + 2H and M + NH4 and for negative ion mode M – H, M – 2H, M + Na – 2H, M + FA – H and M - H2O – H. Adduct selection allows the software to automatically deconvolute the compound ions detected which could then be represented as an ion intensity map. ***Figure 7.2***. shows an example of an ion intensity map, which maps the sample's MS signal by m/z and retention time. The retention time increases from top to bottom (y-axis), while m/z increases from left to right (x-axis), with the darker areas representing a high abundance of ions in the MS signal.

The ion maps were then aligned to correct for any drift in the retention time between the runs, making it possible to accurately compare the compounds in each run. A single set of compound ion outlines could then be added to all the runs, allowing all the compounds to be found, even if missing from one or more sample runs. The Progenesis software used a peak picking algorithm to detect compound ions that made up each compound, including overlapping compound ions, which then helped to generate accurate compound quantification or abundance data. Filters and tags were then applied to the data in the software to elicit the relevant compounds. The results of the quantification and identification were viewed and multivariate statistical analysis including Principal Component Analysis

(PCA) was employed to explore compounds whose abundance differed between the groups investigated.

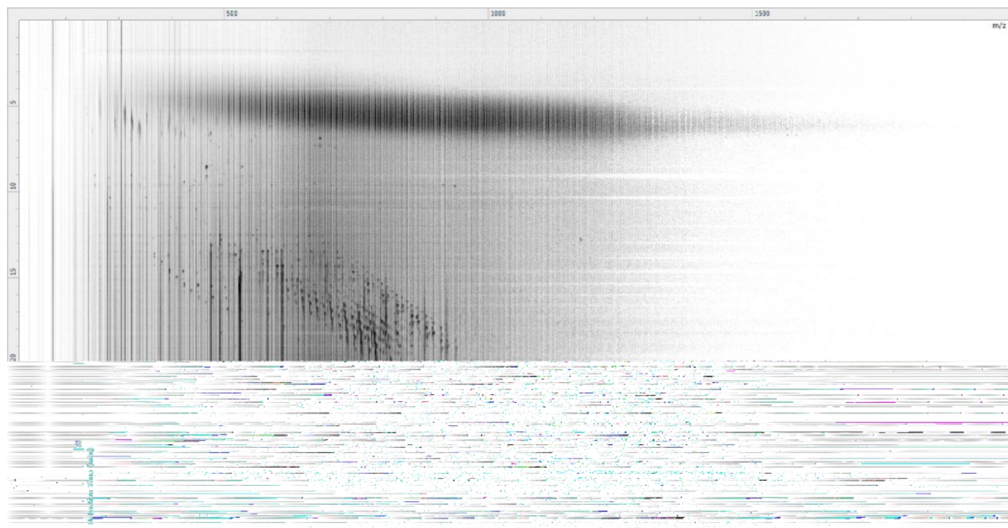


Figure 7.2. An ion intensity map

Compounds with an unlikely structure, a run time less than 6 minutes or an abundance of less than 50 were hidden from the analysis. Tags were applied in order to label low p values ($p<0.05$) or identify where a fold change of >2 had occurred. Filters were also applied to the polar and non-polar compounds present in each ion mode in order to determine any differences between the meibum composition from participants with greater than grade 2 composite meibography score to those with less than or equal to grade 2.

With the use of Progenesis MetaScope and LipidBlast databases, compounds were assigned to a lipid class. Lipid Maps (LipdMaps, 2023) uses a classification system which divides lipids into eight categories including fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids, saccharolipids, and polyketides (Fahy et al., 2005). These classes contain distinct classes and subclasses of molecules. Where several possible compound identities were suggested, the Lipid Maps database could be used to identify them based on exact *m/z* values (mass divided by charge number of ions) and retention times (LipdMaps, 2023). Prior knowledge of likely features, such as theoretical m/z values, possible adducts, fragmentation score, number of carbons and double bonds, and which ion mode the compounds were likely to appear in, were also used for identification. While the exact identities of some compounds were accepted, others had multiple possible identities, which meant it was not always possible to assign an exact identity to them. Therefore, compounds were reported according to their lipid class.

The lipids identified in the study were reported either according to their lipid name or described with reference to the fatty acid chains and number of double bonds which constitute the lipid. For example, (16:0:18:1), the standard OAHFA, which has 16 carbons with zero double bonds in one fatty acid chain and 18 carbons with one double bond in the other fatty acid chain. Fatty acids with no double bonds are known as 'saturated' fatty acids, whereas fatty acids with one or multiple double bonds are termed 'mono-unsaturated' or 'polyunsaturated' fatty acids, respectively. Saturated fatty acids, such as palmitic acid (C16) and stearic acid (C18), and mono-unsaturated fatty acids such as oleic acid (C18) tend to be non-polar, while polyunsaturated fats such as linoleic acid tend to be polar. Cholesterol esters are reported as the number of carbons in the fatty acid that is added, as cholesterol (which has 27 carbons) is always the same.

The raw abundance, normalised abundance, retention time, and highest and lowest mean abundance of each lipid compound were also reported. The progenesis software performs a global normalisation to correct for factors that result in experimental or technical variation when running samples, such as sample size differences. Therefore, correlation analysis with the clinical findings reported in Chapter 4 was possible.

However, the total lipid content for each sample could not be calculated, as the data generated from mass spectrometry is not intrinsically quantitative, with different signal strengths generated by different lipid classes.

7.2.4. Data analysis and sample size

An experiment was set up in the Progenesis software, to compare the lipid compounds between the participants with grade ≤ 2, and grade > 2 meibomian gland loss. An alpha value of 0.05 was adopted for statistical significance when considering individual lipids. However, when analysis was performed on compounds grouped into their lipid classes, rather than as individual lipids, the criteria was relaxed to $p<0.20$, in order to discover any trends. The software also calculated a q-value, an adjusted p-value that incorporated multiple testing correction in order to control the percentage of false positives. A q-value of 0.05 implied that 5% of the significant tests would result in false positives.

Principal Component Analysis (PCA) was performed, using the Progenesis QI software, to examine the compound abundance levels across the sample runs to determine the principal axes of abundance variation. Transforming and plotting the abundance data in principal component space permits separation of the run samples according to abundance variation, in order to identify run outliers.

Data analysis was also performed using IBM SPSS Statistics for Windows, Version 26. Spearman ranked correlation was used to quantify any correlations between the clinical parameters (reported in Chapter 4) and meibum lipids. The strength of any correlation (Spearman's correlation coefficient r) was estimated according to the following coefficient magnitudes: −1 (perfect negative correlation) through 0 (no correlation) to +1 (perfect positive correlation) (Mukaka, 2012). A high correlation was 0.5-1.0, moderate 0.5-0.3, and 0.3 to 0.1 a small if any correlation. Due to the subjective nature of dry eye symptoms used in the diagnosis of DED, a composite meibography score was generated (upper and lower meibography scores combined), in order to apply a more objective measure of MGD. This was used as a single clinical indicator of the level of MGD.

To date there are few previous studies which report data from which a sample size could be calculated. The different methods employed, and the vast number of different lipid compounds present do not allow for an accurate sample size to be calculated from one single lipid compound.

7.3. Results

The meibum lipidomes of 29 participants were analysed from Visit 1. Samples were not collected from two participants at Visit 1, due to the amount of digital pressure required for expression being too great when compared to the other samples collected. Samples were categorised into two groups according to the subjective meibography grading: grade ≤ 2 composite meibography score (n=18) and greater than grade 2 composite meibography score (n=11). Repeat data was obtained for 21 participants at Visit 2: 10 participants with grade ≤ 2 composite meibography score, and 11 with grade > 2. Of the 29 samples a Visit 1, 24 were from females and 25 from participant of Asian ethnicity, while at Visit 2, 17 of the 21 samples were from females and 18 were from participants of Asian ethnicity.

The meibum analysis generated a large amount of raw data. Filters and tags were applied in order to discover the polar and non-polar compounds present in each ion mode and their significance. While non-polar lipids were generally found in the positive ion mode, and polar lipids were found more frequently in the negative ion mode, there was some overlap in this study.

7.3.1. Results for Visit 1

In total, it was possible to categorise 191 compounds, at Visit 1, according to their lipid class, when combining the data from both ion modes and applying filters to discover the lipid classes shown in ***Table 7.1***.

Table 7.2 displays the lipids with a definite identity, at Visit 1. The compounds were separated into polar and non-polar lipids. ***Table 7.3*** presents the compounds which differed significantly between the two gland loss categories. No significant differences were found in the normalised abundance of the non-polar fatty acids; 24 fatty acyls were identified, 11 of which had a higher mean abundance when gland loss was grade ≤ 2. Eight of the ten lipid sterols identified were higher in the participants with grade ≤ 2, but not significantly. However, 8 of the sterols were exogenous and of plant-based origin. The two cholesterol esters identified were higher in the participants with less gland loss, but not significantly.

Six of the eight identified di- and tri- glycerides had a higher mean abundance in the participants with gland loss grade > 2, including a significant finding for 1,2,3-tricaprinoyl-glycerol (p=0.037). However, the diglyceride, DG(20:0/22:0/0:0)[iso2], had a significantly higher mean abundance (p=0.018) in the participants with meibomian gland loss grade ≤ 2. When including the di- and triglycerides which showed significant differences to the p<0.20 level (see ***Table 7.4***), six compounds had a higher mean abundance where gland loss was grade ≤ 2 , while seven were higher when gland loss was lower.

Sixteen polar lipids were identified, including two free cholesterol, three OAHFAs, four glycerophospholipids and six sphingolipids. Seven of the ten phospholipids identified (GP and SP) were present in higher mean abundance in the participants with gland loss ≤ grade 2. Two of the three OAHFAs also followed this trend, although there were no significant differences. However, the glycerophospholipid, PG(21:0/0:0) and the ceramide, CerPE(d14:2(4E,6E)/16:0(2OH)), were significantly more abundant when the gland loss was greater (p=0.036 and p=0.032, respectively). From ***Table 7.3***, which reports the significant differences (p<0.05), it can be seen that four other non-identified phospholipids were also present in significantly higher abundance when the gland loss was greater than grade 2. The free cholesterol, 22:1-Glc-cholesterol, was also significantly more abundant when gland loss was greater than grade 2 (p=0.011).

Table 7.1. Compounds found in various lipid classes at Visit 1

Visit 1	Lipid class	Number of compounds		Number of compounds		Number of compounds
Non-polar lipids	Fatty acyls (FA)	38	Wax monesters (WE)	10		
			Fatty acyls (FA) other	11 (3)		
			Unsaturated fatty acids	5		
			Long chain fatty acids	2 (2)		
			Saturated or straight chain fatty acids	10		
	Sterol lipids (ST)	23	Sterol esters (ST)	12 (3)	Cholesterol esters	2
	Glycerolipids (GL)	76	Diglycerides (DG)	33 (15)		
			Triglycerides (TG)	42 (32)		
			Monoglycerides (MG)	1		
Polar lipids	Free cholesterol	2				
	OAHFAs	3				
	Phospholipids	48	Glycerophospholipids (GP)	16 (11)	Phosphatidic acids (PA)	5 (3)
					Phosphatidylserines (PS)	2 (1)
					Phosphatidylcholines (PC)	0
					Phosphatidylethanolamines (PE)	1 (1)
					Phosphatidylinositols (PI)	0
					Phosphoroglycerols (PG)	5 (5)
			Sphingophospholipids (SP)	32 (5)	Ceramides (CER)	24 (4)
					Sphingomyelins (SM)	2 (1)

Numbers in red brackets specify the number of the compounds reported in black that were found in the positive ion mode

Table 7.2. Lipid compounds identified at Visit 1

Mass to charge ratio(m/z) (Visit 1)	Molecular Formula	Category, main class, subclass	Common name	Highest mean normalised abundance	Significance between gland loss groups (p value)
Non-polar lipids					
255.2331	$C_{16}H_{32}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Palmitic acid	Gland loss > 2	0.530
279.2333	$C_{18}H_{32}O_2$	FA, fatty acids and conjugates, unsaturated fatty acids	Linoleic acid	Gland loss > 2	0.806
281.2486	$C_{18}H_{34}O_2$	FA, fatty acids and conjugates, unsaturated fatty acids	Oleic acid	Gland loss > 2	0.380
283.2645	$C_{18}H_{36}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Stearic acid	Gland loss ≤ 2	0.420
367.3582	$C_{24}H_{48}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Lignoceric acid	Gland loss ≤ 2	0.903
381.3738	$C_{25}H_{50}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Hyenic acid	Gland loss ≤ 2	0.803
393.3740	$C_{26}H_{50}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	11Z-hexacosenoic acid	Gland loss ≤ 2	0.407
395.3895	$C_{26}H_{52}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Cerotic acid	Gland loss ≤ 2	0.725
409.4053	$C_{27}H_{54}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Carboceric acid	Gland loss ≤ 2	0.823
423.4210	$C_{28}H_{56}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Montanic acid	Gland loss ≤ 2	0.866
425.3641	$C_{25}H_{48}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	16Z-pentacosenoic acid	Gland loss > 2	0.702
437.4369	$C_{29}H_{58}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	nonacosanoic acid	Gland loss ≤ 2	0.800
453.3955	$C_{27}H_{52}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	20Z-heptacosenoic acid	Gland loss > 2	0.715

Mass to charge ratio(m/z) (Visit 1)	Molecular Formula	Category, main class, subclass	Common name	Highest mean normalised abundance	Significance between gland loss groups (p value)
549.5614	C37H74O2	FA, fatty acids and conjugates, straight chain fatty acids	heptatriacontanoic acid	Gland loss > 2	0.343
563.5761	C38H76O2	FA, fatty acids and conjugates, straight chain fatty acids	Octatriacontanoic acid	Gland loss > 2	0.340
459.3274	C32H46O3	FA, fatty alcohols	Triangulyne A	Gland loss ≤ 2	0.886
421.4054	C28H54O2	FA, fatty esters, wax monoesters	Myristoleyl myristate	Gland loss ≤ 2	0.373
451.4526	C30H60O2	FA, fatty esters, wax monoesters	Lauryl stearate	Gland loss ≤ 2	0.620
481.4268	C29H56O2	FA, fatty esters, wax monoesters	Tridecyl palmitoleate	Gland loss > 2	0.708
507.5149	C34H68O2	FA, fatty esters, wax monoesters	Arachidyl myristate	Gland loss > 2	0.440
521.5304	C35H70O2	FA, fatty esters, wax monoesters	Octadecyl heptadecanoate	Gland loss > 2	0.347
535.5459	C36H72O2	FA, fatty esters, wax monoesters	Palmityl arachidate	Gland loss > 2	0.352
591.6085	C40H80O2	FA, fatty esters, wax monoesters	Stearyl behenate	Gland loss > 2	0.344
621.5832	C39H76O2	FA, fatty esters, wax monoesters	Tricosanyl palmitoleate	Gland loss > 2	0.601
685.6138	C44H80O2	ST, sterols, steryl esters	Campestanyl-16:0 #	Gland loss > 2	0.856
697.6143	C45H80O2	ST, sterols, steryl esters	18:0 Cholesterol ester	Gland loss ≤ 2	0.938
711.6276	C46H82O2	ST, sterols, steryl esters	19:0 Cholesterol ester	Gland loss ≤ 2	0.518
739.6612	C48H86O2	ST, sterols, steryl esters	20:0 Campesterol ester #	Gland loss ≤ 2	0.394
754.7459	C51H92O2	ST, sterols, steryl esters	22:0 Sitosterol ester #	Gland loss >2	0.488
755.6921	C49H9002	ST, sterols, steryl esters	Sitostanyl-20:0 #	Gland loss ≤ 2	0.555
769.7097	C50H92O2	ST, sterols, steryl esters	Campestanyl-22:0 #	Gland loss ≤ 2	0.194
781.7080	C51H9202	ST, sterols, steryl esters	22:0 Sitosterol ester #	Gland loss ≤ 2	0.570
783.7229	C51H94O2	ST, sterols, steryl esters	Sitostanyl-22:0 #	Gland loss ≤ 2	0.555
916.7940	C57H102O7	ST, sterols, steryl esters	22:0-Glc-Sitosterol #	Gland loss ≤ 2	0.084
701.6453	C46H8805	GL, diradylglycerol, diacylglycerols	DG(21:0/22:1(13Z)/0:0)[iso2]	Gland loss >2	0.843
703.6611	C46H9005	GL, diradylglycerol, diacylglycerols	DG(21:0/22:0/0:0)[iso2]	Gland loss >2	0.855
715.6610	C47H90O5	GL, diradylglycerol, diacylglycerols	DG(22:0/22:1(13Z)/0:0)[iso2]	Gland loss ≤ 2	0.554

Mass to charge ratio(m/z) (Visit 1)	**Molecular Formula**	**Category, main class, subclass**	**Common name**	**Highest mean normalised abundance**	**Significance between gland loss groups (p value)**
717.6768	C47H92O5	GL, diradylglycerol, diacylglycerols	DG(22:0/22:0/0:0)	Gland loss >2	0.803
753.6767	C45H88O5	GL, diradylglycerol, diacylglycerols	DG(20:0/22:0/0:0)[iso2]	Gland loss ≤ 2	**0.018***
554.4554	C33H62O6	GL, triradlyglycerol, triacylglycerol	1,2,3-tricaprinoyl-glycerol	Gland loss >2	**0.037***
675.5542	C40H76O6	GL, triradlyglycerol, triacylglycerol	TG(12:0/12:0/13:0)[iso3]	Gland loss >2	0.706
917.7014	C62H94O6	GL, triradlyglycerol, triacylglycerol	TG(17:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z)/22:6(4Z,7Z, 10Z, 13Z, 16Z,19Z)) [iso6]	Gland loss >2	0.576
Polar lipids					
753.5606	C45H78O7	ST, sterols, cholesterol	12:0-Glc-Cholesterol	Gland loss ≤ 2	0.853
886.7477	C55H96O7	ST, sterols, cholesterol	22:1-Glc-cholesterol	Gland loss >2	**0.011***
729.6765	C48H90O4	FA, fatty esters, fatty acid estolides, unsaturated fatty acids	FAHFA 16:1/32O(FA 32:1) (OAHFA 48:2)	Gland loss >2	0.835
757.7077	C50H94O4	FA, fatty esters, fatty acid estolides, unsaturated fatty acids	FAHFA 16:1/34O(FA 34:1) (OAHFA 50:2)	Gland loss ≤ 2	0.802
785.7389	C52H98O4	FA, fatty esters, fatty acid estolides, unsaturated fatty acids	FAHFA(18:1/34-O-34:1) OAHFA (52:2)	Gland loss ≤ 2	0.739
759.4950	C42H73O8P	GP, glycerophosphates, diacyglycerophosphates	PA(19:0/20:5(5Z,8Z,11Z,14Z,17Z))	Gland loss ≤ 2	0.598
537.3569	C27H53O8P	GP, glycerophosphoglycerols, monoacylglycerophosphoglycerol	PG(21:0/0:0)	Gland loss > 2	**0.036***
749.4352	C39H67O10P	GP, glycerophosphoglycerols, diaglycerophosphoglycerols	PG(18:4(6Z,9Z,12Z,15Z)/15:1(9Z))	Gland loss ≤ 2	0.839
1061.774	C60H111O11P	GP (glycerophospholipid), glycerophosphoglycerol	SLBPA(54:3)	Gland loss ≤ 2	0.872
598.5167	C37H71NO3	SP, ceramide, n-acylsphingosines (ceramides)	Cer(d15:2(4E,6E)/22:0)	Gland loss >2	0.664

Mass to charge ratio(m/z) (Visit 1)	**Molecular Formula**	**Category, main class, subclass**	**Common name**	**Highest mean normalised abundance**	**Significance between gland loss groups (p value)**
663.4339	C32H63N2O7P	SP, phosphosphingolipid, ceramide phosphoethanolamines	CerPE(d14:2(4E,6E)/16:0(2OH))	Gland loss >2	**0.032***
744.6497	C45H89NO5	SP, ceramide, n-acylsphingosines (ceramides)	Cer(t18:1(6OH)/27:0(2OH))	Gland loss ≤ 2	0.101
746.6673	C32H63N2O7P	SP, ceramide, phosphosphingolipids, ceramide phosphoethanolamines	Cer(t18:0/27:0(2OH))	Gland loss ≤ 2	0.096
770.6852	C45H91NO5	SP, ceramide, N-acyl-4-hydroxysphinganines (phytoceramides)	Cer(t18:0/27:0(2OH))	Gland loss ≤ 2	0.222
800.7137	C49H97NO5	SP, ceramide, n-acylsphingosines (ceramides)	Cer(t18:1(6OH)/31:0(31OH))	Gland loss ≤ 2	0.276

*# Plant sterols. * P-values <0.05 were considered significant and are shown in bold.*

Table 7.3. Compound ions significantly different between gland loss categories at Visit 1

Lipid category (Visit 1)	Mass to charge ratio (m/z)	Lipid class	Compound name (if known)	Highest mean normalised abundance	Lowest mean normalised abundance	p value
Fatty acyls (FA)						
Sterol lipids (ST)						
Glycerolipids (GL)	622.5423	DG		Gland loss > 2	Gland loss ≤ 2	0.014*
	753.6644	DG	DG(20:0/22:0/0:0) [iso2]	Gland loss ≤ 2	Gland loss > 2	0.018*
	554.4554	TG	1,2,3-tricaprinoyl-glycerol	Gland loss > 2	Gland loss ≤ 2	0.037*
	913.7281	TG		Gland loss ≤ 2	Gland loss > 2	0.036*
	1025.852	TG		Gland loss ≤ 2	Gland loss > 2	0.021*
Free cholesterol	886.7477	Free Ch	22:1-Glc-cholesterol	Gland loss > 2	Gland loss ≤ 2	0.011*
OAHFAs						
Phospholipids (PL)	537.3569	GP (PG)		Gland loss > 2	Gland loss ≤ 2	0.036*
	593.4200	GP (PA)		Gland loss > 2	Gland loss ≤ 2	0.030*
	911.6725	GP (PG)		Gland loss > 2	Gland loss ≤ 2	0.035*
	663.4339	Cer	CerPE(d14:2(4E,6E)/16:0(2OH))	Gland loss > 2	Gland loss ≤ 2	0.032*
	691.4656	Cer		Gland loss > 2	Gland loss ≤ 2	0.036*

** P-values <0.05 were considered significant*

By relaxing the criteria for significance to p<0.20, whilst grouping the lipids together into their classes, it can be seen that the fatty acyls and sterols (non-polar lipids) have the highest mean abundance where the gland loss is grade ≤ 2, i.e., lower abundance where more gland loss is present. The results for the and di- and triglycerides are equivocal. These results are shown in ***Table 7.4***. Considering the polar lipids, the glycerophospholipids appear to be highest where gland loss is greater, but the sphingolipids do not show a difference.

The lipid compounds that were found to show a significant difference between the two meibomian gland categories were further analysed to explore any correlations with the meibomian gland-related clinical findings reported in Chapter 4. The results of the non-parametric correlation analysis are shown in ***Table 7.5***. Twenty-one lipid compounds are shown, with 15 of them being present in all the samples collected.

Table 7.4. Mean abundance for different lipid classes where p<0.20

Lipid class (Visit 1)	Number of lipids where p< 0.20	Highest mean abundance where gland loss ≤ 2	Highest mean abundance where gland loss > 2
Non-polar lipids			
FA	3	3	
WE	2		2
ST	0		
DG	6	2	4
TG	7	4	3
Polar lipids			
Free Ch	1		1
OAHFAs	0		
GP	6	1	5
SP (ceramides)	7	4	3

Spearman correlation analysis identified several moderate to high positive correlations with the lower lid meibography score, indicating higher levels of these lipids with greater meibomian gland loss. These included one triglyceride, one free cholesterol and three phospholipids (see ***Table 7.5***). A further triglyceride showed this relationship but did not reach significance. Likewise, a significant correlation between the upper meibography score and the unidentified phosphoroglycerol which correlated with lower meibography score was found.

A significant negative correlation between the triglyceride 913.7280 m/z and the quality of the meibum expressed was found (r=-0.465, p=0.013); as the quality of the meibum became thicker the abundance reduced. This trend was found with two further triglycerides and one diglyceride but did not reach significance. The same triglyceride (913.7280 m/z) was also found to positively correlate with the NIKBUT (r=0.404, p= 0.033); as the tear breakup time reduced the abundance of this triglyceride also reduced. No correlations were found between any lipid compound and the number of glands secreting, or the lipid layer thickness.

Although the mean abundance of the OAHFAs did not show any significant differences between meibomian gland categories, correlations were found with NIKBUT; significant positive correlations were found with OAHFA 50:2 (r=0.427, p=0.021), and OAHFA 52:2 (r=0.482, p=0.008).

Table 7.5. Spearman correlation coefficients between specific lipids and meibomian gland-related parameters (Visit 1)

Lipid category and mass to charge ratio (m/z) (Visit 1)	Compound name (if known)	Highest mean normalised abundance	Spearman correlation coefficient & significance	Average NIKBUT	Lipid layer thickness	Meiboscale upper lid	Meiboscale lower lid	Meibomian glands secreting	Meibum quality
FA 439.4130		Gland loss ≤ 2	r p-value	0.045 0.821	0.023 0.906	-0.354 0.065*	-0.228 0.244	-0.209 0.285	-0.136 0.491
FA 467.4468		Gland loss ≤ 2	r p-value	0.082 0.680	0.054 0.787	-0.297 0.125	-0.238 0.224	-0.131 0.506	-0.093 0.638
FA 508.5227		Gland loss ≤ 2	r p-value	0.145 0.463	-0.050 0.800	-0.254 0.191	-0.186 0.342	-0.113 0.566	-0.100 0.612
WE 581.5264		Gland loss > 2	r p-value	0.034 0.859	-0.214 0.264	0.231 0.227	0.011 0.954	-0.122 0.529	-0.014 0.944
WE 609.5576		Gland loss > 2	r p-value	0.029 0.883	-0.153 0.428	0.186 0.333	0.045 0.816	-0.158 0.413	-0.081 0.675
DG# 622.5423		Gland loss > 2	r p-value	-0.292 0.132	-0.112 0.571	0.352 0.066	0.138 0.484	0.230 0.239	0.122 0.536
DG # 753.6644	DG(20:0/22:0/0:0)[iso2]	Gland loss ≤ 2	r p-value	-0.009 0.962	0.184 0.338	-0.278 0.144	-0.014 0.941	-0.171 0.375	-0.325 0.086*
TG # 554.4554	1,2,3-tricaprinoyl-glycerol	Gland loss ≤ 2	r p-value	-0.208 0.288	0.218 0.264	0.314 0.103	0.362 0.059*	0.122 0.538	0.179 0.363
TG 742.6135		Gland loss > 2	r p-value	-0.026 0.897	0.056 0.778	0.169 0.391	0.373 0.050	0.128 0.515	0.179 0.363
TG 745.6701		Gland loss ≤ 2	r p-value	0.167 0.387	0.124 0.523	-0.282 0.138	-0.056 0.774	-0.258 0.176	- 0.365 0.051*
TG 872.6993		Gland loss > 2	r p-value	0.010 0.958	0.030 0.879	0.061 0.759	0.223 0.254	0.070 0.722	0.136 0.491
TG 893.6858		Gland loss ≤ 2	r p-value	0.224 0.244	-0.081 0.674	-0.466 0.011	-0.130 0.502	-0.211 0.273	-0.352 0.061*
TG# 913.7280		Gland loss ≤ 2	r p-value	**0.404** **0.033***	-0.075 0.703	-0.251 0.197	0.284 0.143	-0.145 0.461	**-0.465** **0.013***
TG # 1025.8529		Gland loss ≤ 2	r p-value	0.164 0.405	0.201 0.304	0.199 0.310	**0.560** **0.002***	0.024 0.902	0.093 0.638
Free Ch # 886.7477	22:1-Glc-cholesterol	Gland loss ≤ 2	r p-value	-0.254 0.192	0.312 0.106	0.307 0.112	**0.380** **0.046***	0.109 0.579	0.193 0.325

Lipid category and mass to charge ratio (m/z) (Visit 1)	Compound name (if known)	Highest mean normalised abundance	Spearman correlation coefficient & significance	Average NIKBUT	Lipid layer thickness	Meiboscale upper lid	Meiboscale lower lid	Meibomian glands secreting	Meibum quality
GP (PG) # 537.3569	PG(21:0/0:0)	Gland loss > 2	r p-value	-0.203 0.301	0.229 0.241	**0.375** **0.049***	**0.533** **0.003***	0.122 0.538	0.236 0.227
GP (PA) # 593.4200		Gland loss > 2	r p-value	-0.026 0.895	-0.144 0.455	0.324 0.087*	0.152 0.432	0.007 0.973	0.108 0.576
GP (PS) 762.4572		Gland loss ≤ 2	r p-value	-0.010 0.960	0.224 0.242	-0.088 0.649	-0.115 0.553	0.071 0.715	-0.230 0.230
GP (PG)# 911.6725		Gland loss > 2	r p-value	-0.166 0.399	0.109 0.579	0.269 0.166	**0.407** **0.032***	0.134 0.498	0.264 0.174
Cer # 663.4339	CerPE(d14:2(4E,6E)/16:0(2OH))	Gland loss > 2	r p-value	0.095 0.626	-0.123 0.525	0.302 0.111	0.145 0.454	0.049 0.799	0.014 0.944
Cer # 691.4656		Gland loss > 2	r p-value	0.166 0.399	0.109 0.579	0.269 0.166	**0.407** **0.032***	0.134 0.498	0.264 0.174

** P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance. # indicates compounds where the difference between composite meibography groups was <0.05 (those not marked were significant to p<0.20)*

7.3.2. Results for Visit 2

Table 7.6. Lipid compounds identified at Visit 2

Mass to charge ratio(m/z) (Visit 2)	**Molecular Formula**	**Category, main class, subclass**	**Common name**	**Highest mean normalised abundance**	**Significance between gland loss groups (p value)**
Non-polar lipids					
255.2331	$C_{16}H_{32}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Palmitic acid	Gland loss > 2	0.868
279.2333	$C_{18}H_{32}O_2$	FA, fatty acids and conjugates, unsaturated fatty acids	Linoleic acid	Gland loss > 2	0.806
281.2486	$C_{18}H_{34}O_2$	FA, fatty acids and conjugates, unsaturated fatty acids	Oleic acid	Gland loss > 2	0.380
283.2645	$C_{18}H_{36}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Stearic acid	Gland loss > 2	0.651
367.3582	$C_{24}H_{48}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Lignoceric acid	Gland loss ≤ 2	0.948
381.3738	$C_{25}H_{50}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Hyenic acid	Gland loss ≤ 2	0.937
393.3740	$C_{26}H_{50}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	11Z-hexacosenoic acid	Gland loss ≤ 2	0.775
395.3895	$C_{26}H_{52}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Cerotic acid	Gland loss ≤ 2	0.484
409.4053	$C_{27}H_{54}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Carboceric acid	Gland loss ≤ 2	0.529
423.4210	$C_{28}H_{56}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	Montanic acid	Gland loss ≤ 2	0.490
425.3641	$C_{25}H_{48}O_2$	FA, fatty acids and conjugates, straight chain fatty acids	16Z-pentacosenoic acid	Gland loss > 2	0.185

Mass to charge ratio(m/z) (Visit 2)	**Molecular Formula**	**Category, main class, subclass**	**Common name**	**Highest mean normalised abundance**	**Significance between gland loss groups (p value)**
437.4369	C29H58O2	FA, fatty acids and conjugates, straight chain fatty acids	nonacosanoic acid	Gland loss ≤ 2	0.671
453.3955	C27H52O2	FA, fatty acids and conjugates, straight chain fatty acids	20Z-heptacosenoic acid	Gland loss > 2	**0.012***
549.5614	C37H74O2	FA, fatty acids and conjugates, straight chain fatty acids	heptatriacontanoic acid	Gland loss > 2	0.591
563.5761	C38H76O2	FA, fatty acids and conjugates, straight chain fatty acids	Octatriacontanoic acid	Gland loss > 2	0.906
459.3274	C32H46O3	FA, fatty alcohols	Triangulyne A	Gland loss > 2	0.359
421.4054	C28H54O2	FA, fatty esters, wax monoesters	Myristoleyl myristate	Gland loss ≤ 2	0.358
451.4526	C30H60O2	FA, fatty esters, wax monoesters	Lauryl stearate	Gland loss > 2	0.840
481.4268	C29H56O2	FA, fatty esters, wax monoesters	Tridecyl palmitoleate	Gland loss > 2	0.271
507.5149	C34H68O2	FA, fatty esters, wax monoesters	Arachidyl myristate	Gland loss ≤ 2	0.864
521.5304	C35H70O2	FA, fatty esters, wax monoesters	Octadecyl heptadecanoate	Gland loss > 2	0.134
535.5459	C36H72O2	FA, fatty esters, wax monoesters	Palmityl arachidate	Gland loss ≤ 2	0.836
591.6085	C40H80O2	FA, fatty esters, wax monoesters	Stearyl behenate	Gland loss ≤ 2	0.435
621.5832	C39H76O2	FA, fatty esters, wax monoesters	Tricosanyl palmitoleate	Gland loss > 2	0.595
697.6143	C45H80O2	ST, sterols, steryl esters	18:0 Cholesterol ester	Gland loss > 2	0.912
711.6276	C46H82O2	ST, sterols, steryl esters	19:0 Cholesterol ester	Gland loss ≤ 2	0.962
701.6453	C46H88O5	GL, diradylglycerol, diacylglycerols	DG(21:0/22:1(13Z)/0:0)[iso2]	Gland loss ≤ 2	0.608
703.6611	C46H90O5	GL, diradylglycerol, diacylglycerols	DG(21:0/22:0/0:0)[iso2]	Gland loss ≤ 2	0.766
715.6610	C47H90O5	GL, diradylglycerol, diacylglycerols	DG(22:0/22:1(13Z)/0:0)[iso2]	Gland loss ≤ 2	0.542
717.6768	C47H92O5	GL, diradylglycerol, diacylglycerols	DG(22:0/22:0/0:0)	Gland loss ≤ 2	0.777
753.6767	C45H88O5	GL, diradylglycerol, diacylglycerols	DG(20:0/22:0/0:0)[iso2]	Gland loss ≤ 2	0.074*

Mass to charge ratio(m/z) (Visit 2)	Molecular Formula	Category, main class, subclass	Common name	Highest mean normalised abundance	Significance between gland loss groups (p value)
554.4554	C33H62O6	GL, triradlyglycerol, triacylglycerol	1,2,3-tricaprinoyl-glycerol	Gland loss >2	0.515
675.5542	C40H76O6	GL, triradlyglycerol, triacylglycerol	TG(12:0/12:0/13:0)[iso3]	Gland loss ≤ 2	0.987
917.7014	C62H94O6	GL, triradlyglycerol, triacylglycerol	TG(17:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z)/22:6(4Z,7Z, 10Z, 13Z, 16Z,19Z)) [iso6]	Gland loss > 2	0.205
Polar lipids					
753.5606	C45H78O7	ST, sterols, cholesterol	12:0-Glc-Cholesterol	Gland loss > 2	0.528
886.7477	C55H96O7	ST, sterols, cholesterol	22:1-Glc-cholesterol	Gland loss > 2	0.965
729.6765	C48H90O4	FA, fatty esters, fatty acid estolides, unsaturated fatty acids	FAHFA 16:1/32O(FA 32:1) (OAHFA 48:2)	Gland loss ≤ 2	0.529
757.7077	C50H94O4	FA, fatty esters, fatty acid estolides, unsaturated fatty acids	FAHFA 16:1/34O(FA 34:1) (OAHFA 50:2)	Gland loss ≤ 2	0.712
785.7389	C52H98O4	FA, fatty esters, fatty acid estolides, unsaturated fatty acids	FAHFA(18:1/34-O-34:1) OAHFA (52:2)	Gland loss ≤ 2	0.959
759.4950	C42H73O8P	GP, glycerophosphates, diacyglycerophosphates	PA(19:0/20:5(5Z,8Z,11Z,14Z,17Z))	Gland loss ≤ 2	0.933
537.3569	C27H53O8P	GP, glycerophosphoglycerols, monoacylglycerophosphoglycerol	PG(21:0/0:0)	Gland loss ≤ 2	0.486
749.4352	C39H67O10P	GP, glycerophosphoglycerols, diaglycerophosphoglycerols	PG(18:4(6Z,9Z,12Z,15Z)/15:1(9Z))	Gland loss ≤ 2	0.454
1061.774	C60H111O11P	GP (glycerophospholipid), glycerophosphoglycerol	SLBPA(54:3)	Gland loss ≤ 2	0.070*
598.5167	C37H71NO3	SP, ceramide, n-acylsphingosines (ceramides)	Cer(d15:2(4E,6E)/22:0)	Gland loss > 2	0.265

Mass to charge ratio(m/z) (Visit 2)	**Molecular Formula**	**Category, main class, subclass**	**Common name**	**Highest mean normalised abundance**	**Significance between gland loss groups (p value)**
663.4339	C32H63N2O7P	SP, phosphosphingolipid, ceramide phosphoethanolamines	CerPE(d14:2(4E,6E)/16:0(2OH))	Gland loss > 2	**0.043***
744.6497	C45H89NO5	SP, ceramide, n-acylsphingosines (ceramides)	Cer(t18:1(6OH)/27:0(2OH))	Gland loss ≤ 2	0.844
746.6673	C32H63N2O7P	SP, ceramide, phosphosphingolipids, ceramide phosphoethanolamines	Cer(t18:0/27:0(2OH))	Gland loss ≤ 2	0.243
770.6852	C45H91NO5	SP, ceramide, N-acyl-4-hydroxysphinganines (phytoceramides)	Cer(t18:0/27:0(2OH))	Gland loss ≤ 2	0.200
800.7137	C49H97NO5	SP, ceramide, n-acylsphingosines (ceramides)	Cer(t18:1(6OH)/31:0(31OH))	Gland loss ≤ 2	0.831

** P-values <0.05 were considered significant and are shown in bold. * near significance. Values in red indicate a change in category from Visit 1.*

At Visit 2 the number of compounds found in each lipid class was almost identical to that of Visit 1, with one less wax ester and one additional ceramide only. The compounds with definite identities are reported in ***Table 7.6***. The same sixteen fatty acyls were identified with only one showing a significant difference between gland categories; the straight chain fatty acid 20Z-heptacosenoic acid showed a significantly higher mean abundance where gland loss was greater than grade 2. However, no trend was found in the data to suggest that this difference could indicate a significantly higher abundance of fatty acyls with greater gland loss. No significant differences were found among the identified wax esters either; this was also the case when the criteria were relaxed to p<0.20, see ***Table 7.8***.

At Visit 2, the plant sterols (sitosterol, stigmasterol and campesterol) were again present in the samples, but these were removed from ***Table 7.6*** due to having an exogenous origin. No significant differences between gland loss categories were found for the two cholesterol esters identified.

At Visit 2, all five diglycerides identified had a lower abundance when gland loss was greater than grade 2; one compound which showed a significant difference at Visit 1 (p=0.018) did not reach significance at Visit 2 (p=0.074). No significant differences were found for the identified triglycerides. However, four compounds belonging to the triglyceride class showed significantly lower levels when gland loss was greater than grade 2 (see ***Table 7.7***). Furthermore, when the criteria for significance was relaxed to p<0.20, another nine triglyceride compounds followed this trend (see ***Table 7.8).***

When comparing the polar lipid results, the significant findings for the glycerophospholipids at Visit 1 were not repeated. However, a phosphatidylethanolamine (PE) compound that was not significant at Visit 1, was found to also have a higher mean abundance when gland loss was greater (p=0.012). Of the 11 phospholipids found to differ between gland loss groups, reported in ***Table 7.8***, seven had the highest mean abundance where gland loss was greater than grade 2. The significant finding for the ceramide, CerPE(d14:2(4E,6E)/16:0(2OH)), where the highest mean abundance was found where gland loss was greater the grade 2, was repeated at Visit 2 (p=0.043).

Table 7.7. Compound ions significantly different between gland loss categories at Visit 2

Lipid category Visit 2	Mass to charge ratio (m/z)	Lipid class	Compound name (if known)	Highest mean normalised abundance	Lowest mean normalised abundance	p value
Fatty acyls (FA)	453.3955	Straight chain	20Z-heptacosenoic acid	Gland loss > 2	Gland loss ≤ 2	**0.012***
Sterol lipids (ST)						
Glycerolipids (GL)	622.5423	DG		Gland loss ≤ 2	Gland loss > 2	0.927 (0.014)
	753.6644	DG	DG(20:0/22:0/0:0) [iso2]	Gland loss ≤ 2	Gland loss > 2	0.074* (0.018)
	554.4554	TG	1,2,3-tricaprinoyl-glycerol	Gland loss > 2	Gland loss ≤ 2	0.515 (0.037)
	827.6573	TG		Gland loss ≤ 2	Gland loss > 2	**0.028***
	829.6722	TG		Gland loss ≤ 2	Gland loss > 2	**0.020***
	836.7666	TG		Gland loss ≤ 2	Gland loss > 2	**0.016***
	888.8031	TG		Gland loss ≤ 2	Gland loss > 2	**0.010***
	913.7281	TG		Gland loss ≤ 2	Gland loss > 2	0.130 (0.036)
Free cholesterol	886.7477	Free Ch	22:1-Glc-cholesterol	Gland loss > 2	Gland loss ≤ 2	0.965 (0.011)
OAHFAs						
Phospholipids (PL)	537.3569	GP (PG)		Gland loss ≤ 2	Gland loss > 2	0.486 (0.036)
	593.4200	GP (PA)		Gland loss > 2	Gland loss ≤ 2	0.158 (0.030)
	911.6725	GP (PG)		Gland loss > 2	Gland loss ≤ 2	0.496 (0.035)
	730.4394	GP (PE)		Gland loss > 2	Gland loss ≤ 2	**0.012***
	663.4339	Cer	CerPE(d14:2(4E,6E)/16:0(2OH))	Gland loss > 2	Gland loss ≤ 2	**0.043*** (0.032)
	691.4656	Cer		Gland loss > 2	Gland loss ≤ 2	0.169 (0.036)

** P-values <0.05 were considered significant and are shown in bold. * near significance. Numbers in blue indicate the significant values found at Visit 1*

Table 7.8. mean abundance for different lipid classes where p<0.20

Lipid class (Visit 2)	Number of lipids where p< 0.20	Highest mean abundance where gland loss ≤ 2	Highest mean abundance where gland loss > 2
Non-polar lipids			
FA	3	1	2
WE	2		2
ST	0		
DG	2	3	
TG	14	13	1
Polar lipids			
Free Ch	1		1
OAHFA	0		
GP	4	1	3
SP (ceramides)	6	2	4
SP (SM)	1	1	

Although the highest mean abundance of the three OAHFAs was found in the participants with lower levels of gland loss, no significant differences were found at Visit 2.

Significant correlations were found with the clinical parameters used to define evaporative DED, at Visit 2 (see ***Table 7.9***). When considering meibomian gland expression, the abundance of two fatty acyls, two triglycerides, one wax ester and four polar phospholipids were significantly positively correlated , that is a higher abundance was found when the expression was reduced.

The abundance of the WE, 521.5303 m/z showed a statistically significant positive correlation with the expressibility of the glands at Visit 2; higher abundance was observed when fewer glands expressed. The upper and lower meibography loss was also greater with higher abundance, although the results did not reach statistical significance.

The abundance of four other triglycerides was lower when gland expression reduced, a statistically significant negative correlation. Four triglycerides (one from the latter group) were also significantly negatively correlated with the lower lid meibography score, with lower abundance of each when gland loss was greater. When considering the upper meibography score, no correlations were found with any triglyceride. However, the abundance of one fatty acyl and two phospholipids positively correlated, meaning higher abundance was found where gland loss was greater.

Interestingly two phospholipids were found to significantly negatively correlate with the lipid layer thickness; the abundance in the meibum was greater when the lipid layer thickness was lower. Strong positive correlations were found for two triglycerides, the abundance of which were reduced with lower NIKBUT. Positive and negative correlations were found when the quality of the expressed meibum was explored. A strong negative correlation with the diglyceride DG(20:0/22:0/0:0)[iso2] was

found, which confirms the significant finding at Visit 1 for this lipid; as the quality of the meibum reduced, the abundance values decreased. However, several lipids from different classes also positively correlated with meibum quality, including a significant finding for the polar phosphoroglycerol lipid (PG(21:0/0:0)).

Again, the mean abundance of the OAHFAs did not show any significant differences between meibomian gland categories, and the correlations found at Visit 1 with NIKBUT were not repeated. All the OAHFAs did, however, show strong positive correlations with the number of glands expressing. Therefore, as the number of glands expressing decreased (where grade 0 is ≥75% expression and grade 4 no expression) the abundance of the OAHFAs decreased. A significant negative correlation was also found between OAHFA 50:2 and lower meibography score (r=-0.457, p=0.037), indicating lower abundance with greater gland loss. OAHFA 48:2 also showed a negative correlation with the lower meibography score but did not reach significance (r=-0.431, p=0.051).

Table 7.9. Spearman correlation coefficients between specific lipids and meibomian gland-related parameters (Visit 2)

Lipid category and mass to charge ratio (m/z) (Visit 2)	Compound name (if known)	Highest mean normalised abundance	Spearman correlation coefficient & significance	Average NIKBUT	Lipid layer thickness	Meiboscale upper lid	Meiboscale lower lid	Meibomian glands secreting	Meibum quality
FA 425.3641		Gland loss > 2	r p-value	0.144 0.535	-0.229 0.317	0.272 0.233	0.353 0.117	**0.466** **0.033***	0.135 0.560
FA 439.4130		Gland loss ≤ 2	r p-value	-0.260 0.254	-0.370 0.099*	0.204 0.374	-0.333 0.140	0.382 0.087*	0.089 0.700
FA# 453.3955		Gland loss > 2	r p-value	0.088 0.703	-0.256 0.262	**0.516** **0.017***	0.432 0.050*	**0.486** **0.026***	0.247 0.280
WE 521.5303		Gland loss > 2	r p-value	0.149 0.520	-0.271 0.235	0.389 0.081*	0.418 0.059*	**0.631** **0.002***	0.202 0.379
WE 581.5264		Gland loss > 2	r p-value	0.310 0.171	-.0097 0.675	0.137 0.553	0.343 0.128	0.407 0.067*	0.382 0.087*
DG 598.5782		Gland loss ≤ 2	r p-value	0.084 0.718	-0.352 0.117	-0.012 0.961	-0.078 0.738	0.118 0.609	-0.405 0.069*
DG# 622.5423		Gland loss ≤ 2	r p-value	-0.051 0.827	0.276 0.226	-0.047 0.838	0.383 0.086	0.375 0.094	0.090 0.698
DG# 753.6644	DG(20:0/22:0/0:0)[iso2]	Gland loss ≤ 2	r p-value	0.329 0.145	-0.130 0.574	-0.063 0.786	-0.413 0.062*	-0.377 0.092*	**-0.539** **0.012****
TG # 554.4554	1,2,3-tricaprinoyl-glycerol	Gland loss > 2	r p-value	-0.050 0.830	0.090 0.697	0.272 0.233	0.416 0.061*	**0.564** **0.008***	0.405 0.069*
TG 709.5622		Gland loss > 2	r p-value	0.303 0.182	-0.224 0.329	0.409 0.066*	0.410 0.065*	**0.591** **0.005***	0.180 0.435
TG 742.6135		Gland loss > 2	r p-value	-0.019 0.935	0.425 0.055*	-0.062 0.791	0.239 0.297	0.174 0.452	0.247 0.280
TG 745.6701		Gland loss ≤ 2	r p-value	-0.041 0.860	0.092 0.690	0.003 0.988	**-0.544** **0.011***	**-0.579** **0.006***	-0.405 0.069*
TG 755.6195		Gland loss ≤ 2	r p-value	0.143 0.538	0.262 0.252	-0.169 0.463	-0.379 0.090*	-0.039 0.865	-0.057 0.807
TG 757.6350		Gland loss ≤ 2	r p-value	-0.088 0.705	0.325 0.150	-0.090 0.698	-0.065 0.778	0.219 0.341	0.090 0.698
TG 771.6494		Gland loss ≤ 2	r p-value	**0.559** **0.008***	0.119 0.608	-0.223 0.330	0.102 0.659	0.135 0.558	0.135 0.560
TG 783.6491		Gland loss ≤ 2	r p-value	0.008 0.973	0.361 0.107	-0.167 0.471	-0.103 0.656	0.120 0.605	-0.045 0.847

Lipid category and mass to charge ratio (m/z) (Visit 2)	Compound name (if known)	Highest mean normalised abundance	Spearman correlation coefficient & significance	Average NIKBUT	Lipid layer thickness	Meiboscale upper lid	Meiboscale lower lid	Meibomian glands secreting	Meibum quality
TG 801.6410		Gland loss ≤ 2	r p-value	0.021 0.928	-0.043 0.854	0.013 0.956	**-0.480 0.028***	-0.433 0.050*	-0.407 0.067*
TG# 827.6573		Gland loss ≤ 2	r p-value	-0.018 0.940	-0.131 0.570	-0.250 0.275	**-0.488 0.025***	0.155 0.502	-0.247 0.280
TG# 829.6722		Gland loss ≤ 2	r p-value	-0.084 0.718	0.160 0.489	-0.398 0.074	-0.107 0.644	0.179 0.437	-0.112 0.628
TG# 836.7666		Gland loss ≤ 2	r p-value	0.115 0.619	0.345 0.126	-0.364 0.105	-0.384 0.086*	**-0.532 0.013***	-0.204 0.376
TG 841.6703		Gland loss ≤ 2	r p-value	-0.069 0.767	-0.060 0.795	-0.258 0.259	0.016 0.947	0.270 0.236	0.000 1.000
TG 872.6993		Gland loss > 2	r p-value	-0.249 0.277	0.429 0.052*	-0.139 0.547	0.354 0.115	**-0.512 0.018***	-0.202 0.379
TG 875.7089		Gland loss ≤ 2	r p-value	-0.276 0.226	0.167 0.470	-0.140 0.545	-0.187 0.418	-0.158 0.494	-0.247 0.280
TG# 888.8031		Gland loss ≤ 2	r p-value	-0.012 0.960	0.138 0.550	-0.414 0.062*	**-0.539 0.012***	-0.347 0.124	-0.382 0.087*
TG 893.6858		Gland loss ≤ 2	r p-value	**0.487 0.025***	0.024 0.919	-0.107 0.644	-0.247 0.280	-0.386 0.084*	-0.135 0.560
TG# 913.7280		Gland loss ≤ 2	r p-value	-0.034 0.884	0.295 0.195	-0.327 0.148	0.103 0.656	0.118 0.609	0.270 0.237
TG 933.7913		Gland loss ≤ 2	r p-value	0.357 0.112	0.209 0.363	-0.077 0.741	-0.383 0.086	**-0.561 0.008***	-0.180 0.435
Free Ch # 886.7477	22:1-Glc-cholesterol	Gland loss > 2	r p-value	-0.070 0.765	0.244 0.287	-0.035 0.882	0.364 0.104	0.332 0.141	0.360 0.109
GP (PG)# 537.3569	PG(21:0/0:0)	Gland loss ≤ 2	r p-value	-0.181 0.433	0.234 0.307	0.037 0.873	-0.326 0.150	0.383 0.086*	**0.449 0.041***
GP (PA)# 593.4200		Gland loss > 2	r p-value	0.100 0.665	-0.071 0.758	0.420 0.058	0.357 0.112	**0.499 0.021***	0.383 0.086
GP (PE)# 730.4394		Gland loss > 2	r p-value	0.094 0.687	**-0.550 0.010***	**0.546 0.011***	0.224 0.328	0.350 0.120	0.067 0.772
GP (PS) 762.4572		Gland loss > 2	r p-value	0.002 0.993	-0.005 0.983	-0.010 0.965	0.262 0.251	0.081 0.727	0.112 0.628
GP(PG) 772.4007		Gland loss > 2	r p-value	0.223 0.330	-0.336 0.136	**0.452 0.040***	0.403 0.070*	**0.626 0.002***	0.202 0.379

Lipid category and mass to charge ratio (m/z) (Visit 2)	Compound name (if known)	Highest mean normalised abundance	Spearman correlation coefficient & significance	Average NIKBUT	Lipid layer thickness	Meiboscale upper lid	Meiboscale lower lid	Meibomian glands secreting	Meibum quality
GP (PG)# 911.6725		Gland loss > 2	r p-value	-0.077 0.739	0.188 0.415	0.194 0.399	0.270 0.236	**0.436** **0.048***	0.315 0.165
Cer 654.5811		Gland loss > 2	r p-value	0.181 0.432	-0.150 0.516	0.322 0.154	0.157 0.496	0.391 0.080*	0.157 0.496
Cer# 663.4339	CerPE(d14:2(4E,6E)/16:0(2OH))	Gland loss > 2	r p-value	-0.121 0.602	**-0.455** **0.038***	0.314 0.166	0.150 0.517	0.227 0.322	-0.090 0.698
Cer# 691.4656		Gland loss > 2	r p-value	-0.121 0.602	-0.305 0.179	0.121 0.601	0.157 0.496	0.192 0.404	-0.112 0.628
Cer 695.5465		Gland loss > 2	r p-value	0.214 0.351	-0.349 0.121	0.398 0.074*	0.429 0.052*	**0.621** **0.003***	0.202 0.379
Cer 724.6828		Gland loss ≤ 2	r p-value	-0.031 0.893	-0.176 0.446	-0.322 0.155	-0.372 0.096	-0.029 0.901	-0.090 0.698
Cer 770.6852	Cer(t18:0/27:0(2OH))	Gland loss ≤ 2	r p-value	0.203 0.377	-0.124 0.593	-0.051 0.827	-0.038 0.868	0.236 0.303	-0.022 0.923
SM 862.7703		Gland loss ≤ 2	r p-value	-0.122 0.598	**0.437** **0.047***	-0.370 0.098	0.060 0.797	-0.125 0.588	0.090 0.698

** P-values <0.05 were considered significant and are shown in bold. Significant correlations are shaded. * near significance. # indicates compounds where the difference between composite meibography groups was <0.05 (those not marked were significant to p<0.20). ** near significance also found at Visit 1*

7.3.3. Comparison of results between visits

A significant batch effect was observed between the samples when comparing the compound ions between visits. This is illustrated in ***Figure 7.3.*** and ***7.4***. which show the Principal Component Analysis of data in the negative ion mode and positive ion mode, respectively, for both visits. ***Figure 7.3*** shows the data for the triglyceride compound 913.5865m/z. Data for Visit 1 appears in blue and purple, where blue corresponds to data showing gland loss grade ≤ 2, and purple indicates gland loss grade > 2. The data for Visit 2 is represented by orange (gland loss grade ≤ 2) and green (gland loss grade > 2).

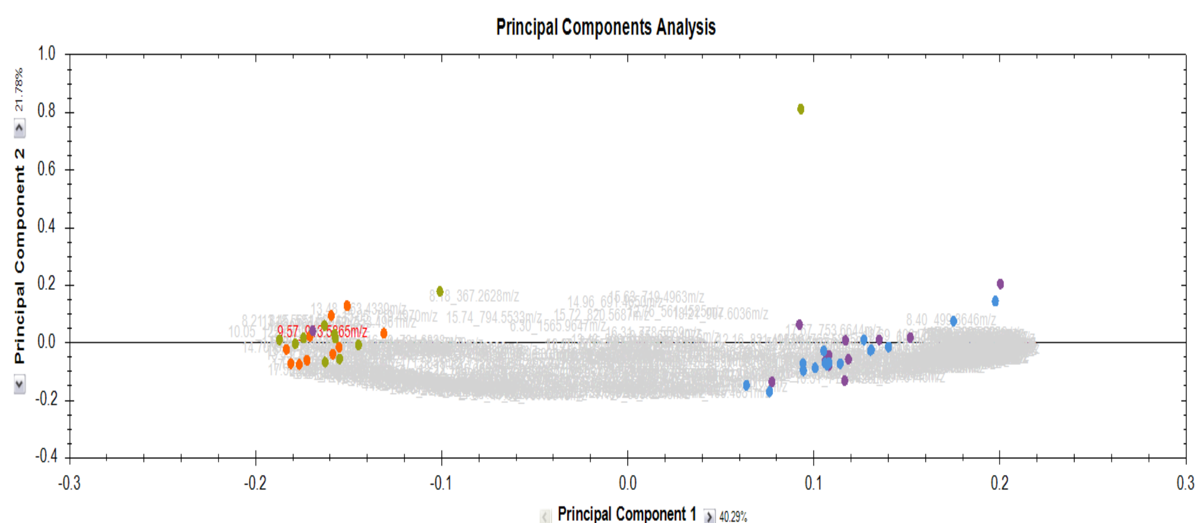


Figure 7.3. Principal component analysis of compound 913.5865 m/z in negative ion mode

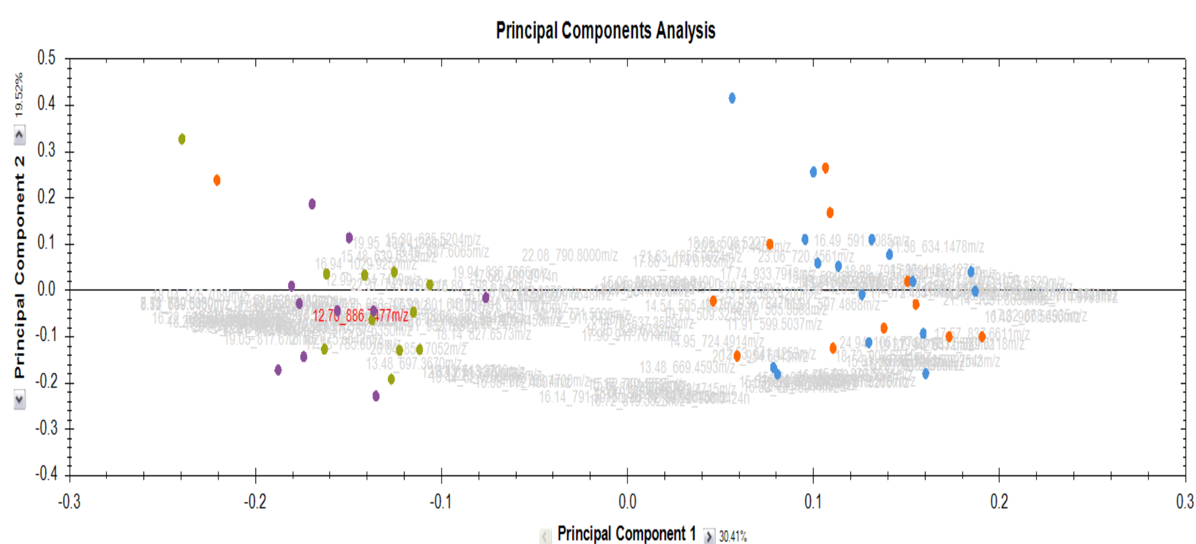


Figure 7.4. Principal component analysis of compound 886.7477 m/z in positive ion mode

Figure 7.4. illustrates the batch effect for compound 886.7477m/z, a free 22:1-Glc-cholesterol. Data for Visit 1 appears in blue and orange, where blue corresponds to data showing gland loss grade ≤ 2, and orange indicates gland loss grade > 2, while data for Visit 2 is represented by purple (gland loss grade ≤ 2) and green (gland loss grade > 2). However, the same batch effect can be observed. Therefore, direct comparison of compound abundance was not possible. However, the lipidome from each visit was almost identical, allowing for a comparison of any significant findings between the two visits, as previously discussed.

7.4. Discussion

The untargeted meibum analysis employed in this study revealed many lipid compounds from several different lipid classes, some of which could be identified exactly. Several statistically significant findings were observed, and trends identified which are in agreement with the findings of previous studies.

A trend towards higher levels of the saturated fatty acids, such as palmitic and stearic acids with greater gland loss was observed. Several unsaturated fatty acids, such as oleic and linoleic acid, and wax esters also followed this pattern, although none were statistically significant. The di- and triglycerides were often found in greater abundance when gland loss was lower, although variations were noted. In agreement with previous studies, increased levels of ceramides and lower abundance of OAHFAs were noted when signs of MGD were greater.

LC-MS/MS, as used in this study, combines the physical separation capabilities of liquid chromatography, with the ability of tandem mass spectrometry to analysis mass (Spickett and Pitt, 2015). Electrospray ionisation (ESI), the passage of a high voltage through a liquid to create an aerosol, was used to cause ionisation, before the analytes were separated and ordered by mass, based on time required to travel through the created electric field to a detector, with the lightest analytes reaching the detector first (Ståhlman et al., 2009).

During MS, lipid molecules can be ionized to form either positively or negatively charged ions. While lipids can be detected in either mode, signals for some are detected more efficiently in the positive ion mode, for example WE, ChE and TGs and free cholesterol (or non-esterized cholesterol), and others such as free fatty acids and OAHFAs in the negative ion mode (Suzuki et al., 2022). For example, triglycerides, which are found in the positive ion mode, have an NH4+ adduct that ionises them, whereas the removal of H+ , in negative ion mode, creates a carboxylic anion in fatty acids and OAHFAs. A difference in molecular mass of ± 1.0073 m/z occurs by the addition or removal of an H+

ion (Pitt, 2023). Therefore, the samples were run in both positive and negative ion modes to allow the best coverage of the lipids.

In this study it was possible to categorise 191 lipid compounds into their lipid classes. However, many more compounds were present which were filtered out or remained unclassified. In total 157 and 315 compounds were identified in the positive and negative ion modes, respectively. Thirty-eight fatty acyls of different types including, saturated or straight chain fatty acids, unsaturated fatty acids, long chain fatty acids, and wax monesters (WE) were identified. Fatty acyls are fatty acids which contain a carboxyl group. Both palmitic acid and stearic acid, saturated fatty acids, were identified in the samples. Oleic acid, a monosaturated lipid and linoleic acid, a polyunsaturated, non-polar fatty acid were also among the fatty acyls identified, and have previously been identified in human meibum (Nichols et al., 2007).

The mean abundance of linoleic acid and oleic acid was higher in the participants with greater gland loss at both visits of this study, but not significantly. Linoleic acid has previously been identified as a potential biomarker in MGD; elevated levels in meibomian gland secretions have been associated with the severity of telangiectasia and plugging of the gland orifices, leading to disruption of the tear lipid layer (Arita et al., 2016a).

While not significant at either visit, the highest mean abundance of palmitic acid occurred in the participants with greater gland loss, while the highest mean abundance of stearic acid was found in the subjects with less gland loss at Visit 1 but more gland loss at Visit 2. These findings do not agree with a previous study which reported lower levels of both of these saturated fatty acids in MGD (Joffre et al., 2008). However, palmitic acid and stearic acid are both saturated fatty acids, which have higher melting points than polyunsaturated fats, and could therefore be expected to be increased in the thicker meibum secretions found in MGD.

Myristic, palmitic, stearic, and oleic fatty acids, are also termed free fatty acids (FFA) or non-esterified fatty acids. Although these fatty acids are considered to be non-polar, the chemical structures contain 2 sections, one section being a long C-H chain that is entirely nonpolar, while the other has a COOH carboxylic acid group, which is polar. This amphiphilic nature means that the moderately long free fatty acids (≤ 18 carbons) are partly soluble in water and have surfactant properties (Arciniega et al., 2011). However, very long chain free fatty acids are poorly soluble in water and tend to float on the surface when mixed. Arciniega et al. (2011), demonstrated that high concentrations of such FFAs could lead to disruption of the tear film lipid layer.

The fatty acyls that were present in higher abundance when more gland loss was present at Visit 2, showed a significant positive correlation with the percentage of glands expressing i.e. abundance was higher when gland expression was poorer. Upper and lower gland loss (where grade '0' indicated no gland loss, and grade '4' indicated ≥ 75% loss) also showed significant or near significant positive correlations with these fatty acyls.

Eight wax esters (WE) were identified in this study. At Visit 1, six had the highest abundance when gland loss was greater than grade 2, although at Visit 2 five had changed categories. None of the individual differences were statistically significant, suggesting that considering wax esters as biomarkers may not be suitable. Wax esters have been shown to display the greatest inter-subject variation within the lipidome (Brown et al., 2016). In agreement with the results of this study, while investigating the effect of age and MGD on the meibum composition, a recent study also found no significant differences in the wax esters (Suzuki et al., 2022). However, Lam et al. (2014b) found a reduction in wax esters in the tears of dry eye subjects compared to a cohort of age-matched control subjects, which correlated with various clinical parameters such as OSDI, tear breakup time, and Schirmer's I test .

Several sterol esters were observed at both visits. However, the majority of these were plant based and therefore removed from the table of identified steryl esters in the Visit 2 data. The remaining two cholesterol esters showed no significant differences between meibomian gland categories. Changes in the levels of cholesterol esters have previously been suggested as a potential biomarker for MGD (Shrestha et al., 2011); the relative amount was 40% lower in MGD. This is in disagreement with a previous study which found an increase in cholesterol esters in MGD (Joffre et al., 2008). However, Borchman et al. (2019) also found lower levels of cholesterol esters in MGD.

This study found two free cholesterols (non-esterified) in the samples collected at both visits. A statistically significant difference was found in the abundance of the free cholesterol, 22:1-Glc-cholesterol, at Visit 2, being higher in abundance when gland loss was greater (p=0.011). Suzuki et al. (2022) also reported increased free cholesterol associated with MGD, along with decreases in non-polar lipids such as ChE and increases in polar lipids.

An increase in the level of free cholesterol in the glands could possibly be attributed to a reduction in cholesterol esters, which are known to regulate the cellular level of free cholesterol (Gonen and Miller, 2020). The quantity of cholesterol esters has previously been shown to be lower in meibum from donors with MGD (Hetman and Borchman, 2020). Shrestha et al. (2011) also suggested that specific features of the ChE (chain length, branching, and degree of unsaturation) may change disease severity.

As well as the non-polar wax and cholesterol esters described above, di and tri- glycerides were also found in the positive ion mode. These are esters of fatty acids with glycerol and tend to be solid at room temperature, whereas wax esters are formed from a long chain fatty acid esterified with an alcohol (Ahmed et al., 2023). While none of the identified di- and tri- glycerides showed a significant difference between gland loss groups, several of those without a definite identity were found to have a significantly higher abundance when the composite gland loss grade was less than or equal to grade 2; lower abundance with increasing signs of MGD. This was more apparent at Visit 2, particularly when a significance level of $p<0.20$ was adopted. This finding is in contrast to a previous study on an Asian population which examined the meibum lipidome (Lam et al., 2011). Although Lam et al. (2011) found similar general lipid profiles in each group, several unsaturated triglycerides and a phospholipid species were found at significantly higher levels in the dry eye patients compared to the healthy eyes.

More recently, Suzuki et al. (2022) also found significantly increased levels of triglycerides in MGD patients. While alterations in the ratio of non-polar and polar lipids are observed in MGD, akin to age-related changes, that is the non-polar lipids significantly decrease, while polar lipids increase, triglycerides did not appear to follow that pattern (Suzuki et al., 2022). An interesting case study of a patient with a metabolic disorder affecting meibogenesis in the meibomian glands, reported a reduction in wax and cholesterol esters, where the total cholesterol to wax esters ratio was doubled, but also a 20-30 times increase in the triglycerides (Butovich and Suzuki, 2020).

In this study, several significant but conflicting correlations were found when considering the triglycerides. At Visit 2, the abundance of several triglycerides was significantly lower when gland loss was greater. Also at Visit 2, when gland loss was greater than grade 2, the abundance of two triglycerides was greater when gland expressibility was poorer, while two other triglycerides negatively correlated when gland loss was > grade 2; the abundance increased when more glands expressed. Meanwhile, when gland loss was greater than grade 2, two further triglycerides showed a statistically significant negative correlation between expressibility and abundance, i.e., the abundance also increased when more glands expressed. It is possible, that depending on the degree of saturation, triglycerides may perform differently. Therefore, further targeted analysis may be needed to provide a clearer answer to this.

When considering the polar lipids, forty-eight compounds were classed as phospholipids at Visit 1; forty-nine at Visit 2, due to the addition of one ceramide. The majority of these were sphingophospholipids (SP), with the majority of those being ceramides. Phosphatidylcholines (PC) and Phosphatidylinositols (PI) were not identified at either visit. The highest mean abundance of phospholipids was observed when gland loss was greater than grade 2 for three identified

glycerophospholipids (GP) and two sphingophospholipids (SP) at Visit 1. This was only repeatable for one ceramide (CerPE(d14:2(4E,6E)/16:0(2OH))) at Visit 2. However, other non-identified phospholipids were also present in a higher abundance where the gland loss was greater than grade 2. The abundance of two GP and one ceramide correlated with lower lid gland loss, at Visit 1, with higher abundance when gland loss was greater. No correlations with gland loss were found at Visit 2. Several phospholipids significantly positively correlated with the percentage of lower lid glands secreting, indicating that the abundance was higher when fewer glands were secreting. This increase in polar lipids with increasing signs of MGD corresponds with the increase in polar lipids previously observed in MGD-related DED (Lam et al., 2011).

An early study in a rabbit model of DED found that the lipids expressible from meibomian glands with clinically evident MGD had an increased ratio of free sterols and ceramides (Nicolaides et al., 1989). It was suggested that this increase could be explained by abnormal keratinization of the meibomian gland ducts occurring in MGD (Jester et al., 1989). Arciniega et al. (2013) were later able to demonstrate disruption and destabilization of meibum lipid films caused by increasing amounts of ceramides, in vivo. It was speculated that increased levels of ceramides could have a dual effect on meibum, by increasing the melting temperature and changing the way it spreads on the ocular surface.

More recently, researchers have shown that by inhibiting an enzyme responsible for ceramide biosynthesis in stearoyl-CoA desaturase-1 (SCD1) mice, improved meibomian gland metabolism and morphology occurred due to reduced inflammation and keratinization (Ji et al., 2021). The results of the study, along with the current study, indicate that elevated levels of endogenous ceramides are indeed a sign of MGD and suggest that early inhibition of ceramide biosynthesis could be a new clinical approach to treating MGD. This would suggest, therefore, that the results obtained in this study are correct. The finding that two phospholipids significantly negatively correlated with the lipid layer thickness (the abundance in the meibum was greater when the lipid layer thickness was lower) also lends support to these findings.

However, the percentage of the identified meibum lipids that were classified as phospholipids in this study appears to be high in comparison to those reported elsewhere. An exact molar percentage could not be calculated, due to the method employed. Previously, phospholipids have been reported to be within the baseline noise or less than 0.006 ± 0.003% of the meibomian lipids (Brown et al., 2013;Butovich et al., 2009). However, Saville et al. (2011) were able to identify 35 phospholipids in the meibum, using electrospray ionisation tandem mass spectrometry, as used in this study. Although care was taken to minimise any contamination of the meibum samples collected in this study, compounds

such as ceramides, can be introduced inadvertently through exogenous sources, such as medical and cosmetic preparations (Arciniega et al., 2013).

Three OAHFAs were found in the meibum samples from all the participants at both visits of this study: OAHFA 48:2 (16:1/32:1) and OAHFA 50:2 (16:1/34:1) and OAHFA 52:2 (18:1/34:1). They were found in the negative ion mode, with the same m/z ratios as those previously reported to be the most abundant OAHFA found in human meibum (Hancock et al., 2018). Lam et al. (2011) found that OAHFAs (18:1/30:1), (16:1/32:1) and (18:1/32:1), were the three species present in highest abundance. Mori et al. (2014) later reported (18:2/30:0) to be among the OAHFAs with the highest abundance, in addition to the previous three OAHFAs.

Previously, OAHFA analysis has proved challenging, due to their low abundance and because they often occur as a mixture of closely related isomers, having the same chemical formula but different arrangements of the atoms within the molecule (Butovich, 2017). Therefore, more OAHFAs may have been present in the samples but in such low abundance as to be below the limit of detection in this study.

The mean abundance of the OAHFAs found did not show any significant differences between meibomian gland categories at either visit in this study. Significant positive correlations were found between two of the OAHFAs (OAHFA 50:2 and OAHFA 52:2) with NIKBUT ($r=0.427$, $p=0.02$ and $r=0.482$, $p=0.008$, respectively), but these were not repeated at Visit 2. At Visit 2, a significant negative correlation was found between OAHFA 50:2 and lower meibography score ($r=-0.457$, $p=0.037$), indicating lower abundance with greater gland loss, as might be expected. Also, as the number of glands expressing decreased, the abundance of all three OAHFAs decreased significantly.

Lipidomic studies are providing growing evidence that OAHFAs, amongst the other polar lipids, are likely to originate from multiple sources (lacrimal gland, conjunctival and corneal cells) (Chen et al., 2019a). Changes in the composition of the tear lipid layer in MGD have been attributed to alterations in OAHFAs. Khanal et al. (2021) detected 76 OAHFAs in human meibum and 78 in tears, with several showing a negative correlation with precorneal tear film (PCTF) thinning i.e., lower levels in MGD compared with healthy controls. Interestingly, they found that the tear-film derived OAHFAs were independent of the PCTF thinning rate. Therefore, the lower abundance with greater gland loss and reduced glands expressing, as found in this study, are in agreement. Lam et al. also reported decreasing levels of several OAHFA species in the tears which correlated with increasing dry eye disease severity (Lam et al., 2014b). This is in line with their proposed physiological role and lower tear film stability in DED.

The meibum samples collected in this study were mainly from female participants of Asian ethnicity. Therefore, it was not possible to conduct sex or ethnicity-based comparisons in the data. However, when the total cohort were considered, as reported in Chapter 4, no clinical associations were found between any meibomian gland related parameter and sex or ethnicity. The prevalence of MGD has previously been found to be higher in studies of Asian populations in contrast to those from mainly Caucasian populations (Schaumberg et al., 2011). The potential predisposition of Asians to the development of DED appears to be related to greater levels of meibomian gland drop out and incomplete blinking (Craig et al., 2016).

Previously, no difference in the ChE/WE ratio have been found when comparing sexes (Borchman et al., 2019); a higher ratio in subjects of black ethnicity, and a lower ratio in Hispanics compared to Caucasians was reported, but the cohorts were small. The lipid ratios reported in other studies are variable, and therefore, further work is needed to determine the contribution, if any of ethnicity to the ChE/WE molar ratio.

Sullivan et al. (2006) observed sex-related differences in the lipid profiles of meibomian gland secretions, particularly in the m/z ratios (molecular ion signals) of the polar lipids, which they attributed to the activity of the sex steroid, androgen. However, Borchman et al. (2019) did not find any sex-related differences in the ChE/WE ratio in a young cohort.

This study used an untargeted LC-MS approach to measure many components simultaneously within the meibum samples, in an unbiased way. While shotgun lipidomics, which omits chromatographic separation, enables simultaneous and fast quantification of diverse lipid classes (Chen et al., 2010a), untargeted LC-MS offers accurate separation and detection of lipids spanning a wide range of classes. The main limitation of untargeted LC-MS measurements is its semi-quantitative nature (Smirnov et al., 2021). Absolute quantification would be challenging to achieve due to the need for extensive internal standards. However, for the purposes of biomarker discovery, the relative differences in lipid abundances provided by untargeted LC-MS are sufficient.

Samples were analysed separately in this study, as pooling of samples has been shown to be unreliable (Brown et al., 2016); in quantifying day-to-day changes in the meibum profiles of individual healthy subjects, substantial inter-subject variations have been reported previously. Specifically, WE showed the greatest inter-subject variations, while individual inter-day variability was smaller.

A limitation of this study was the batch effect between visits, which meant that reliable comparisons of the abundance data obtained between visits could not be made. Reported causative factors for a batch effect include day to day variations due to changes in the performance of the mass spectrometer

or sample preparation, sample storage conditions, and changes in laboratory environmental conditions such as humidity and temperature (Balluff et al., 2021). The samples from visits 1 and 2 were run on different days in this study, although the same method was employed. Therefore, the more likely cause of the batch effect observed would be the possible degradation of the Visit 1 samples due to the longer storage time.

With the use of filters, non-meibum compounds such as prenol lipids, naturally occurring lipids present in certain foods, were excluded. One such example is squalene, which has previously been observed in meibum samples but originates from sebum in the skin (Butovich, 2013). Although this prenol lipid was not identified in the samples in this study, other prenols were found. Also, several plant-based compounds were identified, including free and esterified stigmasterol, sitosterol and campesterol. Other exogenous or xenobiotic compounds such as Coenzyme Q10 were also discovered in some samples, which may have originated from a moisturiser used on the skin.

Care was taken during the collection of meibum samples to avoid possible contamination from the skin and sebum. Freshly expressed meibum may also differ from resident meibum; the simple act of digital expression may alter the normal state of the meibomian secretions (Haworth et al., 2011). However, even with careful and precise collection, inclusion of micro quantities of tears, conjunctival and lid margin epithelial cells and disintegrated meibomian secretory cells may result in contamination (Butovich et al., 2008). Theoretically, bacterial lipases which could breakdown meibomian lipids could have been present in the collected samples. However, the organic solvents in which the samples were placed immediately following collection, should have killed any bacteria, and denatured and diluted any lipase proteins (Butovich et al., 2008). It is possible that some sebum was collected in this study, as the proportion of fatty acyls, which are known to be higher in sebum, appeared to be higher in this study than the cholesterol and wax esters, which have previously been reported to make up 31% and 41% of human meibum, respectively (Butovich, 2013).

With regard to sample size, multiple post hoc calculations would be required to elucidate an optimum sample size for studies exploring the different compound classes of the meibum lipidome. However, a post hoc sample size calculation based on the mean abundance of the diglyceride DG(20:0/22:0/0:0)[iso2], at Visit 1, and using an independent means t-test performed with G-Power (Version 3.1.9.7) (Faul et al., 2007), indicated that the study was underpowered (actual power 0.33). Using the calculated effect size of 0.61, a two-tailed α-level of 5%, and 80% power level, a total sample size of 88 participants would be needed. Therefore, any further study involving analysis of the meibum lipidome requires an increased sample size. Also, the possibility that a significant finding was actually a false positive (Q value), should also be accounted for.

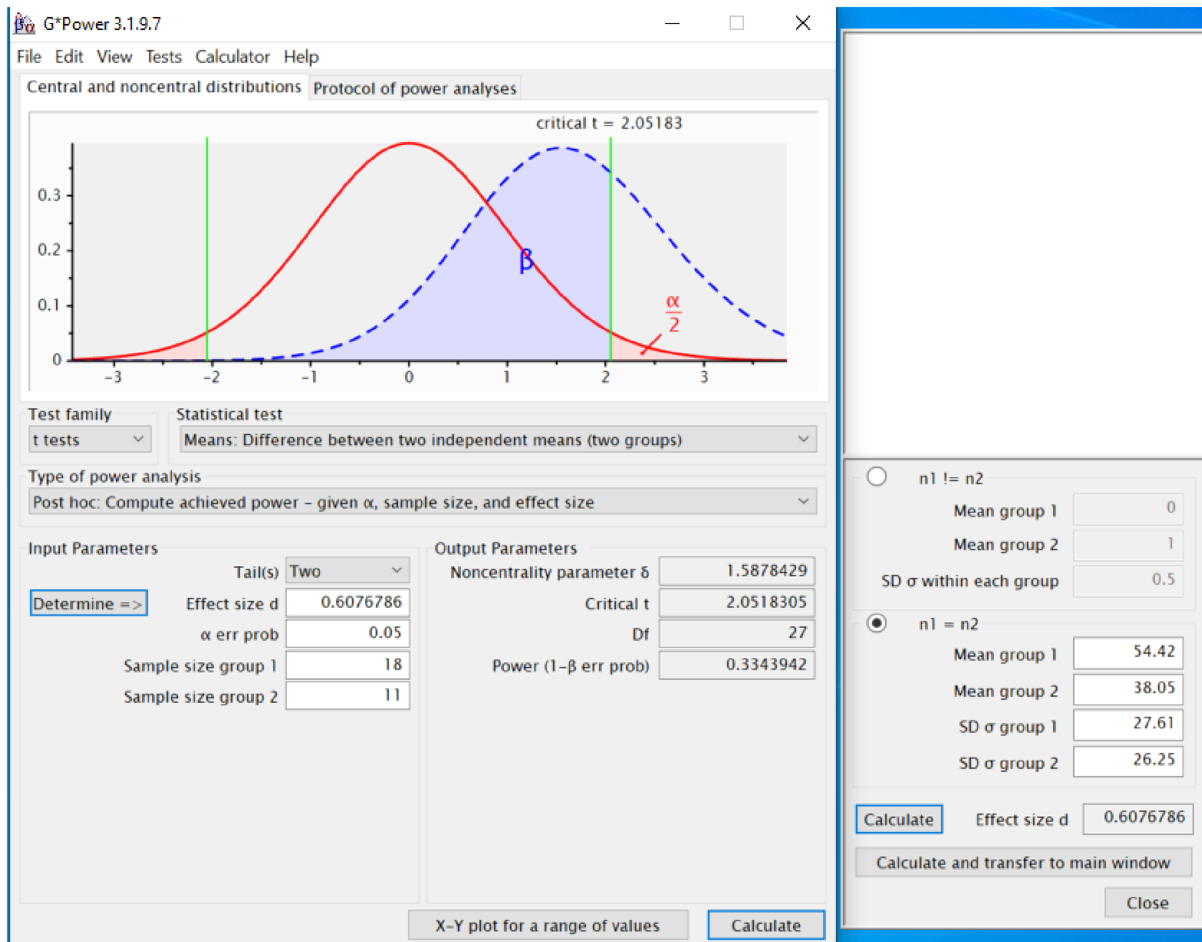


Figure 7.5. Post hoc calculation of the effect size for DG(20:0/22:0/0:0[iso2] using G-Power and an independent means t-test

7.5. Conclusion

The findings of this study indicate that changes to the meibum composition can occur even in early or asymptomatic cases of meibomian gland dysfunction.

A trend towards higher levels of the saturated fatty acids, palmitic and stearic and acids with greater gland loss was observed. Several unsaturated fatty acids, such as oleic and linoleic acid, and wax esters also followed this pattern, although none were statistically significant. The di- and triglycerides were often found in greater abundance when gland loss was lower, although variations were noted.

The increased levels of ceramides that occurred when gland loss expression was reduced, and gland loss levels were greater are in line with recent findings. This finding supports the thinking that phospholipids do in fact mainly originate from the meibomian glands and not from elsewhere as speculated, although ceramides have also been identified in human sebum.

Lower abundance of polar OAHFAs was associated with greater gland loss and fewer glands expressing. These findings are consistent with the likelihood that alterations in the tear lipid layer, leading to more rapid pre-corneal tear film thinning, can be attributed to meibum derived OAHFAs.

Overall, the findings of this study would agree that in young adults with MGD, disruption of the meibum composition occurs due to an increase in certain non-polar fatty acyls and polar phospholipids, and a decrease in the polar OAHFAs. Whether polar lipids originate from multiple sources is still an area for further research. The contribution of exogenous lipids to the disruption of the tear film lipid layer is also an area worthy of further investigation. Further research on meibum should include not only more targeted analysis of the different lipid classes from a larger cohort, but also more detailed investigation into the contribution of diet and causes of exogenous lipids.

Chapter 8 - Thesis summary, conclusions, and recommendations for future work

8.1. Significance of research

The global prevalence of Dry Eye Disease (DED) is reported to range from 5-50% (Stapleton et al., 2017); around a third of the UK population is estimated to have DED (Vidal-Rohr et al., 2023). Evaporative DED is the most prevalent form of DED (Lemp et al., 2012), meibomian gland dysfunction (MGD) being the main contributor (Craig et al., 2017c). Although prevalence and severity are known to increase with age, several recent studies have reported a high prevalence in younger populations (Moon et al., 2016;Asiedu et al., 2017;Vehof et al., 2021), with several modifiable lifestyle factors identified (Wang et al., 2021b). Weak correlations between subjective symptoms and objective signs have been reported; particularly in the early stages of DED, when clinical signs may be mild or absent all together (Nichols et al., 2004). The author is not aware of any previous studies that have investigated biomarkers of DED in young adults, and therefore the main focus of this thesis was to explore evaporative DED in a young population, with no known systemic associations or ADDE.

The multifactorial nature of DED can, however, make diagnosis and management challenging (Bartlett et al., 2015); the poor correlation between signs and symptoms, and the lack of a single 'gold standard' sign or symptom also contribute to the challenge (Wolffsohn et al., 2017). The increasing prevalence of DED not only has the potential to impact a patient's quality of life but can translate into an increasing economic burden and workload for optometrists working in primary care. With this in mind, and the extension of eyecare practitioners roles since the last survey to include UK practitioners 7-8 years ago, (Downie et al., 2016), it was pertinent to elicit current perceptions of DED .

Currently DED treatment is largely based around alleviation of symptoms, with no curative or preventative strategies available (Inomata et al., 2020). Therefore, the search for validated biomarkers to assist with understanding the pathogenesis of DED could lead to more accurate diagnosis and targeted treatment options.

This thesis sought to investigate the tears and meibum for biomarkers of evaporative DED, in a young adult cohort; an age group that has not previously received much attention. It aimed to explore whether any currently available tear biomarker devices are being used in practice, and to identify any new candidate biomarkers in early evaporative DED.

This chapter summarises and discusses the key findings of the previous chapters, as well as the limitations of the research and recommendations for future work.

8.2. Thesis summary

8.2.1. Dry Eye in Clinical Practice Survey for UK Optometrists

Chapter 2 explored the opinions of UK optometrists working in primary care, around their perceptions of DED, their knowledge and confidence in diagnosis and management, and satisfaction with currently available first-line treatment options. While the online survey identified a definite move towards more therapeutic management of DED in the UK over recent years, evidenced by an increase in steroid prescribing, translation of biomarker research findings into optometric practice appears to be limited. Adoption of the commercially available tear biomarker device, InflammaDry®, was low. Practitioners would, however, be willing to incorporate such a point of care device into their practice at a very similar cost. More encouragingly, the survey did show that optometrists are employing a stepwise management approach to the diagnosis and management of DED, indicating an increase in evidence-based practice.

8.2.2. Methodology and repeatability of tear film protein assessment using the Agilent 2100 Bioanalyzer

Chapter 3 described the methodology and repeatability of micro-fluidics based electrophoresis to explore the tear protein profile and total protein content of tears collected by microcapillary tubes. The Agilent 2100 Bioanalyzer was used to measure inter-visit tear protein profiles. Repeatable measurements of seven key tear proteins were found, thus providing a reliable method to investigate for potential biomarkers. The method permitted relative quantification of the main lacrimal proteins in a single analysis from a small quantity of tears (4µL).

The next four chapters described the methodology and findings of a prospective, longitudinal study to explore early evaporative DED in young adults. Tear and meibum samples, as well as clinical data, were collected and analysed in order to investigate early DED.

8.2.3. Prospective, longitudinal study assessing dry eye among young adults - clinical outcomes

Chapter 4 described the clinical findings of the prospective longitudinal study, which aimed to investigate the relationship between lifestyle factors, symptoms, ocular signs, tear inflammatory markers and meibomian gland lipids, in young adults with evaporative DED compared to control participants. While conjunctival staining appeared to be the best single clinical predictor of DED in this study, a significant overlap in clinical signs between the participants diagnosed with and without DED

was identified, further emphasising the need for biomarker studies. A significant number of the young adults were found to have ocular surface signs without symptoms, which would suggest that the term 'pre-clinical' DED would be appropriate. Female sex and contact lens wear were the most significant risk factors. The key modifiable lifestyle risk factors were screen use and stress. Therefore, the study has highlighted the importance of counselling young people for the key modifiable risk factors of DED, regardless of their dry eye status and before the onset of symptoms.

However, in this study the ability of the InflammaDry® rapid test to distinguish MMP-9 between DED and non-DED was inconclusive, with almost half of the non-DED participants at Visit 1 having a positive (trace, weak, positive, or strong positive) result. No correlations were found with any clinical parameter indicative of ocular surface inflammation or tissue breakdown, except for horizontal LWE, which also correlated with screen use hours. Interestingly, two pro-inflammatory cytokines, IL-6 and 8, were later found to correlate with MMP-9 (chapter 6), an indication that inflammation does play a part in the pathogenesis of evaporative DED.

8.2.4. Prospective, longitudinal study assessing dry eye among young adults - tear protein assessment

Chapter 5 reported on the application of the method described in Chapter 3 to analysis of tear samples collected as part of the prospective, longitudinal study on young adults. Differences were observed in the tear protein profiles between the young adults with and without diagnosed evaporative DED, indicating changes that reflect the state of the ocular surface in less advanced DED. The repeatable up-regulation of albumin, present in the tears due to serum leakage, adds to the evidence implicating inflammation in the early pathogenesis of DED. Also, in agreement with previous findings in ADDE, lactoferrin was found to be downregulated, and correlated with reduced meibomian gland expression. Furthermore, the downregulation of ZAG and the correlation with increased signs of MGD at the first visit, suggest this protein could also be useful as an early indicator of EDE.

8.2.5. Prospective, longitudinal study assessing dry eye among young adults - tear cytokine results

Chapter 6 details the tear cytokine assessment of the young DED and non-DED participants over the year, using Luminex. While several of the pro-inflammatory cytokines were repeatedly upregulated in the DED participants (IL-1β, Il-8), the most significant increase was for fractalkine. The upregulation of the anti-inflammatory cytokines, IL-1Ra and IL-10, in the DED participants, provided evidence that a negative feedback mechanism was at play, in order to maintain homeostasis. Several correlations

between cytokine concentrations and clinical findings were also observed, the most notable ones being for NIKBUT and corneal staining at Visit 1 . Interestingly, a significant repeatable association was found between the multifunctional cytokine IL-1β and meibomian gland expression; the quality of meibum and the upper meibography score correlated at Visit 1. The upregulation of this cytokine in DED and its association with signs of MGD warrant further investigation. The results of this chapter showed that both pro-inflammatory and anti-inflammatory cytokines and chemokines are up-regulated in MGD-related evaporative DED, even in the early stages.

8.2.6. Prospective, longitudinal study assessing dry eye among young adults - meibum lipidome analysis

The final chapter 7 reports on the meibum lipid analysis from the longitudinal study. In order to remove the subjective element of a diagnosis of evaporative DED, the lipid composition was compared between participants based on the percentage of meibomian gland loss. Lipidomic analysis is an emerging field and the composition of meibum is a very complex mixture of different polar and non-polar lipid classes. Therefore, identification of changes in different lipid classes, rather than individual lipids were more relevant.

The results showed that changes to the meibum composition can occur even in asymptomatic cases of early meibomian gland dysfunction; disruption of the meibum composition due to an increase in certain polar phospholipids, and a decrease in polar OAFHAs occurred. The findings for the non-polar fatty acyls were variable between compounds and between visits but showed a trend towards higher abundance with increased signs of MGD. However, further targeted analysis is required, with particular regard to the possible effect of lipid compound saturation or unsaturation. The abundance of the di-and triglycerides also showed a variable relationship with signs of MGD. Several correlations were observed between lipids of different classes and lower lid meibography score, percentage of glands excreting, and to a lesser extent the quality of the meibum expressed.

Chapters 4 ,5 and 6 also compared the clinical and tear protein findings from Visit 1 to Visit 2, in order to seek evidence of any progression in the disease over the one year period of the study. Significant increases in ocular redness (bulbar and limbal), and LWE (horizontal and sagittal) at Visit 2, and a general trend towards increased signs of DED (including lower NIKBUT), indicated progression. Ocular redness increased in the non-DED participants, further supporting the evidence of progression, and indicating that the two groups are not mutually exclusive. Other than the clinical findings, no significant differences in the level of any cytokine were found between visits, although several correlations with clinical signs were observed. ***Figure 8.1***. illustrates the key findings of this thesis.

Figure 8.1. Infographic of the key findings of this thesis

8.3. Limitations

There are a few limitations to this thesis, and these are mentioned as follows:

- The survey size was slightly underpowered for making comparisons between the IP and non-IP qualified practitioners, which was not the primary aim of the study.
- Due to the nature of DED, variations were often observed in clinical measures such as tear meniscus height and average NIKBUT, meaning several significant clinical findings were not repeatable at both visits. The sample size was also smaller at Visit 2 due to several dropouts; this meant the study was underpowered for comparison of some tear proteins and cytokines between the DED and non-DED participants at Visit 2.

- Due to the need to collect uncontaminated tear samples by microcapillary tubes and Schirmer strips, measurement of MMP-9 with the InflammaDry® test was performed after these. It is possible that irritation from the Schirmer strips may have induced a low level inflammatory reaction in some participants leading to false positives.
- Some variability was found in both the tear and meibum results between visits. This may be attributed in part to a smaller sample size at Visit 2. However, storage of the tear and meibum samples over the period of the study may have influenced the results obtained. The total protein concentration of the tear samples collected by Schirmer strips was significantly lower at Visit 1 of the study, suggesting possible loss of protein either due to duration of storage or freeze-thaw cycles(Sitaramamma et al., 1998;de Jager et al., 2009). A significant batch effect was observed between the meibum samples collected at each visit, meaning direct comparisons of the lipid abundances could not be made.
- The higher than expected levels of some lipids, including ceramides and several plant sterols in the meibum samples, indicate possible contamination from sebum.

8.4. Future research

Any future survey of the profession to explore the diagnosis and management of DED would benefit from a larger sample size. It would be interesting to discover whether the use of the existing biomarker test for inflammation becomes more widespread in the future, as practitioners become more aware of the role inflammation plays in DED, and the number of Independent Prescribing practitioners grows in the UK. Further information regarding practitioners' awareness of the product and any constraints to its use could be obtained. With the increasing prevalence of DED reported in younger populations, it would also be useful to determine what percentage of their patients with DED practitioners manage in different age categories. Information regarding perceptions around risk factors for DED would also be interesting and whether practitioners counsel their patients for them.

With regard to biomarker research, a larger cohort study to determine the repeatability of the findings reported in this study would add weight to their validity in detecting EDE in the early stages. A larger cohort study would also enable the participants with signs of evaporative DED to be categorised into mild, moderate, and severe groups.

Further investigation into the number of positive semi-quantitative MMP-9 findings in a young cohort would also be useful. However, in any future study, measurement of this biomarker would need to be performed prior to any invasive tear collection procedure.

Also of particular interest, are the tear proteins albumin and ZAG, which were significantly altered in the DED participants. Although lipocalin was not found to show any statistical difference between the participants of this study, or any correlation with meibomian gland parameters, further investigation of this tear protein is also warranted, due to its known lipid binding properties and role in stabilising the meibomian lipid layer (Millar et al., 2009). The pro-inflammatory cytokine Il-1β is also a potential biomarker candidate for evaporative DED and requires further work.

The method used in this study to explore the meibum lipidome, untargeted LC-MS, permitted broad biomarker discovery, but not quantitative analysis. Therefore, for a more targeted approach to explore a particular lipid class, any future analysis would benefit from targeted LC-MS, with the incorporation of appropriate lipid standards. Any future study could also incorporate a more in-depth exploration of the contribution of diet to the composition of meibum and the effect any naturally occurring sebum may have on the tear film.

This study stored tear and meibum samples over a one year period and further studies are required to characterise the effect of long term storage on their compositions. Although the young cohort was followed over a year, a longer longitudinal study would enable monitoring of the clinical parameters, tear protein profiles and meibum composition for change, and for any progression should more severe signs and symptoms develop.

8.5. Conclusions

This thesis has provided a valuable insight into the current use of existing tear biomarkers among eyecare practitioners in the UK, and provided evidence that research is being translated into practice. However, while there is an abundance of existing research exploring the tear proteome, no consensus has been reached on the ideal biomarker which would enable accurate and timely diagnosis, classification, monitoring and even prediction of DED. A more suitable approach might be to include the key biomarkers into a tailored panel of biomarkers, made easily accessible for use in a clinical setting, in order to increase sensitivity and specificity.

The search for the ideal biomarker, be it from the tears or meibum, is confounded by the fact that no standard, validated operating procedures exist, with opinions varying as to the best methods for collection and analysis. Therefore, the development of a clinically applicable test, incorporating a panel of biomarkers, may not be possible until a consensus is reached. However, in the last 2-3 years a network of researchers has been established to collaborate and work on this issue.

This study has identified several potential tear biomarkers, including albumin, zinc-α2-glycoprotein and Il-1β. While several of the findings of this study are in agreement with previous studies, providing further evidence of their significance, further research is still required in order to validate them. The findings reported in this thesis serve to reinforce the need for objective biomarkers to aid in the early detection and differentiation of those subjects who are likely to progress to symptomatic DED.

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Appendices

Appendix 1. Links to Dry Eye in Clinical Practice Survey

Research Study: Dry Eye in Clinical Practice

Dear Colleague

It has been more than six years since UK optometrists were last surveyed concerning their clinical practice patterns in Dry Eye Disease management. However, the previous survey was administered only to practitioners who were members of a contact lens association, which may not reflect the true wider picture of optometric practice. Moreover, during this period of time, optometrists' roles have extended, with many of us becoming more involved in Clinical Commissioning Group Minor Eye Condition Service provision and some gaining IP status.

The current survey is for optometrists working at **all levels within the primary care sector** and aims to gain an insight into perceptions of dry eye as a disease, knowledge and confidence in diagnosis and management, and satisfaction with currently available first-line treatment options.

The survey forms part of my PhD research aimed to optimise the management of Dry Eye Disease. I have worked in primary care myself for over 25 years before starting full-time research at Aston University, in 2020. Over recent years I believe dry eye management has increasingly become part of an optometrist's role and I'm interested to learn about optometrists' perceptions and levels of involvement. It would be great to have a wide range of responses; whether from those who feel dry eye management is often tedious and unsatisfying, or those who find it rewarding and have all the specialist equipment.

If you are willing to participate in the survey, please see the link below to the online survey entitled 'Dry Eye in Clinical Practice' or use the QR code provided. The survey is anonymous and will take around 15 minutes to complete.

https://aston.onlinesurveys.ac.uk/dry-eye-in-clinical-practice

There is the option to enter a **£100 prize draw** at the end of the survey. The winner will be contacted by email at the close of the survey 30th June 2022 at which time all email addresses entered will be deleted (see Participant Information Sheet for details on data confidentiality).Many thanks in advance.

Rachel Casemore (Optometrist and PhD Researcher)

Researcher email: [student ID redacted]@aston.ac.uk

Appendix 2. Participant Information Sheet for the Dry eye in Clinical Practice Survey #1795

Dry Eye in Clinical Practice

A survey of UK optometrists' perceptions of the diagnosis and management of dry eye disease, in a primary care setting

Participant Information Sheet

Invitation

We would like to invite you to take part in a research study.

Before you decide if you would like to participate, take time to read the following information carefully and, if you wish, discuss it with others such as your family, friends, or colleagues.

Please ask a member of the research team, whose contact details can be found at the end of this information sheet, if there is anything that is not clear or if you would like more information before you make your decision.

What is the purpose of the study?

In recent years optometrists' roles have extended, with some gaining independent prescriber status and many more becoming involved in Clinical Commissioning Group minor eye condition service provision.

The purpose of this survey is to gain an insight into optometrists' perceptions of dry eye disease, their knowledge and confidence in diagnosis and their satisfaction with currently available first-line treatment options.

Why have I been chosen?

You are being invited to take part in this study because you are a UK optometrist working in a primary care setting. Please do not complete the survey if you only work in a hospital or secondary care setting.

What will happen to me if I take part?

You will be asked to complete the online questionnaire about your opinions and practice in relation to dry eye disease. The survey should take no longer than 15 minutes to complete. There are a mixture of questions requiring you to indicate your level of agreement to a statement or tick boxes to indicate which options are relevant to you. There are no correct answers to any questions.

Do I have to take part?

No. It is up to you to decide whether or not you wish to take part.

Participation in this study is voluntary. If you do decide to participate in the online survey, you will be asked to confirm your participation using an online consent form. If you start the questionnaire but decide not to complete it and withdraw from the study, you can close the browser and your response will not be saved. If you complete a paper copy of the study, you will be asked to read a consent form. Consent will be implied by completion and submission of the survey. **Please note that you will not be able to withdraw from the study after you submit your responses as the questionnaire is anonymous, so we will not be able to identify individual responses**.

Will my taking part in this study be kept confidential?

Yes. Personal information that could enable participants to be identified will not be collected, unless you wish to enter the prize draw, in which case an email address will be required. Data will be stored on a secure cloud storage device and any emails will be deleted within one month of the prize draw taking place at the completion of the study.

What are the possible benefits of taking part?

While there are no direct benefits for those participating in this study, we hope the information gained will help to inform future clinical practice and research needs.

What are the possible risks and burdens of taking part?

There are no significant risks. You will be volunteering your time but may cease completion of the questionnaire at any time if it becomes inconvenient.

What will happen to the results of the study?

The anonymized results of this study will be collated and analyzed before being published in a thesis and may be published in scientific journals and/or presented at conferences. If the results of the study are published, your identity will remain confidential.

If you are interested, we can provide you with a summary of the results at the conclusion of the study. To enable us to do this please request this by emailing the researcher at [student ID redacted]@aston.ac.uk and we will forward you a summary following completion.

Expenses and payments

None. However, all participants have the option of entering the £100 prize draw.

Who is funding the research?

There is no external funding for this study.

Who is organising this study and acting as data controller for the study?

Aston University is organising this study and acting as data controller for the study. You can find out more about how we use your information in Appendix A.

Who has reviewed the study?

This study was given a favourable ethical opinion by the College of Health and Life Sciences Ethics Committee, Aston University.

What if I have a concern about my participation in the study?

If you have any concerns about your participation in this study, please speak to the research team and they will do their best to answer your questions. Contact details can be found at the end of this information sheet.

If the research team are unable to address your concerns or you wish to make a complaint about how the study is being conducted you should contact the Aston University Research Integrity Office at research_governance@aston.ac.uk or telephone 0121 204 3000.

Research Team

Dr Debarun Dutta Aston University, UK d.dutta@aston.ac.uk

Mrs Rachel Casemore Aston University, UK [student ID redacted]@aston.ac.uk

Thank you for taking time to read this information sheet. If you have any questions regarding the study, please don't hesitate to ask one of the research team.

Aston University
BIRMINGHAM UK

Aston University takes its obligations under data and privacy law seriously and complies with the Data Protection Act 2018 ("DPA") and the General Data Protection Regulation (EU) 2016/679 as retained in UK law by the Data Protection, Privacy and Electronic Communications (Amendments etc) (EU Exit) Regulations 2019 ("the UK GDPR").

Aston University is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study. Aston University will process your personal data in order to register you as a participant and to manage your participation in the study. It will process your personal data on the grounds that it is necessary for the performance of a task carried out in the public interest (GDPR Article 6(1)(e). Aston University may process special categories of data about you which includes details about your health. Aston University will process this data on the grounds that it is necessary for statistical or research purposes (GDPR Article 9(2)(j)). Aston University will keep identifiable information about you for 6 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally identifiable information possible.

You can find out more about how we use your information at https://www.aston.ac.uk/about/statutes-ordinances-regulations/publication-scheme/policies-regulations/data-protection or by contacting our Data Protection Officer at dp_officer@aston.ac.uk.

If you wish to raise a complaint on how we have handled your personal data, you can contact our Data Protection Officer who will investigate the matter. If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO).

Appendix 3. Ethics application #1795 approval

Aston Triangle
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United Kingdom
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Memo

Life and Health Sciences Ethics Committee's Decision Letter

To: **Debarun Dutta**

Cc: Tim Batty

Administrator, Life and Health Sciences Ethics Committee

From: Dr Rebecca Knibb

Chair, Life and Health Sciences Ethics Committee

Date 01/10/21

Subject: **Project #1795 Dry Eye in Clinical Practice**

Thank you for your submission. The additional information for the above proposal has been considered by the Chair of the LHS Ethics Committee.

Please see below for details of the decision and the approved documents.

Reviewer's recommendation: Favourable opinion

Please see the tabled list below of approved documents:

Documentation	Version/s	Date	Approved
Response to reviewers' comments	1	25/9/21	✓
Research protocol	2	25/9/21	✓
Participant information sheet	4	25/9/21	✓
Consent form	4	25/9/21	✓
Survey	7	25/9/21	✓

After starting your research please notify the LHS Research Ethics Committee of any of the following:

Substantial amendments. Any amendment should be sent as a Word document, with the amendment highlighted. The amendment request must be accompanied by all amended documents, e.g. protocols, participant information sheets, consent forms etc. Please include a version number and amended date to the file name of any amended documentation (e.g. "Ethics Application #100 Protocol v2 amended 17/02/12.doc").

New Investigators

The end of the study

Please email all notifications and reports to lhs_ethics@aston.ac.uk and quote the original project reference number with all correspondence.

Statement of Compliance

The Committee is constituted in accordance with the Government Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK. In accord with University Regulation REG/11/203(2), this application was considered to have low potential risk and was reviewed by three appropriately qualified members, including the Chair of the Life and Health Sciences Ethics Committee.

Yours sincerely,

Dr Rebecca Knibb
Chair, LHS Ethics Committee

Appendix 4. Aston University's Standard operating Procedure (SOP) for tear collection

Standard Operating Procedure

Tear Sampling (Capillary or Ophthalmic Sponge without anesthesia

DOCUMENT CHANGE SUMMARY

VERSION	DATE	SUMMARY
1.0	17/2/20	Original

A. PURPOSE

To describe the methodology of the Biomaterials Research Unit to sample tear constituents, in-vivo, either with or without a contact lens in-situ, and without anesthesia.

B. APPLIES TO

All trained Biomaterials Research Unit research staff who sample tear constituents on human subjects in-vivo as part of a clinical study linked to laboratory based analysis.

C. PROCEDURE

1. Prepare the vials.
   a. Use appropriate size and color storage vial, depending on the experiment protocol.
   b. Use a permanent marker to label with subject ID, date, study and other pertinent study information.
2. Prepare the subject.
   a. Seat the subject comfortably.
   b. Explain that the micro capillary or ophthalmic sponge will be inserted over the lower lid margin.
   c. Explain that the subject may have reflex blink/tearing/discomfort when inserting the Schirmer strip, micro capillary or ophthalmic sponge. No pain will be caused.
   d. Open the labelled vial before measurement.
3. Wash your hands and use latex-free gloves.
4. Insert the test over the lower lid margin of right eye, midway between the middle and outer temporal third.
5. Place the capillary or ophthalmic sponge into open labeled vial.
6. Place cap onto vial.
7. Place capped and labeled vial in temporary storage chest containing a layer of dry ice or wet ice or immediately into the appropriate refrigerator or freezer.
8. Repeat the steps 4-8 for left eye.

Note 1: If using Schirmer strip test method please refer to Ophthalmic Research Group SOP A07 Schirmer Strip

Note 2: This method can also be applied to a contact lens (please refer to Ophthalmic Research Group SOP A08 Soft Contact lens Removal and Storage) if being used for tear sampling. In which case the removed lens may be stored in an Eppendorf rather than a vial.

Appendix 5. Aston University Optometry and Vision Sciences Research Group (OVSRG) tear collection protocol

Optometry Translational Research Protocols

Tear collection – basal, reflex and flush

Aston University Optometry and Vision Sciences Research Group (OVSRG)

Prepared by Dr Debarun Dutta

ABBREVIATIONS:

ELISA	Enzyme-linked immuno assay
g	Gravitational force
HA	Hyaluronic acid
HEMA	Hydroxyethyl methacrylate
IRB	Institutional Review Board
µg	Micrograms
µm	Micrometers
µl	Microliters
µM	Micromolar
mg/cm2/min	Milligram per centimeter square per minute
ml	Milliliters
Mm	Millimolar
mOsms/l	Milliosmol per liter
nm	Nanometers
NISDT	Non-invasive surface drying time
NMR	Nuclear magnetic resonance
OAHFA	(O-acyl)-ω-hydroxy fatty acid
OCI	Ocular comfort index
pH	Measure of acidity or basicity
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PS	Phosphatidylserine
pg	Picograms
pmol/µl	Picomole per microliters
PMMA	Polymethyl methacrylate
ROS	Reactive oxygen species
SD	Standard deviation
SE	Standard error
sPLA2	Secretory phospholipase enzyme
SM	Sphingomyelin
SPSS	Statistical package for social sciences
TAG	Triglyceride
TFOS	Tear film and ocular surface society
WE	Wax ester
w/v	Weight per volume
~	Approximately
°C	Degree Celsius
>/<	Greater than/less than
×	Multiplication sign
%	Percentage
®	Registered sign
™	Trademark sign

MATERIALS

- 10 µL microcapillary tube (Blaubrand® intraMark, Wertheim, Germany)
- microcentrifuge tube (0.65 mL; Sigma-Aldrich, Steinheim, Germany)
- Non-preserved unit dose saline (sodium chloride injection 0.9%, AstraZeneca, North Ryde, Australia)
- Icebox
- -80°C degrees freezer

PROTOCOL

- This protocol is based on previously published reports of tear collection, with minor modifications (Dutta et al. 2019).
- Appropriate approval from Institutional Research Board (IRB) is required which will follow the Declaration of Helsinki (1975, amended in 2002).
- All participants should provide written consent prior to the tear collection.

Basal tear collection:
- Basal tears to be collected using a 10 µL microcapillary tube.
- Use sterile latex/vinyl gloves for tear collection.
- Participants will be requested to tilt their head towards the side of the collection while looking in the opposite direction.
- Tear collection will always be from the lateral tear meniscus following gentle resting of the microcapillary tube at the lower lid margin.
- Care should be taken to minimise the touch of the microcapillary tube to the bulbar conjunctiva or the lid margin to avoid stimulation.
- Not more than 30 minutes should be spent on tear collection (symptomatic dry eye patients take up to 30 mins for up to 10 µl basal tear collection).
- Participants should be allowed to blink intermittently during tear collection, this will avoid reflex tearing, during the blink the microcapillary tube should be withdrawn.
- The collection should be paused if any reflex tearing is suspected. Generally, a 5 minutes pause of tear collection is adequate.
- Fresh microcapillary tubes should be used for each eye unless the collected tear is pulled together (based on study design).
- Tear collection rate should be monitored for all samples by noting the time and volume collected.
- The sample should be expelled from the microcapillary tube using a pump into a microcentrifuge tube (0.65 mL) and should be placed on ice until the next step.

Reflex tear collection:
- The tear should be collected similar to basal tear collection as explained earlier, except that the sneeze reflex will be stimulated by gently inserting a sterile cotton bud into the nasal passages to induce tear production.
- All tear should be collected within 1 minute to the last sneeze.

Flush tear collection:
- Non-preserved unit dose saline should be warmed to 35˚C to avid any reflex tearing due to cold stimulus to the ocular surface.
- The flush tear should be collected by instilling a single 40 µL drop of non-preserved unit dose saline into the inferior palpebral fold using an Eppendorf pipette.
- Care should be taken not to touch the eye with the sharp pipette tip.
- Participants should be instructed to gently close their eyes and rotate them twice.

- The ocular fluid then should be collected immediately using a microcapillary tube as described before. Attempts should be made to collect more than 30 µL flush tear (may use 20 µL microcapillary tubes).
- All flush tear fluid should be collected within 1 minute of instillation of non-preservative saline to avoid reflex tear.

Tear sample storage:

- Tear samples should be immediately centrifuged at 5000g (calculate rpm based on the type of centrifuge used) for 10 minutes at 4˚C to remove cellular debris (Masoudi et al. 2017).
- The aqueous supernatant should be collected and aliquoted to a fresh microcentrifuge tube for processing.
- If a larger volume of tear is collected, it may be filtered through 0.22 µm which will completely remove any cellular content.
- For best results tear samples should be analysed within 1 month of the collection (some measurements requires immediate laboratory analysis).
- However, currently there is no known major contraindication for storing tear samples for up to 1 year for many analysis (ELISA, Mass-spectrometry etc; subject to the type of analysis).

References:

Dutta, D., J. Kim, M. Sarkes, S. Nath and M. Markoulli (2019). "The repeatability of subjective and objective tear ferning assessment and its association with lipid layer thickness, non-invasive tear break-up time and comfort." Cont Lens Anterior Eye **42**(4): 420-427.

Masoudi, S., F. J. Stapleton and M. D. P. Willcox (2017). "Differences in Tear Film Biochemistry of Symptomatic and Asymptomatic Lens Wearers." Optom Vis Sci **94**(9): 914-918.

Appendix 6. Ethics application #1756 Approval

Aston University
Birmingham B4 7ET
United Kingdom

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25 August 2021

Dr Debarun Dutta
Student: Rachel Casemore
College of Health and Life Sciences

Dear Rachel,

Study title:	Characterisation of Dry Eye in Young Adults
REC REF:	#1756

Confirmation of Favourable Ethical Opinion

On behalf of the Committee, I am pleased to confirm a favourable opinion for the above research on the basis of the application described in the application form, protocol and supporting documentation listed below.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows.

Document	*Version*	*Date*
Participant Information Sheet	2	18/08/2021
Consent Form	1	21/07/2021
Study advert	2	18/08/2021
OSDI questionnaire		
SoP for Tear Sampling	1	17/02/2020
Risk Assessment Tear Storage	1	13/11/2020

After starting your research please notify the University Research Ethics Committee of any of the following:

- Amendments. Any amendment should be sent as a Word document, with the amendment highlighted or showing tracked changes. The amendment request must be accompanied by a covering letter along with all amended documents, e.g. protocols, participant information

sheets, consent forms etc. Please include a version number and amended date to the file name of any amended documentation (e.g. "Ethics Application #100 Protocol v2 amended 17/02/19.doc").

Amendment requests should be outlined in a "Notice of Amendment Form" available by emailing ethics@aston.ac.uk.

- Unforeseen or adverse events e.g. disclosure of personal data, harm to participants.
- New Investigators
- End of the study

Please email all notifications or queries to ethics@aston.ac.uk and quote your UREC reference number with all correspondence.

Wishing you every success with your research.

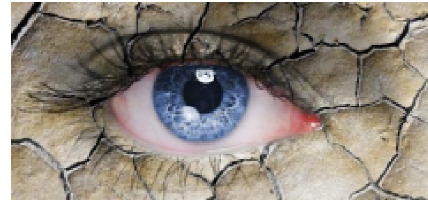
Yours sincerely

Professor James Wolffsohn
Acting Chair, University Research Ethics Committee

Appendix 7. Adverisement for study #1756

Do you suffer from uncomfortable, itchy or watery eyes? Or just feel your eyes are dry any time of a typical day? You might be suffering from Dry Eye Disease (DED)

At the Optometry Clinic, Aston University, Birmingham we are seeking volunteer participants who are suffering from such uncomfortable dry eye problems.
Even if you do not have such eye problems, you can still participate in our study.



What is the study about?

Researchers at the Optometry Clinic, Aston University are investigating dry eye related problems among young adults and how it may change over one year.

Will I be paid to take part in the research study?

If you are eligible, you will be reimbursed with £10 for each visit. The study includes a total of two visits, totalling to £20 over two visits, over approximately 1 year.

Would the research study be a good fit for me?

The study might be a good fit for you if you:

- have or don't have dryness of the eyes (eye grittiness, watering and itchiness)
- are 18-25 years of age
- are able to provide written consent in English
- are able to attend a follow up visit after 1 year ± 2 months
- do not anticipate any major ocular changes over the next one year.

What would happen if I took part in the research study?

If you decide to participate, you would:

- ✓ attend two appointments, one year apart. Each visit will last approximately 45 minutes
- ✓ complete a 12-set questionnaire for assessment of any eye dryness
- ✓ have your vision checked
- ✓ have the health of the front of your eyes and the quality of your tears assessed
- ✓ tears from your eye and naturally secreting oil for your lids collected. This will take approximately 2 minutes per eye.

Who do I contact if I want more information or want to take part in the study?

If you would like more information or are interested in being part of the study, please email reply to us with your convenient time and contact number. We will call you back. You can contact us with the following:

- Mrs Rachel Casemore [redacted].ac.uk)
- Dr Debarun Dutta, (d.dutta@aston.ac.uk)
- Prof. James Wolffsohn (J.S.W.Wolffsohn@aston.ac.uk)
- Ph: +44 (0) 121 204 4400 (dry eye clinic)

REC ID: #1756, Version 2, 20210818

Appendix 8. Participant Information Sheet for study #1756

Characterisation of Dry Eye in Young Adults

Participant Information Sheet

Invitation

We would like to invite you to take part in a research study.

Before you decide if you would like to participate, take time to read the following information carefully and, if you wish, discuss it with others such as your family, friends, or colleagues.

Please ask a member of the research team, whose contact details can be found at the end of this information sheet, if there is anything that is not clear or if you would like more information before you make your decision.

What is the purpose of the study?

Digital screen users often report symptoms of dry eye. Dry eye is a common problem that occurs when the eyes do not make enough tears, or the tears evaporate too quickly. It can be triggered by things like wind, spending time in air conditioned or heated environments, prolonged screen use, wearing contact lenses, certain medications, or conditions such as mild infection of the lids. Symptoms often include sore, gritty, red eyes which can be blurry, sensitive to light or water more frequently.

The prevalence of dryness of the eyes is known to increase with age, but increasingly younger people are being found to have symptoms and signs.

This study is aimed at young adults who spend more than an hour a day using a screen, who may and may not have dryness of the eyes. The symptoms of eye dryness may can lead to mild irritation and itching. Dryness may be felt throughout a day or at a particular time of the day. We will ask you for details of any eye dryness you may or may not experience, including the severity and duration (from all of the time to none of the time), using a questionnaire. The questionnaire has a total of 12 questions, and generally takes 3 minutes to complete. If you do not have eye dryness you can still participate in this study and reflect your experience on the questionnaire.

Investigators will collect approximately one drop of your normal tears with a microcapillary tube placed in the outer canthus of each eye for 2 minutes per eye, following standard operating procedure for the Ophthalmic Research Group, Aston University. These tears will

be placed in small tubes and spun to remove any cells and then stored in the laboratory at -80˚C degrees for future biochemical analysis. We will also collect oils naturally secreted from your eyelids. We will gently clean your lid margin with wet cotton buds as a regular clinical process in the dry eye clinic, to avoid collecting any cells. Then we will gently collect the oil using a sterile loop, and store in -80˚C for future analysis.

During the biochemical analysis we will look to find associations between any dryness of your eyes and various proteins from the tears and oil, comparing the tears of those participants with dry eye signs and symptoms to those without. We will also characterise clinical signs of dry eye, analyse the tears for any signs of inflammation and explore any associations with lifestyle factors, such as screen use. At the end of the study all collected and analysed samples will be disposed of. If we find signs of inflammatory proteins and associations, we will report the results as a group in scientific journals, conferences or in a PhD thesis, keeping all data completely anonymous.

Why have I been chosen?

You are being invited to take part in this study because you have responded to an advert and expressed interest. You have been chosen because you satisfy either of the following two criteria:

1.You are a young person who is likely to spend at least an hour a day using some form of digital device and you do not have dryness of the eyes based on our clinical assessment and your answers to our questionnaire.

OR

2. You are a young person who is likely to spend at least an hour a day using some form of digital device and you do have dryness of the eyes based on our clinical assessments and your answers to our questionnaires.

If any of the following apply, you may not be able to participate in this study:

- A health condition known to affect or alter the dryness of the eyes
- Current use of any medications known to affect the eye
- A pre-existing eye condition or previous eye surgery
- Current involvement in another clinical trial

If you are unsure whether any of these exclusions apply to you, please do not hesitate to contact the researchers (contact emails below).

What will happen to me if I take part?

You will be asked to be available for two appointments, one year ± 2 months apart. Each visit will last approximately 45 minutes and will take place in the Aston University Eye Clinic.

At the initial visit you will be asked to complete a short questionnaire about how your eyes feel. You will also be asked some questions about your lifestyle e.g. hours of screen use and general health.

Routine clinical measurements will be performed to assess your eyes for any signs of dry eye. These include checking your vision, clinical history about your eye health, and assessment of your anterior eye health using standard examination equipment such as the slit lamp biomicroscope. As explained above we will collect tears and naturally secreting oil from your eyelid. We will collect approximately one drop of your normal tears with a microcapillary tube placed in the outer canthus of each eye for 2 minutes per eye. You will be able to blink normally throughout. We will also collect oils naturally secreted from your eyelids. We will gently clean your lid margin with wet cotton buds as a regular clinical process in the dry eye clinic to avoid collecting any cells. Then we will gently collect the oil using a sterile loop. We have used these methods in various previous clinical studies and have never experienced any complications.

You will be contacted again in one year and asked to return to have the same measurements repeated.

Do I have to take part?

No. It is up to you to decide whether or not you wish to take part.

If you do decide to participate, you will be asked to sign and date a consent form. You would still be free to withdraw from the study visits at any time without giving a reason. This means you may withdraw after the first or second visit and ask for the data collected at either visit to be withdrawn. However, we will anonymise your data after each visit and once it has been anonymised and analysed, withdrawal will no longer be possible.

Will my taking part in this study be kept confidential?

Yes. A code will be attached to all the data you provide to maintain confidentiality.

Your personal data (name and contact details) will only be used if the researchers need to contact you to arrange study visits or collect data by phone. Analysis of your data will be undertaken using coded data.

The data we collect will be stored in a secure document store (paper records) or electronically on a secure encrypted mobile device, password protected computer server or secure cloud storage device.

To ensure the quality of the research, Aston University may need to access your data to check that the data has been recorded accurately. If this is required, your personal data will be treated as confidential by the individuals accessing your data.

What are the possible benefits of taking part?

While there are no direct clinical benefits to you of taking part in this study, the data gained will help us to understand how dry eye disease develops in younger people.

What are the possible risks and burdens of taking part?

The researchers do not anticipate any risks during participation in this study; all the clinical procedures are part of a standard dry eye assessment, including collection of samples. The

investigators have substantial experience and will follow their own published method for collection of the tears and oil. The researchers do not anticipate any discomfort or irritation due to these procedures.

With your consent, we will use a couple of staining agents (fluorescein 1.0% strips and lissamine green 1% strips) to aid external examination of your eye. These are used routinely during eye examinations. When applied to the eye, they may sting for a few moments. Due to their colouring (orange/yellow and green) they may cause the vision to take on a coloured appearance, but this will not last long. Sometimes, the eyelids and the skin around the eyes will be coloured by the stain, but this can be removed with cold water. Your vision will not be affected so there will be no problem if you need to drive after the appointment.

In the clinic, we continue to follow the current College of Optometrists COVID-19 guidance for infection control, including the use of PPE and practice disinfection procedures.

What will happen to the results of the study?

The results of this study will form part of a PhD thesis and may be published in scientific journals and/or presented at conferences. If the results of the study are published, your identity will remain confidential.

A lay summary of the results of the study will be available for participants when the study has been completed. If you are interested please provide an email address and we will send a summary to you.

Expenses and payments

You will receive a £10 gift voucher for each visit, (a maximum of £20 for the two visits over 1 year), for taking part in this study

Who is funding the research?

The study is being funded by the Optometry and Vision Sciences Research Group, Aston University.

Who is organising this study and acting as data controller for the study?

Aston University is organising this study and acting as data controller for the study. You can find out more about how we use your information in Appendix A.

Who has reviewed the study?

This study was given a favorable ethical opinion by Aston University Research Ethics Committee.

What if I have a concern about my participation in the study?

If you have any concerns about your participation in this study, please speak to the research team and they will do their best to answer your questions. Contact details can be found at the end of this information sheet.

If the research team are unable to address your concerns or you wish to make a complaint about how the study is being conducted you should contact the Aston University Research Integrity Office at research_governance@aston.ac.uk or telephone 0121 204 3000.

Research Team

Dr Debarun Dutta — Aston University, UK d.dutta@aston.ac.uk

Mrs Rachel Casemore — Aston University, UK [redacted]on.ac.uk

Prof. James Wolffsohn — Aston University, UK J.S.W.Wolffsohn@aston.ac.uk

Thank you for taking time to read this information sheet. If you have any questions regarding the study, please don't hesitate to ask one of the research team.

Appendix A: Transparency statement

Aston University takes its obligations under data and privacy law seriously and complies with the Data Protection Act 2018 ("DPA") and the General Data Protection Regulation (EU) 2016/679 as retained in UK law by the Data Protection, Privacy and Electronic Communications (Amendments etc) (EU Exit) Regulations 2019 ("the UK GDPR").

Aston University is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study. Aston University will process your personal data in order to register you as a participant and to manage your participation in the study. It will process your personal data on the grounds that it is necessary for the performance of a task carried out in the public interest (GDPR Article 6(1)(e). Aston University may process special categories of data about you which includes details about your health. Aston University will process this data on the grounds that it is necessary for statistical or research purposes (GDPR Article 9(2)(j)). Aston University will keep identifiable information about you for 6 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally identifiable information possible.

You can find out more about how we use your information at https://www.aston.ac.uk/about/statutes-ordinances-regulations/publication-scheme/policies-regulations/data-protection or by contacting our Data Protection Officer at dp_officer@aston.ac.uk.

If you wish to raise a complaint on how we have handled your personal data, you can contact our Data Protection Officer who will investigate the matter. If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO).

When you agree to take part in a research study, the information about you may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad.

This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of research and cannot be used to contact you.

Appendix 9. Consent form for study #1756

Personal identification number for study: ________

Consent Form

Characterisation of Dry Eye in Young Adults

Name of Chief Investigator: Dr Debarun Dutta

Investigator: Mrs Rachel Casemore

Please initial boxes

1.	I confirm that I have read and understand the Participant Information Sheet (Version 2, 20210818) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my legal rights being affected.	
3.	I agree to my personal data and data relating to me collected during the study being processed as described in the Participant Information Sheet.	
4.	I agree to my anonymised data being used by research teams for future research.	
5.	I agree to take part in this study.	

______________________	______________	________________
Name of participant	Date	Signature
______________________	______________	________________
Name of Person receiving consent.	Date	Signature

One copy for research participant; one copy for site file

If you would like to receive a summary of the results of the study, please leave an email address here:

____

REC ID: #1756 Version 1, 20210721

Appendix 10. Lifestyle questionnaire for study #1756

Characterisation of Dry Eye in Young Adults

Lifestyle Questions

VISIT ☐ **Date:** ☐☐/☐☐/☐☐

Subject code ☐ **D.O.B** [DD/MM/YYYY] **Ethnicity** ☐

Please place a tick in the relevant boxes

1. How many hours do you spend on a digital screen per day? [hours]
(includes phone, tablet, computer, TV, and other devices)

2. Do you wear contact lens? Never ☐ In the past ☐ Yes ☐

Type [Soft/hard/daily disposable/monthly/EW]

Frequency of wear [Daily/occasional]

3. Do you smoke? No never ☐ No, in the past ☐

Yes ☐ Cigarettes per day [no.]

4. On average, how many hours of exercise do you have per week? [hours]

5. On average, how many hours of outdoor activity do you have per week? [hours]

6. How would you rate your diet? Poor ☐ Fair ☐ Good ☐ Excellent ☐

7. How would you rate your stress levels? Minimal ☐ Mild ☐ Moderate ☐ High ☐

8. How would you rate your health? Poor ☐ Fair ☐ Good ☐ Excellent ☐

Appendix 11. Clinical Record Form for study #1756

Characterisation of Dry Eye in Young Adults

CRF

VISIT ☐ Date: ☐☐/☐☐/☐☐

Subject code ☐ D.O.B (DD/MM/YYYY)

Sex (M/F/ Prefer not to say) Time (__ __/__ __) Inclusion/Exclusion criteria checked ☐

Consent form signed ☐ Room Temperature ☐°C/F Relative Humidity ☐%

OSDI score ☐ Last eye examination ☐ VA (OD) (OS)

Medical History:

Systemic conditions?

Depression ☐ Anxiety ☐ Migraine ☐ Asthma ☐ Rosacea ☐ Psoriasis ☐ IBS ☐

Chronic pain syndrome ☐ Lupus ☐ Arthritis ☐ Thyroid disease ☐ Sjogren's syndrome ☐

Polycystic ovary syndrome ☐ Other ☐ --

Topical/systemic medications ☐ --

Nutritional supplements ☐ --

Ocular conditions/surgery ☐ --

Dry Eye Treatments ☐ --

Allergies ☐ --

Inclusion Criteria:

Aged 18-25 and spend at least 1 hour a day on a digital device ☐

Vision at least 6/12 or better in each eye ☐

Feasible to attend follow up visit in 1 year ☐

Do not anticipate any major ocular changes over 1 year ☐

Exclusion Criteria:

ADDE (defined by a TMH less than 0.20mm or symptoms suggestive of ADDE such as dry mouth or swollen glands) ☐

Use of systemic or topical medications known to affect the eye in the previous 3 months ☐

Previous ocular surgery ☐

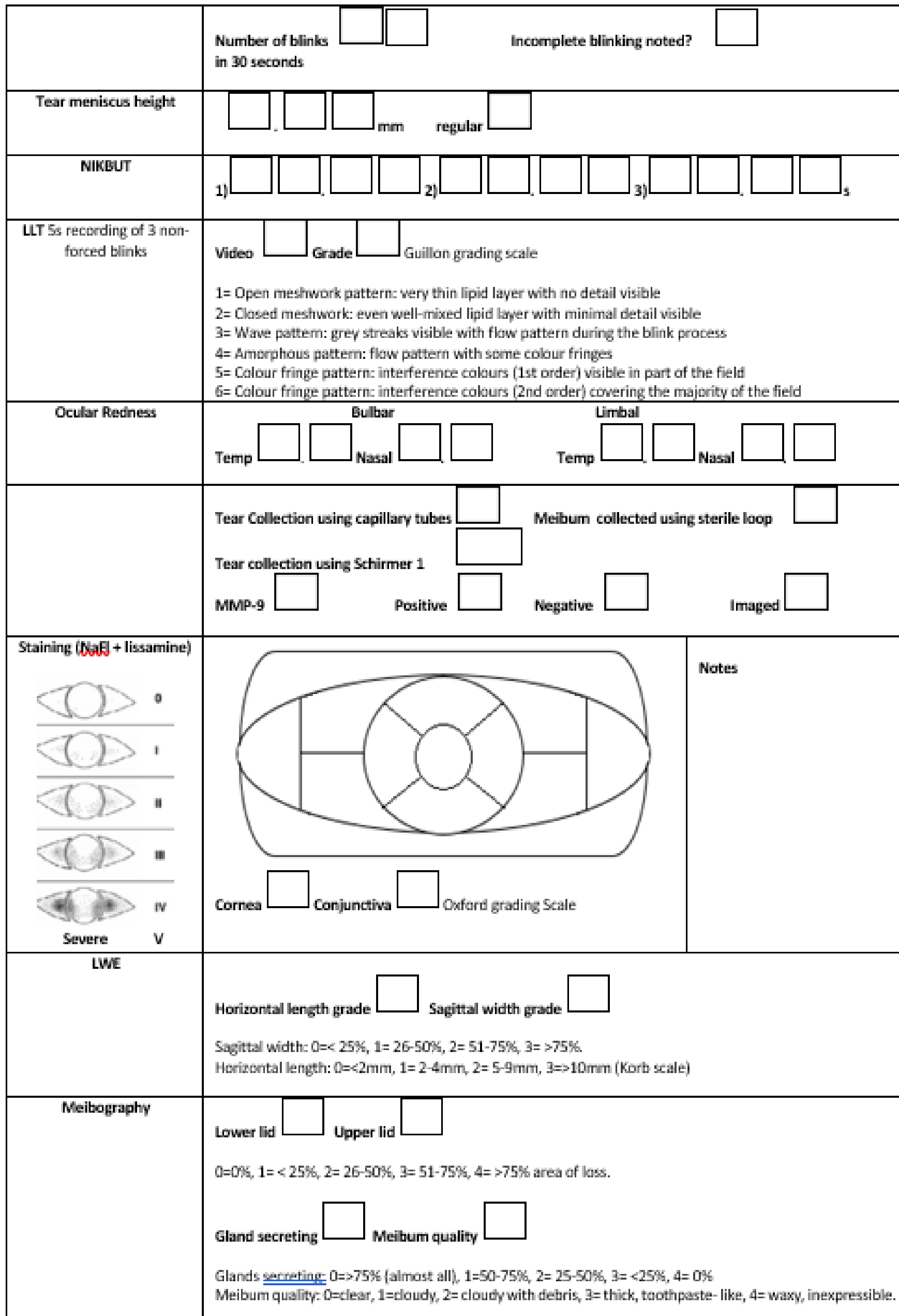
Allergic conjunctivitis with the use of antihistamine drugs ☐

History of major systemic or ophthalmic conditions ☐

Any active infection or inflammation ☐

Currently enrolled on another clinical trial ☐

Eye examined Right ☐ Left ☐



	Number of blinks in 30 seconds ☐☐ Incomplete blinking noted? ☐
Tear meniscus height	☐.☐☐ mm regular ☐
NIKBUT	1) ☐☐.☐☐ 2) ☐☐.☐☐ 3) ☐☐.☐☐ s
LLT 5s recording of 3 non-forced blinks	Video ☐ Grade ☐ Guillon grading scale 1= Open meshwork pattern: very thin lipid layer with no detail visible 2= Closed meshwork: even well-mixed lipid layer with minimal detail visible 3= Wave pattern: grey streaks visible with flow pattern during the blink process 4= Amorphous pattern: flow pattern with some colour fringes 5= Colour fringe pattern: interference colours (1st order) visible in part of the field 6= Colour fringe pattern: interference colours (2nd order) covering the majority of the field
Ocular Redness	Bulbar: Temp ☐.☐ Nasal ☐.☐ Limbal: Temp ☐.☐ Nasal ☐.☐
	Tear Collection using capillary tubes ☐ Meibum collected using sterile loop ☐ Tear collection using Schirmer 1 ☐ MMP-9 ☐ Positive ☐ Negative ☐ Imaged ☐
Staining (NaFl + lissamine) 0 I II III IV Severe V	Cornea ☐ Conjunctiva ☐ Oxford grading Scale Notes
LWE	Horizontal length grade ☐ Sagittal width grade ☐ Sagittal width: 0=< 25%, 1= 26-50%, 2= 51-75%, 3= >75%. Horizontal length: 0=<2mm, 1= 2-4mm, 2= 5-9mm, 3=>10mm (Korb scale)
Meibography	Lower lid ☐ Upper lid ☐ 0=0%, 1= < 25%, 2= 26-50%, 3= 51-75%, 4= >75% area of loss. Gland secreting ☐ Meibum quality ☐ Glands secreting: 0=>75% (almost all), 1=50-75%, 2= 25-50%, 3= <25%, 4= 0% Meibum quality: 0=clear, 1=cloudy, 2= cloudy with debris, 3= thick, toothpaste- like, 4= waxy, inexpressible.

Appendix 12. Aston University's Ophthalmic Vision Science Research Group (OVSRG) Standard Operating Procedure (A07) for Schirmer-1 test (without anaesthesia)

Standard Operating Procedure
Shirmer-1 test – without anesthesia

DOCUMENT CHANGE SUMMARY

VERSION	DATE	SUMMARY
1.0	14.6.13	Original
1.0	02.12.16	Reviewed by JSW
1.0	10.12.18	Reviewed by JSW
1.0	3.12.19	Reviewed by JSW
2.0	28.09. 21	Amendments for 'Characterisation of Dry Eye in Young Adults' Study by RKC

A. PURPOSE

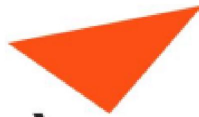
To describe the methodology of the Optometry and Vision Sciences Research Group to measure tear volume and sample tear constituents, in-vivo, either with or without a contact lens in-situ, and without anesthesia.

B. APPLIES TO

All Optometry and Vision Sciences Research Group research staff who measure tear volume or sample tear constituents on human subjects in-vivo as part of a clinical study.

C. PROCEDURE

1. Prepare the vials.
    a. Use appropriate size and color storage vial, depending on the experiment protocol.
    b. Use a permanent marker to label with subject ID, date, study and other pertinent study information.
2. Prepare the subject.
    a. No fluorescein prior to the collection of tears
    b. Seat the subject comfortably.
    c. Explain that the Schirmer test will be inserted over the lower lid margin.
    d. Explain that the subject may have reflex blink/tearing/discomfort when inserting the Schirmer test. No pain will be caused.
    e. Open the labelled vial before measurement.
3. Wash your hands and use latex-free gloves.
4. Take a Schirmer test (5x35mm Haag-Streit, ink-free) and bend its end over with sterile tweezers.



Standard Operating Procedure
Shirmer-1 test – without anesthesia

5. Insert the test over the lower lid margin (by the bent ending) of right eye, midway between the middle and outer temporal third.
6. Ask the subject to keep the eyes closed during tear volume measurement. The eye is closed and remains closed throughout the test to minimize tear stimulation.
7. After 5 minutes of Schirmer test application or when fully saturated (in which case the time is note, remove the test and note and measure the length of the strip which is wet with a calibrated ruler.
8. Place the wetted Schirmer test into an open, labeled 1.5ml Eppendorf tube (containing 250µl PBS for tear analysis purposes).
9. Place cap onto tube and label with Schirmer value in mm, date and participant number.
10. Place capped and labeled vial in temporary storage chest containing a layer of dry ice or wet ice or immediately into the appropriate refrigerator or freezer.

11. Transfer to lab MB327 for storage at -80 C as soon as possible.

Appendix 13. Protocol for tear collection (Department of Ophthalmology, Oslo University Hospital, Norway)

Protocol for proteomics of tears

1. No fluorescein prior to the collection of tears

2. Use gloves and tweezers (to avoid contamination)

3. Schirmer strips – 5 min, standard procedure without anesthesia, to collect tears

Note: Do not use Schirmer strips with ink (due to black particles in the MS). We recommend Haag Streit strips: https://eshop.haagstreituk.com/products/practice-essentials/schirmer-tear-test/schirmer-tear-test

4. Try to avoid excessive tape on the tube

Note: the tubes will be centrifuged - if it is too much tape, the tubes can stuck in the centrifuge. Mark tubes both on the lid and sides with permanent ink or tape label.

5. Put the Schirmer strip into a 1,5 ml Eppendorf Safe-Lock micro test tubes (Catalog No. 0030120094 (Eppendorf). Link: https://online-shop.eppendorf.com/OC-en/Laboratory-Consumables-44512/Tubes-44515/Eppendorf-Safe-Lock-Tubes-PF-8863.html

Note: Add 250 µl PBS, not more! Incubate at room temperature for 5 minutes.

Keep the Schirmer strip inside the tube. Note the Schirmer values in mm when placing the strip in the tube.

6. Put the samples in an ultrafreezer (-80 C) as soon as possible.

Note: If you have access to dry ice that can be put in a well-insulated box, you can transfer all the samples at the end of the day, thus increasing efficacy.

Protocol for lipidomics/metabolomics of tears

As the metabolome is a snapshot of the biochemical phenotype, standardization of the sample collection, preparation, and storage is of uttermost importance. Make sure to write down any deviation from the procedure so that this can be kept in mind if unexpected metabolic profiles are seen in specific samples.

Any diagnostic or therapeutic eye drops prior to sampling of tears should be avoided if possible, as this will influence biochemical processes and thereby the metabolome.

If eye drops must be used prior to sampling, this must be written down.

Use gloves and tweezers to avoid contamination.

1.No fluorescein prior to the collection of tears.

Note: The liquid front on the paper (in mm) must be written down. This is done in order to do quantitative analysis or adjust the amount of solvent when extracting metabolites from the paper.

2. Schirmer strips – 5 min, standard procedure without anesthesia. Do not use Schirmer strips with ink. *We recommend Haag Streit strips: https://eshop.haagstreituk.com/products/practice-essentials/schirmer-tear-test/schirmer-tear-test. Note the Schirmer values in mm when placing the strip in the tube.*

Most biochemical processes stops when biological samples are dry. The complete drying of sample is therefore important to avoid any metabolic changes caused by incomplete drying.

3. Dry the sample by placing the Schirmer strip into a 1,5 ml Eppendorf Safe-Lock micro test tubes (Catalog No. 0030120094 (Eppendorf), https://online-shop.eppendorf.com/OC-en/Laboratory-Consumables-44512/Tubes-44515/Eppendorf-Safe-Lock-Tubes-PF-8863.html) with the cap open and in room temperature for at least 4 hours, or overnight, out of direct sunlight. Mark tubes both on the lid and sides with permanent ink or tape label. Tape, when closing the tubes, but try to avoid excessive tape.

5. Store the dried samples at -80C.

Protocol for cytokine analyses of tears

1. No fluorescein prior to the collection of tears

2. Use gloves and tweezers (to avoid contamination)

3. Schirmer strips – 5 min, standard procedure without anesthesia. Do not use Schirmer strips with ink. *We recommend Haag Streit strips: https://eshop.haagstreituk.com/products/practice-essentials/schirmer-tear-test/schirmer-tear-test.*

4. Tape, when closing the tubes, but try to avoid excessive tape.

5. Storage in a 1,5 ml Eppendorf Safe-Lock micro test tubes (Catalog No. 0030120094 (Eppendorf), https://online-shop.eppendorf.com/OC-en/Laboratory-Consumables-44512/Tubes-44515/Eppendorf-Safe-Lock-Tubes-PF-8863.html) with 250 µl PBS. Keep the Schirmer strip inside the tube. Mark tubes both on the lid and sides with permanent ink or tape label. Note the Schirmer values in mm when placing the strip in the tube.

6. Put the samples in an ultra-freezer (-80 C) as soon as possible. If you have access to dry ice that can be put in a well-insulated box, you may consider transferring all the samples from the box to the ultra-freezer at the end of the day. If the freezer is located far away, this approach will increase the efficacy substantially.

Appendix 14. Aston University Optometry and Vision Research Group protocol for meibum collection

Optometry Translational Research Protocols

Meibum collection

Aston University Optometry and Vision Sciences Research Group (OVSRG)

Prepared by

Mrs Rachel Casemore

Dr Debarun Dutta

MATERIALS

- Meibomian gland expressor
- Sterile metal spatula or loop (no plastic; if reused cleaned with ethanol)
- 2 mL amber glass vials with Teflon-lined cap
- Icebox
- Nitrogen gas
- -80°C degrees freezer
- Sterile water or saline
- Sterile cotton buds
- Methanol or chloroform (HPLC grade)

MEIBUM COLLECTION

- This protocol is based on previously published reports for meibum collection (Haworth et al., 2011) and preparation (Pucker and Nichols, 2012)
- All participants should provide written consent prior to meibum collection.

Method:

- Use sterile latex/vinyl gloves for meibum collection.
- Clean the eyelid margin prior to collection by gently swabbing with a cotton bud soaked in sterile saline. This is done to reduce possible contamination of the sample with tears and to avoid collection of epithelial cells.
- Apply gentle pressure to the lower lids, either with a meibomian gland expressor (preferred) or digital pressure, to express the meibomian glands.
- Collect any expressed meibum using a sterile metal spatula or loop.
- During collection, the lid margin should be rolled away from the globe, where possible, to minimise contamination with tears and conjunctival cells.
- The long sides of the angled portion and the rounded tip of the spatula (if spatula used) should be used to collect meibum.

Meibum storage:

- Collected meibum should be transferred from the sterile staptula or loop directly to an amber glass vial containing the methanol or chlorform and capped immediately.
- Incorrect storage can lead to contamination and breakdown of the samples. Samples require anaerobic storage, avoiding exposure to moisture and light, and use of plastic free containers (Butovich et al., 2008).
- Samples may be kept on ice in the fridge for 1-2 hrs prior to transfer to the lab.
- Samples should then be transferred to lab MB327 for exposure to nitrogen gas
- Following exposure parafilm the cap well to avoid any air-leak and store in a well marked cryo-box at the -80°C freezer for later analysis.

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