Cystic Fibrosis and the eye

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Summary

Cystic Fibrosis (CF) results from the defective function of CF Transmembrane Conductance Regulator (CFTR), an ion channel which facilitates epithelial chloride secretion. Previous observations of dry eye and abnormal visual function in CF subjects have been considered secondary manifestations due to associated vitamin A deficiency (VAD) and CF-related diabetes (CFRD). However, CFTR is fundamentally present in the corneal, conjunctival and retinal pigment epithelium and the corneal endothelium. The hypothesis for this thesis was that abnormal chloride secretion in CF causes reduced basal tear secretion and abnormal photoreceptor function: these investigations aimed to identify primary and secondary ocular manifestations of CF.

Fluorescein tear break-up time was significantly reduced in adult CF subjects compared to healthy controls. Increased signs of ocular surface inflammation and higher tear ferning grades were recorded in CF subjects, although differences failed to reach significance. Tear film stability was further reduced in CF adults with VAD suggesting the aetiology of dry eye appears to be a combination of primary and secondary manifestations of the disease.

Visual function was essentially normal in CF juveniles but was adversely affected in CF adults compared to controls. Impaired distance and near visual acuity (DVA and NVA), contrast sensitivity (CS), dark adaptation (DA) and colour vision (CV) appeared to be a primary manifestation as differences were exaggerated in subjects with predicted increased levels of CFTR disruption and disease severity. These results provide support for the hypothesis and suggest normal rod and cone photoreceptor function are compromised by abnormal CFTR action. DVA, NVA, CS and DA were significantly affected by CFRD status and DA and CV were similarly reduced in VAD subjects. Therefore, abnormal visual function in CF is further modulated by secondary disease characteristics.

These findings present the distinction between primary and secondary ocular manifestations of CF, which is novel to this investigation.

Acknowledgements

I can't believe I'm actually writing this! I was sat in an internet café somewhere on the east coast of Australia when I read an email from Dr Chris Purslow suggesting a PhD. I didn't even consider it at the time, but little did I realise then that 5 years later I would be here. I would like to thank Chris for giving me that initial prompt and for being a fantastic supervisor. Together with Professor Rachel North my supervisors have been a constant source of advice, knowledge and encouragement and they have always remained patient and supportive. Ultimately, this thesis would not have been possible without their help and I am truly thankful to them both.

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Blimey, writing this has been quite a humbling experience- think I need a G&T!

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Abbreviations

AC – Anterior chamber

AMD – Age-related macular degeneration

ANOVA – One-way analysis of variance

AQP – Aquaporin

ASL – Airway surface layer

ATP – Adenosine triphosphate

AUC – Area under curve

B. cepacia – Burkholderia cepacia

BDR – Background diabetic retinopathy

BSA – Bovine serum albumin

BUT – Break-up time

cAMP – Cyclic adenosine monophosphate

CBA – Cytometric bead array

CF – Cystic Fibrosis

CFLD – Cystic fibrosis-related liver disease

CFRD – Cystic Fibrosis-related diabetes

CFTR – Cystic Fibrosis transmembrane conductance regulator

CL – Contact lens

Cl^- – Chloride ion

$Cl^-:HCO_3^-$ – chloride: bicarbonate exchanger

CIC – Voltage gated chloride channels

CLARE – Contact lens induced acute red eye

CLCA – Calcium activated chloride channel

CLCA2 – Calcium activated chloride channel 2

CLDEQ – Contact lens dry eye questionnaire

CLPU – Contact lens induced peripheral ulcer

CPD – Cycles per degree

CS – Contrast sensitivity

CV – Colour vision

CWS – Cotton wool spot

D – Dioptres

DA – Dark adaptation

DD – Disc diameter

DEQ – Dry eye questionnaire

DNA – Deoxyribonucleic acid

DR – Diabetic retinopathy

DVA – Distance visual acuity

ELISA – Enzyme linked immunosorbent sssay

ENaC – Epithelial sodium channel

EOG – Electro-oculogram

ERG – Electroretinogram

FBUT – Fluorescein break-up time

FEV_1 – Forced expiratory volume in one second

FH – Fasting hyperaemia

FO – Fast oscillation

GM-CSF – Granulocyte macrophage colony stimulating factor

GWA – Goldmann-weekers adaptometer

HA – Haemorrhage

H. Influenza – Haemophilus influenzae

HbA1c – Gylcosylated haemoglobin

HCO_3^- – Bicarbonate ion

HLA-DR – Human leukocyte antigen-DR

HPLC – High performance liquid chromatography

IC – Impression cytology

ICAM-1 – Intracellular adhesion molecule-1

IgE – Immunoglobulin E

IGT – Impaired glucose tolerance

IFN-α – Interferon- alpha

IFN-γ – Interferon- gamma

IL-1α – Interleukin-1 alpha

IL-2 – Interleukin-2

IL-4 – Interleukin-4

IL-6 – Interleukin-6

IL-8 – Interleukin-8

IL-10 – Interleukin-10

IPM – Inter-photoreceptor matrix

IQR – Inter-quartile range

IRMA – Intraretinal microvascular abnormalities

K^+ – Potassium ion

LED – Light emitting diode

MA – Micro-aneursym

MIP-1α – Macrophage inflammatory protein-1α

MSD – Membrane-spanning domain

MUC4 – Mucin 4

MUC5AC – Mucin 5AC

MUC16 – Mucin 16

Na^+ – Sodium ion

NaCl – Sodium chloride

Na^+:K^+ – sodium: potassium pump

Na/K-ATPase – Sodium/potassium-ATPase

Na^+:HCO_3^- - sodium: bicarbonate co-transporter

Na^+:K^+:$2Cl^-$ – sodium: potassium: chloride co-transporter

NBD – Nucleotide-binding domain

NCL – Non-contact lens wearer

NDM – No detectable movement

NGT – Normal glucose tolerance

NIBUT – Non-invasive break-up time

NPV – Negative predictive value

NVA – Near visual acuity

NVD – New vessels on disc

NVE – New vessels elsewhere

OCI – Ocular comfort index

OCT – Ocular coherence tomography

ORCC – Outwardly rectifying chloride channel

OSDI – Ocular surface disease index

P. aeruginosa – Pseudomonas aeruginosa

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PKA – Protein kinase A

PPV – Positive predictive value

R domain – Regulatory domain

RH – Relative humidity

RBS – Rose bengal staining

RBP – Retinol binding protein

RPE – Retinal pigment epithelium

RPM – Revolutions per minute

RT-PCR – Reverse transcription polymerase chain reaction

S. aureus – Staphyloccus aureus

SD – Standard deviation

SE – Standard error

SER – Spherical equivalent refraction

SF – spatial frequency

SiH – Silicone hydrogel

SRS – Subretinal space

TBUT – Tear break-up time

TES – Total error score

TF – Tear ferning

TGF-β1 – Transforming growth factor-β1

TNF-α – Tumour necrosis factor- alpha

UV – Ultraviolet

VA – Visual acuity

VAD – Vitamin A deficiency

VAS – Visual analogue scale

VEP – Visual evoked potential

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Chapter 1

Cystic Fibrosis

1.1 Introduction

Cystic Fibrosis (CF) is the most common life-threatening inherited disease in the caucasian population with a frequency of approximately 1 in every 2,500 live births (Ratjen and Doring, 2003). This autosomal recessive disease currently affects over 8,000 babies, children and young adults in the United Kingdom (Dodge et al., 2007), and in the order of 30,000 individuals in the United States of America (Morgan et al., 1999).

1.2 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

CF is caused by the abnormal function of a membrane protein known as Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Rommens et al., 1989). Located on the apical membrane of epithelial cells such as those of the sweat glands, the pancreas and the lungs, its primary function is to regulate the flow of chloride ions (Cl^-) across such epithelia (Schwiebert et al., 1999). CFTR is unusual in its ability to regulate the conductance of other ion channels within the membrane including positive regulation of the outwardly rectifying chloride channel (ORCC) and negative regulation of the epithelial sodium channel (ENaC) (Mall et al., 2004). Therefore, the overall effect of defective CFTR function in CF is reduced epithelial Cl^- permeability and enhanced sodium ion (Na^+) permeability of the cell membrane (Ashcroft, 2000).

1.2.1 CFTR structure

CFTR is composed of five different domains or three-dimensional folded protein structures (Sheppard and Welsh, 1999) (Figure. 1.1). Each membrane-spanning domain (MSD) consists of six transmembrane segments that form the channel pore (Conley, 2000). Attached to each MSD is a single nucleotide-binding domain (NBD), the function of which is to regulate channel gating. The two MSD-NBD units are linked by the regulatory (R) domain. Initially thought to regulate CFTR by closing the channel, the R domain contains multiple potential phosphorylation sites and is now known to exert a stimulatory effect (Ashcroft, 2000). The channel is activated or opened by the catalyst protein kinase A (PKA) which binds to the R domain with adenosine triphosphate (ATP) initiating phosphorylation (Hwang et al., 1994). Once this has occurred channel gating is then regulated by a cycle of ATP hydrolysis at the NBDs (Sheppard and Welsh, 1999). The channel is closed when enzymes, known as phosphotases, dephosphorylate the R domain (Hwang et al., 1994). Therefore, whilst channel opening is controlled by cellular ATP and PKA levels, channel closing is controlled by the level of phophatase activity within the cell (Schwiebert et al., 1999).

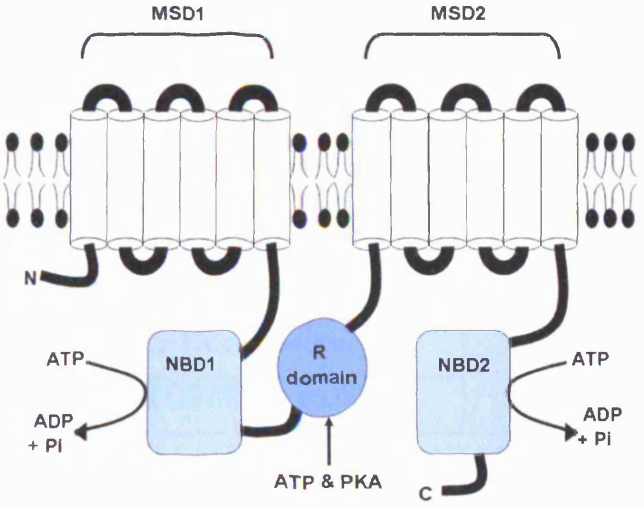


Figure 1.1 Diagram of proposed domain structure of CFTR (Adapted from Sheppard and Welsh, 1999)

1.2.2 CFTR classification

To date, over 1500 gene mutations of CFTR have been identified (Farrell et al., 2008). Mutations fall into five different classifications depending on the effect of CFTR function (Geddes and Alton, 1999). Class 1 mutations, resulting in complete absence of CFTR synthesis, are rare affecting only approximately 7% of CF patients (Table 1.1). The most common Class 2 mutations, such as that causing the ΔF508 mutation, affect approximately 85% of CF patients (Moss, 2001). The defect results in production of misfolded CFTR, which cannot be processed to reach the membrane surface. Class 3 mutations also result in production of defective CFTR, although the protein reaches the surface membrane it causes abnormal regulation of the ion channel (Ratjen and Doring, 2003). These mutations are rare affecting less than 3% of CF patients. Class 4 mutations are similar, the defective CFTR reaches the membrane surface but causes an altered conductance of the channel. Class 5 mutations result in reduced levels of CFTR synthesis (Kulczycki et al., 2003). The incidence of Type 4 and 5 mutations are rare, collectively accounting for approximately 5% of CF patients (Geddes and Alton, 1999).

Table 1.1 Classification of CFTR gene mutations (Kulczycki et al., 2003)

Class	Effect	Incidence within CF population (%)
1	CFTR is not synthesised	7
2	Defective CFTR processing	85
3	Defective CFTR regulation	3
4	Altered CFTR conductance	5
5	Reduced CFTR synthesis	

1.2.3 CF genotype and phenotype

CF shows incredible variation in both genotype and phenotype; primarily depending on how the mutation disrupts CFTR function (Farrell and Fost, 2002). If CFTR is processed incorrectly it may be completely absent from the surface membrane resulting in a total loss of channel activity and a severe form of CF. Mutations which result in a reduced chloride ion conductance, such as the class 5 mutations, are associated with milder forms of the disease. Therefore, phenotypic differences can be related to the functional classification of the CFTR genotype (McKone et al., 2003).

Genotype-phenotype studies have demonstrated the degree of correlation between CFTR genotype and CF phenotype is highest for pancreatic involvement and lowest for pulmonary function (Zielenski, 2000). Therefore, the degree of lung pathology and its rate of progression is less easily defined by genotype and the CF genotype cannot accurately predict disease severity (Gilljam et al., 2004, Kulczycki et al., 2003). There is substantial disease variability within patients with identical genotypes (McKone et al., 2003) suggesting environmental factors and modifying genes, apart from CFTR, may contribute to CF disease severity (Gilljam et al., 2004, Mekus et al., 2000, Mickle and Cutting, 2000, Zielenski, 2000).

1.2.3.1 ΔF508

The most common CFTR mutation, ΔF508, accounts for approximately 70% of CF chromosomes worldwide (Morral et al., 1994). This is caused by a three base pair deletion that removes a phenylalanine residue at position 508 resulting in a particularly severe form of the disease (Morales-Machin et al., 2004). In a recent study of UK CF patients, 74.1% were found to have at least one ΔF508 mutation (McCormick et al., 2002). Homozygous patients have a high frequency of pancreatic insufficiency at an earlier age (McKone et al., 2003). There is a larger variation in lung function however and often a wide phenotypic variation even in homozygous individuals (Lester et al., 1994). In heterozygous individuals the milder mutation is dominant over the ΔF508 mutation resulting in a milder phenotype (Kerem and Kerem, 1995). Therefore, phenotype and mortality are mainly determined by the effects of the non-ΔF508 mutation (McKone et al., 2003).

1.2.3.2 Variability with populations and ethnicity

Studies have revealed different populations or racial groups have a different prevalence of genotypes. Whilst ΔF508 homozygotes account for 57.5% of the UK caucasian CF patients, only 24.7% of the UK Indian Subcontinent CF population are ΔF508 homozygotes (McCormick et al., 2002). It has been observed that often individual or family specific mutations occur within non-caucasian patients (Malone et al., 1998). As the prevalence of different mutations varies between different populations and ethnic groups genetic screening tests must be adapted for the target population (Gilljam et al., 2004).

1.3 Major characteristics of Cystic Fibrosis

1.3.1 Elevated sodium chloride sweat concentration

In the sweat gland, sodium chloride (NaCl) enters the duct lumen by a process of filtration (Ashcroft, 2000). Sodium ions (Na^+) are reabsorbed via the apical membrane due to an electrochemical gradient for Na^+ created by the activity of the Sodium/Potassium-ATPase (Na/K-ATPase) channel in the basolateral membrane. Chloride ions (Cl^-) then follow passively via CFTR in the apical membrane leaving a dilute NaCl solution in the sweat duct lumen. In CF, epithelial Cl^- absorption via CFTR is defective. Continued Na^+ uptake via the apical cell surface causes membrane depolarisation which limits further reabsorption of Na^+ (Massie et al., 2000). The net effect is elevated levels of NaCl in the sweat of patients with CF (Figure. 1.2). This characteristic forms the basis of the sweat test used to diagnose CF.

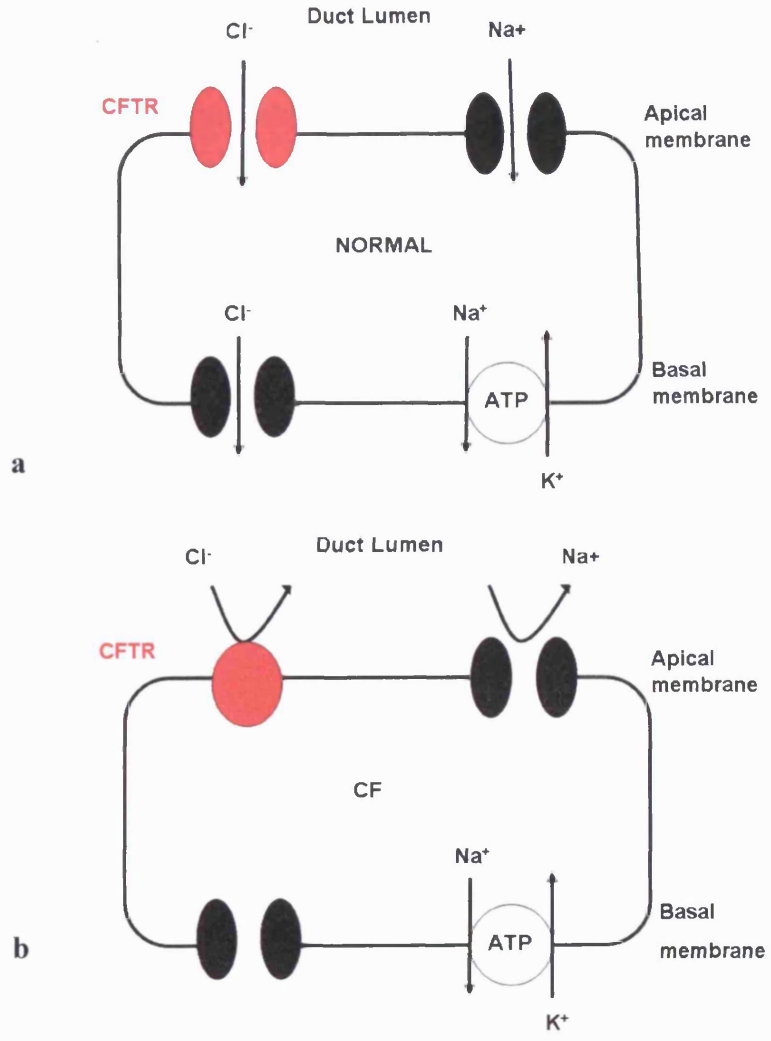


Figure 1.2 Simplified diagram of a normal (a) and CF (b) sweat duct cell

1.3.2 Lung disease

In the lungs, the thickness of the airway surface liquid (ASL) layer lining the ciliated epithelia is determined by osmosis. It is balanced by fluid uptake accompanying Cl^- efflux via CFTR and fluid secretion accompanying Na^+ absorption via ENaC (Frizzell and Pilewski, 2004). In CF, Cl^- efflux is reduced with an accompanying reduction in fluid secretion via the apical membrane (Ashcroft, 2000). However, because the normal inhibitory influence of CFTR on ENaC is also lost the absorption of Na^+ and accompanying fluid uptake is increased (Schwiebert et al., 1999) (Figure 1.3). Therefore, *both* processes cause a depleted ASL volume resulting in a thick hyperviscous mucus layer containing trapped bacteria, deoxyribonucleic acid (DNA)

and immune cells (Gibson et al., 2003) (Figure 1.4). This results in impaired mucociliary clearance, mucus plugging, infection, neutrophil-dominated airway inflammation and fibrosis, which ultimately leads to respiratory failure (Ratjen and Doring, 2003). However, it is uncertain whether CFTR dysfunction itself directly causes increased infection susceptibility or if hyper-inflammation is a primary manifestation of the disease (Jacquot et al., 2008). CF lung disease is further complicated by bacterial colonisation leading to frequent pulmonary exacerbations (Gibson et al., 2003). The most common pathogen is Pseudomonas (P.) aeruginosa although other pathogens include Staphylococcus (S.) aureus, Burkholderia (B.) cepacia infection and Haemophilus (H) Influenzae (Ratjen and Doring, 2003). At present, chronic pulmonary P. aeruginosa infection is responsible for the greatest mortality in CF (Hoiby et al., 2005).

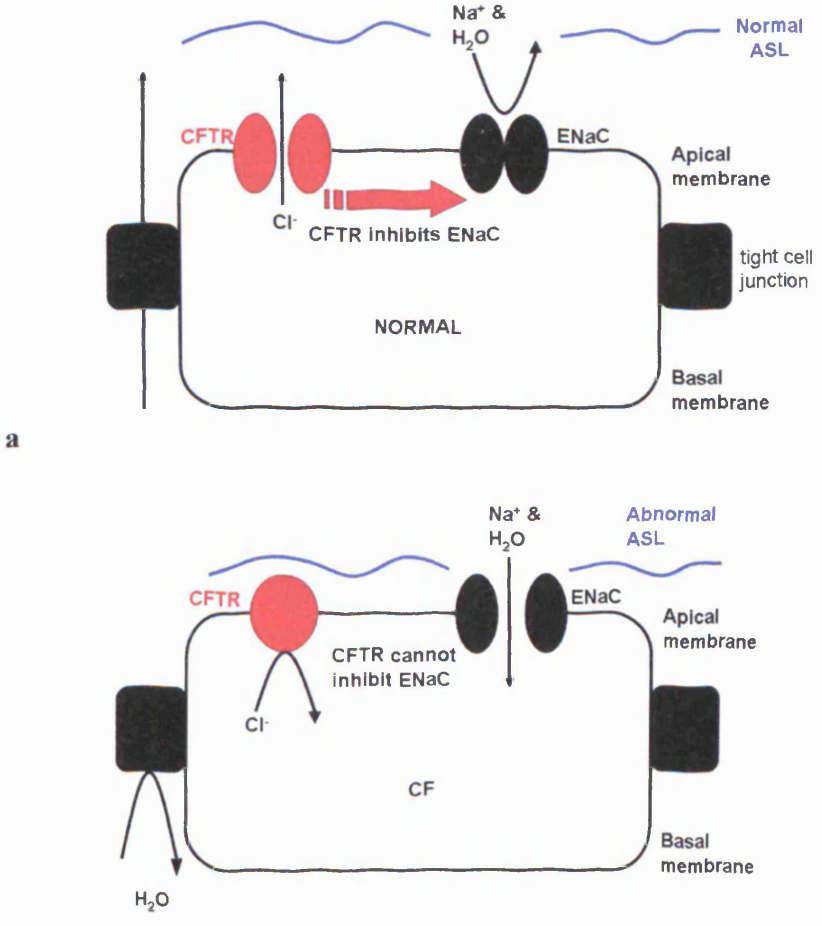


Figure 1.3 Simplified diagram of a normal (a) and CF (b) lung submucosal gland cell

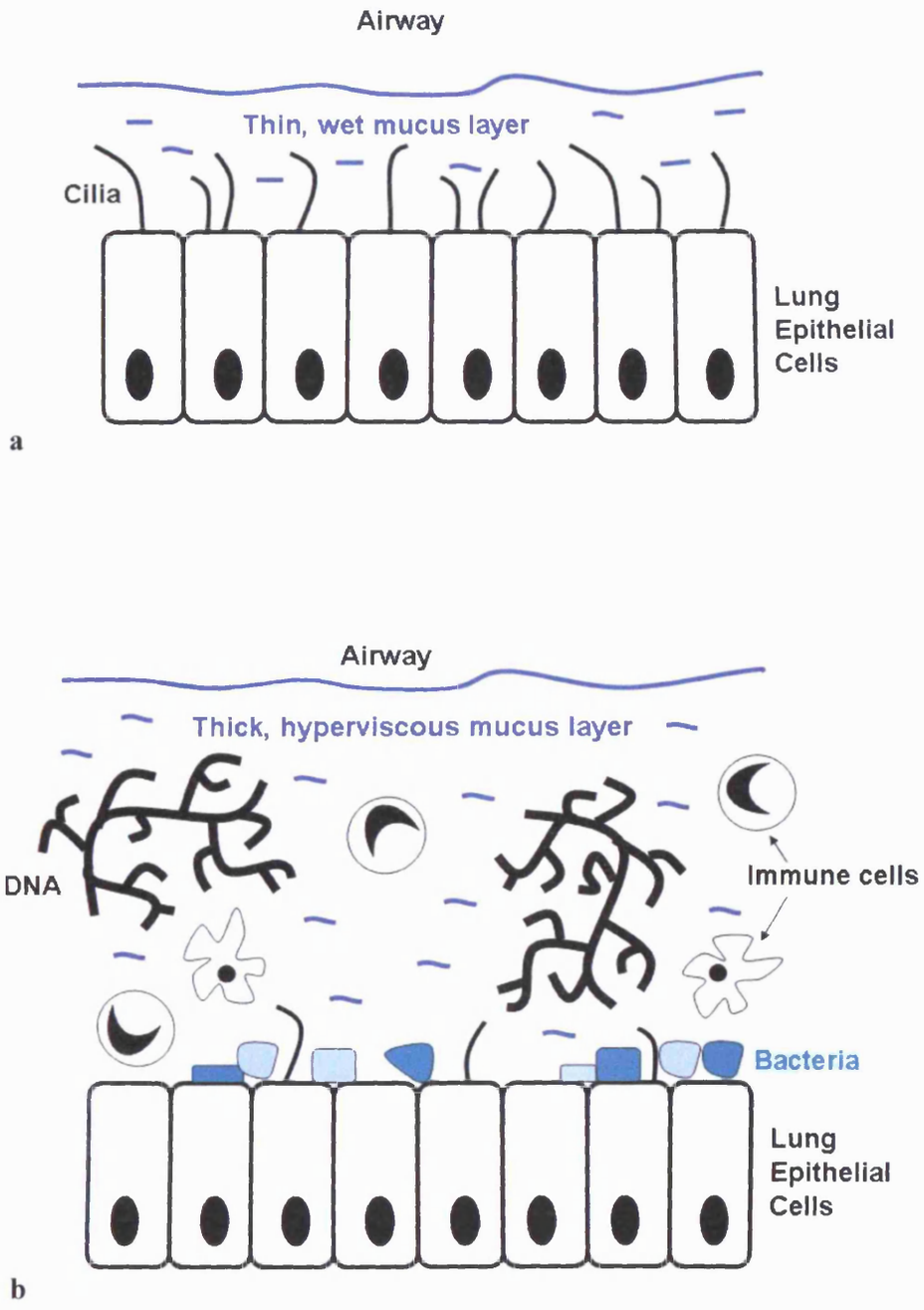


Figure 1.4 Simplified diagram of a normal (a) and CF (b) airway surface layer

Although there is evidence of disrupted Cl^- and Na^+ transport in CF lung epithelia, the mechanism by which they contribute to the pathogenesis of CF lung disease is unresolved. It has been suggested that the high NaCl concentration of the ASL deactivates antimicrobial peptides resulting in chronic infection (Zabner et al., 1998) or the low ASL volume causes ineffective mucociliary clearance (Mall et al., 1998). This is further complicated by hypotheses that CF may modify epithelial inflammatory responses (Heeckeren et al., 1997).

CFTR knockout mice do not demonstrate pulmonary disease that mimics the human disease and cannot be used to study the pathogenesis (Frizzell and Pilewski, 2004). However, a recent investigation found that increased Na^+ absorption via the epithelia of the lung alone could initiate CF lung disease in mice with airway-specific overexpression of ENaC (Mall et al., 2004). Accelerated Na^+ absorption resulted in reduced ASL volume that ultimately caused decreased mucous transport, increased airway mucus obstruction, inflammation and reduced bacterial clearance (Frizzell and Pilewski, 2004). In conclusion it was hypothesised that reduced ASL volume and impaired mucous clearance (due to mucous adhesion of the cilia and ineffective coughing) of the airways play a key role (Mall et al., 2004, Boucher, 2007). Therefore, these findings suggest that loss of the normal inhibitory regulatory effect of CFTR on ENaC may be more influential to the pathogenesis of CF lung disease than the reduced efflux of Cl^- by CFTR itself.

1.3.3 Pancreatic insufficiency

The normal pancreas secretes digestive enzymes, which must be carried by a watery fluid secretion into the small intestine to facilitate digestion. Pancreatic cells secrete a watery fluid into the duct lumen that is high in bicarbonate (HCO_3^-) concentration (Ashcroft, 2000). The apical cell membrane contains a HCO_3/Cl exchanger that transports HCO_3^- out from within the cell in exchange for Cl^-. Cl^- is returned to the lumen via CFTR channels to maintain the negative electrochemical gradient necessary to allow Na^+ to diffuse through extracellular spaces into the lumen. This subsequent increase in ion concentration of the lumen contents results in the movement of water from the blood stream into the lumen by a process of osmosis to produce a dilute, watery secretion (Taylor and Aswani, 2002). In CF however, the normal negative electrochemical gradient cannot be achieved due to the absence or presence of defective CFTR (Figure 1.5). Although the pancreatic enzymes are released normally there is an insufficient fluid vehicle to carry the enzymes to the duodenum (Schibli et al., 2002). The result of reduced pancreatic secretion is impaired digestion and absorption of lipids and lipid-soluble vitamins (specifically vitamin A, D and E), potentially resulting in malnutrition, growth failure and developmental delay (Sinaasappel et al., 2002). Also, obstruction of the pancreatic ducts may occur as pancreatic enzymes become concentrated within the ducts causing atrophy of the pancreatic acinar tissue (Brennan et al., 2004), potentially leading to pancreatic destruction.

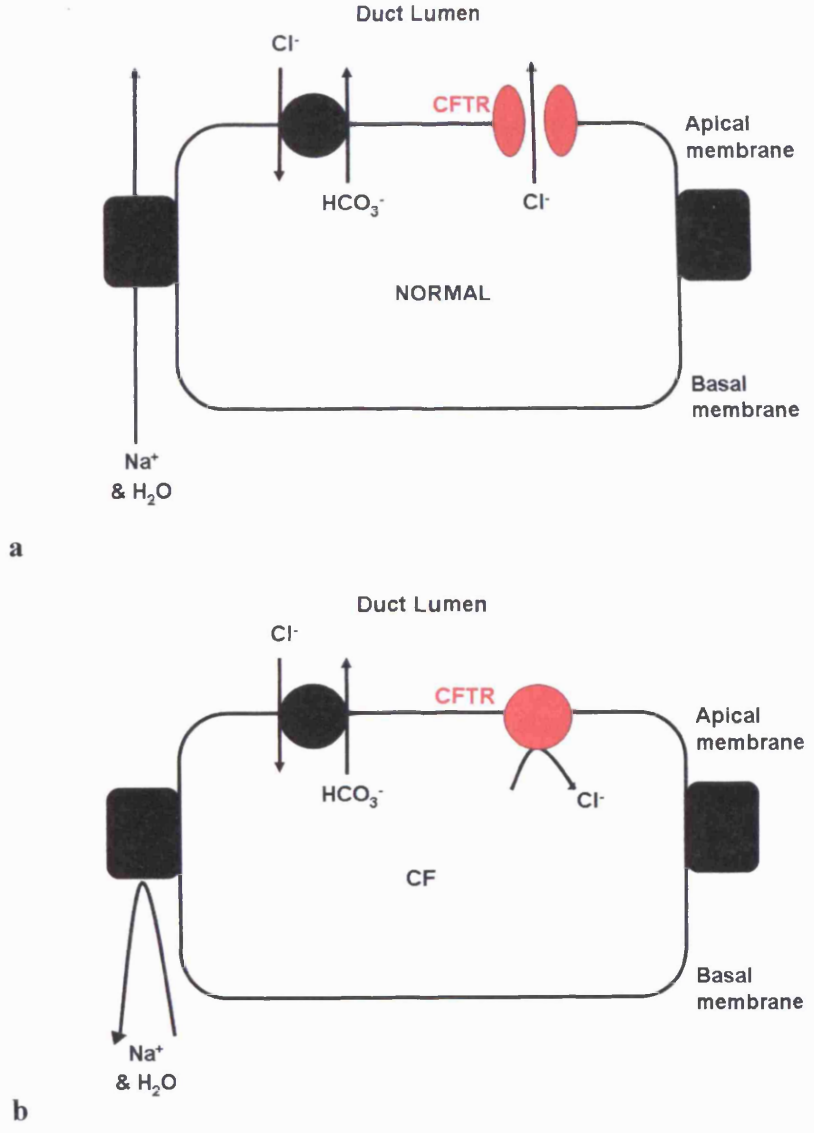


Figure 1.5 Simplified diagram of a normal (a) and CF (b) pancreatic duct cell

1.3.3.1 Vitamin A deficiency

Vitamin A deficiency (VAD) is common in subjects with CF due to impaired digestion and absorption with pancreatic insufficiency (Lancellotti et al., 1996) and despite supplementation many subjects maintain low serum vitamin A concentration (Feranchak et al., 1999). Vitamin A is essential for numerous physiological functions such as maintenance of the retinoid cycle, cellular differentiation and membrane integrity, mucin production and immunity (Sommer, 1998). There are two main dietary sources of vitamin A: pre-formed vitamin A (retinyl esters), from meat and dairy products, or carotenoids, from fruit and vegetables (da Silva Diniz and Santos, 2000). Carotenoids, such as beta-carotene, are precursors or inactive forms of vitamin A. Of the 600 known carotenoids, 50 are referred to as pro-vitamin A compounds as they can be converted to vitamin A within the body (Bellovino et al., 2003).

Ingested vitamin A is stored in the liver and transported to specific target receptors in the body by a carrier protein known as retinol binding protein (RBP). Serum levels can remain low despite adequate liver stores, suggesting defective vitamin A release from the liver in CF (Underwood and Denning, 1972). Furthermore, liver stores may be depleted in subjects with CF-related liver disease (see section 1.5.5) (Cheng et al., 2000). Therefore, circulating serum vitamin A levels are frequently a poor indicator of vitamin A status (Tsinopoulos et al., 2000).

1.3.4 Cystic Fibrosis-Related Diabetes (CFRD)

Cystic Fibrosis-Related Diabetes (CFRD) is distinctly different from Type 1 and Type 2 Diabetes Mellitus although it does share features of both forms (Moran, 2002). The aetiology and development of CFRD is not fully understood. Hyperglycaemia is proposed to be the result of decreased insulin secretion due to blockage of the pancreatic duct and subsequent atrophy and fibrosis of pancreatic islet cells (Brennan et al., 2004). Insulin resistance as a result of the CFTR mutation and prolonged steroid use is also thought to contribute. Female subjects with CFRD have a poorer prognosis compared with male subjects suggesting gender may also be a contributing factor (Milla et al., 2005). It is also proposed that different genotypes may result in

different severities of CFRD, most commonly in those individuals homozygous for the ΔF508 mutation (Koch et al., 2001).

As well as micro and macrovascular complications typically associated with diabetes, individuals with CFRD also exhibit decreased body mass index, reduced pulmonary function and rapid decline in clinical status ultimately resulting in an increased mortality rate (Fredericksen et al., 1996). Due to some residual islet cell function, ketoacidosis is extremely rare in CF at diagnosis or throughout the course of the disease. The incidence of CFRD increases with age (Lanng, 2001). By 20 years of age, 70% of CF patients have impaired glucose tolerance (IGT) and 45% CFRD (Bismuth et al., 2008). This rises to 82% and 70% respectively at 30 years of age (Bismuth et al., 2008). As life expectancy continues to increase more individuals will develop CFRD and the associated complications.

1.3.4.1 Diagnosis of CFRD

The oral glucose tolerance test is considered to be the gold standard for the detection of diabetes mellitus (Brennan et al., 2004). After overnight fasting, a glucose solution is administered orally and blood glucose concentration measured at regular intervals (Moran, 2002). After two hours blood glucose concentration ≤8.8 mmol/l indicates normal glucose tolerance (NGT), 8.9-11.1 mmol/l indicates impaired glucose tolerance (IGT) and ≥11.2 mmol/l is diagnostic of CFRD (Moran, 2002). Elevated levels of plasma glucose cause glycosylation of haemoglobin within the erythrocytes (Mansour, 2000). Gylcosylated haemoglobin (HbA1c) concentration is increased proportionally to the degree of chronic hyperglycaemia and can be used to indirectly measure the average blood glucose concentration over the previous six to ten weeks (McDonald and Davis, 1979). Normal levels range between 2.6-5.8%. HbA1c is now a widely used method for monitoring long term glucose control in subjects with CFRD as well as in Type 1 and 2 diabetes (Brennan et al., 2004).

1.3.4.2 Treatment of CFRD

Treatment is always indicated in patients with CFRD (Allen et al., 1998) although for patients with IGT the benefit of glycaemic control is unclear (Lanng, 2001). The stabilisation of blood glucose in subjects with CFRD should be controlled by insulin and or oral hypoglycaemic drugs rather than any dietary restriction due to malabsorption and malnutrition typically associated with CF (Porter and Barrett, 2004). Evidence suggests hyperglycaemic treatment with insulin before CFRD has even developed may benefit the clinical status and pulmonary function of CF patients (Rolon et al., 2001).

1.4 Diagnosis of Cystic Fibrosis

Early and accurate diagnosis has been shown to improve prognosis significantly, especially for long term nutritional status and growth indices (Farrell et al., 2001). Neonatal screening was introduced in Wales in 2004 and a positive diagnosis of CF is typically made before four weeks of age (Southern et al., 2007). Neonatal screening methods include analysing dried blood for immunoreactive trypsin (IRT) which is known to be significantly elevated in newborns with CF (Crossley et al., 1979). A two-tiered protocol combining IRT and DNA analysis for 20 to 30 of the most common CFTR mutations in patients with elevated IRT, results in rapid diagnosis and improved sensitivity (Gregg et al., 1997). "Sweat Testing" is currently the gold standard for the diagnosis of CF and is generally used to confirm diagnosis of neonates identified during newborn screening programmes, or in patients presenting with clinical features suggestive of CF (Beauchamp and Lands, 2005). Pilocarpine is applied to a small area on the arm or leg then a weak electrical current applied to the area to stimulate sweating. The sweat is collected and the sample analysed for chloride concentration (Crossley et al., 1979).

1.5 Treatment of Cystic Fibrosis

1.5.1 Patient-centred CF services

Patients attend regular hospital outpatient appointments and are assessed by members of a multi-disciplinary team that typically involves a specialised respiratory consultant, CF specialist nurse, physiotherapist, dietician and psychotherapist. Because of potential patient-to-patient transmission, protocols such as separate clinics for individuals with the same isolated infection, such as P. aeruginosa, have been introduced to CF clinics to minimise the risk of cross infection (Schewe et al., 2005).

1.5.2 Nutrition and supplements

The achievement of normal growth and adequate nutrition in CF patients provides a constant challenge (Shepherd, 2002). Typical energy requirements are believed to be up to be 33 to 50% higher than normals (Marin et al., 2004). Generally, a balanced, high-calorie diet with plenty of fat and protein is advocated. Dietary interventions such as oral supplements or enteral tube feeding at night may also be utilised.

Supplementation of the fat soluble vitamins A, D and E is essential (Borowitz et al., 2002). Empirically, the aim is to maintain plasma levels at the upper limit of the normal range. Vitamin K supplementation is also typical as deficiency is common in patients with pancreatic insufficiency (Cheng et al., 2000).

1.5.3 Physiotherapy

Physiotherapy has a pivotal role in the respiratory management of CF and there are various techniques available to facilitate mucociliary clearance and maintain lung function (Davidson, 2002). Airway clearance techniques consist of postural drainage and a combination of manual techniques, including percussion, chest shaking and chest vibrations (Samuels, 2000). The forced expiration technique, a combination of relaxed breathing control and huffing, allow the movement of secretions from smaller airways (Bradley et al., 2005).

1.5.4 Pancreatic enzyme replacement therapy

Pancreatic insufficiency affects approximately 85% of CF patients and is the primary cause of malnutrition due to maldigestion and malabsorption (Schibli et al., 2002). A wide variety of pancreatic enzyme extracts, containing lipase, protease and amylase, are commercially available (Beverley et al., 1987). Modern enteric-coated preparations ensure the enzyme is not deactivated by gastric acid and released at optimum pH in the intestine (Anthony et al., 1999). Contained within the capsule, variable sized microspheres or microtablets allow an even distribution throughout gastric emptying (Durie et al., 1998).

1.5.5 Pharmaceutical therapy

The basis of CF management is the control of respiratory infection (Schidlow, 2000). Many centres treat all patients with prophylactic antibiotics in an attempt to prevent or delay early colonisation (Robinson, 2001). Further oral, intravenous or nebulised antibiotic therapy may be administered for the treatment of pulmonary exacerbations where lung function is decreased due to infection and/or inflammation (Gibson et al., 2003).

Inflammation is a primary characteristic of CF lung disease (Ratjen, 2006). Corticosteroid anti-inflammatory therapy is limited due to adverse side effects (Robinson, 2001). However, treatment with inhaled steroidal bronchodilators is common. More recently, recombinant DNAase or Pulmozyme has been used to decrease the viscoelasticity of mucus by cleaving neutrophil derived DNA (Schidlow, 2000). Inhalation of nebulised hypertonic saline draws water into the ASL, improving mucociliary clearance and lung function (Ratjen, 2006). Up to 20% of patients develop CF-related liver disease (CFLD), caused by abnormal bile secretion and blockage of the bile ducts causing liver cirrhosis (Cheng et al., 2000). A relatively new natural bile acid, ursodeoxycholic acid, has been introduced to improve liver function in these patients.

1.5.6 Gene therapy

Gene therapy involves the addition of a correct, functioning copy of CFTR into epithelial cells and is a promising new treatment. Current transfer vectors, to transport the gene to the desired site, include adenovirus, adeno-associated viruses and liposomes (Schidlow, 2000). However, gene transfer vectors are faced by a number of extracellular barriers within the airways including mucus, a low number of receptor sites and immune responses (Ferrari et al., 2002). Unfortunately, despite significant developments the suitability of gene therapy for the treatment of CF remains limited (Griesenbach and Alton, 2009).

1.5.7 Protein repair therapy

Protein repair therapy involves treatment with compounds which induce normal production, transport or function of CFTR within the cell (Lim and Zeitlin, 2001). However, therapy is likely to be specific for each different CFTR mutation (Kerem, 2006). For example, gentamicin has been shown to suppress some class 1 mutations by allowing normal transcription of messenger ribonucleic acid (mRNA) (Schidlow, 2000). Long term effectiveness and safety are currently under investigation.

1.5.8 Organ transplantation

Organ transplantation, such as heart-lung, liver or pancreatic transplants, are the only definitive treatment for patients with CF (Wise et al., 2001). Due to a shortage of donor organs, waiting times for transplantation are routinely up to two years (Mayer-Hamblett et al., 2002). In the United States only approximately 135 lung transplants are performed in subjects with CF annually (www.cff.org/treatments; accessed on 28/01/2009). Unfortunately transplant associated morbidity increases with age; in the United Kingdom survival decreases from 82% at one year to 53% at 10 years following lung transplant (Meachery et al., 2008). Failure of transplantation is typically due to infection or rejection (Schidlow, 2000).

1.6 Life expectancy

Life expectancy for CF patients has continued to increase; in 1964 the average life expectancy was only five years of age. Today however, in the UK 11% of the CF population are over 30 years of age (McCormick et al., 2002), and worldwide a significant number of patients are surviving beyond 40 years of age (Hodson et al., 2008). Females have been shown to have a shorter survival rate suggesting gender is a prognostic factor (Lai et al., 2002). In the UK, CF population the male/female ratio significantly increases with age from approximately 1.2:1 below five years of age to approximately 1.6:1 above 35 years of age (McCormick et al., 2002). With increasingly sophisticated therapy and interventions it is expected that patients born in 2000 can expect survival beyond 50 years of age, even in the absence of functional gene therapy (Dodge et al., 2007).

1.7 Cause of death

The major cause of death in CF is progressive pulmonary disease (Kulczycki et al., 2003). *P. aeruginosa* respiratory infection particularly has been found to be a major cause of mortality (Elkin and Geddes, 2003, Emerson et al., 2002). CFTR has been characterised as a receptor for *P. aeruginosa*, which when functioning normally, internalises and destroys the pathogen (Zaidi et al., 2004). Untreated patients generally become chronically infected and even with intensive antibiotic regimens the infection cannot be eradicated due to poor antibiotic penetration and resistance due to mutated strains (Worlitzsch et al., 2002). Lung transplantation is ultimately the only option for patients with this type of advanced pulmonary disease (Kerem et al., 1992). Consequently, prevention of bacterial lung infection is paramount for all CF patients.

1.8 Summary

CF remains the most common life-threatening inherited disease in the Caucasian population. The major disease characteristics are summarised in Table 1.2. With improved patient care and therapeutics, CF survival rates are steadily increasing. Therefore, healthcare professionals, such as optometrists, are more likely to encounter these patients. An awareness of the ocular abnormalities associated with CF is imperative and these are reviewed in Chapter 2.

Table 1.2 Summary of major CF disease characteristics

Feature	Cause	Potential outcomes
Lung Disease	Abnormal airway surface (ASL) layer regulation	Impaired mucociliary clearance, mucus plugging, infection and inflammation **= Respiratory failure**
Pancreatic Insufficiency	Insufficient fluid vehicle secretion	Abnormal digestive enzyme secretion **= Malnutrition**
Sweat	Abnormal Cl^- absorption from sweat duct lumen	Elevated sweat NaCl levels **= Electrolyte imbalance**
CF-Related Diabetes (CFRD)	Reduced insulin secretion and increased insulin resistance	Abnormal blood sugar regulation **= Hyperglycaemia**
CF-Related Liver Disease (CFLD)	Abnormal bile secretion	Impaired lipid digestion, blockage of bile ducts **= Liver cirrhosis**

Chapter 2

Cystic Fibrosis and the Eye

2.1 Introduction

This chapter reviews previously published evidence of ocular associations of Cystic Fibrosis (CF) on the eye and visual function which ultimately forms the background and foundation for this thesis. The initial section reviews the literature identifying the presence of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in the ocular epithelia, and the chapter concludes by reviewing previous studies of ocular characteristics in subjects with CF.

2.2 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and the eye

The abnormal function or complete absence of the membrane protein CFTR is known to cause CF (see section 1.2). Initially discovered in 1989 (Rommens, 1989), CFTR has more recently been identified in certain layers of ocular epithelia (of various animal species and cultured cells) including the corneal epithelium and endothelium, the conjunctival epithelium and the retinal pigment epithelium. However, it is not present in the ciliary epithelium (Mitchell, 1998, Coca-Prados, 1995) and does not contribute to aqueous humour production (Do, 2004, McCannel, 1992). Active chloride transport across the ocular epithelia is known to generate the osmotic gradient necessary to drive fluid transport (Levin and Verkman, 2006). CFTR is known to be an essential Cl^- channel in many epithelia (Sheppard and Welsh, 1999); therefore, abnormal CFTR function in the ocular epithelia may adversely affect fluid transport in the eye.

2.2.1 CFTR and the corneal epithelium

The studies that have identified the presence of CFTR in the corneal epithelium are summarised in Table 2.1. Although the precise location of CFTR in corneal epithelial cells is not known, it provides a major pathway for corneal epithelial Cl^- efflux (Levin and Verkman, 2005).

Table 2.1 Detection of CFTR in the corneal epithelium

Author and Year	Species	Method and Observations
(Zaidi et al., 1999)	Wild-type mouse cells	Presence of CFTR detected by immunofluorescence microscopy. Confirmation of CFTR mRNA expression by RT-PCR analysis.
	Cultured human cells	Similar finding as above
	ΔF508 heterozygous mouse cells	Lower abundance of CFTR detected
	ΔF508 homozygous mouse cells	CFTR completely absent
(Al-Nakkash and Reinach, 2001)	Rabbit cells	Patch clamp techniques confirmed CFTR biophysical characteristics. RT-PCR confirmation of CFTR mRNA expression.
(Levin and Verkman, 2005)	Wild type mouse cells*	Open-circuit potential differences used to identify CFTR channel function, then characterised by response to CFTR various transport agonists and inhibitors.
	CF mouse cells*	Absence of such responses in CF mice.

* measurements recorded from the mouse ocular surface (cornea and conjunctiva)

2.2.1.1 Chloride channels in the corneal epithelium

The corneal stroma has a tendency to imbibe fluid. Although major compensation comes from the corneal endothelium, the epithelium also contributes to fluid transport, via various ion channels, from the stroma to the pre-corneal tear film (Candia, 2004, Yang et al., 2000). In addition to CFTR, numerous other Cl^- channels have been found to be present in the corneal epithelium (Figure 2.1). Located on the basolateral membrane the sodium:potassium:chloride (Na^+:K^+:2Cl^-) co-transporter functions in parallel with the sodium:potassium (Na^+:K^+) pump resulting in Cl^- influx (Bildin et al., 2001). The rate of secretion by these channels is regulated by the Cl^- conductance of the apical membrane (Itoh et al., 2000). In addition to CFTR, calcium activated Cl^- channels (CLCA2) are also thought to contribute to Cl^- efflux. Various ClC channels, a family of voltage gated Cl^- channels, have also been detected in the cornea (Davies et al., 2004). However, their location and contribution to Cl^- transport in unknown. The epithelial sodium channel, ENaC, has been detected and located to the apical membrane (Mirshahi et al., 1999) where it is believed to facilitate Na^+ absorption (Levin et al., 2006). $P2Y_2$ receptors are a family of membrane molecules that exert various biological functions. These receptors have been found to be present in the corneal epithelium, where they regulate cellular functions such as ion and fluid transport (Cowlen et al., 2003).

Active trans-epithelial Cl^- transport is known to provide the driving force for subsequent osmotically driven fluid secretion that follows via the apical membrane (Yang et al., 2000). Aquaporins (AQP) are a family of epithelial water channels which facilitate water secretion down an osmotic gradient (Agre, 2004). AQP5, and to a lesser extent AQP3 (Hamann et al., 1998, Thiagarajah and Verkman, 2002, Patil et al., 1997), are known to enable fluid secretion from the corneal stroma (Levin and Verkman, 2006) through the epithelium and could facilitate basal tear production (Hamann et al., 1998, Levin and Verkman, 2004, Verkman, 2003).

Cl^- conductance therefore, plays an essential role in corneal epithelial fluid transport, maintaining normal corneal integrity and contributing to basal tear production (Candia and Zamudio, 1995, Yang et al., 2000, Dartt, 2004, Dartt, 2002). CFTR provides a major pathway for corneal epithelial Cl^- secretion from the ocular surface (Levin and

Verkman, 2005). In CF patients, with hypothesised defective Cl^- efflux from the corneal epithelium (unless other Cl^- channels compensate) basal tear production could be abnormal, contributing to the condition of dry eye.

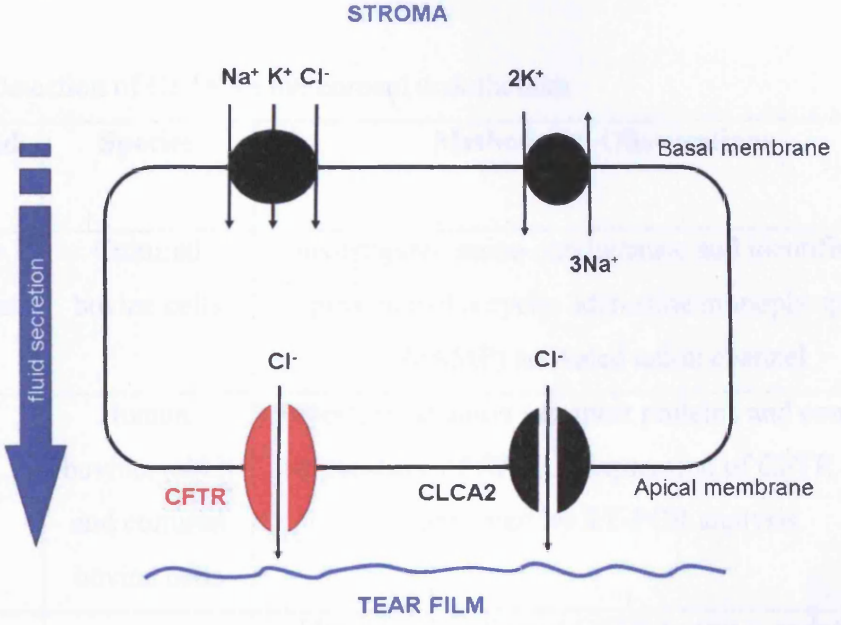


Figure 2.1 Diagram of a simplified corneal epithelial cell

2.2.1.2 CFTR, the corneal epithelium and pseudomonas aeruginosa

Previous studies have shown CFTR to act as a cellular receptor for *Pseudomonas (P.) aeruginosa* in the lung (Pier et al., 1997). CFTR has also demonstrated this function in experimental murine keratitis (Zaidi et al., 1999). Immunoelectron microscopy showed correctly functioning CFTR bound to the bacteria and ingested within the corneal epithelium and stroma. ΔF508 heterozygous mice had an 85% reduction of ingested *P. aeruginosa* and significantly less corneal pathology compared to wild-type mice. ΔF508 homozygous mice were nearly completely resistant to *P. aeruginosa* ingestion and had little subsequent pathology. Under hypoxic conditions increased binding and subsequent internalisation of the bacteria was observed and thought to be caused by increased CFTR expression (Zaidi et al., 2004). The authors concluded CFTR appears to be critical in the pathogenesis of experimental *P. aeruginosa* keratitis (Zaidi et al., 1999). However, a later study concluded CFTR is not the sole receptor responsible for *P. aeruginosa* binding (Yamamoto et al., 2006).

2.2.2 CFTR and the corneal endothelium

CFTR, located on the apical membrane, appears to facilitate Cl^- efflux from the corneal endothelium (Sun and Bonanno, 2002). The studies that have identified the presence of endothelial CFTR are reviewed in Table 2.2.

Table 2.2 Detection of CFTR in the corneal endothelium

Author and Year	Species	Method and Observations
(Bonanno and Srinivas, 1997)	Cultured bovine cells	Investigated anion conductance and identified the presence of a cyclic adenosine monophosphate (cAMP) activated anion channel
(Sun et al., 2001)	Human, bovine, rabbit and cultured bovine cells	Investigated anion transport proteins and concluded the presence of CFTR. Expression of CFTR mRNA confirmed by RT-PCR analysis.
(Sun and Bonanno, 2002)	Fresh and cultured bovine cells	Identified location of CFTR to apical endothelial membrane by indirect immunofluorescence confocal microscopy. Further confirmed by examining changes in Cl^- permeability with various CFTR transport agonists and inhibitors.

2.2.2.1 Chloride channels in the corneal endothelium

Fluid transport across the endothelium, to counteract stromal swelling is secondary to ionic flux (Hara et al., 1999). The Na^+:K^+:$2Cl^-$ co-transporter, present on the basolateral membrane, loads endothelial cells with Cl^- from the stroma (Jelamskii et al., 2000). The sodium:bicarbonate (Na^+:HCO_3^-) co-transporter, also located on the basolateral membrane, similarly loads endothelial cells with bicarbonate ions (HCO_3^-) (Sun et al., 2000). Thus intracellular levels of Cl^- and HCO_3^- are above the electrochemical equilibrium (Sun et al., 2001). CFTR is an important channel for Cl^- transport via the apical membrane and is also found to directly facilitate HCO_3^- flux (Sun and Bonanno, 2002). Calcium activated Cl^- channels (CLCA) are also present in

the apical corneal endothelium and are similarly permeable to Cl^- and HCO_3^- (Bonanno, 2003). ENaC is proposed to be located at the apical membrane although its specific contribution to sodium transport is unclear (Rauz et al., 2003). Five members of the ClC family of voltage gated Cl^- channels are also present in corneal endothelial cells, although the precise location has not been established, these channels are likely to be involved in chloride efflux (Davies et al., 2004). AQP1 channels, present in the basal and apical membranes, (Thiagarajah and Verkman, 2002, Patil et al., 1997, Hamann et al., 1998, Levin and Verkman, 2004) facilitate water movement from the endothelium to regulate cell volume and maintain corneal transparency (Levin and Verkman, 2006, Verkman et al., 2008). Similarly, $P2Y_2$ receptors in the endothelium are likely to regulate fluid secretion (Cowlen et al., 2003). The principle corneal endothelial ion channels are shown in Figure 2.2.

It is well established that both Cl^- and HCO_3^- are essential for trans-endothelial fluid transport and the subsequent maintenance of corneal transparency (Riley et al., 1995, Hodson and Miller, 1976). Therefore, unless other Cl^- channels compensate, CF patients with anticipated defective Cl^- efflux from the corneal endothelium could experience reduced levels of corneal transparency.

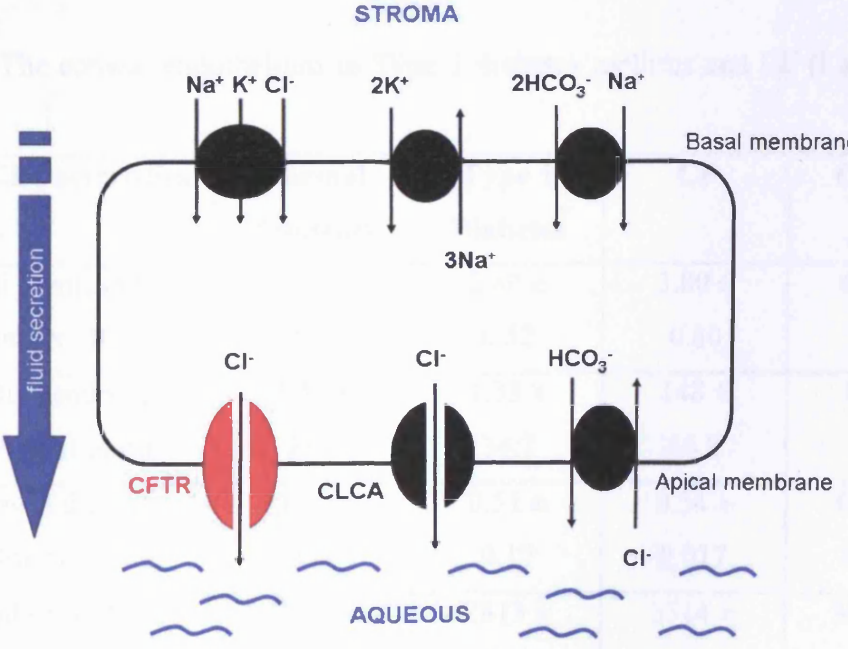


Figure 2.2 Diagram of a simplified corneal endothelial cell

2.2.2.2 Morphologic analysis of the corneal endothelium in CF

Lass and colleagues (1985) investigated corneal endothelial morphological features in subjects with CF, CF-related diabetes (CFRD) and controls with and without Type 1 diabetes. Mean corneal thickness was significantly greater in the CF and CFRD groups compared to both control groups (Table 2.3) and corneal endothelial permeability and mean relative pump rate were significantly higher in the two CF groups and the Type 1 diabetic group. The two CF groups also had significantly higher mean endothelial cell densities and lower mean endothelial cell areas compared to the controls. The increased corneal thickness observed in the CF group suggests there is only partial compensation by the increased pump rate for the increased permeability (Lass et al., 1985). The authors hypothesised that the corneal endothelium in CF is under considerable stress which may be exacerbated by CFRD.

This study was carried out before the discovery of CFTR at the apical corneal endothelial membrane; however these morphological differences could be a sign of compensation for, or adaptation to, the absence of CFTR. The consequence of increased cell density could be greater membrane surface area and greater concentration of other compensatory transporters (Sun and Bonanno, 2002).

Table 2.3 The corneal endothelium in Type 1 diabetes mellitus and CF (Lass et al., 1985)

Clinical Characteristics	Normal Controls	Type 1 Diabetes	CF	CFRD
Mean cell permeability (cm/min x 10^4)	2.14 ± 0.29	2.80 ± 0.52	3.80 ± 0.80	4.50 ± 1.30
Mean relative endothelial pump rate (% of normal)	100 ± 21.2	1.33 ± 34.7	148 ± 46.0	160 ± 45.5
Mean corneal thickness (mm)	0.52 ± 0.033	0.51 ± 0.17	0.54 ± 0.017	0.54 ± 0.033
Mean endothelial cell density (cells/mm^2)	2855 ± 253.9	2813 ± 636.0	3314 ± 651.2	3500 ± 392.3
Mean endothelial cell area (μm^2)	353 ± 28.6	370 ± 71.9	311 ± 51.0	288 ± 31.7

2.2.3 CFTR and the conjunctival epithelium

Similar to the corneal epithelium, CFTR has been located to the apical conjunctival epithelial membrane (Turner et al., 2002) where it mediates Cl^- secretion from the ocular surface (Levin and Verkman, 2005). The studies that have identified conjunctival CFTR are reported in Table 2.4.

Table 2.4 Detection of CFTR in the conjunctival epithelium

Author and Year	Species	Method and Observations
(Itoh et al., 2000)	Human cells	Identified the presence of CFTR mRNA by RT-PCR analysis.
(Turner and Candia, 2001)	Rabbit and pig cells	Identified cAMP stimulated Cl^- channel. Concluded to be CFTR following immunofluorescent and RT-PCR analysis.
(Turner et al., 2002)	Rabbit, pig and rat cells	Presence identified in bulbar and palpebral conjunctival epithelium of all species. Position located on apical membrane of all species. CFTR absent from goblet cells.
(Shiue et al., 2002)	Rabbit cells	Identified presence of CFTR by Western blot, immunostaining microscopy and RT-PCR analysis.
(Levin and Verkman, 2005)	Wild type mouse cells*	Open-circuit potential differences used to identify CFTR channel function, then characterised by response to CFTR various transport agonists and inhibitors.
	CF mouse cells*	Absence of such responses in CF mice.

* measurements recorded from the mouse ocular surface (cornea and conjunctiva)

2.2.3.1 Chloride channels in the conjunctival epithelium

The bulbar and palpebral conjunctival epithelium transports Cl^- across the basal membrane via the $N^+:K^+:CC^-$ co-transporter in conjunction with the $Na^+:K^+$ pump to maintain intracellular chloride above equilibrium (Turner and Candia, 2001, Turner et al., 2000). CFTR, located at the apical membrane in both the bulbar and palpebral conjunctiva, then facilitates Cl^- efflux into the tear film (Turner et al., 2002). CLCA2 also contributes to this Cl^- secretion via the apical membrane (Itoh et al., 2000; Levin et al., 2006). ClC channels have also been identified within the conjunctiva, although their specific contribution to Cl^- transport is unclear (Itoh et al., 2000). Subsequently, the conjunctiva secretes fluid secondary to this active chloride transport (Shiue et al., 2000). ENaC is proposed to facilitate Na^+ absorption at the apical membrane (Levin et al., 2006).

As the conjunctival epithelium has both the surface area and presence of transporters it appears plausible that it is responsible for the secretion of basal tear fluid (Candia, 2004, Dartt, 2002, Shiue et al., 2000, Li et al., 2001). Therefore, it appears that chloride transport has a critical role in the maintenance of the normal tear film (Shi and Candia, 1995). Aquaporin AQP3 is present in the conjunctival epithelium (Hamann et al., 1998, Patil et al., 1997, Levin and Verkman, 2004) and has been suggested to contribute to basal tear production (Hamann et al., 1998). However, unlike in the cornea, osmotically driven water movement across the epithelium is not aquaporin dependant (Levin and Verkman, 2004). $P2Y_2$ receptors have also been detected in the conjunctival epithelium (Cowlen et al., 2003, Jumblatt and Jumblatt, 1998): this receptor is known to mediate Cl^- efflux and net fluid secretion by the conjunctiva, and stimulation of the $P2Y_2$ receptors has been shown to increase tear fluid secretion from the rabbit epithelium (Li et al., 2001, Hosoya et al., 1999, Murakami et al., 2000, Shiue et al., 2000). The principal conjunctival epithelial channels are shown in Figure 2.3.

Conjunctival CFTR mediates Cl^- efflux from the ocular surface (Levin and Verkman, 2005) which could facilitate basal tear production. Therefore, CFTR is likely to have a direct role in tear film homeostasis and CFTR agonists could even be a potential treatment for dry eye (Levin and Verkman, 2005). However, considering subjects

with CF, with predicted defective Cl^- efflux from the conjunctival epithelium, basal tear production is likely to be abnormal unless other Cl^- channels, such as CLCA2 or ClC, allow for compensation.

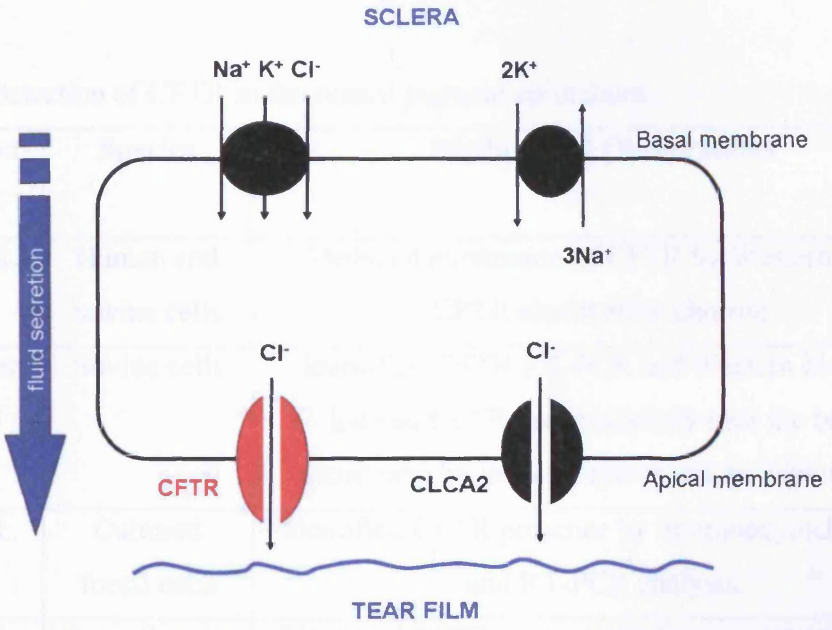


Figure 2.3 Diagram of a simplified conjunctival epithelial cell

2.2.4 CFTR and the retinal pigment epithelium

A number of studies have identified the presence and location of CFTR in the retinal pigment epithelium (RPE), these are summarised in Table 2.5. CFTR is believed to be located on the basal membrane (Peterson et al., 1997) and may in part mediate the apical RPE membrane Cl^- secretion (Weng et al., 2002, Wills et al., 2000b).

Table 2.5 Detection of CFTR in the retinal pigment epithelium

Author and Year	Species	Method and Observations
(Miller et al., 1992)	Human and bovine cells	Detected expression of CFTR by Western blot. CFTR absent from choroid.
(Peterson et al., 1997)	Bovine cells	Identified CFTR RT-PCR and Western blot and located CFTR predominantly near the basal membrane by immunocytochemistry techniques.
(Wills et al., 2000a)	Cultured foetal cells	Identified CFTR presence by immunocytochemical and RT-PCR analysis.
(Wills et al., 2001)	Donor human adult cells	Identified CFTR presence by immunocytochemical and RT-PCR analysis.
(Weng et al., 2002)	Human adult cells	Expression of CFTR confirmed by RT-PCR and Western blot. CFTR located near apical and basal membranes by immunofluorescence microscopy.
(Blaug et al., 2003)	Fresh foetal human cells	CFTR localised to both apical and basolateral membranes by immuno-histochemistry.
(Loewen et al., 2003)	Canine cells	Cl^- efflux via CFTR demonstrated by Ussing chamber electrophysiology.
(Reigada and Mitchell, 2005)	Cultured human and fresh bovine cells	Investigated involvement of CFTR in ATP release. Suggested insertion of CFTR into RPE membrane by vesicular transport is necessary for ATP release.

2.2.4.1 Chloride channels in the retinal pigment epithelium

The area between the RPE and the photoreceptors, known as the subretinal space (SRS), is occupied by the inter-photoreceptor matrix (IPM). The IPM is essential for RPE and photoreceptor interaction and facilitates the exchange of nutrients, ions and metabolites (Wimmers et al., 2007) and is thought to assist photoreceptor maintenance (Mieziewska, 1996). Continuous fluid absorption from the SRS via the RPE is also critical for retinal adhesion (Hamann, 2002, Marmor, 1990). Maintenance of the volume and chemical composition of the SRS and IPM is largely achieved by trans-epithelial transport of Cl^-, and the subsequent passive movement of water, via the RPE to the choroid (Marmor and Wolfensberger, 1998). The N^+:K^+:CC^- co-transporter, which functions in parallel with the N^+:K^+ pump, located on the apical RPE membrane, contributes to Cl^- influx (Kennedy, 1990, Hu et al., 1996, Miller and Edelman, 1990, Quinn and Miller, 1992). The N^+:K^+:CC^- co-transporter is directly activated by a decrease in intracellular RPE volume and is suggested to be critical to the regulation of cell volume (Kennedy, 1994, Kennedy, 1990). A Na^+:HCO_3^- co-transporter is present on the apical membrane (Hughes et al., 1989) and a number of ClC channels have also been detected in RPE cells (Weng et al., 2002, Wills et al., 2000b, Wills et al., 2000a, Wills et al., 2001). CLCA channels are also present on the basal membrane and thought to have substantially greater conductance compared to CFTR (Loewen et al., 2003). ENaC has a widespread distribution in RPE cells (Mirshahi et al., 1999) although its function is currently not understood (Golestaneh et al., 2000). Aquaporins have been identified in the retina, however their exact expression in the RPE is also unknown (Verkman et al., 2008). $P2Y_2$ receptors have also been identified in the RPE (Cowlen et al., 2003, Sullivan et al., 1997) and stimulation has been shown to increase RPE fluid transport (Maminishkis et al., 2002). The principle RPE epithelial ion channels are shown in Figure 2.4.

The basal RPE membrane utilises a "recycling" mechanism via the chloride: bicarbonate (Cl^-:HCO_3^-) exchanger (Gallemore et al., 1997). Thus, Cl^- is recycled at the basolateral membrane and net movement is determined by the balance of efflux, thought to be via CFTR and CLCA, and influx via the Cl^-:HCO_3^- exchanger. When considering the presence of Cl^- channels it is apparent that Cl^- transport is imperative for the maintenance of normal RPE integrity (Gallemore et al., 1997). Therefore,

unless there is compensation from other Cl^- channels in CF subjects, the anticipated defective Cl^- efflux and reduced fluid transport via the RPE could result in impaired regulation of the SRS. Subsequently, this could cause serous retinopathy, retinal detachment or abnormal RPE-photoreceptor interaction potentially leading to impaired visual function.

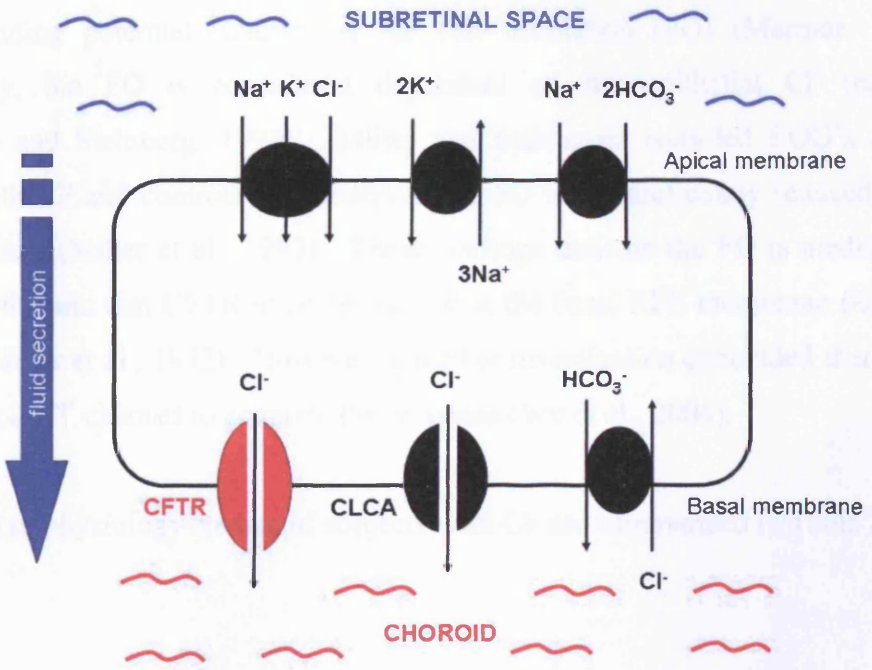


Figure 2.4 Diagram of a simplified retinal pigment epithelial (RPE) cell

2.2.4.2 CFTR and the electro-oculogram

The electro-oculogram (EOG) measures the light response of the RPE (Marmor and Zrenner, 1993). The initial response to light stimulation is shown by a series of oscillations in the standing potential. Light stimulation causes a reduction of potassium (K^+) ion concentration in the SRS with the consequence of hyperpolarising the apical RPE membrane (Wu et al., 2004). This results in a modulation of Cl^- transport, subsequently hyperpolarising the basal RPE membrane causing a reduction in the standing potential, known as the fast oscillation (FO) (Marmor, 1991). Accordingly, the FO is considered dependent on transepithelial Cl^- transport (Gallemore and Steinberg, 1993). Miller and colleagues recorded EOG's among subjects with CF and controls and observed the FO was significantly reduced in the CF individuals (Miller et al., 1992). These findings indicate the FO is mediated by CFTR activity and that CFTR must be located at the basal RPE membrane (Blaug et al., 2003, Miller et al., 1992). However, a further investigation concluded that CFTR is not the sole Cl^- channel to generate the response (Wu et al., 2004).

Further electrophysiology studies of subjects with CF are summarised in Table 2.18.

2.3 Ocular associations of Cystic Fibrosis

2.3.1 Anterior eye

2.3.1.1 CF and blepharitis

A number of studies have investigated the incidence of blepharitis in subjects with CF. The findings indicate some conflict and are summarised in Table 2.6. Sheppard et al. (1989) also assessed eyelid cultures from the two groups. Despite a significantly higher incidence of blepharitis in the CF group no pathogenic organisms were detected. As 60% of the CF group with blepharitis were taking antibiotics, the authors concluded staphylococcal infection did not appear to be a contributing factor to the incidence of blepharitis (Sheppard et al., 1989). Mrugacz et al. (2005b) suggested increased blepharitis could indicate lipid dysfunction in CF and that meibomian dysfunction was consistent with the glandular dysfunction observed in CF (Mrugacz et al., 2005b).

Table 2.6 Summary of the prevalence of blepharitis in CF studies

Author	Number	Incidence	Significant difference
(Sheppard et al., 1989)	17 CF, 17 controls	88% CF, 33% controls	Yes p=0.001
(Kalayci et al., 1996)	23 CF, 20 controls	13% CF, 5% controls	No
(Mrugacz et al., 2005b)	15 CF, 15 controls	60% CF, 7% controls	Yes p=0.03
(Mrugacz et al., 2007c)	25 CF, 25 controls	20% CF, 16% controls	No
(Mrugacz et al., 2007b)	24 CF, 24 controls	21% CF, 17% controls	No
(Mrugacz et al., 2007d)	25 CF, 25 controls	44% CF, 16% controls	No statistical comparison

2.3.1.2 CF and xerophthalmia

Malabsorption of fat-soluble vitamins, especially vitamin A, is a common characteristic of CF (Tsinopoulos et al., 2000). Xerophthalmia refers to the group of ocular signs and symptoms related to Vitamin A deficiency (VAD) (Sommer, 1998). The main clinical manifestations in the ocular system affect the retina, conjunctiva and cornea (da Silva Diniz and Santos, 2000). These include; impaired dark adaptation, conjunctival and corneal xerosis and xerophthalmic fundus (Wamsley et al., 2005).

Dark adaptation is discussed further in Section 2.3.4.4

2.3.1.2.1 Xerosis

Conjunctival xerosis (Figure 2.5a) is characterised by keratinisation and drying of the conjunctiva due to loss of goblet cells and basal cell proliferation (Sommer, 1998). Corneal xerosis (Figure 2.5b) occurs following prolonged VAD and is characterised by punctate keratopathy, corneal haze and keratinisation which potentially leads to corneal ulceration (Hatchell and Sommer, 1984). The incidence of conjunctival xerosis in CF is summarised in Table 2.7. Despite vitamin A supplementation in all subjects of these two studies (Neugebauer et al., 1989, Rayner et al., 1989) the prevalence of xerosis suggests that prescribed values were inadequate for some subjects, or non-compliance may have been an issue.

A number of case reports have indicated xerosis to be a presenting sign in the late diagnosis of CF (Brooks et al., 1990, Lindenmuth et al., 1989, Wamsley et al., 2005, Joshi et al., 2008). A further case study also reported xerosis following poor dietary and supplementary compliance in a subject with CF (Campbell et al., 1998). These case studies highlight the importance of considering VAD in patients with CF presenting with ocular complaints.

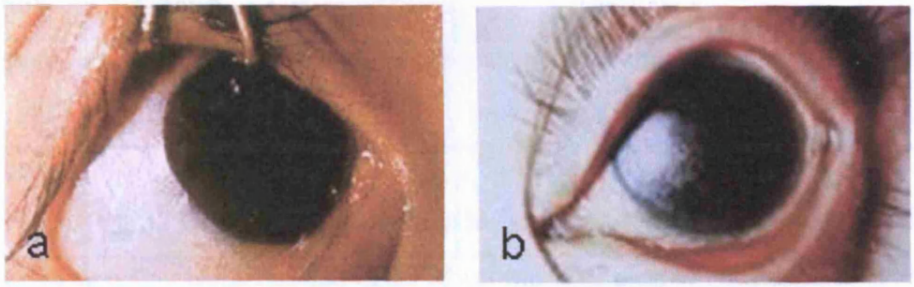


Figure 2.5 Conjunctival xerosis (a) and corneal xerosis (b) observed in vitamin A deficiency (from www.province.moph.go.th and www.unu.edu respectively, accessed on 20/04/2009)

Table 2.7 Summary of the prevalence of conjunctival xerosis in CF studies

Author	Group	Incidence
(Neugebauer et al., 1989)	31 CF patients - all receiving vitamin A supplementation	10% had conjunctival xerosis, despite no ocular symptoms
(Rayner et al., 1989)	43 CF patients - all receiving vitamin A supplementation	19% had conjunctival xerosis, associated with reduced vitamin A and retinol binding protein concentration

2.3.1.2.2 Impression cytology

Impression cytology (IC) is a method used to collect and assess conjunctival epithelial cells and goblet cells. IC has many applications including aiding the diagnosis, differentiation and assessment of various ocular disorders (including conjunctival xerosis) and to allow grading of the extent of conjunctival squamous metaplasia (Singh et al., 2005). Squamous metaplasia refers to the pathologic transition from a non-keratinised squamous epithelium to a non-secretory keratinised epithelium. The process involves a decrease and eventual loss of goblet cells with subsequent increased stratification and keratinisation (Calonge et al., 2004). The use of IC in CF studies is reviewed in Table 2.8.

Table 2.8 Summary of impression cytology in CF studies

Author	Group	Incidence
(Sheppard et al., 1989)	17 CF, 17 controls	No evidence of xerosis; many specimens inadequate for goblet cell density analysis
(Morkeberg et al., 1995)	32 CF	Normal goblet cell density 42% samples indicated cellular characteristics of dry eye
(Castagna et al., 2001)	40 CF, 24 controls	80% CF; 0% controls abnormal conjunctival cytology. 67.5% CF mild-moderate metaplasia; 12.5% severe. Conjunctival metaplasia positively correlated with Schirmer's test and tear break-up time (BUT)
(Mrugacz et al., 2007a)	20 CF, 20 controls	Significantly greater metaplasia; lower goblet cell densities in CF compared to controls 20% CF samples had inflammatory cells- typically neutrophils

Mrugacz and colleagues investigated the expression of human leukocyte antigen-DR (HLA-DR) and intracellular adhesion molecule-1 (ICAM-1) in conjunctival epithelial cells (collected by IC) from CF patients and controls that were all vitamin A sufficient or receiving vitamin A supplementation (Mrugacz et al., 2007b, Mrugacz et al., 2007c, Mrugacz et al., 2006b). HLA-DR is over-expressed by epithelial cells during immune-driven inflammatory responses (Tsubota et al., 1999) and ICAM-1 allows communication between cells during the immune response (Imhof and Dunon, 1997). HLA-DR and ICAM-1 concentration were significantly increased in conjunctival epithelial cells in CF patients compared to controls. HLA-DR expression was positively correlated with Schirmer results and BUT, suggesting the conjunctival epithelium may be subject to an inflammatory response in subjects with CF (Mrugacz et al., 2007c). Inflammation plays a critical role in the pathogenesis of dry eye and is both the *"cause and consequence"* of ocular surface cellular damage (Baudouin, 2001, Johnson and Murphy, 2004). Therefore, the evidence of sub-clinical ocular surface inflammation in vitamin A sufficient subjects with CF suggests that dry eye is likely to be a primary manifestation of the disease.

2.3.2 Tear film

The precorneal tear film offers many functions including; the provision of a smooth optical surface, lubrication, and the supply of nutrients and oxygen to the avascular cornea. Traditionally, the normal tear film is thought to be composed of three distinct layers (Wolff, 1946). The thin superficial lipid layer, which is secreted primarily by the meibomian glands, retards evaporation (Korb et al., 2002). The thick, intermediate aqueous layer contains numerous electrolytes, proteins, enzymes and metabolites. The underlying mucous layer, primarily secreted by the conjunctival goblet cells, provides a wettable surface to facilitate tear film adherence. Reflex or stimulated tears are secreted from the lacrimal gland. Basal tears were originally thought to be solely derived from the accessory glands of Krause and Wolfring. However, recent studies have demonstrated that corneal and conjunctival epithelial fluid transport may be of more significance to basal tear production (Candia, 2004, Dartt, 2002, Candia and Zamudio, 1995, Dartt, 2004, Yang et al., 2000, Li et al., 2001, Shiue et al., 2000).

Dry eye is a common eye disease that can be classified into two main categories namely an aqueous tear deficient state or an evaporative state (Lemp, 1995). Reduced tear film stability and/or increased tear film evaporation in dry eye contributes to tear film hyperosmolarity, ocular surface epithelial damage and inflammation (Baudouin, 2001, Bron, 2001, Gilbard et al., 1988).

A number of clinical methods have been used to assess the integrity of the ocular surface and tear film quality in subjects with CF. These are discussed below:

2.3.2.1 CF and dry eye

2.3.2.1.1 Ocular surface staining

Ocular surface staining is a convenient and readily available method to assess ocular surface damage as a consequence of dry eye disorders (Korb et al., 2002). Tables 2.9-2.11 summarise the incidence of corneal and conjunctival staining in the literature. The results indicate a wide variance between individual studies and the staining criterion in each study may not be comparable.

Table 2.9 Summary of the prevalence of corneal Sodium Fluorescein staining in CF studies

Author	Number	Incidence	Significant difference
(Botelho et al., 1973)	15 CF, 11 unaffected siblings	mild/moderate staining 60% CF and 10% controls initially; 73% and 30% on follow up	No statistical comparison
(Sheppard et al., 1989)	17 CF, 17 controls	≥ 2 punctates 82% CF and 12% controls	Yes p=0.002
(Kalayci et al., 1996)	23 CF, 20 controls	mild punctates 9% CF, 0% controls	No
(Castagna et al., 2001)	40 CF, 24 controls	epithelial defects 10% CF, ? controls	No statistical comparison
(Mrugacz et al., 2005b)	15 CF, 15 controls	60% CF, 13% controls	No

Table 2.10 Summary of the prevalence of Rose Bengal staining in CF studies

Author	Number	Incidence	Significant difference
(Sheppard et al., 1989)	17 CF, 17 controls	mild staining 30% CF, 12% controls	No
(Morkeberg et al., 1995)	35 CF, no controls	23% CF	No statistical comparison
(Ansari et al., 1999)	28 CF, 25 controls	7% CF, 0% controls	No statistical comparison

Table 2.11 Summary of the prevalence of conjunctival Lissamine Green staining in CF studies

Author	Number	Incidence	Significant difference
(Mrugacz et al., 2007d)	25 CF, 25 controls	Abnormal staining 28% CF, 8% controls*	No statistical comparison
(Mrugacz et al., 2007b)	24 CF, 24 controls	0.49 CF, 0.52 controls$^{\Delta}$	No

* staining classified according to van Bijsterveld scoring system

$^{\Delta}$ lissamine green score- no description of detailed methodology

Unfortunately, a number of studies did not report ocular surface staining in controls or did not apply any statistical comparisons. Therefore, only the investigation of Sheppard et al. (1989) report significantly greater corneal sodium fluorescein staining in subjects with CF compared to controls. Conjunctival IC was normal and no significant difference was found for rose bengal staining indicating vitamin A sufficiency. Therefore, the authors suggested tear film aqueous deficiency could be responsible for the increased corneal staining (Sheppard et al., 1989). Reduced basal tear film secretion and/or increased tear evaporation in dry eye contributes to tear film hyperosmolarity (Bron, 2001) resulting in ocular surface epithelial damage (Gilbard et al., 1988). The indication of increased ocular surface staining in subjects with CF suggests reduced basal tear secretion could be a primary manifestation of CF due to reduced Cl^- conductance of CFTR in the corneal and conjunctival epithelia. However, further detailed investigation in subjects with known vitamin A status is necessary.

2.3.2.1.2 Tear break-up time

The tear break-up time (BUT) is recorded as the time taken for an interruption in the fluorescein fluorescence to occur following a blink and indicates tear film stability (Figure 2.6). Generally, a BUT below 10 seconds is considered abnormal and indicative of dry eye (Lemp et al., 1970). Table 2.12 summarises differences in BUT from the literature.

Table 2.12 Summary of CF studies featuring Sodium Fluorescein tear break-up time (BUT)

Author	Number	Details	Significant difference
(Sheppard et al., 1989)	17 CF, 17 controls	Mean 27 seconds (s) CF, 17s controls	No
(Morkeberg et al., 1995)	35 CF, no controls	49% CF abnormal*	No statistical comparison
(Ansari et al., 1999)	28 CF, 25 controls	7% CF, 0% controls abnormal*	No statistical comparison
(Castagna et al., 2001)	40 CF, 24 controls	BUT decreased as level of conjunctival metaplasia increased	
(Mrugacz et al., 2005b)	15 CF, 15 controls	53% CF, 13% controls abnormal*	No
(Mrugacz et al., 2007c)	25 CF, 25 controls	Mean 5.3s CF, 9.9s controls	Yes $p<0.0001$
(Mrugacz et al., 2007d)	25 CF, 25 controls	Mean 5.4s CF, 11.2s controls 60% CF, 4% controls abnormal*	Yes
(Mrugacz et al., 2007b)	24 CF, 24 controls	Mean 5.4s CF, 9.7s controls	Yes $p<0.0001$

* Abnormal BUT < 10 seconds

Whilst the results of Castagna et al. (2001) indicate tear film stability is adversely affected by an indirect measure of VAD, significantly reduced BUT's were also observed in CF subjects with reported vitamin A sufficiency (Morkeberg et al., 1995, Mrugacz et al., 2007b, Mrugacz et al., 2007c, Mrugacz et al., 2007d). Tear film instability is associated with tear deficiency (Lemp and Hamill, 1973) and BUT correlates with tear film thickness (Creech et al., 1998). Morkeberg et al. (1995) suggested increased tear film instability could be a primary manifestation of CF and that the disease could affect any part of the tear production system. Tear film instability is associated with ocular surface inflammation in subjects with dry eye (Khurana et al., 1987) and likewise in CF, increased measures of conjunctival inflammation have been observed in subjects with reduced tear film stability (Mrugacz et al., 2007b, Mrugacz et al., 2007c, Mrugacz et al., 2007d). Therefore, increased levels of tear film instability in subjects with CF gives further support for the hypothesis that dry eye is a primary manifestation of CF.

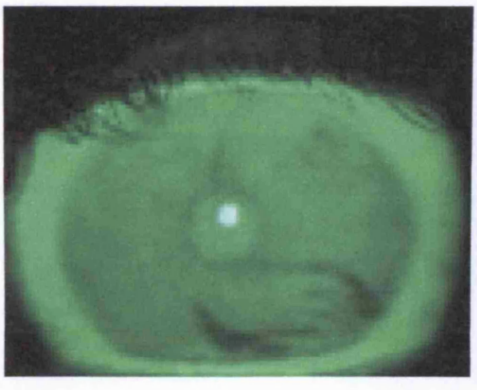


Figure 2.6 Fluorescein tear break-up time (FBUT) (from www.eyeworld.org, accessed on 20/04/2009)

2.3.2.1.3 Schirmer's test

Schirmer's test is used to assess tear secretion or flow. A wetting length less than 15mm within a period of 5 minutes is considered suspect, and a wetting length less than 5mm is clearly indicative of dry eye (Zierhut et al., 2005). A value of 3mm or below confirms the diagnosis of aqueous deficient dry eye (Korb et al., 2002). Table 2.13 summarises differences in Schirmer Test scores between subjects with CF and controls from the literature. There is considerable variance between methodologies for the studies making direct comparison difficult.

A number of studies indicate significantly reduced tear film secretion in subjects with CF compared to controls. The results of Castagna et al. (2001) indicate a correlation of Schirmer wetting and vitamin A status. However, reduced tear secretion was similarly recorded in CF subjects with vitamin A sufficiency (Morkeberg et al., 1995, Mrugacz et al., 2007c, Mrugacz et al., 2007b, Mrugacz et al., 2007d). These findings again provide evidence to suggest impaired tear secretion may be a primary manifestation of the disease.

Table 2.13 Summary of CF studies featuring Schirmer's Test

Author	Group	Incidence	Significant difference
(Fulton et al., 1982)	56 CF, no controls	All results in normal range	No statistical comparison
(Rolando et al., 1988)	13 CF, 13 controls	31.07mm CF, 29.07mm controls	No
(Sheppard et al., 1989)	17 CF, 17 controls	9.5mm CF, 16.0mm controls*	Yes p=0.002
(Morkeberg et al., 1995)	35 CF, no controls	31% abnormal wetting	No statistical comparison
(Kalayci et al., 1996)	13 CF, 19 controls	19.1mm CF, 23.1mm controls	No
(Ansari et al., 1999)	28 CF, 25 controls	0% CF and controls abnormal$^{\Delta}$	No statistical comparison
(Castagna et al., 2001)	40 CF, 24 controls	Wetting decreased as level of conjunctival metaplasia increased. 48% CF abnormal	
(Mrugacz et al., 2005b)	15 CF, 15 controls	33% CF, no controls abnormal#	No
(Mrugacz et al., 2005a)	18 CF, 18 controls	9.6mm CF 25.0mm controls	Yes
(Mrugacz et al., 2007c)	25 CF, 25 controls	9.68mm CF, 25.21mm controls#	Yes p<0.0001
(Mrugacz et al., 2007d)	25 CF, 25 controls	4.6mm CF, 20.12mm controls#	No statistical comparison
(Mrugacz et al., 2007b)	24 CF, 24 controls	9.65mm CF, 25.15mm controls#	Yes p<0.001

* wetting in 5 minutes with topical anaesthetic
$^{\Delta}$ abnormal defined as wetting less that 10mm in 5 minutes
abnormal defined as wetting less than 5mm in 5 minutes without anaesthesia

2.3.2.1.4 The prevalence of dry eye

The Copenhagen criterion is a standard commonly used by ophthalmologists to detect the presence of dry eye (Jacobsson et al., 1989). Dry eye is present if at least two of the following tests are positive; BUT, Rose Bengal staining and Schirmer's Test. Table 2.13 summarises differences in the prevalence of dry eye from the literature.

Table 2.14 Summary of the presence of dry eye according to the Copenhagen criterion in CF studies

Author	Group	Incidence	Significant difference
(Sheppard et al., 1989)	17 CF, 17 controls	18% CF, 12% controls	No statistical comparison
(Morkeberg et al., 1995)	35 CF, no controls	26% CF	No statistical comparison
(Ansari et al., 1999)	28 CF, 25 controls	7% CF, 0% controls	No statistical comparison
(Mrugacz et al., 2005b)	15 CF, 15 controls	33% CF, ? controls	No statistical comparison
(Mrugacz et al., 2007d)	25 CF, 25 controls	48% CF, ? controls	No statistical comparison

The results from the previous studies using tear film tests indicate a considerable incidence of clinical signs of dry eye in subjects with CF. Morkeberg et al. (1995) suggested CF could affect individual or multiple parts of the tear production system with the consequence of increased tear film osmolarity or abnormal mucin production, similar to that of the lung in CF (Morkeberg et al., 1995). Individual aqueous, mucin or lipid tear component abnormalities, or in combination, could account for the dry eye and corneal epithelial disturbance (Sheppard et al., 1989).

Mrugacz and colleagues (2005) observed lower plasma retinol binding protein, a specific plasma transport protein for vitamin A, in their CF patients (Mrugacz et al., 2005b). It was suggested this factor could be associated with the moderate levels of dry eye (33%) observed in the study. A similar conclusion was reached by Castagna

et al. (2001) thereby suggesting dry eye in CF is secondary to VAD. However, dry eye has also been observed in CF patients with normal vitamin A status suggesting dry eye could be a primary manifestation of the disease (Ansari et al., 1999, Morkeberg et al., 1995).

Considering recent discoveries of the presence of CFTR in the corneal and conjunctival epithelium (Turner et al., 2002, Al-Nakkash and Reinach, 2001) and expected subsequent reduced basal tear secretion in subjects with CF, it is plausible that dry eye is a primary manifestation of CF. However, to confirm this hypothesis, further detailed research into the tear film composition and ocular surface in a large cohort of subjects with CF and known vitamin A status is necessary.

2.3.2.2 CF and tear film analysis

2.3.2.2.1 Tear lysozyme concentration

The concentration of lysozyme, a tear film enzyme with antimicrobial activity, is known to decrease with both age and dry eye (Seal et al., 1986). Tear lysozyme concentration has been found to be significantly lower in subjects with CF compared to controls (Sheppard et al., 1989). Significantly reduced Schirmer's test was also recorded in this study indicating considerable tear aqueous deficiency in the CF group. Incidentally, no significant difference in *total* protein concentration and tear pH was observed between the two groups (Sheppard et al., 1989).

2.3.2.2.2 Tear electrolyte levels

Tear film electrolytes are responsible for the osmolarity of the tears (Botelho, 1964), and are essential for the maintenance of normal epithelial integrity (Korb et al., 2002). Botelho et al. (1973) investigated electrolyte concentrations in tear samples collected from subjects with CF and their unaffected siblings (Botelho et al., 1973). Whilst Cl^-, Na^+ and K^+ concentration was the same in both groups, Na^+ concentration was significantly reduced and calcium (Ca^{2+}) concentration was higher in the CF patients compared to their siblings (Botelho et al., 1973).

The authors suggested excessive calcium secretion could result in abnormal mucous production, possibly causing the impaired corneal epithelium wetting indicated by the high level of corneal fluorescein staining (60 and 73%) observed in the CF group (Botelho et al., 1973). This study was conducted several years before the discovery of CFTR. However, it is interesting that chloride ion concentration was the same in both groups considering the primary function of CFTR is to regulate the flow of this ion across epithelia (Schwiebert et al., 1999).

2.3.2.2.3 Tear ferning

Tear ferning (TF) is a novel diagnostic test for the presence of dry eye. Dried tear samples are viewed with a light microscope and classified according to their characteristic fern-like appearance. Whilst Type 1 and 2 are considered normal, Type 3 and 4 are abnormal and indicative of dry eye. TF is discussed in further detail in Chapter 3.

Rolando, Baldi and Calabria (1988) investigated TF in 13 subjects with CF and 13 controls and concluded that whilst tear samples from a CF cohort typically corresponded to Type 3 or 4, those samples from controls were typically of Type 1 appearance (Rolando et al., 1988) and the difference was statistically significant. As a normal secretion rate (Schirmer's Test) had been observed in the CF group it was suggested an increased electrolyte concentration could alter the normal tear mucus properties resulting in abnormal TF.

Kalayci and colleagues (1996) investigated TF in 23 subjects with CF and 20 controls and similarly observed a significantly higher frequency of Type 3 and 4 ferning in CF subjects compared to healthy controls. A negative correlation was found between TF patterns and Shwachman score (a measure of CF clinical disease status). No significant correlation was detected between ferning and vitamin A status. Therefore, it was concluded abnormal TF was the result of primary alteration of the tear fluid by the disease rather than an association with vitamin A levels (Kalayci et al., 1996).

2.3.2.2.4 Tear film inflammatory cytokines

Cytokines are a group of signalling molecules that communicate between cells, including those of the immune system (Hamblin, 1993). Individual cytokines have several different, and apparently unrelated, functions and can be responsible for both harmful and beneficial effects during the immune response (Playfair and Chain, 2001). Inflammation is an important procedure in this response and cytokines are known to mediate the inflammatory process. Cytokines and their role in ocular surface inflammation are discussed in further detail in Chapter 4.

A number of cytokines, Interferon-γ (IFN-γ), Interleukin-8 (IL-8) and macrophage inflammatory protein-1α (MIP-1α), have been observed in higher concentrations in tear samples from subjects with CF compared to controls (n=15 to 25) (Mrugacz et al., 2005c, Mrugacz et al., 2006c, Mrugacz et al., 2007d, Mrugacz et al., 2006a). Disease severity (Shwachman score) correlated significantly with cytokine concentration, and a positive correlation between cytokine concentration and dry eye findings was also observed. The authors suggested the increased cytokine concentration could be an important factor in the pathogenesis of dry eye in patients with CF. It has been suggested tear film concentration of IFN-γ and IL-8 could function as indicators in the evaluation of CF disease clinical status (Mrugacz et al., 2006a). However, further investigation into the presence of a wide profile of pro-inflammatory cytokines in the tear film of patients with CF would be beneficial.

2.3.3 Posterior eye

2.3.3.1 Cystic Fibrosis and the crystalline lens

Majure and colleagues (1989) detected posterior subcapsular cataract formation in two subjects of a cohort of 15 CF patients receiving steroid treatment. However, steroid dose, duration and linear growth pattern during steroid therapy were not predictive factors for cataract development (Majure et al., 1989).

Lens transparency, measured with a Lens Opacity Meter, was assessed in 40 CF and 24 control subjects. Although no clinical evidence of lens opacity was detected by slit lamp examination, lens transparency was significantly higher in the control group compared to that of the CF group. Lens transparency was observed to decrease as the level of conjunctival xerosis and digestive insufficiency increased (Fama et al., 1998, Castagna et al., 2001). As xerosis is a direct manifestation of VAD the authors speculated low serum vitamin A levels could be responsible for the decreased lens transparency (Castagna et al., 2001, Fama et al., 1998). Unfortunately, vitamin A levels were not recorded in this investigation.

Vitamin supplements have been implicated as having a protective function against the development and progression of cataract (Brown et al., 1998). Antioxidants, such as vitamins A, C and E, are associated with reduced cataract formation (Wu and Leske, 2000). Therefore, vitamin deficiency, secondary to pancreatic insufficiency in CF, could contribute to reduced lens transparency. Diabetes also has a long standing association with cataract formation (Harding, 1991, Tan et al., 2008, Leske et al., 1999, Klein et al., 1998). CF subjects with CFRD or fat soluble vitamin deficiency are therefore likely to be at risk from a reduction in lens transparency. To fully understand the effect of CF on crystalline lens transparency further detailed studies in CF patients with known diabetic and vitamin A status and steroid history are necessary.

2.3.3.2 Cystic Fibrosis and macula pigment density

Macular pigment is largely derived from two carotenoids, lutein and zeaxanthin. Schupp et al. (2004) hypothesised that the combined association of reduced carotenoid absorption and increased oxidative stress in CF could result in a decreased serum concentration of these carotenoids. Serum lutein and zeaxanthin concentration and macular pigment optical density were observed to be significantly lower in a CF group compared to controls (Schupp et al., 2004). However, no significant difference in visual function was recorded. Reduced serum and retinal carotenoid concentration is associated with an increased incidence of AMD. In the study by Schupp et al. (2004) the age range of the CF patients was 21-47 years of age. However, AMD typically causes visual loss in older individuals (Smith et al., 2001). The authors concluded it would be appropriate to conduct the same protocol longitudinally in CF patients over the age of 50 to investigate the incidence of AMD (Schupp et al., 2004). As the survival rate of individuals with CF continues to increase it could be expected the incidence of AMD, secondary to reduced carotenoid uptake, at an earlier age in these individuals may also increase. As a consequence, recommendation of a supplement containing lutein and zeaxanthin to all CF patients may be pertinent.

2.3.3.3 Cystic Fibrosis and diabetic retinopathy

The incidence of diabetic retinopathy (DR) in CFRD is summarised in Table 2.15. The prevalence of CFRD increases with age; at 30 years of age 50% of patients with CF are diabetic (Lanng, 2001). As the survival rate of patients with CF continues to increase the incidence of CFRD is similarly likely to increase. The incidence of DR following diabetes duration of 10 years ranges between 67-89% for American Type 2 and Type 1 diabetics respectively (Klein et al., 1994). Whilst Anderson et al. (2006) observed a 10 year incidence of DR in 36% subjects with CFRD, Schwarzenberg et al. (2007) recorded DR in only 16%. Although the incidence of DR in CFRD appears to be lower than that observed in Type 1 or 2 diabetes, the findings indicate the importance of regular optometric and/or ophthalmological examinations, especially in those with CFRD duration of 10 years or more.

Table 2.15 Summary of diabetic retinopathy (DR) in subjects with CFRD

Author	CFRD Group	Incidence
(Rodman et al., 1983)	24	2 subjects (8%) had DR- one with microaneurysm, one with macular oedema
(Spaide et al., 1987)	5	1 subject (20%) DR; vitreous haemorrhage & macular oedema.
(Sullivan and Denning, 1989)	19 patients reviewed during 9 year period	1 subject (5%) – reportedly poorly compliant developed DR
(Scott et al., 2000)	Case report	1 subject with proliferative DR requiring laser therapy
(Lanng, 2001)	79	2 (3%) subjects had DR
(Yung et al., 1998)	34 patients CFRD ≥ 5 years, all insulin treated	5 subjects (15%) had DR; 3 background, 1 maculopathy, 1 proliferative. 23% incidence of DR in CFRD duration ≥ 10 years
(Andersen et al., 2006)	38 patients, all insulin treated	9 subjects (24%) had DR; 6 mild, 1 mod, 2 proliferative. 36% incidence of DR in CFRD duration ≥ 10 years
(Schwarzenberg et al., 2007)	84	7% DR. 16% incidence of DR in CFRD duration ≥ 10 years

2.3.4 Visual function

2.3.4.1 Cystic Fibrosis and visual acuity, refractive error and binocular vision

A number of studies have recorded reduced visual acuity (VA) in individuals with CF. Suggested causes have included: chloramphenicol associated optic neuritis (Spaide et al., 1987), VAD (Campbell et al., 1998, O'Donnell and Talbot, 1987, Suttle and Harding, 1998) and diabetic retinopathy (Spaide et al., 1987). Other investigations have recorded normal VA (6/6 or 20/20 and above) in the vast majority of the participants (Fama et al., 1998, Fulton et al., 1982, Castagna et al., 2001, Morkeberg et al., 1995). However, no study has directly compared VA in subjects with CF to matched healthy controls. Fama et al. (1998) and Morkeberg et al. (1995) reported refractive error in CF patients: 28% myopic and 13% hypermetropic; and 31% myopic respectively (Fama et al., 1998, Morkeberg et al., 1995). Similarly, no comparisons were made to a control group. Whilst a number of studies have recorded CF subjects with strabismus and/or amblyopia there has been no detailed study to compare binocular vision status in CF subjects and controls (Fulton et al., 1982, Morkeberg et al., 1995, Spaide et al., 1987).

CF is associated with prematurity and low birth weight (Festini et al., 2005, Muller et al., 1999), which are themselves associated with a greater incidence of ametropia, strabismus and amblyopia (Huynh et al., 2006, O'Connor et al., 2006, Robaei et al., 2006a). Therefore, a large scale investigation of visual acuity, refractive and binocular vision status in a large cohort of CF patients and controls would be invaluable for clinical management.

2.3.4.2 Cystic Fibrosis and colour vision

Some form of colour vision defect is observed in approximately 8% male and 0.5% females within a normal caucasian population (Tovee, 1994). However, Spaide et al. (1987) recorded colour vision (CV) deficiencies in 29% subjects with CF. These patients had been treated with the antibiotic chloramphenicol, had reduced VA and

hyperaemic optic discs. Therefore, the defects were believed to be caused by chloramphenicol associated optic neuritis (Spaide et al., 1987). Morkeberg et al. (1995) reported three male patients had CV deficiencies. These included; a deuteranormal, a deuteranope and a protanope (Morkeberg et al., 1995). However, it was not indicated if these were congenital or acquired defects. Tinley et al. (2008) reported a case study where a vitamin A deficient subject complained of abnormal colour perception where "colours assumed a green hue" (Tinley et al., 2008). However, no diagnostic CV test was performed. More recently, CV was assessed in a small CF cohort (n=10) with a computerised Cambridge Colour Test. Whilst one deuteranomalous subject was excluded all remaining subjects had normal CV (Schupp et al., 2004).

Although it is not believed any patients in these previous studies had CFRD, diabetes is known to adversely affect colour vision, even in the absence of diabetic retinopathy (Stavrou and Wood, 2003, Di Leo et al., 1992, North et al., 1997, Banford et al., 1994). To examine if CFRD has a similar effect, further detailed studies investigating CV in CF patients with known diabetic status is necessary.

2.3.4.3 Cystic Fibrosis and contrast sensitivity

A number of studies have recorded reduced levels of contrast sensitivity (CS) in subjects with CF. These are reviewed in Table 2.16 later in the chapter. In the study by Spaide et al. (1987), even after the exclusion of nine CF patients with decreased VA, CS remained significantly reduced. The authors suggested optic nerve disease could be responsible, which could be a primary effect of CF itself or a secondary effect from other factors including; antibiotics, vitamin deficiencies, hypoxia (Spaide et al., 1987). In the Leguire case study, CS was shown to significantly improve following vitamin A supplementation (Leguire et al., 1991). Therefore, the authors suggested the observations of both their case report and Spaide et al. were caused by subtle loss of photoreceptor function secondary to VAD (Leguire et al., 1991). However, subjects with good vitamin A status have also been shown to have significantly reduced CS (Morkeberg et al., 1995, Ansari et al., 1999). It was suggested the abnormal pre-corneal tear film observed in many patients with CF could

cause a glare phenomenon adversely affecting the CS function (Morkeberg et al., 1995). However, no significant correlation between the incidence of dry eye and reduced CS was observed in this study. More recently, a smaller study found no significant difference in CS between subjects with CF and controls (Schupp et al., 2004).

A number of other factors beside VAD could contribute to abnormal CS in subjects with CF. A reduction in CS in Type 1 and Type 2 diabetics, even in the absence of diabetic retinopathy has been observed (North et al., 1997, Di Leo et al., 1992, Dosso et al., 1996). This could similarly be observed in subjects with CFRD. CS is also reduced in patients who have reduced lens transparency due to increased light scatter, even though high contrast visual acuity may remain unchanged (Thomson, 2002, Brown, 1993, Elliott et al., 1989, Cheng et al., 2001) and reduced lens transparency and cataract have been observed in CF previously (Fama et al., 1998, Castagna et al., 2001, Majure et al., 1989). It is unknown if any of the previous studies included subjects with CFRD or lens abnormalities. Therefore, a detailed study of CS in patients with known vitamin A and diabetic status and crystalline lens physiology would be invaluable.

Table 2.16 Summary of contrast sensitivity (CS) in CF studies

Author	Groups	Method	Results
(Spaide et al., 1987)	29 CF, 12 controls	Bekesy interactive CS technique, measured at 6 spatial frequencies (sf) range 0.5-22.8 cycles/degree (cpd)	CS decreased at every sf in CF. CS still reduced when subjects with reduced VA were excluded ($p<0.1$)
(Leguire et al., 1991)	case report, 16 year old CFRD subject	Wall mounted chart; 8 contrast levels at 5 sf. Compared to CS of 15 controls	CS abnormal at all sf. CS improved by 94% following vitamin A supplementation
(Morkeberg et al., 1995)	35 CF	Mesopter, using Landolt ring at 2 luminance levels (0.1 & 0.03cd/m^2) also measured after 10 second "blinding" light Results compared to reference population	CS significantly reduced in CF for all tests ($p<0.001$), CS was most affected by light "blinding"
(Ansari et al., 1999)	28 CF, 25 controls	Vistech chart, measured at 5 sf range 1.5-18 cpd	8 (29%) CF patients had reduced CS at intermediate and high sf.
(Schupp et al., 2004)	10 CF, 10 controls	Gratings presented on computer screen at 0.55, 1.125, 2.25, 2.5, 9 & 18 cpd	No significant difference in CS for all sf

2.3.4.4 Cystic Fibrosis and dark adaptation

The earliest clinical manifestation of xerophthalmia is impaired dark adaptation (DA) and night blindness (Brooks et al., 1990). Initially, a decrease in the rate of DA and the rate of pigment regeneration in the rod system is observed (Lamb and Pugh, 2004). As the deficiency continues DA becomes slowed and thresholds elevated. Following prolonged and severe deficiency the photoreceptors degenerate. Provided the deficiency has not been too severe or prolonged the effects can be typically reversed within days following vitamin A supplementation (Congdon and West, 2002). Table 2.17 summarises a number of studies that have investigated DA in CF later in this chapter.

Several studies have reported reduced serum vitamin A levels in CF patients with abnormal DA (Fulton et al., 1982, Neugebauer et al., 1989, Rayner et al., 1989). DA thresholds have been shown to improve following vitamin A supplementation in a number of subjects with CF (Fulton et al., 1982, Huet et al., 1997, Rayner et al., 1989). A number of case reports have been published indicating abnormal DA in vitamin A deficient CF patients that has been reversed following intervention (Eid et al., 1990, Brooks et al., 1990, O'Donnell and Talbot, 1987, Joshi et al., 2008).

Considering the more recent investigations (Ansari et al., 1999, Morkeberg et al., 1995), the incidence of abnormal DA secondary to VAD appears to have significantly reduced. This undoubtedly reflects modern supplementary management and appropriate intervention. However, assessment of DA appears to be a relatively simple method to evaluate and monitor the effects and subsequent improvement of VAD in patients with CF.

There is no recorded indication of CFRD in any subjects with abnormal dark adaptation, however there is substantial evidence that individuals with diabetes generally have abnormal dark adaptation thresholds (Holopigian et al., 1997) and take longer to adapt (Henson and North, 1979) even in the absence of diabetic retinopathy (Arden et al., 1998). Consequently, it is of interest to compare dark adaptation thresholds in CF patients, with normal vitamin A levels, both with and without CFRD.

Table 2.17 Summary of dark adaptation (DA) in CF studies

Author	Group	Method	Results
(Fulton et al., 1982)	56 CF, 8 controls	Maxwellian-view adaptometer	Average thresholds & vitamin A lower in CF group ($p<0.05$ & $p<0.001$).
(Neugebauer et al., 1989)	31 CF, 28 controls	Friedmann Field Analyser and in house computer system. DA abnormal if threshold >2 standard deviation above controls	19% CF abnormal DA
(Rayner et al., 1989)	43 CF, 4 controls	Modified Friedmann Field Analyser and computer system	19% CF abnormal DA, vitamin A & RBP significantly lower in affected patients
(Huet et al., 1997)	10 CF	Beyne optometer	3 CF poor and 2 CF pathological DA 90% subjects low vitamin A levels
(Morkeberg et al., 1995)	35 CF	Goldmann-Weekers Adaptometer. Threshold determined after 15 minutes adaptation. Values below 20μcd/m^2 normal	All subjects normal vitamin A levels & normal DA
(Ansari et al., 1999)	28 CF	Friedmann Field Analyser, pupils dilated, 30 minute test period	All subjects normal vitamin A levels & normal DA

2.3.4.5 Cystic Fibrosis and electrophysiology

A summary of electrophysiological studies in subjects with CF is presented in Table 2.18 later in this chapter. The visual evoked potential (VEP) is an electrophysiological response to light or pattern stimulation recorded from encephalographic activity and can be used to assess the functional integrity of the visual system (Odom et al., 2004). Reduced VEP's in CF subjects treated with chloramphenicol supports the theory that the antibiotic causes optic nerve disease in CF (Spaide et al., 1987). There is limited use of chloramphenicol in CF patients currently (Schupp et al., 2004). Therefore, the incidence of chloramphenicol associated optic neuritis has certainly reduced. A number of studies highlight the electrophysiological consequences of VAD in CF, indicating abnormal photoreceptor function (Leguire et al., 1991, Leguire et al., 1992).

Vitamin E deficiency also appears to have detrimental consequences, particularly for the VEP (Messenheimer et al., 1984, Kaplan et al., 1988, Willison et al., 1985). The antioxidant vitamin provides retinal protection from free radical damage and oxidative destruction of stored vitamin A (Lu et al., 2006). Tinley et al. (2008) reported a case study where a vitamin A deficient CF subject showed restoration of the rod response (scotopic electroretinogram (ERG)) following zinc supplementation (Tinley et al., 2008). Zinc deficiency has been implicated in rod dysfunction and may cause secondary VAD (Christian and West, 1998). These studies indicate vitamin A is not the sole nutrient essential for normal ocular physiology.

Table 2.18 Summary of Electrophysiology in CF studies

Author	Groups	Method	Results
(Messenheimer et al., 1984)	case report, vitamin E deficient subject	VEP	Pattern reversal VEP abnormal, became normal after 2 month period of vitamin E administration
(Willison et al., 1985)	case report, vitamin A & E deficient subject	VEP & ERG	VEP's bilaterally delayed Pattern reversal ERG not detectable
	10 CF	ERG & VEP	2 subjects abnormal flash ERG's, 1 subject prolonged VEP latency These subjects vitamin A sufficient but vitamin E deficient
(O'Donnell and Talbot, 1987)	case report, vitamin deficient subject	ERG	ERG initially abnormal, normalised following 4 months of vitamin A supplementation
(Spaide et al., 1987)	17 CF	VEP	29% CF reduced bilateral delays, associated with chloramphenicol associated optic nerve disease
(Kaplan et al., 1988)	10 CF, all vitamin E deficient	VEP	3 subjects abnormal VEP's
(Leguire et al., 1991)	case report 1 vitamin A deficient CF patient	Scotopic & photopic ERG's	ERG's reduced, normalised following vitamin A supplementation

(Leguire et al., 1992)	case report 1 vitamin A deficient CF patient	EOG	Initially abnormal, normalised following vitamin A supplementation
(Miller et al., 1992)	13 CF, 15 controls	EOG	FO significantly reduced ($p<0.001$) in CF
(Suttle and Harding, 1998)	case report, CF infant	Scotopic & photopic ERG	Scotopic ERG absent before 19 weeks of age, photopic ERG normal. Restoration of scotopic ERG following vitamin A treatment
(Tsinopoulos et al., 2000)	41 CF, 41 controls	Scotopic & photopic ERG	Vitamin A significantly lower in CF group, 66% abnormal. No significant differences in ERG responses between two groups.
(Schupp et al., 2004)	9 CF, extensive age matched controls	mfERG	Two CF patients showed delayed latencies but normal response densities
(Constable et al., 2006)	6 CF, 9 controls	light-EOG & acohol-EOG	Alcohol and light-EOG's amplitudes normal. FO amplitude significantly higher & time to dark troughs significantly slower in ΔF508 heterozygotes compared to both controls and homozygotes.
(Tinley et al., 2008)	case report 1 vitamin A deficient patient	Scotopic ERG	Rod response restored following zinc supplementation

2.4 Summary

CFTR is a significant channel for Cl^- efflux in the eye and is present in the corneal epithelium, corneal endothelium, conjunctival epithelium and the retinal pigment epithelium. CFTR is likely to facilitate a number of ocular processes (Figure 2.7) that rely on active Cl^- transport to allow passive movement of water such as:

- basal tear production by the cornea and conjunctiva
- maintenance of normal corneal integrity by the corneal endothelium
- maintenance of the SRS by the RPE and normal photoreceptor function

Therefore, abnormal or complete absence of CFTR function in individuals with CF may result in abnormal basal tear production and dry eye, increased corneal thickness and reduced corneal transparency, increased SRS and abnormal photoreceptor function.

A number of small studies have investigated the ocular surface, posterior eye and visual function in subjects with CF. These studies have identified a number of ocular characteristics associated with the disease such as; dry eye and tear film abnormality, reduced lens transparency and contrast sensitivity and colour vision irregularities. However, it is unclear if these characteristics are primary or secondary manifestations of CF. These investigations have frequently been of a small scale, have not used healthy matched controls for comparison, or are dated in terms of modern CF therapy and management. Many have included confounding factors such as VAD or unknown serum vitamin A levels and others have undoubtedly included subjects with CFRD.

As the life expectancy of individuals with CF continues to increase eyecare professionals will encounter these subjects more frequently. In order to offer optimal care to these patients the ocular associations of CF must be researched.

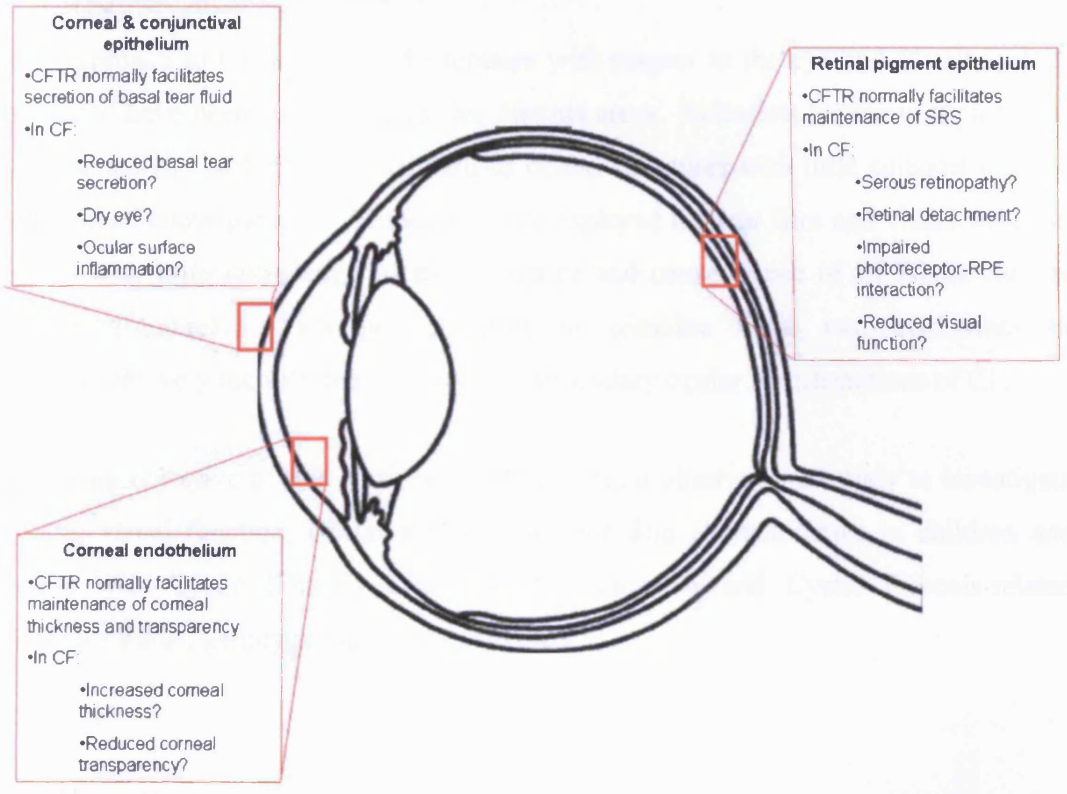


Figure 2.7 A summary of the presence of CFTR, proposed function and potential ocular consequences in subjects with CF

2.5 Objectives for this Ph.D

The approach to CF studies and literature with respect to the eye and visual system appear to have been considered in two distinct areas. Scientists have investigated the cellular activity of CFTR within various ocular structures with little consideration of the clinical consequences. Clinicians have explored the tear film and visual function in CF with little knowledge of the existence and consequence of CFTR in various ocular structures. This project aims to combine these two disciplines to comprehensively identify the primary and secondary ocular manifestations of CF.

The main objective of this work is to implement an observational study to investigate vision, visual function, ocular surface and tear film characteristics in children and adults with Cystic Fibrosis with known vitamin A and Cystic Fibrosis-related Diabetes status, genotype and disease severity.

2.6 Hypotheses

1. Dry eye is a primary manifestation of CF, even in vitamin A sufficient subjects: abnormal Cl^- efflux by CFTR from the corneal and conjunctival epithelium results in reduced basal tear fluid secretion with a subsequent increase in tear film osmolarity. Increased tear film inflammatory cytokine concentration indicates sub-clinical inflammation of the ocular surface. Dry eye severity is likely to be correlated with CF genotype and therefore disease progression.

2. Abnormal visual function is a primary manifestation of CF due to anomalous Cl^- secretion by CFTR from the RPE resulting in impaired regulation of the SRS and IPM leading to abnormal retinal photoreceptor function. However, visual function is proposed to be confounded by secondary disease characteristics including vitamin A deficiency and CFRD.

3. There is a greater incidence of ametropia and abnormal binocular vision in subjects with CF, associated with low birth weight and/or prematurity.

Chapter 3

Preliminary Experiments and Development of Protocols

3.1 Introduction

In order to investigate the tear film in subjects with Cystic Fibrosis (CF) it is essential to collect tear samples with proficiency. Therefore, the aims of these preliminary experiments were to:

- Identify suitable alternative tear collection material to glass capillary tubes for use in children and young adults with CF and controls
- Investigate subject comfort and sample absorbency of alternative tear collection material
- Optimise tear sample storage and recovery

Tear ferning, a novel diagnostic dry eye test, was found to be abnormal in CF subjects (Rolando et al., 1988, Kalayci et al., 1996). However, a detailed methodology for the tear ferning technique is not available from the literature. It is important to consider the effect of tear collection technique on tear film composition (Stuchell et al., 1984). Many components, such as cytokine concentration, are sensitive to the method of tear sample collection (Cook et al., 2001) and tear film osmolarity and various protein concentrations are dependant on factors such as stimulation (Stuchell et al., 1981, Farris et al., 1981). Therefore, further aims for this work included:

- Development of a detailed tear ferning protocol
- Assessment of the effect of the new tear collection material on tear ferning samples

3.2 Suitable materials for tear sample collection in children and young adults

3.2.1 Introduction

Glass capillary tubes have traditionally been utilised to collect tear samples for tear ferning, osmolarity, cytokine analysis, refractive index measurement, mucin concentration and protein analysis (Patel et al., 2000, Norn, 1988, Nichols and Sinnott, 2006, Yoon et al., 2007, Argueso et al., 2002, Glasson et al., 2003). Although the technique is well cited, the safe and effective use for tear collection in children, young adults or subjects lacking co-operation is questionable. Relatively few alternatives have been suggested in the literature and are summarised in Table 3.1.

Absorbent sponges have proved to be effective alternatives to glass capillaries. Small and colleagues concluded surgical sponges were both easy to use, allowed rapid collection and the collected tear sample easily analysed (Small et al., 2000). In adults, cellulose sponges have been used successfully to collect tear samples from neonates (Esmaeelpour et al., 2008) and patient comfort has been cited as an advantage when using polyurethane minisponges in adults (Lopez-Cisternas et al., 2006). Therefore, a variety of alternative tear collection materials, including a number of sponge devices, were investigated for their suitability of tear collection in children and young adults.

3.2.2 Method

A number of suitable tear collection materials (Table 3.1) were sourced and their relative advantages and disadvantages investigated. Bench-top comparisons considered factors such as expense and material sterility. Materials were trialled for tear collection on a single subject (the author); features considered included; ease of use, if forceps were necessary to hold the material, relative material absorbency or capillary attraction. Absorbent sponges were centrifuged (method described in 3.4.2.1.1) to investigate sample recovery.

Table 3.1 Alternative tear collection materials to glass capillary tubes

Author and Year	Alternative method for tear collection	Purpose
(Botelho et al., 1973)	Plastic sponge	Tear flow rate and ion analysis
(Webster and Kairys, 1984)	Polyethylene tube with syringe	Improved tear collection for protein analysis
(van Agtmaal et al., 1987)	Cellulose sponges	Tear collection in neonates for protein analysis
(Boonstra et al., 1988)	Schirmer strips & sponges	High performance liquid chromatography (HPLC) analysis of tear protein profile
(Tuft and Dart, 1989)	Cellulose sponges	Immumoglobulin E (IgE) analysis
(Norn, 1992)	Cotton, Spongostan & Schirmer strips	Analysis of various tear constituents eg. albumin, glucose
(Jones et al., 1997)	Porous polyester rods	Tear protein analysis
(Small et al., 2000)	Surgical sponges & Schirmer strips	Pharmaceutical drug recovery from tears
(Zhao et al., 2001)	Schirmer strips	Quantification of the mucin MUC5AC
(Lopez-Cisternas et al., 2006)	Polyurethane minisponges	Electrophoresis
(Esmaeelpour et al., 2008)	Cellulose rods	Tear collection in neonates for protein analysis

3.2.3 Results

Tear collection materials and their properties are summarised in Table 3.2. In particular the cellulose rods and triangular sponges appear to have optimal qualities for tear collection in children and young adults. The cellulose spears, although ideal for tear collection, must be excluded due to discolouring the tear sample after centrifuging, indicating contamination by the tear collection material.

Table 3.2 Relative properties of the alternative tear collection materials

Material	Relative Advantages	Relative Disadvantages
10µl glass pre-calibrated pipette tubes (LIP Ltd.)	-Well referenced, established method -Excellent collection and retrieval of sample -Minimal stimulation	-Not suitable for use with children -Difficult to obtain ethical approval for use in children?
Plastic capillary bores (Advanced Instruments Inc., MA, USA)	-Safer alternative to glass capillary?	-Very poor capillary attraction
Absorbent dental paper points (Dentsply Int., PA, USA)	-Sterile -Pointed tip easy to place in tear meniscus	-Poor absorbency -Too small for easy use -Difficult to retrieve sample, must be eluted -Need to be held with forceps
Sno Strips (Chauvin Pharmaceuticals Ltd., Kingston-upon-Thames, UK)	-Allows simultaneous assessment of tear flow rate -Widely accepted clinically	-Difficult to use for tear collection in children -Stimulated tears only -Difficult to retrieve sample, must be eluted

Cellulose Spears (Eyetec, Network Ophthalmic Products, Rippon, UK)	-Sterile -Easy to use, no forceps needed -Pointed tip easy to place in tear meniscus -Excellent absorbency	-Discolour tear samples when centrifuged -Relatively expensive
* Cellulose Rods (Filtrona Filter Products, Milton Keynes, UK)	-Have been used to collect tears in neonates -Inexpensive	-Not sterile (can be UV sterilised) -Small surface area for tear collection -Need to be held with forceps
Sugi steril rectangular sponges (Kettenbach GmbH & Co., Eschenburg, Germany)	-Sterile -Excellent absorbency	-Relatively expensive -Too large to accurately place in tear meniscus -Need to be held with forceps
* Sugi steril triangular sponges (Kettenbach GmbH & Co., Eschenburg, Germany)	-Sterile -Pointed tip easy to place in tear meniscus -Excellent absorbency	-Relatively expensive -Need to be held with forceps

* Two suitable tear collection materials

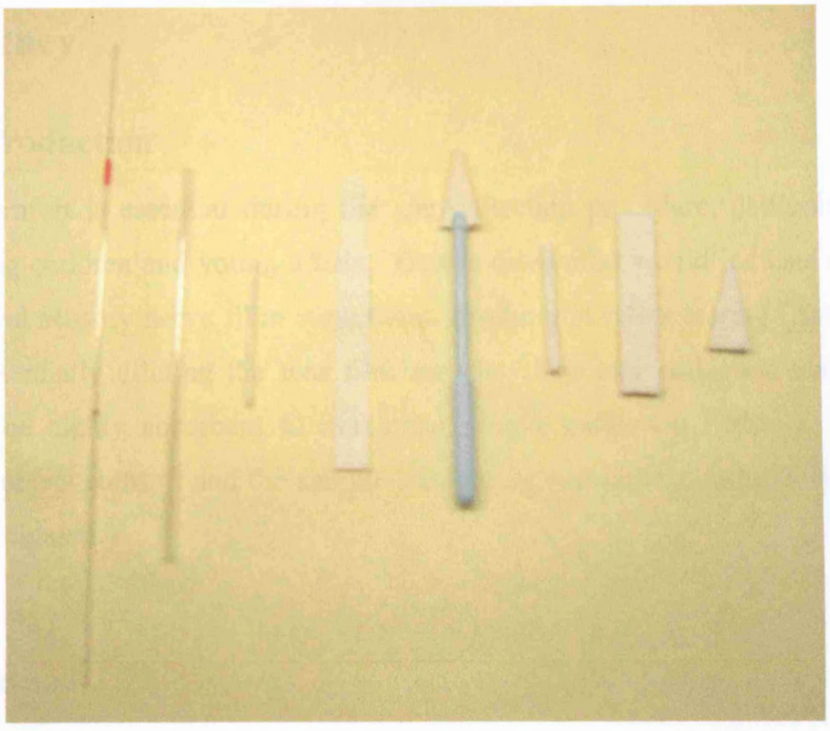


Figure 3.1 Alternative tear collection materials (from left to right); glass capillary tube, plastic capillary bore, dental paper point, Sno Strip, cellulose spears, cellulose rods, Sugi Steril rectangular sponge, Sugi Steril triangular sponge.

3.2.4 Discussion

A number of cellulose based materials have been cited in the literature as suitable alternatives to glass capillary tubes for tear film collection (van Agtmaal et al., 1987, Tuft and Dart, 1989, Esmaeelpour et al., 2008). Therefore, it is unsurprising that the two most appropriate materials identified were cellulose rods (Filtrona Filter Products, Milton Keynes, UK) and triangular cellulose sponges (Kettenbach GmbH & Co., Eschenburg, Germany). Each product is highly absorbent and ideal to place at the inferior tear meniscus with forceps. However, further investigation into patient comfort and tear film sample absorbency was necessary to identify which would ultimately be the most suitable.

3.3 Investigating subject comfort and tear film sample absorbency

3.3.1 Introduction

Patient comfort is essential during the tear collection procedure, particularly when considering children and young adults. Ocular discomfort would indicate corneal or conjunctival sensory nerve fibre stimulation resulting in reflex tearing (Acosta et al., 2004), potentially diluting the tear film sample. The tear collection material also needs to be highly absorbent to maximise sample collection within a short time period. Subject comfort and the sample size during tear collection for both materials were investigated.

3.3.2 Method

3.3.2.1 Subjects

Healthy subjects (n=20; 7 male, 13 female: mean age 23.7 years ± SD 5.13 years) were recruited from the School of Optometry and Vision Sciences, Cardiff University. Informed consent was obtained from all subjects; favourable approval had been obtained from the School of Optometry and Vision Sciences Research Ethics Committee and all procedures conformed with the tenets of the Declaration of Helsinki. Subjects were free from ocular and systemic pathology known to affect the eye (apart from dry eye) and contact lenses were not worn two hours prior to tear collection. Subjects were excluded if they were using topical ocular medication, pregnant or breast feeding. These criteria apply to all experiments within this chapter.

3.2.2.2 Ultraviolet sterilisation of cellulose rods

Cellulose rods were ultraviolet (UV) sterilised before use by being placed into a UV Crosslinker for five minutes, energy 120 micro-Joules/centimetre^2 ($\mu J/cm^2$), according to the manufacturer's instructions (Jencons (Scientific) Limited, Leighton Buzzard, UK).

3.3.2.3 Experimental design

Cellulose sponges (Kettenbach GmbH & Co., Eschenburg, Germany) and cellulose rods (Filtrona Filter Products, Milton Keynes, UK) were placed into individual 0.5ml Eppendorf tubes (Alpha Laboratories Ltd., Hampshire, UK) and the combined weight for each recorded. Tear samples were collected from the inferior tear meniscus with either a cellulose rod or cellulose sponge. Initial tear collection material and the first eye selected for tear collection were randomised. The subject was directed to fixate superior-nasally and the collection material applied to the outer third of the inferior tear meniscus with forceps for a period of three minutes. Care was taken to avoid stimulation and subjects were instructed to blink as necessary. Following tear collection the subject was asked to indicate ocular comfort on a 10 centimetre visual analogue scale (VAS) (Figure 3.2) and the tear collection material was returned to the Eppendorf tube. After a resting period of five minutes the process was repeated with the second tear collection material. All Eppendorf tubes were re-weighed and the weight difference calculated to give tear sample weight. The indication mark was measured from the VAS with a ruler to the nearest millimetre and recorded as subject discomfort.

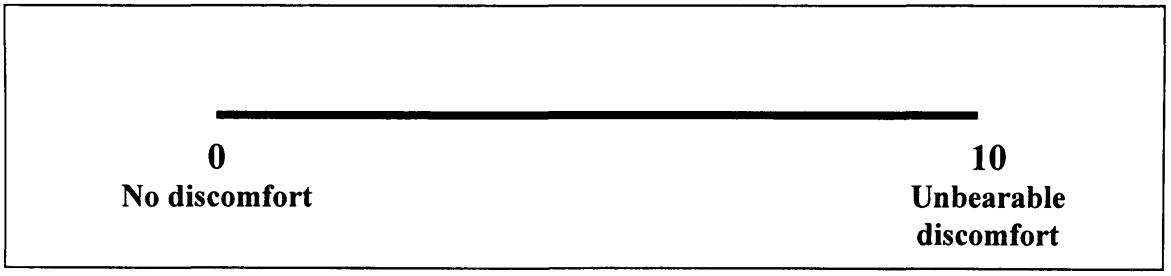


Figure 3.2 Visual Analogue Scale (VAS)

3.3.2.4 Standard curve

In order to accurately calculate the volume of each tear sample a standard curve was plotted. Saline was used to simulate tears due to the large volumes necessary. Known volumes of saline (Bausch & Lomb, Surrey, UK), from 1 to 20 microlitres (μl), were pipetted into pre-weighed Eppendorf tubes and re-weighed. The weight difference for each volume was calculated and plotted on a graph (Figure 3.3). The equation of the best fit line could then be used to convert tear sample weight into tear sample volume.

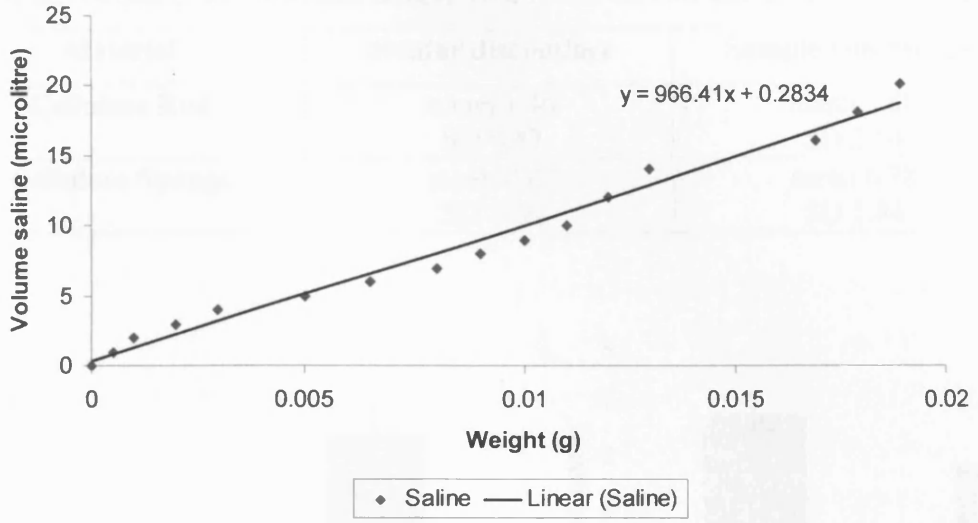


Figure 3.3 Standard curve used to calculate tear sample volume

3.3.2.5 Statistical analysis

A paired-samples (2-tailed) t-test (SPSS version 16, Incorporated, Chicago, Illinois, USA) was used to compare ocular comfort and tear sample volume for the two tear collection materials. Significance was set at the 0.05 level.

3.3.3 Results

Ocular discomfort for the 20 subjects ranged from a minimum of 0.7 and maximum of 2.4 for tear samples collected with cellulose rods, and 0.4 to 1.7 with the cellulose sponges. Mean ocular discomfort for the two collection materials is shown in Figure 3.4a and Table 3.3. The difference was statistically significant (Paired-samples t-test; $p<0.005$), indicating tear collection was more comfortable with the sponges compared to the rods. Considering sample volume collected with the two materials, a range of 2.22 to 11.88µl was recorded for the rods and 3.20 to 10.91µl with the sponges. Mean sample size was significantly greater for tears collected with cellulose sponges compared to cellulose rods (Paired-samples t-test; $p<0.0001$). Mean sample size for the two tear collection materials is shown in Figure 3.4b and Table 3.3.

Table 3.3 Ocular discomfort and sample volume for the two tear collection materials

Material	Ocular discomfort	Sample volume (µl)
Cellulose Rod	mean 1.46 SD 0.47	mean 5.80 SD 2.14
Cellulose Sponge	mean 0.84 SD 0.36	mean 6.78 SD 1.84

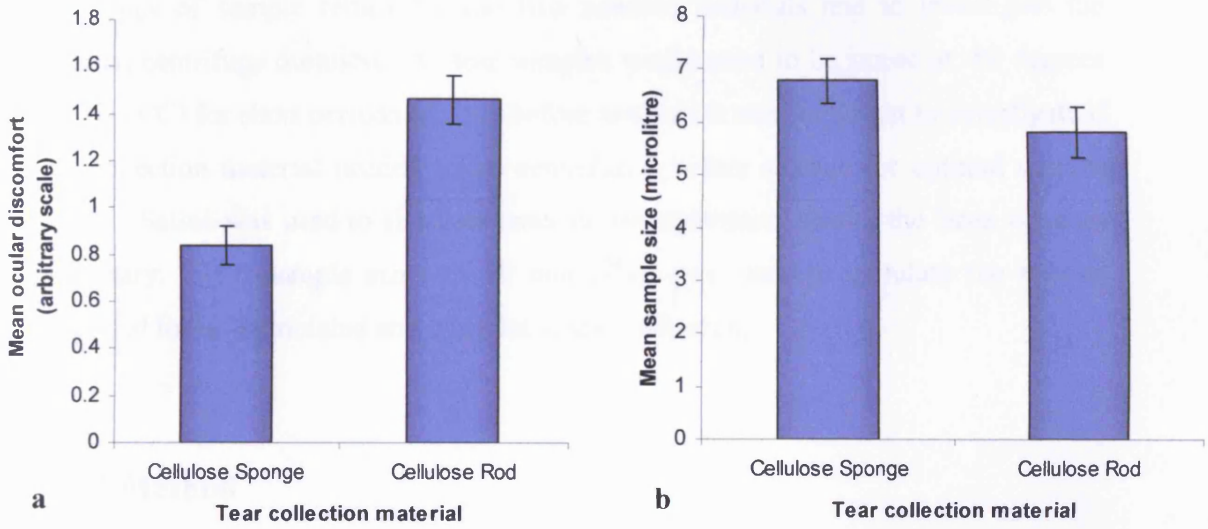


Figure 3.4 Mean ocular discomfort (a) and mean sample size (b) for the two collection materials (Paired-samples t-test; $p<0.005$ and $p<0.0001$ respectively)

3.3.4 Discussion

Cellulose sponges appear significantly more comfortable and yield greater sample volume return than the cellulose rods. During tear collection with the cellulose sponges the triangular tip expanded as the material became saturated, improving comfort and increasing surface area for greater absorption. Furthermore, the material expansion gives a useful visual aid to the volume of tear sample recovered, unlike the cellulose rods which do not alter with saturation. Whilst the results indicate cellulose sponges are a favourable alternative to glass capillary tubes, the properties of the two materials for sample recovery must be investigated.

3.4 Optimisation of tear film sample storage and recovery

3.4.1 Introduction

For tear samples to be analysed they must be extracted from the tear collection medium. Centrifuging has typically been the technique used previously (Lopez-Cisternas et al., 2006, Jones et al., 1997). The aim of this study was to compare the efficiency of sample return for the two selected materials and to investigate the optimal centrifuge duration. As tear samples would need to be stored at -80 degrees Celsius (°C) for short periods of time before analysis it was important to investigate if the collection material needed to be centrifuged before storage for optimal volume return. Saline was used to simulate tears in the experiment due to the large volumes necessary. Two sample sizes of 10 and 20µl were used to simulate the volume expected for un-stimulated and stimulated tear collection.

3.4.2 Method

3.4.2.1 Experimental design

3.4.2.1.1 Sample recovery

Aliquots of 10µl or 20µl saline (Bausch & Lomb Inc., Surrey, UK) were pipetted onto each tip of 25 cellulose rods. Each rod was placed into a 0.2ml micro-centrifuge tube (Alpha Laboratories Ltd., Hampshire, UK) (all had the apex of the tube removed previously with a scalpel) and the cap closed. Each was placed into a pre-weighed 0.5ml Eppendorf tube and into a tube rack on ice to minimise evaporation. Five of the 10µl and five of the 20µl samples were centrifuged (GS-15R, Beckman Coulter Ltd, Buckinghamshire, UK) for a period of three minutes at 14,000 revolutions per minute (RPM) at 4°C. This was repeated for centrifuge durations of five, 10, 15 and 30 minutes with the other samples. The micro-centrifuge tubes were removed and each Eppendorf tube re-weighed and the regression curve ($y = 966.41x + 0.2834$) created in 3.2.2 (Figure 3.3) was used to calculate sample volume. Finally, the entire process was repeated with the cellulose sponges.

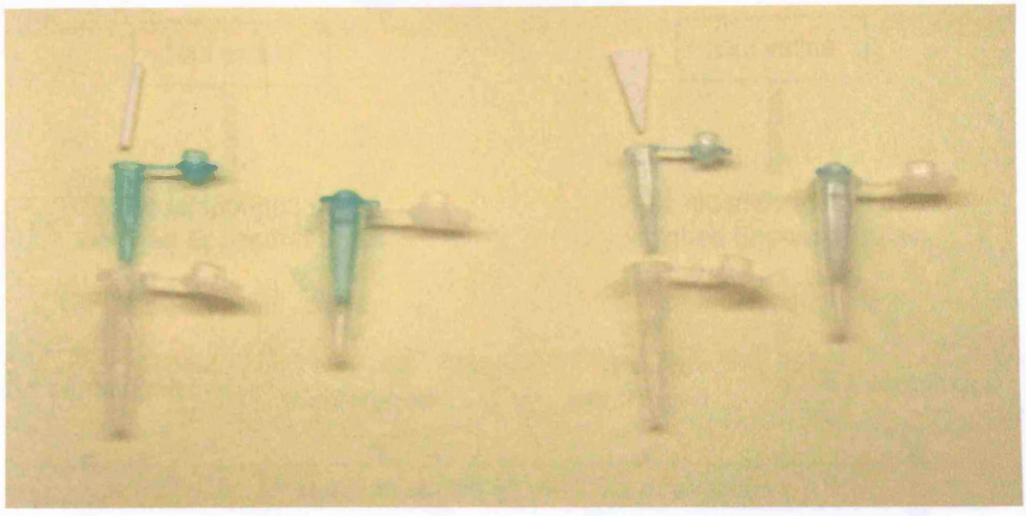


Figure 3.5 Eppendorf set up for centrifuging tear collection material

3.4.2.1.2 Sample storage

Aliquots of 10µl or 20µl saline were pipetted onto each tip of 10 cellulose rods and 10 cellulose sponges and placed within a de-tipped 0.2ml micro-centrifuge tube and the cap closed and subsequently placed into a pre-weighed 0.5ml Eppendorf tube. Half of the samples were centrifuged at 14,000 RPM for a period of three minutes at 4 °C then all the samples placed into a tube rack inside a sealed container and stored at -80°C for a period of one month. Following storage, the remaining un-centrifuged tubes were then centrifuged (Figure 3.6). All micro-centrifuge tubes were removed and each Eppendorf tube re-weighed and the standard curve (Figure 3.3) created in 3.2.2 was utilised to calculate sample volume.

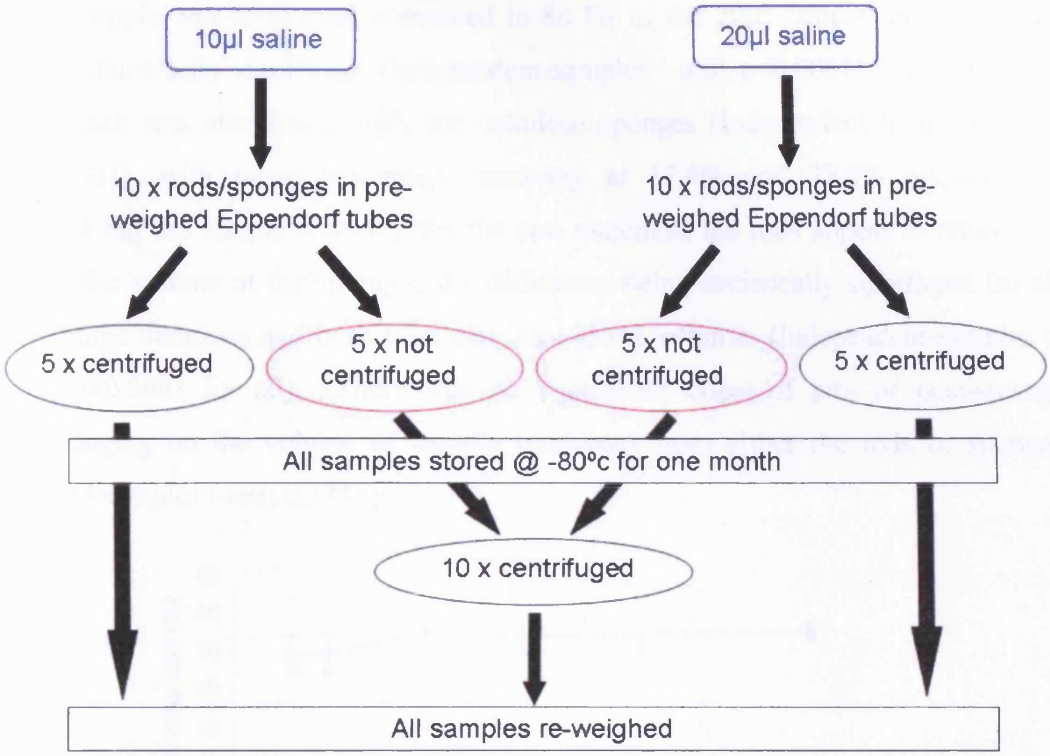


Figure 3.6 Flow diagram of experimental design investigating sample storage

3.4.2.2 Statistical analysis

One-way analysis of variance (ANOVA) was used to investigate differences in sample return from increasing centrifuge duration. A paired-samples (2-tailed) t-test was used to investigate the effect of storage on sample recovery from the two materials and an independent-samples (2-tailed) t-test compared the volume recovered from the rods to the sponges. Significance was set at the 0.05 level.

3.4.3 Results

Figures 3.7a and b demonstrate the percentage of sample recovery with increasing centrifuge duration. There was no significant increase in the volume of saline recovered with increased centrifuging for either the rods (ANOVA; $p=0.074$ and $p=0.055$ for 10µl and 20µl saline respectively) or the sponges (ANOVA; $p=0.465$ and $p=0.413$). For both collection materials it can be seen the greater the initial sample volume the greater the sample return. For the cellulose rods a mean of 74.2% of the

10µl sample was recovered, compared to 88.2% of the 20µl sample, the difference being statistically significant (Independent-samples t-test; $p<0.0001$). A significant difference was also found with the cellulose sponges (Independent-samples t-test; $p<0.0001$), with mean percentage recovery at 17.4% and 37.8% respectively. Comparing the sample recovery for the two materials, the rods appear to return 2-3 times the volume of the sponges, the difference being statistically significant for all centrifuge durations and both 10µl and 20µl saline volumes (Independent-samples t-test; $p<0.0001$ for all). There was no significant effect of pre- or post-storage centrifuging on the volume of sample recovered from either the rods or sponges (Paired-samples t-test; $0.374<p<0.717$).

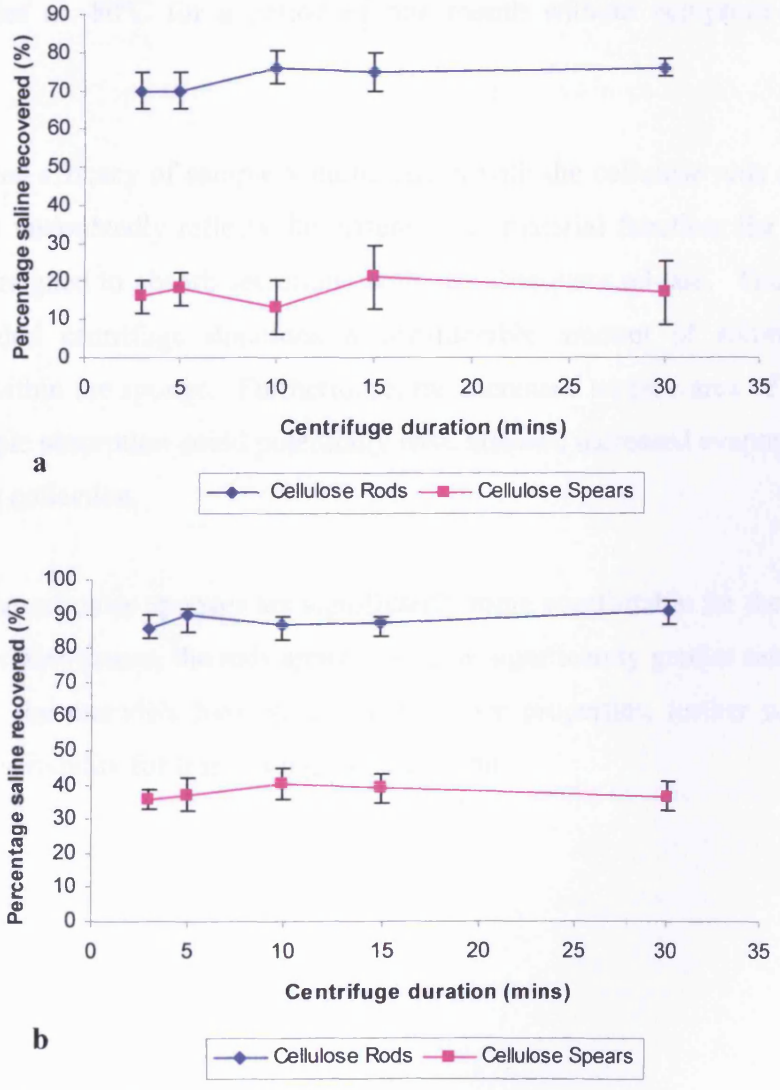


Figure 3.7 The percentage of sample recovered from 10µl (a) and 20µl (b) saline following various centrifuge durations for the two collection materials

(error bars represent standard error of the mean)

3.3.4 Discussion

For both collection materials, increased centrifuging had an insignificant effect at increasing volume return. Therefore, centrifuging tear samples for a period of three minutes appears sufficient for maximal sample recovery. Greater volume return was observed with the 20µl samples compared to the 10µl samples for both collection materials suggesting increased material saturation yields greater sample return. This implies that collection of larger volumes of stimulated tears or longer periods of un-stimulated tear collection may be more effective. There was no significant effect on sample volume return with storage of the materials at -80°C, either pre or post centrifuging. Therefore, for convenience it is considered acceptable to immediately store samples at -80°C for a period of one month without compromising sample volume.

The increased efficacy of sample volume return with the cellulose rods compared to the sponges undoubtedly reflects the difference in material function; the sponges are primarily designed to absorb secretions without subsequent release. Therefore, even with extended centrifuge durations a considerable amount of solution remains "trapped" within the sponge. Furthermore, the increased surface area of the sponges during sample absorption could potentially have allowed increased evaporation of tear fluid during collection.

Although the cellulose sponges are significantly more comfortable for the subject and have greater absorbance, the rods appear to allow significantly greater sample volume return. As the materials have such contradictory properties, further investigation, such as the suitability for tear ferning, is paramount.

3.5 Optimisation of the tear ferning protocol

3.5.1 Introduction

A number of bodily secretions produce a distinctive ferning pattern when allowed to air dry on a glass microscope slide (Abou-Shabanah and Plotz, 1957). This ferning phenomenon was originally utilised to determine the ovulation period in women by evaluating cervical mucus ferning characteristics (Papanicolau, 1946). However, Tabbara and Okumoto used conjunctival scrapings to devise a test to aid differential diagnosis of acute forms of conjunctivitis by the presence or absence of tear ferns (Tabbara and Okumoto, 1982). Rolando (1984) later devised a qualitative grading scale based on the appearance of four different tear ferning (TF) patterns (Table 3.4 and Figure 3.8). Type 1 and 2 patterns are considered normal and Type 3 and 4 abnormal, typically observed in subjects with dry eye (Rolando, 1984). According to this criterion, the TF technique has demonstrated good sensitivity and specificity for the diagnosis of dry eye (Norn, 1994, Vaikoussis et al., 1994, Maragou et al., 1996). Although a number of other grading scales have been suggested (Norn, 1994, Vaikoussis et al., 1994), Rolando's original scale is highly repeatable (Pensyl and Dillehay, 1998) and remains popular (Srinivasan et al., 2007).

Table 3.4 Rolando's tear ferning grading scale (Rolando, 1984)

Type		Characteristics
1	**Normal Tear Ferning**	uniform and closely branching arborisation
2		single, smaller ferns with less branching and empty spaces beginning to appear
3	**Abnormal Tear Ferning**	small ferns with almost no branching and numerous empty spaces
4		ferns absent and clusters of mucus may be present

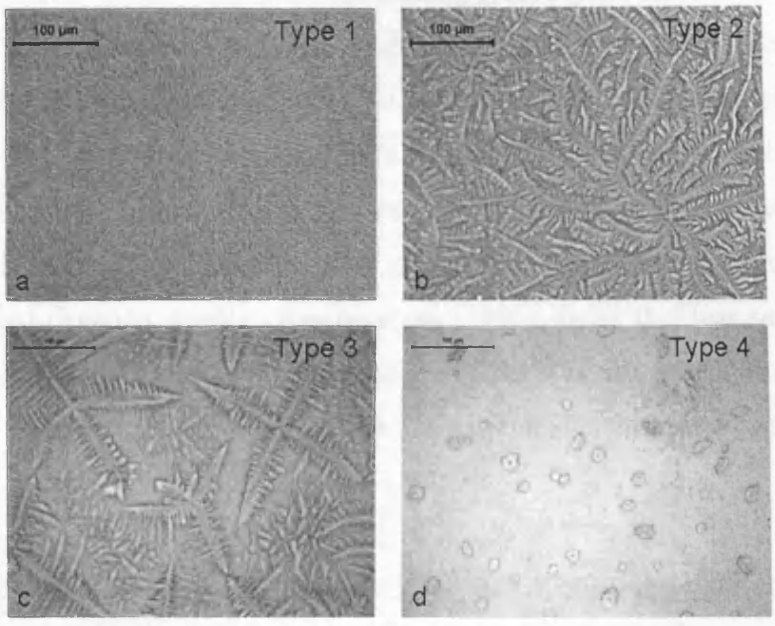


Figure 3.8 Tear Ferning Grades for the four ferning "types" defined by Rolando (1984)

TF methodology within the literature varies considerably and is summarised in Table 3.5 later in the chapter. Tear samples of capricious size have been collected from the fornix or tear meniscus with an assortment of implements such as spatulas (Tabbara and Okumoto, 1982), pipettes (Norn, 1987), loops (Filipello et al., 1992) and glass rods (Jackson and Perrigin, 1999). Norn (1988) concluded a greater repeatability of ferning grades could be achieved with glass capillary tubes (Norn, 1988). Golding and Brennan (1989) further recommended sampling a fixed volume of minimally stimulated tears from the inferior tear meniscus with a glass capillary (Golding and Brennan, 1989).

Collected tear samples were subsequently allowed to dry onto a clean, glass microscope slide nevertheless, the drying period, temperature for drying and the time for grading the sample after drying was inconsistent. Whilst the slide was typically viewed with phase contrast microscopy, magnification also varied from 10x - 400x (Srinivasan et al., 2007, Vaikoussis et al., 1994). Horwath et al. (2001) investigated

the effect of environmental factors, such as temperature and relative humidity (RH), on the reproducibility of Rolando's tear ferning grades (Horwath et al., 2001). It was concluded a temperature range of 20-26°C and RH not greater than 50% were necessary for ensuring ferning quality. The time for viewing the sample for grading once dried is also relevant. Whilst Norn found ferns were stable in the refrigerator (4°C) for a period of up to four days (Norn, 1988), it was suggested ferns should be examined five to ten minutes after the sample was placed on the microscope slide (Golding and Brennan, 1989). Kogbe et al. (1991) found that tear samples formed reproducible ferning patterns after storage at 4°C for a period of one month (Kogbe et al., 1991). However, reproducibility after storage at -80°C has not been investigated previously.

Increased levels of abnormal TF have been observed in subjects with CF (Kalayci et al., 1996, Rolando et al., 1988). However, it is apparent a number of factors needed to be investigated regarding the TF technique to produce a comprehensive protocol before TF could be investigated in subjects with CF. These include:

- Tear collection material
- The effect of magnification
- The effect of tear film sample volume
- The effect of stimulation
- Inter-eye comparisons
- The effect of diurnal variation
- The effect of tear film sample storage

Table 3.5 Summary of tear ferning methodology in the literature

Author	Collection method	Sample size (µl)	Magnification (X)	Sample Preparation
(Tabbara and Okumoto, 1982)	Conjunctival scrapings with platinum spatula		63	
(Rolando, 1984)	Lower fornix with spatula		40-100	Dried at 20°C ± 2°C
(Rolando et al., 1986)	Lower fornix with cataract loop		40-100	Dried at 20°C ± 2°C, graded within 5-7 minutes
(Kogbe and Liotet, 1987)	Glass capillary conjunctival cul de sac	2-3	40	Dried at 20°C for 5-7minutes
(Norn, 1987)	Pipette, inferior fornix		40-100	
(Norn, 1988)	Glass rod/pipette/capillary tube	Glass capillary 2.5	100	Dried at room temperature, viewed within 12 hours
(Rolando et al., 1988)	Cataract loop			
(Kogbe et al., 1991)	30-50µl stimulated tears Micropipette or capillary tube	2-4	40-100	Dried at room temperature for 5-7minutes
(Puderbach and Stolze, 1991)	Iron loop		100	Graded 5-7 minutes after collection
(Filipello et al., 1992)	Plastic loop, inferior fornix	4	40-100	Graded 6-7 minutes after collection
(Golding et al., 1994)	Onion-stimulated, glass pipette			

(Vaikoussis et al., 1994)	Lower fornix capillary tube		100-400	Graded within 15minutes of collection
(Norn, 1994)	Lacrimal meniscus, glass capillary	2.5		
(Kalayci et al., 1996)	Spatula		100 & 400	Dried at room temperature for 10 minutes
(Ravazzoni et al., 1998b)	Micropipette, inferior meniscus		40-100	Dried at 23°C ± 2°C for 5-10 minutes
(Pensyl and Dillehay, 1998)	Micropipette, non-stimulated	1	20	Graded within 10 minutes drying
(Jackson and Perrigin, 1999)	Glass rod, inferior palpebral cul de sac		10 & 40	
(Oguz et al., 2001)	2-3 µl capillary tube from lower fornix	2-3	400	Dried at room temperature
(Peponis et al., 2002)	Glass capillary, lateral part of inferior tear meniscus		40-100	Dried at room temperature for 5-10 minutes
(Peponis et al., 2004)	Lateral tear meniscus capillary		40-100	Dried at room temperature for 10 minutes
(Li et al., 2005)	2-3µl non-stimulated capillary pipette from inferior fornix		400	
(Versura et al., 2007)	Outer canthus, 5 µl micropipette			
(Srinivasan et al., 2007)	2µl inferior tear meniscus, glass capillary tube	0.5	10	Dried at room temperature

3.5.2 Method

3.5.2.1 Tear collection procedure

Tear fluid was collected from the right eye (and also the left eye in experiment 3.5.2.3.5) with a glass capillary tube by placing the tube tip at the outer two thirds of the inferior tear meniscus whilst the patient fixated away. Care was taken to avoid stimulation. Tear samples were aspirated into labelled 0.5ml Eppendorf tubes and centrifuged for 60 seconds at 4°C, 14,000 RPM to collect the tears at the bottom of the tube for ease of pipetting.

3.5.2.2 Slide preparation procedure

With the exception of experiment 3.5.2.4.3, 1.5µl tear samples were pipetted onto clean, glass microscope slides and allowed to air dry at 20-26°C and RH <50% (L55AJ Hygro Thermometer, Maplin, UK). Samples were observed at 20x magnification with a light microscope (Leica Microsystems GmbH, Wetzlar, Germany) and photographed within a 10 minute period of drying and categorised according to Rolando's grading scale (Rolando,1984).

3.5.2.3 Experimental design

3.5.2.3.1 Tear collection material

A 10µl tear sample was collected from a single subject. To investigate ferning in a number of solutions 5µl volumes of the tear sample, saline (Bausch & Lomb, Surrey, UK) and artificial tear solution (Vismed Light, TRB Chemedica Ltd., Staffordshire, UK) were each pipetted onto the tip of individual sponges and rods. The rods and sponges were prepared as in 3.2.2 and centrifuged for three minutes at 14,000 RPM. Slides were prepared for all three solutions, examined and photographed.

3.5.2.3.2 The effect of magnification

A single 1µl tear sample was prepared, observed and photographed at various magnifications (10, 20 and 40x) to identify which allowed for the greatest ease of grading.

3.5.2.3.3 The effect of tear film sample volume

Volumes of 2.5, 2.0, 1.5, 1.0, 0.5, and 0.25µl, from a single tear sample, were pipetted onto a labelled microscope slide. Observations were recorded to identify which tear sample volume allowed for the greatest ease of grading.

3.5.2.3.4 The effect of stimulation

Minimally stimulated tear samples were collected from the right eye of 10 subjects. Stimulated tear samples were subsequently collected by asking the subject to stare at a bright light to induce reflex tearing. 1.5µl samples were pipetted onto labelled microscope slides and graded to allow comparison.

3.5.2.3.5 Inter-eye comparisons

Tear samples were collected from each eye of 10 subjects. 1.5µl samples were pipetted onto labelled microscope slides and graded to allow comparison.

3.5.2.3.6 The effect of diurnal variation

Tear samples were collected from the right eye of 10 subjects in the morning, within a two hour period of waking. Tear samples were subsequently prepared and graded. This was repeated in the afternoon, following a minimum period of six hours, after which the TF grades from the two samples were compared.

3.5.2.3.7 The effect of tear film sample storage

A number of excess tear samples remaining from previous experiments were used to investigate the stability of tear samples stored at 4°C, twenty of these samples were refrigerated at 4°C and the TF pattern checked after overnight, one week and one month storage to allow comparison. To investigate storage at -80°C, 5µl tear samples were collected from 10 subjects and TF patterns were then recorded for each. The remaining samples were stored at -80°C for a period of one month. Samples were thawed at 4°C and centrifuged at 4°C, 14000RPM for 60 seconds, slides prepared and ferning patterns graded and compared to original patterns.

3.5.3 Results

3.5.3.1 Tear collection material

Microscope images of the three solutions collected by the various methods are shown in Figure 3.9. Considering the tear film samples; the glass capillary and sponge-collected tears appear similar, forming uniform and closely branched ferns typical of the Type 1 pattern (Figure 3.9a & c). The rod-collected tears show limited ferning more typical of the Type 3 pattern (Figure 3.9b). The saline samples collected with the capillary tube and sponge formed long "twig-like" crystals (Figure 3.9d & f); the rod-collected saline formed a clumped crystal arrangement however (Figure 3.9e). Considering the Vismed light samples the difference is more apparent. Whilst the capillary and sponge samples produced regular "fan-like" formations (Figure 3.9g & i), the rod-collected sample formed a "bubble-like" crystal pattern (Figure 3.9h).

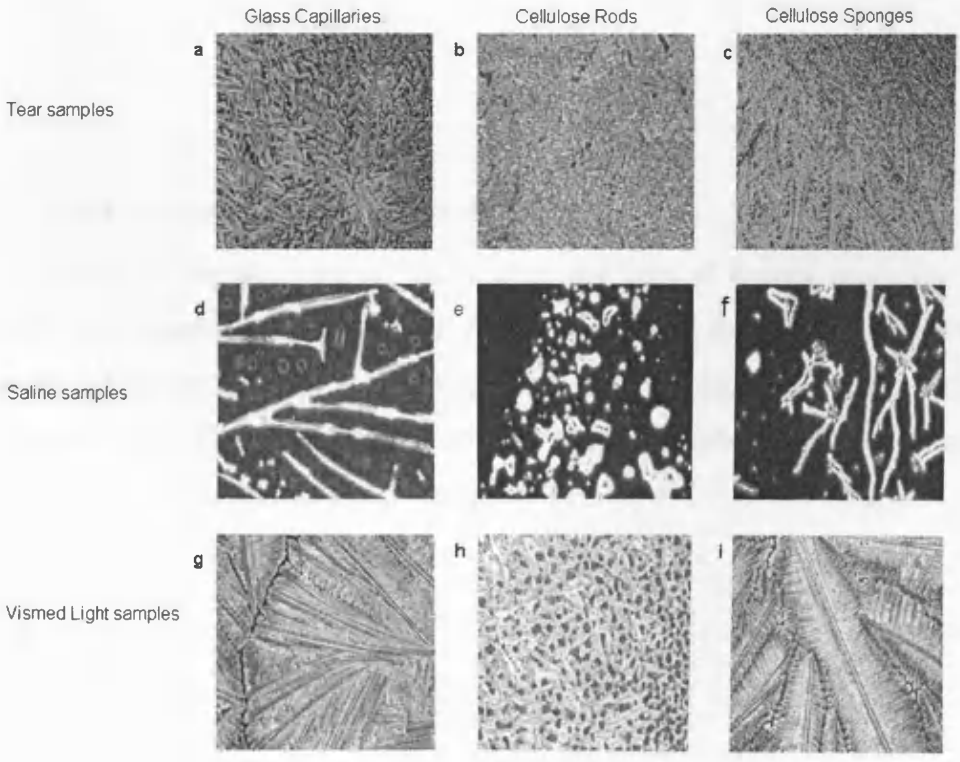


Figure 3.9 Ferning images of the solutions collected with the three different materials

3.5.3.2 The effect of magnification

TF images photographed at different magnifications are shown in Figure 3.10 (Figure 3.10). Ideal magnification of the tear ferns was observed at 10x or 20x magnification. Above this point there was too little of the sample within the field of view to allow precise grading. With escalating magnification the images appeared increasingly dissimilar to Rolando's grading scale (Figure 3.8) and hindered accurate grading.

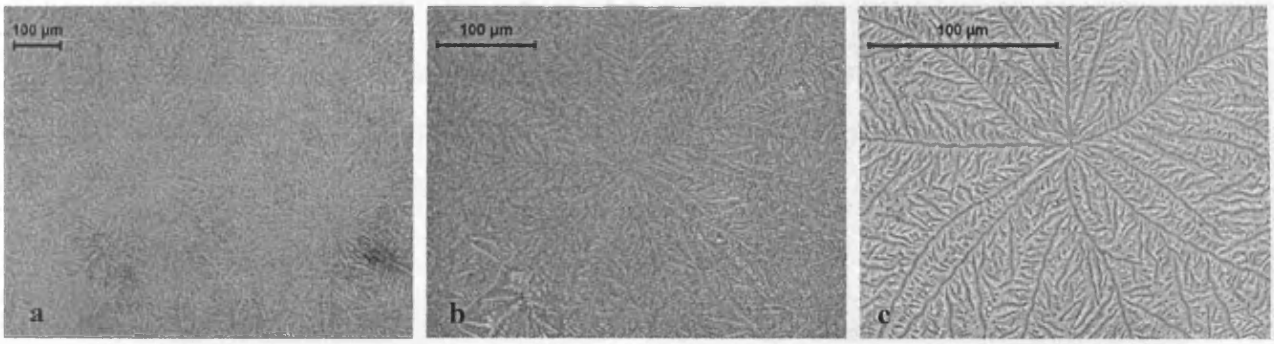


Figure 3.10 Tear ferning pattern from a single tear sample imaged at different magnifications; 10x (a), 20x (b), 40x (c)

3.5.3.3 The effect of tear film sample volume

The smaller the tear sample volume, the smaller the area of ferning available to examine. It was observed the TF pattern varied throughout the sample area, with ferning at the periphery typically inconsistent with ferning within the centre of the sample (Figure 3.11). Figure 3.12 shows smaller sample volumes gave less central area within the field of vision impairing assessment.

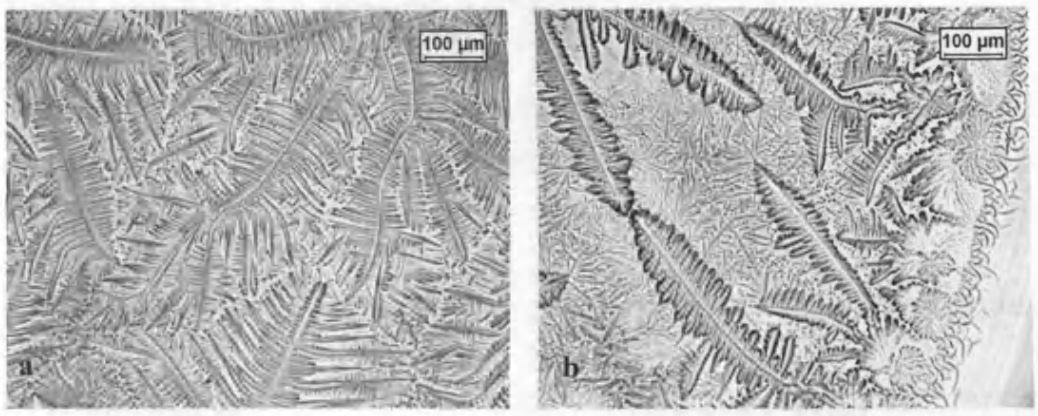


Figure 3.11 The difference in ferning pattern from a single tear sample imaged at the centre indicating type 2 ferning (a); and the periphery indicating type 3 ferning (b)

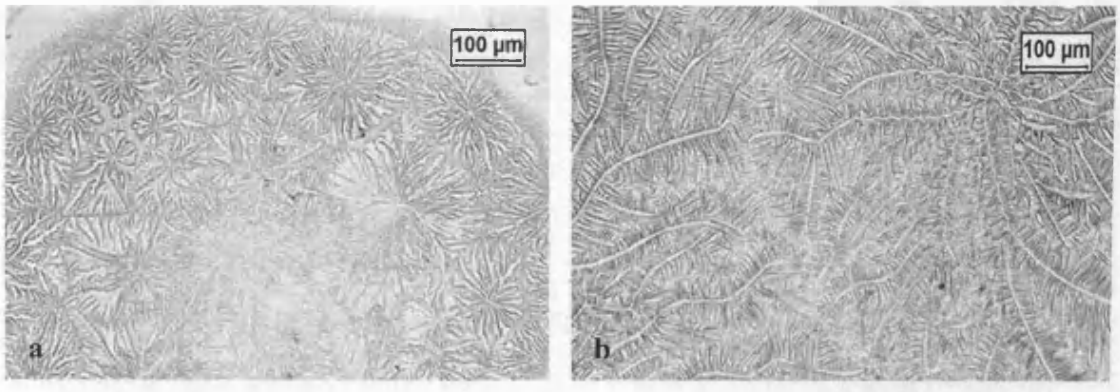


Figure 3.12 Tear ferning patterns from an identical tear sample of 0.25µl (a) and 1.0µl (b) volume. Both samples indicate type 2 ferning although the larger volume has a greater central area within the field of view allowing easier grading

3.5.3.4 The effect of stimulation

The ferning patterns for stimulated and non-stimulated tears were identical for all 10 subjects as listed in Table 3.6. Therefore, there appeared to be no effect of stimulation on the TF pattern.

Table 3.6 The comparison of tear ferning patterns in stimulated and non-stimulated tear samples

Sample number	Tear ferning grade (Type)	
	Non-stimulated tear sample	Stimulated tear sample
1	1	1
2	1	1
3	2	2
4	1	1
5	1	1
6	1	1
7	1	1
8	2	2
9	1	1
10	1	1

3.5.3.5 Inter-eye comparisons

Table 3.7 indicates TF grades were identical for each eye of the 10 subjects.

Table 3.7 Inter-eye comparisons of tear ferning patterns

Sample number	Tear ferning grade (Type)	
	Right Eye	Left Eye
1	2	2
2	1	1
3	1	1
4	1	1
5	1	1
6	2	2
7	1	1
8	1	1
9	1	1
10	1	1

3.5.3.6 The effect of diurnal variation

The TF patterns were identical at both time intervals for all subjects (Table 3.8).

Table 3.8 Diurnal variations in tear ferning patterns

Sample number	Tear ferning grade (Type)	
	Morning tear collection (8:30 – 10:30am)	Afternoon tear collection (2:30 – 4:30pm)
1	1	1
2	1	1
3	2	2
4	1	1
5	1	1
6	1	1
7	1	1
8	1	1
9	1	1
10	2	2

3.5.3.7 The effect of tear film sample storage

With increasing observer experience it became apparent TF patterns could be subdivided into 0.5 increments of Rolando's grading scale. There was no alteration in the pattern following overnight storage (Table 3.9). After a week at 4°C two tear samples (10%) had altered. A greater change was observed for samples stored for a period of one month, where a change in ferning was observed in 45% of samples. A change of normal to abnormal grading was observed in two (10%) samples. No difference in TF patterns was observed for the samples stored at -80°C for a period of one month (Table 3.10).

Table 3.9 The effect of storage at 4°C on tear ferning patterns

Sample number	Tear ferning grade (Type)			
	Original	Overnight storage	One week storage	One month storage
1	2	2	2	2
2	2	2	2	2
3	1	1	1	2*
4	1	1	1	2*
5	1	1	1	1
6	1	1	1	1
7	1	1	1	1
8	1	1	1	1
9	1	1	1	1
10	1	1	1	2
11	1	1	1.5*	2*
12	2	2	2	2.5**
13	1	1	1	1.5*
14	1	1	1	1
15	1	1	2*	2
16	1	1	1	2*
17	2	2	2	2
18	2	2	2	3**
19	1	1	1	2*
20	1	1	1	1.5*

* change in tear ferning pattern

** tear ferning pattern changed from normal to abnormal

Table 3.10 The effect of storage at -80°C on tear ferning patterns

Sample number	Tear ferning grade (Type)	
	Initial	Following storage
1	1	1
2	1.5	1.5
3	1	1
4	1	1
5	2	2
6	1	1
7	1	1
8	1	1
9	2	2
10	1	1

3.5.4 Discussion

The rod collected samples produce atypical ferning results compared to the "gold standard" glass capillaries. The TF pattern is believed to be determined by the proportion of various ions and macromolecules within the tear film (Pearce and Tomlinson, 2000). After centrifuging the rods the biochemical composition of the sample appears to have been altered, perhaps by retaining certain components within the cellulose matrix and adversely affecting the ferning pattern. Although a considerably greater proportion of the sample is retained within the cellulose sponge, the composition is unaffected allowing for consistent ferning patterns when compared with glass capillary collected samples. Therefore, cellulose sponges appear to be a suitable alternative to glass capillary tubes to collect tear samples in children for the tear ferning technique.

A wide variety of microscope magnifications used to image tear ferns have been quoted in the literature, ranging from 10-400x (Table 3.3). It was observed that increasing the magnification beyond 20x actually hindered accurate grading by reducing the field of view of the ferns. A number of recent publications have similarly suggested either 10x or 20x (Srinivasan et al., 2007, Jackson and Perrigin, 1999, Pensyl and Dillehay, 1998).

Kogbe et al. (1991) investigated the effect of sample droplet size on tear ferning and whilst they concluded droplets smaller than 4µl dried rapidly and uniformly, they did not investigate the effect on sample grading (Kogbe et al., 1991). As cellulose sponges are particularly inefficient for sample return when centrifuged it was reassuring that small sample volumes still allowed for accurate tear ferning. It was observed the ferning pattern varied from the centre to the periphery. To maintain consistency, it was concluded grading should be assessed in the centre of the droplet. Although adequate grading could be achieved for a minimum volume of 0.5µl a larger droplet of at least 1.0-1.5µl is preferable due to the larger ferning area within the centre of the droplet available for observation.

Stimulated tears have been used previously to investigate factors responsible for tear ferning and x-ray and scanning electron microscopy of tear ferns (Kogbe et al., 1991,

Golding et al., 1994). Due to the poor efficiency of sample return by the cellulose sponges it may be essential to stimulate patients in order to collect sufficient volumes for TF. This study showed no alteration of the ferning pattern following tear stimulation in these subjects. The TF grade is thought to be the consequence of the ratio of inorganic salt to macromolecule (protein and mucin) concentration within the tear sample (Golding and Brennan, 1989, Golding et al., 1994, Pearce and Tomlinson, 2000). Whilst the concentration of the major lacrimal gland proteins, such as lysozyme and lactoferrin, are unaltered by tear stimulation (Fullard and Tucker, 1991, Kijlstra et al., 1983, Fullard and Snyder, 1990, Jones et al., 1997, Sitaramamma et al., 1998) a significant increase in the serum derived proteins, such as IgG and tansferrin, has been observed (Fullard and Snyder, 1990). A number of mucins are known to be secreted by the lacrimal gland (Paulsen and Berry, 2006), although alteration of concentration following tear stimulation is unknown. The crystallisation process that forms the tear fern is the product of water subtraction (Battaglia Parodi and Giusto, 1993). An equal increase in the tear film aqueous phase, serum derived protein and possibly mucin concentration following stimulation may allow the salt to macromolecule concentration to be identical to that prior to stimulation. Norn similarly observed no difference in stimulated and non-stimulated tear ferning patterns (Norn, 1988). However, his choice of stimulation method (inhalation of ammonia vapour) would not be suitable for use in children.

No inter-eye differences in TF were observed. A similar finding was reported in an investigation of tear ferning in subjects with Down's syndrome (Filipello et al., 1992). Therefore, only a single tear sample needs to be collected from the child to determine the tear ferning grade. Ocular comfort is known to be subject to diurnal variation in patients with dry eye (Begley et al., 2002), suggesting that the open-eye state significantly affects the tear film and associated ocular comfort. However, there were no diurnal effects on the tear ferning pattern within the six hour tear collecting period of this study.

Kogbe et al. (1991) successfully stored tears for one month at 4°C without altered tear ferning (Kogbe et al., 1991). However in our investigation, after a period of one week, the tear ferning pattern of a number of tear samples had altered. Tear samples were significantly altered after a period of one month, with two samples actually

changing from normal to abnormal grades. Tear samples could be safely stored for a period of one month at -80°C however. It was also noted that with increasing observer experience of the tear ferning patterns, smaller increments of Rolando's grading scale could be used.

3.6 Summary

Cellulose sponges are a suitable material for tear collection in children. They are comfortable for the patient and highly absorbent but unfortunately offer poor efficiency when centrifuged for sample release. Sponges can be centrifuged for three minutes at 14000 RPM at 4°C for maximum sample return. The collected tear sample can be stored at -80°C either pre- or post-centrifuging. The sponges do not appear to alter the chemical composition of the tear sample, unlike the cellulose rods, and are a suitable alternative to glass capillary tubes for the TF technique.

The TF protocol was finalised. The tear sample can be collected from a single eye with a cellulose sponge, stimulation is acceptable and collection time is not a factor. A sample of at least 1µl should be pipetted onto a microscope slide and allowed to air dry at 20-26°C and RH <50%. Tears can be stored overnight at 4°C and up to a month at -80°C. The sample should be viewed at objective magnification x20 and graded at the centre of the droplet within a 10 minute period of drying.

Chapter 4

Investigating Dry Eye and Ocular Surface Inflammation

4.1 Introduction

Ocular surface inflammation contributes to the pathogenesis of dry eye. Increased tear film cytokine concentration, indicating sub-clinical levels of ocular surface inflammation, have been observed in subjects with CF previously (Mrugacz et al., 2006a, Mrugacz et al., 2006c). However, quantitative analysis of the tear film requires sophisticated biochemical techniques and the methodology typically requires development and refinement. Before implementing tear film investigations in subjects with CF it was important to practise these techniques and optimise the methodology to compare subjective and objective tests of ocular surface inflammation in a group of readily available subjects who could simulate the hypothesised dry eye anticipated in the CF patients.

Contact lens (CL) wearers are ideal candidates: in a recent survey, 43% of hydrogel CL wearers reported symptoms of CL-related dry eye (Guillon and Maissa, 2005). Symptoms of discomfort and drying are regularly cited as the principal cause of CL wear drop out (Pritchard et al., 1999), and subjective symptoms of drying are reported in much higher frequency compared to objective, clinical signs of dry eye (Begley et al., 1999). Alterations of various tear film constituents indicate sub-clinical levels of ocular inflammation following CL wear (Thakur and Willcox, 2000), even in asymptomatic subjects (Pisella et al., 2001).

Therefore, the aim of the study was to investigate the relationships between tear film cytokine concentration, tear fernin and traditional tear film stability tests amongst experienced contact lens wearers and non-contact lens wearers, expressing varying degrees of ocular comfort. A selection of the results from this study have been published in a peer reviewed journal (see Appendix).

4.1.1 Tear ferning

Tear ferning (TF) is a sensitive diagnostic dry eye test (Vaikoussis et al., 1994, Maragou et al., 1996, Norn, 1994). The technique has also demonstrated impressive sensitivity and specificity (78.95% and 78.35% respectively) for the prediction of successful contact lens (CL) tolerance in new CL wearers (Ravazzoni et al., 1998). The authors concluded that whilst CL patients with TF Types of 2, 3 and 4 often encountered tolerance problems, those with Type 1 TF patterns displayed good tolerance over a six month wearing period. Despite its apparent value, TF remains a relatively uncommon diagnostic test amongst traditional examinations of the tear film, and TF in *habitual* CL wearers has not been investigated previously.

4.1.2 Cytokines

The function of the immune system is to recognise the presence of a pathogen and eliminate infection and is essential for survival (Parham, 2000). The leukocytes (white blood cells) that mediate the immune response are shown in Figure 4.1. Cytokines are a collection of signalling molecules that promote communication between cells of the immune system (Hamblin, 1993). A group of cytokines, known as Interleukins, are released by certain leukocytes and exert a specific effect on other leukocytes. Cytokine activity can be autocrine (act on itself) or paracrine (act locally on another type of cell) (Meager, 1990). Individual cytokines are secreted by a number of different cells of the immune system and similarly, their receptors are found on a number of different cell types. Consequently, there is frequent overlap of their biological activities. Individual cytokines have several different and apparently unrelated functions, and can be responsible for both harmful and beneficial effects during the immune response (Playfair and Chain, 2001). Inflammation is an important procedure in this response and cytokines are known to mediate the inflammatory process.

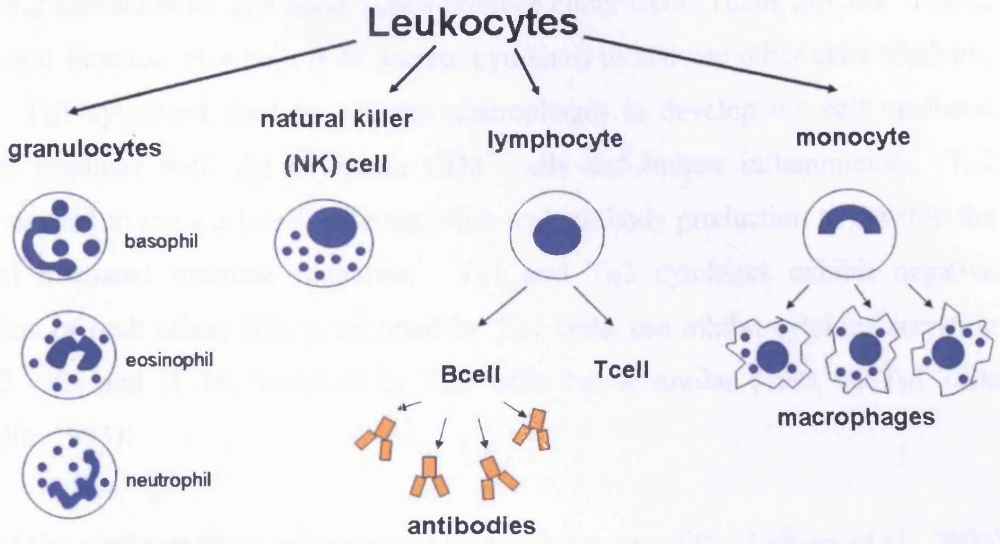


Figure 4.1 A schematic diagram of the leukocytes involved in the immune response

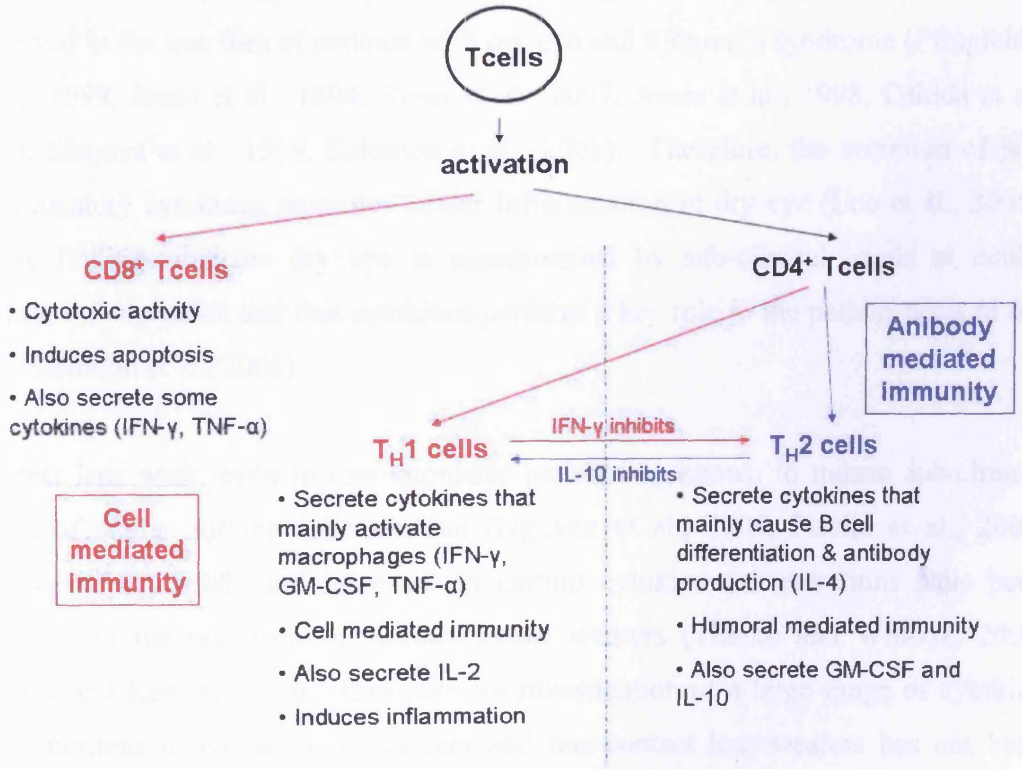


Figure 4.2 T_H1 and T_H2 cytokines

Figure 4.2 demonstrates activated Tcells produce either $CD8^+$ Tcells or $CD4^+$ Tcells; the general function of which is to secrete cytokines to activate other cells (Parham, 2000). T_H1 cytokines tend to activate macrophages to develop the cell mediated immune response with the cytotoxic $CD8^+$ cells and induce inflammation. T_H2 cytokines tend to induce Bcell differentiation and antibody production to develop the humoral mediated immune response. T_H1 and T_H2 cytokines exhibit negative regulation of each other; IFN-γ, secreted by T_H1 cells, can inhibit cytokine secretion by T_H2 cells and IL-10, secreted by T_H2 cells has a similar effect on T_H1 cells (Hamblin, 1993).

Cells of the ocular surface secrete cytokines into the tear film (Uchino et al., 2006) (Malvitte et al., 2007) consequently, tear film cytokine concentrations reflect levels of *in vivo* ocular surface inflammation (Leonardi et al., 2006). Hyperosmotic stress has been demonstrated to function as a stimulant for inflammatory cytokine production (Shapiro and Dinarello, 1997) and elevated cytokine concentrations have been observed in the tear film of patients with dry eye and Sjögren's syndrome (Pflugfelder et al., 1999, Jones et al., 1994, Yoon et al., 2007, Jones et al., 1998, Oshida et al., 2004, Slepova et al., 1998, Solomon et al., 2001). Therefore, the secretion of pro-inflammatory cytokines promotes ocular inflammation in dry eye (Luo et al., 2005). These findings indicate dry eye is accompanied by sub-clinical levels of ocular surface inflammation and that cytokines perform a key role in the pathogenesis of dry eye (Solomon et al., 2001).

Contact lens wear, even in asymptomatic patients, is known to induce sub-clinical levels of ocular surface inflammation (Nguyen et al., 2004, Pisella et al., 2001, Versura et al., 2000) and increases in certain cytokine concentrations have been observed in the tear film of contact lenses wearers (Thakur and Willcox, 2000, Schultz and Kunert, 2000). However, an investigation of a large range of cytokine concentrations in contact lens wearers and non-contact lens wearers has not been investigated previously.

Cytokines also mediate airway inflammation observed in subjects with CF and evidence suggests that cytokine production is altered or dysregulated in CF (Sagel and Accurso, 2002). The concentrations of three tear film cytokines, IFN-γ, IL-8 and

MIP-1α, have been found to be up-regulated in tear film samples from subjects with CF compared to controls (Mrugacz et al., 2005c, Mrugacz et al., 2006a, Mrugacz et al., 2007d). However, there has been no previous investigation of a wide range of different cytokine concentrations in the tear film of subjects with CF.

4.1.2.1 Luminex cytokine analysis

Enzyme-Linked Immunosorbent Assay (ELISA) and Cytometric Bead Array (CBA) are biochemical techniques traditionally employed to quantify cytokine concentrations in tear film samples (Kallinikos et al., 2006, Solomon et al., 2001, Sonoda et al., 2006). However, multicytokine assays are becoming more popular (Uchino et al., 2006, Uchino et al., 2005). The Luminex 100 analyser (R&D Systems Europe Ltd, Abingdon, UK) can measure multiple cytokines simultaneously in small sample volumes. Specific sample cytokines bind to spectrally distinct micro-particle beads; each is then coated with a capture specific antibody and streptavidin-phycoerythrin (method shown in Figure 4.3). The analyser consists of two lasers; the first is bead specific and identifies the analyte, the second laser then detects the amount of bound analyte. The system can quantify selected cytokines with a sensitivity range of approximately 0-32,000 picograms/millilitre (pg/ml). The Luminex system is a suitable alternative method to ELISA and has a number of advantages such as smaller sample volume and lower cost (Dupont et al., 2005). Recently, Malvitte and colleagues successfully used the Luminex to analyse very small tear samples (2μl) from patients treated with topical drugs (Malvitte et al., 2007).

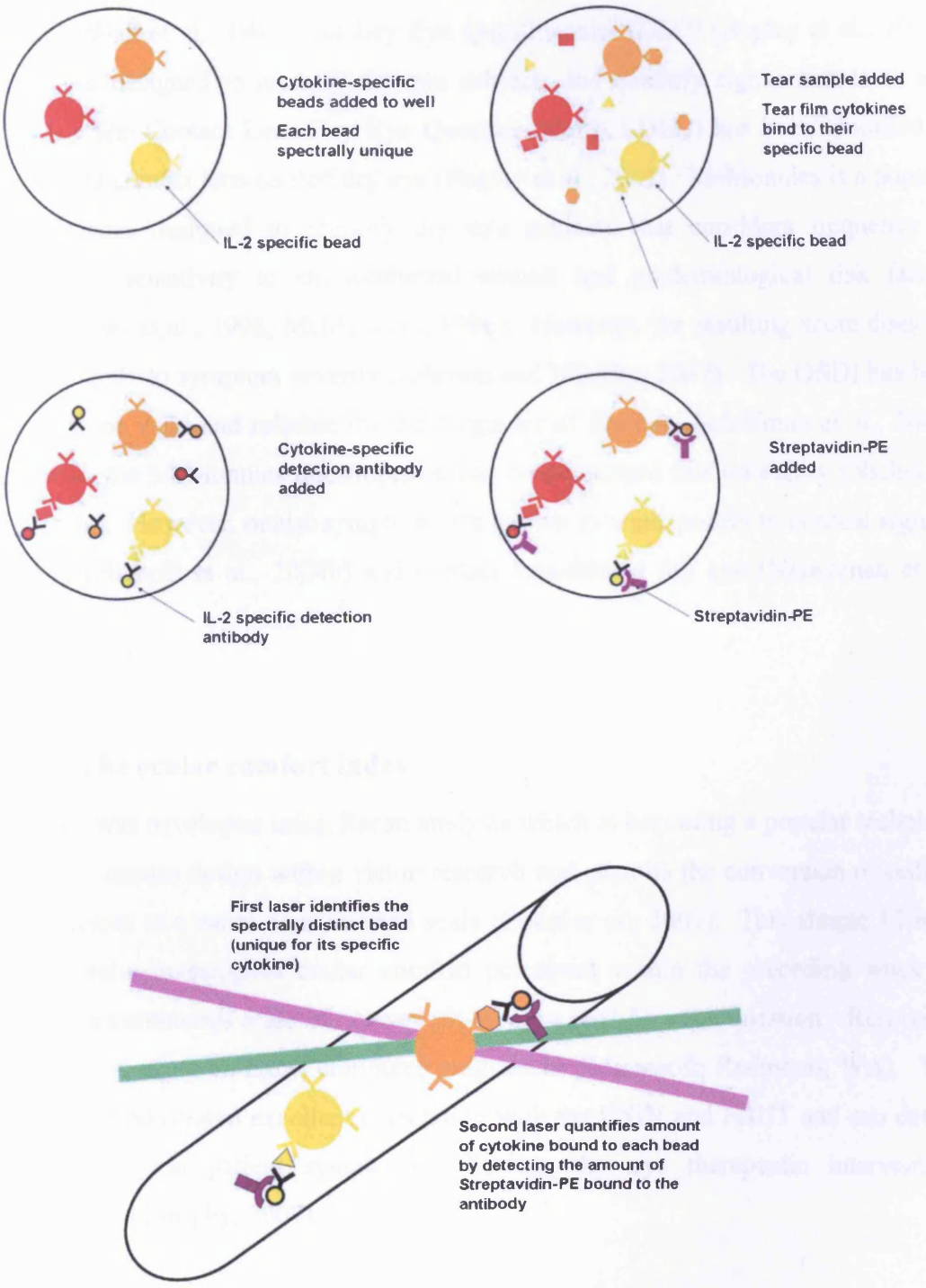


Figure 4.3 Simplified schematic of the Luminex methodology, adapted from www.rndsystems.com

4.1.3 Dry eye questionnaires

A number of dry eye questionnaires, including the Ocular Surface Disease Index (OSDI) (Walt et al., 1997) and Dry Eye Questionnaire (DEQ) (Begley et al., 2001), have been designed to identify dry eye subjects and quantify signs, symptoms and stimuli. The Contact Lens Dry Eye Questionnaire (CLDEQ) has been modified to investigate contact lens-related dry eye (Begley et al., 2001). McMonnies is a popular questionnaire designed to identify dry eye subjects that considers frequency of symptoms, sensitivity to environmental stimuli and epidemiological risk factors (McMonnies et al., 1998, McMonnies, 1986). However, the resulting score does not relate directly to symptom severity (Johnson and Murphy, 2007). The OSDI has been shown to be valid and reliable for the diagnosis of dry eye (Schiffman et al., 2000). Likewise, the McMonnies questionnaire has demonstrated fair reliability (Nichols et al., 2004c). However, ocular symptoms are known to relate poorly to clinical signs of dry eye (Nichols et al., 2004b) and contact lens-related dry eye (Narayanan et al., 2005).

4.1.3.1 The ocular comfort index

The OCI was developed using Racsh analysis which is becoming a popular technique for questionnaire design within vision research and permits the conversion of ordinal Likert scores to a continuous interval scale (Court et al., 2007). This simple 12 item questionnaire investigates ocular comfort perceived within the preceding week by utilising a continuous scale of "Never (0)-Always (6)" for each question. Responses are analysed using an Excel computer programme (Microsoft; Redmond, WA). The OCI has demonstrated excellent correlation with the OSDI and FBUT and can detect improvements in patient symptoms following dry eye therapeutic intervention (Johnson and Murphy, 2007).

4.1.4 Tear film stability

Tear break-up time (BUT) is typically used to assess tear film stability and is defined as the time interval for the occurrence of interruptions to the pre-corneal tear film following a blink (Norn, 1969). Two distinct methods can be employed; invasive, where sodium fluorescein is instilled into the tear film, and non-invasive, where a grid or other image is projected onto the tear film and observed.

4.1.4.1 Fluorescein break-up time

To assess the fluorescein tear break-up time (FBUT) a small volume of sodium fluorescein, from either a minim or strip pre-moistened with saline, is instilled into the tear film. Ocular surface fluorescence is observed with a slit lamp and cobalt blue filter. The subject is instructed to blink and the time recorded for dark, dry spots to develop on the cornea recorded as the FBUT. Typically, values below 10 seconds are considered abnormal and indicative of dry eye (Lemp et al., 1970, Lemp et al., 1971).

Clinically, FBUT remains a popular diagnostic test (Korb, 2000, Nichols et al., 2000, Smith et al., 2008) and has been used successfully to predict contact lens intolerance (Andres et al., 1987). When applying a cut off value for the diagnosis of dry eye as less than 10 seconds, Vitali et al. reported a sensitivity and specificity of 77.8% and 38.9% respectively (Vitali et al., 1994). The technique is considered an invasive assessment as fluorescein instillation is known to reduce tear film stability (Mengher et al., 1985). FBUT is dependant on the volume of fluorescein instilled (Johnson and Murphy, 2005) and results are frequently variable and unrepeatable (Mengher et al., 1985, Vanley et al., 1977). Therefore, to improve diagnostic reliability a published methodology was followed whereby a fixed amount of fluorescein is delivered into the inferior fornix with minimal stimulation using a micro-pipette (Johnson and Murphy, 2005).

4.1.4.2 Non-invasive break-up time

Estimation of the non-invasive tear break-up time (NIBUT) involves the observation of a projected image onto the pre-corneal tear film, whereby distortion of the image indicates localised thinning of the tear film. Frequently, the image of a keratometer mires have been utilised (Patel et al., 1985). The technique has demonstrated sensitivity and specificity of 82% and 86% respectively when applying cut off value of less than 10 seconds for the diagnosis of dry eye (Mengher et al., 1986). A commercial instrument, the Tearscope Plus (Keeler Ltd., Windsor, Berkshire) utilises a grid to allow indirect NIBUT assessments (Guillon, 1998). Elliott et al. (1998) reported a greater repeatability with the device compared to the fluorescein break-up time (Elliott et al., 1998).

4.2 Method

4.2.1 Subjects

Measurements were recorded from the right eyes of 60 young, healthy subjects (17 male and 43 female, mean age 23.2 ± SD 4.8 years). Thirty six subjects were non-contact lens (NCL) wearers (11 males, 25 females) and 24 habitual hydrogel contact lens (CL) wearers (6 males, 18 females). CL wearers wore contact lenses for a minimum of three days per week for a period of at least six months; contact lenses were removed one hour prior to tear film tests. Subjects were excluded if they had any other ocular or systemic condition known to affect the tear film, including pregnancy, conjunctivitis and blepharitis. Informed consent was obtained from all subjects; favourable approval had been obtained from the Human Science Research Ethics Committee, School of Optometry and Vision Sciences, Cardiff University and all procedures conformed to the tenets of the Declaration of Helsinki.

4.2.2 Experimental design

Non-invasive tear break up time (NIBUT), measured with the Tearscope Plus (Keeler, UK), was recorded as the time taken after three natural blinks to disruption of the projected grid mires of the pre-corneal tear film (Guillon, 1998). The median of three readings was recorded for each subject. Using glass capillary tubes, up to 5µl non-stimulated tear samples were collected from the inferior tear meniscus within a five minute period (to minimise stimulation) and expelled into 1.5ml eppendorf tubes. Tear samples were prepared and TF graded according to the protocol described in Chapter 3. Subjects then completed the Ocular Comfort Index (OCI) Questionnaire. Remaining tear samples were stored at -80°C for cytokine analysis.

After a recovery period of 10 minutes following tear collection, fluorescein tear break up time (FBUT) was recorded following instillation of 1.0µl 2% sodium fluorescein with a micropipette into the inferior fornix. Care was taken to avoid contact with the ocular surface or eyelid margin. The tear film was scanned for indications of disruption during the recording period using a slit lamp (SL.2F, Topcon, Tokyo,

Japan); x16 magnification, 10mm wide beam with cobalt blue filter. FBUT was recorded as the time taken after three blinks before disruption of the pre-corneal tear film and the median of three recordings was recorded for each subject. For both BUT measurements, subjects were reminded not to stare but hold their eyes open naturally. Measurement was repeated if the subject blinked before tear film break-up was observed.

4.2.3 Cytokine analysis

The tear film concentration of eight different cytokines, available as a pre-prepared multi-plex assay panel (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK), was assessed. An overview of different cytokine sources and effects is shown in Table 4.1. Sufficient tear film samples for cytokine analysis were obtained from 31 subjects (9 male, 22 female); 18 were NCL wearers (6 male, 12 female) and 13 CL wearers (3 male, 10 female). Tear film samples were thawed at 4°C and 2µl of each was diluted with 48µl of phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) giving a 1:25 dilution. 50µl PBS BSA solution was used as a negative control. The plate design is shown in Figure 4.4.

120µl stock beads were diluted with 2880 µl Bio-Plex assay buffer. Forty wells of a 96-well filter plate were each wetted with 100µl of assay buffer and placed on a calibrated filter plate vacuum manifold (pressure 1-2" Hg) to remove the buffer. 50µl of vortexed bead solution was added to each well and the plate vacuum filtered. 100µl Bio-Plex wash buffer (Bio-Rad) was added to each well, removed by vacuum filtration and repeated.

500µl PBS containing 1% BSA was pipetted into the cytokine standard, vortexed and incubated on ice for 30 minutes. Eight 1.5ml Eppendorf tubes were labelled and 12.8µl of standard added to the first tube containing 187.2µl of PBS containing 1% BSA and vortexed. Serial dilutions of the standard were prepared and vortexed after each dilution (shown in Figure 4.5).

Table 4.1 An overview of the principle cellular sources and effects of the eight cytokines analysed in the investigation (Hamblin, 1993, Mire-Sluis and Thorpe, 1998, Parham, 2000)

Cytokine	T_H group	Sources	Effects
IL-2 Interleukin-2	T_H1	Activated Tcells	Stimulates proliferation and cytotoxic activity of activated CD8 Tcells
IL-4 Interleukin-4	T_H2	T cells, eosinophils, basophils	Bcell proliferation and differentiation
IL-6 Interleukin-6	T_H2	Monocytes/macrophages	Stimulates Bcell antibody production
IL-8 Interleukin-8 (chemokine)	-	Monocytes/macrophages, lymphocytes	Neutrophil chemotaxis/activation, trigger basophils, Tcell chemotaxis, macrophage production
IL-10 Interleukin-10	T_H2	Monocytes, Tcells, Bcells	Inhibit pro-inflammatory cytokine production. Inhibition of IL-2 production by Tcells
GM-CSF Granulocyte-Macrophage Colony Stimulating Factor	$T_H1>T_H2$	T lymphocytes, macrophages	Stimulates granulocytes and macrophages
IFN-γ Interferon-γ	T_H1	Leukocytes	Macrophage and Tcell activation
TNF-α Tumour Necrosis Factor-α	T_H1 and T_H2	Macrophages, Tcells	Cytotoxic for tumour cells, antiviral/antibacterial activity, activates macrophages

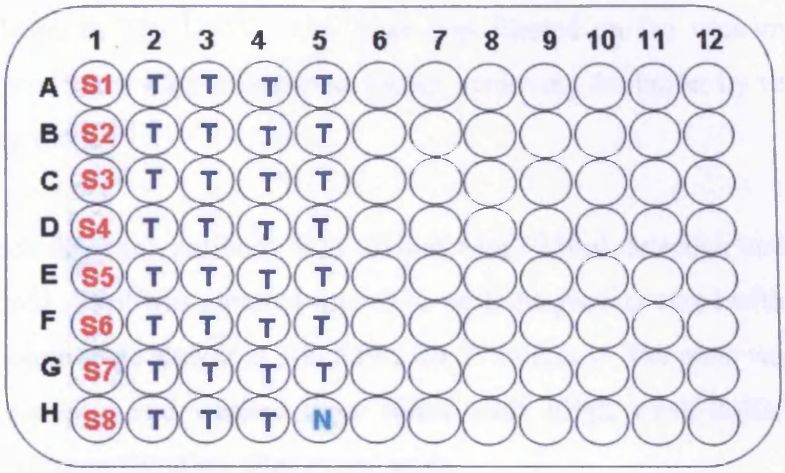


Figure 4.4 Diagram of 96 well plate design (S = standard, T = tear film sample, N = negative control)

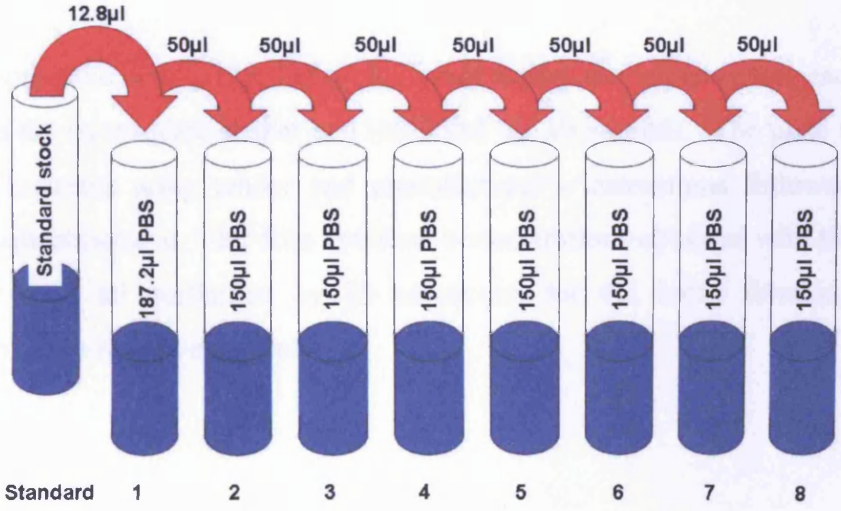


Figure 4.5 Diagram of the serial dilution procedure used to generate the standard curve

50µl of each sample or standard was pipetted per well. The plate was covered with aluminium foil, placed onto a micro-plate shaker and incubated at room temperature for two hours at 300 RPM. The plate was filtered on the vacuum manifold and washed three times with 100µl wash buffer, removing the buffer by vacuum filtration after every wash.

150µl stock detection antibody was diluted with 1350µl detection antibody diluent A and vortexed. 25µl was pipetted into each well, the plate covered with foil and placed onto the micro-plate shaker at 300 RPM for 30 minutes. The plate was filtered on the vacuum manifold and washed three times with 100µl wash buffer, removing the buffer by vacuum filtration after every wash.

30µl Streptavidin-PE was diluted with 2970µl Bio-Plex assay buffer and vortexed. 50µl was pipetted into each well, the plate covered with foil and incubated on the micro-plate shaker at 300 RPM for 10 minutes at room temperature. The plate was filtered on the vacuum manifold and washed three times with 100µl wash buffer, removing the buffer by vacuum filtration after every wash.

To re-suspend the beads, 125µl Bio-Plex assay buffer was pipetted into each well and placed on the micro-plate shaker at 1100 RPM for 30 seconds. The plate was loaded into the Luminex array reader and manufacturer's instructions followed to allow cytokine quantification. Tear film cytokine concentrations obtained with the Luminex Analyser were all multiplied by 25 to correct for the initial dilution, with the exception of the negative control.

4.2.4 Statistical analysis

Results were not normally distributed (Kolmogorov-Smirnov; $0.070<p<0.200$) with the exception of tear film stability ($p<0.05$; NIBUT and FBUT). Therefore, non-parametric and parametric statistical analysis was applied as appropriate. An Independent-samples t-test (2-tailed) was used to compare tear film stability between CL and NCL wearers and the Mann-Whitney U test (2-tailed) was used to compare cytokine concentration, TF and ocular comfort between the two groups. Pearson's product-moment correlation coefficient or Spearman's rank order correlation was used as appropriate to investigate the relationship between tear film tests and ocular comfort. Significance was set at the 0.05 level.

4.3 Results

4.3.1 Tear ferning

The effect of gender was not significant across all tests ($0.099<p<0.982$). Significantly higher grades of TF pattern and ocular discomfort (higher OCI scores) were observed in CL wearers compared to NCL wearers (Mann-Whitney U test, $p<0.005$ and $p<0.05$ respectively) (Table 4.2). However, the difference in tear film stability between the two groups was not significant (Independent-samples t-test; NIBUT, p=0.752; FBUT, p=0.243) (Table 4.3).

Table 4.2 Results for tear ferning (TF) and ocular comfort index (OCI) scores for non-contact lens (NCL) and contact lens (CL) wearers

Diagnostic Test	**NCL Wearers** median	**CL Wearers** median	**Sig. (Mann-Whitney U Test)**
TF (Type)	1.25 IQR 1.00-1.750	2.13 IQR 1.50-2.50	0.001*
OCI (score)	31.07 IQR 26.97-34.33	34.02 IQR 31.10-37.54	0.044*

*statistically significant at 0.05 level

Table 4.3 Results for non-invasive break up time (NIBUT) and fluorescein break-up time (FBUT) for non-contact lens (NCL) and contact lens (CL) wearers

Diagnostic Test	**NCL Wearers** mean	**CL Wearers** mean	**Sig. (Independent-sample t-test)**
NIBUT (secs)	9.61 SD ± 2.45	9.28 SD ± 4.64	0.752
FBUT (secs)	7.34 SD ± 3.78	6.33 SD ± 2.86	0.243

Using Rolando's criteria of abnormal TF above Type 2, Figure 4.6 indicates 14% (5 subjects) of NCL wearers and 50% (12 subjects) of CL wearers exhibited abnormal TF. TF of Type 3 or above was observed in 8% (2 subjects) of CL wearers (Fig. 4.7). The highest TF pattern observed in the NCL subjects was Type 2.75, recorded in a single subject. For all subjects, there was poor correlation between TF and NIBUT

(Spearman's; r=0.140, p=0.287), FBUT (Spearman's; r=-0.053, p=0.690) and OCI scores (Spearman's; r=0.002, p=0.989). Even when subjects were grouped according to CL status, correlations remained similar. For all subjects, FBUT and NIBUT displayed a significant positive correlation (Pearson's; r=0.407, p<0.005). The correlation remained significant for CL wearers (Pearson's; r=0.502, p<0.005) but not for NCL wearers alone (Pearson's; r=0.391, p=0.059) (Fig. 4.8).

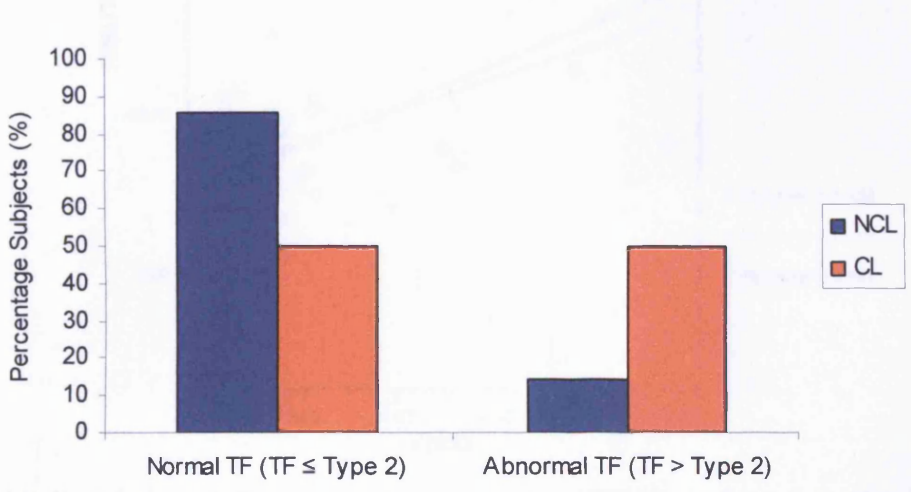


Figure 4.6 Normal (≤ Type 2) and abnormal tear ferning (> Type 2) in non-contact lens (NCL) and contact lens (CL) wearers

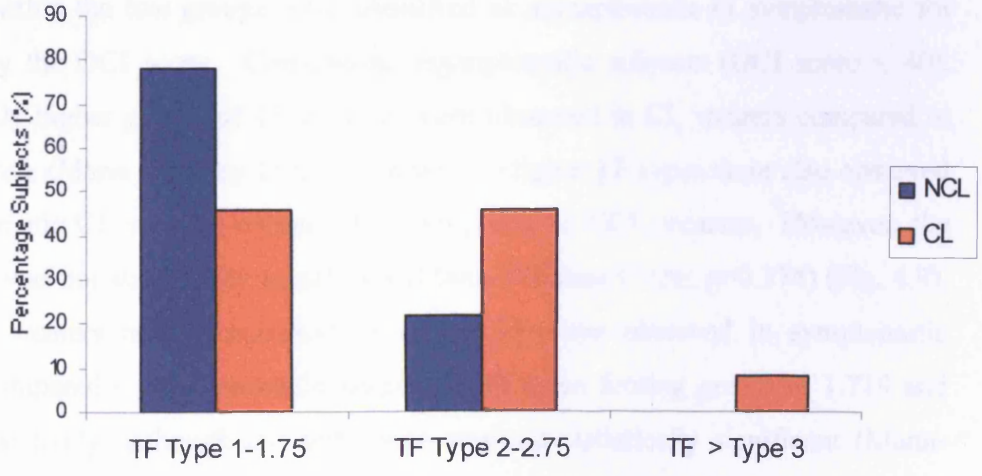


Figure 4.7 The distribution of tear ferning (TF) Types for non-contact lens (NCL) and contact lens (CL) wearers

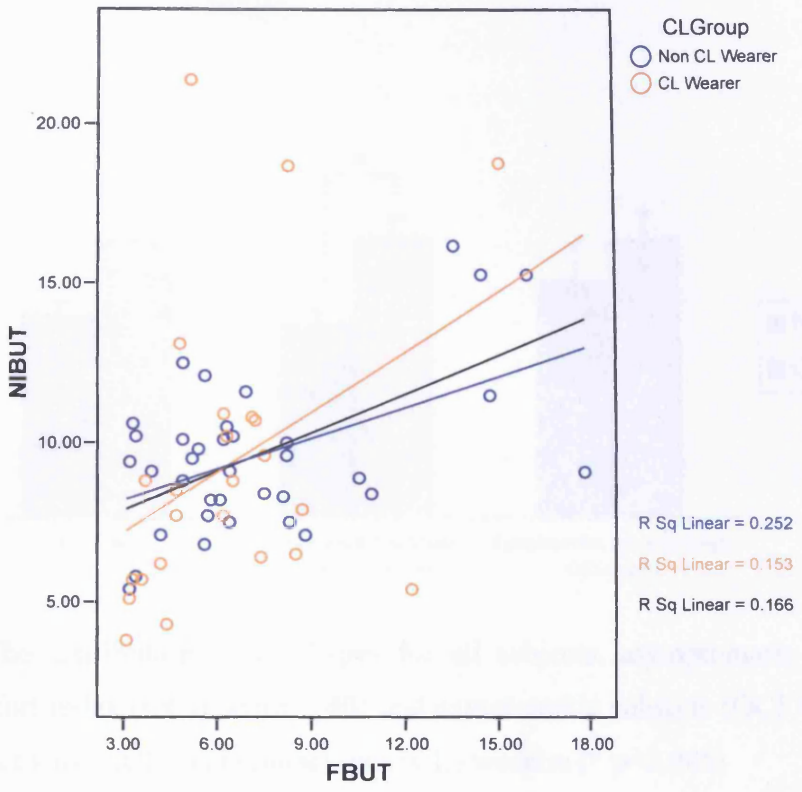


Figure 4.8 Correlation of fluorescein break-up time (FBUT) and non-invasive break-up time (NIBUT) in all subjects, contact lens (CL) wearers and non-contact lens (NCL) wearers (Pearson's; all subjects r=0.407, p<0.005: CL r=0.502, p<0.005: NCL r=0.391, p=0.059)

Subjects within the two groups were identified as asymptomatic or symptomatic for dry eye by the OCI score. Considering asymptomatic subjects (OCI score $\leq$ 40), significantly higher grades of TF patterns were observed in CL wearers compared to NCL wearers (Mann-Whitney U test; p<0.005). Higher TF types were also observed in symptomatic CL wearers compared to symptomatic NCL wearers. However, the difference was not statistically significant (Mann-Whitney U test, p=0.374) (Fig. 4.9). For NCL wearers alone, increased levels of TF were observed in symptomatic subjects compared to asymptomatic subjects with mean ferning grades of 1.719 and 1.411 respectively, although the difference was not statistically significant (Mann-Whitney U test; p=0.371). Considering CL wearers, similar patterns were observed for symptomatic and asymptomatic subjects, indicating TF in CL wear appears to be independent of ocular comfort.

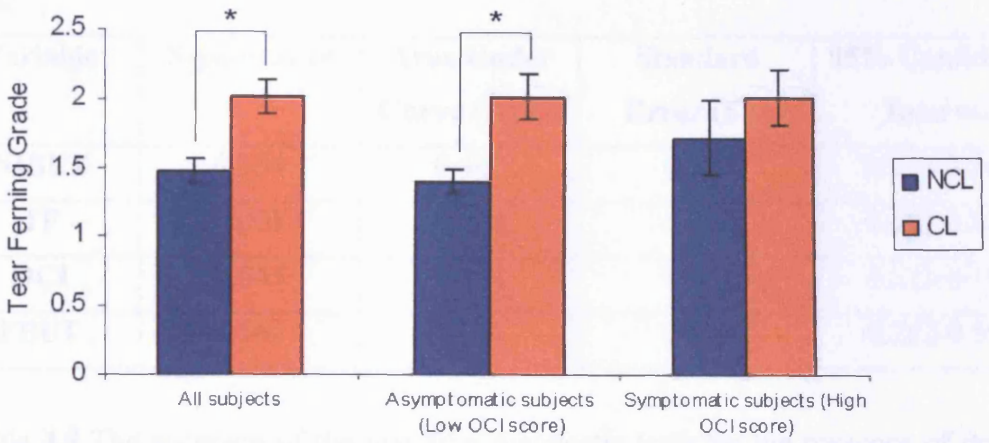


Figure 4.9 The distribution of TF Types for all subjects, asymptomatic subjects (Ocular Comfort Index (OCI) score ≤ 40) and symptomatic subjects (OCI > 40) for the non-contact lens (NCL) and contact lens (CL) wearers (* $p<0.005$)

The TF technique demonstrates superior accuracy for the discrimination of CL and NCL wearers by tear film assessment (Table 4.4). Applying cut off values according to Rolando's criterion where Type 1 and 2 are considered normal and Type 3 and 4 abnormal (Rolando, 1984), TF demonstrated a sensitivity of 50% and specificity of 86% for the discrimination of tear film samples between CL and NCL wearers, with a positive predictive value (PPV) and negative predictive value (NPV) of 71% and 72% respectively. For the prediction of dry eye symptoms (OCI score >40), TF displayed sensitivity and specificity of 33% and 73% respectively (Table 4.5). The PPV was 18% indicating a large number of false positives however; the NPV was 86% indicating normal TF to be an accurate predictor of good ocular comfort (low OCI score).

Table 4.4 The accuracy of the tear film diagnostic tests (non-invasive break-up time (NIBUT), Tear Ferning (TF), Ocular Comfort Index (OCI) and fluorescein break-up time (FBUT)) for the discrimination of contact (CL) wear and non-contact lens (NCL) wear

Variable	Significance	Area Under Curve (AUC)	Standard Error (SE)	95% Confidence Interval
NIBUT	0.194	0.400	0.082	0.239-0.562
TF	0.001	0.748	0.064	0.623-0.874
OCI	0.045	0.654	0.072	0.512-0.796
FBUT	0.369	0.431	0.076	0.282-0.580

Table 4.5 The accuracy of the tear film diagnostic tests for the presence of dry eye symptoms based on Ocular Comfort Index (OCI) score

Variable	Significance	Area Under Curve (AUC)	Standard Error (SE)	95% Confidence Interval
NIBUT	0.419	0.585	0.098	0.393-0.777
TF	0.992	0.501	0.105	0.296-0.707
FBUT	0.033	0.276	0.092	0.096-0.455

4.3.2 Cytokine concentration

The negative control sample contained levels of cytokines: 0.26pg/ml IL-4, 0.17pg/ml IL-8 and 23.32pg/ml IFN-γ. Five (IL-2, IL-4, IL-8, GM-CSF and IFN-γ) of the eight different cytokines tested were detected by the Luminex Analyser. Levels of IL-6, IL-10 and TNF-α were below the sensitivity range of the standard curves. Different cytokine concentrations were not detected in all analysed tear samples and results for the NCL and CL groups are shown in Table 4.6. The concentration of the five detected analytes (IL-2, IL-4, IL-8, GM-CSF and IFN-γ) were greater in the CL group compared to the NCL wearers, only the difference of IL-4 is statistically significant (Mann-Whitney U test, $p<0.05$). IL-2 and GM-CSF were detected in only three and eleven of the 31 tear film samples analysed respectively, and variance for all detected cytokines was considerable (Figure 4.10).

Table 4.6 Median results for the cytokine concentration for the non-contact lens (NCL) and contact lens (CL) wearers.

Cytokine	CL status	Number of samples	Median Concentration (pg/ml)	Inter-quartile range	P value (Mann-Whitney U Test)
IL-2	NCL	1	627.75	-	1.000
	CL	2	690.75	487.50-894.00	
IL-4	NCL	16	38.00	22.75-119.69	0.039*
	CL	13	107.25	52.75-177.13	
IL-8	NCL	17	269.00	136.50-870.00	0.305
	CL	13	462.50	321.38-627.88	
GM-CSF	NCL	5	860.50	512.13-1507.75	0.273
	CL	6	991.38	807.625-2617.75	
IFN-γ	NCL	15	2182.75	1222.25-7257.00	0.124
	CL	12	6125.63	2184.19-8501.88	

*statistically significant at 0.05 level

For all cytokines, there was a poor correlation of analyte concentration, tear film tests and ocular comfort (Spearman's; $-0.500<r<0.258$, $0.177<p<0.974$). Correlations remained poor when subjects were grouped for CL wear (Spearman's; CL - $0.617<r<0.229$, $0.101<p<0.957$; NCL $-0.445<r<0.247$, $0.073<p<0.876$). A number of cytokine combinations displayed a significant positive correlation including; IFN-γ and IL-4 (Spearman's; $r=0.837$, $p<0.0001$), IFN-γ and GM-CSF (Spearman's; $r=0.851$, $p<0.005$), GM-CSF and IL-4 (Spearman's; $r=0.890$, $p<0.005$) (Figure 4.11). However, sample numbers for certain analytes, particularly IL-2, are small. Considering all subjects, cytokine concentrations were not significantly different between the asymptomatic and symptomatic groups. This was also the case when subjects were grouped according to CL wear status.

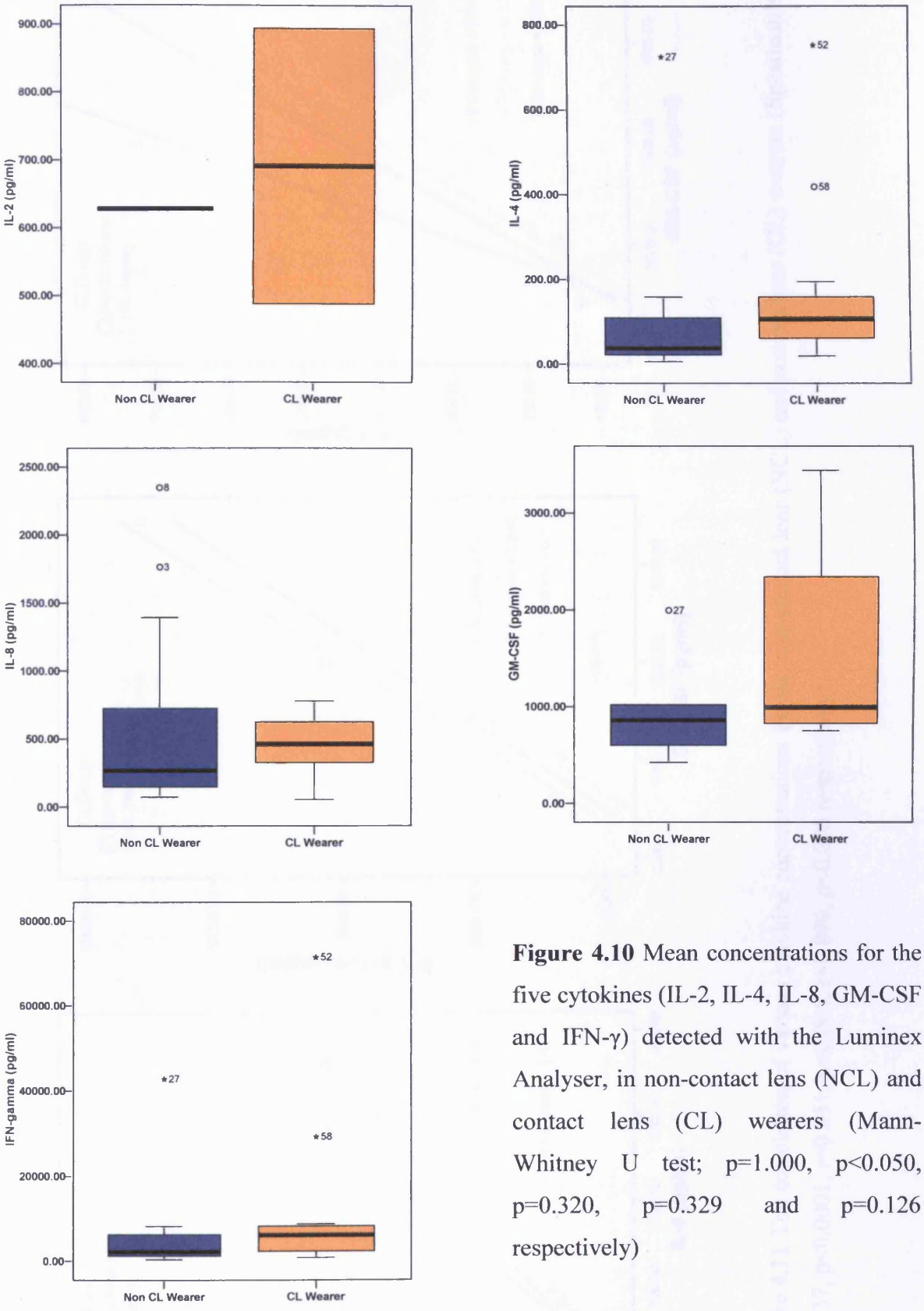


Figure 4.10 Mean concentrations for the five cytokines (IL-2, IL-4, IL-8, GM-CSF and IFN-γ) detected with the Luminex Analyser, in non-contact lens (NCL) and contact lens (CL) wearers (Mann-Whitney U test; $p=1.000$, $p<0.050$, $p=0.320$, $p=0.329$ and $p=0.126$ respectively)

(Box length indicates interquartile range containing the median value; whiskers represent the range of values)

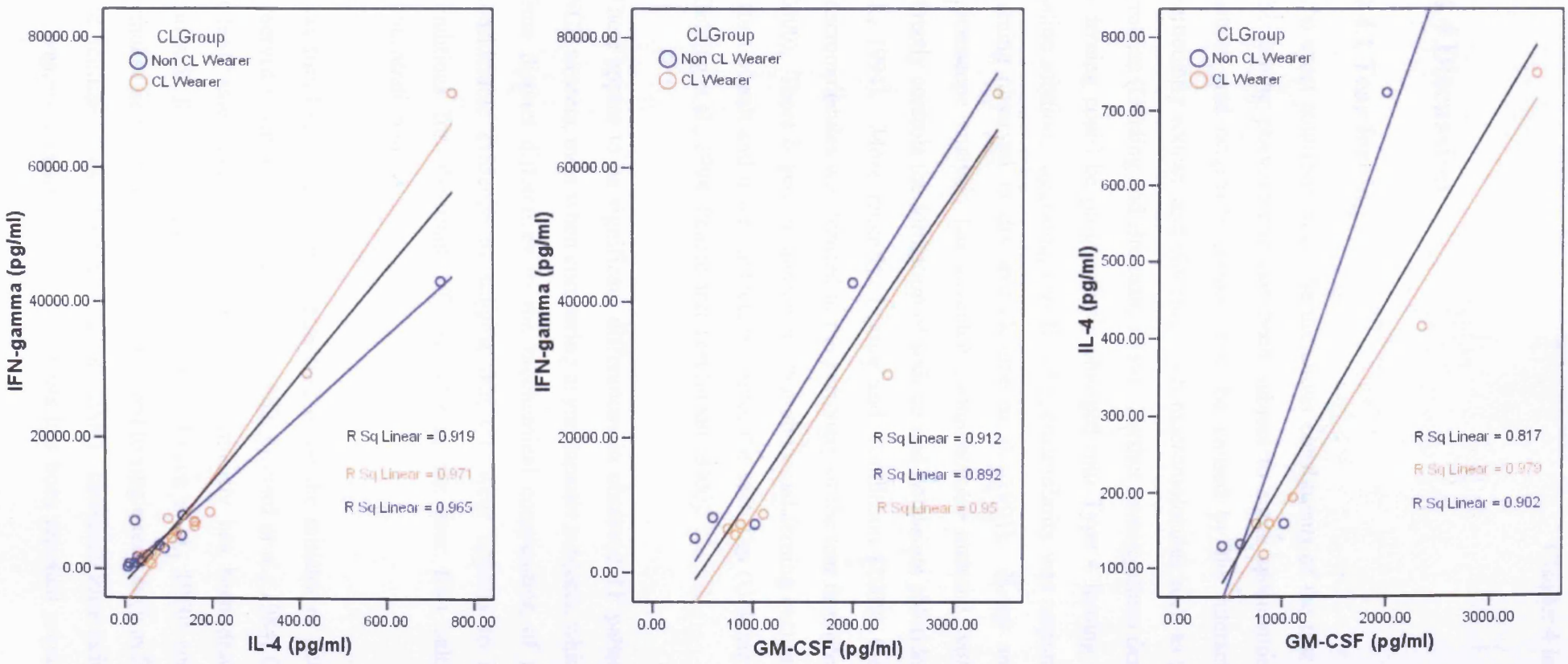


Figure 4.11 The correlation of various cytokine concentrations for the non-contact lens (NCL) and contact lens (CL) wearers (Spearman's; r=0.837, p<0.0001; r=0.851, p<0.005; r=0.890, p<0.005 respectively)

4.4 Discussion

4.4.1 Tear ferning

The exact contribution of the numerous constituents of the tear film components to the ferning phenomenon has been subject to some speculation. The tear ferning pattern was originally proposed to be caused by the interaction of electrolytes, particularly sodium and chloride, with macromolecules such as tear film mucins and proteins (Golding and Brennan, 1989). Further investigations demonstrated that Type 1 ferning could be progressively changed into Type 4 ferning with the addition of saline solution, suggesting tear film hyperosmolarity was responsible for altered tear ferning observed in dry eye (Kogbe et al., 1991). X-ray and scanning electron microscope analysis has revealed proteinaceous material within the tear sample directly controls the formation of sodium and potassium chloride crystals (Golding et al., 1994). More recently, Pearce and Tomlinson (2000) found the presence of macromolecules was limited to the periphery of the tear fern (Pearce and Tomlinson, 2000). There is general agreement that abnormal ferning occurs due to unfavourable ratios of salt and macromolecules within the tear film (Golding and Brennan, 1989, Golding et al., 1994, Pearce and Tomlinson, 2000).

There appear to be significant differences in observed TF patterns between CL and NCL wearers, even when comparing asymptomatic subjects, which are likely to result from distinct differences in the biochemical composition of the tears. There is considerable evidence to suggest that CL wear appears to induce "favourable" conditions for abnormal TF by altering the tear film salt to macromolecule concentration ratios.

Tear film hyperosmolarity is proposed to be the initiator of ocular surface damage observed in dry eye (Gilbard et al., 1988, Gilbard et al., 1984, Gilbard et al., 1987). Indeed, assessment of the tear film osmolarity has been described as the "gold standard" for the diagnosis of dry eye (Lucca et al., 1990) and the technique has demonstrated superior accuracy compared to single tests such as Rose Bengal staining and Schirmer's test (Tomlinson et al., 2006). Increased osmolarity of the tear film of CL wearers compared to NCL wearers has been reported previously (Miller et al.,

2004), and different mechanisms have been suggested. Reduced corneal sensitivity induced by CL wear (causing a reduction of tear secretion) could subsequently increase tear film osmolarity (Gilbard et al., 1986). Further studies have demonstrated increased tear film thinning and evaporation are likely to be responsible for tear film hyperosmolarity during CL wear (Thai et al., 2002). More recently, however, Srinivasan and colleagues found no correlation between TF and tear film osmolality in a cohort of postmenopausal women (Srinivasan et al., 2007). The authors suggested this was because the TF pattern is defined by the interaction of the tear film salts and macromolecules rather than the tear film osmolarity.

A reduction of total mucin concentration in the tear film of CL wearers has been observed previously (Yasueda et al., 2005). In particular, MUC5AC is significantly reduced in tear film samples from symptomatic CL wearers (Berry et al., 2007) and altered forms of ocular mucins have been found adhered to the contact lens surface (Berry et al., 2003). This binding could be responsible for the reduction of tear film mucin concentration observed. Hori and colleagues reported similar tear film mucin concentrations between CL and NCL wearers, although the total tear protein concentration was significantly reduced in CL wearers (Hori et al., 2006). An alteration of mucin distribution has also been observed in the tear film of patients with dry eye (Danjo et al., 1998) and Sjögren's syndrome (Argueso et al., 2002).

Traditional tear film stability tests are known to relate poorly to patient symptoms (Nichols et al., 2004b) and TF, like other diagnostic tests, appears to correlate poorly with ocular comfort symptoms. Increased levels of ocular discomfort (higher OCI scores) were observed in CL wearers compared to NCL wearers. Similar findings have been published previously; with almost three times as many CL wearers deemed to be symptomatic and over five times as many reporting moderate to severe discomfort compared to non-lens wearers (Chalmers and Begley, 2006, Guillon and Maissa, 2005). Increased TF grades were observed whenever contact lenses were worn or symptom scores increased, but these differences lacked significance within each cohort (CL and NCL), except where all subjects were essentially comfortable. Such results have several possible interpretations. Successful, asymptomatic CL wear is likely to induce a significant alteration of the tear film salt to macromolecule concentration which is detectable by the TF technique. The lack of significant

difference in TF between symptomatic CL and NCL wearers could suggest similar aetiology (tear film hyperosmolarity) in each cohort, or may be related to small sample size.

To summarise, although TF has been shown to be a good predictor of successful CL wear tolerance (Ravazzoni et al., 1998) the technique displayed a poor correlation with traditional tear film tests and ocular comfort in CL and NCL wearers. Significantly increased TF grades were observed in CL wearers compared to NCL wearers even when comparing those asymptomatic patients with good ocular comfort. This study demonstrates that TF potentially identifies differences between patient groups, but further significant findings may be somewhat restricted by the sample size and the semi-quantitative nature of the grading scale.

4.4.2 Tear film cytokines

The study detected the presence of five cytokines (IL-2, IL-4, IL-8, GM-CSF and IFN-γ) in tear film samples from NCL and CL wearers. Of the eight cytokines tested with the Luminex Analyser, three remained undetected (IL-6, IL-10 and TNF-α). These cytokines have been detected in tear film samples from contact lens wearers previously. IL-6, IL-10 and TNF-α have been detected in the tear film of both CL and NCL wearers and TNF-α has been noted to be significantly increased in CL wearers (Lakkis et al., 2007). IL-6 has also been detected in CL wearers suffering from CL induced acute red eye (CLARE) and CL induced peripheral ulcer (CLPU), although the concentration was not significantly different to reflex tears from NCL wearers (Thakur and Willcox, 1998).

IL-10, typically secreted by T_H2 cells, can inhibit macrophage activation, controlling the T_H1 response (Parham, 2000) and is often described as an anti-inflammatory cytokine (Cook et al., 2001). TNF-α is typically secreted by T_H1 cells and activates macrophages and increases their phagocytic ability leading to inflammation. IL-6 is also a pro-inflammatory cytokine but primarily activates Bcells (Parham, 2000). The literature demonstrates the three analytes have been found to be present in tear film samples previously. Lack of detection could have occurred following excessive dilution of the tear film samples. However, this appears unlikely as a published

methodology was followed where IL-6, IL-10 and TNF-α have been successfully detected (Malvitte et al., 2007). It is likely the analytes were undetected in this study because for each of these analytes the generated standard curves were poor with a number of the most diluted standards being out of range for the Luminex Analyser. Sensitivity was significantly reduced to a minimum detection level of 139.63 pg/ml, 220.95 pg/ml and 59.46 pg/ml for IL-6, IL-10 and TNF-α respectively. Therefore, any of the three analytes present in the tear samples below these concentrations would remain undetected in this investigation.

The five detected cytokines (IL-2, IL-4, IL-8, GM-CSF and IFN-γ) were all found to be up-regulated in the tear film of CL wearers compared to NCL wearers, although only IL-4 was significantly increased. The lack of significance observed here is likely to be caused by the small cohort size, especially for IL-2 and GM-CSF, and the high variance of the results. This wide variance of cytokine concentration in tear film samples has been observed previously (Uchino et al., 2006, Uchino et al., 2005).

An increased concentration of IL-8 has been observed previously in tear film samples from habitual silicone hydrogel (SiH) CL wearers following overnight wear when compared to neophytes or NCL wearers (Thakur and Willcox, 2000). IL-8 has also been found to be up-regulated in patients with CLARE and CLPU (Thakur and Willcox, 1998). Whilst IL-2, IL-4 and IFN-γ have been detected in tear film samples from patients suffering from various forms of conjunctivitis and control tear samples (Cook et al., 2001, Leonardi et al., 2006) they have not been analysed in CL wearers previously. The increase in IL-2 and IFN-γ here indicates a cell mediated immune response and increased inflammation in the CL group.

IL-8 is described as a pro-inflammatory chemotactic cytokine or chemokine and has powerful chemotactic properties on neutrophils and Tcells. The ratio of pro-inflammatory to anti-inflammatory cytokines may be informative (Uchino et al., 2006, Uchino et al., 2005) however, this cannot be investigated in this study as IL-10 was not detected. A number of positive cytokine-cytokine correlations were observed. The correlation of IFN-γ and GM-CSF could be expected, as both are primarily T_H1 cytokines. A positive correlation of T_H1 and T_H2 cytokine concentrations (IFN-γ and IL-4, GM-CSF and IL-4) is perhaps unexpected but has

been observed previously in patients with seasonal and chronic allergic conjunctivitis (Leonardi et al., 2006).

For all detected cytokines there was a poor correlation between concentration and tear film tests. Yoon and colleagues demonstrated a significant correlation of IL-6 with BUT and Schirmer's test for subjects with dry eye (Yoon et al., 2007), but no correlation was observed for TNF-α. Correlations have also been observed for various cytokines (IL-1α, TNF-α, IL-6, IL-8 and TGF-β1) with fluorescein staining and Schirmer's test (Pflugfelder et al., 1999). Narayanan and colleagues concluded that whilst dry eye subjects had significantly different symptom scores, tear film osmolarity and FBUT, the cytokine profiles were not different from control subjects (Narayanan et al., 2006). Unfortunately, ocular comfort also correlated poorly with cytokine concentration in this study and no significant differences were observed when patients were grouped according to ocular comfort symptoms or CL status. Again, the high variability and low cohort size may have masked any significant patterns in this study.

CL wear is known to induce an increase in tear film osmolarity (Miller et al., 2004). Tissue cultures and the mouse ocular surface have been shown to significantly up-regulate various cytokines following hyperosmotic stress (Luo et al., 2005, Shapiro and Dinarello, 1997). Therefore, increased tear film hyperosmolarity is likely to be the cause of the increased ocular surface inflammation observed in the CL group. Kallinikos and colleagues found IL-8 to be significantly increased in rigid gas permeable CL wearers compared to SiH CL wearers (Kallinikos et al., 2006) and in tear samples from rubbed eyes when compared to controls (Kallinikos and Efron, 2004). They hypothesised mechanical stimulation of the ocular surface by the CL could account for the increase in cytokine concentration (Kallinikos and Efron, 2004, Kallinikos et al., 2006). It has also been suggested up-regulation may be caused by a response to the CL material, CL solutions or even bacterial contaminants (Lakkis et al., 2007). In reality, it is probable a combination of a number of these factors that induce levels of allergy and sub-clinical inflammation in CL wearers.

4.5 Summary

Tear ferning (TF) identified fundamental differences in the tear film of NCL and CL wearers. Abnormal TF has been observed in CF patients previously. As the technique appears to have some diagnostic value it is justified to investigate TF in the CF cohort. Whilst the Luminex system detected five different cytokines from the tear samples, three remained undetected due to inaccurate standard curves. Cytokines were also observed in the negative control sample. These findings indicate the technique requires a greater level of biochemical competence and further optimisation particularly regarding tear film dilution. The high variance of the results from a relatively small subject group is also likely to mask any statistical significance. As the kit is relatively expensive the Luminex does not appear to be viable method for tear film cytokine quantification in tear samples from CF patients.

Chapter 5

Investigating the Tear Film and Ocular Surface in subjects with Cystic Fibrosis

5.1 Introduction

A number of previous investigations have indicated the tear film and ocular surface is abnormal in vitamin A sufficient subjects with CF, with evidence of sub-clinical ocular surface inflammation in CF (Sheppard et al., 1989, Mrugacz et al., 2007b, Mrugacz et al., 2007c, Mrugacz et al., 2006b, Mrugacz et al., 2007d). Dry eye has been suggested to be a primary manifestation of CF (Morkeberg et al., 1995, Ansari et al., 1999). However, the suggestion that ocular surface CFTR is responsible for the pathogenesis of dry eye in CF is novel. It is hypothesised that corneal and conjunctival epithelial Cl^- secretion via CFTR is reduced or absent in subjects with CF, depending on the class of CFTR mutation. Active trans-epithelial Cl^- transport is known to provide the driving force for subsequent osmotically driven fluid secretion, such as basal tear production (Yang et al., 2000, Candia, 2004, Dartt, 2004, Dartt, 2002). Therefore, unless alternative epithelial Cl^- channels compensate for defective CFTR function, subjects with CF would have reduced basal tear secretion with subsequent increased tear film osmolarity, ocular surface epithelial damage and inflammation.

If dry eye is a primary manifestation of CF the degree of CFTR dysfunction, would be expected to correlate with dry eye severity. The most common ΔF508 class 2 mutation (see section 1.2.2) results in defective CFTR production, which cannot be subsequently processed to reach the membrane surface (Kulczycki et al., 2003). In the ΔF508 mutation, CFTR is completely absent from the epithelia resulting in a complete loss of normal channel activity. In other, less severe mutations, CFTR may reach the apical membrane but cause abnormal channel conductance or the volume of CFTR synthesis may be reduced resulting in a milder phenotype (Ratjen and Doring, 2003). Therefore, whilst ΔF508 homozygotes have a particularly severe phenotype,

in ΔF508 heterozygotes, the other milder mutation is dominant over the ΔF508, typically resulting in a milder phenotype (Kerem and Kerem, 1995). A greater disruption to normal basal tear secretion would be expected in ΔF508 homozygotes compared to ΔF508 heterozygotes where some CFTR activity within the ocular epithelia is likely to be maintained. Therefore, increased levels of dry eye would be expected in ΔF508 homozygous subjects compared to ΔF508 homozygotes.

CF genotype correlates poorly with phenotype and there is considerable disease variability within patients of identical genotype (McKone et al., 2003, Zielenski, 2000) and CF genotype may not accurately predict disease severity in this study. Measures of clinical severity, such as lung function or Shwachman score could circuitously indicate the level of CFTR disruption. Therefore, a correlation of disease clinical severity with signs and symptoms of dry eye could be expected.

Disruption of the tear film and ocular surface has been observed secondary to vitamin A deficiency (VAD) in subjects with CF (Neugebauer et al., 1989, Rayner et al., 1989, Castagna et al., 2001, Mrugacz et al., 2005b). Xerophthalmia refers to ocular signs of vitamin A deficiency, such as conjunctival and corneal xerosis (Sommer, 1998). Conjunctival xerosis is characterised by keratinisation and loss of goblet cells and precedes corneal manifestations of VAD. A number of tear film mucins are secreted by the goblet cells and ocular surface apical cells (Spurr-Michaud et al., 2007, Gipson, 2004, Argueso and Gipson, 2001). Mucins facilitate ocular surface wetting and tear film stability (Watanabe, 2002, Sharma, 1998a, Sharma, 1998b). Other functions include prevention of pathogen invasion and lubrication (Gipson and Argueso, 2003, Argueso and Gipson, 2001). Two ocular surface mucins, MUC5AC and MUC4, are down-regulated in experimental VAD in studies of the rat ocular surface demonstrating secretion is directly or indirectly regulated by vitamin A (Tei et al., 2000). Similarly, MUC16 secretion is also thought to be modulated by vitamin A (Hori et al., 2005).

In CF subjects with reduced serum vitamin A concentration or VAD, reduced goblet or apical cell densities within the cornea and conjunctiva could result in reduced mucin secretion with consequential tear film disruption. Therefore, observations of dry eye in patients with CF are likely to be increased in vitamin A deficient subjects

or could correlate with serum vitamin A concentration. Serum vitamin A concentration is known to poorly reflect vitamin A liver stores in subjects with CF (Underwood and Denning, 1972). VAD in CF occurs following impaired absorption of the fat soluble vitamin in subjects with pancreatic insufficiency (Sinaasappel et al., 2002) and subjects with CF liver disease may have compromised vitamin A liver stores (Lindblad et al., 1997). Therefore, a more accurate reflection of vitamin A status may be to compare individuals based on pancreatic and liver function.

Dry eye is a common ocular feature of both adults and children with Type 1 and Type 2 diabetes (Manaviat et al., 2008, Rahman et al., 2007, Akinci et al., 2007, Kaiserman et al., 2005, Grus et al., 2002). Tear film changes have been shown to increase with diabetes duration (Ramos-Remus et al., 1994) and the presence of dry eye correlates with glycaemic control (HbA1c) (Seifart and Strempel, 1994). However, no previous studies have investigated dry eye when subjects with CF were grouped according to diabetic status.

5.1.1 Objectives for this study

This study aimed to examine juveniles and adults with CF of known disease severity and status with respect to ocular surface signs and symptoms. The hypothesis was that dry eye is a primary manifestation of CF.

5.2 Method

5.2.1 Subjects

Children and young adults with CF (referred to as juveniles for the purpose of this chapter) (n=30; 20 male, 10 female; mean age 11.77 ± SD 3.39 years) were recruited from the Children's Hospital for Wales, Cardiff. Age and gender matched juvenile controls (n=30; 20 male, 10 female; mean age 11.83 ± SD 3.25 years) were recruited through friends and colleagues.

Adults with CF (n=28; 19 male, 9 female; 27.07 ± SD 7.36 years) were recruited from the All Wales Cystic Fibrosis Centre, Llandough Hospital. Age and gender matched controls (n=28; 19 male, 9 female; 27.04 ± SD 7.29 years) were recruited from the School of Optometry and Vision Sciences.

Informed consent was obtained from all subjects or their parent/guardian as necessary; favourable approval had been obtained from the Cardiff and Vale Trust Research and Development Office, South East Wales Research Ethics Committee and the Human Science Research Ethics Committee, School of Optometry and Vision Sciences, Cardiff University and all procedures conformed to the tenets of the Declaration of Helsinki.

5.2.1.1 Inclusion and Exclusion Criteria

Juveniles were required to be between 5 to 18 years of age. Adults were required to be between 18 to 50 years of age. For subjects with CF the inclusion criteria included a positive diagnosis of CF, regular outpatient attendance at the Children's Hospital for Wales or the All Wales Adult CF Centre, and valid informed consent from the participant or parent/guardian where necessary.

For gender matched control subjects, inclusion involved date of birth within six months of their CF match, and good general health, free from systemic disease and a negative family history of CF. Exclusion for all subjects included pregnancy, breast feeding, and signs of ocular infection or topical medication use.

5.2.2 Experimental Design

Adult subjects were seen for a single session whilst juveniles were seen on two to three separate sessions due to out-patient clinic constraints. Data collection involved completion of the Ocular Comfort Index (OCI) for adults or face-to-face comfort questionnaire for juveniles which included complaints of soreness/grittiness, tiredness, watering or frequency of eye rubbing.

Non-invasive tear break-up time (NIBUT) was measured with the Tearscope Plus (Keeler, UK) (as described in Section 4.2.2). The anterior eye was examined with a slit lamp (Juveniles SL.2F, Topcon, Tokyo, Japan; Adults SM-70N, Takagi Seiko Co., Ltd., Nagano, Japan) for evidence of ocular surface xerophthalmia such as; conjunctival or corneal xerosis, punctate keratopathy and corneal ulceration (Sommer, 1998). Blepharitis, conjunctival hyperaemia and limbal hyperaemia were graded according to 0.25 increments of the Efron grading scale (Efron, 1998).

Tear samples were collected from the right eye of adults using glass capillary tubes (Section 4.2.2) and sterile cellulose sponges with juveniles (Chapter 3). Each tear collection device was applied to the inferior tear meniscus for a maximum period of up to 5 minutes. Saturated sponges and expelled tears from capillary tubes were placed in 1.5ml eppendorf tubes and stored at -80°C until analysis.

After a recovery period of at least 10 minutes, fluorescein BUT (FBUT) was assessed (Section 4.2.2). Corneal fluorescein staining was subsequently investigated (x16 magnification, 3mm wide beam, cobalt blue filter) and graded according to 0.25 increments of the Efron grading scale.

Collected tear samples were stored at -80°C and subsequently thawed at 4°C for analysis. Sponge collected tear samples were centrifuged for three minutes at 14000 RPM at 4°C to extract the tear sample and glass capillary collected tear samples were similarly centrifuged for one minute to facilitate sedimentation and ease of pipetting. Tear ferning samples were prepared and analysed according to the protocol stated in chapter 3 (Section 3.6).

Information regarding subjects with CF was recorded from their medical records. This included:

- Genotype
- Medications and supplements
- Serum vitamin A concentration – an indication of VAD; normal values range between 1.10-2.60 millimoles/litre (mmol/l)
- Glucose tolerance – typically used for the detection of diabetes where <8.8 mmol/l indicates normal glucose tolerance (NGT), 8.9-11.1 mmol/l indicates impaired glucose tolerance (IGT) and ≥11.2 mmol/l is diagnostic of Cystic Fibrosis-Related Diabetes (CFRD) (Moran, 2002)
- Glycosylated haemoglobin (HbA1c) – an indication of metabolic control, normal levels range between 2.6-5.8% (Mansour, 2000)
- Forced Expiratory Volume in one second (FEV_1 %) – an indication of lung function and is expressed as a percentage of the predicted value
- Shwachman score - a general score of clinical severity, assessed at annual review. Scoring consists of assessment of the patient's general activity, nutritional status, a physical examination, and x-ray findings; there is a maximum score of 100 where above 86 is considered excellent and below 40 severe (Shwachman and Kulczycki, 1958)
- Chrispin-Norman score - a chest radiograph scoring system to assess respiratory disease progression in CF using the severity of 5 disease characteristics in 4 zones of a chest x-ray with a maximum score of 38 (Chrispin and Norman, 1974)
- Northern score - based on the appearance of the lungs from a chest x-ray where severity is marked out of a maximum score of 20 (Conway, 1994)
- Premature birth and gestation period in juveniles

To enhance subject participation and due to lack of access to a phlebotomist and laboratory analysis, blood samples were not drawn at the time of data collection. Therefore, vitamin A and HbA1c levels were recorded from the most recent blood test. FEV_1 was recorded from the most recent outpatient appointment. Chrispin-

Norman, Shwachman or Northern scores were recoded from the preceding annual review.

5.2.3 Statistics

Data was checked for normality using the Kolmogorov-Smirnov test (SPSS Version 16). Results for the anterior eye assessment (blepharitis, conjunctival hyperaemia, limbal hyperaemia and corneal fluorescein staining) and OCI scores in adults were not normally distributed ($p<0.005$ for all) although tear break up times (NIBUT and FBUT) were (0.092 and 0.200 respectively). Therefore, non-parametric and parametric statistics were applied as appropriate.

Inter-ocular differences were explored with Wilcoxon Signed Rank (2-tailed) test or Paired-samples (2-tailed) t-test. The Mann-Whitney U (2-tailed) test or Independent samples (2-tailed) t-test was applied to compare data from the CF and control cohorts as appropriate. Correlation was assessed with Spearman's Rank Order Correlation or Pearson's Product Moment Correlation as appropriate. The Kruskal-Wallis test or One-Way Analysis of Variance was used to compare the effect of vitamin A status, pancreatic status, CFLD, CFRD and genotype. Bonferroni's test was used for post-hoc comparisons. Significance was set at the 0.05 level.

5.3 Results

Measures of anterior eye assessment, ocular comfort and tear film stability were not significantly different when comparing values for each eye (Wilcoxon Signed Rank test, $0.053<p<1.00$; Paired-samples t-test, $0.944<p<0.948$). Therefore, only values from the right eye will be presented.

5.3.1 Subjects

An overview of CF disease status in juveniles and adults with CF is shown in Table 5.1 and 5.2 respectively.

Due to varying levels of co-operation a small number of subjects did not participate in all procedures:

Juveniles

- 30 CF and 30 control subjects participated in the ocular comfort questionnaire
- 27 CF subjects took part in any tear film or ocular surface examination (3 CF subjects dropped out following initial recruitment due to poor health or personal choice)
- 24 CF and 23 control subjects had corneal staining assessed (3 CF and 7 controls declined to have fluorescein inserted with the pipette)
- 25 CF and 28 control subjects performed NIBUT (2 subjects were excluded from each group due to a lack of understanding or uncontrolled blinking)
- 24 CF and 22 control subjects performed FBUT (a number did not have fluorescein inserted and 1 control subject was excluded due to inadvertent blinking)

Adults

- 27 control subjects had corneal staining and FBUT assessed (one control subject declined to have fluorescein inserted with the pipette)

Table 5.1 Overview of subject characteristics and clinical disease severity for the *juvenile* CF group

Variable	Description
Genotype	n=11 ΔF508 homozygous n=17 ΔF508 heterozygous n=2 non-ΔF508
Pancreatic function	n=6 pancreatic sufficient n=24 pancreatic deficient
Serum vitamin A concentration	range 0.59 – 2.04 μmol/l mean 1.25 μmol/l (SD ± 0.35)
Vitamin A status	n=19 vitamin A sufficient n=8 vitamin A deficient (3 subjects unmeasured)
CFRD	None
HbA1c	5 subjects assessed, range 5.2 – 5.9% mean 5.58% (SD ± 0.26)
FEV_1	range 19 – 123% mean 89.64% (SD ± 13.08)
Shwachman Score	score >86 (excellent) n=19 score 71-85 (good) n=8 no score n=3 range 100 – 71 mean score 88.17 (SD ± 8.43)
Chrispin-Norman Score	range 0 – 10 no score n=7 mean 5.22 (SD ± 2.76)

Table 5.2 Overview of subject characteristics and clinical disease severity for the *adult* CF group

Variable	Description
Genotype	n=13 ΔF508 homozygous n=11 ΔF508 heterozygous n=4 non-ΔF508
Pancreatic function	n=3 pancreatic sufficient n=25 pancreatic deficient
Serum vitamin A concentration	range 0.35 – 2.34 μmol/l mean 1.31 μmol/l (SD ± 0.58)
Vitamin A status	n=16 vitamin A sufficient n=12 vitamin A deficient
CFRD	n=11 CFRD n=17 non-CFRD
HbA1c	range 4.7 – 13.2% mean 6.68% (SD ± 0.2.16)
CFLD	n=6 CFLD, all taking ursodeoxycholic acid
FEV_1	range 24 – 103% mean 58.61 % (SD ± 24.11)
Northern Score	range 0/20 – 12/20 mean 6.00 (SD ± 3.58)

5.3.2 Ocular comfort

A total of 43% of juveniles (n=13) with CF and 50% of controls (n=15) reported at least one symptom of ocular discomfort including; soreness/grittiness, tiredness, watering or eye rubbing. The frequency of reported ocular comfort symptoms is shown in Table 5.3. No significant difference was observed for all symptoms (Mann-Whitney U test; $0.200<p<1.000$). No significant difference in the total number of subjects reporting symptoms of ocular discomfort was observed when control subjects were compared to vitamin A deficient and sufficient CF subjects (Kruskal-Wallis test; $p=0.159$). Similarly, no difference was observed when subjects were grouped

according to pancreatic status or genotype (Kruskal-Wallis test; p=0.082 and p=0.214 respectively).

Table 5.3 The frequency of reported ocular symptoms in *juveniles* with CF and healthy controls

Feature	Control (no. of subjects)	CF (no. of subjects)	Significance (Mann-Whitney U-test)
Soreness/Grittiness	4	4	1.000
Rubbing	6	9	0.375
Tiredness	5	2	0.232
Watering	8	4	0.200
Total no. of *different* subjects reporting ocular symptoms	15	13	0.608

In the adults, median Ocular Comfort Index (OCI) scores were slightly higher (indicating greater ocular discomfort) with greater variability in the CF group compared to the controls at 29.24 and 28.57 respectively, although the difference was not statistically significant (Mann-Whitney U test; p=0.491) (Figure 5.1). Four outliers can be observed in Figure 5.1 (two from each group) where subjects responded "Never" to all questions of the OCI resulting in a negative score. An insignificant difference remained when these outliers were removed (Mann-Whitney U test; p=0.442).

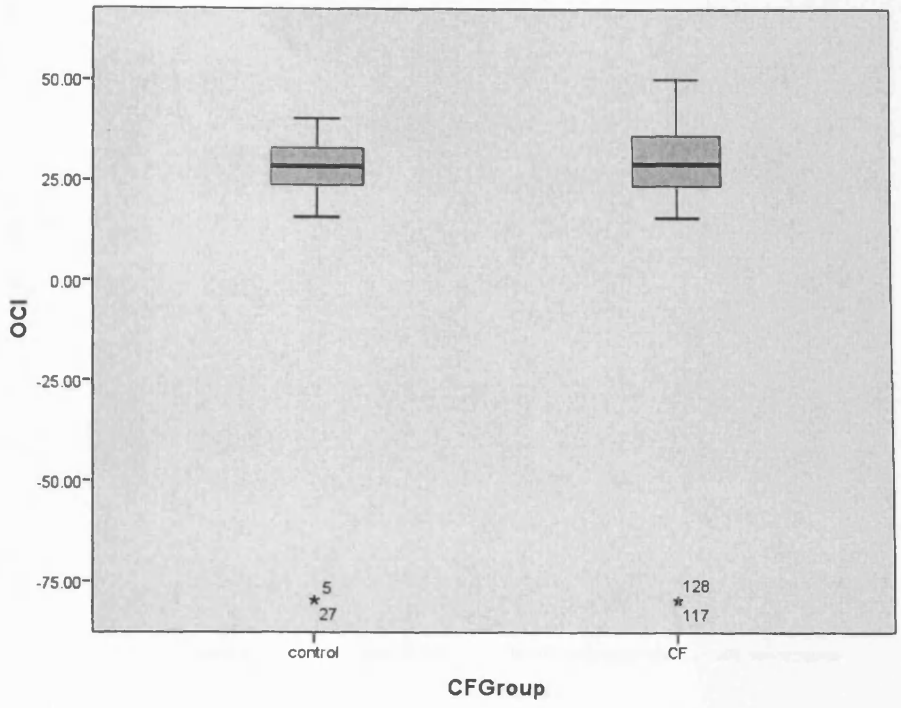


Figure 5.1 Ocular Comfort Index scores in *adults* with CF and healthy controls (Mann-Whitney U test; p = 0.491)

(Box length indicates interquartile range containing the median value; whiskers represent the range of values)

Significantly greater levels of ocular discomfort were observed in the non-ΔF508 subjects compared to the ΔF508 heterozygotes and the controls (Kruskal-Wallis; $p<0.05$) (Figure 5.2). Two negative OCI score outliers were recorded in both the ΔF508 heterozygote and the control group and median OCI scores were not significantly different when the four outliers were removed (Kruskal-Wallis; p=0.053) (Figure 5.3). Higher OCI scores can be observed in the ΔF508 homozygotes compared to the heterozygotes although the difference is not significant. Considering clinical severity in the CF cohort, the OCI scores showed poor correlation with the Northern Score and lung function (FEV_1) (Spearman; r=0.032, p=0.684 and r=-0.11, p=0.956 respectively).

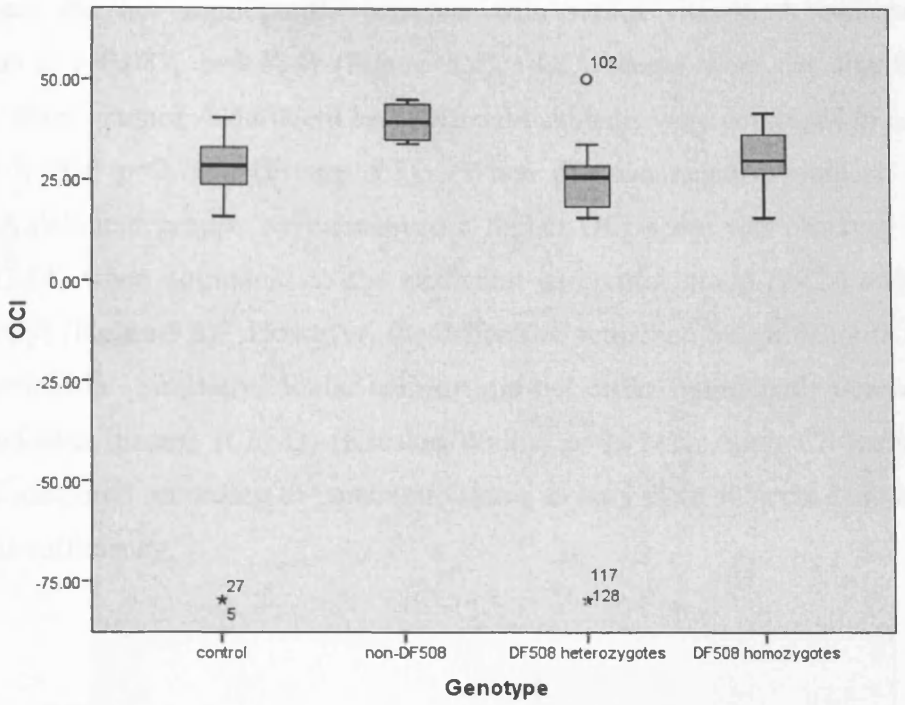


Figure 5.2 Ocular comfort index (OCI) scores in the three different *adult* CF genotype groups and healthy controls (Kruskal-Wallis; $p<0.05$)

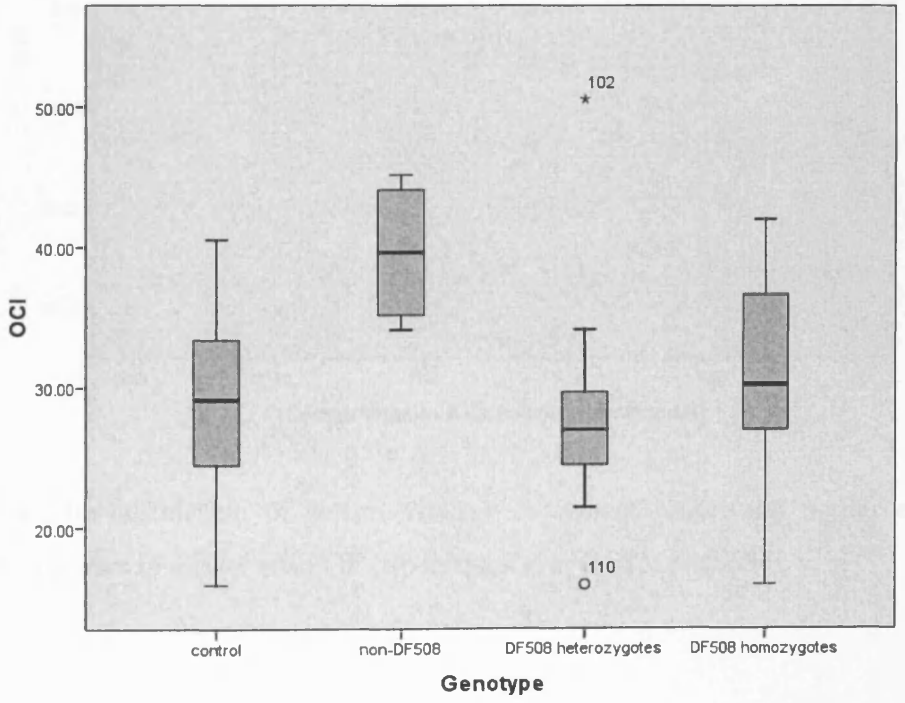


Figure 5.3 Ocular comfort index (OCI) scores in the three different *adult* CF genotype groups and healthy controls following the removal of four outliers (Kruskal-Wallis; $p=0.053$)

OCI scores did not significantly correlate with serum vitamin A concentration (Spearman's; r=0.081, p=0.684) (Figure 5.4). OCI scores were not significantly different when vitamin A deficient and sufficient subjects were compared to controls (Kruskal-Wallis; p=0.769) (Figure 5.5). When the two negative outliers, in the vitamin A deficient groups were removed a higher OCI score was observed in this group (32.14) when compared to the sufficient or control group (29.24 and 29.11 respectively) (Figure 5.6). However, the difference remained insignificant (Kruskal-Wallis; p=0.603). Similarly, ocular comfort did not differ significantly according to CF-related liver disease (CFLD) (Kruskal-Wallis; p=0.711). Adult CF participants were not compared according to pancreatic status as only three subjects demonstrated pancreatic sufficiency.

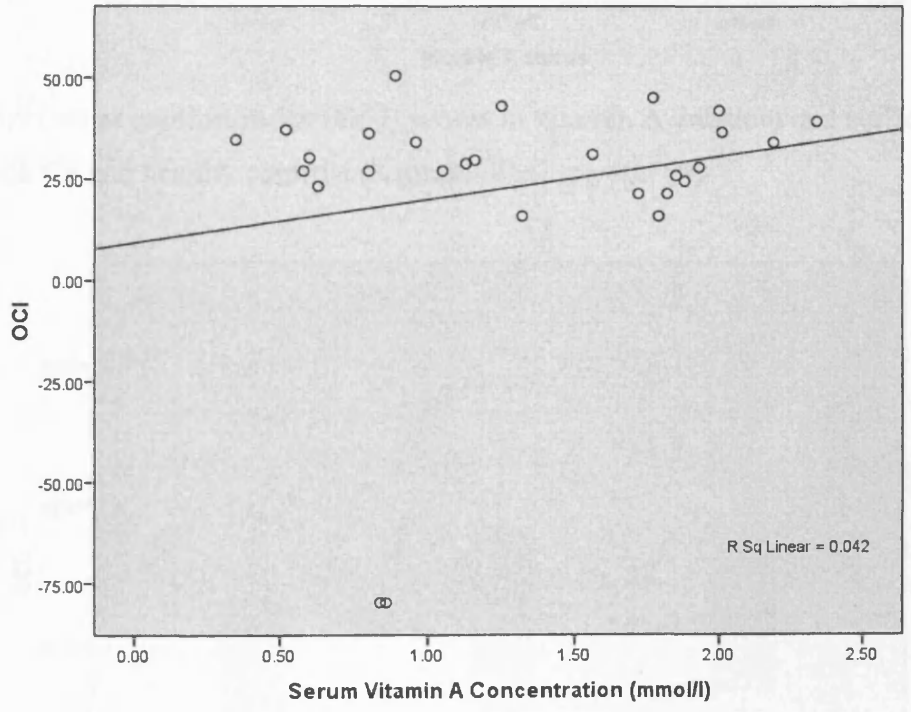


Figure 5.4 The correlation of serum vitamin A concentration and ocular comfort index (OCI) scores in *adults* with CF (Spearman's; r=0.081, p=0.684)

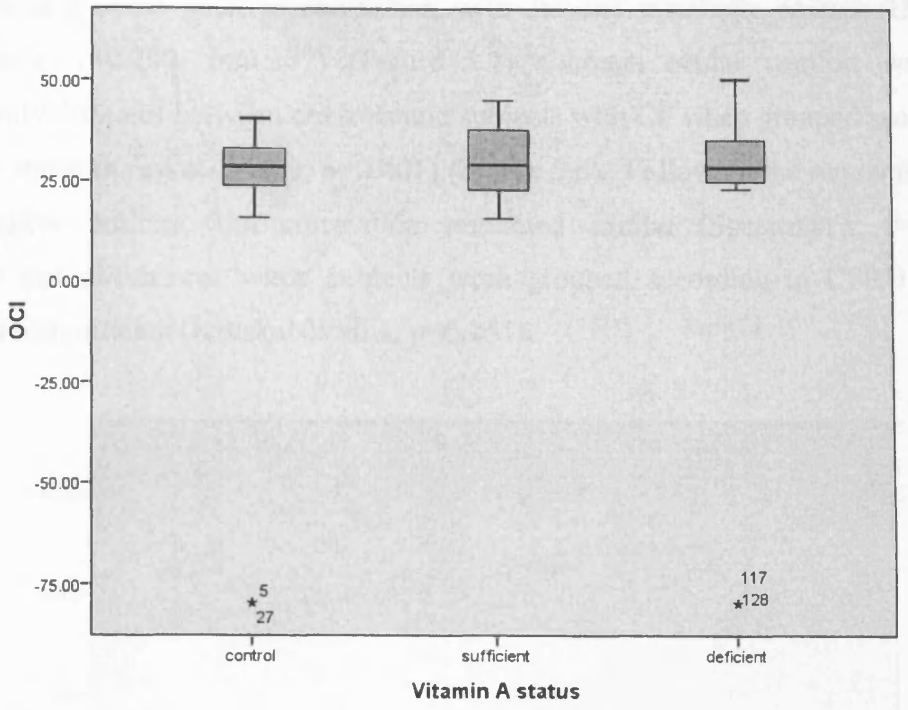


Figure 5.5 Ocular comfort index (OCI) scores in vitamin A deficient and sufficient *adults* with CF and healthy controls (Kruskal-Wallis; p=0.769)

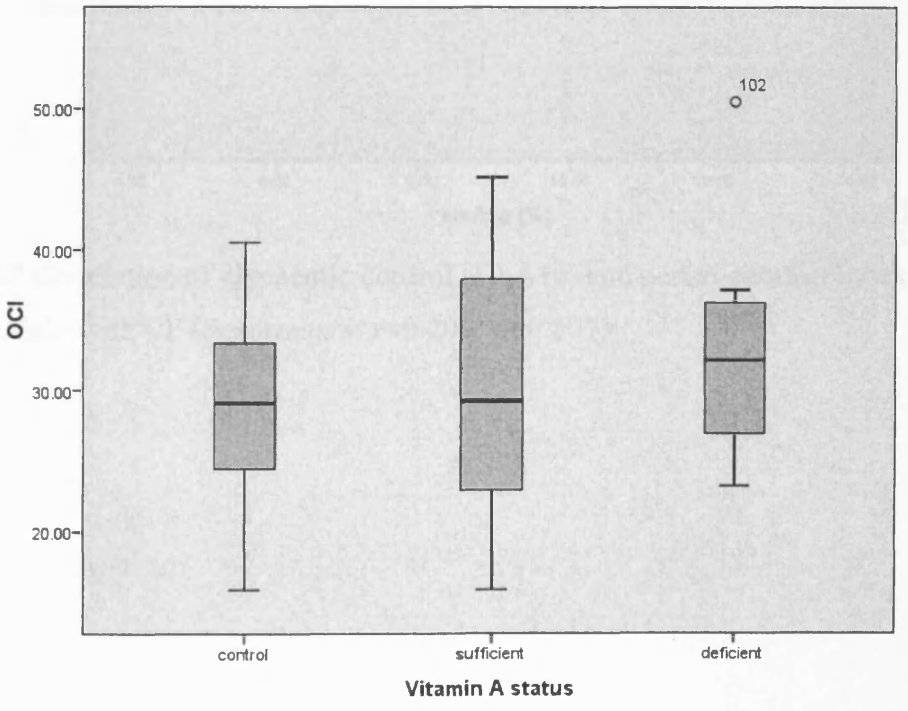


Figure 5.6 Ocular comfort index (OCI) scores in vitamin A deficient and sufficient *adults* with CF and healthy controls following the exclusion of four outliers (Kruskal-Wallis; p=0.603)

OCI showed a small positive correlation with diabetic metabolic control (HbA1c) (Spearman's; r=0.200, p=0.307) (Figure 5.7) although ocular comfort was not significantly different between controls and subjects with CF when grouped according to CFRD status (Kruskal-Wallis; p=0.401) (Figure 5.8). Following the removal of the two negative outliers, the correlation remained similar (Spearman's; r=0.199, p=0.329) and differences when subjects were grouped according to CFRD status remained insignificant (Kruskal-Wallis; p=0.251).

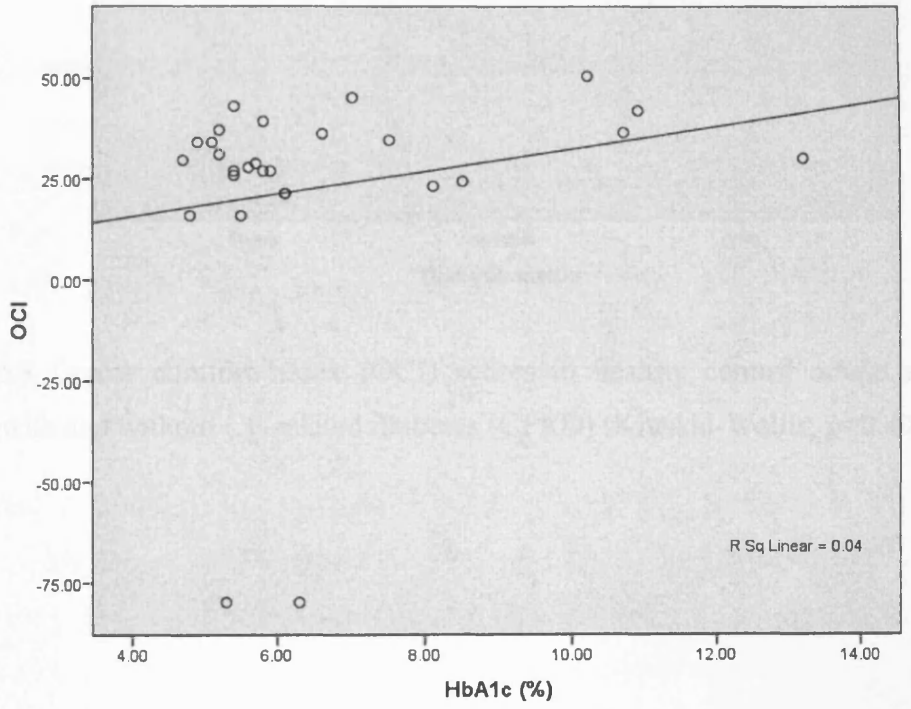


Figure 5.7 Correlation of glycaemic control (HbA1c) and ocular comfort index (OCI) score in *adults* with CF (Spearman's; r=0.200, p=0.307)

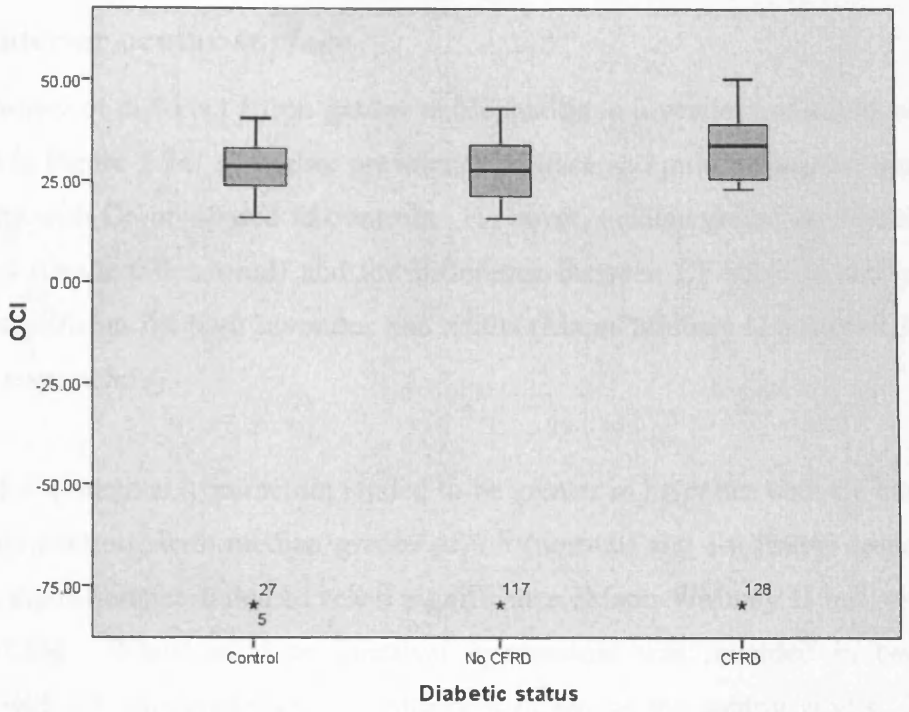


Figure 5.8 Ocular comfort index (OCI) scores in healthy control *adults* and CF subjects with and without CF-related diabetes (CFRD) (Kruskal-Wallis; p=0.401)

5.3.3 Anterior ocular surface

The frequency of different Efron grades of blepharitis in juveniles and adults with CF is shown in Figure 5.9a. A higher prevalence of trace and mild blepharitis were seen in subjects with CF compared to controls. However, median grades are identical for all groups (Grade 0.0 normal) and the difference between CF subjects and controls was not significant for both juveniles and adults (Mann-Whitney U test; p=0.347 and p=0.422 respectively).

Levels of conjunctival hyperaemia tended to be greater in juveniles with CF compared to juvenile controls, with median grades of 0.5 (normal) and 1.0 (trace) respectively although the difference failed to reach significance (Mann-Whitney U test; p=0.187) (Figure 5.9b). Whilst mild conjunctival hyperaemia was recorded in two adult subjects with CF, this grade was not observed in any of the control adults. Median grades were identical (Grade 1.0 trace) and no significant statistical difference detected (Mann-Whitney U test; p=0.474).

Considering both adults and juveniles, levels of limbal hyperaemia tended to be greater in subjects with CF compared to controls (Figure 5.10a). A median hyperaemia of Grade 0.5 was observed for all groups, and differences failed to reach significance (Mann-Whitney U test; juveniles p=0.866 and adults p=0.248).

Adults with CF exhibited a higher median level of corneal staining (1.0) compared to both controls (0.0) and juveniles (0.0). Mild corneal staining was observed only in the CF adult group: median levels of corneal staining were 1.0 in this group and 0.0 in the healthy adult controls (Figure 5.10b). Median grades were identical in the juvenile group (Grade 0.0). Whilst the difference in corneal staining approached significance in the adult cohort, the difference was not significant in juveniles (Mann-Whitney U test; juveniles p=0.794 and adults p=0.079 respectively).

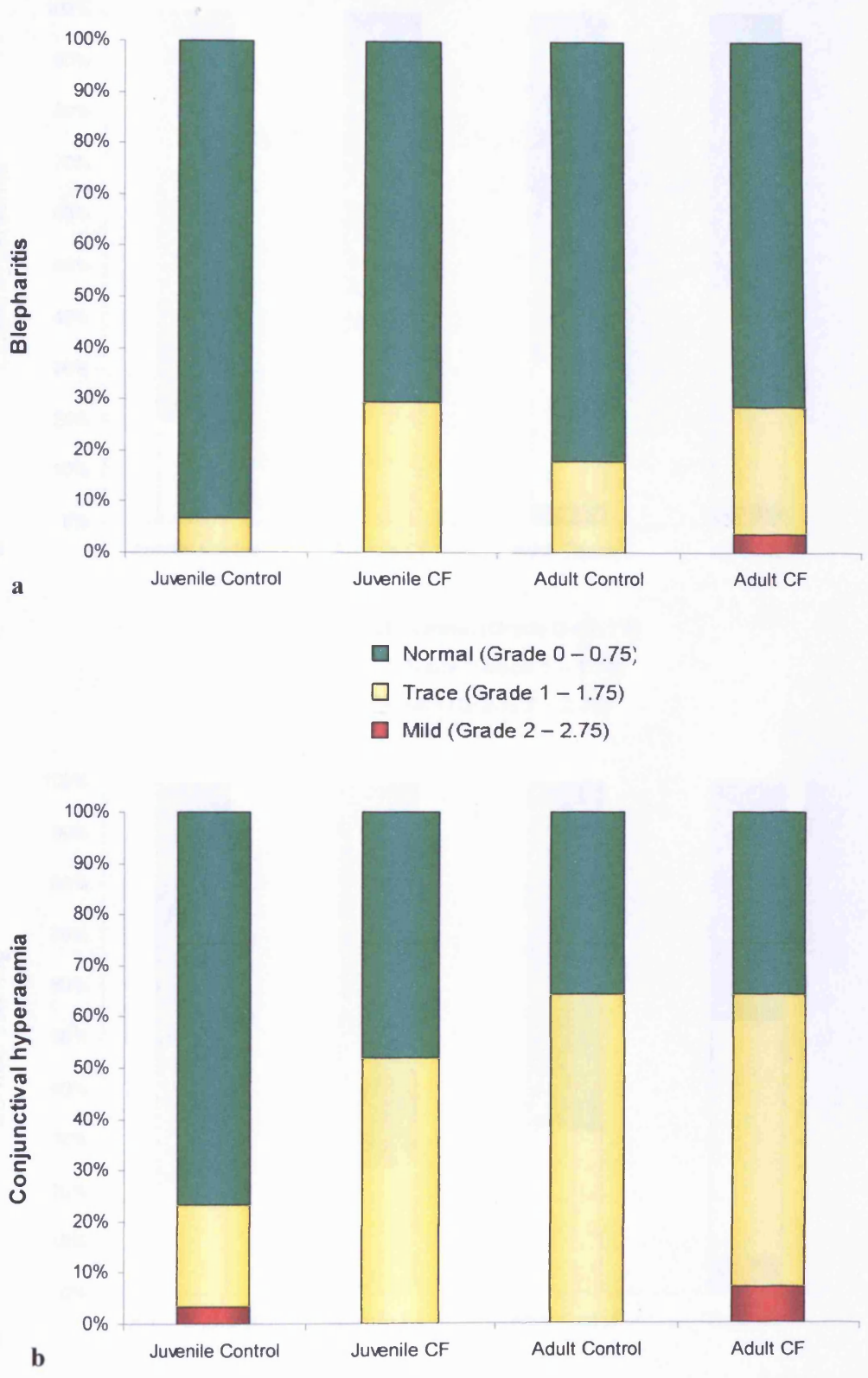


Figure 5.9 The prevalence of blepharitis (a) and conjunctival hyperaemia (b) in *juveniles* and *adults* with CF and healthy controls according to the Efron grading scale

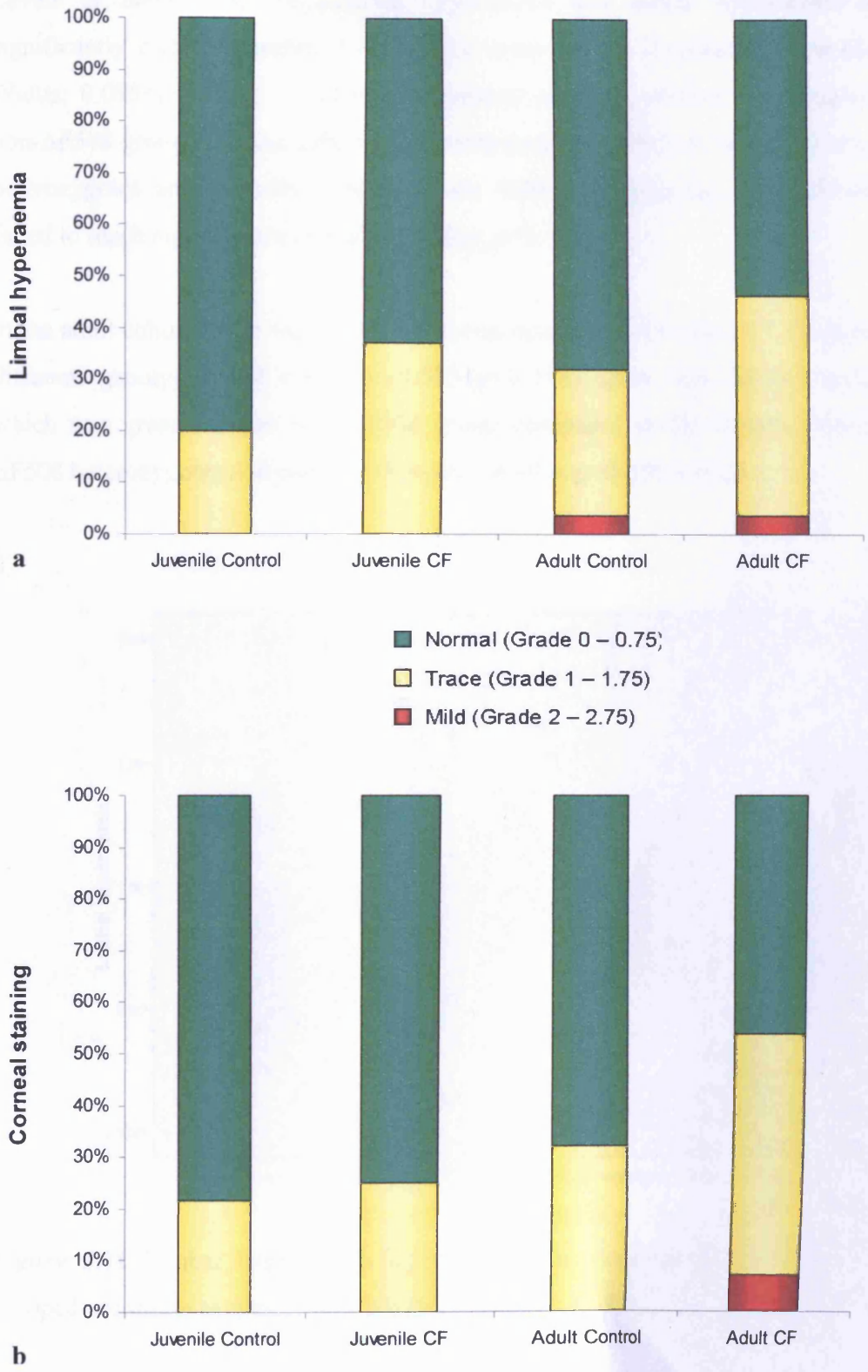


Figure 5.10 The prevalence of limbal hyperaemia (a) and corneal staining (b) in *juveniles* and *adults* with CF and healthy controls graded according to the Efron grading scale

Levels of blepharitis, conjunctival hyperaemia and limbal hyperaemia did not significantly differ according to genotype amongst the juvenile subjects (Kruskal-Wallis; $0.098<p<0.526$). Considering corneal staining, severity was greater in the non-ΔF508 group (median grade 1.00) compared to the ΔF508 homozygotes, ΔF508 heterozygotes and controls (median grade 0.00 each) although the difference just failed to reach significance (Kruskal-Wallis; $p=0.051$).

In the adult cohort, there was no difference in ocular surface signs in CF subjects with different genotypes and controls ($0.073<p<0.268$) apart from limbal hyperaemia, which was greater in the non-ΔF508 group compared to the ΔF508 homozygotes, ΔF508 heterozygotes and controls (Kruskal-Wallis; $p<0.05$) (Figure 5.11).

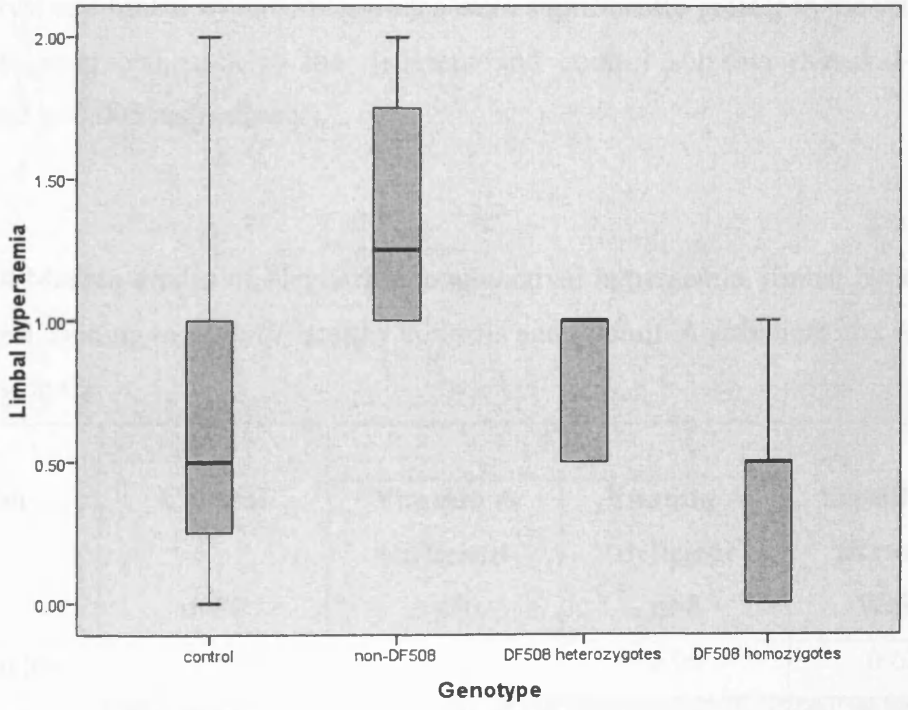


Figure 5.11 Limbal hyperaemia in *adult* healthy controls and subjects with CF grouped according to genotype (Kruskal-Wallis; $p<0.05$)

In the juvenile cohort, disease clinical severity (FEV_1, Crispin-Norman and Shwachman score) correlated poorly with measures of anterior ocular surface (Spearman's; $-0.350<r<0.315$, $0.141<p<0.968$). Considering the adults, corneal staining significantly correlated with Northern score (Spearman's; $r=0.451$, $p<0.05$). Blepharitis, corneal hyperaemia and limbal hyperaemia showed a poor correlation with Northern score and FEV_1 (Spearman's; $r=-0.266<r<0.275$, $0.157<p<0.960$).

Although no subjects showed any ocular surface signs of clinically significant xerophthalmia, results were still examined for differences according to vitamin A status. Considering the juvenile cohort, blepharitis and corneal staining was not significantly different between the vitamin A deficient, sufficient and control cohorts (Table 5.4) (Kruskal-Wallis; $p=0.631$ and $p=0.642$ respectively). Median conjunctival and limbal hyperaemia grades were significantly greater in the vitamin A sufficient group compared to the deficient and control subjects (Kruskal-Wallis; $p<0.01$ and $p<0.005$ respectively).

Table 5.4 Median grades of blepharitis, conjunctival hyperaemia, limbal hyperaemia and corneal staining in *juvenile* healthy controls and vitamin A sufficient and deficient subjects with CF

Factor	Control	CF		Significance (Kruskal-Wallis)
	n=30	Vitamin A sufficient n=16	Vitamin A deficient n=8	
Blepharitis	0.00	0.00	0.00	0.631
Conjunctival hyperaemia	0.50	1.00	0.50	**<0.010**
Limbal hyperaemia	0.50	1.00	0.50	**<0.005**
Corneal staining	0.00 n=24	0.00 n=15	0.00 n=6	0.642

A significant correlation was observed with serum vitamin A concentration and limbal hyperaemia (Spearman's; r=0.459, p<0.05) (Figure 5.12). There was a poor correlation between serum vitamin A concentration and blepharitis (Spearman's; r=-0.27, p=0.902) and a small positive correlation with conjunctival hyperaemia and corneal staining (Spearman's; r=0.322, p=0.125 and r=0.105, p=0.652 respectively). The anterior ocular surface appearance was not significantly different when juvenile subjects were grouped according to pancreatic status (Kruskal-Wallis; 0.167<p<0.966).

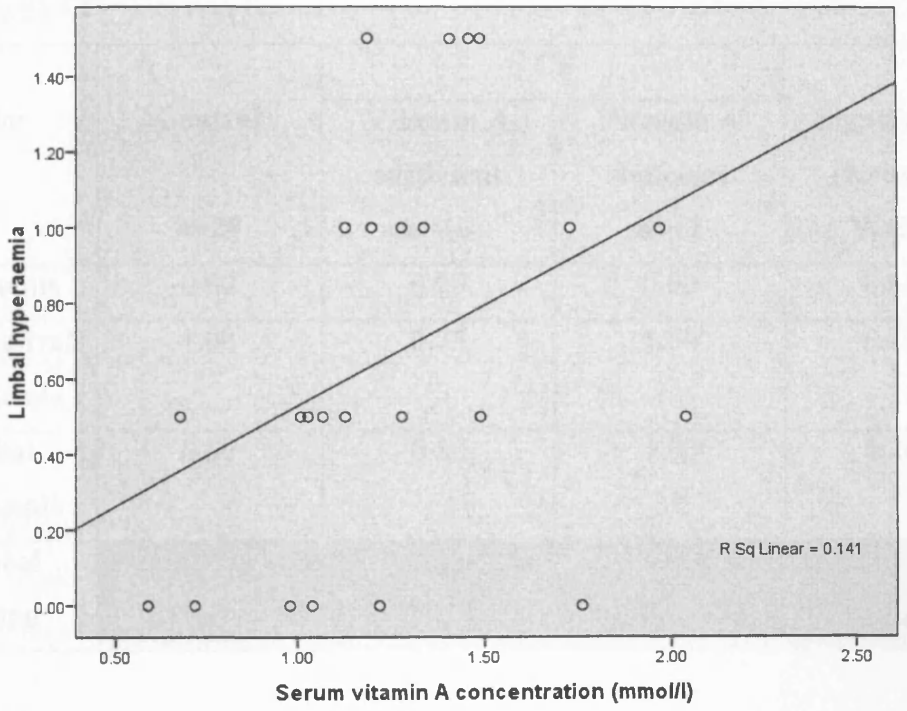


Figure 5.12 The correlation of limbal hyperaemia and serum vitamin A concentration in *juveniles* with CF (Spearman; r=0.459, p<0.05)

In adults, median blepharitis grades were identical in the vitamin A deficient and sufficient groups (Table 5.5). Conjunctival and limbal hyperaemia and corneal staining grades were greater in the vitamin A deficient group compared to the other groups, but these differences only reached significance for corneal staining (Kruskal-Wallis, $p<0.05$).

Table 5.5 Median grades of blepharitis, conjunctival hyperaemia, limbal hyperaemia and corneal staining in *adult* healthy controls and vitamin A sufficient and deficient subjects with CF

Factor	Control	CF		Significance (Kruskal-Wallis)
		Vitamin A sufficient	Vitamin A deficient	
	n=28	n=16	n=12	
Blepharitis	0.00	0.00	0.00	0.681
Conjunctival hyperaemia	1.00	0.75	1.50	0.204
Limbal hyperaemia	0.50	0.50	1.00	0.264
Corneal staining	0.00	0.00	1.00	**<0.050**

Measures of anterior ocular surface positively correlated with serum vitamin A concentration (Spearman's; $0.066<r<0.189$, $0.334<p<0.740$) and a significant correlation was observed with bulbar conjunctival hyperaemia (Spearman's; $r=0.405$, $p<0.05$) (Figure 5.13). The anterior ocular surface appearance was not significantly different when subjects were compared according to CFLD status (Kruskal-Wallis; $0.248<p<0.616$).

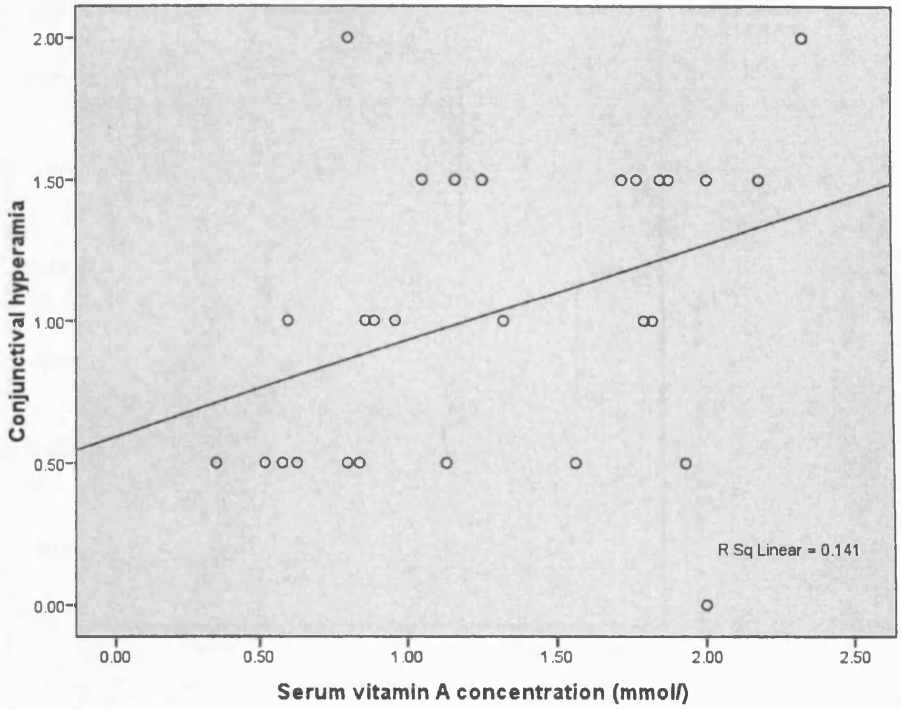


Figure 5.13 The correlation of conjunctival hyperaemia and serum vitamin A concentration in *adults* with CF (Spearman; r=0.405, p<0.05)

The presence/absence of CFRD did not seem to relate to any ocular surface findings; there were no significant differences observed between controls and those subjects with CFRD and those without (Kruskal-Wallis; 0.082<p<0.458).

5.3.4 Tear film stability

For the juvenile cohort, mean non-invasive break-up time (NIBUT) and fluorescein break-up time (FBUT) were only marginally greater in the control group compared to the CF group (Independent-samples t-test; p=0.357 and 0.154 respectively (Figure 5.14a). Tear film stability was significantly reduced in the adult cohort compared to the juveniles for both CF subjects and controls (ANOVA; NIBUT p<0.001 and FBUT p<0.001). In the adult group mean tear film stability times were greater in the controls compared to the subjects with CF (Figure 5.14b). Whilst NIBUT was not significantly different, the difference in FBUT reached significance (Independent-samples t-test; p=0.509 and p<0.05 respectively).

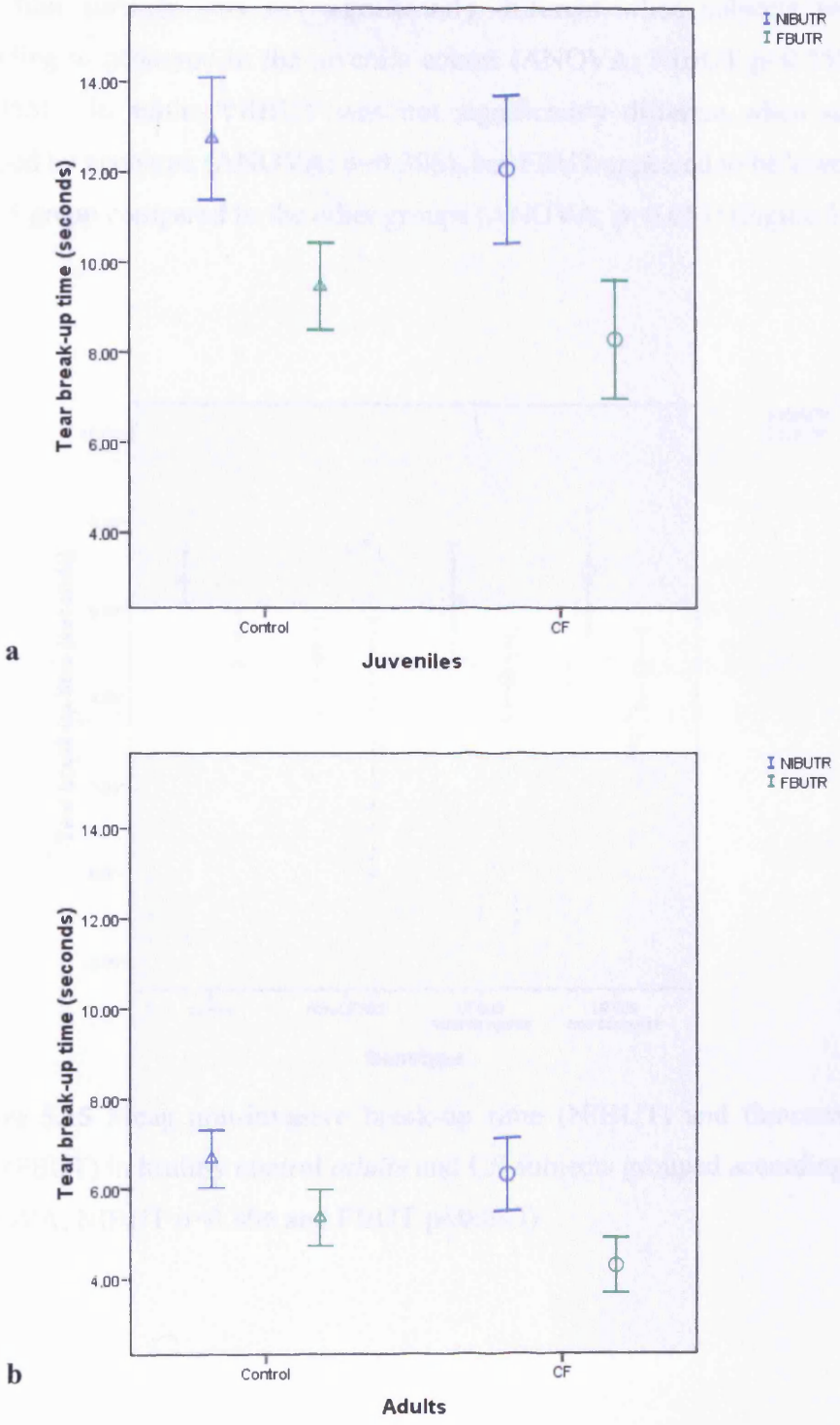


Figure 5.14 Mean non-invasive tear break up time (NIBUT) and fluorescein tear break up time (FBUT) in *juveniles* with CF and healthy controls (a) (Independent-samples t-test; p= 0.357 and p=0.154 respectively) and *adults* with CF and healthy controls (b) (Independent-samples t-test; p= 0.509 and p<0.05 respectively)

(Error bars indicate 95% confidence intervals)

Tear film stability was not significantly different when subjects were grouped according to genotype in the juvenile cohort (ANOVA; NIBUT p=0.555 and FBUT p=0.355). In adults, NIBUT was not significantly different when subjects were grouped by genotype (ANOVA; p=0.306), but FBUT appeared to be lower in the non-ΔF508 group compared to the other groups (ANOVA; p=0.053) (Figure 5.15).

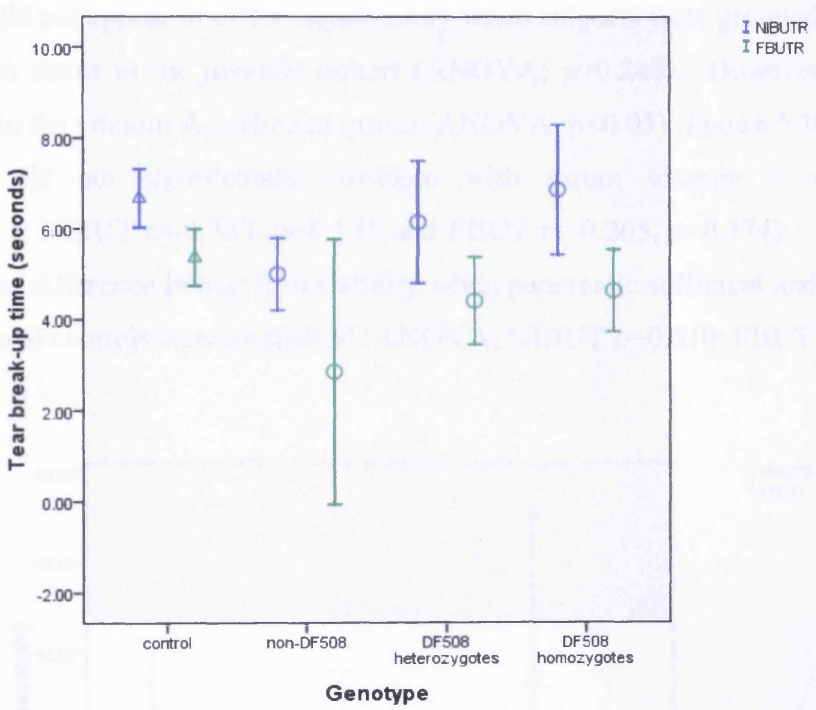


Figure 5.15 Mean non-invasive break-up time (NIBUT) and fluorescein break-up time (FBUT) in healthy control *adults* and CF subjects grouped according to genotype (ANOVA; NIBUT p=0.306 and FBUT p=0.053)

Considering measures of clinical severity in the juvenile group, tear film stability did not significantly correlate with lung function (FEV_1), Shwachman score or Chrispin-Norman score (Pearson's; $-0.015<r<0.277$; $0.224<p<0.952$). In adults, tear film stability showed a negative correlation with Northern score and a positive correlation with FEV_1 however, the correlations were not significant (Pearson's; NIBUT; r=-0.110, p=0.577 and r =0.280, p=0.149 respectively and FBUT; =-0.331, p=0.085 and r=0.343, p=0.074 respectively).

NIBUT did not appear to differ significantly when subjects were grouped according to vitamin A status in the juvenile cohort (ANOVA; p=0.240). However, FBUT was reduced in the vitamin A sufficient group (ANOVA, $p<0.05$) (Figure 5.16). Tear film stability did not significantly correlate with serum vitamin A concentration (Pearson's; NIBUT r=-0.333, p=0.131 and FBUT r=-0.205, p=0.374). There was no significant difference in tear film stability when pancreatic sufficient and deficient CF subjects and controls were compared (ANOVA; NIBUT p=0.610; FBUT p=0.349).

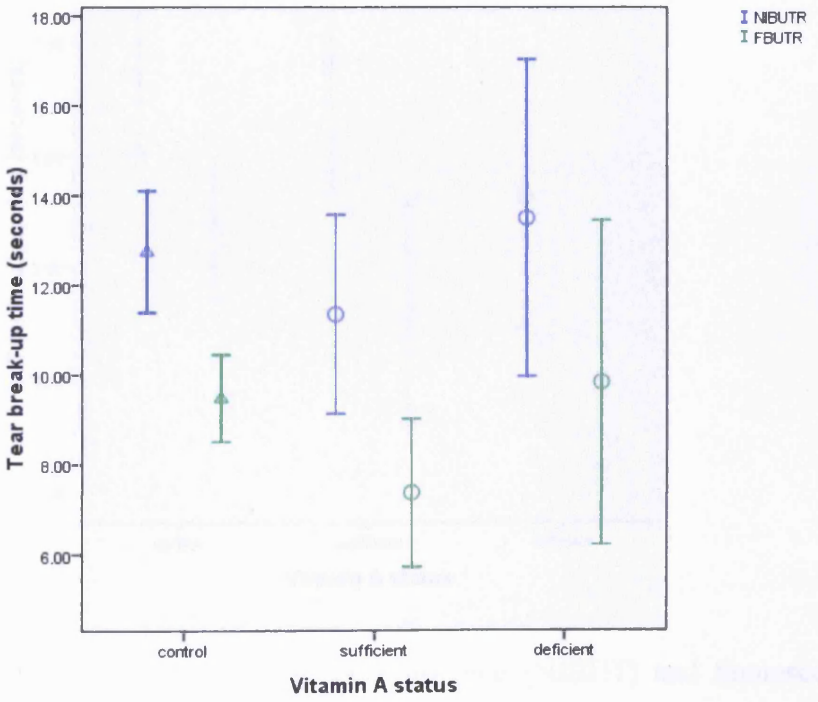


Figure 5.16 Mean non-invasive break-up time (NIBUT) and fluorescein break-up time (FBUT) in healthy control *juveniles* and CF subjects grouped according to vitamin A status (ANOVA; NIBUT p=0.240 and FBUT $p<0.05$)

Although tear film stability was reduced in the vitamin A deficient cohort compared to the sufficient group or the controls the difference failed to reach significance (ANOVA; NIBUT p=0.570; FBUT p=0.881) (Figure 5.17). Serum vitamin A concentration did not significantly correlate with tear film stability (Pearson's; NIBUT r=0.189, p=0.336; FBUT r=0.235, p=0.229). NIBUT and FBUT were not significantly different when subjects were grouped according to CFLD status (ANOVA; p=0.533 and p=0.109 respectively).

Measures of tear film stability negatively correlated with HbA1c, although the correlation was not significant (Pearson's; NIBUT; r=-0.118, p=0.551: FBUT; r=-0.228, p=0.243). NIBUT and FBUT were not significantly different amongst controls, and CF subjects with and without CFRD (Kruskal-Wallis; p=0.805 and p=0.108 respectively).

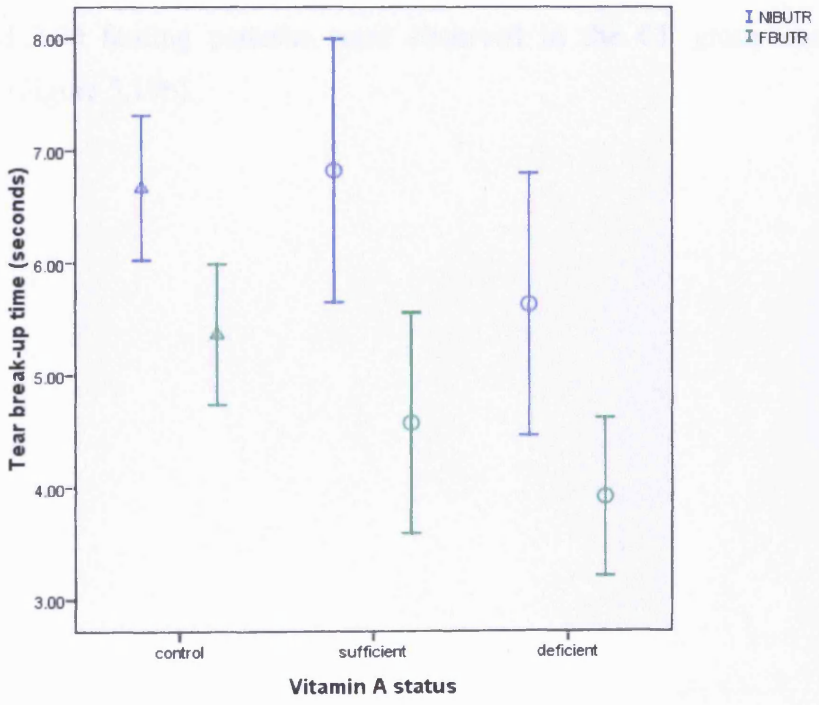
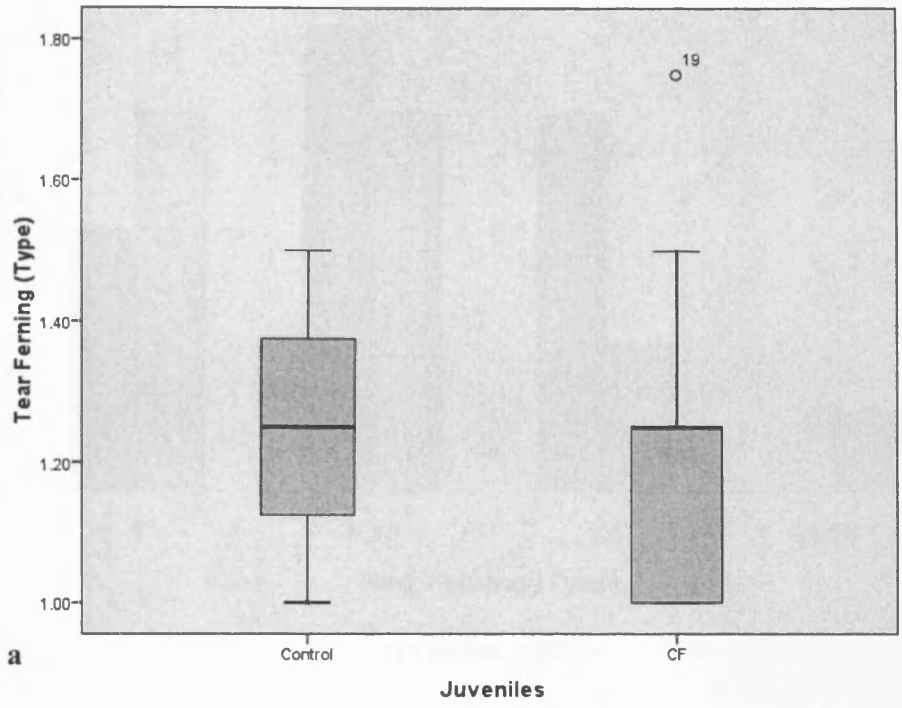


Figure 5.17 Mean non-invasive break-up time (NIBUT) and fluorescein break-up time (FBUT) in healthy control *adults* and CF subjects grouped according to vitamin A status (ANOVA; NIBUT p=0.570 and FBUT p=0.881)

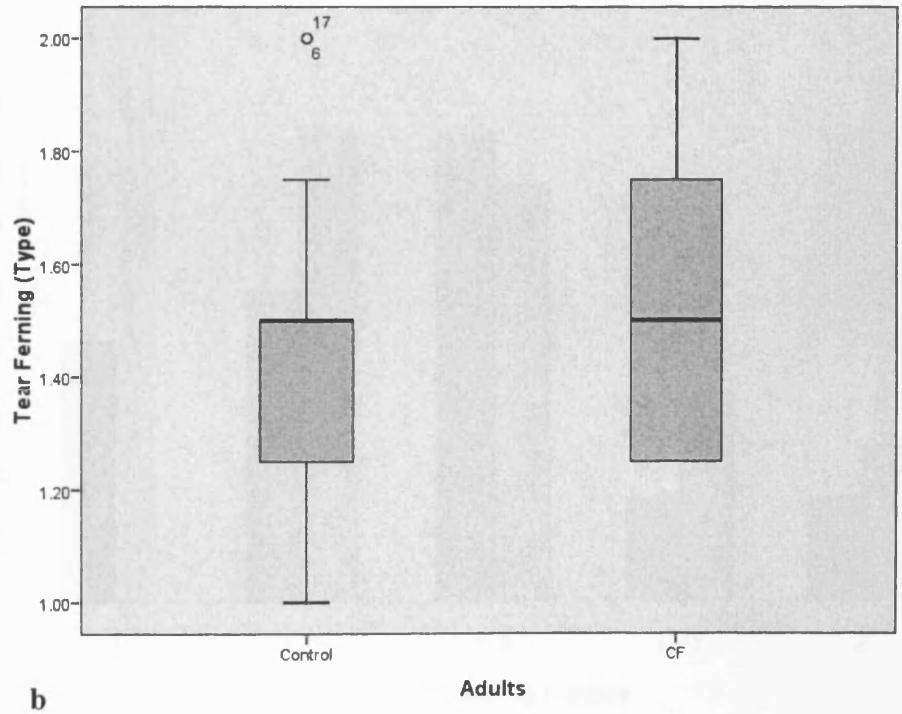
5.3.5 Tear Ferning

Sufficient tear film samples to produce tear ferning (TF) images were collected from 13 juveniles with CF and 15 healthy controls. All subjects had normal TF grades (Type 1 and Type 2) according to Rolando's grading scale. Median TF grades of 1.25 were observed in both the CF and control groups, the difference was not significant (Mann-Whitney U test; p=0.658) (Figure 5.18a). The incidence of the different TF Types in the two groups is shown in Figure 5.19a).

Sufficient tear film samples were collected from 24 adults with CF and 24 control subjects. All subjects had normal TF grades (Type 1 and 2) according to Rolando's grading scale. Median TF grades were slightly greater compared to the juveniles. Identical median grades were observed in the adult CF subjects and controls groups at 1.50 respectively (Figure 5.18b). Although there was no significant difference between the two groups (Mann-Whitney U test; p=0.190), a greater number of Type 1.75 and 2.00 ferning patterns were observed in the CF group compared to the controls (Figure 5.19b).

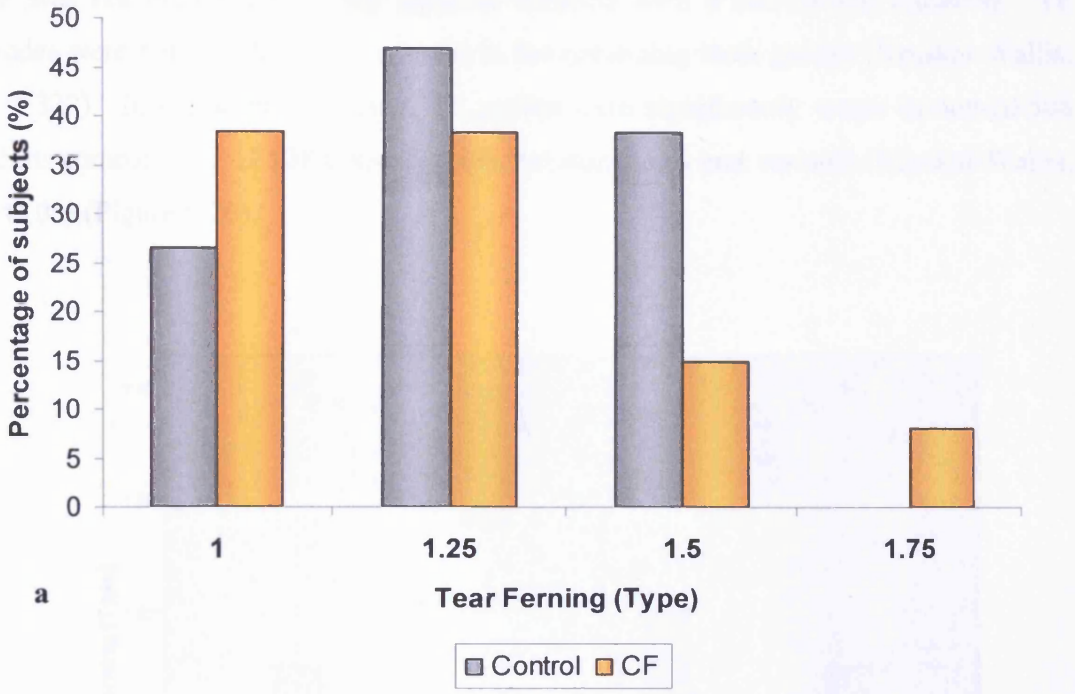


a



b

Figure 5.18 Tear ferning in juveniles with CF and healthy controls (Mann-Whitney U test; p=0.658) (a) and adults with CF and healthy controls (b) (Mann-Whitney U test; p=0.190)



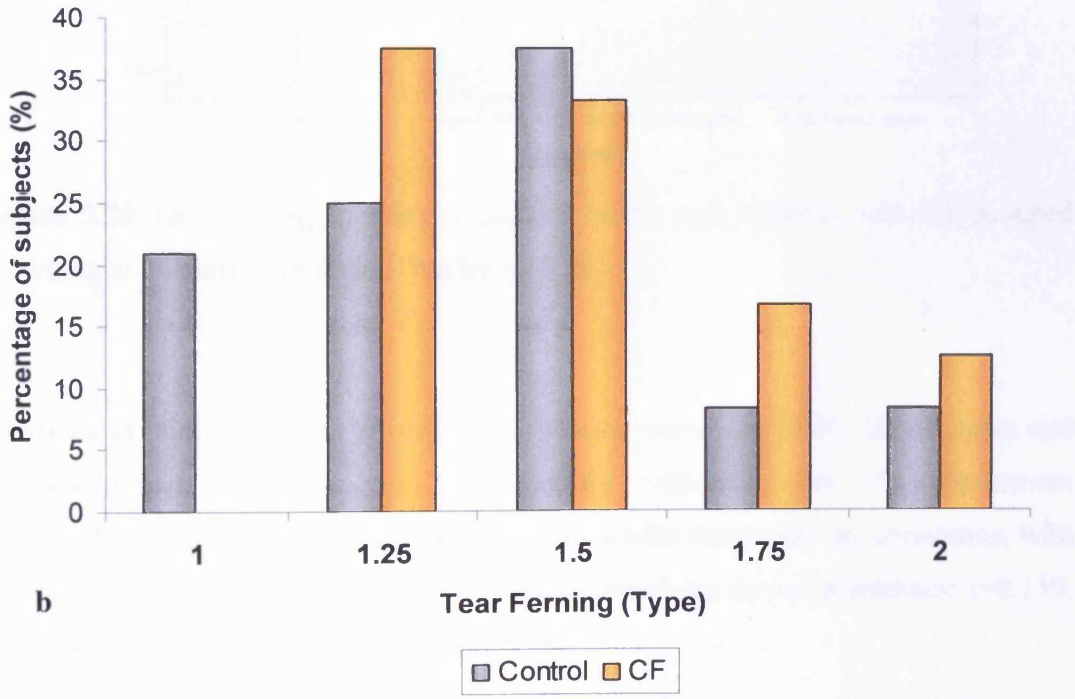


Figure 5.19 The prevalence of different tear ferning "Types" in juveniles with CF and healthy controls (a) and adults with CF and controls (b)

TF was not performed in any juvenile subjects with a non-ΔF508 mutation. TF grades were not significantly different in the remaining three groups (Kruskal-Wallis; p=0.332). In the adults however, TF grades were significantly worse in non-ΔF508 adults compared to ΔF508 homozygotes, heterozygotes and controls (Kruskal-Wallis; p<0.05) (Figure 5.20).

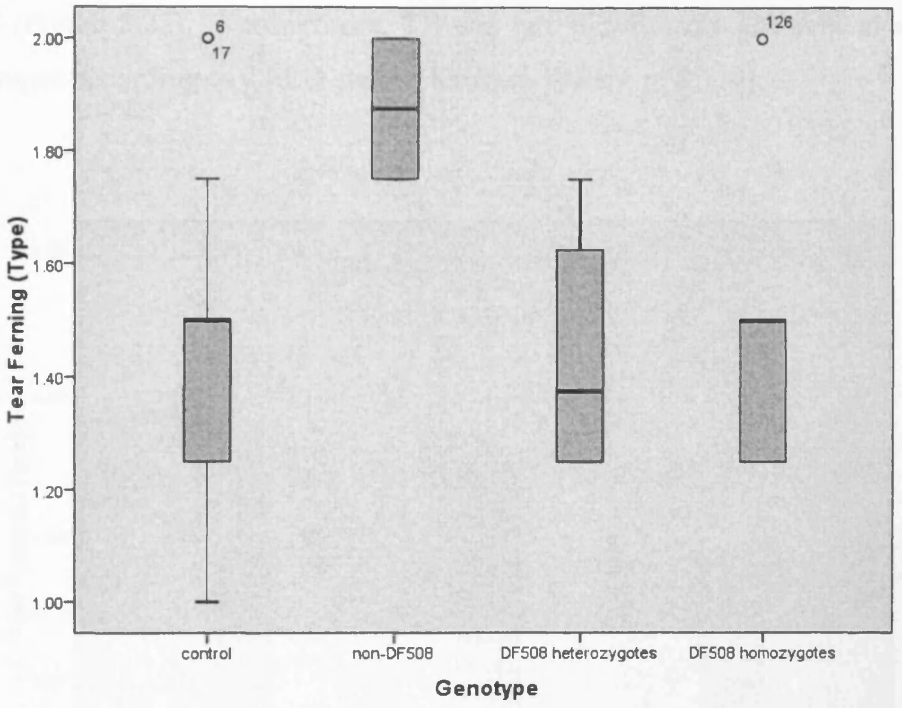


Figure 5.20 Tear ferning in healthy control *adults* and subjects with CF grouped according to phenotype (Kruskal-Wallis; p<0.05)

Measures of clinical disease severity in juveniles, including FEV_1, Shwachman and Crispin-Norman score did not significantly correlate with TF (Spearman; 0.152<r<0.475, 0.140<p<0.636). Similarly in adults there was no correlation with measures of disease severity such as FEV_1 or Northern Score (Spearman; r=0.150, p=0.484 and r=-0.226, p=0.289 respectively).

In the juvenile cohort, TF was not significantly different when controls, vitamin A deficient and vitamin A sufficient subjects were compared (Kruskal-Wallis; p=0.224) (Figure 5.21) and there was no significant correlation of TF grade with serum vitamin

A concentration (Spearman; r=0.491, p=0.105). Similarly, TF was not significantly different when subjects were grouped according to pancreatic status (Kruskal-Wallis; p=0.224).

TF did not significantly correlate with serum vitamin A concentration in the adults with CF (Spearman; r=0.214, p=0.314) and TF grades were not different between the vitamin A deficient and sufficient adults and the adult controls (Kruskal-Wallis; p=0.392) (Figure 5.22). Furthermore, TF was not significantly different in subjects when grouped according to CFLD status (Kruskal-Wallis; p=0.257).

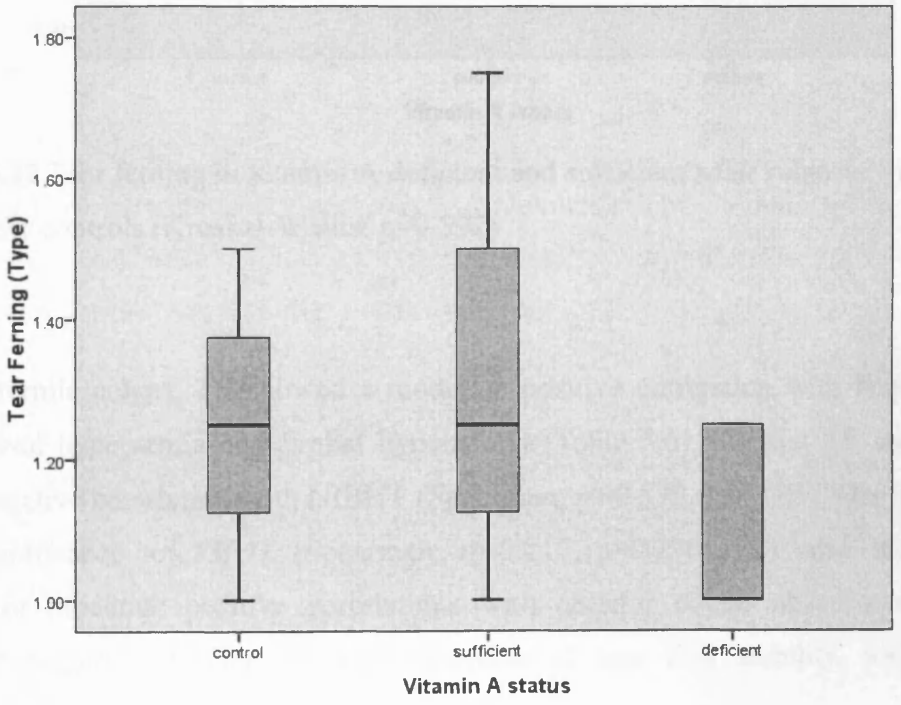


Figure 5.21 Tear ferning in vitamin A deficient and sufficient *juvenile* subjects with CF and healthy controls (Kruskal-Wallis; p=0.224)

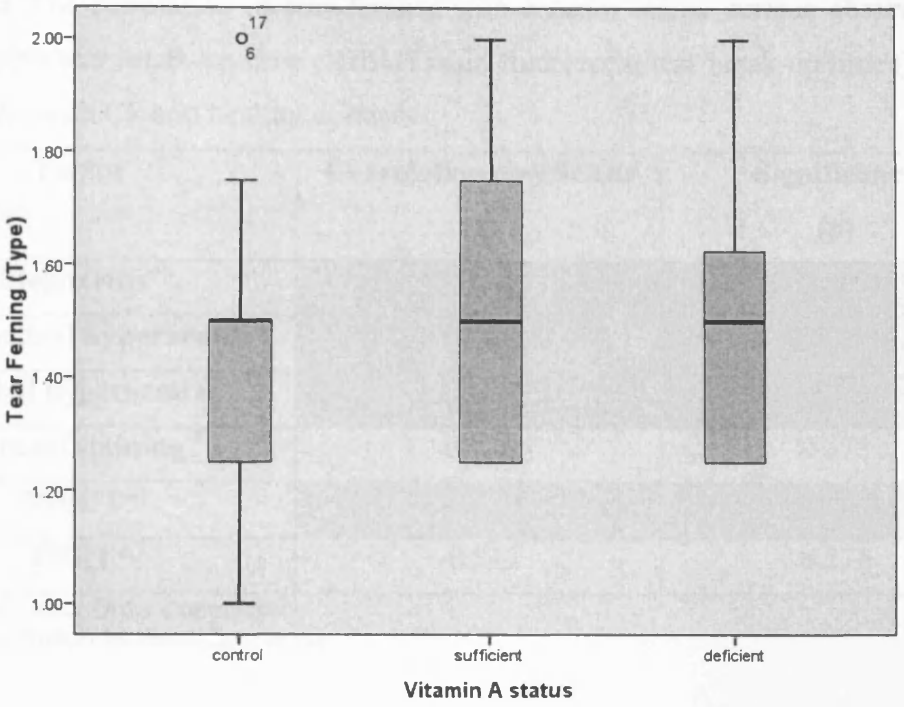


Figure 5.22 Tear ferning in vitamin A deficient and sufficient a*dult* subjects with CF and healthy controls (Kruskal-Wallis; p=0.392)

In the juvenile cohort, TF showed a moderate positive correlation with blepharitis, conjunctival hyperaemia and limbal hyperaemia (Table 5.6). Whilst TF showed a strong negative correlation with NIBUT (Spearman; r=-0.579, p<0.005), this failed to reach significance for FBUT (Spearman; r=-0.217, p=0.276). TF also showed a number of moderate positive correlations with anterior ocular observations and moderate negative correlations with measures of tear film stability which are summarised in Table 5.7.

Table 5.6 The correlation of tear ferning with anterior ocular surface observations, non-invasive tear break-up time (NIBUT) and fluorescein tear break-up time (FBUT) in *juveniles* with CF and healthy controls

Factor	Correlation coefficient (r)	Significance (p)
Blepharitis #	**0.410**	**<0.050**
Conjunctival hyperaemia #	**0.401**	**<0.050**
Limbal hyperaemia #	**0.480**	**<0.050**
Corneal staining #	0.175	0.373
NIBUT*	**-0.579**	**<0.005**
FBUT*	-0.217	0.276

Spearman's Rank Order Correlation
* Pearson's Product Moment Correlation

Table 5.7 The correlation of tear ferning with the ocular comfort index (OCI), anterior ocular surface observations, non-invasive tear break-up time (NIBUT) and fluorescein tear break-up time (FBUT) in *adults* with CF and healthy controls

Factor	Correlation coefficient (r)	Significance (p)
OCI #	0.61	0.679
Blepharitis #	**0.380**	**<0.01**
Conjunctival hyperaemia #	**0.445**	**<0.005**
Limbal hyperaemia #	0.271	0.062
Corneal staining #	-0.023	0.877
NIBUT*	**-0.442**	**<0.005**
FBUT*	**-0.427**	**<0.005**

Spearman's Rank Order Correlation
* Pearson's Product Moment Correlation

5.3.6 Major findings of this study

- Significantly higher levels of ocular discomfort in non-ΔF508 adult subjects compared to ΔF508 heterozygotes

- Generally increased levels of hyperaemia and staining in subjects with CF compared to controls although the differences were not significant

- Significantly higher grades of limbal hyperaemia in non-ΔF508 adult subjects compared to ΔF508 heterozygotes and homozygotes

- Significantly higher conjunctival and limbal hyperaemia in vitamin A sufficient juveniles compared to vitamin A deficient subjects and controls

- Significantly higher grades of corneal staining in vitamin A deficient adults compared to vitamin A sufficient adults and controls

- Significant correlation of corneal staining and Northern score in CF adults

- FBUT was significantly reduced in adult CF subjects compared to controls

- Tear film stability further reduced in vitamin A deficient adults compared to vitamin A sufficient adults although the differences were not significant

- FBUT was significantly reduced in juvenile vitamin A sufficient subjects compared to controls

- Generally higher TF grades in CF subjects compared to controls although the differences were not significant

- TF showed a number of significant correlations with measures of tear film stability and anterior ocular surface observations in juvenile and adults groups

5.4. Discussion

5.4.1 Ocular comfort

The results indicate juvenile subjects with CF have similar perceived levels of ocular discomfort symptoms compared to controls. This is the first investigation to quantitatively assess ocular comfort in subjects with CF and controls. In adults, with increased levels of disease severity and duration, it could be expected that dry eye symptoms may be more prevalent. Whilst median OCI scores were higher in the CF cohort, this was not significantly different. It is well documented that clinical signs and symptoms correlate poorly in dry eye (Nichols et al., 2004b) and this could be true in CF similarly. However, if dry eye is a primary manifestation it is likely to be a chronic condition in CF subjects and adults may be well adapted or tolerant of symptoms. Significance could also be hindered by higher levels of variance in the CF adult group or relatively small cohort sizes.

Specifically, the presence of VAD, CFRD or CFLD did not appear relevant to ocular comfort. However, when adult subjects were grouped according to genotype, greater levels of ocular discomfort were observed in the non-ΔF508 subgroup. The non-ΔF508 genotype group consisted of four individuals, three of unknown CFTR mutations and an individual with a R553X/R553X mutation. The R553X class 1 mutation results in complete absence of CFTR synthesis and is associated with a severe CF phenotype (Zielenski and Tsui, 1995, Aznarez et al., 2007). Therefore, the non-ΔF508 group could consist of subjects with severe CFTR mutations resulting in complete absence of CFTR activity in the apical corneal and conjunctival epithelium and maximally impaired basal tear secretion and more severe symptoms of dry eye in these subjects.

5.4.2 Anterior ocular surface

This is the first study to utilise a quantitative grading scale to assess the anterior eye in subjects with CF. Blepharitis is frequently observed in dry eye subjects and meibomian gland dysfunction is the primary cause of evaporative dry eye (Lemp and Nichols, 2009). Previous investigations of the prevalence of blepharitis in CF have been varied with a reported range of 20-88% CF subjects affected (Mrugacz et al., 2007c, Sheppard et al., 1989). However, the clinical definition of blepharitis was inconsistent in these studies. In this investigation, 30% juveniles and 29% adults with CF had trace or mild blepharitis (Grade 1-2.75) compared to 7% and 18% controls respectively. The results of this investigation support previous reports that despite an increased incidence of blepharitis in CF, the difference fails to reach significance (Kalayci et al., 1996, Mrugacz et al., 2005b, Mrugacz et al., 2007b).

Corneal staining, conjunctival and limbal hyperaemia are frequently observed in ocular surface inflammation. Corneal fluorescein staining is regarded as a popular diagnostic test for dry eye (Smith et al., 2008, Nichols et al., 2000). Whilst CF subjects tended to demonstrate higher levels of hyperaemia and staining, particularly in the adults, differences failed to reach significance, although this could be a reflection of sample size. This study is in concurrence with earlier reports in that whilst the incidence of corneal staining was greater in the CF cohort compared to healthy controls the difference was not significant (Kalayci et al., 1996, Mrugacz et al., 2005b).

Whilst no genotype differences were observed in the juvenile group, limbal hyperaemia was significantly increased in the non-ΔF508 adults. This could be due to reasons outlined previously and the lack of associated significance in the increase of conjunctival redness and staining could be due to small subgroup size. According to disease severity, pancreatic, liver and diabetic status, there were no ocular surface differences apart from a correlation of Northern Score and staining in adults. This could indicate disease severity contributes to the pathogenesis of dry eye in CF. Reassuringly, features of ocular surface xerophthalmia which occur following prolonged VAD were not observed in any subjects with CF. Whilst increased corneal staining was observed in vitamin A deficient adults, increased hyperaemia in juvenile

vitamin A sufficient subjects was somewhat unexpected. This may have occurred because vitamin A status at the time may not have been represented by serum levels recorded from the patient's medical records, or this could be an incidental finding in a relatively small cohort. VAD results in reduced goblet cell density and mucin secretion causing reduced ocular surface wetting (Sharma, 1998a) which is likely to be responsible for the increased epithelial damage observed in the vitamin A deficient adults.

5.4.3 Tear film stability

Whilst a number of previous studies have assessed FBUT in CF, NIBUT is novel to this investigation. In general, tear film stability was lower in CF subjects compared to controls although only FBUT in the adult cohort reached significance. The variances amongst measures of NIBUT tend to be larger than those of FBUT which would limit statistical significance of differences. Measures of tear film stability in juveniles are similar to published values in normal adults (mean NIBUT 11.2 and FBUT 7.6 seconds) (Nichols et al., 2002). In this investigation tear film stability was significantly lower in the adults compared to juveniles. Whilst tear film stability is known to decrease with age (Ozdemir and Temizdemir, 2009, Cho and Yap, 1993), the difference compared to the findings of Nichols et al. (2002) could reflect environmental differences as juvenile and adult data were collected in different locations.

The instillation of fluorescein into the tear film is known to reduce tear film stability (Mengher et al., 1985) and mean FBUT was lower than NIBUT in all cohorts. FBUT in subjects with CF were similar to previous reports where tear film stability was also significantly reduced (Mrugacz et al., 2007b, Mrugacz et al., 2007d, Mrugacz et al., 2007c). The lack of significance in NIBUT could also be associated with poor repeatability of measures of tear film stability (Nichols et al., 2004a). Tear film stability was not significantly different amongst juveniles, and whilst increased levels of variance may have hindered significance this could also indicate disease duration or severity may contribute to the pathogenesis dry eye.

Tear film stability was not different when subjects were grouped according to CFRD, CFLD, pancreatic status or genotype apart from reduced FBUT in the non-ΔF508 adults. FBUT was significantly reduced in vitamin A sufficient juveniles, further suggesting that vitamin A levels of this group may not have been truly representative of their status at the time of this investigation. In the adult cohort, FBUT and NIBUT were reduced in the vitamin A deficient subjects, and tear film stability positively correlated with serum vitamin A concentration. The results did not reach significance however and again, may have been hindered by small subgroup size and time differences between blood analysis and tear film assessment.

5.4.4 Tear ferning

Significantly higher grades of abnormal TF have been observed in subjects with CF compared to controls previously (Rolando et al., 1988, Kalayci et al., 1996). In this study, abnormal TF (above Type 2) was not observed in any subjects. Kalayci et al. (1988) observed almost 74% of CF subjects in their investigation also had normal TF. In the juvenile cohort, whilst the highest TF grade of 1.75 was only observed in subjects with CF, there was no significant difference in TF patterns between CF and control subjects. In the adult cohort, a shift towards higher TF types was observed in the CF group compared to the controls although the difference failed to reach significance. The increased presence of higher TF grades in adults with CF compared to controls could reflect increased tear film osmolarity in these subjects.

TF was not significantly different when subjects were grouped according to genotype, CFRD or CFLD. Similarly, TF grades were not significantly different when subjects were grouped according to vitamin A status, and TF did not significantly correlate with vitamin A concentration. However, the categorical nature of the TF grading scale could be insensitive to such relationships. Rolando et al. (1988) suggested abnormal tear film mucin was responsible for the altered TF grades observed in subjects with CF (Rolando et al., 1988). Evidence suggests mucin secretion may be decreased in VAD (Tei et al., 2000, Hori et al., 2005) and reduced mucin secretion is likely to impair tear film stability leading to increased tear film evaporation and increased osmolarity. Therefore, increased electrolyte and reduced glycoprotein

concentrations in the tear film of vitamin A deficient subjects with CF would adversely affect the TF pattern. This suggests the abnormal TF observed by Rolando et al. was secondary to VAD. However, there was no indication of vitamin A status in this study. Kalayci et al. (1996) also recorded a poor correlation of TF and vitamin A concentration. Whilst serum vitamin A is known to be a poor measure of vitamin A liver stores (Underwood and Denning, 1972) statistical significance could also be restricted by sample size and the fact vitamin A was not measured at the point of tear film collection.

A significant negative correlation of TF and measures of tear film stability was observed in this study. Although a correlation with the Schirmer test has been documented previously (Puderbach and Stolze, 1991) a correlation of TF with tear break-up time is novel to this investigation. Similarly, this is the first report of a correlation of ocular surface observations and TF. TF did not correlate with ocular symptoms in adults and similar findings have been reported (Evans et al., 2009, Srinivasan et al., 2007, Jackson and Perrigin, 1999).

5.5 Summary

This study provides some support for the hypothesis that dry eye is a primary manifestation of CF although substantial significant evidence is limited. Whilst CF subjects tended to demonstrate increased signs of ocular surface inflammation (hyperaemia and staining) differences failed to reach significance. Genotypic differences did not predict dry eye intensity and limited correlations were recorded with clinical disease severity. However, results could be hindered by relatively small cohort sizes and the variable nature of the disease. CF subjects, particularly the juvenile cohort, were generally of relative "good health". This undoubtedly reflects modern therapeutic treatment and disease management.

The results could suggest alternative epithelial Cl^- channels provide some level of compensation for defective CFTR function leading to sub-clinical levels of dry eye that were undetectable in this study. Dry eye may only be manifest during periods of exacerbation, poor health or following prolonged disease duration.

Tear film stability appears reduced in CF, but the finding that it was further reduced in adult CF subjects who were vitamin A deficient provides some evidence that vitamin A deficiency is likely to contribute to the pathogenesis of dry eye in CF. Therefore, the aetiology of dry eye in CF is likely to be a combination of these primary and secondary manifestations.

Chapter 6

Investigating Visual Function in Children and Young Adults with Cystic Fibrosis

6.1 Introduction

Chapter 2 identified common ocular associations of Cystic Fibrosis (CF). However, existing studies have frequently been small scale or included confounding factors such as unknown vitamin A or diabetic status. Abnormal visual function in CF is hypothesised to be caused by impaired Cl^- secretion from the apical retinal pigment epithelial membrane via CFTR. This primary manifestation of CF is proposed to cause abnormal regulation of the subretinal space (SRS) and inter-photoreceptor matrix (IPM) resulting in loss of normal photoreceptor function and reduced visual acuity, colour vision and contrast sensitivity.

6.1.1 Objectives for this study

This study aimed to examine juveniles with CF of known disease status and severity with respect to refractive error, binocular vision and visual function. The hypotheses were that higher levels of ametropia, strabismus and amblyopia would be expected in subjects with CF due to associated prematurity and low birth weight, and that abnormal visual function is a primary manifestation of CF.

6.2 Method

6.2.1 Subjects

See Section 5.2.1

6.2.2.1 Inclusion and exclusion criteria

See Section 5.2.1.1

6.2.2 Experimental design

Subjects with CF were seen at the Children's Hospital for Wales, and due to out-patient clinic constraints juveniles were seen for two or three different data collection sessions each lasting approximately 10 to 15 minutes. This allowed subjects to be examined between different outpatient appointments to minimise inconvenience and increase participation. Control subjects were assessed during a single 30 to 40 minute session at the School of Optometry and Vision Sciences, Cardiff University.

All subjects completed a face-to-face ocular health questionnaire with their parent or guardian which investigated previous and current ocular health, vision, colour vision and family ocular health. Medications and reports of general health were also recorded in control subjects. Further information regarding subjects with CF was recorded from their medical record (see Section 5.2.2).

6.2.2.1 Refractive error and binocular vision

Distance vision (right, left and binocular) was recorded with a Bailey-Lovie chart (Clement Clarke International Ltd., Essex, UK) set at three metres (luminance 160 candelas/ metre^2 (cd/m^2), range 80-320 cd/m^2, (Sheedy et al., 1984)). Patients were refracted and distance visual acuity was similarly recorded. Near visual acuity (right, left and binocular) was recorded with a Bailey-Lovie word near vision chart (Clement Clarke International Ltd., Essex, UK) chart at 25cm. Spherical equivalent refraction (SER) was calculated as spherical correction plus half the negative cylindrical correction. Myopia was defined as a mean sphere of -0.50 Dioptres (D) and above and hypermetropia as a mean sphere of +2.00D and above (Fan et al., 2004). Correction between these values was recorded as emmetropia.

A cover-uncover and alternating distance and near cover test was performed whilst the patient fixated a single distance LogMAR letter or letter on a budgie stick two lines above that of their distance or near visual acuity with spectacle correction. Stereopsis was assessed with a Titmus Fly Test (Clement Clarke International Ltd., Essex, UK) with spectacle correction and polarised glasses.

6.2.2.2 Visual function

Contrast sensitivity (right, left and binocular) was recorded with spectacle correction using a Peli-Robson chart (Clement Clarke International Ltd., Essex, UK) at three metres (m) (luminance 160 cd/m^2), which corresponds to a spatial frequency of approximately three cycles per degree (Pelli et al., 1988). Subjects completed saturated and de-saturated Farnsworth D15 colour vision tests (Clement Clarke International Ltd., Essex, UK) illuminated within a light box (CIE source C, 320-400 lux). A BASIC computer programme was used to calculate quantitative scores (Vingrys and King-Smith, 1988) where the output included a Total Error Score (TES), Angle (indicating the type of colour vision defect), S-index (indicating the lack of randomness) and C-index (indicating the degree of colour vision (CV) defect).

6.2.3 Statistics

Data was checked for normality using the Kolmogorov-Smirnov test. Results for vision, visual acuity, measures of colour vision, contrast sensitivity, mean sphere and stereopsis were not normally distributed ($0.000<p<0.016$). However, the results were a small sample of continuous data which is representative of a normal distribution. Therefore, Bland and Altman (2009) have suggested parametric statistics can be applied (Bland and Altman, 2009). A Paired-samples (2-tailed) t-test was applied to compare results from right and left eyes. An Independent-samples (2-tailed) t-test was used to compare data from the CF and control cohort. Correlation was assessed with Pearson's product moment correlation coefficient. One-Way Analysis of Variance (ANOVA) analysis was used to examine data based on vitamin A status, pancreatic status and genotype. Bonferroni's test was used for post-hoc comparisons. Significance was set at the 0.05 level.

6.3 Results

All subjects participated fully, with the exception of colour vision assessment where:

- 25 subjects with CF participated (3 of the younger subjects were excluded due to lack of understanding, 2 subjects were lost to follow up)
- 27 control subjects participated (3 of the younger subjects were excluded due to lack of understanding)

A Paired-samples t-test indicated distance vision, distance visual acuity, near visual acuity, mean spherical equivalent refraction and contrast sensitivity were not significantly different when comparing the right and left eyes of all subjects ($0.065<p<1.000$). From this point only values from the right eye are reported.

6.3.1 Overview of subjects with CF

Table 6.1 Overview of subject characteristics and disease severity for the CF group

Variable	Description
Genotype	n=11 ΔF508 homozygous n=17 ΔF508 heterozygous n=2 non ΔF508
Pancreatic function	n=6 pancreatic sufficient n=24 pancreatic deficient
Serum vitamin A	range 0.59 – 2.04 μmol/l mean 1.25 μmol/l (SD ± 0.35)
Vitamin A status	n=19 vitamin A sufficient, n=8 vitamin A deficient (3 subjects unmeasured)
HbA1c	5 subjects assessed range 5.2 – 5.9% mean 5.58% (SD ± 0.26)
CFRD	None
FEV_1	range 19 – 123 % mean 89.64 % (SD ± 13.08)
Shwachman Score	score >86 (excellent) n=19 score 71-85 (good) n=8 no score n=3 range 100 – 71 mean score 88.17 (SD ± 8.43)
Chrispin-Norman Score	range 0 – 10 no score n=7 mean 5.22 (SD ± 2.76)
Premature birth	n=2 at 31 weeks and 36 weeks gestation

6.3.2 Refractive error

Of the CF cohort, 43% had undergone an eye examination with an optometrist within the previous 2 years and 20% were currently prescribed some form of spectacle correction. In the control cohort, these values were 70% and 23% respectively (Figure 6.1). Considering refractive error, in the CF group 25 subjects were emmetropic, 2 hypermetropic and 3 myopic. In the control group, 25 subjects were emmetropic and 5 were myopic (Figure 6.2). Whilst mean distance vision was identical in the two groups at -0.21 in each group, SER was higher in the CF group (Figures 6.3 and 6.4). Differences were not significant however (Independent-samples t-test; p=0.911 and p=0.153).

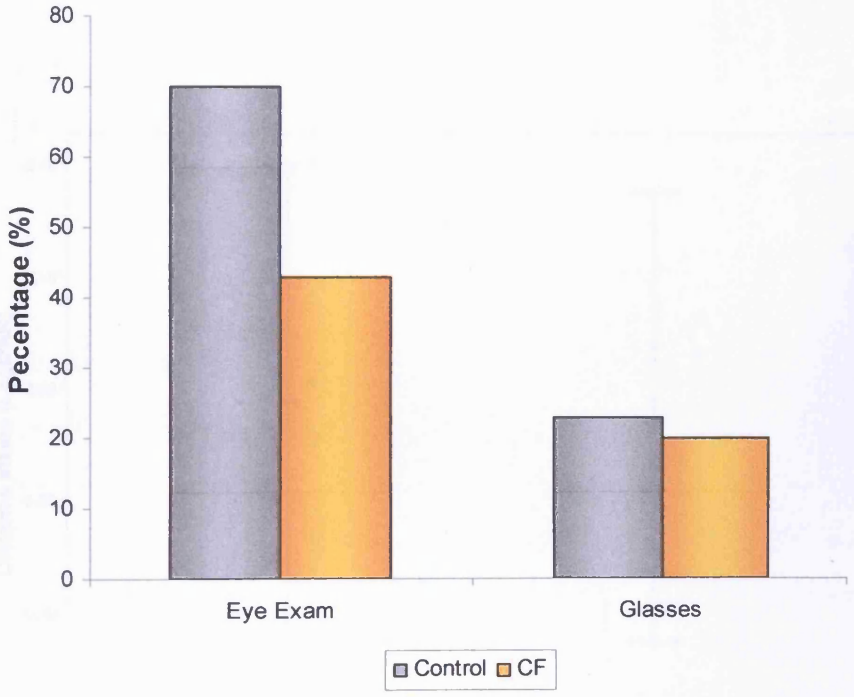


Figure 6.1 The percentage of subjects who had undergone an eye examination within the previous two years and percentage of subjects currently prescribed glasses in the CF and healthy control groups

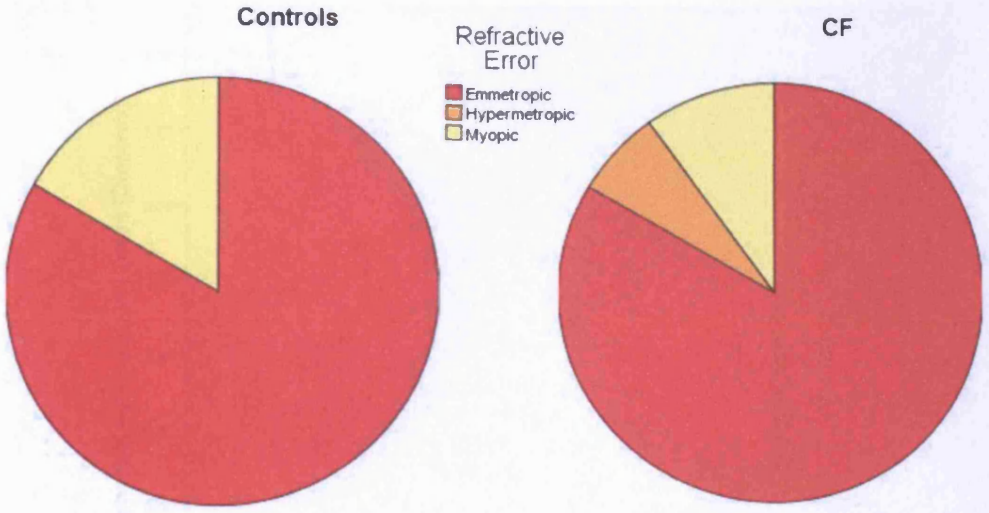


Figure 6.2 The distribution of emmetropia, hypermetropia and myopia in subjects with CF and healthy controls

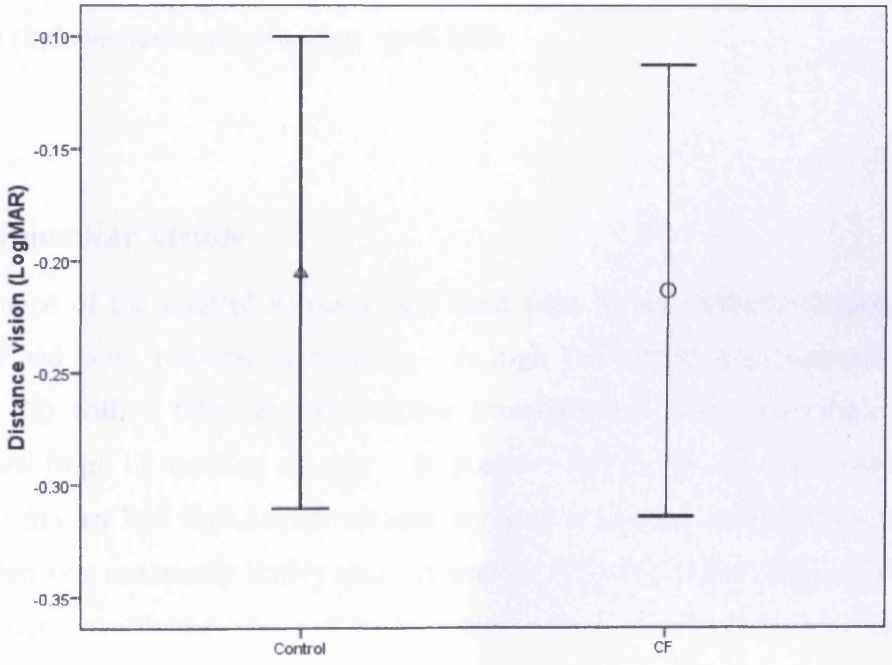


Figure 6.3 Distance vision in subjects with CF and healthy controls (Independent-samples t-test; p=0.911)

(Error bars indicate 95% confidence intervals)

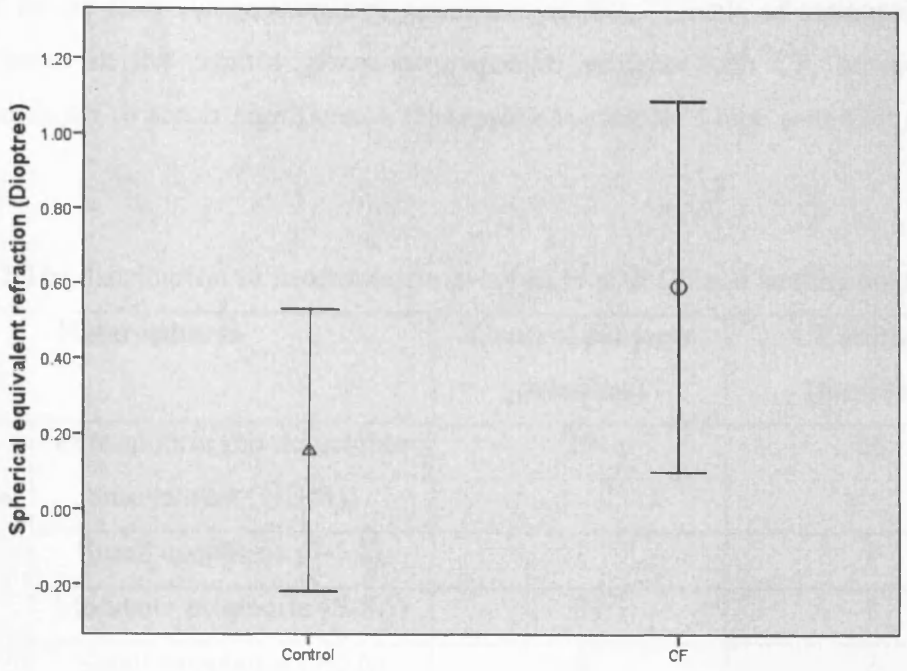


Figure 6.4 Spherical equivalent refraction (SER) in subjects with CF and healthy controls (Independent-samples t-test; p=0.153)

6.3.3 Binocular vision

Whilst none of the control subjects had been seen by an ophthalmologist, two CF subjects had been referred previously. A high (>+6.00D) hypermetrope (subject number 23) with a fully accommodative esotropia had been prescribed spectacle correction from 15 months of age. A positive family history was reported; the subject's mother had high hypermetropia and had undergone strabismus surgery and his brother was reportedly amblyopic. A second subject (subject number 20) was an anisometropic amblyope who had been treated with previous occlusion therapy. VA was slightly reduced in the affected right eye although a moderate level of stereopsis was recorded. There was no family history in this case.

With the exception of CF subject number 23, heterotropia was not detected in any participants, although a number of individuals demonstrated heterophorias. The distribution is summarised in Table 6.2 and appears similar for the two cohorts. A moderate distance and near heterophoria was recorded in a single CF subject only;

observed in the fully accommodative esotropic subject. Levels of stereopsis were slightly better in the control group compared to subjects with CF, however the difference failed to reach significance (Independent-samples t-test; p=0.426) (Figure 6.5).

Table 6.2 The distribution of heterophoria in subjects with CF and healthy controls

Heterophoria		Control subjects (number)	CF subjects (number)
Distance	Orthophoria (no detectable movement (NDM))	29	26
	Small esophoria (3-5Δ)	1	1
	Moderate esophoria (5-8Δ)	0	1
	Small exophoria (3-5Δ)	0	2
Near	Orthophoria (NDM)	21	20
	Small esophoria (3-5Δ)	5	4
	Moderate esophoria (5-8Δ)	0	1
	Small exophoria (3-5Δ)	4	5

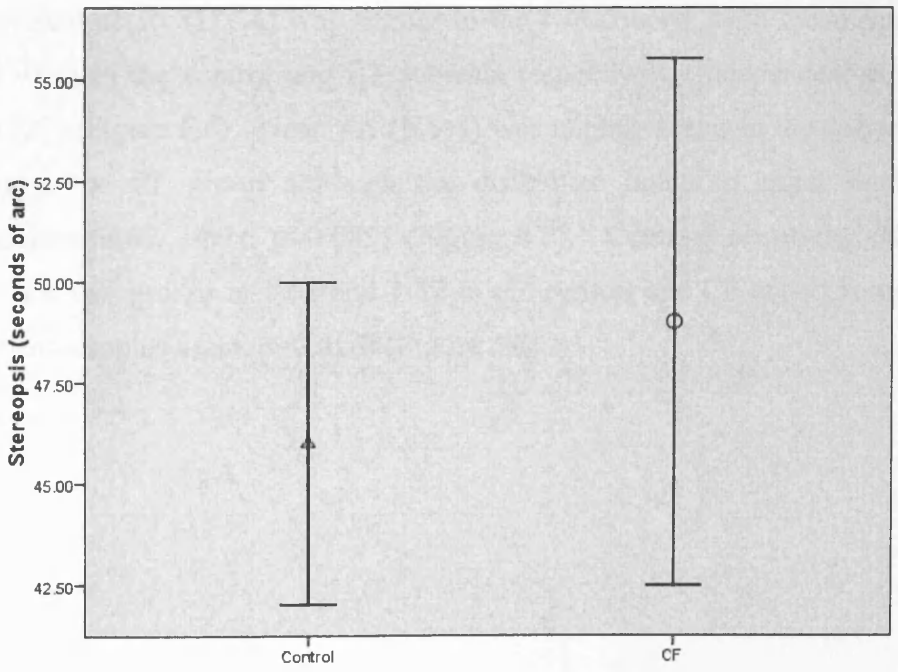


Figure 6.5 Stereopsis in subjects with CF and healthy controls (Independent-samples t-test; p=0.426)

Two CF subjects were known to be born prematurely, at 31 weeks and 36 weeks gestation. A single control subject was born 8 weeks premature. Two subjects had low levels of myopia but all had normal binocular and visual function and are summarised in Table 6.3.

Table 6.3 Visual function and binocular vision in subjects known to be born prematurely

Subject	Group	Gestation (weeks)	SER (D)	DVA (LogMAR)	NVA (N)	Stereopsis (sec of arc)
6	CF	31	+0.625	-0.42	4	40
12		36	-1.125	-0.30	5	40
35	Control	32	-1.75	-0.40	3	50

6.3.4 Visual Function

Distance visual acuity (DVA) was similar in the two cohorts, with mean acuity of -0.36 and -0.35 in the control and CF subjects respectively (Independent-samples t-test; p=0.721) (Figure 6.6). Near VA (NVA) was slightly better in the control group compared to the CF group although the difference failed to reach significance (Independent-samples t-test; p=0.089) (Figure 6.7). Contrast sensitivity (CS) was similar in the two groups at 1.61 and 1.59 in the control and CF cohort respectively (Independent-samples t-test; p=0.416) (Figure 6.8).

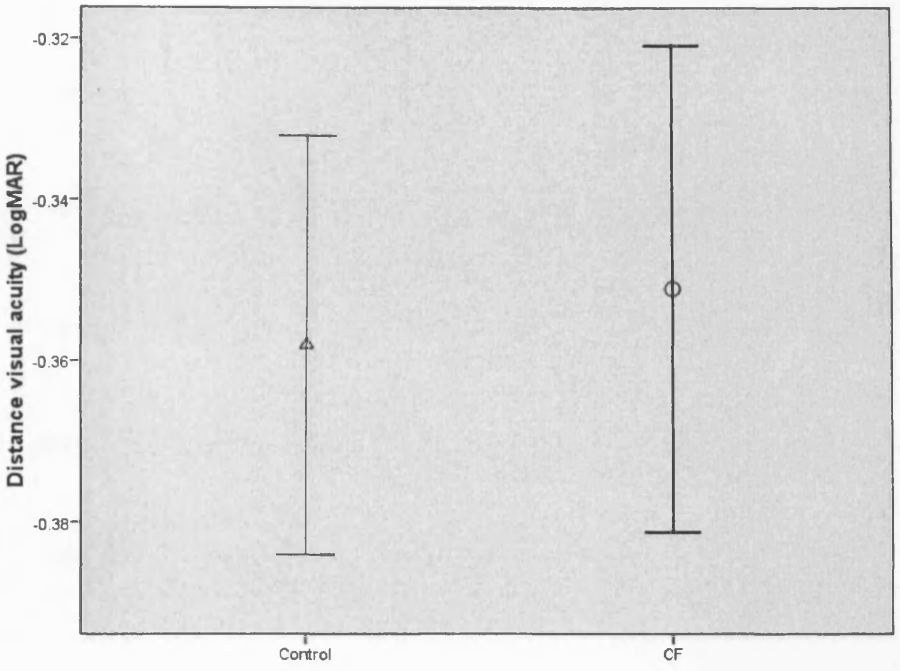


Figure 6.6 Distance visual acuity in subjects with CF and healthy controls (Independent-samples t-test; p=0.721)

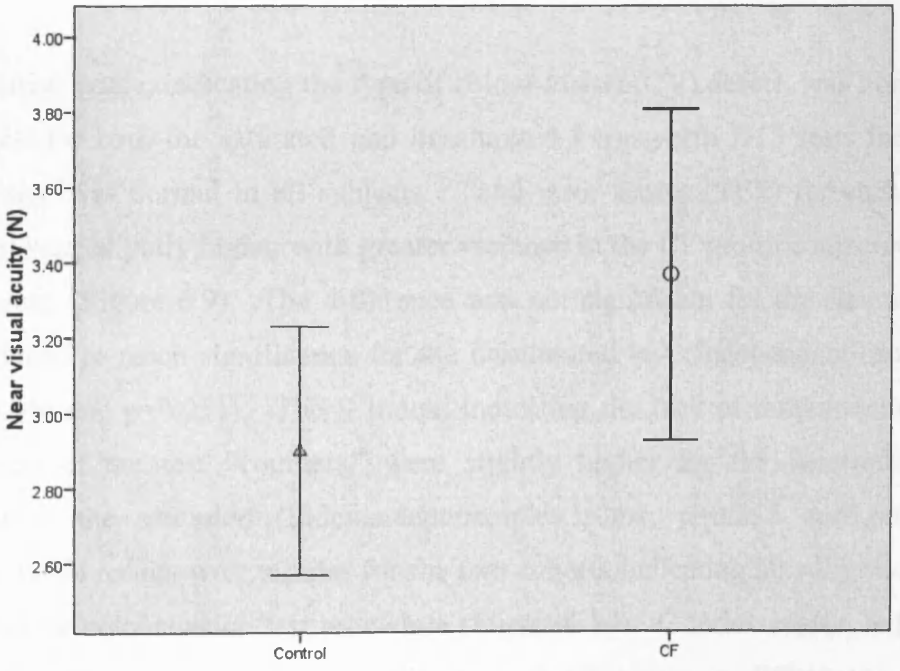


Figure 6.7 Near visual acuity in subjects with CF and healthy controls (Independent-samples t-test; p=0.089)

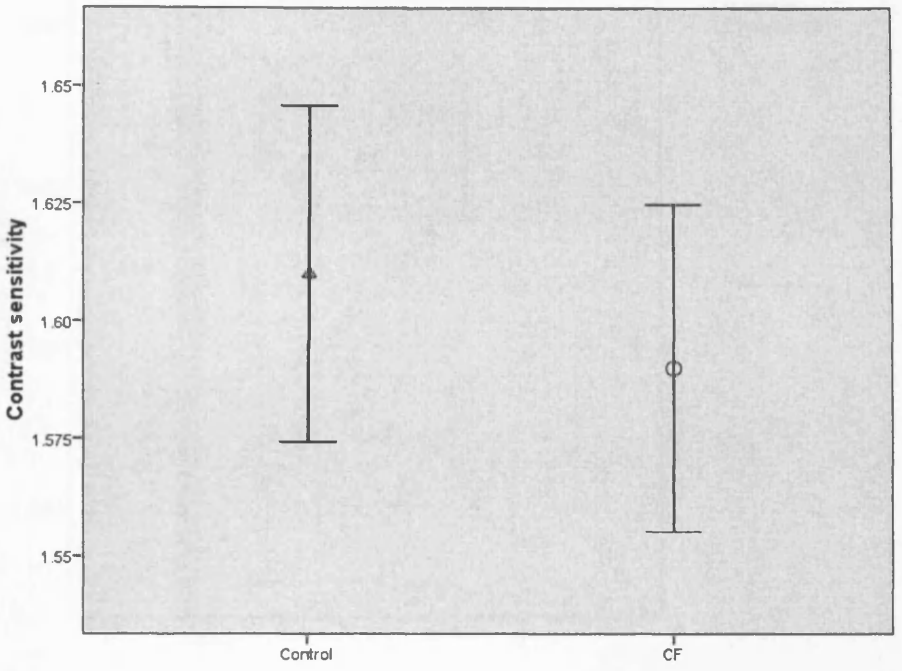


Figure 6.8 Contrast sensitivity in subjects with CF and healthy controls (Independent-samples t-test; p=0.416)

The confusion angle, indicating the type of colour vision (CV) defect, was normal for all subjects for both the saturated and desaturated Farnsworth D15 tests indicating colour vision was normal in all subjects. Total error scores (TES) for each colour vision test were slightly higher with greater variance in the CF group compared to the control group (Figure 6.9). The difference was not significant for the saturated test and just failed to reach significance for the desaturated test (Independent-samples t-test; p=0.446 and p=0.051). The S Index, indicating the lack of randomness of the arrangement of the test "counters" were slightly higher for the desaturated test compared to the saturated (Independent-samples t-test; p=0.555 and p=0.772). However, mean results were similar for the two cohorts indicating all subjects clearly understood the colour vision test procedure (Figure 6.10). C Index scores, indicating the degree of defect, were higher in subjects with CF however, differences did not reach significance (Independent-samples t-test; p=0.470 and p=0.084 respectively) (Figure 6.10). Differences in visual function between subjects with CF and healthy controls are summarised in Table 6.4.

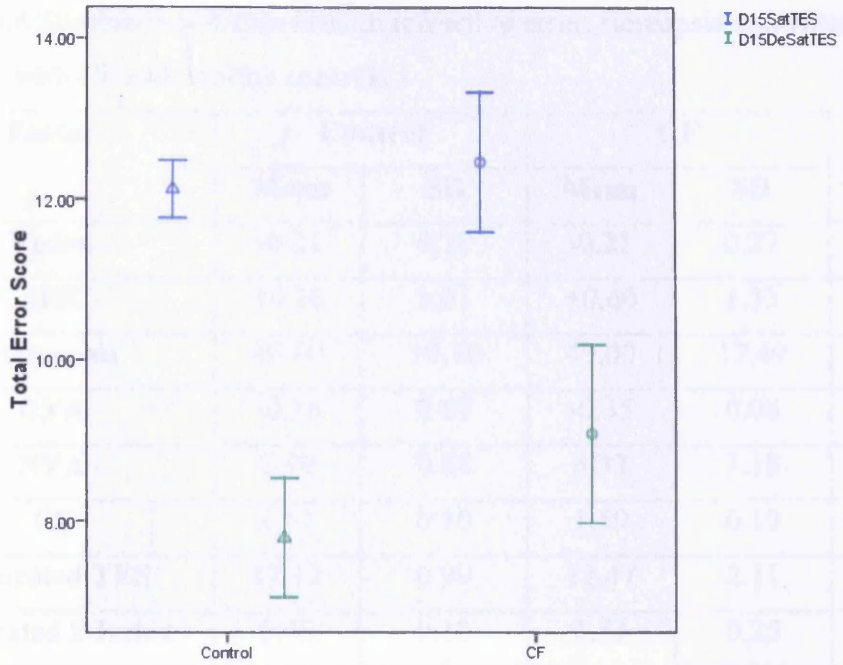


Figure 6.9 Total error score (TES) for the saturated and desaturated Farnsworth D15 test in subjects with CF and healthy controls (Independent-samples t-test; p=0.446 and p=0.051 respectively)

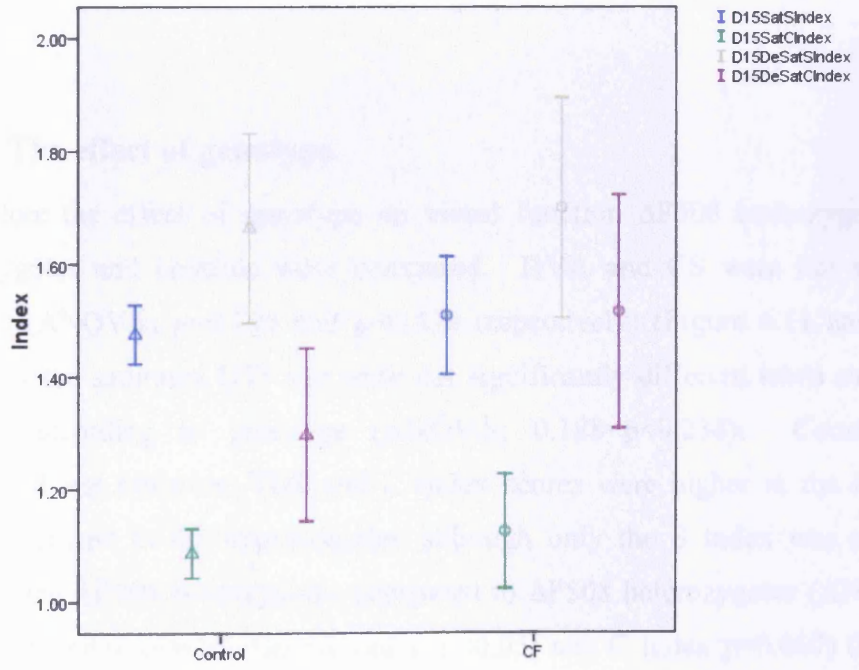


Figure 6.10 S Index and C Index values for the saturated and desaturated Farnsworth D15 test in subjects with CF and controls (Independent-samples t-test; S Index p=0.555 and p=0.772 and C Index p=0.470 and p=0.084)

Table 6.4 Summary of differences in refractive error, stereopsis and visual function in subjects with CF and healthy controls

Factor	Control		CF		Sig. (Ind t-test)
	Mean	SD	Mean	SD	
Vision	-0.21	0.28	-0.21	0.27	0.911
SER	+0.16	1.01	+0.60	1.33	0.153
Stereopsis	46.00	10.70	49.00	17.49	0.426
DVA	-0.36	0.07	-0.35	0.08	0.721
NVA	2.90	0.88	3.37	1.18	0.089
CS	1.61	0.10	1.59	0.10	0.416
Saturated TES	12.13	0.90	12.47	2.11	0.446
Saturated S Index	1.48	0.13	1.51	0.25	0.555
Saturated C Index	1.09	0.11	1.12	0.25	0.470
Desaturated TES	7.79	1.87	9.09	2.67	0.051
Desaturated S Index	1.66	0.42	1.70	0.47	0.772
Desaturated C Index	1.30	0.39	1.52	0.50	0.084

6.3.2.1 The effect of genotype

To explore the effect of genotype on visual function ΔF508 homozygotes, ΔF508 heterozygotes and controls were compared. DVA and CS were not significantly different (ANOVA; $p=0.705$ and $p=0.578$ respectively) (Figure 6.11 and 6.12) and values for the saturated D15 test were not significantly different when subjects were grouped according to genotype (ANOVA; $0.188<p<0.238$). Considering the desaturated test however, TES and C Index scores were higher in the homozygote group compared to the heterozygotes although only the S Index was significantly worse in the ΔF508 homozygotes compared to ΔF508 heterozygotes (ANOVA; TES $p=0.075$, S Index $p<0.05$ (Bonferroni's; $p<0.05$) and C Index $p=0.067$) (Figure 6.13 and 6.14). NVA was significantly worse in ΔF508 heterozygotes compared to controls (ANOVA; $p<0.05$: Bonferroni's; $p<0.05$). Differences in visual function in the three groups are summarised in Table 6.5.

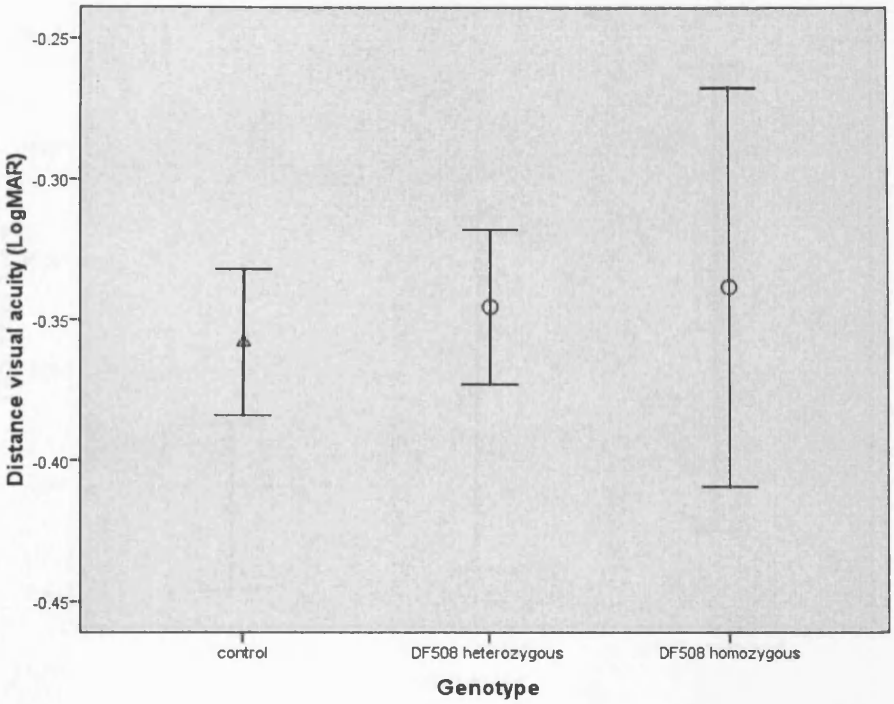


Figure 6.11 Distance visual acuity in ΔF508 homozygotes, ΔF508 heterozygotes and healthy controls (ANOVA; p=0.705)

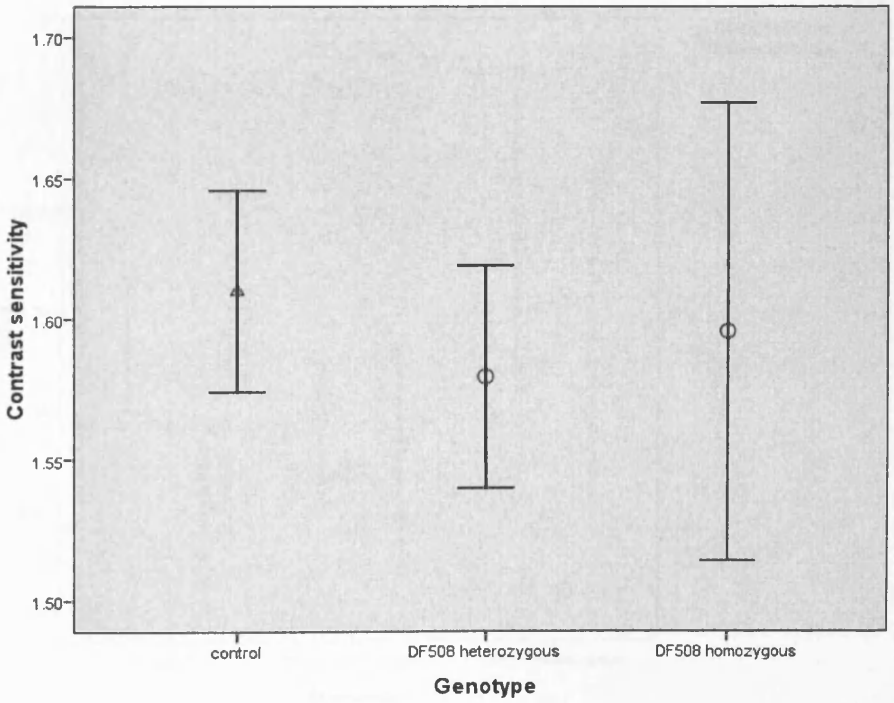


Figure 6.12 Contrast sensitivity in ΔF508 homozygotes, ΔF508 heterozygotes and healthy controls (ANOVA; p=0.578)

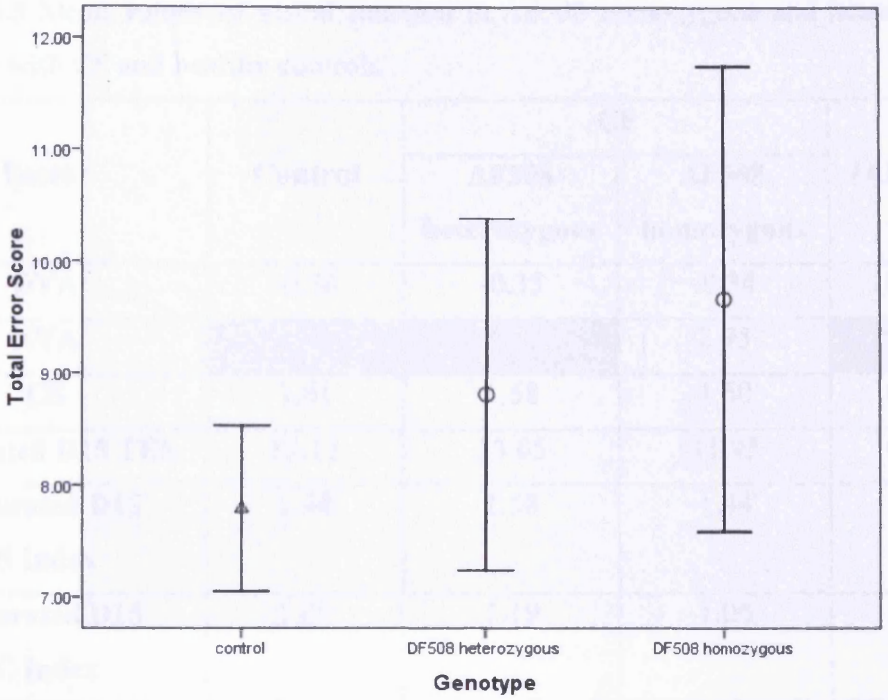


Figure 6.13 Desaturated D15 Total Error Score (TES) in ΔF508 homozygotes, ΔF508 heterozygotes and healthy controls (ANOVA; p=0.075)

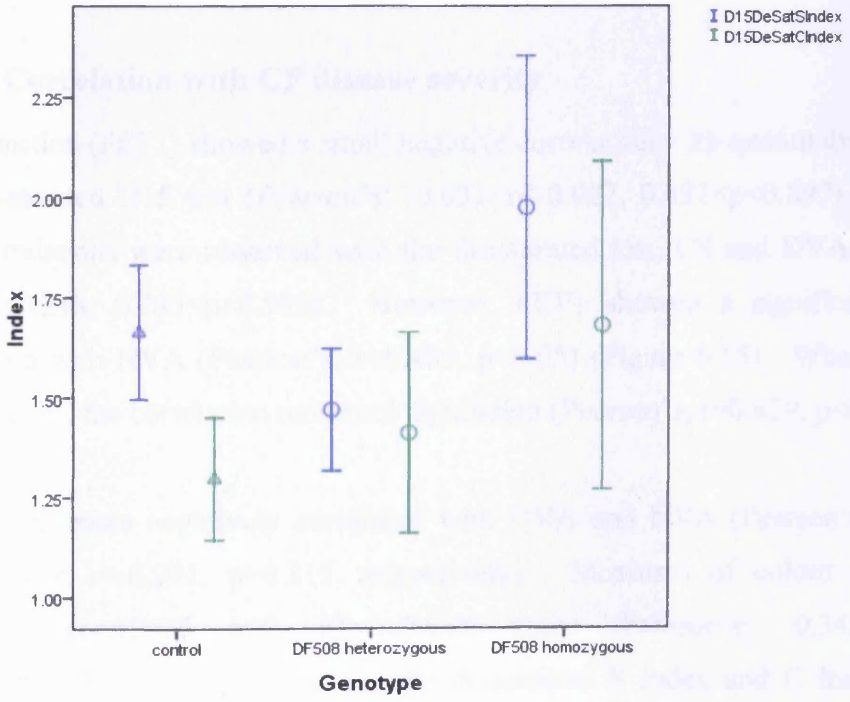


Figure 6.14 Desaturated D15 S Index and C Index in ΔF508 homozygotes, ΔF508 heterozygotes and healthy controls (ANOVA; p<0.05 and p=0.067 respectively)

Table 6.5 Mean values of visual function in ΔF508 homozygous and heterozygous subjects with CF and healthy controls

Factor	Control	CF		Sig. (ANOVA)
		ΔF508 heterozygous	ΔF508 homozygous	
DVA	-0.36	-0.35	-0.34	0.705
NVA	2.90	3.68	2.95	**<0.050**
CS	1.61	1.58	1.60	0.578
Saturated D15 TES	12.13	13.05	11.95	0.188
Saturated D15 S Index	1.48	1.58	1.44	0.238
Saturated D15 C Index	1.09	1.19	1.06	0.199
Desaturated TES	7.79	8.81	9.67	0.075
Desaturated S Index	1.66	1.47	1.97	**<0.050**
Desaturated C Index	1.30	1.41	1.68	0.067

6.3.2.2 Correlation with CF disease severity

Lung function (FEV_1) showed a small negative correlation with quantitative measures of the saturated D15 test (Pearson's; $-0.031<r<-0.027$, $0.882<p<0.897$). Similarly, poor correlations were observed with the desaturated test, CS and DVA (Pearson's; $0.004<r<0.218$, $0.295<p<0.985$). However, FEV_1 showed a significant positive correlation with NVA (Pearson's; $r=0.405$, $p<0.05$) (Figure 6.15). When an outlier was removed, the correlation remained significant (Pearson's; $r=0.429$, $p<0.05$).

Shwachman score negatively correlated with DVA and NVA (Pearson's; $r=-0.110$, $p=0.586$ and $r=-0.201$, $p=0.315$ respectively). Measures of colour vision also negatively correlated with Shwachman score (Pearson's; $-0.343<r<-0.028$, $0.119<p<0.902$; and correlations for the desaturated S Index and C Index reached significance (Pearson; $r=-0.578$, $p<0.01$ and $r=-0.428$, $p<0.05$ respectively) (Figure

6.16 and 6.17). CS did not significantly correlate with Shwachman score (Pearson's; r=0.163, p=0.417).

Whilst Chrispin-Norman score negatively correlated with CS and measures of the saturated D15 test, these failed to reach significance (Pearson's; $0.095<r<0.188$, $0.428<p<0.666$). Similarly, DVA and NVA and measures of the desaturated D15 test showed a weak correlation (Pearson's; $-0.320<r<-0.132$, $0.169<p<0.549$).

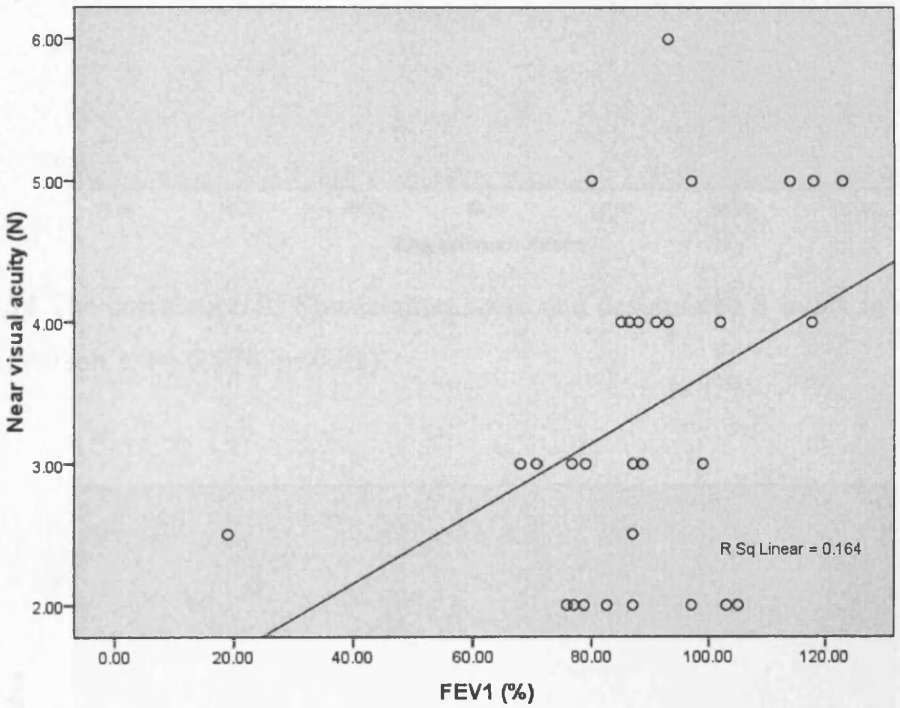


Figure 6.15 The correlation of FEV_1 and near visual acuity (Pearson's; r=0.405, $p<0.05$)

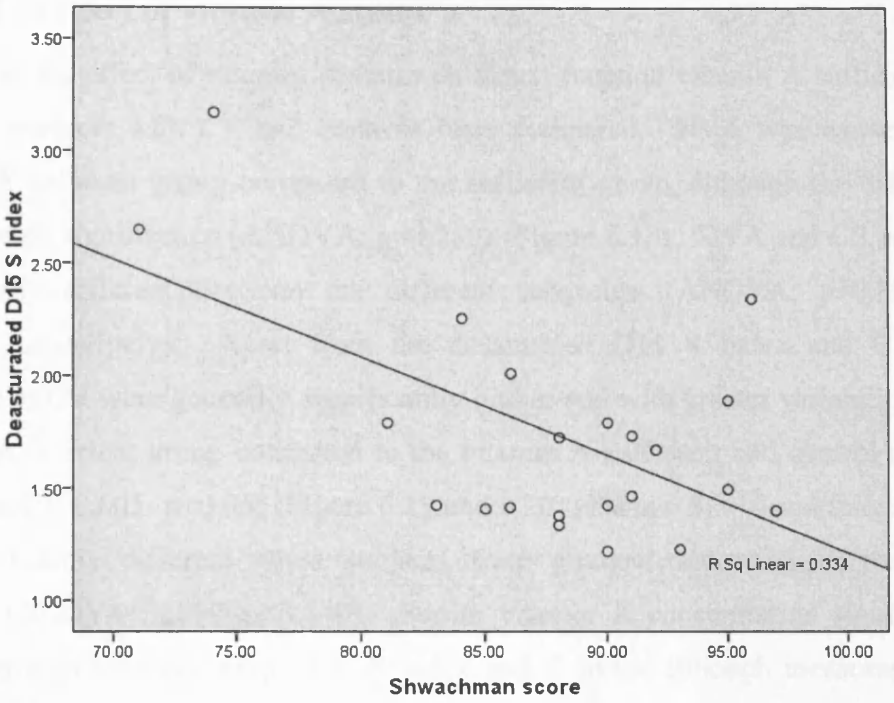


Figure 6.16 The correlation of Shwachman score and desaturated S Index in subjects with CF (Pearson's; r=-0.578, p<0.01)

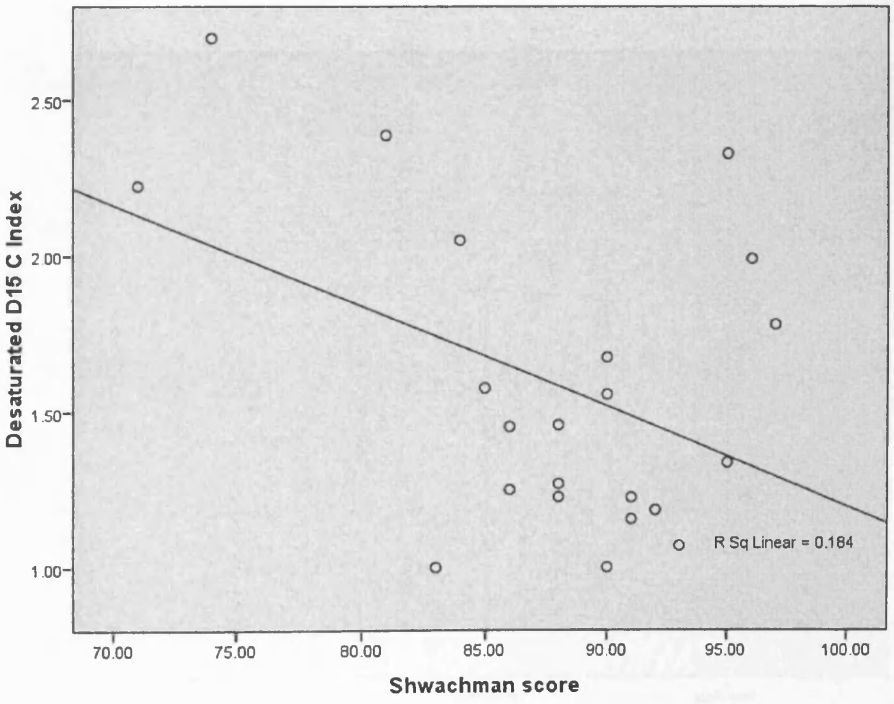


Figure 6.17 The correlation of Shwachman score and desaturated C Index in subjects with CF (Pearson's; r=-0.428, p<0.05)

6.3.2.3 The effect of vitamin A status

To explore the effect of vitamin A status on visual function vitamin A sufficient and deficient subjects with CF and controls were compared. NVA was worse in the vitamin A deficient group compared to the sufficient group, although the difference did not reach significance (ANOVA; p=0.250) (Figure 6.18). DVA and CS were not significantly different between the different subgroups (ANOVA; p=0.882 and p=0.679 respectively). Apart from the desaturated D15 S Index and C Index, measures of CV were generally significantly higher and with greater variability in the vitamin A deficient group compared to the vitamin A sufficient and control subjects (Bonferroni's; 0.005<p<0.05) (Figure 6.19 and 6.20) (Table 6.6). Visual function was not significantly different when subjects were grouped according to pancreatic function (ANOVA; 0.059<p<0.840). Serum vitamin A concentration significantly correlated with saturated D15 TES, S Index and C Index although measures of the desaturated D15 colour vision test did not significantly correlate (Table 6.7). Vitamin A showed a negative correlation with DVA and NVA however a small positive correlation was recorded with CS although these correlations failed to reach significance (Table 6.7).

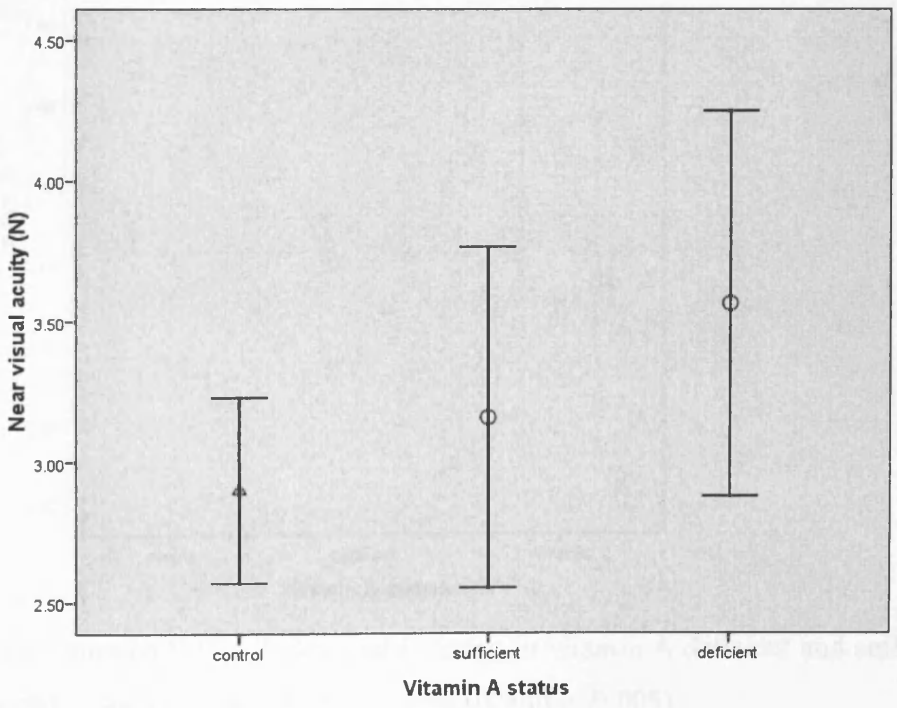


Figure 6.18 Near visual acuity in vitamin A deficient and sufficient subjects with CF and healthy controls (ANOVA; p=0.250)

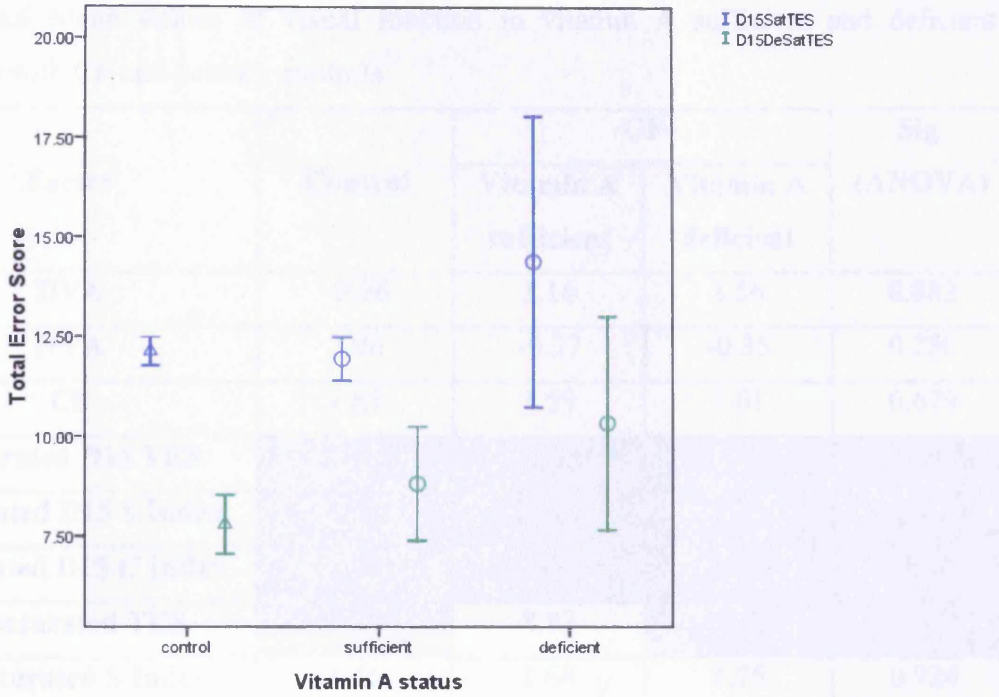


Figure 6.19 Saturated and desaturated D15 Total Error Score (TES) in vitamin A deficient and sufficient subjects with CF and controls (ANOVA; $p<0.005$ and $p<0.05$)

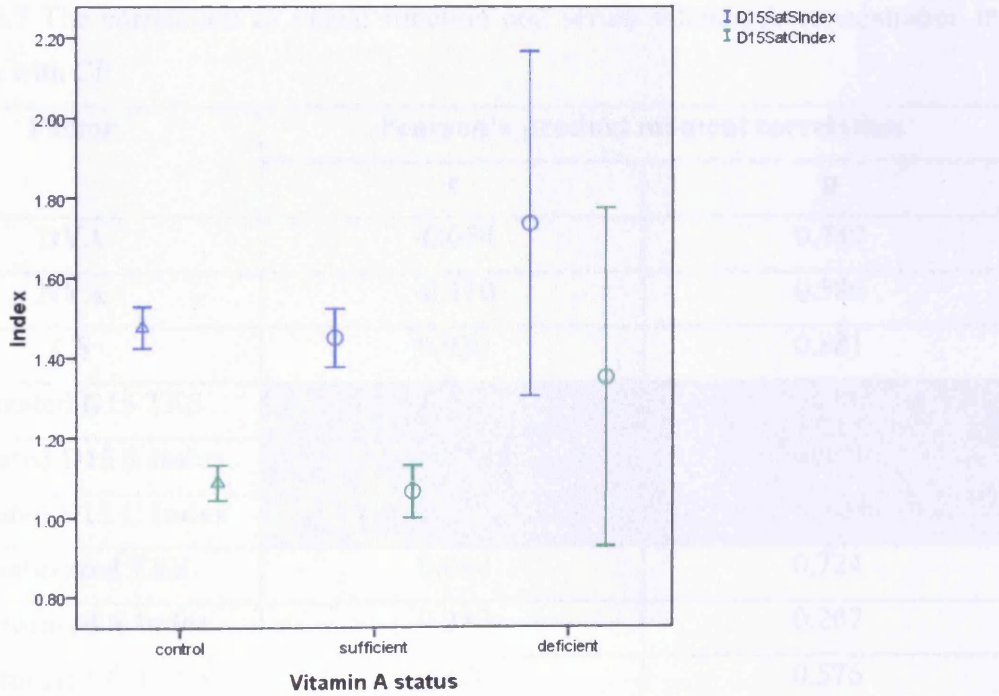


Figure 6.20 Saturated D15 S Index and C Index in vitamin A deficient and sufficient subjects with CF and controls (ANOVA; $p<0.01$ and $p<0.005$)

Table 6.6 Mean values of visual function in vitamin A sufficient and deficient subjects with CF and healthy controls

Factor	Control	CF		Sig. (ANOVA)
		Vitamin A sufficient	Vitamin A deficient	
DVA	-0.36	3.16	3.56	0.882
NVA	2.90	-0.37	-0.35	0.250
CS	1.61	1.59	1.61	0.679
Saturated D15 TES	12.13	11.95	14.40	**<0.005**
Saturated D15 S Index	1.48	1.45	1.73	**<0.010**
Saturated D15 C Index	1.09	1.06	1.35	**<0.005**
Desaturated TES	7.79	8.82	10.34	**<0.050**
Desaturated S Index	1.66	1.68	1.75	0.924
Desaturated C Index	1.30	1.47	1.73	0.086

Table 6.7 The correlation of visual function and serum vitamin A concentration in subjects with CF

Factor	Pearson's product moment correlation	
	r	p
DVA	-0.054	0.789
NVA	-0.110	0.586
CS	0.030	0.881
Saturated D15 TES	-0.584	**<0.005**
Saturated D15 S Index	-0.568	**<0.010**
Saturated D15 C Index	-0.581	**<0.010**
Desaturated TES	0.080	0.724
Desaturated S Index	0.280	0.207
Desaturated C Index	0.126	0.576

6.3.5 Major findings of this study

- Visual function was essentially normal in juvenile subjects with CF compared to healthy controls
- NVA was significantly worse in ΔF508 heterozygotes compared to controls
- Desaturated D15 S Index was significantly worse in ΔF508 homozygotes compared to ΔF508 heterozygotes
- Saturated D15 TES, S Index, and C Index was significantly worse in vitamin A deficient subjects compared to vitamin A sufficient subjects and controls
- Desaturated D15 TES was significantly worse in vitamin A deficient subjects compared to controls
- Saturated D15 TES, S Index and C Index showed a significant negative correlation with serum vitamin A concentration
- Desaturated S Index and C Index showed a significant negative correlation with Shwachman score

6.4 Discussion

Whilst a similar number of CF and control subjects were prescribed some form of spectacle correction, a lower percentage of CF subjects had undergone an eye examination within the previous two years. This suggests eyecare may be neglected due to demand of the prevailing healthcare regime in CF. Mean SER indicated low levels of hypermetropia in each group. Mean SER in this investigation is similar to that for 12 year old children (the mean age of the CF and control subjects) in a normal population (Saunders, 1981). Ideally, a cycloplegic refraction would have been employed but this was not implemented due to time restraints within the hospital clinic. Two subjects with CF had binocular vision abnormalities. However, mean levels of stereopsis and the prevalence of heterophoria were similar in the two groups. Despite reports of prematurity and low birth weight in subjects with CF (Festini et al., 2005, Muller et al., 1999), which in themselves are associated with ametropia, strabismus and amblyopia (O'Connor et al., 2006, Robaei et al., 2006a, Huynh et al., 2006), the incidence in this CF cohort appears to be low. Therefore, this could imply that normal emmetropisation (Brown et al., 1999) and orthophorisation (Dowley, 1987) processes appear to take place in subjects with CF, but caution must be applied due to the relatively small sample size.

This is the first investigation to compare visual acuity in subjects with CF and controls. DVA in this study was similar to that of reported logMAR values for 12 year old children (Robaei et al., 2006b) but slightly lower than that of healthy adults aged 25-29 years (Lovie-Kitchin, 2002). Levels of CS were similar to those reported for 10 year old children with the Peli-Robson chart (Myers et al., 1999). A number of studies have reported abnormal levels in subjects with CF (Spaide et al., 1987). After improvement in CS function following vitamin A supplementation, Leguire *et al.* (1991) suggested vitamin A deficiency (VAD) was responsible (Leguire et al., 1991). However, abnormal CS has been observed in vitamin A sufficient subjects with CF (Ansari et al., 1999, Morkeberg et al., 1995) suggesting abnormal visual function could be a primary manifestation of CF caused by abnormal CFTR activity within the apical retinal pigment epithelium. In this study, VA and CS were only slightly reduced in subjects with CF, and significance when subjects were grouped according

to genotype was limited. Similarly, VA and CS generally weakly correlated with measures of clinical severity.

Normal visual function in subjects with CF in this investigation suggests compensation for defective CFTR within the RPE. The abundance of alternative Cl^- secreting channels such as CLCA or ClC channels could provide an alternative route for Cl^- efflux from the basal membrane. In particular, CLCA has been shown to have greater conductance compared to CFTR (Loewen et al., 2003). However, a lack of significance could also indicate sub-clinical levels of abnormal visual function in CF that remained undetected by the methods employed in this study. Electrophysiology techniques may be more sensitive and have recorded abnormal results from CF subjects receiving vitamin A supplementation (Schupp et al., 2004, Constable et al., 2006). Statistical significance in this study could be hindered by small cohort size and phenotype-genotype variability (McKone et al., 2003, Zielenski, 2000). However, the recruitment of 30 subjects with CF is still an achievement. The subjects in this study are essentially healthy (in terms of CF) and differences in visual function may only become manifest following prolonged disease duration or periods of exacerbation.

Abnormal CV, attributed to chloramphenicol associated optic neuritis has been recorded in CF previously (Spaide et al., 1987). As indicated by the confusion angle, no subjects had congenital or acquired colour vision defects in this study. Measures of CV were slightly worse in the CF group compared to the controls, however vitamin A deficient CF subjects had significantly reduced CV scores compared to sufficient CF subjects and controls. Abnormal colour vision has been reported in non-CF subjects with VAD previously (Reddy and Vijayalaxmi, 1977) but is not a documented feature of xerophthalmia (Sommer, 1998). These results could indicate subtle levels of cone photoreceptor dysfunction secondary to VAD. The negative correlation of serum vitamin A concentration and CV scores supports this theory. Similarly, Leguire et al. (1991) suggested reduced CS in CF was caused by altered cone function as an improvement was observed following vitamin A supplementation (Leguire et al., 1991).

6.5 Summary

To conclude, visual function, refractive error and binocular vision appear to be normal in juveniles with CF in this relatively small study although CV appears to be impaired by VAD. Due to modern therapeutics and considerable improvements in disease management, this juvenile CF cohort is relatively healthy with high Shwachman scores, good levels of lung function and generally adequate vitamin A levels. Also no subjects were diagnosed with CF-Related Diabetes (CFRD). If previously observed ocular effects of CF are secondary manifestations of the disease, caused by diabetes or VAD, the relative "good health" of the subjects could be responsible for the lack of significant findings in this study.

Further studies are necessary in CF subjects with longer disease duration and greater clinical severity in order to investigate secondary manifestations of CF. Adults with CF are more likely to have CFRD and could be less compliant without parental supervision leading to increased levels of vitamin A deficiency. Furthermore, assessment of rod photoreceptor function, such as dark adaptation, is likely to be revealing as impaired dark adaptation is the earliest clinical manifestation of vitamin A deficiency. However, this could only really be implemented in adults in a controlled environment.

Chapter 7

Investigating Visual Function and Morphological Characteristics in Adults with Cystic Fibrosis

7.1 Introduction

In the previous study, none of the juveniles had been diagnosed with Cystic Fibrosis Related Diabetes (CFRD), and only a small proportion of the cohort was vitamin A deficient. A number of clinical studies have observed a wide range of ocular characteristics of CF but many were of a small scale and included confounding factors, such as CFRD and vitamin A deficiency (VAD). Published literature indicates that these conditions adversely affect visual function and they are reviewed below. As life expectancy increases in the adult CF population it is more important to understand which ocular effects may be considered primary manifestations of the condition or secondary to CFRD or VAD.

7.1.1 The ocular effects of diabetes

Diabetic retinopathy (DR) is a common micro-vascular complication observed in diabetic patients. Hyperglycaemia damages retinal vessels by causing basement membrane thickening and loss of pericytes leading to microaneursym formation (Cai and Boulton, 2002). Small areas of retinal non-perfusion result in capillary dilation with subsequent leakage and haemorrhage (Kohner, 1993): this is described as background DR (Figure 7.1). Retinal ischaemia, caused by capillary drop out and non-perfusion, is characterised by cotton wool spots and intra-retinal microvascular abnormalities (Garner, 1993). Venous loop formation, reduplication and venous beading indicate imminent neovascularisation (Kohner, 1993). In combination with signs of retinal ischaemia, these changes are described as pre-proliferative DR. Proliferative DR, however, is a serious sight threatening complication and is characterised by neovascularisation, vitreous detachment and haemorrhage (Kanski,

1999). Diabetic maculopathy is a further clinical feature and is the most common cause of visual impairment in diabetic patients. DR adversely affects visual function and is currently the most prevalent cause of blind registration in the working population in England and Wales (Bunce and Wormald, 2008).

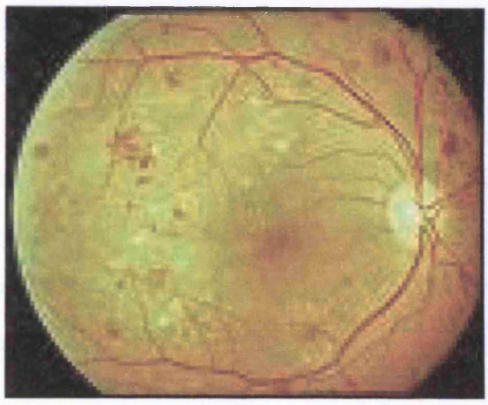


Figure 7.1 Diabetic retinopathy (from www.cehjournal.org; accessed on 13/03/2009)

Increased levels of cataract, crystalline lens density and thickness have been observed in diabetes (Beneyto et al., 2007, Klein et al., 1998, Fledelius and Miyamoto, 1987, Logstrup et al., 1997). Lens thickness appears to be positively correlated with diabetes duration (Pierro et al., 1996, Logstrup et al., 1996, Sparrow et al., 1990, Fledelius and Miyamoto, 1987); increased lens hydration following alterations in the osmotic gradient from raised blood glucose is thought to be responsible (Logstrup et al., 1996). Differing observations have been made when comparing Type 1 and Type 2 diabetics (Weimer, 2008), and are likely to reflect pathophysiological differences (Sparrow et al., 1992). Whilst correlations with metabolic control have not been observed, poor control is a risk factor for nuclear and cortical cataracts in diabetics (Klein et al., 1998). Visual function is adversely affected by cataract formation; contrast sensitivity (CS) in particular, may be reduced despite normal visual acuity (VA) (Elliott et al., 1989, Cheng et al., 2001, Brown, 1993).

A number of studies have observed abnormal colour discrimination in diabetic patients, even in the absence of diabetic retinopathy (North et al., 1997a, Hardy et al., 1992, Verrotti et al., 1995). Tritan (blue-yellow) defects have typically been

described (Muntoni et al., 1982, Kurtenbach et al., 2002, Bresnick et al., 1985, Fong et al., 1999, Utku and Atmaca, 1992, Ismail and Whitaker, 1998), and the degree of colour vision abnormality has been shown to correlate with glycaemic control (HbA1c) (Hardy et al., 1995, Trick et al., 1988, Muntoni et al., 1982) and disease duration (Kurtenbach et al., 1999). Similarly, CS is reduced in subjects with Type 1 and Type 2 diabetes compared to healthy controls, even in those without retinopathy (Stavrou and Wood, 2003, Di Leo et al., 1992, North et al., 1997, Dosso et al., 1996, Ismail and Whitaker, 1998). Reduced CS has been observed despite normal VA (Sokol et al., 1985) and the effect on CS also correlates with HbA1c (Banford et al., 1994). These abnormalities cannot be solely caused by diabetes-associated increases in crystalline lens optical density (Dosso et al., 1996), and have been suggested to be retinal or neural in nature (Hardy et al., 1994).

The slow recovery of visual sensitivity following photoreceptor bleaching is termed dark adaptation (DA). Elevated DA thresholds are observed in diabetics compared to healthy controls (Henson and North, 1979, Amemiya, 1977, Abraham et al., 1988). Reports of a correlation with diabetes longevity are variable however (Greenstein et al., 1993, Henson and North, 1979) and thresholds appear adversely affected in diabetic subjects with little or no diabetic retinopathy (Greenstein et al., 1993). Similarly, elevated thresholds occur following panretinal photocoagulation for the treatment of diabetic retinopathy (Pender et al., 1981). The DA function in diabetes has been suggested to be adversely affected by retinal ischaemia, hypoxia, metabolic or physiological abnormalities (Greenstein et al., 1993, Henson and North, 1979, Abraham et al., 1988).

7.1.2 The ocular effects of vitamin A deficiency

Xerophthalmia refers to the group of ocular conditions caused by vitamin A deficiency (VAD) (Sommer, 1998). Impaired DA is the earliest clinical manifestation of xerophthalmia (Brooks et al., 1990). DA is primarily determined by the retinoid cycle of photopigment regeneration (Lamb and Pugh, 2004) (Figure 7.2). The rod pigment, rhodopsin, is composed of *11-cis retinal* (an oxidised form of retinol which is a compound of vitamin A) bound to a protein known as opsin. When a photon of

light is captured the complex undergoes a conformational change to *all-trans retinal* resulting in a phototransduction cascade and neuronal signalling. Retinal is released from opsin and reduced to *all-trans retinol* before being transported to the retinal pigment epithelium (RPE). Within the RPE, it is converted to *11-cis retinol* before being oxidised to *11-cis retinal* and travelling back to the rod outer segment where it can bind to opsin to reform as rhodopsin (da Silva Diniz and Santos, 2000). In VAD, initially, the rate of pigment regeneration and the rate of dark adaptation decreases (Lamb and Pugh, 2004). However, following prolonged deficiency the threshold becomes permanently elevated and rod function may be lost. In severe deficiency, cone function can be similarly affected and after prolonged periods the photoreceptors may eventually degenerate (Kemp et al., 1988).

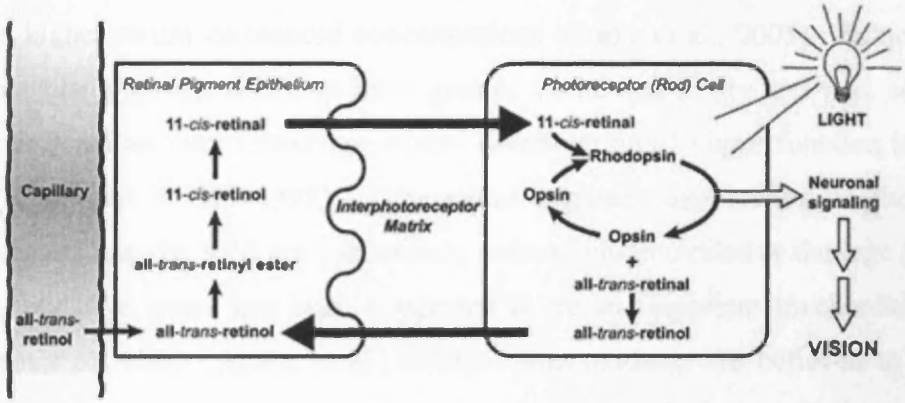


Figure 7.2 A simplified diagram of the retinoid cycle
(from http://lpi.oregonstate.edu/infocenter/vitamins/vitaminA/visualcycle.html; accessed on 13/03/2009)

Vitamin supplements have been implicated as providing a protective function against the development and progression of cataract (Brown et al., 1998, Leske et al., 1998, Hodge et al., 1995). There is evidence that the carotenoid, beta-carotene (a pro-vitamin A compound) protects against nuclear, cortical and mixed cataract (Leske et al., 1991, Leske et al., 1995) and the risk of cataract surgery is elevated in subjects with low serum beta-carotene levels (Knekt et al., 1992). Vitamin A has anti-oxidant properties and protects cells from free radical damage, and supplementation has been shown to provide protection against nuclear sclerosis and cortical lens opacities

(Kuzniarz et al., 2001, Mares-Perlman et al., 2000). Intraperitoneal vitamin A injections have been demonstrated to promote lens regeneration in animal models (Shekhawat et al., 2001, Jangir et al., 2005). A higher concentration of retinol is observed in the younger, more metabolically active lens epithelium and cortex in comparison to the older nuclear lens tissue (Yeum et al., 1999). Retinoic acid, a product of retinol, and its receptors have been shown to play a critical role in the regulation of certain genes within the lens (Gopal-Srivastava et al., 1998). Therefore, beta-carotene, vitamin A and its derivatives appear to have a role in normal lens physiology and provide some protection against cataract formation.

In the National Health and Nutritional Examination Survey, consumption of vitamin A rich foods was negatively correlated with AMD (Goldberg et al., 1988). A higher density of macular pigment has been observed in individuals with carotenoid-rich diets and higher serum carotenoid concentrations (Burke et al., 2005). Subjects with higher macular pigment densities have greater visual sensitivity and it is suggested that higher densities may retard age-related deterioration of visual function including AMD (Hammond et al., 1998). The outer segment membranes of the retinal photoreceptors and the RPE are particularly susceptible to oxidative damage (Strauss, 2005). Oxidative stress has been suggested to be an important mechanism in the pathogenesis of AMD (Alfaro et al., 2006). Anti-oxidants are believed to prevent cellular damage by functioning as singlet oxygen and free radical scavengers (Sperduto et al., 1990). Beta-carotene has been shown to provide the RPE with protection from oxidative stress in cultured cells (Chichili et al., 2006). It has been hypothesised a high dietary or supplementary intake of certain vitamins and carotenoids may be associated with a reduced incidence of AMD. The risk of exudative AMD is reduced in subjects with high levels of carotenoids (Group, 1993). A combination of zinc and anti-oxidants, including beta-carotene, showed a significant reduction in the rates of moderate visual acuity loss in the Age-Related Eye Disease Study (Kassoff et al., 2001). Therefore, vitamin A appears to provide protection for the RPE and is important for preserving normal macular function.

7.1.3 Objectives for this study

Whilst CFRD is known to be different to Type 1 and Type 2 diabetes, it does share common features of the disease (Moran, 2002). Therefore, CFRD may similarly affect the crystalline lens and visual function in subjects with CF. Despite supplementation, VAD remains relatively common in subjects with CF (Rayner et al., 1989) and visual function may be adversely affected in CF. The aim of this study was to investigate the visual function and corneal and lenticular morphological characteristics in subjects with CF with known diabetic and vitamin A status to allow comparison with healthy, matched controls. Furthermore, this prospective study aimed to distinguish between the primary and secondary ocular manifestations of CF.

7.2 Method

7.2.1 Subjects

See Section 5.2.1

7.2.2.1 Inclusion and exclusion criteria

See Section 5.2.1.1

7.2.2 Experimental design

Subjects attended the School of Optometry and Vision Sciences for a single session of data collection. All participants completed a face-to-face ocular health questionnaire which investigated previous and current ocular health, vision, colour vision and family ocular health. Medications and reports of general health were also recorded in control subjects. Information regarding subjects with CF was recorded from their medical record (see Section 5.2.2).

7.2.2.1 Refractive error and binocular vision

Refractive error and binocular vision were assessed according to the methodology in Section 6.2.2.1.

7.2.2.2 Visual function

Contrast sensitivity (CS) and colour vision (CV) were assessed according to the methodology in Section 6.2.2.2.

Dark adaptation (DA) was assessed monocularly (right eye with the exception of one CF subject with right diabetic maculopathy) with a Goldmann-Weekers Adaptometer (GWA) (Haag-Streit, Bern, Switzerland) following previously described methods (Peters et al., 2000, Boemel, 1999, Abraham et al., 1988). Pupils were not dilated and spectacle correction was worn as appropriate. Room lights were extinguished and after 5 minutes of light adaption to the illuminated GWA bowl the subject was instructed to maintain primary gaze fixation and indicate when they could detect the presence of an 11° achromatic flashing stimulus. The light intensity was gradually increased by the examiner and a semi-logarithmic score sheet (on a rotating drum) marked when the subject identified the stimulus. This was repeated continuously for a 5 minute period. Fixation was subsequently changed to a small red light 10° superior to the original stimulus and thresholds recorded every minute for the remaining 20 minutes. Final log relative threshold was recorded to allow comparison (Greenstein et al., 1993).

7.2.2.3 Fundus photography

Fundus photographs were recorded from both eyes of CF subjects using a Canon CR-DGi Non-Mydriatic Retinal Camera (Canon Inc., USA). Images were assessed by three registered optometrists and diabetic images were reviewed by an independent experienced grader according to the Diabetic Screening Service for Wales grading protocol (Table 7.1).

Table 7.1 Diabetic retinopathy screening services for Wales screening grading protocol

Level	Abbreviation	Description
No retinopathy	**R0**	No retinopathy
Background Diabetic Retinopathy (BDR)	**R1.1 Mild BDR**	<5 MA >1 DD from fovea <4 dot/blot HM >1DD from fovea Any exudates outside the macula with/without <5 CWS
	R1.2 Mod BDR	≥5 MA >1 DD from fovea <8 dot/blot HM >1 DD from fovea <3 MA within 1DD from fovea (VA >6/12) Circinate/grouped exudates outside macula but within temporal arcade Questionable IRMA only in presence of MA/HM
Severe BDR	**R2**	>8 HM outside 1 DD from fovea Venous irregularities, beading, reduplication, venous loops Definite IRMA With/without CWS
Proliferative BDR	**R3**	NVD or NVE Pre-retinal HM or Vitreous HM Pre-retinal fibrosis Traction retinal detachment
Maculopathy	**M1**	Any exudates/blot HM within macula (within 2 DD but outside 1 DD from fovea) ≥3 MA or any HM within 1 DD of fovea only with VA ≤ 6/12
	M2	Any exudates within 1 DD of fovea Retinal thickness changes within 1 DD of fovea (stereo)

Background Diabetic Retinopathy (BDR), Micro-Aneurysm (MA), Disc Diameter (DD), Haemorrhage (HM), Cotton Wool Spot (CWS), Visual Acuity (VA), Intraretinal Microvascular Abnormalities (IRMA), New Vessels on Disc (NVD), New Vessels Elsewhere (NVE)

7.2.2.4 Corneal and lenticular morphological characteristics

The Oculus Pentacam (Oculus Optikgerate GmbH, Wetzlar, Germany) utilises a rotating Scheimpflug camera to image the anterior ocular surface and was used to record corneal thickness, corneal density and crystalline lens density. The Pentacam has been used previously to assess lens density abnormalities in subjects with diabetes (Beneyto et al., 2007, Tkachov et al., 2006) and has shown impressive accuracy and repeatability for the measurement of corneal thickness (Miranda et al., 2009, Al-Mezaine et al., 2008, Lackner et al., 2005). A three dimensional (3D) Scan, consisting of 25 images per scan, was used to evaluate pachymetry. An Enhanced Dynamic Scheimpflug Image, consisting of the mean of 15 images taken from a single camera position, allowed densitometric assessment of the lens and cornea. A densitogram, a cross section of ocular density along the plane from the apex of the cornea, is automatically generated. This can be altered manually to identify lens density at any point. Densitometry is based on a grey scale of the image with a standardised numerical range from zero to 100, where 100 is completely opaque.

For pachymetric analysis, corneal thickness at the pupil centre, minimum corneal thickness and peripheral corneal thickness at four locations (superior, inferior nasal and temporal cornea) were recorded. Anterior chamber volume, anterior chamber depth and angle were also recorded from the 3D scans. For densitometric analysis, maximum corneal density and anterior surface lens density along the axis of the corneal apex were recorded from locations automatically identified by the Pentacam software. Maximum lens density along the visual axis was identified manually from the densitogram.

7.2.3 Statistics

All data were checked for normality with the Kolmogorov-Smirnov test. Results recorded with the Pentacam were normally distributed ($0.077<p<0.200$) whereas results for visual function were not ($0.000<p<0.019$). Despite a non-normal distribution of the visual function results, the results are a small sample of continuous data which is representative of a normal distribution. Therefore, Bland and Altman (2009) suggest parametric statistics may be applied.

A Paired-samples (2-tailed) t-test was applied to compare results from right and left eyes. The Independent-samples (2-tailed) t-test was used to compare data from the CF and control cohort. Correlation was assessed with Pearson's product moment correlation coefficient. One-Way Analysis of Variance (ANOVA) analysis was used to examine data based on genotype, vitamin A status and diabetes. Bonferroni's test was used for post-hoc comparisons. Significance was set at the 0.05 level throughout.

7.3 Results

7.3.1 Subjects

An overview of the disease involvement in adults with CF is shown in Table 7.2.

All subjects participated fully, with the exception of:

- Dark adaptation - 26 subjects with CF and 28 controls participated (2 subjects with CF could not participate due to time restrictions)
- Colour vision - 27 subjects with CF and 28 controls participated (1 subject with CF was excluded due to a congenital colour vision defect)

A Paired-samples t-test indicated measures of refractive error, binocular vision, visual function and morphological recordings were not significantly different when comparing the right and left eyes of all subjects (Paired-samples t-test; $0.063<p<0.859$). Therefore, results are presented for the right eye only.

Table 7.2 Overview of disease involvement for the CF group

Variable	Description
Genotype	n=13 ΔF508 homozygous n=11 ΔF508 heterozygous n=4 non-ΔF508
Pancreatic function	n=3 pancreatic sufficient n=25 pancreatic deficient
Serum vitamin A concentration	range 0.35 – 2.34 μmol/l mean 1.25 μmol/l (SD ± 0.35)
Vitamin A status	n=16 vitamin A sufficient n=12 vitamin A deficient
CFRD	n=11 CFRD n=17 non-CFRD
HbA1c	range 4.7 – 13.2 % mean 6.68 % (SD ± 2.16)
CFLD	n=6 CFLD, all taking ursodeoxycholic acid
FEV_1	range 24 – 103 % mean 58.61 % (SD ± 24.11)
Northern Score	range 0/20 – 12/20 mean 6.00 (SD ± 3.58)

7.3.2 Refractive error

In the CF cohort, 50% of subjects had undergone an eye examination within the previous two years compared to 64% of controls. Some form of spectacle correction was prescribed to 23% and 70% of CF subjects and controls respectively (Figure 7.3). Distance vision was better and near vision worse in the CF group compared to the control cohort, although the differences were not significant (Independent-samples t-test; p=0.144 and p=0.199 respectively) (Figure 7.4 and 7.5). A higher prevalence of myopia was recorded in the control group (Figure 7.6) and whilst the mean SER was more myopic with greater variance in the control group compared to the CF cohort, the difference was not significant (Independent-samples t-test; p=0.146) (Figure 7.7).

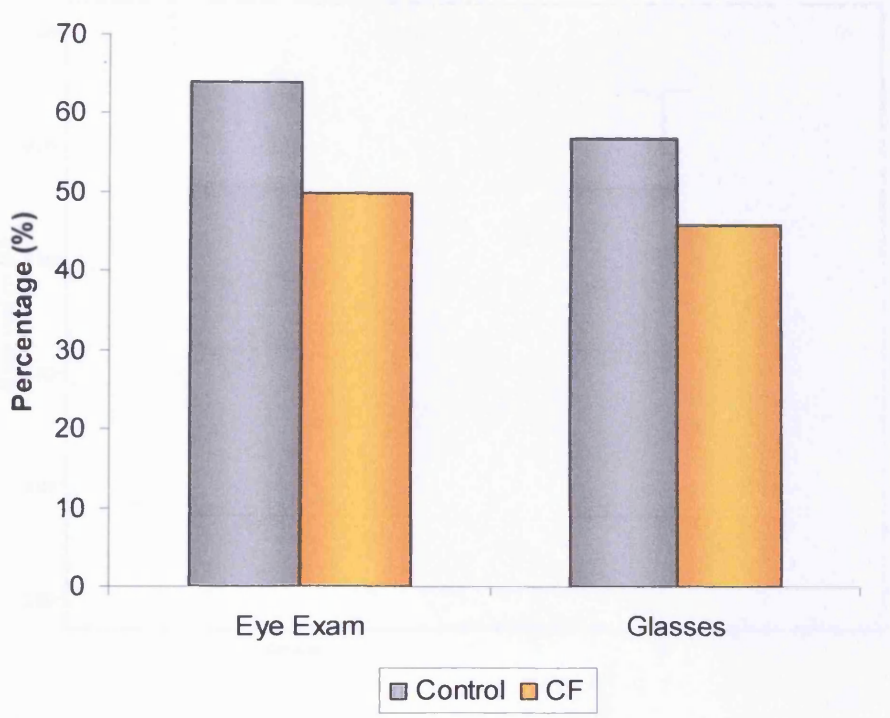


Figure 7.3 The percentage of subjects who had undergone an eye examination within the previous two years and the percentage of subjects currently prescribed spectacle correction in the CF and healthy control groups

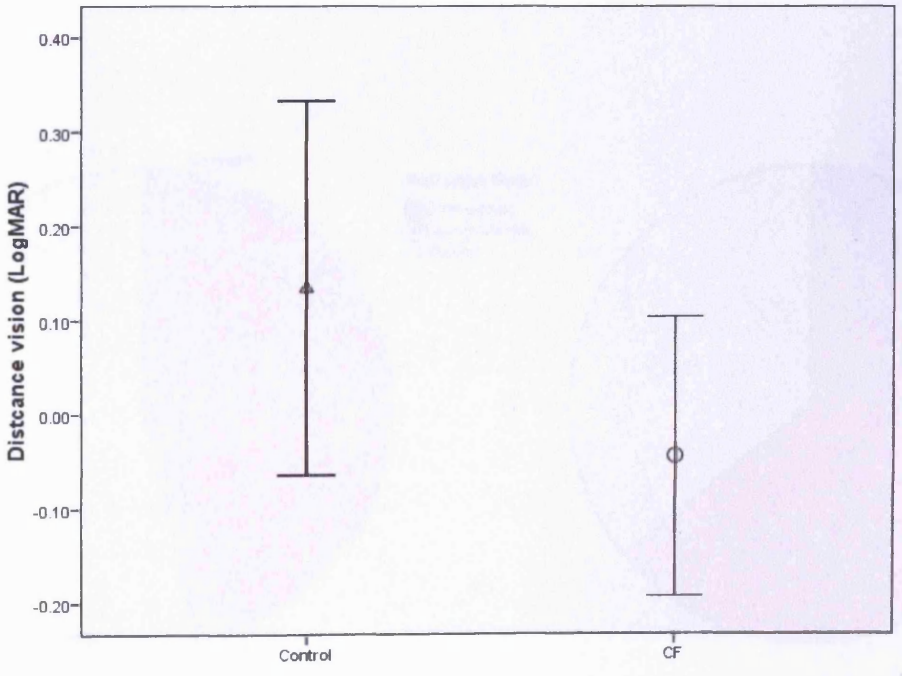


Figure 7.4 Distance vision in subjects with CF and healthy controls (Independent-samples t-test; p=0.144)

(Error bars indicate 95% confidence intervals)

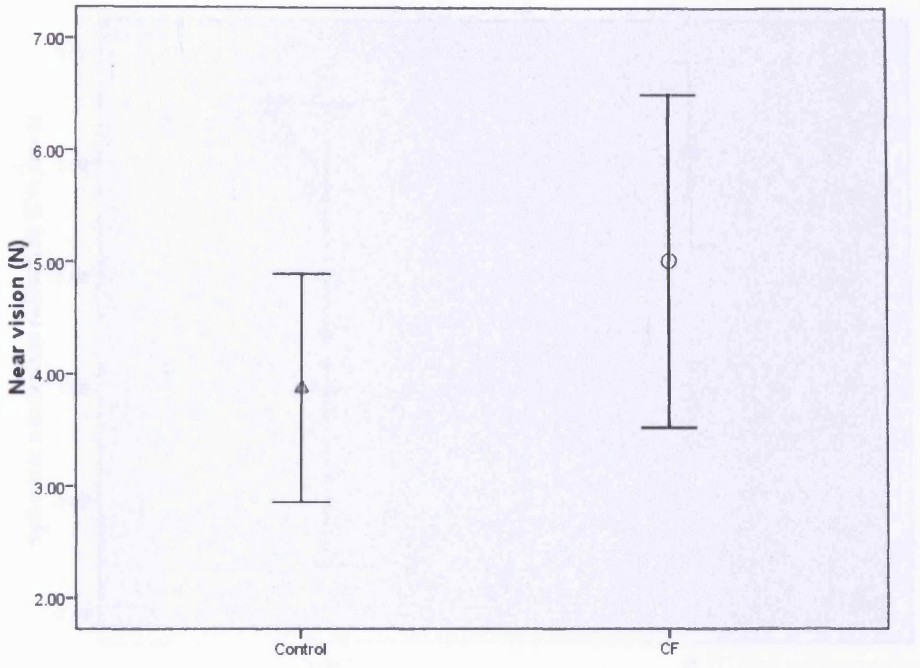


Figure 7.5 Near vision in subjects with CF and healthy controls (Independent-samples t-test; p=0.199)

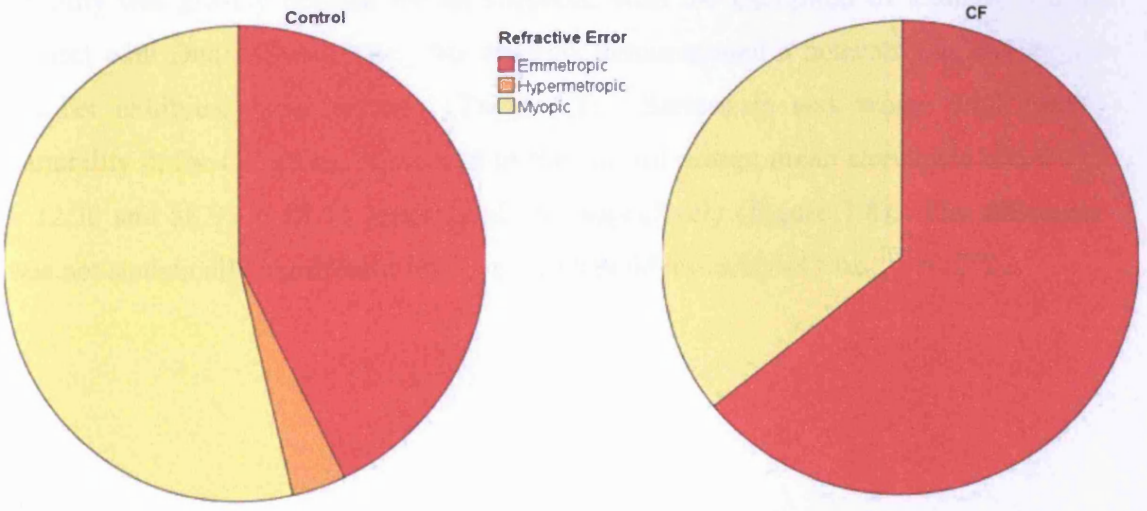


Figure 7.6 The distribution of emmetropia, hypermetropia and myopia in subjects with CF and healthy controls

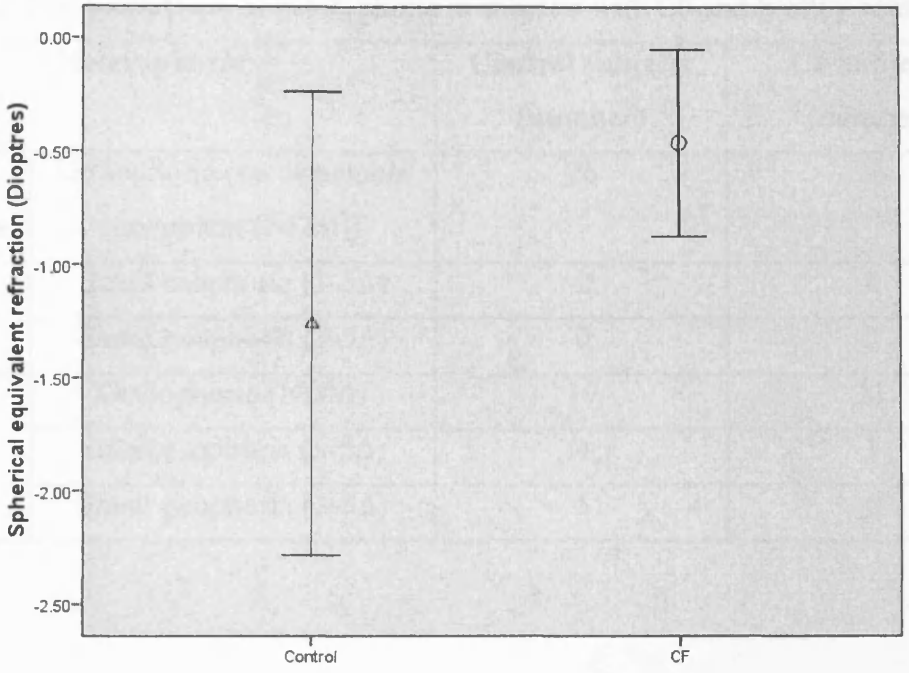


Figure 7.7 Spherical equivalent refraction (SER) in subjects with CF and healthy controls (Independent-samples t-test; p=0.146)

7.3.3 Binocular vision

Motility was grossly normal for all subjects, with the exception of a single control subject with Duane Syndrome. No subjects demonstrated a heterotropia, although a number exhibited heterophorias (Table 7.3). Stereopsis was worse with greater variability in the CF group compared to the control group; mean stereopsis was 44.29 ± 12.30 and 58.93 ± 68.71 seconds of arc respectively (Figure 7.8). The difference was not statistically significant however (Independent-samples t-test; p=0.272).

Table 7.3 The distribution of heterophoria in subjects with CF and healthy controls

Heterophoria		Control subjects (number)	CF subjects (number)
Distance	Orthophoria (no detectable movement (NDM))	26	26
	Small esophoria (3-5Δ)	2	0
	Small exophoria (3-5Δ)	0	2
Near	Orthophoria (NDM)	19	21
	Small esophoria (3-5Δ)	4	1
	Small exophoria (3-5Δ)	5	6

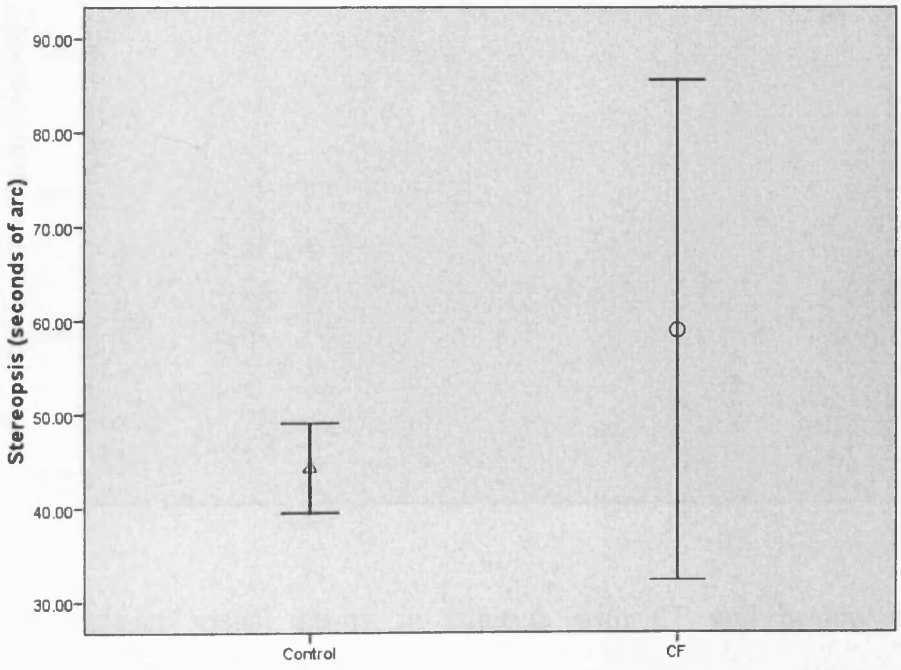


Figure 7.8 Stereopsis in subjects with CF and healthy controls (Independent-samples t-test; p=0.272)

7.3.4 Visual function

Distance VA (DVA) and near VA (NVA) were significantly better in the control cohort compared to the CF cohort (Independent-samples t-test; $p<0.05$ each) (Figure 7.9 and 7.10). Similarly, CS was significantly increased and DA thresholds significantly lower in the control group (Independent-samples t-test; $p<0.005$ each) (Figure 7.11 and 7.12). Statistical significance remained when a single CF subject with a history of diabetic maculopathy was excluded (Independent-samples t-test; DVA $p<0.05$, NVA $p<0.05$, CS $p<0.005$, DA $p<0.005$).

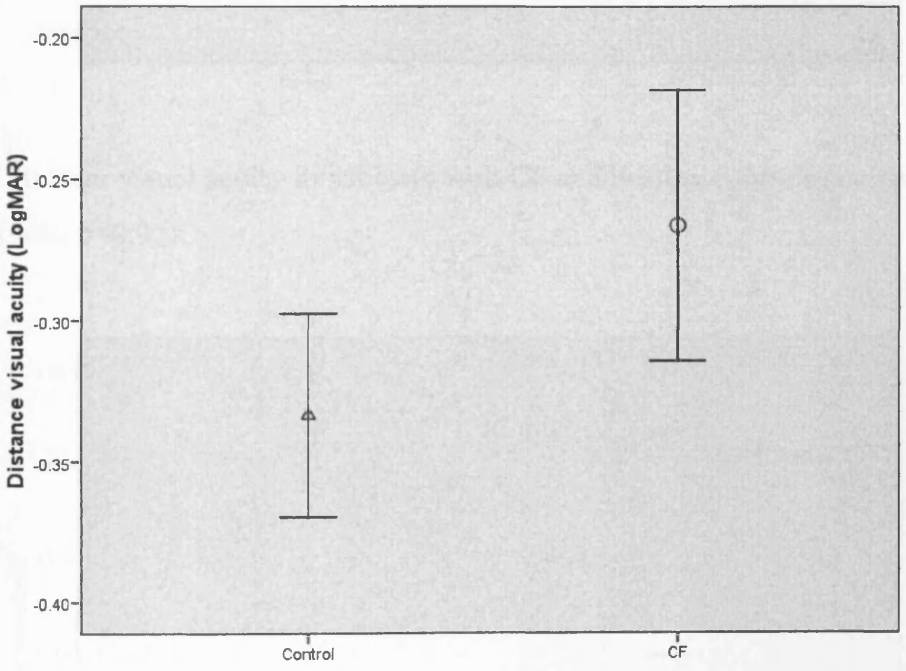


Figure 7.9 Distance visual acuity in subjects with CF and healthy controls (Independent-samples t-test, $p<0.05$)

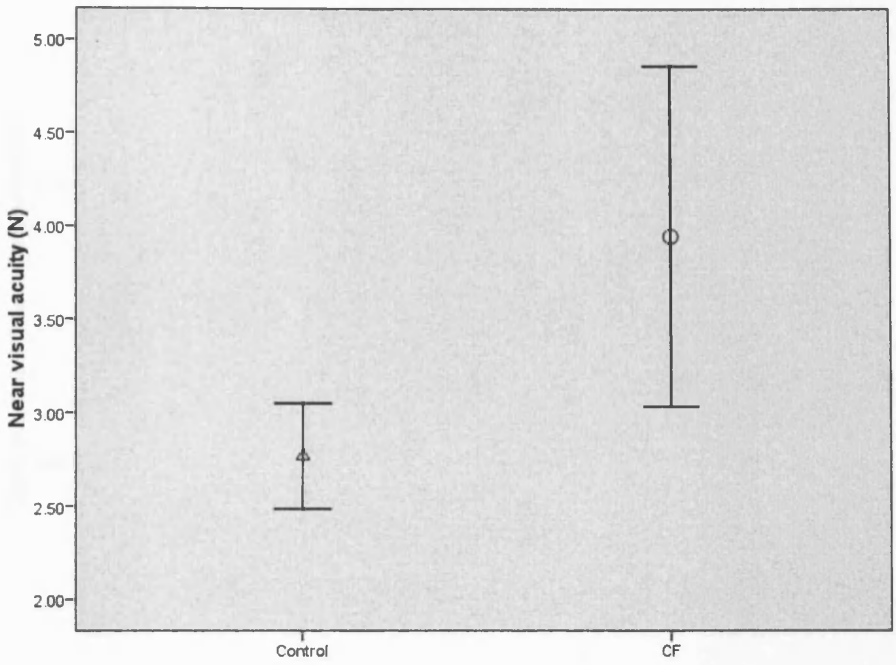


Figure 7.10 Near visual acuity in subjects with CF and healthy controls (Independent-samples t-test; $p<0.05$)

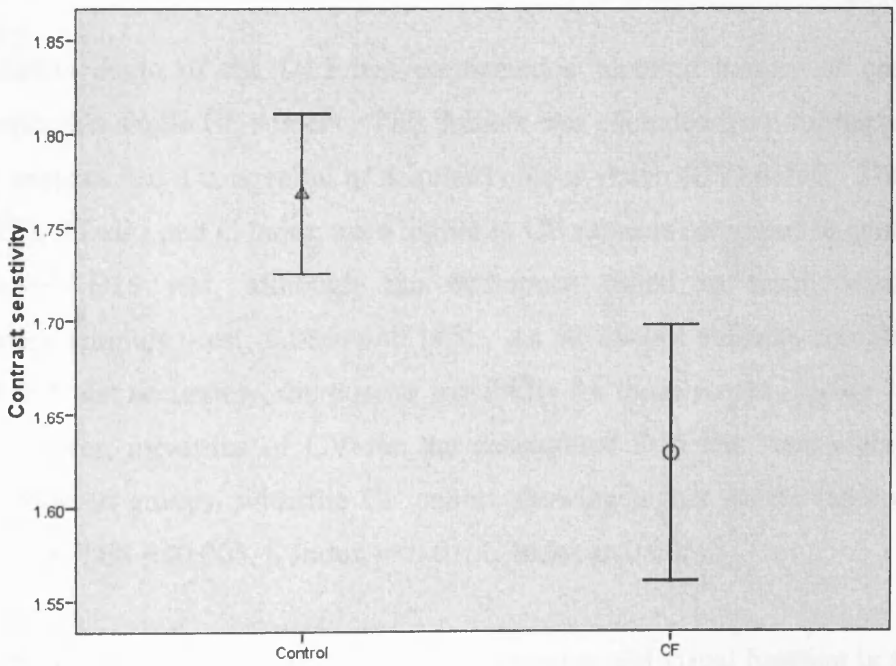


Figure 7.11 Contrast sensitivity in subjects with CF and healthy controls (Independent-samples t-test; $p<0.005$)

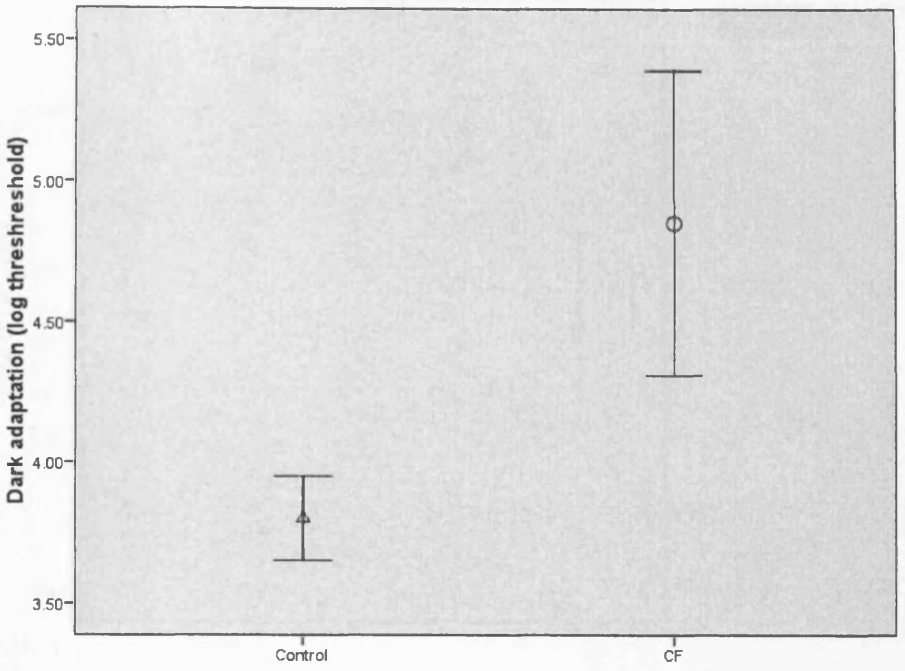


Figure 7.12 Dark adaptation log thresholds in subjects with CF and healthy controls (Independent-samples t-test; $p<0.005$)

The confusion angle of the D15 test confirmed a reported history of congenital deuteranopia in a single CF subject. This subject was excluded from further analysis. No other subjects had a congenital or acquired colour vision (CV) defect. Total error score (TES), S Index and C Index were higher in CF subjects compared to controls for the saturated D15 test, although the difference failed to reach significance (Independent-samples t-test; $0.086<p<0.145$). As all control subjects completed the saturated D15 test accurately, there is no variability for those scores (Figure 7.13 and 7.14). However, measures of CV for the desaturated D15 test were significantly different between groups, with the CF cohort showing higher scores (Independent-samples t-test; TES $p<0.005$, S Index $p<0.01$, C Index $p<0.005$).

A summary of differences in refractive error, stereopsis and visual function in subjects with CF and healthy controls is presented in Table 7.4.

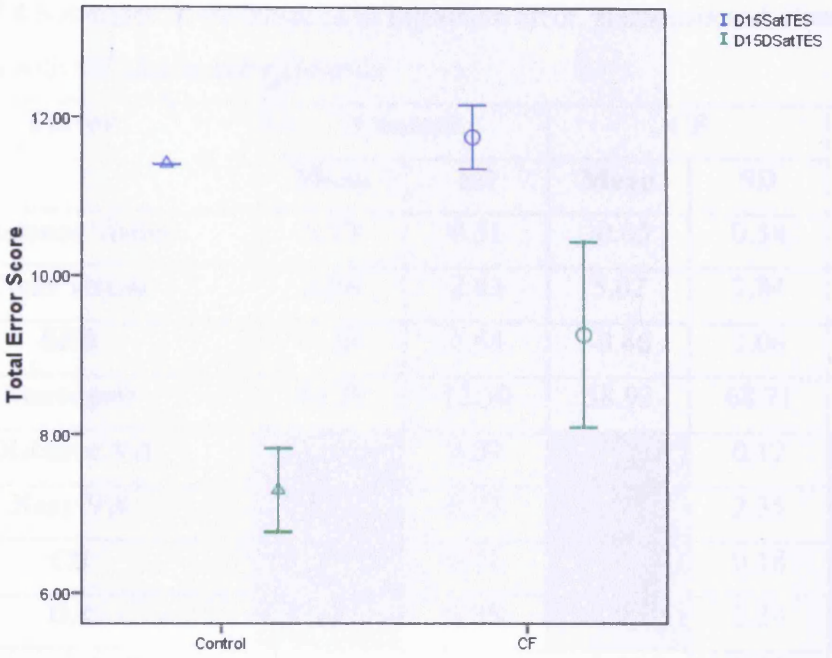


Figure 7.13 Total error score (TES) for the saturated and desaturated Farnsworth D15 test in subjects with CF and healthy controls (Independent-samples t-test; p=0.086 and p<0.005 respectively)

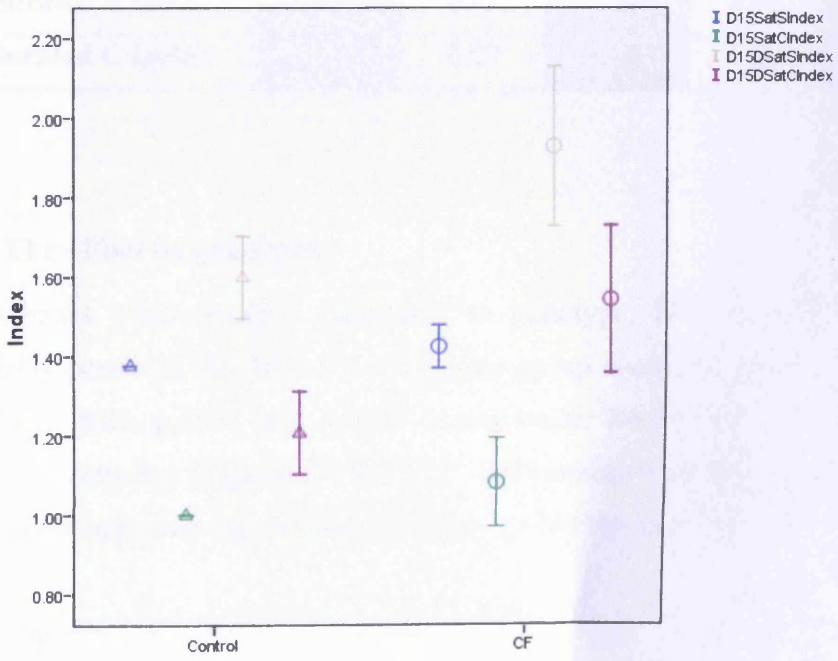


Figure 7.14 S Index and C Index values for the saturated and desaturated Farnsworth D15 test in subjects with CF and healthy controls (Independent-samples t-test; S Index p=0.084 and p<0.01 and C Index p=0.145 and p<0.005 respectively)

Table 7.4 Summary of differences in refractive error, stereopsis and visual function in subjects with CF and healthy controls

Factor	Control		CF		Sig.
	Mean	SD	Mean	SD	(Ind. t-test)
Distance vision	0.13	0.51	-0.05	0.38	0.144
Near vision	3.88	2.63	5.02	3.84	0.199
SER	-1.26	2.64	-0.46	1.06	0.146
Stereopsis	44.29	12.30	58.93	68.71	0.272
Distance VA	-0.33	0.09	-0.27	0.12	**<0.050**
Near VA	2.77	0.73	3.95	2.35	**<0.050**
CS	1.77	0.11	1.63	0.18	**<0.005**
DA	3.80	0.38	4.85	1.34	**<0.005**
Saturated TES	1.14	0.00	1.18	1.02	0.084
Saturated S Index	1.38	0.00	1.42	0.14	0.145
Saturated C Index	1.00	0.00	1.08	0.28	0.086
Desaturated TES	7.30	1.36	9.27	2.96	**<0.005**
Desaturated S Index	1.60	0.27	1.93	0.51	**<0.010**
Desaturated C Index	1.21	0.27	1.54	0.47	**<0.005**

7.3.4.1 The effect of genotype

When subjects were grouped according to genotype, NVA, CS and DA were significantly poorer in the ΔF508 homozygote group compared to the control group (ANOVA; $p<0.01$, $p<0.01$ and $p<0.05$ respectively: Bonferroni's; $p<0.05$, $p<0.05$, $p<0.005$ respectively) (Figures 7.15-7.17). Differences in DVA amongst the three groups did not approach significance, however (ANOVA; $p=0.082$).

Considering the saturated D15 test, the C index was not significantly different between the groups (ANOVA; $p=0.053$). The TES and S index were significantly higher in the ΔF508 heterozygote group compared to the controls (ANOVA; $p<0.05$ each: Bonferroni's; $p<0.05$ each). For the desaturated D15 test, TES was significantly worse in the control group compared to the ΔF508 heterozygotes (ANOVA, $p<0.005$:

Bonferroni's; $p<0.005$), the S index was significantly worse in the ΔF508 homozygotes compared to the controls (ANOVA; $p<0.01$: Bonferroni's; $p<0.05$) and the C Index was significantly worse in both CF genotype groups compared to the controls (ANOVA; $p<0.005$: Bonferroni's; $p<0.05$ each).

These results are summarised in Table 7.5.

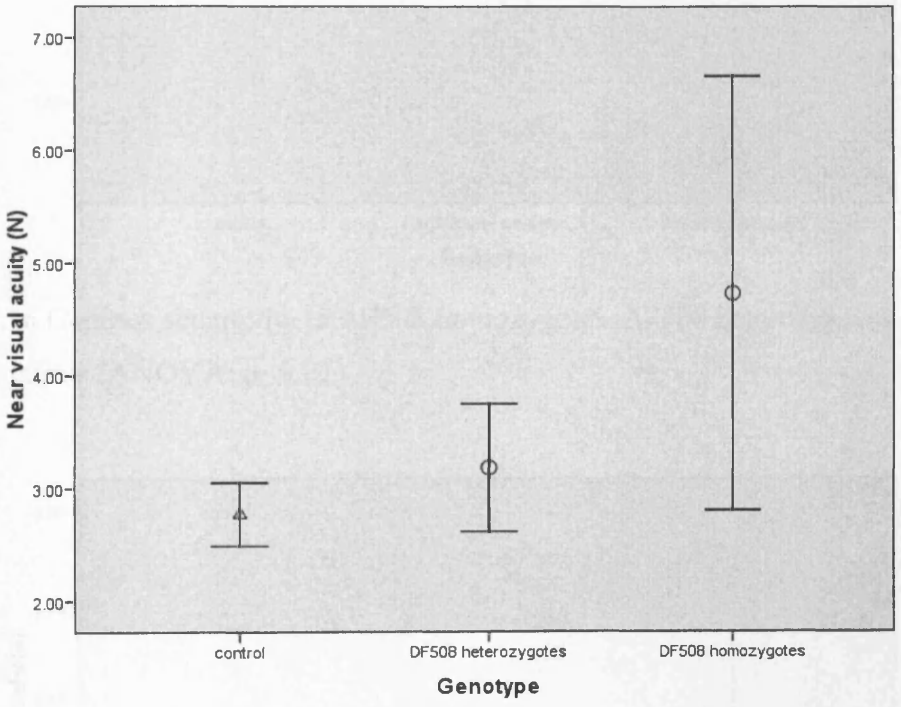


Figure 7.15 Near visual acuity in ΔF508 homozygotes, ΔF508 heterozygotes and healthy controls (ANOVA; $p<0.01$)

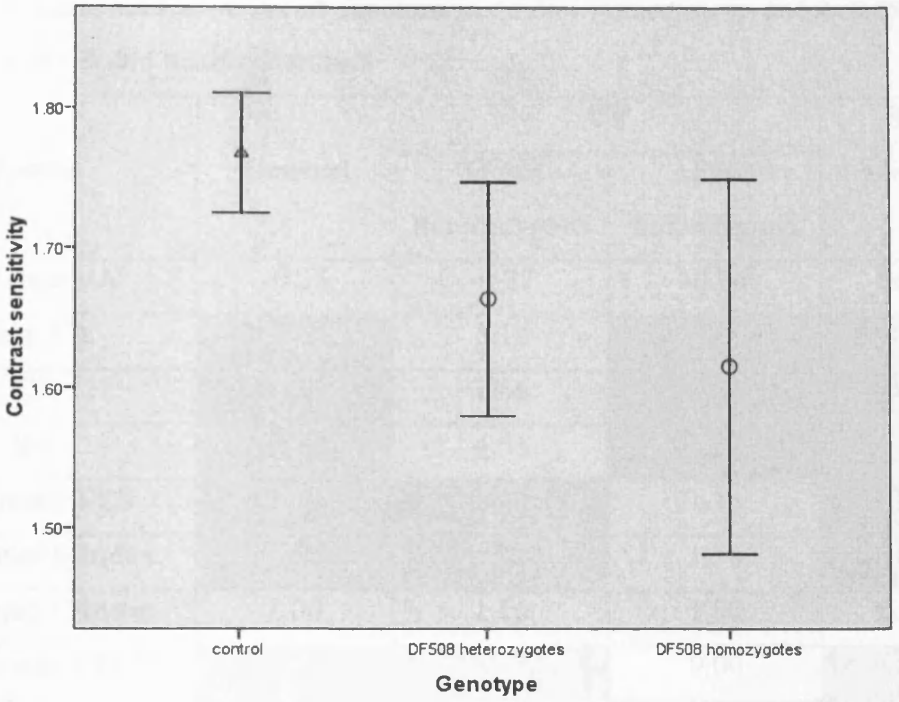


Figure 7.16 Contrast sensitivity in ΔF508 homozygotes, ΔF508 heterozygotes and healthy controls (ANOVA; $p<0.01$)

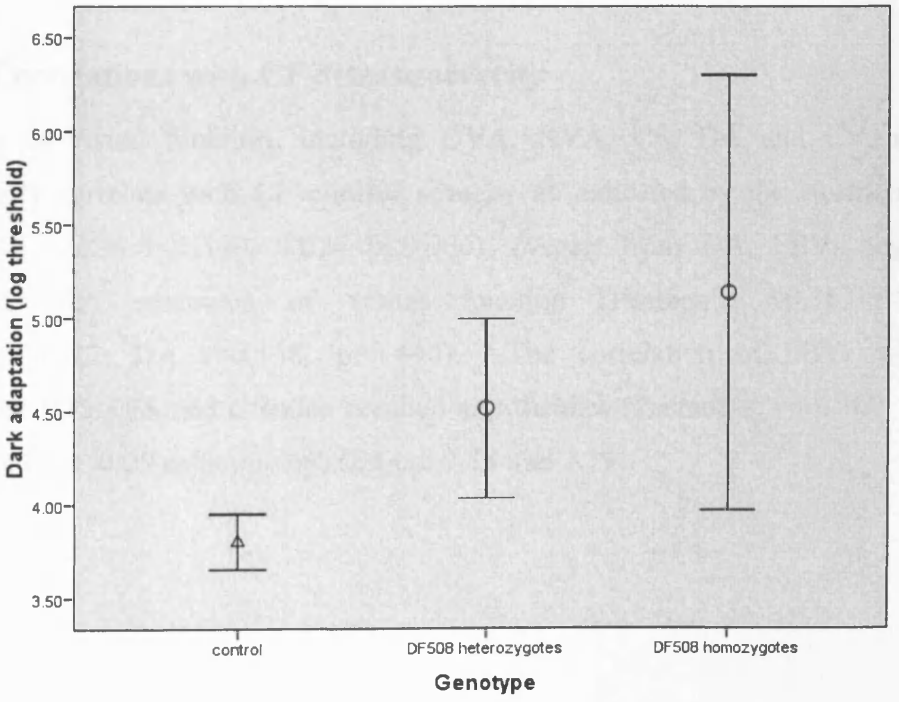


Figure 7.17 Dark adaptation in ΔF508 homozygotes, ΔF508 heterozygotes and healthy controls (ANOVA; $p<0.005$)

Table 7.5 Mean values of visual function in ΔF508 homozygous and heterozygous subjects with CF and healthy controls

Factor	Control	CF		Sig. (ANOVA)
		ΔF508 heterozygous	ΔF508 homozygous	
Distance VA	-0.33	-0.27	-0.26	0.082
Near VA	2.77	3.18	4.73	**<0.010**
CS	1.77	1.66	1.62	**<0.010**
DA	3.80	4.51	5.13	**<0.005**
Saturated TES	1.14	1.20	1.14	**<0.050**
Saturated S Index	1.38	1.45	1.38	**<0.050**
Saturated C Index	1.00	1.16	1.00	0.053
Desaturated TES	7.30	1.02	9.00	**<0.005**
Desaturated S Index	1.60	1.95	2.00	**<0.010**
Desaturated C Index	1.21	1.63	1.56	**<0.005**

7.3.4.2 Correlations with CF disease severity

Measures of visual function, including DVA, NVA, CS, DA and CV, did not significantly correlate with CF clinical severity as indicated by the Northern score (Pearson's; $-0.236<r<0.349$, $0.074<p<0.966$). Apart from DA, FEV_1 negatively correlated with measures of visual function (Pearson's; $-0.353<r<-0.125$, $0.071<p<0.0.522$; DA $r=0.158$, $p=0.440$). The correlation of FEV_1 with the desaturated D15 TES and C Index reached significance (Pearson's; $r=-0.500$, $p<0.01$ and $r=-0.451$, $p<0.05$ respectively) (Figure 7.18 and 7.19).

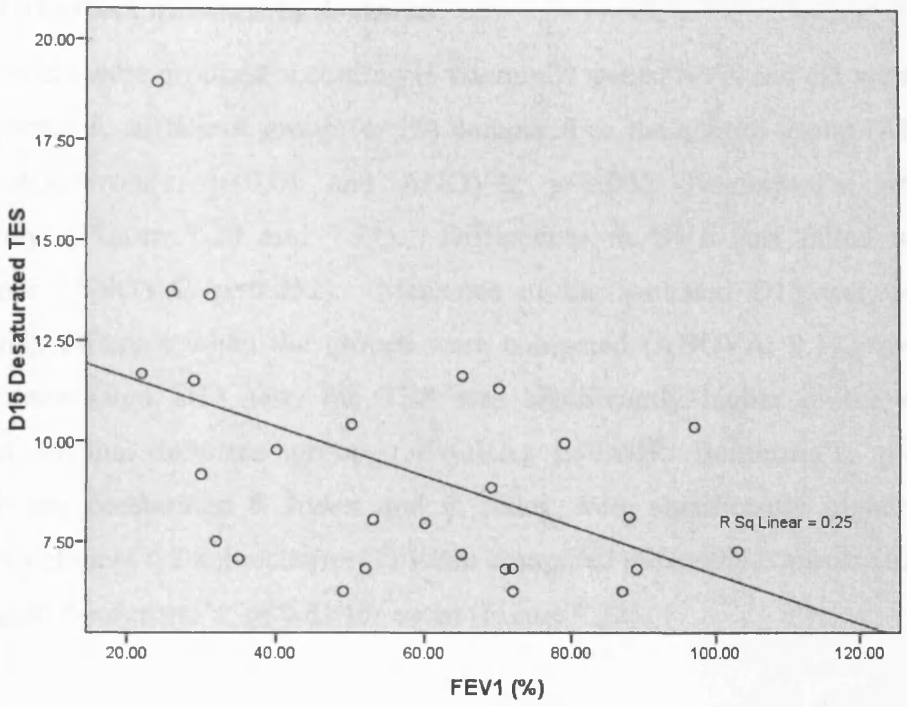


Figure 7.18 The correlation of lung function (Forced Expiratory Volume in 1 second (FEV_1)) and Farnsworth desaturated D15 colour vision test Total Error Score (TES) in subjects with CF (Pearson's; r=-0.500, p<0.01)

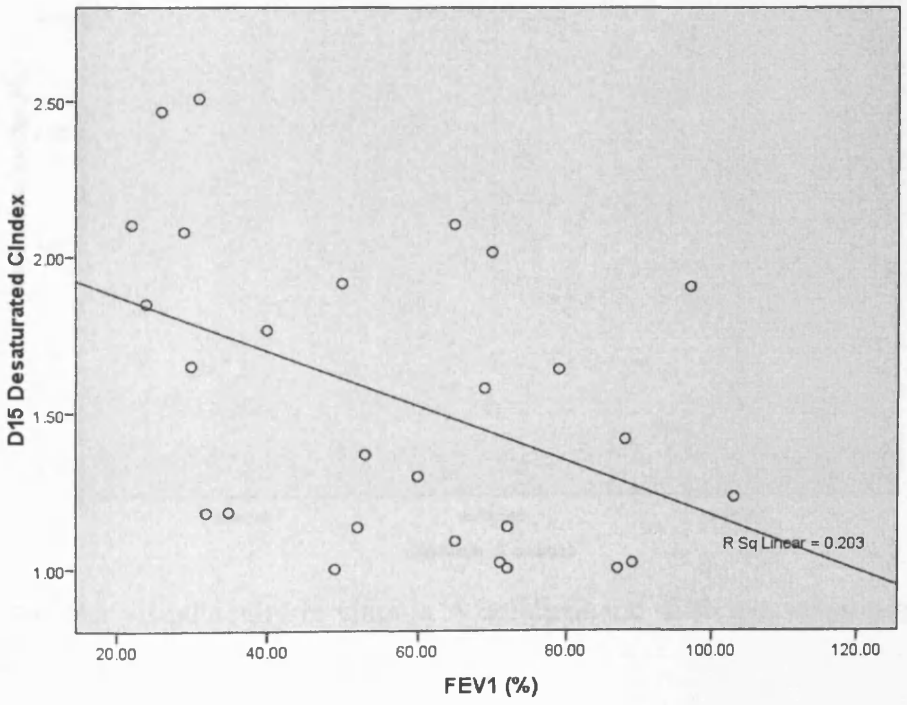


Figure 7.19 The correlation of lung function (Forced Expiratory Volume in 1 second (FEV_1)) and Farnsworth desaturated D15 colour vision test C Index in subjects with CF (Pearson's; r=-0.451, p<0.05)

7.3.4.3 The effect of vitamin A status

When subjects were grouped according to vitamin A status, NVA and CS were worse in the vitamin A sufficient group (n=16) compared to the control group (ANOVA; $p<0.01$: Bonferroni's; $p<0.01$ and ANOVA; $p<0.005$: Bonferroni's; $p<0.0001$ respectively) (Figure 7.20 and 7.21). Differences in DVA just failed to reach significance (ANOVA, $p=0.052$). Measures of the saturated D15 test were not significantly different when the groups were compared (ANOVA; $0.142<p<0.238$). For the desaturated D15 test, the TES was significantly higher in the controls compared to the deficient group (ANOVA; $p<0.005$: Bonferron's; $p<0.005$). However, the desaturated S Index and C Index were significantly higher in the vitamin A deficient CF subjects (n=12) when compared to healthy controls (ANOVA, $p<0.01$ each: Bonferroni's; $p<0.01$ for each) (Figure 7.22).

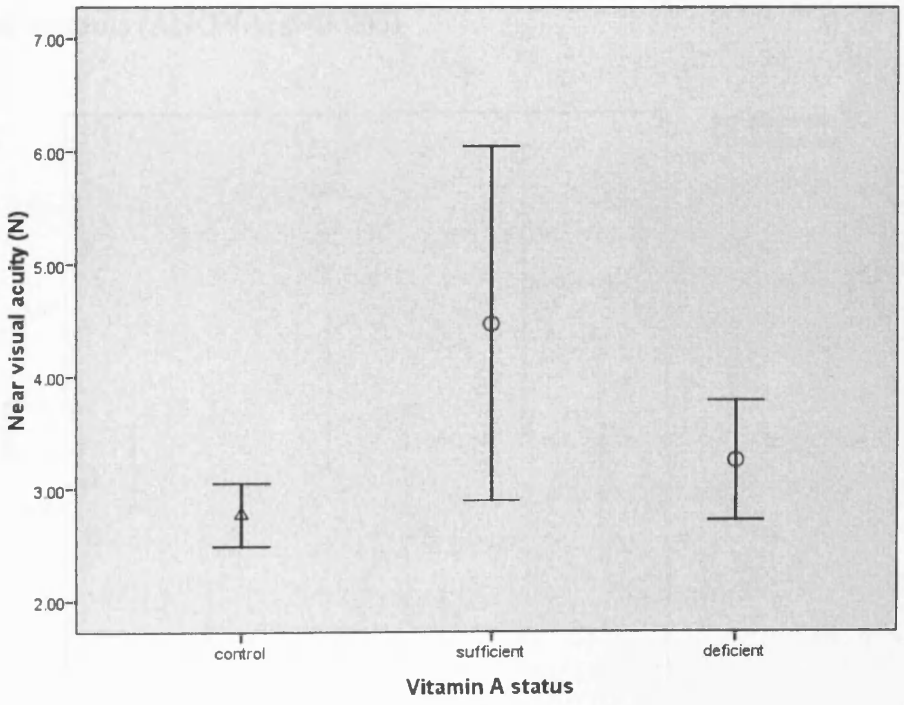


Figure 7.20 Near visual acuity in vitamin A deficient and sufficient subjects with CF and healthy controls (ANOVA; $p<0.01$)

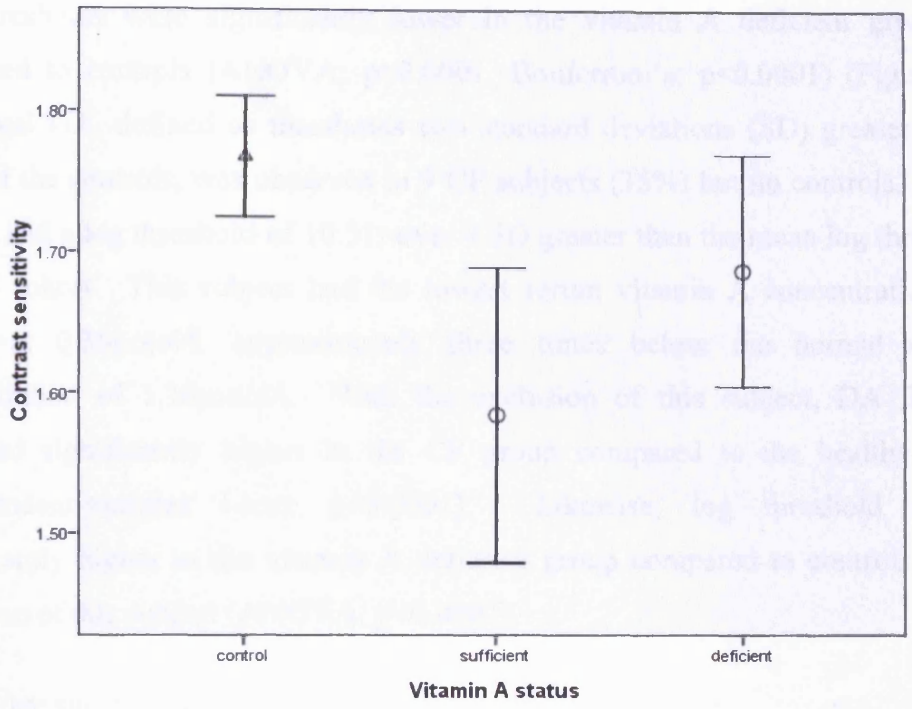


Figure 7.21 Contrast sensitivity in vitamin A deficient and sufficient subjects with CF and healthy controls (ANOVA; $p<0.005$)

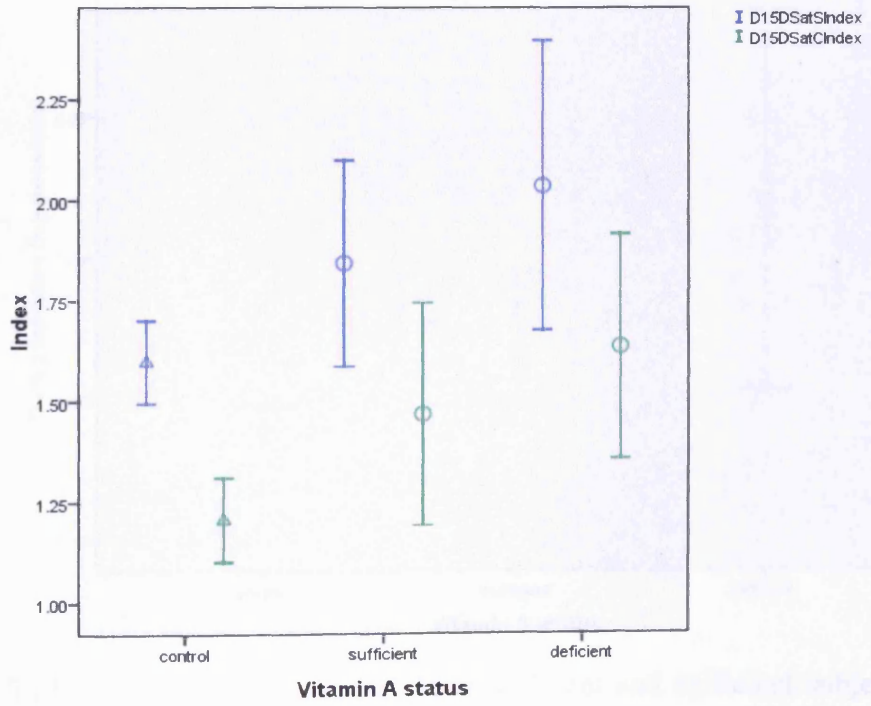


Figure 7.22 Desaturated D15 S Index and C Index in vitamin A deficient and sufficient subjects with CF and controls (ANOVA; $p<0.01$ each)

Log thresholds were significantly lower in the vitamin A deficient group when compared to controls (ANOVA; $p<0.0001$: Bonferroni's; $p<0.0001$) (Figure 7.23). Abnormal DA, defined as thresholds two standard deviations (SD) greater than the mean of the controls, was observed in 9 CF subjects (35%) but no controls. A single subject had a log threshold of 10.31; over 4 SD greater than the mean log threshold of the CF cohort. This subject had the lowest serum vitamin A concentration of the cohort at 0.35μmol/l, approximately three times below the normal minimum concentration of 1.10μmol/l. With the exclusion of this subject, DA thresholds remained significantly higher in the CF group compared to the healthy controls (Independent-samples t-test; $p<0.0001$). Likewise, log threshold remained significantly higher in the vitamin A deficient group compared to controls with the exclusion of this subject (ANOVA; $p<0.0001$).

Results are summarised in Table 7.6.

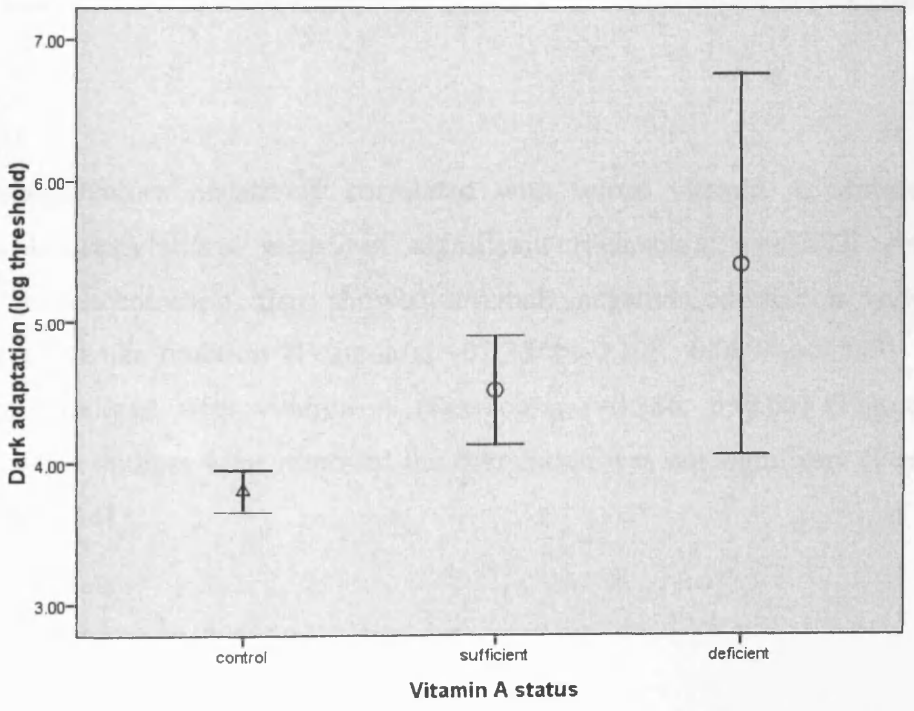


Figure 7.23 Dark adaptation in vitamin A deficient and sufficient subjects with CF and healthy controls (ANOVA; $p<0.0001$)

Table 7.6 Mean values of visual function in vitamin A sufficient and deficient subjects with CF and healthy controls

Factor	Control	CF		Sig. (ANOVA)
		Vitamin A sufficient	Vitamin A deficient	
Distance VA	-0.33	-0.25	-0.29	0.052
Near VA	2.77	4.47	3.25	**<0.010**
CS	1.77	1.58	1.69	**<0.010**
DA	3.80	4.51	5.40	**<0.0001**
Saturated TES	1.14	1.19	1.16	0.142
Saturated S Index	1.38	1.44	1.41	0.157
Saturated C Index	1.00	1.05	1.11	0.238
Desaturated TES	7.30	8.58	1.01	**<0.005**
Desaturated S Index	1.60	1.84	2.04	**<0.010**
Desaturated C Index	1.21	1.47	1.63	**<0.010**

DA threshold values negatively correlated with serum vitamin A concentration, although the correlations were not significant (Pearson's; r=-0.282, p=0.162). Vitamin A concentration also showed a small negative correlation with other measures of visual function (Pearson's; $-0.333<r<-0.109$, $0.089<p<0.589$). NVA positively correlated with vitamin A (Pearson's; r=0.386, $p<0.05$) (Figure 7.24) however, when outliers were removed the correlation was not significant (Pearson's; r=-0.198, p=0.344).

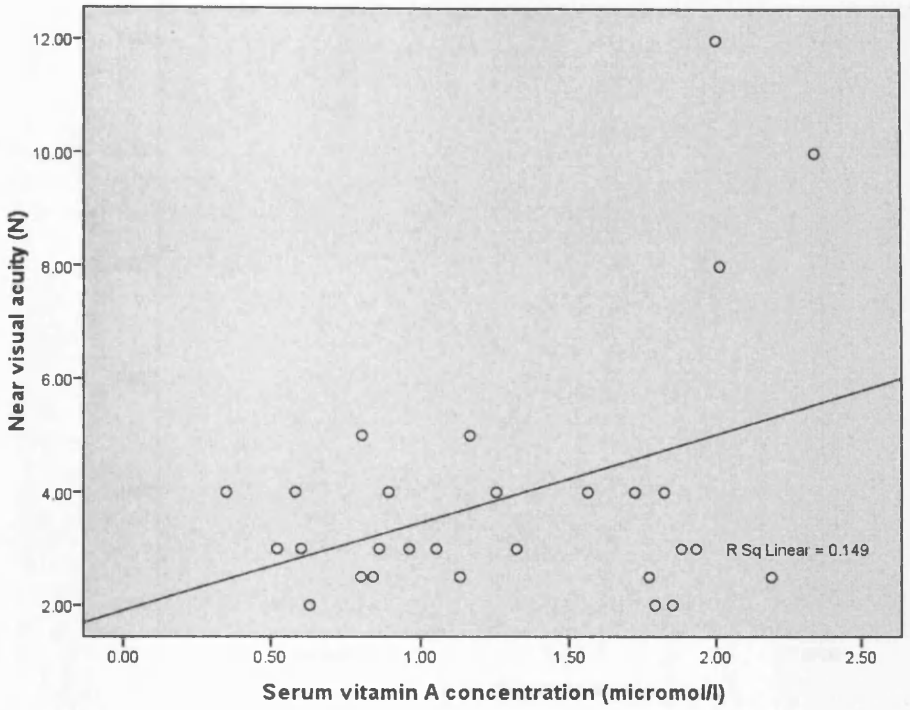


Figure 7.24 The correlation of near visual acuity and serum vitamin A concentration in subjects with CF (Pearson's; r=0.386, p<0.05)

7.3.4.4 The effect of diabetic status

When subjects were grouped according to diabetic status, subjects with CFRD had significantly worse NVA, CS and DA compared to controls (ANOVA; p<0.05, p<0.0001 and p<0.0001 respectively) (Figures 7.25-7.27) (Bonferroni's; 0.0001<p<0.05). DA was also significantly worse in the CFRD group compared to the non-CFRD subjects (Bonferroni's; p<0.005). The difference for DVA approached significance (ANOVA; p=0.071). Measures of CV with the saturated D15 test were not significantly different when subjects were grouped according to diabetic status (ANOVA; 0.164<p<0.205). However, considering the desaturated test, TES, S index and C Index were significantly worse in the non-CFRD group compared to the controls (ANOVA; p<0.01, p<0.005 and p<0.005 respectively: Bonferroni's; 0.001<p<0.05). Results are summarised in Table 7.7. Glycaemic control (HbA1c) positively correlated with DVA and DA (Pearson's; r=0.163, p=0.408 and r=0.104, p=0.612 respectively) and the correlation with NVA approached significance (Pearson's; r=0.364, p=0.057). For other measures of visual function correlations remained weak (Pearson's; -0.085<r<0.213, 0.286<p<0.926).

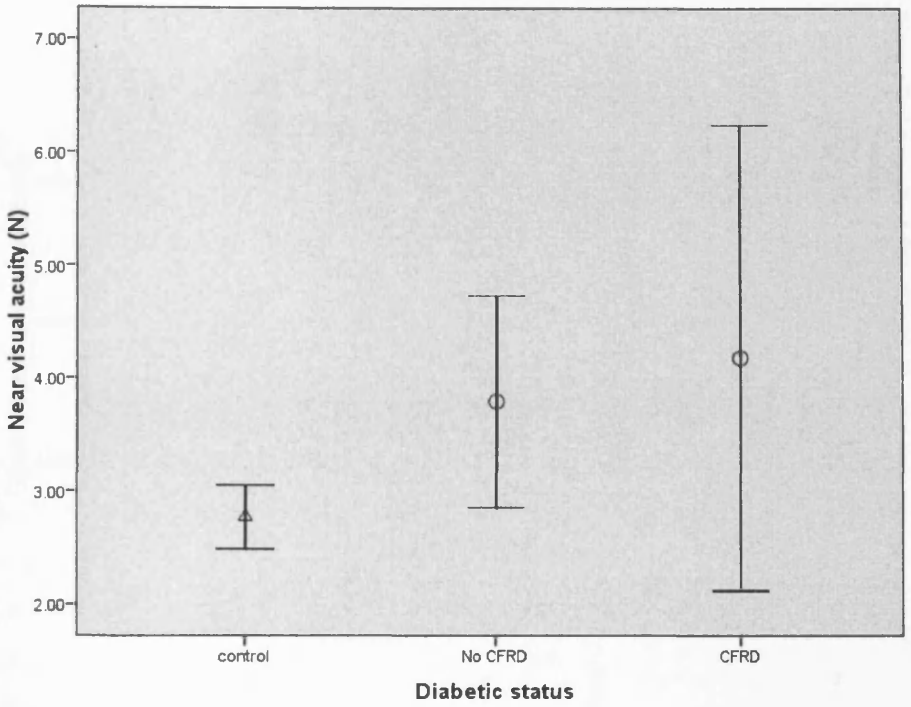


Figure 7.25 Near visual acuity in CF subjects with and without CFRD and healthy controls (ANOVA; $p<0.05$)

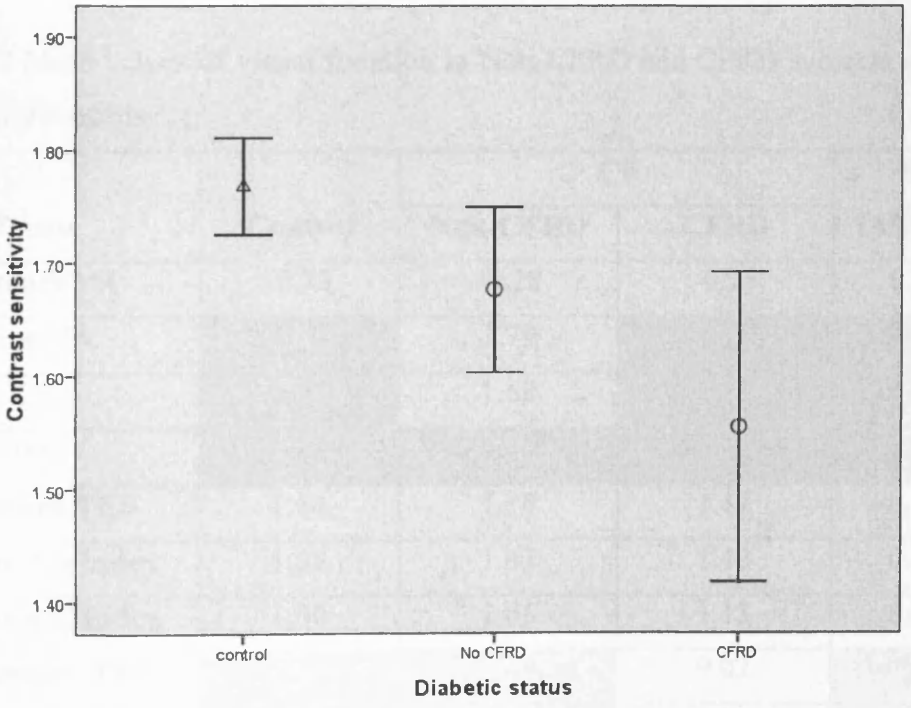


Figure 7.26 Contrast sensitivity in CF subjects with and without CFRD and healthy controls (ANOVA; $p<0.0001$)

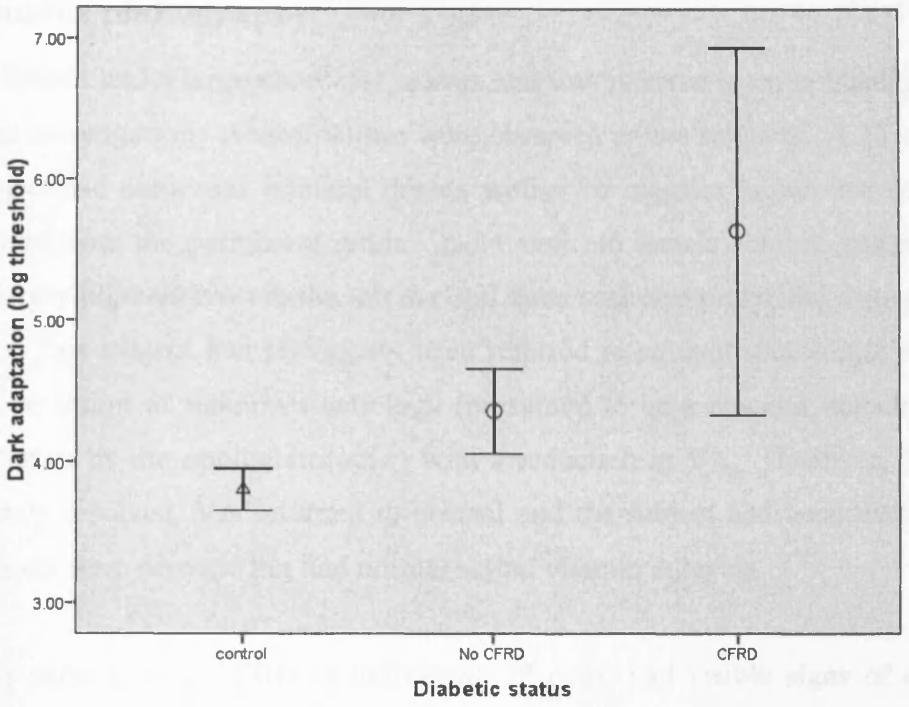


Figure 7.27 Dark adaptation in CF subjects with and without CFRD and healthy controls (ANOVA; $p<0.05$)

Table 7.7 Mean values of visual function in Non-CFRD and CFRD subjects with CF and healthy controls

Factor	Control	CF		Sig. (ANOVA)
		Non-CFRD	CFRD	
Distance VA	-0.33	-0.28	-0.25	0.071
Near VA	2.77	3.79	4.18	**<0.050**
CS	1.77	1.68	1.55	**<0.0001**
DA	3.80	4.36	5.64	**<0.0001**
Saturated TES	1.14	1.18	1.17	0.164
Saturated S Index	1.38	1.43	1.42	0.176
Saturated C Index	1.00	1.05	1.13	0.205
Desaturated TES	7.30	9.44	9.02	**<0.010**
Desaturated S Index	1.60	2.09	1.70	**<0.005**
Desaturated C Index	1.21	1.66	1.38	**<0.005**

7.3.5 Fundus photography

One CF subject had a large choroidal naevus and was referred to an ophthalmologist for further investigation. Abnormalities were observed in two subjects: A 23 year old male subject had numerous bilateral drusen within the macular region and scattered drusen throughout the peripheral retina. A 24 year old female subject, had areas of macular hyper-pigmentation in the left eye and three scattered peripheral drusen in the right eye. This subject had previously been referred to an ophthalmologist due to a left macular lesion of unknown aetiology (presumed to be a macular branch retinal vein occlusion by the ophthalmologist) with a reduction in VA. However, this had subsequently resolved, VA returned to normal and the subject had been discharged. Both subjects were diabetic but had normal serum vitamin A levels.

Of the 11 patients with CFRD, 4 individuals (7 eyes) had visible signs of diabetic retinopathy (DR). The specific features are summarised in Table 7.8. Subject number three had been treated with right sectorial pan-retinal photocoagulation and left grid and subsequent sectorial therapy photocoagulation. The highest HbA1c levels were observed in the subjects with DR. The mean diabetes duration of subjects with DR was 13.3 years, compared to 6.6 years for those without DR. At the time of examination, 73% (n=7) of the diabetic patients were currently part of a retinal screening programme.

Whilst DVA, NVA, CS and DA were lower in CF subjects with DR compared to those without DR, the difference was not significant (Independent-samples t-test; $0.110<p<0.359$). Similarly, CV was not significantly different when subjects were grouped according to DR status (Independent-samples t-test; $0.149<p<0.693$).

Table 7.8 Diabetic retinopathy features and grading according to the diabetic retinopathy screening services for Wales protocol

Subject	Eye	Grading	Features	Diabetes duration (years)	HbA1c (%)
2	R	R1.1 Mild BDR	1 HM	10	10.2
	L	R1.2 Mod BDR	1 MA within 1 DD from fovea		
3	R	R2 M1 Severe BDR with Maculopathy	Numerous dot and blot HM, exudates, venous beading, MA and HM (>3) within 1 DD from fovea	20	10.9
	L	R1.2 Mod BDR	Blot and dot HMs, 2 within 1DD from fovea		
23	R	R1.2 Mild BDR	1 MA within 1 DD from fovea	5	13.2
	L	R0 No DR	-		
27	R	R1.1 Mild BDR	1 HM	5	10.7
	L	R1.2 Mod BDR	1 MA within 1 DD from fovea, 2 HM		

Background Diabetic Retinopathy (BDR), Micro-Aneurysm (MA), Disc Diameter (DD), Haemorrhage (HM)

7.3.6 Corneal and lenticular morphological characteristics

7.3.6.1 Pachymetry

Corneal thickness values are presented in Table 7.9. For all subjects corneal thickness was increased in peripheral locations compared to the pupil centre and is greatest at the superior corneal location. Corneal thickness was not significantly different when subjects with CF and controls were compared (Independent-samples t-test; $0.370<p<0.997$). Corneal thickness was not significantly different when subjects were grouped according to genotype (ANOVA; $0.229<p<0.824$), vitamin A status (ANOVA; $0.667<p<0.969$) or diabetic status (ANOVA; $0.638<p<0.877$).

In all subjects, corneal thickness at the pupil centre negatively correlated with SER (Pearson's; r=-0.168, p=0.216) and mean corneal thickness was significantly greater in myopic subjects (569.16μm) compared to emmetropic subjects (549.50μm) (Independent-samples t-test; $p<0.05$).

Table 7.9 Mean corneal thickness measurements at various corneal locations in subjects with CF and controls

Corneal location	Mean corneal thickness (μm)				Sig. (Ind-samples t-test)
	Control		CF		
	Mean	SD	Mean	SD	
Pupil centre	560.43	37.18	558.50	36.52	0.845
Minimum	555.93	36.39	555.21	36.76	0.942
Superior	672.64	46.50	678.18	38.02	0.628
Nasal	645.89	44.39	634.93	37.47	0.997
Inferior	618.71	43.63	622.78	43.33	0.370
Temporal	631.71	42.13	600.68	33.14	0.621

7.3.6.2 Anterior chamber analysis

Anterior chamber (AC) volume, depth and angle were significantly greater in the control cohort compared to the CF group (Independent-samples t-test; $p<0.005$ each) (Table 7.10). AC volume and AC depth significantly correlated with SER (Pearson's; $r=-0.463$, $p<0.0001$ and $r=-0.449$, $p<0.005$ respectively). Although AC angle also negatively correlated with SER, the relationship was not significant (Pearson's; $r=-0.231$, $p=0.087$). AC volume was significantly greater in myopes compared to emmetropes (Independent-samples t-test; $p<0.05$). Similarly, AC depth and angle were greater in the myopic group although the differences failed to reach significance (Independent-samples t-test; $p=0.066$ and $p=0.125$ respectively).

Table 7.10 Mean anterior chamber (AC) measurements in subjects with CF and controls

Factor	Control		CF		Sig. (Ind-samples t-test)
	Mean	SD	Mean	SD	
AC Volume (mm^3)	209.11	33.15	178.96	41.19	**<0.005**
AC Depth (mm)	3.31	0.29	3.03	0.34	**<0.005**
Angle (°)	42.03	6.17	36.95	7.32	**<0.005**

7.3.6.3 Densitometry

Mean densitometry values are shown in Table 7.11. Anterior lens surface and maximum lens density were significantly higher in the subjects with CF compared to healthy controls (Independent-samples t-test; $p<0.05$ each). Similarly, maximum corneal density was higher in the CF group, although the difference did not reach significance (Independent-samples t-test, $p=0.089$). Densitometry was not significantly different between myopic and emmetropic subjects (Independent-samples t-test; $0.157<p<0.714$) and values did not significantly correlate with SER (Pearson's; $-0.006<p<0.175$). Similarly, when the two cohorts were analysed independently, there was no significant difference in corneal or lens density in the

myopic or emmetropic subjects (Independent-samples t-test; $0.171<p<0.963$ and $0.228<p<0.771$ respectively).

Table 7.11 Mean densitometry values in subjects with CF and healthy controls

Density	Mean density (relative units)				Sig. (Ind-samples t-test)
	Control		CF		
	Mean	SD	Mean	SD	
Maximum corneal	31.49	10.07	37.44	15.13	0.089
Anterior lens surface	9.54	1.43	11.03	3.30	**<0.05**
Maximum lens	11.22	1.90	12.62	2.99	**<0.05**

Densitometry values were generally higher in the CFRD group compared to the non-CFRD group and controls, although differences failed to reach significance (ANOVA; $0.099<p<0.220$) (Table 7.12). Unexpectedly, values were higher in the vitamin A sufficient subgroup compared to the deficient and control groups and differences approached significance (ANOVA; $0.060<p<0.105$) (Table 7.12). Densitometry values did not correlate with serum vitamin A concentration (Pearson's; $-0.102<r<0.218$, $0.265<p<0.689$) or HbA1c (Pearson's; $-0.178<r<0.129$, $0.365<p<0.532$). Anterior lens density and maximum lens density were not significantly different when CF subjects were grouped according to current steroid use and compared to control subjects (ANOVA; $p=0.098$ and $p=0.109$).

Table 7.12 Mean densitometry values in subjects with CF grouped according to diabetic and vitamin A status compared to healthy controls

Density	Mean density (relative units) ± SD			Sig. (Ind-samples t-test
	Control	CF		
		Non-CFRD	CFRD	
Maximum corneal	31.49 ± 10.07	36.63 ± 14.11	38.68 ± 17.22	0.220
Anterior lens surface	9.54 ± 1.43	10.82 ± 2.78	11.35 ± 4.11	0.099
Maximum lens	11.22 ± 1.90	12.66 ± 2.90	12.55 ± 3.26	0.108
Density	**Control**	**CF**		**Sig. (Ind-samples t-test**
		Vit A suff	**Vit A def**	
Maximum corneal	31.49 ± 10.07	40.91 ± 15.58	32.80 ± 13.78	0.060
Anterior lens surface	9.54 ± 1.43	11.42 ± 4.18	10.50 ± 1.57	0.074
Maximum lens	11.22 ± 1.90	12.73 ± 3.86	12.48 ± 1.23	0.105

Density values did not significantly correlate with any aspect of visual function (Pearson's; $-0.207<r<0.257$, $0.088<p<0.956$). Abnormal lens density was defined as a two SD above the mean maximum lens density of the control group. According to this criterion, two control subjects and four CF subjects had abnormal lens density. Mean DVA, NVA and CS were worse in the abnormal lens density group compared to normal group although the difference was not significant (Independent-samples t-test; $0.151<p<0.381$). Similarly, DA and CV were not significantly different in the two groups (Independent-samples t-test; $0.464<p<0.939$).

7.3.7 Major findings of this study

- Visual function (DVA, NVA, CS, DA and desaturated D15 CV TES, S Index and C Index) was significantly reduced in CF subjects compared to controls

- NVA, CS and DA were significantly reduced in ΔF508 homozygotes compared to controls

- Saturated D15 TES and C Index were significantly worse in ΔF508 heterozygotes compared to controls

- Lung function showed a significant negative correlation with desaturated D15 TES and C Index

- DA and desaturated TES, S Index and C Index were significantly reduced in vitamin A deficient subjects compared to controls

- NVA, CS and DA were significantly worse in CFRD subjects to controls

- DA was significantly worse in CFRD subjects compared to non-CFRD subjects

- Anterior lens surface and maximum lens density were significantly higher in CF subjects compared to controls

7.4 Discussion

7.4.1 Refractive error and binocular vision

Distance vision was better in the CF group compared to the control group and is likely to reflect the higher incidence and greater severity of myopia in the control cohort. A large proportion of the control group were undergraduates and a high prevalence of myopia has been observed in university students previously (Loman et al., 2002, Fledelius, 2000). Approximately 36% of the CF subjects were myopic, which is similar to previous findings (Fama et al., 1998, Morkeberg et al., 1995). No subjects with CF were hypermetropic and binocular function was similar to the control group. Therefore, similar to the juvenile CF group, normal emmetropisation and orthophorisation appear to have taken place in these adult CF subjects.

7.4.2 Visual function

Unlike the juvenile cohort, all aspects of visual function were adversely affected in adults with CF suggesting rod and cone photoreceptor dysfunction in these subjects. CFTR, within the apical RPE membrane, mediates Cl^- secretion to facilitate regulation of the SRS and IPM (Marmor and Wolfensberger, 1998, Wills et al., 2000a). In CF, CFTR function may be defective or completely absent depending on the level of mutation or genotype (McKone et al., 2003). Without compensation from other apical Cl^- channels normal RPE and photoreceptor function may be affected. Whilst the lack of significance in the juvenile study could have been due to high variability or small cohort size, the juvenile subjects were "relatively" healthy. Therefore, disease severity and/or duration may have a cumulative adverse effect on normal RPE function and subsequent retinal photoreceptor maintenance.

Besides regulation of the SRS, the RPE has a number of functions essential for the maintenance of normal visual function including; absorption of scattered light, transport of nutrients to the photoreceptors, retinal regeneration, photoreceptor outer segments phagocytosis and recycling of essential substances for the photoreceptors (Strauss, 2005). Failure of any one of these functions could result in impaired photoreceptor function, photoreceptor degradation and reduced visual function. In

CF, normal RPE metabolism and/or function may be altered in response to cellular compensation to abnormal CFTR Cl^- transport. CF subjects with a more severe CFTR mutation are hypothesised to have greater levels of disruption to normal RPE Cl^- efflux. In general, visual function appeared to be worse in subjects with the ΔF508 homozygote mutation giving support to the hypothesis that abnormal visual function is a primary manifestation of CF. There were no levels of metamorphopsia and no indication of serous retinopathy in subjects with CF however, the level of RPE disturbance could be sub-clinical and remain undetected by retinal photographs.

Impaired RPE function results in drusen formation between the RPE and Bruch's membrane. An early feature of age-related macular degeneration (AMD), drusen are composed of metabolic waste products and are rarely observed in individuals before 45 years of age (Hageman and Mullins, 1999). Two young CF subjects had bilateral scattered drusen indicating impaired RPE function. This atypical presentation could be caused by abnormal RPE function or metabolism in response to compensation of abnormal or absent CFTR function or through premature oxidative damage to the RPE following reduced carotenoid concentration. Altered trans-epithelial transport has been implicated in AMD pathogenesis (Strauss, 2005). Therefore, abnormal Cl^- efflux by CFTR in CF could contribute to the development of premature drusen in subjects with CF. Incidentally, both subjects are diabetic, which is a known risk factor for AMD (Holz et al., 2004).

Similar to the juvenile group, certain measures of CV were significantly reduced in vitamin A deficient CF subjects suggesting subtle levels of cone dysfunction. However, other measures of visual function, such as NVA and CS, were worse in the vitamin A sufficient group. This may result from inaccurate assessment of current vitamin A status; it was recorded from the last known blood test, not directly at the time of assessment. Alternatively, this could reflect an overlap of the CF subgroups: DVA and CS were also significantly reduced in CFRD subjects and a third of the vitamin A sufficient subjects had CFRD, potentially skewing significance. Impaired DA and CS have been observed in CF subjects with VAD and supplementary vitamin A has been shown to improve both DA and CS in these subjects (Leguire et al., 1991, Fulton et al., 1982, Huet et al., 1997, Rayner et al., 1989). Whilst CS was not significantly different, impaired DA thresholds were observed in the vitamin A

deficient CF group indicating rod photoreceptor dysfunction in these subjects. Other recent investigations have demonstrated DA function to be normal in subjects with CF (Morkeberg et al., 1995, Ansari et al., 1999), however this study indicates that despite intervention patients are still at risk of xerophthalmia. In particular, this finding highlights the importance of vitamin A supplementation and patient compliance in CF in order to maintain normal visual function.

Certain aspects of visual function, including NVA, CS, and DA, were significantly reduced in the CFRD group. Although some correlations were observed with glycaemic control (HbA1c), these failed to reach significance and are likely to be caused by the fact levels were not recorded at the time of assessment. DA thresholds and CS were particularly reduced in subjects with CFRD and similar findings have been observed in patients with Type 1 and Type 2 diabetes (Amemiya, 1977, Henson and North, 1979, Greenstein et al., 1993, Abraham et al., 1988, Di Leo et al., 1992, Dosso et al., 1996, Ismail and Whitaker, 1998, North et al., 1997, Stavrou and Wood, 2003). Retinal hypoxia, caused by abnormal retinal perfusion and ischaemia, is thought to be responsible (Drasdo et al., 2002, Arden et al., 1998, Kurtenbach et al., 2006). Temporary improvements in DA, CS and CV have been observed following the inhalation of oxygen in diabetic patients (Kurtenbach et al., 2006, Arden et al., 1998, Harris et al., 1996, Dean et al., 1997). In this study, certain measures of CV were negatively correlated with lung function in CF subjects and both CV and DA have previously been shown to deteriorate in experimental hypoxic conditions in healthy subjects (Connolly et al., 2008, Vingrys and Garner, 1987, Karakucuk et al., 2004, Ernest and Krill, 1971, McFarland and Evans, 1939). Therefore, visual function may be reduced in subjects with CFRD due to retinal hypoxia. DA may be particularly affected by CFRD as scotopic conditions are known to further increase the metabolic demands of the rods (Arden et al., 2005).

7.4.3 Retinal photography

In this study, 36% CFRD patients displayed varying degrees of DR. The reported incidence of diabetic retinopathy (DR) in subjects with CFRD is variable ranging from 5 to 24% (Sullivan and Denning, 1989, Andersen et al., 2006). It is plausible that subjects with diagnosed DR were more motivated to participate in the study, hence potentially skewing the incidence. Poor metabolic control, a known risk factor for DR in Type 1 and Type 2 diabetes (Rodriguez-Fontal et al., 2009) was also observed in these subjects. Diabetes duration is a further risk factor for DR development in Type 1 and 2 diabetes (Lind et al., 2007). Mean CFRD duration in those with DR in this study was approximately 13 years. Therefore, this study supports previous findings that the incidence of DR in CFRD is greater in subjects with a diabetes duration greater than 10 years (Andersen et al., 2006, Yung et al., 1998).

The 10 year incidence of DR in insulin treated diabetic patients, diagnosed before 30 years of age, is 89%. In comparison, the incidence of DR in CFRD is considerably lower. Although CFRD shares features with Type 1 and 2 diabetes it is distinctly different (Moran, 2002). It is probable CFRD subjects maintain better metabolic control, and a reduced DR risk, due to the persistence of some insulin secretion (Schwarzenberg et al., 2007). Whilst Schwarzenberg (2007) suggested annual retinal screening should commence after a duration of 5 years (Schwarzenberg et al., 2007), two subjects in this study had mild and moderate background DR before that milestone. Reassuringly, the majority of subjects were receiving annual retinal screening.

7.4.4 Corneal and Lenticular morphological characteristics

Anterior chamber (AC) depth and volume were significantly increased in healthy controls. However, a negative correlation with refractive error and significant differences between myopes and emmetropes indicate the difference is due to the higher levels of myopia and hence increased axial length in the control cohort. A correlation of refractive error and anterior chamber depth have been observed previously (Xu et al., 2008, Rabsilber et al., 2003, Touzeau et al., 2003, Ucakhan et

al., 2008). Incidentally, CFTR is not present in the ciliary epithelium (Mitchell et al., 1998, Coca-Prados et al., 1995) and does not facilitate aqueous humor production (Do and Civan, 2004, McCannel et al., 1992). Therefore, smaller AC depth and volume would not appear to be a primary manifestation of CF due to reduced aqueous secretion.

CFTR, located at the apical corneal endothelial membrane, facilitates fluid efflux to prevent corneal desturgesence (Sun and Bonanno, 2002). In CF, unless other Cl^- channels provide compensation for reduced Cl^- secretion via CFTR the cornea is likely to be thicker with increased density. Whilst Lass and colleagues (1985) observed increased corneal thickness in subjects with CF compared to controls with a contact video specular microscope (Lass et al., 1985), a significant difference was not observed in either central or peripheral corneal locations in this study. Mean central corneal thickness values were comparable to previous studies that utilised the Pentacam (Fujioka et al., 2007, Al-Mezaine et al., 2008, Miranda et al., 2009). Corneal thickness was significantly greater in myopes and negatively correlated with refractive error. A similar finding with the Pentacam has been documented (Ucakhan et al., 2008), although axial length and corneal thickness do not appear to correlate (Shimmyo and Orloff, 2005). As the control group consisted of a greater proportion of myopes any significance could be masked. This highlights the importance of matching subjects according to refractive error in further investigations.

Although not significantly different, corneal density was increased in CF. Significance could be hampered by high levels of variation and the relatively small cohort size. Lass et al. (1985) recorded significantly increased endothelial cell density in subjects with CF compared to healthy controls (Lass et al., 1985). Increased endothelial cell density in CF could be responsible for the slight corneal density differences observed in this study. The findings by Lass et al. (1985) and this present study could indicate a level of compensation or adaptation by the corneal endothelium to CFTR dysfunction in CF leading to sub-clinical differences in corneal thickness and density. An increased cell density could allow for a greater concentration of compensatory transporters (Sun and Bonanno, 2002), such as CLCA and ClC channels which also facilitate Cl^- efflux from the apical membrane (Sun et al., 2003, Davies et al., 2004).

Increased corneal thickness has been observed in Type 1 and Type 2 diabetic subjects previously suggesting hyperglycaemia impairs normal corneal endothelial transport (Su et al., 2008, Lass et al., 1985). However, this was not observed in CFRD subjects in this study, and pachymetry did not correlate with glycaemic control. Similarly, corneal density was not significantly different in subjects with CFRD. A lack of significance could be due to small sub-group size or differences in pathogenesis of diabetes mellitus and CFRD.

Increased levels of lens opacity have been observed in subjects with CF previously (Fama et al., 1998, Castagna et al., 2001, Majure et al., 1989). Similar findings of increased anterior surface and maximum lens density in CF were observed in this study. To date, CFTR has not been detected in the lens epithelium and fluid transport within the lens is widely uncharacterised (Candia, 2004). Therefore, it is debatable if increased lens density is a primary manifestation of CF. Increased levels or abnormal lens density did not appear to impair visual function however. Lens density did not correlate with refractive error in subjects with CF and was similar in myopes and emmetropes, suggesting increased lens density does not induce index myopia in these subjects. In the normal population, steroid use and diabetes are known risk factors for cataract formation (Beneyto et al., 2007, Fledelius and Miyamoto, 1987, Klein et al., 1998, Logstrup et al., 1997, Harding, 1991, Harding et al., 1993) and vitamin A and beta-carotene have been shown to provide protection against some lens opacities (Leske et al., 1991, Leske et al., 1995, Mares-Perlman et al., 2000, Kuzniarz et al., 2001). In this study, increased lens density in CFRD subjects approached significance; with a larger cohort size this could reach significance. Increased lens density also neared significance in the vitamin A sufficient group. This unexpected result could reflect an overlap of the CF subgroups as discussed earlier. Lens density was not significantly different when subjects were grouped according to current steroid use. However, current steroid use does not reflect previous steroid history and the risk to increased lens density may be cumulative.

7.5 Summary

Impaired DVA, NVA, CS, DA and certain measures of CV appear to be a primary manifestation of CF as differences were exaggerated in ΔF508 homozygous subjects. This suggests normal rod and cone photoreceptor function are compromised by abnormal or absent CFTR function in the RPE. However, DVA, NVA, CS and DA were also significantly affected by diabetic status indicating complications from this secondary disease characteristic. DA and certain measures of CV were also reduced in vitamin A deficient CF subjects demonstrating further secondary manifestations. Therefore, it appears vitamin A deficiency has less detrimental impact on normal visual function in these CF subjects compared to CFRD. The cause of increased lens density is less apparent although CFRD appears to be influential.

This investigation demonstrates binocular vision, stereopsis and refractive error are not significantly different in subjects with CF compared to healthy controls. Similarly, AC depth, volume and angle were also not significantly different. In conflict to the hypothesis, corneal thickness and density were not significantly different in CF and this could indicate corneal compensation for defective CFTR function.

DR is a common feature amongst subjects with CFRD and highlights the necessity of regular dilated fundus examination. The incidence of CFRD increases with age (Lanng, 2001). Therefore, the incidence of DR in CFRD is likely to increase in the future due to improving longevity. Similarly, subjects are increasingly susceptible due to persistent low carotenoid levels and further supplementation may be pertinent, especially for subjects with physical signs of early AMD.

It is a concern that only half of the CF cohort had had an eye examination within the previous two years, and this was typically in those requiring visual correction. In order to deliver optimal eye care to subjects with CF, eye care practitioners and CF consultants must be aware of the ocular associations of CF. Likewise patients with CF need to be educated of the importance of regular, thorough eye examinations.

Chapter 8

Discussion

8.1 Discussion

8.1.1 The corneal and conjunctival epithelium in CF

Active trans-epithelial Cl^- transport is known to provide the driving force for subsequent osmotically driven fluid secretion that follows via the apical membrane (Yang et al., 2000). Cl^- conductance plays an essential role in corneal and conjunctival epithelial fluid transport, maintaining normal corneal integrity and contributing to basal tear production (Candia and Zamudio, 1995, Shiue et al., 2000, Yang et al., 2000, Li et al., 2001, Dartt, 2002, Candia, 2004, Dartt, 2004). Therefore, it appears that Cl^- transport has a critical role in the maintenance of the normal tear film (Shi and Candia, 1995). CFTR provides a major pathway for corneal epithelial Cl^- secretion from the ocular surface (Levin and Verkman, 2005). In CF patients, with hypothesised defective Cl^- efflux from the corneal and conjunctival epithelium (unless other Cl^- channels compensate) basal tear production could be abnormal, contributing to the condition of dry eye.

Increased signs of ocular surface hyperaemia, corneal staining and tear ferning were observed in CF subjects compared to healthy controls. Whilst tear film stability was similar in juvenile CF subjects, fluorescein tear break-up time was significantly reduced in adult CF subjects compared to controls. This study provides some support for the hypothesis that dry eye is a primary manifestation of CF although substantial significant evidence is limited. The results could suggest alternative epithelial Cl^- channels provide some level of compensation for defective CFTR function. CLCA2 and a number of ClC channels are known to be present in the corneal and conjunctival epithelium (Itoh et al., 2000, Davies et al., 2004). Although the precise location and function of a number of these channels remain largely unknown, they could provide an alternative route for epithelial Cl^- efflux in CF (Figure 8.1). CLCA2, in particular

may provide compensation for defective CFTR, as studies have shown it to be the most abundant Cl^- channel in the human corneal epithelium (Itoh et al., 2000).

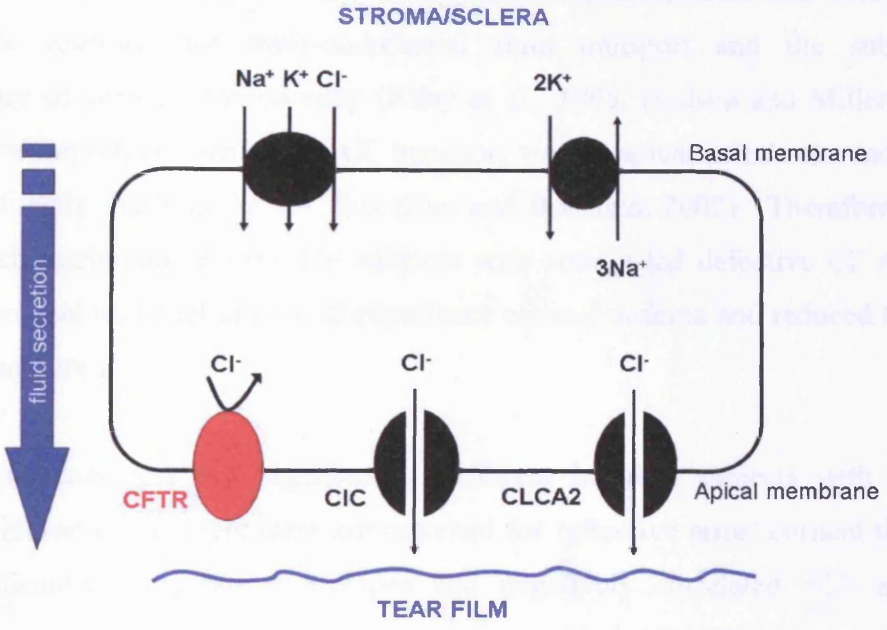


Figure 8.1 Diagram of a simplified corneal/conjunctival epithelial cell in CF with proposed compensation for defective CFTR function by CLCA2 and ClC channels

Cellular levels of compensation could result in sub-clinical levels of dry eye in CF that were undetectable in this study. Certainly, sophisticated biochemical techniques have previously shown increased levels of sub-clinical, ocular surface inflammation in subjects with CF (Mrugacz et al., 2005c, Mrugacz et al., 2006a, Mrugacz et al., 2006b, Mrugacz et al., 2006c, Mrugacz et al., 2007b, Mrugacz et al., 2007c, Mrugacz et al., 2007d). Furthermore, dry eye may only be manifest during periods of exacerbation, poor health or following prolonged disease duration. This theory could explain the lack of significant difference in tear film stability in the relatively "healthy" juvenile cohort. However, the finding that tear film stability was further reduced in adult CF subjects who were vitamin A deficient provides some evidence that vitamin A deficiency is likely to contribute to the pathogenesis of dry eye in CF.

8.1.2 The corneal endothelium in CF

Fluid transport across the corneal endothelium, to counteract corneal swelling is secondary to ionic flux (Hara et al., 1999). It is well established that both Cl^- and HCO_3^- are essential for trans-endothelial fluid transport and the subsequent maintenance of corneal transparency (Riley et al., 1995, Hodson and Miller, 1976). CFTR is an important channel for Cl^- transport via the apical membrane and is also found to directly facilitate HCO_3^- flux (Sun and Bonanno, 2002). Therefore, unless other Cl^- channels compensate, CF subjects with anticipated defective Cl^- secretion from the corneal endothelium could experience corneal oedema and reduced levels of corneal transparency.

Corneal thickness was not significantly different in adult subjects with CF and controls. However, subjects were not matched for refractive error: corneal thickness was significantly increased in myopes and negatively correlated with spherical equivalent refraction. As the control group consisted of a greater proportion of myopes, potential differences may be masked. Corneal density however, was increased in subjects with CF compared to healthy controls, although differences failed to reach significance. Therefore, these findings could indicate a level of compensation within the corneal endothelium to CFTR dysfunction in CF, leading to sub-clinical differences in corneal thickness and density. CLCA and ClC channels are likely compensatory candidates as they are believed to facilitate Cl^- secretion from the apical membrane (Sun et al., 2003, Davies et al., 2004) (Figure 8.2).

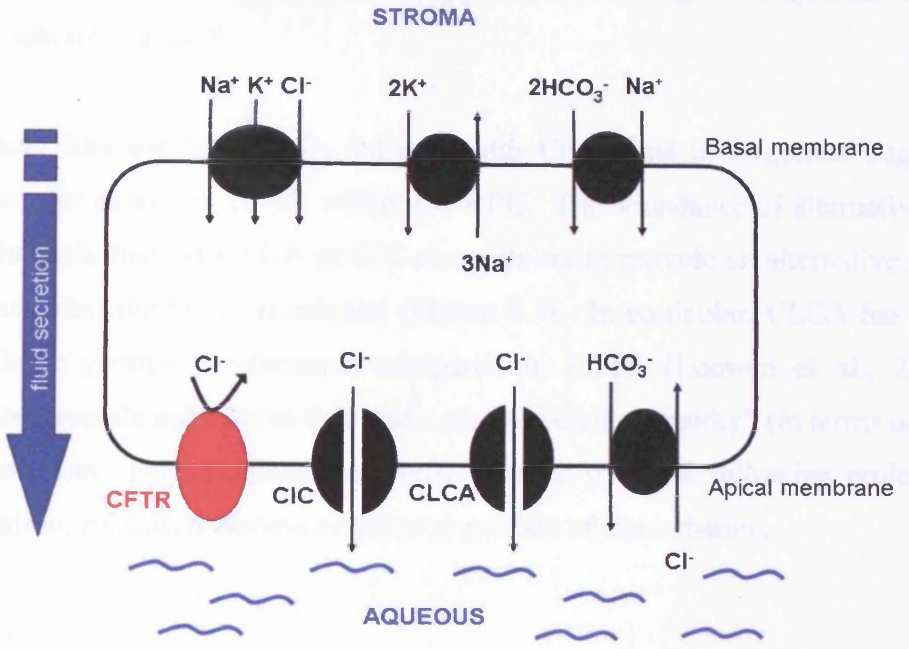


Figure 8.2 Diagram of a simplified corneal endothelial cell in CF with proposed compensation for defective CFTR function by CLCA and ClC channels

8.1.3 The retinal pigment epithelium in CF

The inter-photoreceptor matrix (IPM) within the subretinal space (SRS) is essential for RPE and photoreceptor interaction and facilitates the exchange of nutrients, ions and metabolites (Wimmers et al., 2007) and is thought to assist photoreceptor maintenance (Mieziewska, 1996). Maintenance of the volume and chemical composition of the SRS and IPM is largely achieved by trans-epithelial transport of Cl^-, and the subsequent passive movement of water, via the RPE to the choroid (Marmor and Wolfensberger, 1998). Therefore, Cl^- transport is imperative for the maintenance of normal RPE integrity (Gallemore et al., 1997). CFTR, believed to be located on the basal membrane (Peterson et al., 1997), may in part mediate apical RPE membrane Cl^- secretion (Weng et al., 2002, Wills et al., 2000b). Unless there is compensation from other Cl^- channels in CF subjects, the anticipated defective Cl^- efflux and reduced fluid transport via the RPE could result in impaired regulation of the SRS. Subsequently, this could result in serous retinopathy, retinal detachment or

abnormal RPE-photoreceptor interaction potentially leading to impaired visual function in subjects with CF.

Normal visual function in juvenile subjects with CF in this investigation suggests compensation for defective CFTR within the RPE. The abundance of alternative Cl^- secreting channels such as CLCA or ClC channels could provide an alternative route for Cl^- efflux from the basal membrane (Figure 8.3). In particular, CLCA has been shown to have greater conductance compared to CFTR (Loewen et al., 2003). However, the juvenile subjects in this study are essentially "healthy" (in terms of CF) and differences in visual function may only become manifest following prolonged disease duration, increased disease severity or periods of exacerbation.

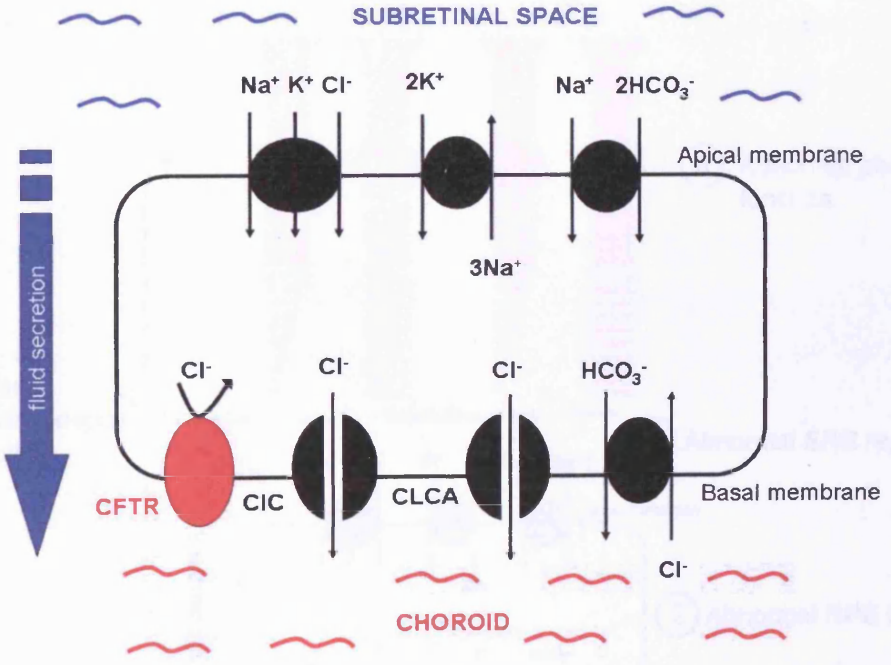


Figure 8.3 Diagram of a simplified retinal pigment epithelial cell in **juvenile** CF subjects with proposed compensation for defective CFTR function by CLCA and ClC channels

Results from adults with CF would seem to support this argument: visual function was demonstrated to be adversely affected in adults with CF compared to healthy controls. Impaired visual function appears to be a primary manifestation as differences were exaggerated in ΔF508 homozygotes with predicted increased levels of CFTR disruption and disease severity. These results suggest proposed compensatory Cl^- secretion mechanisms in juveniles are not sufficient to recompense for abnormal CFTR function in adults. A cumulative effect from prolonged disease duration could be accountable for these cellular differences. Abnormal regulation of the SRS by the RPE appears to compromise normal RPE function or RPE-photoreceptor interaction. Therefore, normal rod and cone function is likely to be impaired with the ultimate consequence of impaired visual function in adults with CF (Figure 8.4). However, visual function was significantly affected by Cystic Fibrosis-related diabetes and vitamin A deficiency. Therefore, the visual function in adults with CF is further modulated by secondary disease characteristics.

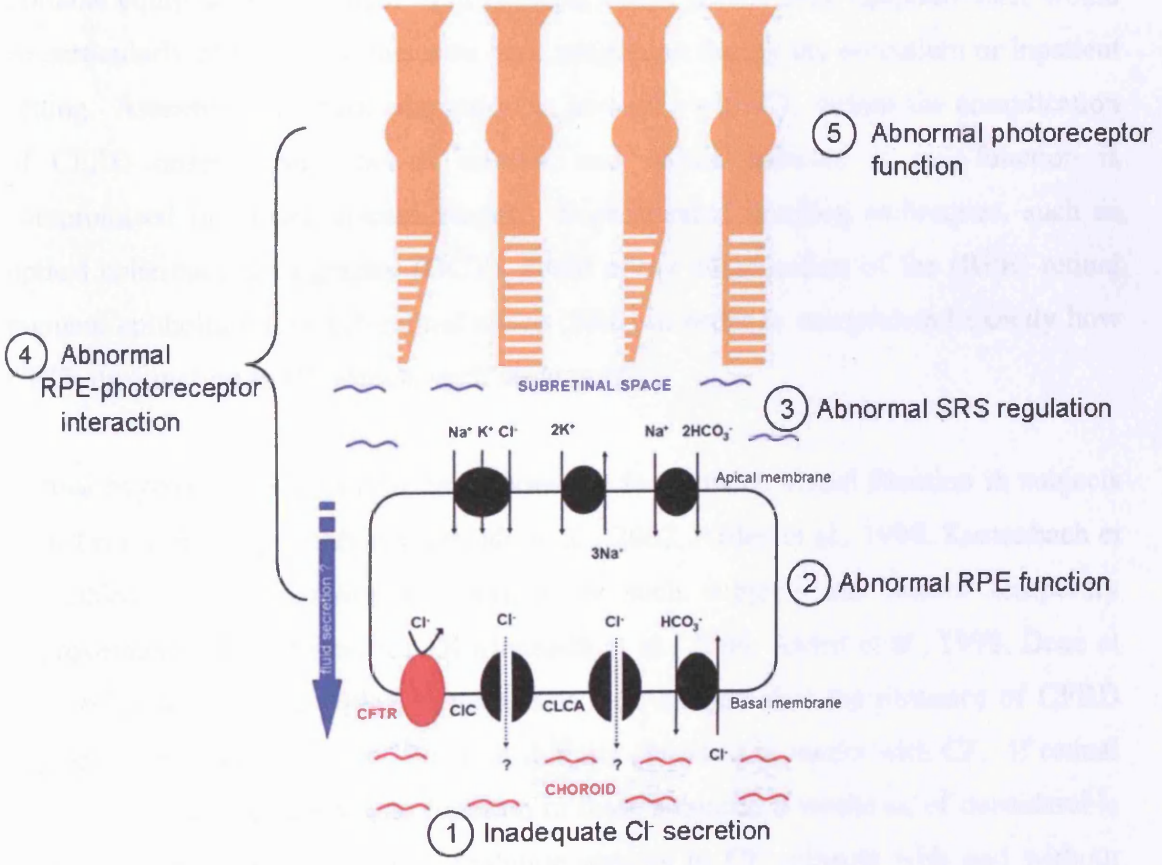


Figure 8.4 Diagram of a simplified retinal pigment epithelial cell in **adult** CF subjects with proposed mechanisms for abnormal visual function in such subjects

8.2 Future work

There is considerable scope for further research of the effect of CF on the ocular surface, visual function and ocular structures.

It will always be of benefit to examine larger numbers of subjects with CF in order to strengthen these novel findings, or to confirm that where no difference has been found, that one does not truly exist. However, recruitment will always remain problematic and multicentre studies should be considered in future. The cohorts recruited in this thesis represent almost 25% of the CF population in South Wales. To maximise future participation, assessment during in-patient admission for intravenous antibiotic therapy would be favourable. Assessment could be co-ordinated to allow direct assessment of serum vitamin A concentration, carotenoid concentration and HbA1c levels to allow for accurate analysis.

Portable equipment, such as a light emitting diode (LED) dark adaptatometer would be particularly of benefit to measure dark adaptation during the outpatient or inpatient setting. Assessment of dark adaptation in juveniles with CF, before the complication of CFRD onset, would be of interest and would indicate if rod function is compromised in earlier disease stages. Sophisticated imaging techniques, such as optical coherence tomography (OCT), could allow visualisation of the (RPE) retinal pigment epithelium and sub-retinal space (SRS) in order to comprehend exactly how CFTR dysfunction in CF affects such structures.

Retinal hypoxia is proposed to be responsible for reduced visual function in subjects with Type 1 and Type diabetes (Drasdo et al., 2002, Arden et al., 1998, Kurtenbach et al., 2006). The inhalation of oxygen by such subjects has shown temporary improvement of visual function (Kurtenbach et al., 2006, Arden et al., 1998, Dean et al., 1997, Harris et al., 1996). The results here suggest that the presence of CFRD appears to enhance the visual function deficits observed in adults with CF. If retinal hypoxia similarly affects visual function in these subjects, it would be of considerable interest to repeat these oxygen inhalation studies in CF subjects with and without CFRD, to further understand how much of the visual deficit is related to hypoxia.

Furthermore, detailed assessment of visual function with the inclusion of Type 1 and Type 2 diabetics for comparison could identify differences in the aetiology of CFRD.

Significant differences in corneal thickness in the myopic and hypermetropic participants highlight the necessity for the comparison of results in refractive error matched CF and control subjects. Furthermore, confocal microscopy could allow for detailed assessment of the corneal endothelium in order to observe structural changes as a consequence of CFTR dysfunction in CF.

Tear film hyperosmolarity is considered the "gold standard" for dry eye diagnosis (Farris, 1994). However, determination of tear film osmolarity requires careful tear sample collection and specialist analytical equipment. OcuSense (San Diego, CA, USA) recently introduced the TearLab®, a handheld portable osmometer which utilises a disposable, microfluidic lab card giving instant tear film osmolarity from a 50 nanolitre tear sample. The TearLab® device would be ideal to assess dry eye in an outpatient setting and the non-invasive nature would allow for significant subject participation.

Reduced mucin concentrations have been observed in CF airway mucus (Henke et al., 2004). Similarly, tear film mucin distribution is altered in subjects with dry eye (Danjo et al., 1998, Berry et al., 2004). Mucin secretion is also thought to be regulated by ocular surface epithelial vitamin A (Tei et al., 2000). Therefore, it would be of interest to investigate tear film and ocular surface mucins in subjects with CF and healthy controls.

CFTR has been suggested as a potential target for dry eye therapy in non-CF subjects to increase tear secretion (Levin et al., 2006, Levin and Verkman, 2005). A number of studies report CFTR agonist activity or increased CFTR expression in animal studies or cell cultures (Al-Nakkash et al., 2008, Schmidt et al., 2008, Levin and Verkman, 2005, Rowe et al., 2007, Wang et al., 2005, Zegarra-Moran et al., 2002, Pedemonte et al., 2005). Therefore, further studies could investigate if such activity can modulate tear film secretion from the ocular surface.

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Appendix

Publications

Publications

Peer reviewed papers

EVANS, K. S., NORTH, R. V., PURSLOW C. (2009) Tear ferning in contact lens wearers. *Ophthalmic Physiol Opt*, 29, 199-204.

Conference presentations

EVANS, K. S., PURSLOW, C., NORTH, R. V., KETCHELL, R. I. (2009) Characteristics of visual function in adults with cystic fibrosis. Poster and Oral Presentation, *Thirty second European Cystic Fibrosis Conference*, Brest, France; 10-13 June.

EVANS, K. S., NORTH, R. V., PURSLOW C. (2008) The relationship between tear ferning, tear film stability and ocular comfort. Poster Presentation, *ARVO Annual Meeting*, Fort Lauderdale, Florida, USA; 27 April-1 May.

EVANS, K. S., NORTH, R. V., PURSLOW C. (2007) An investigation of the relationships between tear ferning, tear film stability and ocular comfort. Poster Presentation, *Fifth International Conference on Tear Film and Ocular Surface: Basic Science and Clinical Relevance*, Taormina, Sicily, Italy; 5-8 September.

Other publications

EVANS, K. S., NORTH, R. V., PURSLOW C. (2007) The ocular surface and tear film characteristics in cystic fibrosis. *Optician*, 233, 16-18.

Nominated for Contribution to CET Award, 2008 Optician Awards.

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Tear ferning in contact lens wearers

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Abstract

Tear ferning (TF) has shown good sensitivity and specificity in the diagnosis of dry eye, but is a relatively uncommon test, especially in contact lens wearers. The aim of this study was to investigate the relationship between TF, ocular comfort and tear film stability amongst contact lens (CL) wearers and non-contact lens (NCL) wearers. Subjects (36 NCL, 24 CL; mean age 23.2 ± 4.8 years) underwent assessment of non-invasive tear break up time (NIBUT), fluorescein tear break up time (FBUT) and completed the Ocular Comfort Index (OCI) questionnaire. Non-stimulated tears were collected from the inferior tear meniscus with a glass capillary. Samples of 1.5 μL were air dried, observed by light microscopy and the TF pattern quantified according to Rolando's grading scale. Significantly higher grades of TF pattern and discomfort (higher OCI scores) were observed in CL wearers compared to NCL wearers (Mann–Whitney *U*-test; $p < 0.005$ and $p < 0.05$ respectively). Differences in tear film stability were not significant between groups. Even when asymptomatic (low OCI scores) CL and NCL subjects were compared, TF remained significantly different ($p < 0.005$). In both CL and NCL subjects, TF displayed poor correlation with tear film stability tests and OCI scores. Higher TF grades in CL wearers, even if asymptomatic, indicate an unfavourable ratio of salt to macromolecule concentration within the tear film of such subjects. The lack of significant difference in TF between symptomatic CL and NCL wearers could suggest similar aetiology (tear film hyperosmolarity) in each cohort. The TF technique demonstrates limited sensitivity and specificity for the prediction of ocular surface comfort in both CL and NCL wearers.

Keywords: contact lens, contact lens related dry eye, dry eye, ocular comfort, tear ferning, tear film stability

Introduction

A number of bodily secretions produce a distinctive ferning pattern when allowed to air dry on a glass microscope slide (Abou-Shabanah and Plotz, 1957). This ferning phenomenon was originally utilised to determine the ovulation period in women by evaluating cervical mucus ferning characteristics (Papanicolau, 1946). However, Tabbara and Okumoto (1982) used conjunctival scrapings to devise a test to aid differential diagnosis of acute forms of conjunctivitis by the presence or absence of tear ferns. Rolando (1984) later devised a qualitative grading scale based on the appearance of four different tear ferning (TF) patterns (Table 1 & Figure 1). Type 1 and 2 patterns are considered normal and Type 3 and 4 abnormal, typically observed in subjects with dry eye (Rolando, 1984). Although a number of other grading scales have been suggested (Norn, 1994; Vaikoussis *et al.*, 1994), Rolando's original scale is highly repeatable (Pensyl and Dillehay, 1998) and remains popular (Srinivasan *et al.*, 2007).

The TF technique has progressed from scrapings of mucus from the inferior fornix with a spatula (Tabbara and Okumoto, 1982) to simple collection of the tear sample from the inferior tear meniscus with a glass capillary tube (Norn, 1988). The sample is pipetted onto a clean microscope slide and allowed to air dry by evaporation (Kogbe *et al.*, 1991). Within a 10 min period of drying, the sample must be observed by phase

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Table 1. Rolando's tear ferning grading scale (Rolando, 1984)

Type		Characteristics
1	Normal tear ferning	Uniform and closely branching arborisation
2		Single, smaller ferns with less branching and empty spaces beginning to appear
3	Abnormal tear ferning	Small ferns with almost no branching and numerous empty spaces
4		Ferns absent and clusters of mucus may be present

Table 2. The sensitivity and specificity of tear ferning within the literature: cut-off values based on Rolando's classification (Type 1 and 2 normal, Type 3 and 4 abnormal) (Rolando, 1984)

Author	Sensitivity (%)	Specificity (%)
Maragou *et al.*, 1996	90 Primary Sjögren's syndrome (SS) 81 Secondary SS	89
Norn, 1994	86 Keratoconjunctivitis sicca	75
Vaikoussis *et al.*, 1994	90 Primary SS 80 Secondary SS	88

contrast light microscopy and quantified accordingly (Golding and Brennan, 1989). Magnification levels used in reported studies range from 10–100X (Norn, 1987; Golding and Brennan, 1989; Ravazzoni *et al.*, 1998; Srinivasan *et al.*, 2007). TF has demonstrated good sensitivity and specificity (Table 2) for the diagnosis of dry eye (Norn, 1994; Vaikoussis *et al.*, 1994; Maragou *et al.*, 1996). The TF technique has also demonstrated impressive sensitivity and specificity (78.95% and 78.35% respectively) in the prediction of successful contact lens tolerance (Ravazzoni *et al.*, 1998). The authors concluded that whilst contact lens (CL) patients with TF Types of 2, 3 and 4 often encountered tolerance problems, those with Type 1 TF patterns displayed good tolerance over a 6-month wearing period.

Symptoms of discomfort and drying are regularly cited as the principal cause of CL wear drop out (Pritchard *et al.*, 1999) and subjective symptoms of drying are reported in much higher frequency compared to objective, clinical signs of dry eye (Begley *et al.*, 1999). In a recent survey, the McMonnies questionnaire identified 43% of hydrogel CL wearers reporting symptoms of CL-related dry eye (Guillon and Maissa, 2005). Despite its apparent value, TF remains a relatively uncommon diagnostic test amongst traditional tests of the tear film. The diagnosis of CL-related dry eye appears to be hindered by the lack of an accurate, reliable tear film test and TF in experienced CL wearers has not been investigated previously.

The aim of this study was to investigate the relationship between TF and tear film stability amongst experienced contact lens wearers and non-contact lens wearers, expressing varying degrees of ocular comfort.

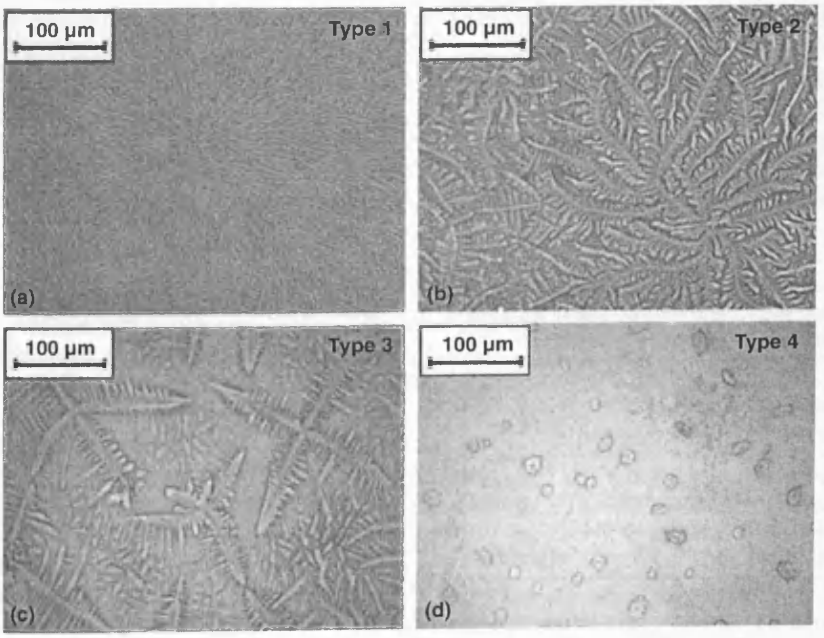

Figure 1. Tear ferning grades for the four ferning 'Types' reproduced according to Rolando's original grading scale (Rolando, 1984).

Methods

Subjects

All measurements were recorded from the right eyes of 60 young, healthy subjects, 17 males and 43 females, mean age 23.2 ± 4.8 years. 36 subjects were non-contact lens (NCL) wearers (11 males, 25 females) and 24 were habitual hydrogel contact lens (CL) wearers (six males, 18 females). CL wearers wore contact lenses for a minimum of 3 days per week for a period of at least 6 months; contact lenses were removed 1 h prior to tear film tests. Subjects were excluded if they had any other ocular or systemic condition known to affect the tear film, including pregnancy, conjunctivitis and blepharitis. Informed consent was obtained from all subjects; favourable approval had been obtained from the Human Science Research Ethics Committee, School of Optometry and Vision Sciences, Cardiff University and all procedures conformed to the tenets of the Declaration of Helsinki.

Procedure

Non-invasive tear break up time (NIBUT), measured with the Tearscope Plus (Keeler, Windsor, Berks, UK), was recorded as the time taken after a blink until disruption of the projected grid mires of the pre-corneal tear film (Guillon, 1998). The median of three readings was recorded for each subject. Using glass capillary tubes, 2 μL non-stimulated tear samples were collected from the inferior tear meniscus and expelled into 1.5 μL eppendorf tubes. 1.5 μL tear samples were pipetted onto clean microscope slides and allowed to air dry at room temperature. Samples were observed by light microscopy (Leica DMRA2; Leica Microsystems, Wetzlar, Germany), photographed within a 10 min period of drying (20 × magnification) and the TF pattern quantified according to 0.25 increments of Rolando's original grading system. Tear ferning grading was performed blind. Subjects then completed the Ocular Comfort Index (OCI) questionnaire. This 12 item questionnaire investigates ocular comfort perceived within the preceding week and has a continuous scale of 'Never (0)-Always (6)' for each question. The OCI was developed using Rasch analysis which is becoming a popular technique for questionnaire design within vision research and permits the conversion of ordinal Likert scores to a continuous interval scale (Court *et al.*, 2007). The OCI has demonstrated excellent correlation with the Ocular Surface Disease Index (OSDI) and FBUT (Johnson and Murphy, 2007). After a recovery period of 10 min following tear collection, fluorescein tear break up time (FBUT) was recorded following instillation of 1.0 μL 2% sodium fluorescein with a micropipette into the inferior fornix (Johnson and Murphy, 2005). FBUT was recorded as the time taken after a blink before disruption of the pre-corneal tear film was observed, and the median of three readings was recorded for each subject.

Statistical analyses

Data were checked for normality (SPSS, Version 14); as all distributions were not normal non-parametric statistical analysis was applied. Mann–Whitney *U*-test was used to compare TF, tear film stability and ocular comfort between CL and NCL wearers. Spearman's rank order correlation was used to investigate the relationship between tear film tests and ocular comfort. Significance was set at the 0.05 level.

Results

The effect of gender was not significant across all tests. Mean TF types, BUT and OCI scores for the two groups are shown in Table 3. Significantly higher grades of TF pattern and ocular discomfort (higher OCI scores) were observed in CL wearers compared to NCL wearers (Mann–Whitney *U*-test, $p < 0.005$ and $p < 0.05$ respectively). However, the difference in tear film stability between the two groups was not significant.

Using Rolando's criteria of abnormal TF above type 2, Figure 2 indicates 14% (five subjects) of NCL wearers and 50% (12 subjects) of CL wearers exhibited abnormal TF. TF of type 3 or above was observed in 8% (two subjects) of CL wearers (Figure 3). The highest TF pattern observed in the NCL subjects was Type 2.75, recorded in a single subject. For all subjects, there was poor correlation between TF and NIBUT ($r = 0.140$), FBUT ($r = -0.053$) and OCI scores ($r = -0.002$). Even when subjects were grouped according to CL status, correlations remained similar.

Subjects within the two groups were identified as asymptomatic or symptomatic for dry eye by the OCI score. Considering asymptomatic subjects (OCI

Table 3. Mean results for tear ferning, non-invasive break up time, fluorescein break up time and Ocular Comfort Index scores for the non-contact lens and contact lens wearers

	NCL wearers mean ± S.D.	CL wearers mean ± S.D.	*p* value (Mann–Whitney *U*-test; 2 tailed)
TF (type)	1.48 ± 0.53	2.02 ± 0.60	0.001*
NIBUT (s)	9.61 ± 2.45	9.28 ± 4.64	0.194
FBUT (s)	7.34 ± 3.78	6.33 ± 2.86	0.369
OCI (score)	31.28 ± 7.31	33.88 ± 6.48	0.044*

NIBUT, non-invasive break up time; TF, tear ferning; OCI, ocular comfort index; FBUT, fluorescein break up time.
*statistically significant at 0.05 level.

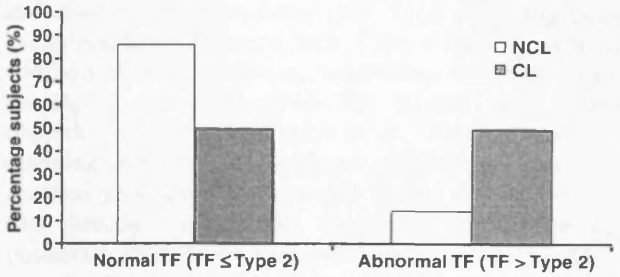

Figure 2. The distribution of normal tear ferning (TF) (≤type 2) and abnormal tear ferning (>type 2) in non-contact lens (NCL) and contact lens (CL) wearers.

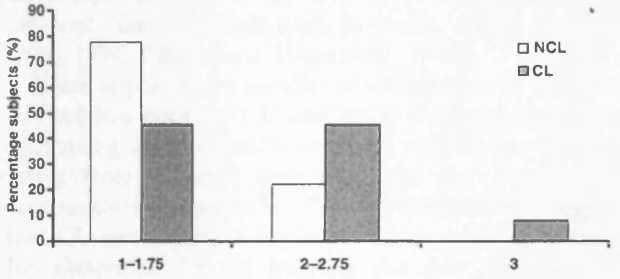

Figure 3. The distribution of tear ferning (TF) types for non-contact lens (NCL) and contact lens (CL) wearers.

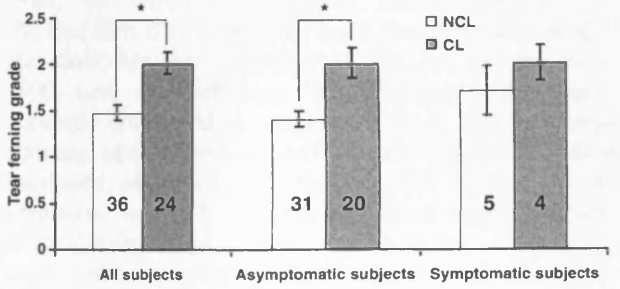

Figure 4. The distribution of TF types for all subjects, asymptomatic subjects (ocular comfort index (OCI) score ≤ 40), and symptomatic subjects (OCI > 40), for the non-contact lens (NCL) and contact lens (CL) wearers (*p < 0.005). Numbers indicate the number of subjects within each subgroup.

score ≤ 40), significantly higher grades of TF patterns were observed in CL wearers compared to NCL wearers (Mann–Whitney U-test, $p < 0.005$). Higher TF Types were also observed in symptomatic CL wearers compared to symptomatic NCL wearers. However, the difference was not statistically significant (Mann–Whitney U-test, $p = 0.374$) (Figure 4). For NCL wearers alone, increased levels of TF were observed in symptomatic subjects compared to asymptomatic subjects with mean ferning grades of 1.719 and 1.411 respectively, although the difference was not statistically significant ($p = 0.371$). Considering CL wearers, similar patterns were observed for symptomatic and asymptomatic subjects, indicating TF in CL wear is independent of ocular comfort.

Table 4. The accuracy of the tear film diagnostic tests (non-invasive break up time, tear ferning, ocular comfort index and fluorescein break up time) for the discrimination of contact wear and non-contact lens wear

Variable	Significance	Area under curve	Standard error	95% Confidence interval
NIBUT	0.194	0.400	0.082	0.239–0.562
TF	0.001	0.748	0.064	0.623–0.874
OCI	0.045	0.654	0.072	0.512–0.796
FBUT	0.369	0.431	0.076	0.282–0.580

NIBUT, non-invasive break up time; TF, tear ferning; OCI, ocular comfort index; FBUT, fluorescein break up time.

Table 5. The accuracy of the tear film diagnostic tests (non-invasive break up time, tear ferning and fluorescein break up time) for the presence of dry eye symptoms based on ocular comfort index score

Variable	Significance	Area under curve	Standard error	95% Confidence interval
NIBUT	0.419	0.585	0.098	0.393–0.777
TF	0.992	0.501	0.105	0.296–0.707
FBUT	0.033	0.276	0.092	0.096–0.455

NIBUT, non-invasive break up time; TF, tear ferning; FBUT, fluorescein break up time.

The TF technique demonstrates superior accuracy for the discrimination of CL and NCL wearers by tear film assessment (Table 4). Applying cut off values according to Rolando's criterion where Type 1 and 2 are considered normal and Type 3 and 4 abnormal (Rolando, 1984), TF demonstrated a sensitivity of 50% and specificity of 86% for the discrimination of tear film samples between CL and NCL wearers, with a positive predictive value (PPV) and negative predictive value (NPV) of 71% and 72% respectively. For the prediction of dry eye symptoms (OCI score > 40) (Table 5), TF displayed sensitivity and specificity of 33% and 73% respectively. The PPV was 18% indicating a large number of false positives however; the NPV was 86% indicating normal TF to be an accurate predictor of good ocular comfort (low OCI score).

Discussion

The exact contribution of the numerous constituents of the tear film components to the ferning phenomenon has been subject to some speculation. The tear ferning pattern was originally proposed to be caused by the interaction of electrolytes, particularly sodium and chloride, with macromolecules such as tear film mucins and proteins (Golding and Brennan, 1989). Further

investigations demonstrated that Type 1 ferning could be progressively changed into Type 4 ferning with the addition of saline solution, suggesting tear film hyperosmolarity was responsible for altered tear ferning observed in dry eye (Kogbe *et al.*, 1991). X-ray and scanning electron microscope (SEM) analysis has revealed proteinaceous material within the tear sample that directly controls the formation of sodium and potassium chloride crystals (Golding *et al.*, 1994). More recently, Pearce and Tomlinson found the presence of macromolecules was limited to the periphery of the tear fern (Pearce and Tomlinson, 2000). However, there is general agreement that abnormal ferning occurs due to unfavourable ratios of salt and macromolecules within the tear film (Golding and Brennan, 1989; Golding *et al.*, 1994; Pearce and Tomlinson, 2000).

There appear to be significant differences in observed TF patterns between CL and NCL wearers, even when comparing asymptomatic subjects, which are likely to result from distinct differences in the biochemical composition of the tears. There is evidence to suggest that CL wear appears to induce 'favourable' conditions for abnormal TF by altering the tear film salt to macromolecule concentration ratios. Tear film hyperosmolarity is proposed to be the initiator of ocular surface damage observed in dry eye (Gilbard *et al.*, 1984, 1987 Gilbard *et al.*, 1988). Indeed, assessment of the tear film osmolarity has been described as the 'gold standard' for the diagnosis of dry eye (Lucca *et al.*, 1990) and the technique has demonstrated superior accuracy compared to single tests such as rose bengal staining and Schirmer's test (Tomlinson *et al.*, 2006). Increased osmolarity of the tear film of CL wearers compared to NCL wearers has been reported previously (Miller *et al.*, 2004), and different mechanisms have been suggested. Reduced corneal sensitivity induced by CL wear (causing a reduction of tear secretion) could subsequently increase tear film osmolarity (Gilbard *et al.*, 1986). Further studies have demonstrated increased tear film thinning and evaporation are likely to be responsible for tear film hyperosmolarity during CL wear (Thai *et al.*, 2002). More recently however, Srinivasan *et al.* (2007) found no correlation between TF and tear film osmolality in a cohort of post-menopausal women.

A reduction of total mucin concentration in the tear film of CL wearers has been observed previously (Yasueda *et al.*, 2005). In particular, MUC5AC is significantly reduced in tear film samples from symptomatic CL wearers (Berry *et al.*, 2007) and altered forms of ocular mucins have been found adhered to the contact lens surface (Berry *et al.*, 2003). This binding could be responsible for the reduction of tear film mucin concentration observed. Hori *et al.* (2006) reported similar tear film mucin concentrations between CL and NCL wearers, although the total tear protein concentration was significantly reduced in CL wearers.

Traditional tear film stability tests are known to relate poorly to patient symptoms (Nichols *et al.*, 2004). TF, like other diagnostic tests, appears to correlate poorly with ocular comfort symptoms. Increased levels of ocular discomfort (higher OCI scores) were observed in CL wearers compared to NCL wearers. Similar findings have been published previously; with almost three times as many CL wearers deemed to be symptomatic and over five times as many reporting moderate to severe discomfort compared to non-lens wearers (Guillon and Maissa, 2005; Chalmers and Begley, 2006). Increased TF grades were observed whenever contact lenses were worn or symptom scores increased, but these differences lacked significance within each cohort (CL and NCL), except where all subjects were essentially comfortable. Such results have several possible interpretations. Successful, asymptomatic CL wear is likely to induce a significant alteration of the tear film salt to macromolecule concentration which is detectable by the TF technique. The lack of significant difference in TF between symptomatic CL and NCL wearers could suggest similar aetiology (tear film hyperosmolarity) in each cohort, or may be related to small sample size.

To summarise, although TF has been shown to be a good predictor of successful CL wear tolerance (Ravazzoni *et al.*, 1998) the technique displayed a poor correlation with traditional tear film tests and ocular comfort in CL and NCL wearers. Significantly increased TF grades were observed in CL wearers compared to NCL wearers even when comparing those asymptomatic patients with good ocular comfort. This small study demonstrates that TF potentially identifies differences between patient groups, but further significant findings may be somewhat restricted by the sample size and the semi-quantitative nature of the grading scale.

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