A Multifaceted Approach to Improving Management of Sight-threatening Non-infectious Uveitis: Patient Perspectives and Disease Phenotyping for Individualised Patient Therapy

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Declaration

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, all material which is not my own has been properly acknowledged.
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Abstract

Non-infectious uveitis is a clinically heterogenous disease, which can cause chronic relapsing and remitting chorioretinal inflammation and vision loss. The disease is incurable but long-term clinical remission of inflammation may be induced with high dose ‘blockbuster’ systemic immunosuppression, which has multiple side effects. Personalised immunosuppressive therapy is currently not feasible, partly due to limitations in disease phenotyping.

The aims of this thesis were to individualise management of patients with sight-threatening non-infectious uveitis by investigating:

1. Clinical phenotypes of two similar subtypes of sight-threatening non-infectious uveitis: multifocal choroiditis (MFC) and punctate inner choroidopathy (PIC).
2. Immunological phenotypes of patients with sight-threatening non-infectious uveitis.
3. Perspectives of patients with sight-threatening retinal disease.

Clinical and imaging data from 343 eyes of 185 subjects with MFC and PIC were found have some significant differences, with PIC showing a greater predilection for young, myopic women and tendency to secondary choroidal neovascularisation. Fifty patients with intermediate uveitis, posterior uveitis and panuveitis and 10 control subjects were prospectively recruited for immunological analysis of blood samples. Patients in clinical remission had significantly higher levels of Treg, polarised towards TIGIT and T-bet, associated with higher serum cytokine levels of IL-10 of TGF-β, and significantly lower methylation levels at key Treg epigenetic sites, compared to active patients and controls. Qualitative data was collected from patients.
with sight-threatening retinal disease and used to design a pilot intervention to increase subjects’ capability and opportunity for healthy lifestyle change.

Results suggest that the phenotyping of non-infectious uveitis could be improved and that immunological biomarkers could assist in disease management. The visual and psychosocial consequences of sight-threatening retinal disease may also be addressed with tailored behavioural interventions. These findings could improve management of patients with uveitis by supporting the transition towards a more individualised therapeutic approach.
Impact Statement

The impact of the research contained within this thesis is pertinent to the development of patient-centred therapies as we enter an era of ‘personalised’ or ‘stratified’ approaches to disease management. Individualised treatment approaches to inflammatory disease are, in theory, favourable to standard ‘blockbuster’ systemic immunosuppression approaches (which have significant associated morbidity). In order to best utilise the increasing range of targeted immunomodulating drugs coming to market, it is important to have methods of disease phenotyping which are supported by scientific evidence and are relevant to outcomes prioritised by patients. The results of the clinical and laboratory studies, outlined in this thesis, contribute to the body of knowledge within this specialist area of sight-threatening non-infectious uveitis.

The results of the lab-based immunology study, in particular, provide evidence that the T-cell surface-expressed coinhibitory molecule, TIGIT, has utility as a marker of functional Treg, and that low levels of TIGIT may be a biomarker for increased risk of disease relapse of sight-threatening non-infectious uveitis. This could help to individualise patient treatment by assisting in determining whether systemic immunosuppressive drugs are inducing functionally suppressive Treg, and by helping to inform clinical decision-making about tapering off immunosuppression. These data are also relevant to the recent interest in quantitative identification and isolation of viable, functional Treg for downstream clinical purposes, which includes their generation \textit{ex vivo} for immunomodulating therapies.

The public engagement and qualitative analysis project presented in this thesis involved engagement with patients who have sight-threatening, progressive retinal disease. The project identified an interventional approach
which has the potential to increase an individual’s sense of agency and self-efficacy in the context of this type of chronic sight-threatening disease; and possibly improve their experience of the disease. This project demonstrated how public engagement might help to guide research, by developing the interface between healthcare/research institutions and their local communities.

A final important impact of this thesis is the example of interdisciplinary thinking applied to a medical problem, in order to facilitate patient-centred solutions. Scientific and medical collaborations with teams specialising in rheumatology and cancer epigenetics, for example, were formed to assist in the design and conduct of this clinical ophthalmology and immunology research. Furthermore, social science theory and methods were explored in addition to conventional biomedical theory and quantitative methods. I believe that the value of drawing upon diverse expertise, in the development of individualised therapeutic approaches for sight-threatening non-infectious uveitis, has been demonstrated in this PhD thesis.
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</thead>
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa Fluor dye</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>APC (cell)</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APC (dye)</td>
<td>allophycocyanin dye</td>
</tr>
<tr>
<td>BUV</td>
<td>brilliant ultra-violet</td>
</tr>
<tr>
<td>BV</td>
<td>brilliant violet</td>
</tr>
<tr>
<td>Cy</td>
<td>cyanine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EAU</td>
<td>experimental autoimmune uveitis</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FoxP3</td>
<td>forkhead box protein</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>IRBP</td>
<td>interphotoreceptor binding protein</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>Th</td>
<td>helper T cell</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Tr1</td>
<td>type 1 T regulatory cells</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>Tresp</td>
<td>responder T cells</td>
</tr>
<tr>
<td>VKH</td>
<td>Vogt-Koyanagi-Harada syndrome</td>
</tr>
<tr>
<td>vol/vol</td>
<td>volume to volume ratio</td>
</tr>
</tbody>
</table>
Chapter 1 Background and Introduction

1.1 Definition of uveitis and anatomy of the uveal tract

Uveitis is a term that refers to inflammation of the uveal tract, which is the highly vascular pigmented middle layer of the three concentric layers that make up the eye (Figure 1). The uvea lies between the sclera (superficial to it) and the retina (deep to it). The uveal tract is composed of the iris and ciliary body anteriorly and the choroid posteriorly (Foster and Vitale 2002).

![Diagram of the eye showing the uveal tract](image)

Figure 1: The uveal tract, consisting of the iris, ciliary body and choroid (highlighted in red) (Hu 2009)

The term ‘uveitis’ may derive from the Latin ùva, meaning grape, possibly because of the deep purple/pigmented appearance which characterises uveal tissue. The term ‘uveitis’ has now become almost synonymous in
ophthalmic clinical practice with any inflammation involving structures inside the eye, including vitreous gel and the retina. When the posterior uvea is inflamed there is a high likelihood of developing sight-threatening ‘retinitis’, defined as follows:

‘Retinitis is an inflammation of the retina, most commonly related either to an infectious or an inflammatory cause. It is a nonspecific sign that accompanies many diverse causes of ocular inflammation and indicates the inflammation is involving the retina, either in isolation or in association with other structures, such as the choroid (chorioretinitis) or the optic nerve (neuroretinitis).’ (Gilbert, et al. 2016a)

Therefore, sight-threatening posterior uveitis may also be referred to as ‘choroiditis’, ‘retinitis’, ‘chorioretinitis’ or ‘neuroretinitis’ and these terms are sometimes used interchangeably.

1.2 Classification of uveitis

Uveitis is a clinically heterogenous disease: at least 150 disorders are known to be associated with intraocular inflammation. Individually, these disorders have a relatively low prevalence. In order to gain understanding of commonalities between subtypes the uveitis and develop uniformity in clinical research, diagnosis and management, the International Uveitis Study Group (IUSG) developed clinical criteria for classifying uveitis (Bloch-Michel and Nussenblatt 1987). In 2004, the Standardization of Uveitis Nomenclature (SUN) workshop analysed the IUSG criteria, found them very useful and added criteria for onset, duration and course of the disease.

The SUN classification, as is it now known, is the most commonly used classification system for uveitis (Jabs, et al. 2005b). It classifies uveitis according to the anatomical location of intraocular inflammation, as follows (with a visual depiction of this shown in Figure 2):
1. Anterior
2. Intermediate
3. Posterior
4. Pan uveitis

The SUN group also recommended consistent terminology to grade intraocular inflammation in the anterior chamber (AC), which presents as floating cells or ‘flare’ in the anatomical area of the AC. This may be graded
on a scale of 0 to 4, according to its severity (Tables 1-2) (Jabs, et al. 2005b). Cells or cloudiness (haze) within the vitreous gel may be graded in a similar way to AC activity, although this is not currently part of the SUN classification (Davis, et al. 2010; Nussenblatt, et al. 1985). The clinical presentation of uveitis will be described in more detail, later in this chapter.

<table>
<thead>
<tr>
<th>Table 1 SUN working group classification of anterior chamber cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0.5+</td>
</tr>
<tr>
<td>1+</td>
</tr>
<tr>
<td>2+</td>
</tr>
<tr>
<td>3+</td>
</tr>
<tr>
<td>4+</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Table 2 SUN working group classification of anterior chamber flare</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1+</td>
</tr>
<tr>
<td>2+</td>
</tr>
<tr>
<td>3+</td>
</tr>
<tr>
<td>4+</td>
</tr>
</tbody>
</table>

Whilst the SUN criteria have been helpful for anatomically sub-classifying uveitis and grading active inflammation in clinical and research contexts, they
are not comprehensive clinical criteria. Therefore, uveitis is often further classified according to the clinical course of the inflammation, that is, into acute or chronic and, according to aetiology, that is, into infectious or non-infectious.

1.3 Epidemiology of uveitis
In the Western world, the current incidences of uveitis vary between 38 and 200 per 100,000 (Sangwan 2010; Williams, et al. 2007). Uveitis can affect people of all ages but occurs most frequently in the working age population (20 to 50 years), with 35% affected remaining visually disabled and resulting in a socio-economic impact comparable to diabetic retinopathy (Suttorp-Schulten and Rothova 1996). A population-based study from the United States (US) reported three-fold increment in the incidence of uveitis, especially in the older age group, compared to the previous studies. The same study reported higher prevalence of uveitis in females compared to males (Gritz and Wong 2004). Anterior uveitis is the most common type of uveitis followed by pan uveitis, posterior uveitis and, finally, intermediate uveitis (Foster, et al. 2016a). Many cases of uveitis are chronic and may lead to numerous sight-threatening complications, which have been historically underestimated due of the paucity of data concerning their incidence.

1.4 Prevalence and causes of vision loss in uveitis
Uveitis is thought to be responsible for around 10-15% of all causes of vision loss worldwide and up to 22% of legal blindness in the UK (Durrani, et al. 2004). Uveitis causes visual loss from direct inflammation, but also substantially from macular oedema, epiretinal membrane, cataract (opacity of the crystalline lens), glaucoma (optic neuropathy with characteristic visual field defect), choroidal neovascular membrane (CNV) and retinal
detachment. A recent study from Brazil found that uveitis was the second most common cause of vision impairment (15.7%) (Silva, et al. 2013) and, in Singapore, 7.5% of uveitis patients had severe vision loss, most commonly from cataract and glaucoma (Yeo, et al. 2013). In a recently published study of the causes of visual impairment in England and Wales, ‘uveitis’ did not feature as a distinct category (Quartilho, et al. 2016). The current UK Certificate of Vision Impairment (CVI) registration form, which formally certifies someone as visually impaired and functions as a record of data about visual impairment, contains only ‘chorioretinitis (unspecified) H30.9’, as a specific uveitis category. It has been noted that in addition to the 0.43% of patients in the UK recorded in this study with chorioretinitis (Quartilho, et al. 2016), “many other patients with visual loss from uveitis are ‘hiding in plain sight’ within ‘secondary glaucoma’, ‘cataract’, ‘other retinal disorders’ and other categories” (Jones 2016). It was also observed that in a recent large study from a tertiary centre in Manchester, only 671 of 3000 uveitis patients (21%) could be labelled as having chorioretinitis, if severely affected (Jones 2015; Jones 2016).

‘At a time when great advances in the control of uveitis by immunosuppression and biologic therapy are being thwarted by funding restrictions, it would mean a disservice to affected patients if their disease cannot be adequately represented in vision impairment statistics.’ (Jones 2016).

Another study of uveitis patients attending a specialist clinic at Moorfields Eye Hospital indicates that vision loss was noted in 19.2% of eyes with uveitis, with chronic cystoid macular oedema being the most common cause of ‘moderate’ vision loss (3.55%) and macular scarring, the most common cause for irreversible ‘severe’ visual loss (4%) (Table 3) (Tomkins-Netzer, et al. 2014). Other causes of vision loss from uveitis include retinal vasculitis with or without ischaemia, retinopathy, hypotony (an eye with intraocular
pressure (IOP) of 5 mm Hg or less) and phthisis bulbi (an atrophic, non-functional eye as a result of chronic inflammation) (Daniel, et al. 2012; Foster, et al. 2016a)

Table 3 Causes of vision loss among all patients with uveitis attending a specialist at a tertiary centre in London (moderate vision loss corresponded to a best corrected Snellen visual acuity (BCVA) of 20/50 to 20/120 and severe vision loss to a BCVA of 20/200) (Tomkins-Netzer et al., 2014).

<table>
<thead>
<tr>
<th>Causes</th>
<th>Moderate Vision Loss</th>
<th>Severe Vision Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic CME</td>
<td>64 (3.55)</td>
<td>21 (1.16)</td>
</tr>
<tr>
<td>Macular scarring</td>
<td>36 (2.00)</td>
<td>72 (4.00)</td>
</tr>
<tr>
<td>ERM</td>
<td>25 (1.39)</td>
<td>2 (0.11)</td>
</tr>
<tr>
<td>Glaucoma</td>
<td>8 (0.44)</td>
<td>8 (0.44)</td>
</tr>
<tr>
<td>Ocular media opacities</td>
<td>8 (0.44)</td>
<td>2 (0.11)</td>
</tr>
<tr>
<td>Optic neuropathy</td>
<td>8 (0.44)</td>
<td>14 (0.78)</td>
</tr>
<tr>
<td>Retinal detachment</td>
<td>6 (0.33)</td>
<td>24 (1.33)</td>
</tr>
<tr>
<td>Macular ischemia</td>
<td>2 (0.11)</td>
<td>9 (0.5)</td>
</tr>
<tr>
<td>Hypotony &amp; phthisis</td>
<td>1 (0.05)</td>
<td>12 (0.67)</td>
</tr>
<tr>
<td>Total</td>
<td>158 (8.8)</td>
<td>164 (9.1)</td>
</tr>
</tbody>
</table>

CME = cystoid macular edema; ERM = epiretinal membrane.
Data are number of eyes (% of all cases).

1.5 Visual impairment, disability and stereotypes
Eye disease may lead to visual impairment and/or disability. Reduced visual acuity and reduced visual contrast sensitivity, in particular, have been found to be independently associated with self-reported difficulty with a wide variety of everyday visual activities and an increase in overall vision disability score (RUBIN, et al. 1994). The attitudes of sighted persons, which includes the majority of practising clinicians, are thought to have a major influence on the successful adjustment of severely sight-impaired individuals to disability (Allen and Bellstedt 1996). The General Medical Council states that its standards and guidance about disability are informed by the social model, that is, ‘the problem of disability lies with society, not with the disabled person’, as opposed to the medical model where ‘the impairment is seen as the problem’ (GMC 2017). However, there is still a paucity of opportunity
within medical training to learn about the psycho-social aspects of disability, and in particular, the consequences of visual impairment. This may lead to deficiencies in provision of health-care and adequate support for patients with eye disease.

The Stereotype Content Model (Fiske, et al. 2002) describes how universal dimensions of social cognition, namely, perceptions of others’ ‘warmth and competence’ can cause stereotyping (widely held but fixed and oversimplified images or ideas) of individuals and influence behaviour towards these ‘other’ individuals:

‘People perceived as warm and competent elicit uniformly positive emotions and behavior, whereas those perceived as lacking warmth and competence elicit uniform negativity. People classified as high on one dimension and low on the other elicit predictable, ambivalent affective and behavioral reactions. These universal dimensions explain both interpersonal and intergroup social cognition.’

(Fiske, et al. 2007)

Thus, patients with visual impairment might be perceived by clinicians as ‘well-intentioned and warm but incompetent’, particularly if they are in the younger or older age groups:

‘Groups seen as well-intentioned and warm, but incompetent, include older people and younger people in Canada and elsewhere, and in places where they are salient, also people with disabilities. They evoke pity and sympathy, as well as active help and protection, but also passive neglect and demeaning. Pity itself is a top-down, paternalistic response, and this mix of active help and passive harm reflects it. These ambivalent responses would seem a chilling description of institutionalizing people to ensure their physical care but isolating them from the rest of society.’

(Fiske 2012)
Furthermore, research on ‘stereotype threat’ which refers to ‘the risk of confirming, as self-characteristic, a negative stereotype about one's group’ (Steele and Aronson 1995), suggests that members of a stigmatised group, such older people with a disability, are more likely to act in stereotypical ways when their ‘identity’ is made salient (St Claire 2009). Therefore, unconscious bias generated through negative stereotypes of individuals with visual impairment could be an important influence on clinicians’ behaviour towards these groups, which has relevance to the quality of the doctor-patient relationship and the provision of patient-centred care. This problem could be addressed through ‘interventions aimed at increasing the relative salience to doctors of the human worth of individuals’, which have been shown to assist in increasing unconditional respect towards individuals under clinical care (Clucas and Claire 2017).

1.6 Aetiology of uveitis
The various forms of uveitis represent a common manifestation of multiple underlying causes of ocular inflammation:

- Inflammatory/Idiopathic (when evaluation has failed to find an underlying cause for inflammation)
- Infectious (including opportunistic infection caused by immunosuppression)
- Infiltrative (invasive neoplastic processes)
- Trauma
- Iatrogenic
- Inherited (secondary to metabolic or dystrophic disease)
- Ischaemic
Infectious agents include bacteria, viruses, fungi and protozoa and examples of infectious pathogens well known to cause uveitis include:

- Toxoplasma
- Tuberculosis
- Toxocara
- Herpes viruses – Simplex, Zoster and Cytomegalovirus (CMV)
- Candida
- Bartonella

Newer pathogens being implicated in the aetiology of infectious uveitis, include the Zika (Furtado, et al. 2016) and Ebola (Shantha, et al. 2016) viruses. However, during the past century, the aetiology of inflammation has swung from infective to non-infectious inflammation and in the majority of cases of uveitis, the aetiology of inflammation is non-infectious and idiopathic. The inflammatory process can be apparently localized to the eye or be part of a systemic inflammatory disease.

Important examples of systemic non-infectious inflammatory disease associations with uveitis include:

- Sarcoidosis
- Multiple sclerosis
- Vogt-Koyanagi-Harada Syndrome
- Behcet syndrome
- Spondylarthroses with and without HLA B27⁺ antigen

In addition, there are two specific childhood systemic disease associations with uveitis:
• Juvenile idiopathic arthritis
• Juvenile HLA B27+ arthritis

The aetiology of sight-threatening retinitis may be described, as follows:

‘Retinitis has inflammatory and infectious causes, which are either parasites, bacteria, viruses, fungi. The most common cause of infectious retinitis is related to Toxoplasma gondii which is an obligate intracellular protozoan parasite. Ocular toxoplasmosis may be congenital due to transplacental transmission of tachyzoites or acquired following consumption of raw or undercooked meat containing cysts or by ingestion of fruits, vegetables, or water contaminated by cat faeces rich in oocysts. Other parasites which can cause retinitis include toxocariasis, onchocerciasis, and diffuse unilateral subacute neuroretinitis can be caused by several different parasites. Cytomegalovirus (CMV) retinitis is seen predominantly in immunocompromised patients and especially those with acquired immunodeficiency syndrome (AIDS). It may also occur following organ transplantation in patients receiving systemic immunosuppression or following chemotherapy for malignant disease. Following primary CMV infection, which is usually asymptomatic in immunocompetent people, the virus remains latent and only reactivates when the immune system is suppressed. Acute retinal necrosis (ARN) is caused by herpes simplex or varicella zoster virus (VZV) infections. In immunocompetent patients, these viruses result in a progressive peripheral necrotizing retinitis. In immunocompromised patients, infections can cause a rapidly progressive disease, progressive outer retinal necrosis (PORN), which can involve the posterior pole and has a very poor visual prognosis. Noninfectious retinitis can be related to systemic diseases such as Behçet disease and sarcoidosis’ (Gilbert, et al. 2016a), reproduced with permission.
1.7 The ‘white dot syndromes’

The ‘white dot syndromes’ are a heterogeneous subgroup of posterior uveitides, so called because they clinically manifest as multiple whitish-yellow inflammatory lesions at the level of the outer retina, retinal pigment epithelium and choroid. To date, no specific underlying cause (infectious, autoimmune or otherwise) has been identified for these inflammatory chorioretinal disorders and they are, therefore, defined by their clinical presentation and idiopathic aetiology. These white dot syndromes typically affect relatively young patients, who are often ‘healthy’ in the sense that they do not have any known or identifiable chronic systemic inflammatory disease.

Multifocal choroiditis and punctate inner choroidopathy are specific idiopathic non-infectious uveitic diseases within the ‘white dot syndromes’ category. Along with other white dot syndromes, they are considered part of the ‘AZOOR complex’, which Gass and others (Gass 1993; Gass, et al. 2002; Gass and Wilkinson 1972; Jampol and Wiredu 1995; Mrejen, et al. 2014; Taira, et al. 2006) have speculated are aetiologically and pathogenically related. MFC was initially described in patients who had ‘punched-out’ fundal lesions similar to presumed ocular histoplasmosis syndrome (POHS), a form of multifocal choroidal inflammation in Histoplasma-endemic areas of the United States (Nozik and Dorsch 1973). POHS tends to affect men and women equally, is not significantly associated with myopia, presents a little later in life, and is rarely associated with clinically evident recurrent episodes of choroiditis (Dreyer and Gass 1984; Gass and Wilkinson 1972). The original phenotypic description of MFC was later expanded to include a variant, MFC with panuveitis, which included recurrent episodes of anterior chamber and vitreous inflammation associated with active chorioretinal lesions, ranging from 50µm to 350µm in diameter (Dreyer and Gass 1984; Morgan and Schatz 1986; Nozik and Dorsch 1973). Around the same time,
PIC was first described in a series of 10 young, myopic women (aged 21 to 37 years), whose multifocal choroidal lesions, ranging from 100µm to 300µm in diameter, evolved into atrophic scars, also similar in appearance to POHS, although 7 out of the 10 women who underwent skin testing for histoplasmosis were negative (Watzke, et al. 1984). Since no anterior chamber or vitreous inflammation was clinically detected in these young women, the term PIC was used to highlight the lack of evident intraocular inflammation associated with the choroidal lesions. Many clinicians believe that the ‘AZOOR complex’ diseases, and especially, MFC and PIC, represent a spectrum of the same disease and that there is no clinical utility in differentiating between them (Essex, et al. 2010; Essex, et al. 2013; Fung, et al. 2014; Morgan and Schatz 1986; Spaide, et al. 2013)

1.8 Overview of the Immune System

1.8.1 Innate and adaptive immunity

Immune responses are typically classified as innate or acquired, which represent non-specific or specific responses, respectively, to foreign macromolecules (antigens). Innate immunity is characterised by physical barriers, fixed receptors based on pathogen molecular patterns, limited immunological memory and the fact that it does not require immunisation (priming) (Rich 2013). Acquired immunity is characterised by clonally variable receptors based on gene rearrangement, development of immunological memory, B-cell and/or T-cell activation, cytotoxic T-cells and antibody production (Rich 2013). Common features of both systems include: cytokines and chemokines, the complement cascade, phagocytic cells, NK cells and ‘natural’ autoantibodies (Rich 2013)
1.8.2 Cells of the immune system

Pluripotent stem cells in the bone marrow give rise to immune cells from myeloid and lymphoid lineages (Figure 3):

- Myeloid lineage:
  - Granulocytes, erythrocytes, monocytes-dendritic cells and megakaryocytes

- Lymphoid lineage:
  - B-lymphocytes, T-lymphocytes and NK cells

Figure 3: Origins of myeloid and lymphoid cells lineages of the immune system
1.8.3 Tissues of the immune system
The tissues of the lymphoid immune system (Figure 4) consist of ‘central’
locations of primary lymphoid organs, where immune cell production and
differentiation occur, and ‘peripheral’ secondary lymphoid organs where
immune cells encounter and respond to antigens. B-cells and T-cells develop
in the bone marrow and thymus respectively (Lewis 2013). The cells of the
immune system come into contact with foreign antigen primarily at the
mucosal surfaces and the skin, where they first interact with antigen-
presenting cells (APC) (Lewis 2013). Immune reactions subsequently occur
in the secondary lymph organs, which include the spleen, lymph nodes,
tonsils and Peyer's patches of the bowel (Figure 4) (Lewis 2013).
1.8.4 Inflammation and the immune system

The immune system primarily evolved to protect the host against infectious pathogens and other ‘invasive’ processes. The purpose of inflammation is to localize and remove the presumed cause of damage, that is, the invading pathogen. Specialized immune cells and proteins exist in the bloodstream to mediate this inflammatory response. Host infection by pathogens, which include bacteria, fungi, viruses or parasites, activates the immune system and the inflammatory response. Bacteria products, especially endotoxin or LPS, have an ‘adjuvant’ effect on the innate immune response, which effectively activates an acute inflammatory response. Acute inflammation lasts for a short period during which immune cells, such as leukocytes,
accumulate at the site and remove the pathogen. Following this, a resolution phase should occur, during which there is repair of the surrounding tissue (Serhan, et al. 2007). Chronic inflammation, on the other hand, ‘refers to a prolonged inflammatory response that involves a progressive change in the type of cells present at a site of inflammation. It is characterised by the simultaneous destruction and repair of tissue from the inflammatory process. It can follow an acute form of inflammation or be a prolonged low-grade form’ (Nature 2018). Infection is a putative predisposing factor to some forms of chronic inflammation. The exact mechanisms by which infection might progress to chronic non-infectious inflammatory disease are not fully understood, but various mechanisms have been proposed (Fairweather, et al. 2001), which include:

1. Molecular ‘antigen’ mimicry
2. Epitope spreading
3. Bystander activation
4. Processing and presentation of cryptic antigens

A general trend has been observed in the evolution of human infectious disease, which has implications for the prevalence of chronic inflammatory disease:

‘The growing success of experimental treatment of auto-immune conditions with live helminths is giving weight to the controversial ‘hygiene hypothesis’, which posits that hosts exposed to too few infections become prone to immune-mediated diseases...It may indeed prove true that some non-zero burden of both germs and worms is optimal for human health.’ (Restif and Graham 2015)
1.8.5 Lymphocytes
Lymphocytes represent about 20-30% of the leukocytes in circulation and are a central cell type of the adaptive immune system (Lewis 2013). The three types of lymphocyte exist: T-lymphocytes (T-cells), B-lymphocytes (B-cells) and NK cells, which circulate in the peripheral blood, constituting approximately 80, 10 and 10% of the total lymphocyte population respectively (Lewis 2013).

1.8.5.1 T-cell development and natural Treg
T-cells develop in the thymus, a primary lymphoid organ, consisting of three major regions: the cortex, the medulla, and the corticomedullary region. T-cells arise from CD34+ lymphoid progenitor cells in the bone marrow. These 'double negative' progenitor cells, lacking expression of CD4 and CD8 surface antigen, migrate in waves to the thymus during embryogenesis and enter the thymus at the corticomedullary junction (Swainson 2013)(Figure 5). Subsequent to migration, the immature T-cells, termed thymocytes, are mainly located in the cortex of thymus. Transcription of relevant genes and commitment of thymocytes to the T cell lineage is mediated by a Notch1 signal. In order to reach the mature T-cell stage, successful rearrangement of the pair of genes encoding a heterodimeric T-cell receptor (TCR) (αβ or γδ) must occur, cells must survive thymic selection and undergo lineage commitment (Swainson 2013). As thymocytes mature, they migrate to the thymic medulla in a chemokine-dependent manner (Swainson 2013).

Notch1 signaling is critical for driving developing thymocytes through the first β-selection point (Swainson 2013). Positive selection results in the survival of thymocytes which have a surface TCR that interacts with self-peptides complexed with Major Histocompatibility Complex (MHC) antigens (Swainson 2013) (Figure 5). Negative selection results in the deletion of thymocytes with high affinity TCRs to self-antigen, so called ‘central
tolerance’, which prevents the development of autoreactive T-cells (Swainson 2013) (Figure 5). It is thought that only 3% of thymocytes survive the thymic selection process. Lineage choice results from interaction of thymocytes with MHC Class I and II- presented antigens. The αβ thymocytes undergo maturation and development stages, in which both CD4 and CD8 are expressed (double positive thymocytes), which further differentiate to cells expressing CD4 (‘helper’ T-cells) or CD8 (‘cytotoxic’ T-cells) but not both (Lewis 2013). Thymus-derived CD4+FoxP3+ ‘natural’ regulatory T-cells (nTreg) regulate tolerance in the periphery, so called ‘peripheral tolerance’, by controlling self-reactive T-cells that escaped deletion in the thymus during the negative selection process (Swainson 2013) (Figure 5).
1.8.5.2 Autoimmunity and T-cell subsets

Human autoimmune disease is thought to occur secondary to imbalances between effector and regulatory immune responses, where the fundamental underlying mechanism is defective elimination and/or control of self-reactive
lymphocytes (Rosenblum, et al. 2015). Autoimmunity typically develops through stages of initiation and propagation, and often shows phases of resolution (indicated by clinical remissions) and exacerbations (indicated by symptomatic flares) (Rosenblum, et al. 2015). It is, therefore, useful to examine the development of autoimmunity through these three phases: initiation, propagation and resolution (Figure 6), which are preceded by a susceptibility phase (Cottrell 2013). Mechanisms underlying susceptibility to autoimmune disease include:

- Incomplete induction of tolerance in the thymus to peripherally expressed auto-antigens
- Impaired clearance and tolerance induction by apoptotic cells
- Defective production of Treg
- Altered immune signalling thresholds

(Cottrell 2013)

Figure 6: Phases of human autoimmunity (Rosenblum, et al. 2015). ‘Teff’ are T-effector cells and ‘T-reg’ are T-regulatory cells.

Autoreactive effector CD4+ T cells have been associated with the pathogenesis of autoimmune disorders (Damsker, et al. 2010). T-helper
(Th)1 and Th17 subsets, characterised by expression of T-bet (Tbx21) and retinoic-acid related orphan receptor (ROR) γ-t (Rorc) transcription factors and secretion of the ‘signature’ pro-inflammatory cytokines, interferon (IFN)-γ and IL-17, respectively, are thought to be causal agents in the pathogenesis of autoimmunity (Caspi 2008; Damsker, et al. 2010) (Figure 7). Th17 effector cells may be induced in parallel to Th1, and, like Th1, polarized Th17 cells have the capacity to cause inflammation and autoimmune disease (Damsker, et al. 2010). The Th2 subset, characterised by the transcription factor, GATA-3, and production of cytokines IL-4, IL-5, and IL-13, was first described at the same time as the Th1 subset (Mosmann and Coffman 1989), and before the Th17 subset was discovered (Figure 7). Although both Th1 and Th2 have important roles in host defence (with Th2 being particularly important in allergic responses and the clearance of extracellular pathogens), only the Th1 subset has been widely implicated in autoimmunity (Damsker, et al. 2010; Mosmann and Coffman 1989). Phenotypically categorised CD4⁺CD25⁺FoxP3⁺ T-regulatory (Treg) exist as a naturally occurring mechanism to suppress autoreactive T-cells through production of cytokines such as IL-10 and transforming growth factor β (TGF-β), in addition to other mechanisms (Agarwal and Caspi 2004; Caspi 2008; Caspi 2011; Caspi 2014; Zheng, et al. 2004) (Figure 7).
Figure 7: Antigen-activated naive CD4⁺ T cells adopt T-helper (Th) and T-regulatory (Treg) phenotypes as directed by cytokine cues and transcriptional factors. Professional illustration by Kenneth X. Probst. (Teshima 2011)
Therefore, it is thought that some forms of autoimmune disease may occur as a result of imbalances between pro-inflammatory Th1 and Th17 effectors and anti-inflammatory Treg (Figure 8).

![Figure 8: In immune tolerance there is a balance between T-effectors and Tregs, whereas in autoimmunity, there is an imbalance, which could be due to inadequate numbers of Treg, defects in Treg function or phenotype or reduced susceptibility of T-effectors to Treg-mediated suppression (Chavele and Ehrenstein 2011).](image)

When discussing chronic inflammation, immunologists make a distinction between 'autoinflammatory' and 'autoimmune' disease, which refers to the specific effectors of tissue damage in each case. In autoinflammation, the innate immune system directly causes tissue inflammation, whereas in autoimmunity the innate immune system activates the adaptive immune system, which then mediates the inflammatory process (Figure 9) (Doria, et al. 2012).
Figure 9: Schematic pathway of pathogenesis of autoimmune and autoinflammatory diseases – adapted from “Autoinflammation and autoimmunity: bridging the divide” (Doria, et al. 2012).

Animal models suggest that the inflammation in non-infectious uveitis is mediated by the adaptive immune system and, specifically, the Th1 and Th17 subsets of T-cells (Caspi 2010; Caspi 2011; Caspi 2014; Damsker, et al. 2010). Although the specific ‘self-antigens’ activating the immune system in the human forms of uveitis have not yet been determined, the immune-mediated chronic inflammation in human uveitis is thought to be of autoimmune aetiology.

1.8.5.3 Epigenetic regulation of T-cells
The word ‘epigenetic’ means ‘around changes in genetic sequence’ and the term ‘has evolved to include any process that alters gene activity without changing the DNA sequence, and leads to modifications that can be transmitted to daughter cells (although experiments show that some epigenetic changes can be reversed)’ (Weinhold 2006).
The eukaryotic nuclear genome is linear, conforming to the Watson-Crick double-helix structural model, and embedded in nucleosomes, which are complex DNA-protein structures which pack together to form chromosomes (Pray, 2008) (Figure 10). Processes such as transcription and replication require free access to the DNA template (Bhat, et al. 2017). Chromatin is substantially modulated by epigenetic modifications, notably DNA and histone modifications (Bhat, et al. 2017). Nucleosomes, which consist of
DNA and a heterodimer of histones (H1, H2, H3 and H4), can act as a barrier to DNA-binding proteins (Bhat, et al. 2017). Nucleosome remodelling involves changes in DNA–protein interactions by disrupting, disassembling or moving nucleosomes and thus allowing DNA to get accessible for further processes (Bhat, et al. 2017). Consequently, chromatin accessibility and nucleosome remodelling are highly important for gene expression.

Epigenetic mechanisms are associated with gene expression and ultimately protein expression (Bhat, et al. 2017). Types of epigenetic processes include methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation (Weinhold 2006). However, the most studied epigenetic process is DNA methylation, which is the addition or removal of a methyl group (CH$_3$), predominantly where cytosine bases occur consecutively (Weinhold 2006).

Within the immune system, epigenetic control is a well-established means of gene regulation. Epigenetic mechanisms are essential during haematopoiesis to establish a specific transcription profile for each gene, which in turn may influence the cell phenotype. Furthermore, in response to certain environmental stimuli, such as viral infection, fully differentiated cells have the capacity to modify their gene expression to adapt to new conditions. Differentiation of naïve CD4+ cells into T-cell subsets such as effector Th1/Th17 or induced Tregs (iTregs) may depend on epigenetic regulation in response to changing environmental conditions, in addition to determination by cell fate programmes (Murphy and Stockinger 2010; Wilson, et al. 2009). This suggests that inflammatory environments may induce T-cell plasticity, in which phenotypically unstable peripherally induced CD4*CD25*FoxP3* Treg subsets (iTreg or pTreg) may co-exist with the more stable naturally occurring CD4*CD25*FoxP3* Tregs (nTregs).

The addition of a methyl group (-CH3) to DNA is a common epigenetic mechanism that cells use to switch genes ‘off’ and extensive methylation of cytosine in DNA is known to correlate with reduced gene transcription.
Furthermore, it has been observed that epigenetic mechanisms play a crucial role in controlling the expression of FoxP3 locus. Studies have revealed that both nTregs and pTreg cells possess a specific DNA hypomethylation pattern, the Treg-specific demethylated region (TSDR), within the FOXP3 gene, which is associated with enhancement and stabilization of FoxP3 expression (Floess, et al. 2007; Huehn, et al. 2009; Polansky, et al. 2008; Zheng, et al. 2010). DNA methylation, in general, is thought to play an essential role in T-cell function and failure to maintain DNA methylation patterns in mature T-cells has been implicated in the development of autoimmune disease (Richardson 2003).

1.9 Ocular immune privilege

Immune privilege was first described by Sir Peter Medawar, who observed that skin homografts survived for prolonged periods when grafted into the anterior chamber of the eye (or brain) (Medawar 1956). He attributed this phenomenon to the absence of vascular supply, which was in keeping with the historical observation that human cornea, an avascular tissue, could be grafted without rejection (Zirm 1989). Medawar was awarded a Nobel Prize for these discoveries (Medawar 1991).

Ocular immune privilege has been subsequently studied in more detail and is described as:

‘A complex phenomenon that involves multiple components, starting with sequestration behind an efficient blood–retina barrier, through active local inhibition by soluble and surface-bound molecules that actively inhibit activation and function of adaptive and innate immune cells, and culminating in systemic regulation via induction of T regulatory cells.’ (Stein-Streilein and Streilein 2002).
Key features of the eye pertinent to maintenance of immune privilege may be summarised as follows (Streilein 1995; Streilein and Cousins 1990):

- the anatomical integrity of the blood-ocular barrier
- the (virtual) absence of lymphatics
- an afferent drainage pathway that is almost exclusively via the blood vasculature
- an immunosuppressive intraocular microenvironment, including immunomodulatory cytokines, for example, TGF-β in the aqueous humour, and anterior chamber-associated immune deviation (ACAID)

Immune privilege is thought to have evolved to protect the eye from infectious and inflammatory threats, which could have potential deleterious effects on vision. Conversely, it may also leave the eye vulnerable to autoimmune attack by T-cells that have been primed by chance encounter with a self or with mimic antigens in the systemic circulation (Caspi 2008).

1.10 Pathophysiology of non-infectious uveitis

Much of our knowledge of the pathogenesis of human uveitis has derived from animal models. Experimental autoimmune uveitis (EAU) can be induced by immunising animals, most commonly, rodents, with a retinal antigen (Ag), such as interphotoreceptor retinoid binding protein (IRBP) or retinal soluble Ag (S-Ag) (Agarwal and Caspi 2004; Agarwal, et al. 2012) (Figure 11).
Figure 11: Murine retinal histology sections showing cross-sections of: (a) normal murine retina and (b) inflamed retina in murine model of EAU (courtesy of Dr Virginia Calder).

These retinal Ags are involved in the visual cycle and are typically unique to the eye, therefore serving as targets for the immune system in EAU. The blood-retinal barrier can be an effective barrier to small molecules but it is not a very effective barrier to cells. For example, circulating IFN-γ producing Th1 cells and monocytes, which have been activated systemically, are able to cross the blood vessels and penetrate the barrier in the context of inflammation (Xu, et al. 2005; Xu, et al. 2004). When previously ‘unknown’ retinal Ag are exposed to the immune system, a break in immune tolerance may occur and then an inflammatory response ensues.

Inflammation in EAU is mediated by Th1 and Th17 T-cell subsets and may be suppressed by Treg (Agarwal and Caspi 2004; Caspi 2008; Caspi 2011; Caspi 2014; Damsker, et al. 2010; Zheng, et al. 2004). A study in rats found that during resolution of the first acute attack of EAU, the number of ocular Tregs increased (Ke, et al. 2008). Interestingly, the suppressor function of Tregs was weaker in those rats who went on to develop recurrent EAU (Ke, et al. 2008). In mouse models of EAU, a significantly increased frequency and immunoregulatory action of CD4+CD25+ Treg cells has been associated with the development and regression of EAU, suggesting that CD4+CD25+ Treg cells are induced during EAU and may be involved in its regression.
(Sun, et al. 2010). This is further supported by murine EAU evidence demonstrating that retina-specific functionally suppressive FoxP3+ Tregs accumulated in inflamed eyes and persisted for several months after disease remission (Silver, et al. 2015). Depletion of Tregs at the peak of uveitis delayed resolution and, following resolution, (when mice displayed a low grade chronic inflammation), Treg depletion precipitated disease relapse (Silver, et al. 2015).

1.11 Clinical presentation and features of human uveitis
To make a diagnosis of uveitis, examination of the anterior and posterior chambers of the eye with slit lamp biomicroscopy or indirect ophthalmoscopy is required, in order to identify the characteristic signs of intraocular and chorioretinal inflammation. Figure 12 shows a normal view of a retinal fundus.
Figure 12: Clinical retinal photography image showing the normal appearance of the retina with the following structures: (a) optic disc, (b) macular area of the retina (responsible for central vision) and (c) retinal blood vessels (superior arcade).

1.11.1 Anterior uveitis

Anterior uveitis occurs when the anterior part of the uvea, the iris, is inflamed. On examining the eye, the cornea may be clear or keratic precipitates (KPs), inflammatory deposits from the inflamed iris, may be present on the posterior corneal surface, if inflammation is severe. The anterior chamber is often of normal depth but ‘cells’, ‘fibrin’ and ‘flare’, which occur secondary to exudative protein from the inflamed iris, may be seen in the slit light beam of the slit lamp. If a number of inflammatory cells have infiltrated the aqueous humour, these may settle and form a ‘hypopyon’ in the anterior chamber. Synaechia may occur in anterior uveitis, where the iris forms inflammatory adhesions to either the cornea (anterior synaechia) or lens (posterior synaechia). ‘Posterior synaechia’ may appear as a fixed
irregular-shaped pupil (Figure 13), which can become permanent if the pupil is not dilated with a mydriatic eyedrop. The intraocular pressure may also become elevated, leading to secondary glaucoma.

![Image](image1.jpg)

**Figure 13: Posterior synechiae, where the iris has become adherent to the crystalline lens posterior to it, as a result of inflammation in the anterior chamber of the eye (photo courtesy of Professor Sue Lightman)**

### 1.11.2 Intermediate uveitis

Intermediate uveitis occurs when the main site of ocular inflammation is the pars plana and/or the vitreous gel which fills the posterior chamber of the eye. The inflamed vitreous may appear hazy, due to the infiltration of inflammatory cells from adjacent structures, which is called ‘vitritis’ (Figure 14). Inflammation of the pars plana portion of the ciliary body is known as ‘pars planitis’. Whilst there may be associated inflammatory macular oedema, there should be no chorioretinitis. The fundal view, however, may be obscured by the vitritis.
Figure 14: Severe vitritis obscuring the view of the retina (fundus) (courtesy of Professor Sue Lightman)

1.11.3 Posterior uveitis

Posterior uveitis occurs when the choroid (posterior uvea) is inflamed, in which case the retina and retinal vessels overlying the choroid may be affected leading to a chorioretinitis, neuroretinitis or vasculitis. Sight-threatening retinitis may manifest as either focal or diffuse areas of pale-white retina accompanied by retinal hemorrhages, vascular sheathing, retinal ischemia and retinal necrosis (Figure 15) (Gilbert, et al. 2016a). Non-infectious retinitis in Behçet disease, for example, can be isolated or multifocal and is ischaemic in type, usually occurring with significant vitritis.
Following an episode of chorioretinal inflammation, chorioretinal atrophy or scarring may occur (Figures 16-17). If this affects the macular area of the retinal, it is likely to have a significant effect on the patient’s central vision. If the uveitis is inactive at the time of examination, the presenting signs of uveitis may be scarring or post-inflammatory fibrotic changes, rather than the inflammation itself.
Figure 16: Multiple pale chorioretinal lesions in various phases of evolution (from active inflammatory lesions with indistinct edges to inactive scars with discrete borders and exposure of the underlying black retinal pigment epithelium and/or white/pale sclera).
Figure 17: Inactive chorioretinal scars (with discrete borders and exposure of the underlying black retinal pigment epithelium and/or white/pale sclera).

1.11.4 Panuveitis
In panuveitis, the inflammation is not confined to a single anatomic location of the eye, and any, or all, of the above clinical signs may occur.

1.12 Diagnosis and differential diagnosis of uveitis
Ocular inflammation is a nonspecific sign related to many conditions and therefore a detailed history and clinical examination are important in guiding the diagnostic work-up, particularly with regard to the exclusion of infectious aetiologies (Gilbert, et al. 2016a). Retinal imaging tests, such as fluorescein angiography, may be helpful in identifying the extent of retinal involvement and, depending on the clinical presentation, blood tests, may be warranted. In many cases, the clinical appearance of the fundus and patient history can
direct diagnostic investigations. Table 4 lists the recommended systemic investigations in cases of retinitis.

**Table 4: Recommended systemic investigations in suspected cases of uveitis (Gilbert, et al. 2016a)**

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Systemic investigations in retinitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoidosis</td>
<td>Serum angiotensin-converting enzyme (ACE), chest X-ray</td>
</tr>
<tr>
<td>Syphilis</td>
<td>Specific treponemal (FTA-ABS) and nonspecific treponemal antibody (VDRL)</td>
</tr>
<tr>
<td>Toxoplasmosis</td>
<td>Toxoplasma serology</td>
</tr>
<tr>
<td>Cat scratch disease</td>
<td>Bartonella serology</td>
</tr>
<tr>
<td>HIV infection</td>
<td>HIV serology</td>
</tr>
<tr>
<td>Behçet disease</td>
<td>HLA-B51</td>
</tr>
<tr>
<td>Birdshot chorioretinopathy</td>
<td>HLA-A29</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>Interferon-γ release assay (QuantiFERON Gold, Mantoux, or T-spot tests), chest X-ray</td>
</tr>
</tbody>
</table>

If viral aetiology or a masquerade syndrome is suspected, a vitreous biopsy should be obtained in order to perform polymerase chain reaction (PCR) testing (for the presence of viral DNA) and/or cytology and immunology tests (for suspected haematological malignancy).

**1.13 Pharmacotherapy of uveitis**

Chronic relapsing remitting non-infectious uveitis is challenging to manage and severe cases require treatment with high dose oral immunosuppressive drugs. Corticosteroids and other immune-modulating treatments do not cure uveitis but, rather, suppress immune system activity. Control of inflammation is the most critical step in the management of uveitis and the prevention of vision loss, which can result from the cumulative damage to ocular structures (Sangwan 2010). The term ‘disease activity’ refers to the ongoing immune response, which can usually be reversed with immunosuppression, whereas
‘damage’ refers to the irreversible effects of inflammation on tissue (Barry, et al. 2014). In the clinical setting, after a diagnosis of uveitis has been made, patients are followed up regularly to determine disease activity, ocular complications, and side effects of treatment. The clinical follow-up schedule is individualised according to the aetiology of the uveitis and its activity, the threat to vision, and type of treatment required (McCluskey, et al. 2000). Disease activity is routinely clinically assessed by examination (using slit-lamp biomicroscopy) and clinical ocular imaging methods, such as optical coherence tomography (OCT). A long-term ‘stepladder approach’ is used, involving initiation of the lowest appropriately aggressive therapy for the specific disease process and severity, and advancement up the ladder to other modalities as needed, because of subjective intolerance or failure to control inflammation (Foster, et al. 2016a). Initiating treatment with immunosuppressants aims to suppress pathogenic immune system over-activity rather than ‘cure’ the underlying disease. This can lead to a durable drug-free ‘remission’ of uveitic disease, which is the overall treatment aim.

1.13.1 Corticosteroids
The mainstay immunosuppressant treatment for uveitis is the glucocorticoid subgroup of corticosteroids, sometimes with additional immunosuppressive drug therapy. Medical therapy should include topical, regional and systemic corticosteroids, as well as topical cycloplegics and mydriatics for symptomatic relief and prevention of synaechiae formation, when appropriate.

Glucocorticoids are produced endogenously in response to adrenocorticotrophic hormone (ACTH)-induced conversion of cholesterol to pregnolone in the adrenal cortex (Castiblanco and Foster 2014). They regulate gluconeogenesis and inhibit the inflammatory response through broad and non-selective inhibition of the immune system, notably by
inhibiting phospholipase A2 and thereby affecting the conversion of phospholipid to arachidonic acid (Castiblanco and Foster 2014). The end result is inhibition of prostaglandin production through the cyclooxygenase pathway and inhibition of production of leukotrienes through the lipooxygenase pathway (Castiblanco and Foster 2014). The following effects on immune cells have been observed:

- Inhibition of leukocyte trafficking
- Interference of functions of leukocytes, fibroblast and endothelial cells
- Suppression of the production of humoral factors, such as, cytokines, involved in the inflammatory process

Topical corticosteroids eyedrops are primarily used to treat anterior uveitis. The frequency of installation depends on the severity of inflammation and the agents vary in their formulation and potency (Table 5).
Periocular or intraocular steroid (local) therapy may be given when patients are unresponsive or poorly compliant to systemic treatment or for patients in whom systemic therapy is contraindicated, for example pregnant women. Local therapy may also be a preferable choice for some patients, as it avoids the systemic side effects of oral therapy. The eye is an ideal organ for local therapy due to the fact that the intraocular space can be directly accessed, for example, through intravitreal steroid injections or implants, achieving high drug concentrations on target (Sharief, et al. 2018). The ocular side effects of corticosteroids include raised intraocular pressure, glaucoma and cataracts. Raised intraocular pressure may prove particularly problematic if there is an
existing clinical history of raised intraocular pressure associated with steroid use.

The Multicentre Uveitis Steroid Treatment (MUST) trial was a multicentre clinical trial to compare local and systemic treatment for non-infectious uveitis, which examined the effect of systemic immunosuppression treatment on long-term control with long-acting local therapy in the form of a surgically inserted fluocinolone acetonide implant (Retisert; Bausch & Lomb, Bridgewater, NJ) (Kempen, et al. 2011). The interventional trial ran for 24 months, with 255 patients (479 eyes with uveitis) randomised to either systemic immunosuppression or the long-acting intraocular Retisert implant, and followed up for an additional 5 years as part of an observational study. The primary results demonstrated that at 24 months, the implant resulted in comparable visual improvement to systemic immunosuppression with greater inflammatory control and macular oedema resolving in two thirds of eyes from both groups (Kempen, et al. 2011). Overall control of inflammation was superior in the implant group at every time point assessed, although most eyes in the systemic therapy arm also showed substantial improvement, achieving complete control or low levels of inflammation (Kempen, et al. 2015). Eyes receiving the implants had a greater likelihood of ocular side effects developing, with 90% of phakic eyes requiring cataract surgery and 45% undergoing surgery to control raised intraocular pressure (IOP) (Sharief, et al. 2018).

In the following cases of non-infectious uveitis, systemic immunosuppressive therapy might be considered (Jabs, et al. 2000):

- Where there is poor response to local treatment or contraindications to local treatment e.g. raised IOP
- Frequent relapse or recurrence of inflammation
- Bilateral cases of inflammation
Furthermore, in all cases of uveitis of suspected infectious aetiology, antimicrobial treatment must be given along with the corticosteroid treatment, and local steroid injections should not be used (Gilbert et al., 2016).

High loading doses of corticosteroids are often the best option for rapid control of inflammation in the case of a uveitic relapse and the clinical response should be carefully monitored through examination and imaging of the eye. The initial loading dose of oral prednisone typically is 1 mg per kg per day, for example, 60 to 80 mg per day in an adult (Jabs, et al. 2000). If uveitis clinically responds to high dose prednisolone, with reduction in the clinical signs of inflammation, the medication dose is tapered (by 1 to 10mg per week depending on the initial dose) until the lowest dose that maintains the vision, without adverse drug effects, is determined (Table 6).
Table 6: Suggested guidelines for the use of prednisone for chronic ocular inflammation (Jabs, et al. 2000)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Suggested Guideline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial dose</td>
<td>1 mg/kg/day*</td>
</tr>
<tr>
<td>Maximum adult oral dose</td>
<td>60–80 mg/day</td>
</tr>
<tr>
<td>Maintenance dose (adult)</td>
<td>≤10 mg/day</td>
</tr>
<tr>
<td>Tapering schedule</td>
<td>Over 40 mg/day, decrease by 10 mg/day every 1–2 weeks</td>
</tr>
<tr>
<td></td>
<td>40–20 mg/day, decrease by 5 mg/day every 1–2 weeks</td>
</tr>
<tr>
<td></td>
<td>20–10 mg/day, decrease by 2.5 mg/day every 1–2 weeks</td>
</tr>
<tr>
<td></td>
<td>10–0 mg/day, decrease by 1 to 2.5 mg/day every 1–4 weeks</td>
</tr>
<tr>
<td>Monitor</td>
<td>Blood pressure, weight, glucose every 3 months</td>
</tr>
<tr>
<td></td>
<td>Lipids (cholesterol and triglycerides) annually</td>
</tr>
<tr>
<td></td>
<td>Bone density within first 3 months and annually thereafter</td>
</tr>
<tr>
<td>Supplemental treatment</td>
<td>Calcium 1500 mg daily and vitamin D 800 IU daily</td>
</tr>
<tr>
<td></td>
<td>Estrogens and antiresorptive agents as needed</td>
</tr>
</tbody>
</table>

*In selected situations, where an immediate effect is needed, some investigators will begin with intravenous methylprednisolone at a dosage of 1 gm/day for 3 days and then start oral prednisone.
In addition to the risk of infection associated with immunosuppression, given systemically, these drugs together have significant side effects which include hypertension, diabetes, liver dysfunction, osteoporosis and potential malignancy (Figure 18).

**Figure 18: Side effects of systemic corticosteroids (Gilbert, et al. 2017).**

Prescription of gastroprotective prophylaxis, such as a proton pump inhibitor drug, should be considered for patients on high dose or long-term treatment (Table 6). Bone density should be regularly monitored with Dual Energy X-ray Bone Scan (DEXA) due to the risk of osteopenia and/ or osteoporosis, in addition to prescription of oral bone protection supplements, such as calcium and vitamin D tablets, or bisphosphonates in higher risk patients (Foster, et al. 2016a) (Table 6).
A previous study showed that approximately half of the patients (525 out of 1076 patients) attending a specialist uveitis service at Moorfield’s Eye Hospital, London UK required systemic steroids to control their ocular inflammation (Tomkins-Netzer, et al. 2014). Around 60% of cases required a corticosteroid dose of more than 40 mg per day, and around 25% of patients required one or more second line agents to control their inflammation (Tomkins-Netzer, et al. 2014).

On tapering high dose corticosteroid medication, it is not uncommon for relapses of ocular inflammation to occur. Such patients might require maintenance corticosteroid treatment or addition of a ‘steroid sparing agent’, that is, an immunosuppressive drug to reduce the corticosteroid dose and associated side effects. The Fundamentals Of Care for Uveitis (FOCUS) initiative recommended that non-corticosteroid systemic immunomodulatory therapy (NCSIT) be introduced to control persistent or severe inflammation or to prevent ocular structural complications that present a risk to visual function (Dick, et al.). Other indications for introducing NCSIT included contraindications or intolerance to other therapies or a need for corticosteroid-sparing effect to maintain disease remission. There are several categories of NCSIT, which include agents such as mycophenolate, methotrexate, azathioprine or cyclosporine, all of which act by targeting one or more aspects of the immune response (Table 7).
Table 7: Immunosuppressive drugs for ocular inflammation (Jabs, et al. 2000)

<table>
<thead>
<tr>
<th>Class</th>
<th>Generic Name/Trade Name</th>
<th>Oral Formulation</th>
<th>Initial Dose</th>
<th>Maximum Dose</th>
<th>Mechanism</th>
<th>Expected Onset</th>
<th>Recommended Use</th>
<th>Side-effects</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cell inhibitor</td>
<td>Cyclosporine</td>
<td>Capsule, oral suspension</td>
<td>2.5-5 mg/kg/d</td>
<td>10 mg/kg/d</td>
<td>IMMUNE SUPPRESSION</td>
<td>3-6 weeks</td>
<td>Bone marrow suppression</td>
<td>Cataracts, nephrotoxicity, hypertension, anaphylaxis, liposomal type, uveitis</td>
<td>Follow BP</td>
</tr>
<tr>
<td>T-cell inhibitor</td>
<td>CsA</td>
<td>Capsule, oral suspension</td>
<td>5-10 mg/kg/d</td>
<td>20 mg/kg/d</td>
<td>IMMUNE SUPPRESSION</td>
<td>3-6 weeks</td>
<td>Bone marrow suppression</td>
<td>Cataracts, nephrotoxicity, hypertension, anaphylaxis, liposomal type, uveitis</td>
<td>Follow BP</td>
</tr>
<tr>
<td>T-cell inhibitor</td>
<td>Tacrolimus</td>
<td>Capsule, oral suspension</td>
<td>0.1-0.3 mg/kg/d</td>
<td>0.3 mg/kg/d</td>
<td>IMMUNE SUPPRESSION</td>
<td>3-6 weeks</td>
<td>Bone marrow suppression</td>
<td>Cataracts, nephrotoxicity, hypertension, anaphylaxis, liposomal type, uveitis</td>
<td>Follow BP</td>
</tr>
<tr>
<td>Lymphokinese inhibitor</td>
<td>Leflunomide</td>
<td>Capsule, oral suspension</td>
<td>20 mg Qd</td>
<td>100 mg Qd</td>
<td>IMMUNE SUPPRESSION</td>
<td>3-6 weeks</td>
<td>Bone marrow suppression</td>
<td>Cataracts, nephrotoxicity, hypertension, anaphylaxis, liposomal type, uveitis</td>
<td>Follow BP</td>
</tr>
<tr>
<td>Lymphokinesis inhibitor</td>
<td>Azathioprine</td>
<td>Capsule, oral suspension</td>
<td>2-5 mg/kg/d</td>
<td>25 mg/kg/d</td>
<td>IMMUNE SUPPRESSION</td>
<td>3-6 weeks</td>
<td>Bone marrow suppression</td>
<td>Cataracts, nephrotoxicity, hypertension, anaphylaxis, liposomal type, uveitis</td>
<td>Follow BP</td>
</tr>
</tbody>
</table>

Note: The table above provides a summary of immunosuppressive drugs commonly used in ocular inflammation, including their mechanism of action, expected onset, recommended use, and potential side-effects. The table also includes comments on the drugs, such as the need for bone marrow suppression or the potential for uveitis.

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Unfortunately, some patients may have to progress through various drug combinations over time, due to the various side or adverse effects of the drugs that they experience. It is worth noting that if patients respond inadequately to NCSIT, there are a number of factors to consider before changing clinical management (these are summarised in Table 8).

### Table 8: Management of patients with an inadequate response to noncorticosteroid systemic immunomodulatory therapy (Dick, et al.)

<table>
<thead>
<tr>
<th>Considerations for Management of Patients with an Inadequate Response to Noncorticosteroid Systemic Immunomodulatory Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Consider differential diagnosis</td>
</tr>
<tr>
<td>2A. Dose escalation of current therapy</td>
</tr>
<tr>
<td>2B. Transition to alternative noncorticosteroid systemic agent</td>
</tr>
<tr>
<td>2C. Local or regional therapies</td>
</tr>
<tr>
<td>2D. Nonmedical therapy (vitrectomy, cryotherapy, etc.)</td>
</tr>
<tr>
<td>2E. Biologic therapy</td>
</tr>
<tr>
<td>3. Therapies should be individualized based on history, cause of uveitis, and patient preference</td>
</tr>
<tr>
<td>Currently limited evidence exists to support adding an additional agent; safety and cost implications should be considered.</td>
</tr>
</tbody>
</table>

#### 1.13.2 Antimetabolites

Antimetabolites are a class of compounds, exemplified by methotrexate and azathioprine, that inhibit nucleic acid synthesis and cell proliferation (Table 7) (Tomkins-Netzer, et al. 2012). Methotrexate is an analogue of folic acid, which inhibits purine and pyridine synthesis, therefore suppressing cellular inflammation. Its actions depend on the inhibition of dihydrofolate reductase, therefore, it is prescribed in combination with folic or folinic acid. It reduces cellular and humoral immune responses by affecting proliferation, inducing T-cell apoptosis, altering B-cell response, inhibiting cytokine production and
also with antiangiogenic properties (Castiblanco and Foster 2014).

Azathioprine is an imidazolyl derivative of 6-mercaptopurine. In its original form, it is a prodrug, which is metabolized in the liver to the active form, 6-mercaptopurine, that is then converted to thioinosine 5-phosphate, that serves as a purine analogue that interferes with RNA and DNA synthesis (Castiblanco and Foster 2014). It has been shown to inhibit T-lymphocyte function by interfering with targeting of circulating T cells, decreasing the development of monocyte precursors and suppressing type IV hypersensitivity reaction, in addition to interfering with the participation of natural killer (NK) cells in the antibody-dependent cytotoxic reactions (Castiblanco and Foster 2014)

The Systemic Immunosuppressive Therapy for Eyes (SITE) study was a recent, large survey of the use of NCSIT in uveitis, which provided valuable information about many of these immunosuppressive agents (Jabs 2017). In the SITE study, 639 eyes were treated with methotrexate and, at 12 months, 66% achieved corticosteroid-sparing control and 58% were able to reduce their steroid treatment to under 10mg (Tomkins-Netzer, et al. 2012). However, many patients required a full six months of therapy before the full steroid-sparing effect was seen. 16% of patients in this study discontinued methotrexate treatment due to side effects, the most common being gastrointestinal upset, bone marrow suppression or elevated liver enzymes (Tomkins-Netzer, et al. 2012).

Mycophenolate mofetil is a selective purine synthesis inhibitor which is used to treat organ transplant rejection, in addition to autoimmune diseases. By inhibiting the de novo purine pathway, it selectively inhibits B and T lymphocytes, and is widely used at a dose of 500-1500mg twice a day (Table 7). It may achieve inflammation control faster than methotrexate or azathioprine, and it is also generally effective and well tolerated. The SITE study showed that systemic corticosteroid use was reduced to 10 mg/day or
less in 41% and 55% of patients respectively (Tomkins-Netzer, et al. 2012). 20% of patients discontinued the drug, with the most frequent side effects being gastrointestinal disturbance and bone marrow suppression (Tomkins-Netzer, et al. 2012). Comparative studies support the use of mycophenolate mofetil and methotrexate for control of inflammation and maintenance of vision, with, thus far, no evidence of superiority of one drug over the other (Dick, et al.; Jabs 2017).

1.13.3 Alkylating agents
Alkylating agents, specifically, cyclophosphamide and chlorambucil, are potent immunomodulatory agents (Table 7). Their mechanism of action is via alkylation of nucleotide bases, thereby inhibiting DNA synthesis and, thus, suppressing the cell replication rate. They appear to be the most effective conventional immunosuppressive agents for inducing remission of uveitis (Jabs 2017). In the SITE study, cyclophosphamide was shown to be effective in achieving inflammation control in over 75% of uveitis patients at 12 months (Tomkins-Netzer, et al. 2012). Chlorambucil was reported to be useful in treating Behçet’s uveitis and Vogt-Koyanagi-Harada syndrome (Tomkins-Netzer, et al. 2012). However, alkylating agents are also the most toxic of conventional immunosuppressants and are associated with myelosupression and the development of malignancies (Castiblanco and Foster 2014). Cyclophosphamide is furthermore associated with high risk of sterile hemorrhagic cystitis and bladder carcinoma (Foster, et al. 2016a). Therefore, their use is restricted to a small number of cases of severe treatment-refractory uveitis and weekly blood test monitoring is warranted to monitor drug toxicity and severe bone marrow suppression.
1.13.4 T-cell inhibitors
Uveitis is considered a T-cell mediated disease and thus T-cell inhibitors, such as, cyclosporine, tacrolimus and sirolimus have entered common usage as steroid-sparing agents (Table 7). These agents affect the signal transduction of T lymphocytes, resulting in a reduction in T-cell activity and resultant suppression of inflammation. Cyclosporine is a calcineurin inhibitor and cytostatic agent and its primary effect is to inhibit T cell activation and recruitment. Because of its rapid onset of action, cyclosporine is usually the treatment of choice for sight-threatening and progressive uveitis. However, the long-term use of cyclosporine is limited by a range of side effects, including neurological side effects, hirsutism, hyperglycaemia, hepatotoxicity, nephrotoxicity and hypertension. In the SITE study, over 50% of patients treated with cyclosporine showed inflammation control at 12 months (Tomkins-Netzer, et al. 2012). Tacrolimus is also thought to be efficacious in uveitis with a similar side-effect profile to cyclosporine, although the incidence of hypertension, hyperlipidemia, hirsutism and gingival hyperplasia are purportedly lower (Jabs 2017; Tomkins-Netzer, et al. 2012).

1.13.5 Biologic anti-inflammatory agents
When antimetabolite therapy is not effective or well tolerated, or when disease is more advanced or severe, biologic response modifiers, also known as ‘biologics’, are the next medication in the ‘stepladder’ approach to medical therapy of ocular inflammation (Foster, et al. 2016b). These consist of biological molecules, typically antibodies, developed to target and alter certain areas or deactivate specific cytokines within the immune response. The biological agents most widely used for treating uveitis are tumour necrosis factor alpha (TNF-α) inhibitors, specifically infliximab and adalimumab. These monoclonal antibodies both have specificity for TNF-α, but whereas infliximab is a chimeric molecule containing non-human rodent
antigens, adalimumab is fully humanised. Infliximab has been found to be effective in treating Behçet’s uveitis patients, reducing the number of uveitis attacks compared with cyclosporine and other conventional agents (Tomkins-Netzer, et al. 2012). Adalimumab is also recommended for the treatment of nonanterior non-infectious uveitis in adults (Dick, et al.). Previous RCT studies have shown that adalimumab significantly lowered uveitic flare and loss of vision and that, in severe forms of adult sight-threatening non-infectious uveitis, it is superior to placebo in improving vision and reducing flares (Dick, et al.). Furthermore, adalimumab is given by subcutaneous injection rather than intravenous infusion, making it more convenient for the patient and cheaper to administer. Both drugs are associated with reactivation of latent tuberculosis infection and unmasking of multiple sclerosis (associated with intermediate uveitis), so careful pre-treatment screening, including patient history and QuantiFERON blood tests are recommended (Tomkins-Netzer, et al. 2012). Additionally, infliximab may require additional immunosuppression, with methotrexate for example, to reduce the risk of developing antibodies to the murine fragment of the antibody, unlike the fully-humanised adalimumab (Tomkins-Netzer, et al. 2012).

Interferon α-2a (IFNα2a) is an immunomodulatory cytokine which has also been studied in the treatment of refractory uveitis, and is thought to be capable of inducing disease remission through the activation of regulatory T-cells, leading to the potential for its immunosuppressive effects for 6 to 12 months after the drug is discontinued (Lightman, et al. 2015). The most common side effects, reported in almost all patients, are flu-like symptoms, which may be quite debilitating, and more severe side-effects include leucopenia, thrombocytopenia and central nervous system effects (psychosis, depression) (Tomkins-Netzer, et al. 2012).
1.14 Clinical course of uveitis and prognosis

Uveitis often responds well to high dose immunosuppressive treatment, however, some patients may relapse on tapered withdrawal of immunosuppression and a further proportion go on to develop chronic disease (Gupta and Murray, 2006). For the majority of patients with acute anterior uveitis, that shows a good clinical response to intensive topical corticosteroid treatment, the risk of irreversible sight threatening complications is low and the visual prognosis is good. However, those with chronic anterior uveitis, posterior uveitis, and panuveitis have a worse visual prognosis (Gutteridge and Hall, 2007).

Chronic uveitis can be defined as active uveitis that persists longer than three months (McCluskey, et al. 2000). In the UK, sarcoidosis is the commonest disease that presents as chronic uveitis in adults (McCluskey, et al. 2000). As with many chronic inflammatory diseases, non-infectious uveitis may follow a relapsing and remitting disease course.

The prognosis of sight-threatening retinitis, ‘depends on the aetiology, the visual acuity at the time treatment is started, the degree of involvement of the macula and the optic disk, and the development of subsequent complications, such as retinal detachment. In cases of noninfectious retinitis, prompt treatment and long-term immunosuppressive control of the disease result in a stable long-term visual outcome, with most patients maintaining the vision at presentation. Causes of vision loss include macular scaring and atrophy, chronic cystoid macular oedema, epiretinal membranes, and macular ischemia. In cases of ARN, the extensive retinal necrosis can lead to the development of retinal detachment, posterior pole involvement, and vitreous haemorrhages, all of which result in a poor long-term visual outcome. In CMV retinitis, treatment is effective in arresting the progression
of retinitis and minimizing the complications related to that. Anti-retroviral therapy is required to control CMV replication in the long term and prevent further recurrence. However, patients may develop significant visual impairment secondary to large areas of retinal involvement, recurrent disease, epiretinal membranes, cystoid macular oedema, cataract, or retinal detachment. In toxoplasmosis, the visual prognosis depends on the location of the retinal involvement. Posterior pole involvement in the form of macular scars, optic nerve involvement, or vascular occlusion can lead to severe vision loss.’ (Gilbert, et al. 2016a), reproduced with permission.

1.15 Improving management of non-infectious uveitis
Non-infectious uveitis is a clinically heterogenous disease and it has been suggested that ‘many of the constraints in generating evidence for the efficacy of treatments have been due to the heterogeneity and spectrum of disorders of uveitis, their relatively low prevalence, and the lack of robust outcome measures’ (Lee and Dick 2012). As in the case of autoimmune disease, critical to improving diagnosis, monitoring and treatment of the disease, is application of some of the principles of personalised or stratified medicine, that is, the precise clinical, molecular/genetic and immunological phenotyping of patients at various disease stages of non-infectious uveitis (Figure 19) (Cottrell 2013; Lonergan, et al. 2017). Understanding the mechanisms of disease amplification, propagation and regulation will enable the development of effective targeted therapies (Cottrell 2013).
Currently, the inability to precisely define the potentially separate clinical entities which are currently labelled as ‘uveitis’ severely limits the application of individualised or stratified therapies. However, the need to develop better biomarkers and surrogate endpoints of disease activity for uveitis, in line with the United States Food and Drug Administration (FDA)/ National Institutes of Health (NIH) criteria (Figure 20), has been recognised (Denniston, et al. 2017). Clinical outcome measures can also be developed to incorporate the impact of an eye condition on a patient’s life, so-called, patient-reported outcome measures (Denniston, et al. 2014).
• **Biomarker**
  - A characteristic that is objectively measured and evaluated as an indicator of:
    - Normal biologic processes;
    - Pathogenic processes; or
    - Pharmacologic response(s) to a therapeutic intervention

• **Surrogate Endpoint**
  - A biomarker that is intended to substitute for a clinical endpoint
  - Expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence

Figure 20 Summary of FDA/NIH Guidance on biomarkers and surrogate endpoints

1.16 **Patient and public involvement in uveitis research**

Priority Setting Partnerships (PSPs) enable clinicians, patients and carers to identify and prioritise uncertainties about the effects of treatments that could be answered by research, through a collaborative approach (JLA 2017a).

The James Lind Priority Setting Partnership (PSP) on Sight Loss and Vision was led by a Steering Group headed by representatives from Fight for Sight, the College of Optometrists, the UK Vision Strategy, and the NIHR Biomedical Research Centre at Moorfields Eye Hospital (JLA 2017c; Rowe, et al. 2014). The consultation also included representatives of patient/service user groups and healthcare professionals from ophthalmology, optometry, orthoptics, ophthalmic nursing and social care (JLA 2017c):

‘2,220 people responded to the survey, generating 4,461 questions. With questions across different eye diseases and conditions, it was decided that 12 final priority setting workshops would take place during April and May 2013. 17% of survey respondents identified themselves as
healthcare professionals and over 60% of the respondents were people with sight loss or an eye condition.’ (Figure 21) (JLA 2017c)

The final workshop of a Priority Setting Partnership (PSP) enables patients, carers and clinicians to agree on the order of priority of a shortlist of unanswered questions, which is used to create a list of the Top 10 priorities for future research (JLA 2017d). A Top 10 priorities list was created for ocular inflammatory diseases (Figure 22), which included infectious and non-infectious uveitis, in addition to other inflammatory diseases such as scleritis and thyroid eye disease.
Figure 21: Flow chart showing the steps of the process from stage 1 when establishing the Priority Setting Partnership (PSP) through to stage 5 at the final prioritisation (PICO, Population, Intervention, Comparison, Outcome) (Rowe et al., 2014)
1. What are the most effective treatments for ocular and orbital inflammatory diseases?
2. What causes thyroid eye disease?
3. Can the severity of ocular and orbital inflammatory disease in an individual be predicted?
4. Is it possible to prevent further occurrences of retinal damage caused by toxoplasmosis?
5. What causes birdshot retinopathy?
6. Why does disease burn out in patients with ocular and orbital inflammatory diseases?
7. Can early detection methods be developed for ocular and orbital inflammatory diseases?
8. What medications best prevent the development of eye disease in Behcets?
9. What causes scleritis?
10. Can diet or lifestyle changes prevent uveitis from developing?

Figure 22: Top 10 priorities list for ocular inflammatory diseases (JLA, 2017b; Rowe et al., 2014)

1.17 **Definitions and models of health and disease**

The World Health Organization (WHO) defines health as ‘a state of complete physical, mental and social well-being’ and ‘not merely the absence of disease’ (WHO 1946). Researchers from the Louis Bolk Institute in the
Netherlands felt that this definition was inadequate because it represents health as a static rather than a dynamic state. They observed that this was particularly relevant for individuals with long term health conditions and disability, as it renders them ‘unhealthy’ despite efforts to cope and live with their condition:

‘The WHO definition becomes counterproductive as it declares people with chronic diseases and disabilities definitively ill. It minimises the role of the human capacity to cope autonomously with life’s ever changing physical, emotional, and social challenges and to function with fulfilment and a feeling of wellbeing with a chronic disease or disability.’ (Huber, et al. 2011)

The researchers developed a new definition of health ‘as the ability to adapt and self-manage, in light of the physical, emotional and social challenges of life’ (Huber, et al. 2016). The medical perspective of health has typically had a ‘negative’ disease-focus rather than a ‘positive’ health focus. Conventional medical treatment is based on a scientific process involving observation, description and differentiation, which moves from recognising and treating symptoms to identifying disease aetiologies and developing specific treatments (Clare 1980, 2001). This viewpoint has been criticized as mechanistic and reductionist, with illness understood in terms of causation and remediation, in contrast to holistic and social models. In his landmark paper on ‘The Biopsychosocial Model’, Engel observed that:

“The dominant model of disease today is biomedical, and it leaves no room within its framework for the social, psychological, and behavioral dimensions of illness...We are now faced with the necessity and the challenge to broaden the approach to disease to include the psychosocial without sacrificing the enormous advantages of the biomedical approach”

(Engel 1977)
Positive wellbeing can be defined as ‘optimal psychological experience and functioning’, which has an important relationship with health (Steptoe 2011). Studies suggest that when individuals engage in health promoting actions, their sense of well-being, satisfaction, and performance are greater and their incidence of symptoms and problems associated with disease and infirmity are lower because of a better ability to adapt and self-manage in the face of challenges (Becker, et al. 2009; Becker, et al. 2015). The concept of ‘positive health’, which takes into account an individual’s psycho-social wellbeing, in addition to their physical health and body functioning, may be addressed through community engagement. The National Institute for Health and Care Excellence (NICE) has noted that, ‘Community engagement is a highly complex area with several important purposes. These include empowering people within communities to gain more control over their lives and to play a part in decisions that affect their health and wellbeing’. One of the difficulties, however, in evaluating the contribution of positive wellbeing to health is that it occurs with a range of other psycho-social characteristics that may have intrinsically positive influences on health (Steptoe 2011).

There is a role for individual social cognitions (the cognitive model), for example, self-efficacy, in determining health and wellbeing (Bandura 2000; Steptoe 2011). Self-efficacy may be defined by one’s belief in one’s ability to succeed in specific situations or accomplish a task, which can play a major role in how one approaches health goals and challenges (Bandura 1977).

1.18 Designing interventions to change health behaviours

Health-related behaviours can contribute to major causes of mortality and morbidity. In the early 1950s, the focus of public health service was on understanding the factors which might motivate asymptomatic individuals to take up free or low cost tests of early disease detection (Rosenstock 1974). At that time, theories to explain health behaviours were being developed by
social psychologists, who did not have in depth knowledge of prevalent diseases or the health system. The Health Belief Model, the earliest Social Cognition Model (SCM), was designed to have a phenomenological orientation and to be adapted, in consultation with other social psychologists, for application to different health problems, if required (Rosenstock 1974). Notably, SCMs do not specifically mention individuals’ past experiences (Rosenstock 1974). Whilst SCMs help to identify target cognitions, which is particularly useful for preventative health behaviours, they do not describe how to change them, with the possible exception of ‘self-efficacy’ (Steptoe 2011).

The SCMs assume a rational cognitive approach to health behaviour, with intention being a strong predictor. Studies, however, indicate existence of an ‘intention-behaviour’ gap, suggesting that intention formation does not always follow through to action (Armitage and Conner 2001; Webb and Sheeran 2006). It is known that past behaviour might influence future behaviour (Ouellette 1998) but this is not accounted for within the models. Therefore, SCMs are less suitable for predicting behaviours which have a habitual or addictive component, where habit strength will moderate the intention behaviour relationship (Triandis, 1977) and motivation is viewed as part of a complex system, subject to instability and fluctuation (West 2006). Furthermore, SCMs fail to incorporate wider information about physical environment, biological processes, past behaviour and the system in which the individual exists.

A methodological approach which classifies behaviour change interventions (Abraham and Michie 2008) and uses models to understand individual behavior in the context of a wider system (Michie, et al. 2011) is likely to be more useful in informing behaviour change interventions, in comparison to using individual social cognitions, alone. A simple model, such as the COM-B model of behaviour in context (Michie, et al. 2011) is potentially amenable to
use by health-care researcher without a specialist psychology background, and might assist in the generation of evidence to explain behaviour in varied healthcare settings. Behaviour change taxonomy (Abraham and Michie 2008) might also help to identify which models are suitable for targeting specific types of health cognition and/or behaviour, to promote change.

1.19 Aims of the project
The original plan of investigation for the research, as proposed by my supervisors and submitted with PhD registration, was to investigate whether peripheral blood levels of T-regulatory and B-regulatory cells in patients with non-infectious uveitis are induced by treatment with immunosuppressive and/or immunomodulating drugs when patients go into clinical remission. This line of investigation was informed by previous research by the group in a subset of patients with ocular Behcets disease (chronic non-infectious uveitis associated with systemic Behcets disease) showing that:

1) The risk of severe sight loss is significantly reduced in patients with uveitis associated with Behcets’ Disease given biological anti-inflammatory drugs, such as anti-tumour necrosis factor (-TNF) agents (Taylor, et al. 2011)

2) In ocular Bečhets Disease, Treg levels are increased by 3 months treatment with pegylated Interferon-α (pegIFNα), and the increased levels of Tregs are still detectable at 12 months, 6 months after cessation of therapy (Lightman, et al. 2014)

These clinician-developed research aims were reviewed in the context of the James Lind PSP for research on ocular inflammatory disease and found to have relevance to the following questions:
1. What are the most effective treatments for ocular and orbital inflammatory diseases?

2. Can the severity of ocular and orbital inflammatory disease in an individual be predicted?

3. Why does disease burn out in patients with ocular and orbital inflammatory diseases?

4. Can early detection methods be developed for ocular and orbital inflammatory diseases?

With regard to Question 1 (what are the most effective treatments for ocular and orbital inflammatory diseases?), it is known that high dose ‘blockbuster’ systemic immunosuppression is the most effective available treatment, in terms of suppressing ocular inflammation (and thereby preventing severe sight loss) for many patients with sight-threatening uveitis. Systemic immunosuppression also has multiple side effects, which we know are undesirable to patients, and have long-term implications for health. With regard to Questions 2 to 4, the challenges in predicting the severity and prognosis of non-infectious uveitis could be in part attributed to limitations in phenotyping uveitis. Improvements in disease phenotyping and identification of disease biomarkers could help to predict prognosis and/or stratify management.

Subsequently, the aims of the lab-based PhD research were changed by the supervisory team to focus on Treg only (rather than both Treg and Breg) and an additional clinical research project with a focus on clinical phenotyping of non-infectious uveitis was added by the primary supervisor. Furthermore, a public engagement project with a focus on sight-threatening retinal disease and behavioural science was carried out alongside the clinical and lab-based research, to support the patient-centred aims of the PhD.
Therefore, the revised aims of research and this PhD thesis were to individualise management of patients with non-infectious uveitis by investigating:

1. Potential differences in clinical phenotypes of multifocal choroiditis (MFC) and punctate inner choroidopathy (PIC) (two subtypes of non-infectious uveitis with similar clinical characteristics) at presentation and whether these are stable over time.

2. Immunophenotyping of peripheral blood of patients with sight-threatening non-infectious uveitis in sustained drug-induced clinical remission to investigate whether there are associations between levels of Treg, Th1, Th17, their related cytokines and the methylation levels of their epigenetic loci.

3. Patient perspectives of sight-threatening retinal disease, with a view to identifying and targeting individual health behaviours for a pilot intervention.

The research undertaken to achieve these in the following chapters, is as follows:

1.9.1 Summary of Chapter 2

The aim of this clinical study was to determine whether two subtypes of non-infectious uveitis with similar clinical characteristics: multifocal choroiditis (MFC) and punctate inner choroidopathy (PIC) are distinct diseases or the same. This is important because of a growing clinical trend towards grouping MFC and PIC together as a single disease (Spaide, et al. 2013), which could have detrimental implications for clinical phenotyping and management.

This chapter has been submitted for publication as an original research paper and is currently undergoing revisions (Gilbert, Niederer et al, under review). A concurrent research paper, with a focus on PIC, has been published (Niederer, et al. 2017).
1.19.2 Summary of Chapters 3 & 4

The aim of these laboratory studies were to improve the phenotyping of uveitis to allow more individualised patient management, with a focus on immunophenotypic analysis of patients in clinical remission, using different approaches: Flow cytometric analysis of Treg, Th1 and Th17 levels and functional studies of Treg; and evaluation of serum cytokine levels of Treg, Th1 and/ or Th17 related cytokines and methylation changes at epigenetic loci related to these T-cell lineages; and statistical modelling of results.

These chapters have been presented at ARVO Congress 2016, the German Ophthalmological Congress (DOG) 2016 and published as an original research paper (Gilbert, et al. 2018; Gilbert, et al. 2016b)

1.19.3 Summary of Chapter 5

The aim of this public engagement and qualitative analysis work was to engage with patients who have sight-threatening non-infectious retinal disease (which does not have curative treatment) and identify suitable interventional approach(es) which could serve to increase sense of agency and/or self-efficacy of affected individuals, in order to potentially change the course of their disease and/or its consequences.

Sections of this chapter have been published as a short report of a pilot medical education intervention [Gilbert, Rose] (Rose, et al. 2018) and have been provisionally accepted as a social science review article (Gilbert, Rawlings et al, in press).
1.19.4 Summary of Chapter 6
This is a general summary discussion of the results from the previous chapters, including summary of conclusions from and limitations to the research, with suggestions for future work.
Chapter 2 Multifocal Choroiditis and Punctate Inner Choroidopathy: An Epidemiological Survey of Phenotypical Differences

2.1 Introduction

2.1.1 Background summary
Non-infectious uveitis is a clinically heterogeneous disease. The aim of this study was to improve the phenotyping of uveitis to allow more individualised patient management, with a focus on clinical phenotyping of idiopathic multifocal choroiditis (MFC) and punctate inner choroidopathy (PIC).

2.1.2 Study aim
To determine whether two subtypes of non-infectious uveitis with similar clinical characteristics: multifocal choroiditis (MFC) and punctate inner choroidopathy (PIC) are distinct diseases or, in fact, the same disease.

2.1.3 Objectives
1. Review of the literature to date to determine the clinical consensus for categorizing patients as MFC or PIC
2. Identify eligible cases with clinical diagnoses of MFC or PIC by a search of electronic hospital records.
3. Classify study eyes as MFC or PIC based on objective clinical criteria identified from the literature review.
4. Investigate the clinical trajectory of patients in each category, MFC or PIC, over 5 years with respect to the development of peripheral lesions.
5. Analyse and compare clinical and imaging features of MFC and PIC for phenotypic differences

6. Determine if cases identified as MFC or PIC, using above standardised definition, did have a distinct clinical trajectory justifying their categorization as clinically distinct diseases.

2.1.4 Rationale for study

The study was devised in response to a contemporaneously published paper (Spaide, et al. 2013), which investigated the clinical phenotyping and definitions of multifocal choroiditis (MFC) and punctate inner choroidopathy (PIC), two subtypes of non-infectious uveitis with similar clinical characteristics. MFC and PIC are thought to be the most closely related of the white dot syndromes due to their similarity in clinical presentation with symptoms of field defects and photopsias, scar appearance and peripapillary location, tendency towards bilateral ocular involvement and frequency of development of choroidal neovascular membranes (CNV) (Gerstenblith, et al. 2007; Kaiser and Gragoudas 1996; Khorram, et al. 1991). They share the same haplotype associations with interleukin-10 and tumour necrosis factor loci (Atan, et al. 2011). However, there are no definitive tests or standardised classifications for MFC and PIC and, as a result, diagnoses are subject to clinical bias.

The authors of the aforementioned study obtained data from newer imaging modalities such as enhanced depth imaging OCT scans in order to try to re-define MFC and PIC in a retrospective, consecutive, observational case series of 38 eyes of 22 patients (Spaide, et al. 2013). They found that the principle sites of disease involvement in eyes with these diagnoses were the subretinal pigment epithelium and outer retinal spaces (Spaide, et al. 2013). In this small group of patients with MFC or PIC, they found no difference in
the demographics and clinical characteristics, such as visual acuity, age, sex and degree of myopic refractive error, and concluded that there seemed to be ‘limited clinical utility in trying to differentiate them’ (Spaide, et al. 2013). This was an interesting conclusion because MFC and PIC have been historically considered different diseases, with the term ‘PIC’ often being used to describe eyes with smaller lesions clustered at the posterior pole in young, myopic women (Atan, et al. 2011; Brown, et al. 1996; De Meyer, et al. 1999; Kedhar, et al. 2007). The findings of Spaide et al coincided with a developing clinical trend towards grouping MFC and PIC together as a single disease, with much discussion in the literature on this topic (Ahnoood, et al. 2017; Essex, et al. 2010; Essex, et al. 2013; Kedhar, et al. 2007; Morgan and Schatz 1986; Spaide, et al. 2013).

The epidemiological data on PIC and MFC have been limited due to the low incidence of these diseases. Grouping these two diseases together as a single entity, if they are in fact different diseases, could have detrimental consequences for patient management. Therefore, the aim of this study was to provide clinical epidemiological data to assist in disease phenotyping and the determination of whether MFC and PIC should be regarded as different diseases. It has been previously observed that imaging modalities did not differentiate between MCP and PIC, but it was also conceded that this might be due to the disease definitions used in the study (Spaide, et al. 2013). Therefore, in this study, a comparison of demographic and clinical characteristics was undertaken for a large cohort of patients who were observed over a 5-year period and classified as MFC or PIC, according to chorio-retinal lesion location and associated inflammation. A standardised approach was used to minimise subjective clinical diagnostic bias.

2.1.5 Non-invasive clinical imaging of the eye
Colour images of the ocular fundus (the part of the posterior segment of the eye, which is opposite to, and viewed through, the pupil) may assist with
diagnosis and documentation of abnormalities observed in the retina and posterior choroid on clinical examination, such as retinitis and/or choroiditis (Foster and Vitale 2002). Ultra-widefield images, such as those captured by the Optos fundus camera (Optos, Dunfermline, Scotland, UK) are being increasingly used in the clinical management of uveitis. These images, which can capture up to 200-degrees of the ocular fundus in a single photograph, reveal a wider and more peripheral view of the fundus than the standard 30-to 50-degree fundus images.

Fundus autofluorescence (AF) imaging is based on the principle that increased (hyper) or decreased (hypo) autofluorescence emitted from ocular fluorophores, such as lipofuscin, in the retinal pigment epithelium, may indicate retinal pathology. AF imaging has value in that it may detect retinal abnormalities beyond those detected on clinical funduscopic examination.

Optical coherence tomography (OCT) imaging is analogous to ultrasound B-scan imaging, except that it uses light waves rather than sound waves to obtain images of retinal structures of a higher longitudinal resolution. OCT is based on the principle of white light or low coherence interferometry, that is, that light reflected or scattered off the subject under investigation interferes with light from a reference arm (Figure 23):

- The light originates from a light source and is split into two arms by the beam splitter.
- The light incident on the tissue undergoes partial backscattering, due to the presence of discrete, as well as a continuum of, reflection sites at different depths within the ocular tissue.
- The two beams will interfere if the path length difference of the two arms in the interferometer is within the coherence length of the optical signal,
- At the output of the interferometer the backscattered light from the tissue is then recombined with the light from the reference arm.
• The interference signal recorded at the detection end is then used to extract axial structural information of the tissue, which can be represented as an A-scan.
• The beam incident on the tissue is scanned laterally and a series of A-scans are collected and then used to obtain the cross-sectional image of the tissue.
• The coherence gating allows the detection system to discriminate between reflections from closely spaced reflectors, thus enabling high resolution imaging.

Figure 23: Principles of Optical Coherence Tomography (OCT)

Spectral-domain OCT (SD-OCT), also known as Fourier-domain OCT (FD-OCT), is a subtype of OCT imaging where the reference mirror is fixed and the interference pattern is detected spectrally and converted to spatial information by the Fourier transformation (Figure 23). This allows greater tissue resolving power, higher scan density, and faster data acquisition than previous versions of the technology, allowing better visualisation of retinal
Enhanced depth imaging (EDI) OCT (Figure 25) allows enhanced visualisation of choroid and sclera, in addition to the retinal layers. This is achieved by displacing the zero delay point, the point of maximal OCT signal sensitivity, closer to the choroid rather than the inner retinal layers (Baltmr, et al. 2014). This technique facilitates quantitative measurement of choroidal thickness with high reliability and reproducibility (Baltmr, et al. 2014).

Figure 24: SD-OCT showing normal retinal layers: NFL: Nerve fiber layer. GCL: Ganglion cell layer. IPL: Inner plexiform layer. INL: Inner nuclear layer. OL: Outer plexiform layer. ONL: Outer nuclear layer. ELM: External limiting membrane. PIS: Photoreceptors inner segments. POS: Photoreceptors outer segments. RPE: Retinal pigment epithelium
Figure 25: Enhanced depth optical coherence tomographic B-scan using a Heidelberg Spectralis OCT (Heidelberg Engineering, Germany). (a) Near-infrared fundus image, (b) corresponding EDI-OCT demonstrating normal retinal and choroidal anatomy at the macula. Note that both retinal and choroidal layers can be clearly identified on the same scan. (Baltmr, et al. 2014)

2.2 Methods

2.2.1 Ethical approvals
The study adhered to the tenets of the Declaration of Helsinki and was approved by the local institutional review boards (National Health Service Research Ethics Committee Approval, R&D reference: 16039, in the UK and The Institutional Review Board of Rabin Medical Center in Israel).

2.2.2 Literature search
A literature search was conducted on MEDLINE by combining searches for the terms ‘multifocal choroiditis’ with (OR) ‘punctate inner choroidopathy’ in any part of the publication text (Figure 26).
2.2.3 Review of literature and determination of clinical consensus regarding disease definitions

MFC was initially described in patients who had 'punched-out' fundal lesions similar to presumed ocular histoplasmosis syndrome (POHS), a form of multifocal choroidal inflammation in Histoplasma-endemic areas of the United States (Nozik and Dorsch 1973). POHS tends to affect men and
women equally, is not significantly associated with myopia, presents a little later in life, and is rarely associated with clinically evident recurrent episodes of choroiditis (Dreyer and Gass 1984; Gass and Wilkinson 1972). The original phenotypic description of MFC was later expanded to include a variant, MFC with panuveitis, which included recurrent episodes of anterior chamber and vitreous inflammation associated with active chorioretinal lesions, ranging from 50µm to 350µm in diameter (Dreyer and Gass 1984; Morgan and Schatz 1986; Nozik and Dorsch 1973). It was later found that MFC lesions might range in size from 500µm to 1000µm and, therefore, characterised by chorioretinal lesions that were larger than initially described (Kedhar, et al. 2007). Around the same time that MFC was originally reported, PIC was first described in a series of 10 young, myopic women (aged 21 to 37 years), whose multifocal choroidal lesions, ranging from 100µm to 300µm in diameter, evolved into atrophic scars, also similar in appearance to POHS, although 7 out of the 10 women who underwent skin testing for histoplasmosis were negative (Watzke, et al. 1984). Since no anterior chamber or vitreous inflammation was clinically detected in these young women, the term PIC was used to highlight the lack of evident intraocular inflammation associated with the choroidal lesions.

A review of the literature to date suggests that authors of at least 11 previous publications have agreed that MFC and PIC are idiopathic white dot syndromes which might be clinically differentiated on the basis of their distinctive ‘fundus pattern’ (Eldem and Sener 1991), that is, morphology of chorioretinal lesions, which in the case of PIC, are focal, predominantly in the posterior pole area of the retina, whereas they extend to the periphery of the retina in MFC (Amer and Lois 2011; Brown and Folk 1998; De Meyer, et al. 1999; Gerstenblith, et al. 2007; Hirooka, et al. 2014; Hirooka, et al. 2006; Kedhar, et al. 2007; Raven, et al. 2017; Scheider 1993; Shakoor and Vitale 2012; Zarranz-Ventura, et al. 2014). There is also a consensus amongst
authors of 12 previous publications that, in cases diagnosed as PIC, there should be no history or other evidence of intraocular inflammation, in the form of anterior or vitreous cells (Ahnood, et al. 2017; Amer and Lois 2011; Atan, et al. 2011; Brown and Folk 1998; Brown, et al. 1996; De Meyer, et al. 1999; Essex, et al. 2010; Flaxel, et al. 1998; Kedhar, et al. 2007; Reddy, et al. 1996; Shimada, et al. 2008; Spaide, et al. 2013). Evidence to strengthen this clinical consensus was provided by a study, which categorised cases of MFC and PIC by morphology only, and found that cases diagnosed as PIC lacked inflammation (Kedhar, et al. 2007). It has been frequently noted that PIC has a demographic predilection for young, myopic women (Brown, et al. 1996; Flaxel, et al. 1998; Hirooka, et al. 2014; Raven, et al. 2017) and observed that patients presenting with focal chorioretinal lesions around the posterior pole area, diagnosed as PIC, are young females (Amer and Lois 2011; Chen and Hwang 2014; Eldem and Sener 1991; Gerstenblith, et al. 2007; Reddy, et al. 1996; Saito, et al. 2007; Wachtlin, et al. 2003). This predilection of PIC for young females was confirmed by a survey of 77 subjects with PIC (Gerstenblith, et al. 2007) and a larger group of 136 subjects, which included ‘atypical’ PIC with larger POHS-like lesions (Essex, et al. 2010). A few studies have also differentiated between MFC and PIC on the basis of lesion size, with being PIC diagnosed by the presence of ‘small’ yellowish white lesions, measuring 100- to 300-μm in diameter (Leung, et al. 2014) and MFC diagnosed by the presence of lesions larger than 300μm (Shimada, et al. 2008). Authors who have prospectively followed cases which they have differentiated as MFC and PIC, based on clinical features, have noted that their clinical courses are different (Atan, et al. 2011; Kedhar, et al. 2007; Shen, et al. 2011).

It is noted that there is no standardised classification criteria used to describe MFC and PIC and, therefore, where they have been differentiated,
inconsistent combinations of the above criteria have been used to define them.

Based on the described review of literature, the greatest level of clinical consensus supports that PIC is characterised by posterior pole lesions without an associated intraocular inflammatory response, which differentiates it from cases of MFC, characterised by peripheral chorioretinal lesions, with or without an intraocular inflammatory response. There appeared to be a lesser degree of consensus regarding the size of the chorioretinal lesions which characterise MFC and PIC. Many authors agreed about the predilection of PIC for young women but this was not considered a definitive characteristic for diagnosis because PIC was observed to affect subjects outside this demographic.

For the purposes of this study, it was determined that defining criteria for MFC or PIC would be based on a sequential classification approach, as follows:

1. Disease morphology, determined by chorioretinal lesion distribution, that is, whether lesions predominantly involve the posterior pole, or extend to the peripheral retina.

2. Presence of identifiable intraocular inflammation, in eyes with lesions predominantly in the posterior pole area.

Therefore, if chorio-retinal lesions were confined to the posterior pole AND there was no inflammation, the diagnosis would be ‘PIC’; If there were peripheral chorio-retinal lesions OR intraocular inflammation, the diagnosis would be ‘MFC’.

2.2.4 Study design, setting and participants
This was a cross-sectional and longitudinal observational study. The setting was tertiary referral centres for clinical management of uveitis in the United Kingdom (UK) and Israel. A total of 343 eyes of 185 study participants were...
identified as having a diagnosis of MFC or PIC from clinically coded electronic hospital records.

2.2.5 Subject selection
Subjects were identified by searching electronic hospital databases for cases of uveitis containing the terms ‘multifocal choroiditis’ and ‘punctate inner choroidopathy’ in all subjects seen at two tertiary centres, Moorfields Eye Hospital, UK and Rabin Medical Centre, Israel between September 1992 and July 2017. The database search yielded a total number of 2399 subjects with uveitis and, of these, 396 (16.5%) cases contained the relevant search terms. Subjects were then excluded if they had fewer than 5 years of follow-up records or if there was evidence of underlying infective or a systemic inflammatory diagnosis including: a fundal appearance suspicious for POHS and/or a history of travel to Histoplasma-endemic regions; a history of travel to known viral, for example, Zika, endemic regions; positive blood serology for an underlying systemic condition, such as tuberculosis or sarcoidosis, which could mimic an idiopathic white dot syndrome; or if there was any doubt, otherwise, as to the diagnosis. Following exclusion of cases, as described, a total of 185 eligible subjects were identified.

2.2.6 Data collection
All available paper casenotes, electronic records and clinical imaging, including wide field colour fundus photography, fundus autofluorescence (AF) imaging, spectral domain (SD) and enhanced depth (EDI) optical coherence tomography (OCT) imaging, were reviewed for the subjects included in the study, between March 2014 and July 2017. Clinical information, including: age; gender; best corrected visual acuity (BCVA) at baseline presentation and 5 years later; presence of myopia; refractive error
(spherical equivalent); presence of choroidal neovascularisation (both active and inactive); and whether treatment was received, was entered into an Excel (Microsoft Inc.) spreadsheet. BCVA was converted to logarithm of the minimum angle of resolution (LogMAR) units for analysis (Lange, et al. 2009).

2.2.7 Identification of peripheral retinal lesions
Subjects’ contemporaneous clinical notes and imaging, including, wide field colour fundus and AF imaging, where available, were reviewed at baseline presentation and observed over a 5-year period to identify the presence of peripheral retinal lesions. A peripheral retinal lesion was defined as any lesion outside the standardized template for the combined Early Treatment Diabetic Retinopathy Study (ETDRS) 7 standard fields on the Optos 200 degree widefield image (ResMax; Optos plc) (Figures 27-28) (Silva, et al. 2015).
Figure 27: Image (adapted from Silva, Cavallerano et al) showing ultrawide (OPTOS) field view of the retina, with the standardized template for the combined ETDRS 7 standard fields demarcated by the boundary highlighted in yellow. For the purposes of the current study, the entire fundal area outside the yellow boundary represented the peripheral retinal field. Chorioretinal lesions in the peripheral area represented ‘peripheral retinal lesions’.
Figure 28: Further image for comparison of imaging areas within the Optos 200 widefield and within the EDTRS 30 7-field (Silva, et al. 2012).

2.2.8 Identification of ‘active’ disease and intraocular inflammation

Current methods for clinical assessment of the inflammatory status of eyes with uveitis are predominantly based on clinical estimates of inflammatory signs according to standardized scales (Jabs, et al. 2005a; Nussenblatt, et al. 1985). For example, the ‘Nussenblatt scale’ for grading of vitreous haze is based on estimation of the clarity of the fundus when viewed with the indirect ophthalmoscope and a 20D lens in comparison to a set of reference photographs (Nussenblatt, et al. 1985). These scales have introduced some level of standardisation into clinical grading of inflammation but are, nevertheless, subjective systems. More recent objective methodologies of vitreous inflammation measurement, in eyes with uveitis, have compared the signal intensity of the vitreous compartment with that of the RPE on SD-OCT, to generate an optical density ratio with arbitrary units (Keane, et al. 2015a). These ‘VIT/RPE-relative intensity values’ have been shown to provide good objective measurements of vitreous inflammation, which do not appear to be
influenced by phakic status and previous vitrectomy surgery (Keane, et al. 2015b; Zarranz-Ventura, et al. 2014). OCT-derived clinical parameters are considered ‘well-defined and reliable’ endpoints for research studies and currently being developed for use in clinical trials investigating the management of uveitis (Denniston, et al. 2017). These objective methods for clinical assessment of inflammation are likely to be valuable but they require further validation and were, therefore, not included in the methodology of the current study.

In the current study, uveitic disease activity was determined by standard clinical methods used in a specialist hospital setting: clinical assessment of subject history; physical examination; retinal and choroidal imaging (including wide field imaging); and previous clinical documentation of disease activity. ‘Active’ disease included new visual symptoms in combination with anterior chamber or vitreous chamber activity and/or new chorioretinal lesions, but excluded choroidal neovascularisation. ‘Inactive’ disease was the absence of disease activity, as described. Prior history of intraocular inflammation was determined by reviewing all previous clinical documentation and imaging for evidence of vitreous and/or anterior chamber activity.

**2.2.9 Classification of eyes as ‘PIC’ or ‘MFC’, according to presence of peripheral lesions and intraocular inflammation**

Following clinical data collection and image analysis of all cases clinically coded as idiopathic MFC and PIC, eyes were classified as PIC or MFC only according to the location and distribution of their lesions and identifiable evidence of inflammation (as detailed above). An approach was used in which eyes with lesions at the level of the RPE or inner choroid, in the absence of any other evidence of intraocular inflammation, and the absence of peripheral lesions were designated as PIC (Figure 29A). Following this, the remaining cases with peripheral multifocal lesions at the level of the RPE
or inner choroid, with or without evidence of anterior chamber or vitreous inflammation, were identified as MFC (Figure 29B). Observers were masked to the clinical diagnosis during image analysis to minimise bias.

![Figure 29: Colour images showing A: typical fundal appearance of ‘PIC’ B: typical fundal appearance of ‘MFC’ classified using the study criteria.](image)

2.2.10 Chorioretinal lesion size analysis from fundus AF images

The size of chorioretinal lesions at the posterior pole retinal area (defined as the area within the retinal arcades) of each eye on was determined from fundus autofluorescence (AF) images. All AF images included a standardised scale in the bottom left hand corner of the image. Colour fundus images, on the other hand, did not include a standardised scale. Furthermore, lesions could be detected on AF images, which were not visualised on colour fundus image and lesions on AF images had clear boundaries, which constituted defined endpoints for measurement. An image analysis software package (ImageJ, National Institutes of Health, USA) was used to measure chorioretinal lesion size as follows: the horizontal diameter (X) of each lesion at the posterior pole was measured images taken during
an inactive phase of disease (Figure 30A). The measurement was transformed according to the standardised 500µm image scale, as follows:

**Mean lesion diameter** \( \times 500(\mu m) \)

\( \chi \)

The horizontal diameter of every lesion in the posterior pole region of an eye was determined and these measurements were used to calculate mean posterior pole lesion size for this eye. Choroidal neovascular (CNV) lesions were excluded from measurement, as these were considered to represent a secondary complication of the disease, rather than a chorioretinal lesion characterising the primary disease. CNV lesions were identified and excluded from analysis on the basis of their AF imaging features, which included: their foveal location; their larger size in comparison to the typical chorioretinal lesions which characterised the primary disease; and their heterogenous features on AF imaging, including areas of hypo- and hyper-autofluorescence (Figure 30B).
Figure 30: The horizontal diameter (X) of each lesion at the posterior pole was measured using images taken during an inactive phase of disease (Figure A) and transformed according to the standardised 500µm image scale. CNV lesions were identified and excluded from analysis on the basis of their AF imaging features, which included: their foveal location; their larger size in comparison to the typical chorioretinal lesions which characterised the primary disease; and their heterogenous features on AF imaging, including areas of hypo- and hyper-autofluorescence (Figure B).

2.2.11 Qualitative analysis of anatomical disease features using SD-OCT images

Imaging was acquired by experienced technicians using SD-OCT technology (Spectralis, Heidelberg Engineering, Germany). This imaging device captures near infrared fundus photographs with a 55° field of view. A standardized, qualitative analysis of retinal and choroidal morphology on SD-OCT imaging was performed, as per previously described qualitative analyses of MFC and/or PIC (Spaide, et al. 2013; Zarranz-Ventura, et al. 2014), evaluating for:

- presence of infiltration into the outer retinal and/or subretinal space (Figures 31a and 33)
- subretinal exudation (Figure 32)
- loss of anatomical integrity of the retinal layers (Figure 31b)
• retinal pigment epithelium (RPE) elevation: homogenous (Figure 33) or heterogenous (Figure 34)

• intraretinal spaces or cystoid macular oedema (Figure 35)

Figure 31: SD-OCT image showing: a) infiltration into subretinal and outer retinal space and b) loss of anatomical integrity of the retinal layers (Spaide, et al. 2013)

Figure 32: SD-OCT image showing subretinal exudation (Spaide, et al. 2013)

Figure 33: SD-OCT image showing a hyperreflective dome-shaped homogenous elevation of the RPE (white arrow) and an adjacent infiltration into the subretinal space (triangular arrowheads) (Spaide, et al. 2013)
Figure 34: SD-OCT image showing a hyperreflective heterogenous elevation of the RPE

Figure 35: SD-OCT image showing cystoid macular oedema in a uveitic eye (courtesy of Dr Lazha Sharief)

2.2.12 Quantitative analysis of choroidal thickness using EDI-OCT images

EDI scans were acquired on the SD-OCT machine by experienced technicians, using a custom EDI scanning protocol described in a previous study by another group at the same institution: ‘each EDI-OCT image set consisted of 7 OCT B-scans obtained in a 20° x 5° horizontal raster pattern centred around the fovea and each individual B-scan was generated from 50 averaged scans’ (Zarranz-Ventura, et al. 2014). The aforementioned study performed a quantitative analysis of ‘PIC’ cases using custom software (OCTOR; Doheny Image Reading Center, Los Angeles, CA), which allowed manual delineation of any morphologic compartment of interest and then provided detailed quantitative analysis of this compartment’ (Zarranz-Ventura, et al. 2014). The OCTOR software was not available for general
use, so was not used for the current study. It is of note that the automated method of choroidal thickness measurement, using OCTOR software, requires the boundaries for the neurosensory retina, RPE and choroid to be manually segmented (Zarranz-Ventura, et al. 2014) and is, therefore, still subject to introduction of human error at this level of analysis. The current study used the following methods to measure choroidal thickness, which are based on similar principles of quantitative analysis used in the study by Zarranz-Ventura, Sim et al, except that measurements were performed manually, using the caliper function of the SD-OCT software on the EDI-OCT scans.

Determination of the boundaries for the neurosensory retina, RPE and choroid and estimation of the choroidal thickness within the central macular area were, additionally, informed by previously published methodology for manual evaluation of choroidal thickness in ocular inflammation (Baltmr, et al. 2014; Géhl, et al. 2014). Briefly, choroidal thickness was measured in the central subfield (CSF) of the standardised Early Treatment Diabetic Retinopathy Study (ETDRS) grid, a region with a diameter of 1.0 mm around the fovea, on the EDI-OCT scan. A central choroidal thickness measurement was taken (CSF) in addition to two nasal (N1 and N2) and two temporal (T1 and T2) measurements at the ETDRS grid outer ring diameters of 3 and 6 mm respectively. Choroidal thickness measurements were performed perpendicular to the RPE, using the outer border of the hyper-reflective line corresponding to the RPE/Bruch membrane complex as the inner boundary and outer border of the choroid stroma as the outer boundary (Figure 36).
Figure 36: Choroidal thickness was measured in the central subfield (CSF) of the standardised Early Treatment Diabetic Retinopathy Study (ETDRS) grid, a region with a diameter of 1.0 mm around the fovea, on the EDI-OCT scan.
This method of manual choroidal thickness measurement using EDI-OCT scans has been shown to be the most reproducible (Vuong, et al. 2016). The mean thickness of the choroid at the 3 mm and 6 mm rings (the mean of N1, N2, T1, T2 and CSF choroidal thickness measurements) was calculated and used for further statistical analysis. Image analysis was performed by a single experienced observer and independently reviewed by a further experienced observer.

2.2.13 Main study outcome measure
The main outcome measure used in this study was the difference in observed clinical and imaging characteristics between eyes classified as MFC or PIC, as above.

2.2.14 Data analysis and statistics
Study data was analysed with frequency and descriptive statistics using Instat software package version 3 (GraphPad Inc.) and SPSS software package version 22 (SPSS Inc, Chicago, IL, USA). Cohen's kappa coefficient was used to calculate inter-rater agreement for qualitative measurements. Continuous data were plotted as frequency histograms and statistical tests for normality were performed. Data is presented in mean ± standard deviation for normally distributed data, and median ± interquartile range for skewed data. Categorical variables are presented as a percentage (%) of n. It is of note that, in this study, no discordant diagnoses of MFC and PIC were found in eyes from the same subject. Intraclass correlation coefficients (ICC) were calculated for paired eye data and high correlations between pairs of eyes were found for some variables. The dataset was not appropriate for generalised linear modelling with adjustment for correlation by a general estimating equation (GEE), so data was analysed using
univariate statistical tests: student T-test, Mann Whitney U test or Fisher’s exact test, as appropriate (Fan, et al. 2011). The assumptions for these tests include the assumption of independence, and so, in cases where subjects had both eyes included in the study and there were high correlations between pairs of variables, the right eye was included in the analysis (Fan, et al. 2011). P-values obtained from statistical tests were adjusted using the Benjamini-Hochberg correction for false discovery rate (FDR) in multiple analyses. All tests were two-tailed, and an adjusted P-value of < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Demographics and clinical characteristics of the entire cohort
The age range across the entire cohort of 185 subjects was 11 to 89 years with final (5-year) BCVA of 2.3 to -0.2 LogMAR (light perception to 20/12.5 Snellen) (Figures 37-38). Eighty-two percent of eyes were from female subjects and 74% were myopic, with refractive errors across the whole group varying from +3.00 to -17.00D (spherical equivalent) (Figure 39). Fifty-eight percent had a previous history of choroidal neovascularisation. During the 5-year period of observation, one eye from the cohort (0.3%), which had chorioretinal lesions confined to the posterior pole at baseline, was observed to develop peripheral lesions associated with anterior chamber inflammation.
No eyes developed peripheral lesions in the absence of inflammation over this period of observation.

Figure 36: Frequency distribution of age (years) across the cohort

Figure 37: Frequency distribution of right eye refractive error (spherical equivalent) across the cohort
2.3.2 Comparison of demographics and clinical characteristics

Following classification as PIC or MFC, 169 eyes had MFC and 174 eyes had PIC. Figures 36-38 show the comparative frequencies of visual acuity, age and refractive error at baseline across the group following classification as MFC or PIC. Table 9 summarises the demographic and clinical differences between MFC and PIC groups.

There was no significant difference in the prevalence of bilateral disease in the subjects with PIC in comparison to subjects with MFC. The median BCVA at baseline presentation was logMAR 0.0 (20/30) in both the PIC and MFC groups (p= 0.235); and logMAR 0.2 (20/32) in the PIC and logMAR 0.0 (20/30) in the MFC group at 5 years (p=0.235). However, eyes with PIC were from significantly younger subjects than eyes with MFC (33 years vs 40 years, p=0.006); the proportion of eyes in females was significantly higher in the PIC group (93% vs 71%, p=0.001); a significantly higher proportion of
PIC eyes had CNV (73% vs 43%, p=0.001); and a significantly higher proportion of PIC eyes also had myopia (any myopic refractive error) (91% vs 59%, p=0.001), with a significantly higher degree of myopic refractive error (spherical equivalent) (-7.50D vs -2.75D, p=0.001).
Table 9: Demographics and clinical characteristics of subjects with PIC and MFC

<table>
<thead>
<tr>
<th></th>
<th>Punctate Inner Choroidopathy</th>
<th>Multifocal Choroiditis</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of eyes</td>
<td>174</td>
<td>169</td>
<td></td>
</tr>
<tr>
<td>Subjects with bilateral disease (%)</td>
<td>80 (85)</td>
<td>78 (86)</td>
<td>0.255</td>
</tr>
<tr>
<td>Baseline logMAR right VA (Snellen)</td>
<td>0.0±0.5 (20/20)</td>
<td>0.0±0.5 (20/20)</td>
<td>0.235</td>
</tr>
<tr>
<td>5 year logMAR right VA (Snellen)</td>
<td>0.2±0.5 (20/32)</td>
<td>0.0±0.5 (20/20)</td>
<td>0.235</td>
</tr>
<tr>
<td>Age (years) at baseline</td>
<td>33±15</td>
<td>40±21</td>
<td>0.006*</td>
</tr>
<tr>
<td>Females (%)</td>
<td>162 (93)</td>
<td>120 (71)</td>
<td>0.001*</td>
</tr>
<tr>
<td>CNV (%)</td>
<td>127 (73)</td>
<td>73 (43)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Myopia (%)</td>
<td>158 (91)</td>
<td>100 (59)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Median spherical equivalent (D)</td>
<td>-7.50±0.5</td>
<td>-2.75±0.6</td>
<td>0.001*</td>
</tr>
<tr>
<td>Received any treatment (%)</td>
<td>84 (48)</td>
<td>137 (81)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Systemic immunosuppression (% of treatment)</td>
<td>50 (60)</td>
<td>125 (91)</td>
<td>0.001*</td>
</tr>
<tr>
<td>Anti-VEGF injections (% of treatment)</td>
<td>64 (76)</td>
<td>25 (15)</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

2.3.3 Comparison of treatment received

Table 9 summarises the differences in treatment received by the two groups. The overall proportion of eyes receiving any treatment (laser photocoagulation, photodynamic therapy, surgical excision, intravitreal injections and/or oral immunosuppression) was significantly larger in the MFC group than the PIC group (81% vs 48%, p= 0.001), which included a significantly greater proportion of patients who received systemic immunosuppression (91% vs 60%, p=0.001). Indications for immunosuppressive treatment included intraocular inflammation, new chorioretinal lesions, recurrent secondary CNV, decreased BCVA and visual symptoms in combination with evidence of intra- or sub-retinal fluid or lesion progression on clinical imaging. Anti-VEGF injections were received by a higher proportion of eyes with PIC compared to MFC (76% vs 15% p=0.001).
2.3.4 Comparison of imaging features

Spectralis imaging data was collected from 55 eyes in the active phase of disease, which included 25 eyes with PIC and 30 eyes with MFC; and from 259 eyes in the inactive phase of disease, which included 128 eyes with PIC and 131 eyes with MFC (not all of the study eyes had Spectralis imaging). Table 10 summarises the findings from the fundus AF, SD-OCT and ED-OCT analyses in active and inactive phases of disease, respectively. Inter-rater agreement between measurements was high ($\kappa = 0.78$). Qualitative analysis of anatomical features on OCT imaging did not demonstrate any significant differences in the presence of infiltration into the subretinal or outer retinal spaces, subretinal exudation, anatomical integrity of the retinal layers, retinal pigment epithelium (RPE) elevation, intraretinal spaces or cystoid macular oedema or alterations in the choroidal reflective pattern between eyes classified as MFC or PIC. Furthermore, there was no significant difference in the frequency of lesions in the perifoveal (within the ETDRS macular grid) area of eyes with PIC or MFC in the active and inactive phases of disease. Although there was no observed difference in frequency of ERMs between MFC and PIC in the active phase of disease, in the inactive phase of disease, a greater proportion of eyes in the MFC group had ERM (50% vs 12%, $p=0.011$). The median central choroidal thickness, during the active phase of disease was greater in eyes diagnosed with PIC (238µm) compared to eyes diagnosed with MFC (189µm), however this was not found to be statistically significant ($p=0.690$). There was no difference in the median central choroidal thickness, during the inactive phases of disease, between MFC and PIC (182µm vs 189µm, $p=1.000$). The median diameter of lesions within the posterior pole area of the retina during the inactive phases of disease ranged from 100µm to 833µm in the PIC group and from 101µm to 1140µm in the MFC group. The median lesion diameter in MFC
group (378µm) was not significantly greater (p=0.276) than that of the PIC group (296µm).

Table 10: Multi-modal imaging features of eyes with PIC and MFC during active and inactive phases of disease

<table>
<thead>
<tr>
<th>Eyes imaged in active phase of disease</th>
<th>Punctate Inner Choroidopathy</th>
<th>Multifocal Choroiditis</th>
<th>Adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subretinal and/or outer retinal infiltration (%)</td>
<td>25 (85)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Subretinal exudation (%)</td>
<td>8 (31)</td>
<td>13 (44)</td>
<td>0.702</td>
</tr>
<tr>
<td>Loss of anatomical integrity (%)</td>
<td>25 (100)</td>
<td>26 (86)</td>
<td>0.690</td>
</tr>
<tr>
<td>RPE elevation by material (%)</td>
<td>21 (84)</td>
<td>21 (70)</td>
<td>0.690</td>
</tr>
<tr>
<td>Homogenous (%)</td>
<td>10 (48)</td>
<td>8 (37)</td>
<td>0.690</td>
</tr>
<tr>
<td>Heterogenous (%)</td>
<td>11 (52)</td>
<td>13 (63)</td>
<td>0.690</td>
</tr>
<tr>
<td>Intraretinal spaces or cystoid macular oedema (%)</td>
<td>10 (39)</td>
<td>19 (63)</td>
<td>0.690</td>
</tr>
<tr>
<td>Epiretinal membrane (%)</td>
<td>4 (14)</td>
<td>19 (63)</td>
<td>0.121</td>
</tr>
<tr>
<td>Median central choroidal thickness (µm)</td>
<td>238±107</td>
<td>189±89</td>
<td>0.690</td>
</tr>
<tr>
<td>Perifoveal lesions (%)</td>
<td>20 (80)</td>
<td>19 (63)</td>
<td>0.690</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eyes imaged in inactive phase of disease</th>
<th>128</th>
<th>131</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median lesion size (µm)</td>
<td>296±103</td>
<td>378±200</td>
</tr>
<tr>
<td>Subretinal and outer retinal infiltration (%)</td>
<td>82 (64)</td>
<td>92 (70)</td>
</tr>
<tr>
<td>Subretinal exudation (%)</td>
<td>8 (6)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Loss of anatomical integrity (%)</td>
<td>105 (82)</td>
<td>107 (82)</td>
</tr>
<tr>
<td>RPE elevation by material (%)</td>
<td>105 (82)</td>
<td>86 (66)</td>
</tr>
<tr>
<td>Homogenous (%)</td>
<td>64 (61)</td>
<td>56 (65)</td>
</tr>
<tr>
<td>Heterogenous (%)</td>
<td>41 (39)</td>
<td>30 (35)</td>
</tr>
<tr>
<td>Intraretinal spaces or cystoid macular oedema (%)</td>
<td>23 (18)</td>
<td>34 (26)</td>
</tr>
<tr>
<td>Epiretinal membrane (%)</td>
<td>15 (12)</td>
<td>66 (50)</td>
</tr>
<tr>
<td>Median central choroidal thickness (µm)</td>
<td>182±87</td>
<td>189±119</td>
</tr>
<tr>
<td>Perifoveal lesions (%)</td>
<td>78 (61)</td>
<td>73 (56)</td>
</tr>
</tbody>
</table>
2.4 Discussion

This study examining eyes with diagnoses of MFC or PIC represents the largest to date and provides some evidence that these diseases are phenotypically different, with PIC showing a predilection for younger, more highly myopic women and secondary CNV development. Subjective clinical diagnostic bias was minimised in the methods by classifying eyes as having a diagnosis of PIC or MFC using a standardised approach, based on clinical consensus of disease characteristics (fundal distribution of lesions and presence of intraocular inflammation) in the literature to date. Eyes which may have been previously diagnosed with ‘atypical PIC’ (Essex, et al. 2010) were classified as having MFC. There were no discordant diagnoses between eyes. Eyes with chorioretinal lesions confined to the posterior pole were observed over 5 years for phenotypic stability and, in the absence of intraocular inflammation, none of these eyes developed peripheral lesions. It was determined from our data that MFC and PIC differ significantly in demographic and clinical characteristics. Our findings contrast with those from Spaide et al, who compared MFC with panuveitis and PIC in a smaller retrospective case series of 38 eyes of 22 patients, using multi-modal imaging and a disease definition based on clinical evidence of inflammation only (and not lesion location), and did not observe any clinical differences (Spaide, et al. 2013). However, similarly to Spaide et al, we found that lesions in both groups involved the outer retina and sub-RPE space.

A higher proportion of eyes in our MFC group received treatment, of any kind, compared to the PIC group. The PIC group, however, received more anti-VEGF injections, due to the higher association of CNV. This did not result in any significant differences in the BCVA between the PIC and MFC groups five years after baseline presentation. These findings are partly supported by research concurrent to this study, which suggested that visual
loss is common in subjects with PIC, predominantly secondary to late development of CNV; and that treatment with oral corticosteroids may help to reduce the risk of CNV development, with anti-VEGF therapy for CNV being associated with better clinical outcomes (Niederer, et al. 2017).

These data show that PIC was associated with a significantly higher proportion of eyes with myopia and a higher degree of myopic refractive error than MFC. It was originally suggested that the lesions in PIC were secondary to myopia, representing ‘a lacquer-crack’ type scar (Watzke, et al. 1984). However, PIC pathogenesis is now thought to be immune-mediated and SD-OCT findings demonstrate that the breach in Bruch’s membrane occurs later in PIC lesion development, potentially as a consequence of inflammatory disruption (Channa, et al. 2012; Zhang, et al. 2013a). Furthermore, it has been shown that ocular inflammation, caused by Kawasaki Disease, independently increased the risk of myopia (in a population with a high prevalence of myopia) (Kung, et al. 2017). This might suggest that the myopia observed in PIC subjects could be a consequence of the inflammation rather than the predisposing cause for the observed lesions.

Against this assertion, however, is the fact that the inflammation in Kawasaki disease occurs in childhood, and there is no substantial evidence, as yet, which demonstrates that chorio-retinal inflammation onset occurs during childhood, in subjects with PIC. In the current study, the youngest subject age at first presentation with PIC was 15 years and the median age of presentation, 33 years.

Subjects with PIC have been reported to have personal and/or family history of autoimmune disease with a prevalence of 3-26% (Atan, et al. 2011; Gerstenblith, et al. 2007). It is well known that autoimmune disease is more common in women, which may be due to the effect of sex hormones, microchimerism, gene encoded by X or Y chromosomes, X chromosome inactivation and differing innate immune responses to environmental factors,

It could be argued that since PIC manifests with posterior pole lesions and a higher prevalence of secondary CNV, these patients are more likely to notice visual symptoms and present at an earlier age. However, our SD-OCT analysis did not reveal any significant difference in the prevalence of perifoveal lesions in the active and inactive phases of disease between eyes with MFC or PIC.

Evidence of anterior chamber or vitreous inflammation, required for our classification of eyes as MFC or PIC, was determined from clinical examination and review of clinical documentation and imaging. Since AC and vitreous inflammation (if it occurs in MFC) is episodic, it is possible that this may not have been captured during a clinic visit. Nevertheless, the fact that the MFC group with inactive disease had a higher proportion of subjects with ERM than the PIC group suggests that there was a higher prevalence of previous intraocular inflammation in this group.

It was observed that the mean choroidal thickness in active PIC was greater than in active MFC but this difference was not statistically significant, probably since fewer eyes were imaged during the active phase of disease compared to the inactive phase. A standardised manual caliper approach was used to measure central choroidal thickness in subjects with MFC and PIC. Due to the influence of myopia on chorioretinal thickness and its association with PIC, the effect of inflammatory activity on chorioretinal thickness was likely underestimated. A previous study has demonstrated that, in subjects with a diagnosis of PIC, choroidal thickness increased throughout the active phase in 30% of the 27 eyes in which lesions progressed (Zhang, et al. 2013a). Although not convincingly demonstrated in the current study, it is possible that inflammation in active PIC involves the choroid to a greater extent than the inflammation in MFC. Mean lesion size in
this study was based on AF images. Due to the clear delineation of lesion borders on AF images, a standardised image scale and a higher number of images available for analysis, this meant that the measurements taken had greater reliability and validity than measurements taken from colour fundus photos. However, given that areas of chorioretinal atrophy may enlarge over time in myopic eyes, retrospective clinical differentiation between these two diseases, based on lesion size, remains challenging.

Studies to date comparing MFC and PIC have been limited by small sample size and lack of standardisation in disease definitions. Currently, many clinicians do not believe that there is any clinical utility in differentiating between MFC and PIC, which predisposes towards diagnostic bias and away from individualised disease management. Results based on ultrastructural electron microscopy (Pachydaki, et al. 2012) support the hypothesis that PIC is an inflammatory disease originating in the choroid which predominantly affects young myopic women. In another study of 42 patients with PIC, the authors conclude, based on SD-OCT findings, that PIC is a chorioretinitis rather than an inner choroidopathy. They describe a 5 stage evolution process initially involving the inner choroid and progressing to disrupt the RPE and Bruch’s membrane, with the formation of a chorioretinal nodule being ‘the core event of PIC inflammation’ (Zhang, et al. 2013a). Other studies have shown the value of AF imaging in monitoring progression of PIC (Hua, et al. 2014; Kramer and Priel 2014). Although advances in multi-modal imaging have allowed us to analyse the posterior pole at a greater level of detail, this has not thus far facilitated more specific differentiation between these two syndromes. Detailed clinicopathological studies of these diseases are not often feasible but our group and others have previously demonstrated that clinical inflammation in MFC is associated with infiltration of B-lymphocytes, particularly in the CNV, which has not been observed in PIC (Dunlop, et al. 1998; Shimada, et al. 2008). As yet, there is no further
evidence to support distinct pathophysiological processes in PIC and MFC, apart from the proposed differences in clinical phenotype.

In summary, these data provide some evidence to support clinical differentiation between MFC and PIC on the basis of chorioretinal lesion location and associated inflammation. It is demonstrated here that subjects with diagnoses of PIC or MFC, based on these criteria, had significantly different demographics and showed phenotypical stability over a 5-year period. These could represent important differences in disease pathophysiology and phenotype, which have relevance for individualised management. The phenotypical differences identified here between cases classified as PIC or MFC could be further investigated in conjunction with pathophysiological data and/or assessment of effects on retinal function in a future study.
Chapter 3 An Investigation into the Role of Treg in Clinical Remission of Sight-threatenig Non-infectious Uveitis: Materials and Methods

3.1 Background to methods

3.1.1 Study background and rationale
Non-infectious uveitis is a clinically heterogeneous disease. The overall aim of the laboratory studies was to improve the phenotyping of uveitis through identification of immunological biomarkers, such as T-regulatory cells, associated with clinical remission, which could allow more individualised patient management by helping to predict which patients are at risk of inflammatory disease relapse.

3.1.2 Study aims
1. To investigate the association of immunological biomarkers (peripheral blood levels of Treg, Th1 and Th17, their related cytokines and methylation changes at epigenetic loci of their signature transcription factors) with sustained clinical remission of non-infectious uveitis, in patients who have previously received treatment with oral immunosuppression.

2. To develop a clinically relevant statistical model for predicting clinical remission in non-infectious sight-threatening uveitis.
3.1.3 Hypothesis:
Clinical remission of non-infectious uveitis in patients, who have previously received treatment with oral immunosuppression, is associated with an ‘immunoregulatory’ signature, characterised by upregulation of phenotypically stable T-reg

3.1.4 Objectives
- To isolate peripheral blood mononuclear cells (PBMC) from patients from non-infectious uveitis in clinical remission for comparison of Treg, Th1 and Th17 population frequency in peripheral blood with those from patients with active uveitis and control subjects.
- To compare the immunosuppressive function of Treg isolated from patients with non-infectious uveitis in clinical remission with those from patients with active uveitis and control subjects.
- To isolate peripheral blood sera from study subjects for analysis of serum cytokine levels of IL-10, IL-22, IL-17A, IFNγ and TGFβ.
- To isolate high quality DNA from PBMC from study subjects for CpG methylation site analysis of epigenetic loci, relevant to Treg, Th1 and Th17 lineages.
- To compare serum cytokine levels and CpG methylation levels in samples from patients in clinical remission with those from patients with active uveitis and control subjects.
- To analyse relationships between the serum cytokine levels and percentage (%) DNA methylation with previous immunosuppressive treatment duration, % frequency of Treg, TIGIT+ Treg, Th1 and Th17 across the whole study population.
• To statistically model the relationship variables analysed above, in order to assist individualised clinical management of patients with sight-threatening non-infectious uveitis.

3.1.5 Immunophenotyping clinical remission of sight-threatening non-infectious uveitis

Treatment paradigms of systemic autoimmune diseases, such as rheumatoid arthritis (RA) and inflammatory bowel disease, have evolved beyond partial symptom control, alone, towards the induction and maintenance of sustained biological remission. This is also known as a ‘treat to target’ strategy, with the goal of improving long-term disease outcomes (Colombel, Louis, Peyrin-Biroulet, Sandborn, & Panaccione, 2012). A working definition of sustained ‘deep remission’, which includes long-term biological remission and symptom control with defined patient outcomes, including no disease progression, has been proposed for Crohn’s disease (Colombel et al., 2012; Zallot & Peyrin-Biroulet, 2013). Clinical remission in uveitis, however, has not been defined or investigated as an immunologically distinct phase of disease. Understanding the immunological mechanisms underlying disease remission, in addition to the focus on inhibiting inflammation, could have significant implications for clinical phenotyping of uveitis and determining the best therapeutic approach to induce remission for each patient.

3.1.6 Immunophenotyping ocular inflammation using peripheral blood samples

This study was designed to detect peripheral blood biomarkers associated with ocular inflammation. All recruited patients had forms of sight-threatening non-infectious uveitis severe enough to require systemically administered immunosuppressive treatment, which is known to be effective in ocular treating inflammation. The response of the eye to immunosuppressive treatment, in contrast to other organs, such as the bowel, can be directly
visualised, without necessitating an invasive procedure. Biopsies of aqueous or vitreous humour are occasionally performed as part of routine clinical management of sight-threatening uveitis and are required in cases when clinical presentation and/or screening blood tests point towards an infective or neoplastic cause of inflammation.

The supervisors of this research have previously analysed aqueous humour samples from the eyes of patients with uveitis (Ooi, Galatowicz, Towler, Lightman, & Calder, 2006). However, the procedure of direct intraocular sampling, itself, carries risks of introducing infection, triggering or exacerbating inflammation and causing retinal detachment to the eye being sampled. Even when it is deemed clinically necessary, sequential/ repeated intraocular sampling should be avoided. In this study, all patients were screened for known common infective causes of uveitis and, therefore, additional intraocular sampling for immunophenotypic analysis was not undertaken as part of this study.

The study methods for collection of peripheral blood samples excluded patients with known systemic diagnoses, for example, sarcoidosis or Behçet’s disease, and any other identifiable systemic inflammation. Patients with anterior uveitis were also excluded from recruitment, as this form of ocular inflammation is confined to the anterior segment of the eye, and therefore, tends not be sight-threatening and/or necessitate systemic immunosuppression.

The blood components of immunological interest were collected from the plasma and leukocytes fractions of the samples.

The typical composition of human blood is as follows:

- 55% plasma
- 45% cells
95% erythrocytes
5% platelets
marginal – 1% leukocytes

The typical composition of the leukocyte compartment is as follows:

- granulocytes (polymorphonuclear leukocytes)
  - neutrophils 30-80%
  - basophils 0.4%
  - eosinophils 2.3%

- agranulocytes (mononuclear leukocytes)
  - lymphocytes 30-40%
  - monocytes/ macrophages 2-12%
  - dendritic cells 0.2-1%

Differential blood counts give relative percentages of each type of leukocyte within the blood sample. Approximate reference ranges for differential leukocyte counts in normal adults are as follows:

- Neutrophils - 2.0–7.0×10⁹/L (40–80%)
- Lymphocytes - 1.0–3.0×10⁹/L (20–40%)
- Monocytes - 0.2–1.0×10⁹/L (2–10%)
- Eosinophils - 0.02–0.5×10⁹/L (1–6%)
- Basophils - 0.02–0.1×10⁹/L (<1–2%)
3.1.7 Flow cytometric immunophenotyping of peripheral blood for T-reg, Th1, Th17 and related cytokines

3.1.7.1 Immunophenotyping by flow cytometry

Flow cytometry has become a standard laboratory tool for identifying homogeneous subsets of cells within the complex immune system. Identification and enumeration of immune cell subsets is referred to as ‘immunophenotyping’. This technology makes use of monoclonal antibody reagents (which recognise specific epitopes on immune cells), directly conjugated to a ‘fluorochrome’ dye. The dyes have characteristic photochemical properties which allow them to absorb and emit electromagnetic radiation of different wavelengths in the visible spectrum, based on the energy lost during the return of excited electrons to their ground state (Fleisher, 2013). The energy absorbed and emitted is associated with their illumination by a specific wavelength of light, where the emitted light has a longer wavelength (lower energy) than the wavelength of the excitation beam (Fleisher, 2013).

Flow cytometry can be used to test for the presence and absence of cell surface antigens and the use of panels of monoclonal reagents can help address questions regarding cell differentiation (Fleisher, 2013). The clinical application of this technology has been facilitated by a series of developments in flow cytometry instruments (hardware), systems for data analysis (software) and fluorescent dye chemistry and the range of monoclonal antibody-dye conjugates now available.

The older systems could only incorporate two- to four-color flow cytometric measurements, however it is now possible to simultaneously measure up to 18 distinct fluorescence and two scattered light parameters on each cell.
Multicolour flow cytometry can, therefore, provide a comprehensive overview of immunophenotypic information, for each sample studied.

Flow cytometric data is analysed by acquisition of cells on a flow cytometer, followed by a method known as cell ‘gating’, a process by which cell populations of interest are defined based on two non-fluorescent parameters (forward and side light scatter) in addition to mean fluorescent intensities (MFI) for the immune markers of interest. The information that flow cytometry provides about the immune cells populations within the samples can be very useful for diagnosing and/or managing disease in addition understanding disease pathogenesis.

3.1.7.2 Considerations for immunophenotypic analysis of Treg, Th1 and Th17

T-regulatory cells (Treg) were first identified as a circulating subset of CD4+ T cells expressing high levels of CD25 (the IL-2 receptor α-chain), which, by adoptive transfer, demonstrated the ability to protect the host from autoimmune pathology, such as multi-organ autoimmune diseases and/or rodent graft-versus-host disease (GVHD)-like wasting disease (Powrie and Mason 1990; Sakaguchi, et al. 1982; Smith, et al. 1991; Suri-Payer, et al. 1998). IL-2, itself, has important, non-redundant effects on Treg maintenance and survival and CD25 is present on activated T cells and B cells, in addition, to some thymocytes and myeloid precursors in humans. Approximately 30% of human CD4+ T cells exhibit continuous low expression of CD25 and these levels increase during activation. Whereas CD4+CD25+ T-cells in mice were found to exhibit regulatory function, only the CD4+ T-cells expressing high levels of CD25 (CD25hi) demonstrated similar regulatory function in humans (Baecher-Allan, et al. 2001).

The identification of the Forkhead family transcription factor, (Fox)P3, (Brunkow, et al. 2001; Chatila, et al. 2000) and its specific expression in CD4+CD25+ regulatory T cells (Fontenot, et al. 2003), led to FoxP3
expression becoming a defining characteristic of Treg. The FoxP3 gene contains a forkhead (FKH) domain at the C terminus, critical for nuclear localisation and DNA binding, an N terminus transcriptional repressor domain, in addition to C2H2 zinc finger and leucine zipper domains, which mediate DNA binding and dimerization (Figure 39) (Li, et al. 2006). The basal, T cell-specific promoter contains six NF-AT and AP-1 binding sites, which positively regulate the transactivation of the FoxP3 promoter after the TCR is triggered (Mantel, et al. 2006).

**Figure 39:** Schematic diagram of the X Chromosome, (a) FoxP3 gene and (b) FoxP3 protein, modified from NCBI Reference Sequence (RefSeq) (Marques, et al. 2015). (a) shows two isoforms of the gene, with a difference in exon 2. (b) shows the FoxP3 protein with Forkhead (FKH) Forkhead family transcription factor domain at the C terminus, the Repressor domain at the N terminus; and in between lie the Leucine zipper (LZ) and Zinc finger (ZF) domains.

FoxP3 is considered the Treg ‘master transcription factor’ because it is critically required for Treg-cell development and function and for suppressing autoimmunity (Bennett, et al. 2001; Fontenot, et al. 2003; Hori, et al. 2003a; Hori, et al. 2003b). Deficiencies in FoxP3 were shown to cause lymphoproliferation and multiorgan autoimmunity in *scurfy* mutant mice and human IPEX patients, which could be rescued by transfer of Treg cells reproduced by inactivation of FoxP3 uniquely in T cells (Benoist and Mathis 2012). The large majority of FoxP3-expressing Treg are found within the CD4^+^CD25^+^ T-cell population, which constitutes approximately 5-10% of total CD4^+^ T cells in humans.
Natural CD4⁺CD25⁺FoxP3⁺ Treg (nTreg) cells differentiate in the thymus, where they undergo selection determined by high-avidity interactions with APCs, following which they migrate to peripheral tissues (Figure 40). Adaptive CD4⁺CD25⁺FoxP3⁺ Treg (iTreg) cells, on the other hand, are thought to differentiate from naïve, activated effector or proliferating memory CD4⁺ T cells outside the thymus, in the ‘periphery’ (Akbar, et al. 2007) (Figure 40). The peripheral population of FoxP3⁺ Treg cells, therefore, consists of both nTreg and iTreg cells, which have a similar phenotype and function (Curotto de Lafaille and Lafaille 2009).

Figure 40: ‘Natural Treg (nTreg) cells differentiate in the thymus and migrate to peripheral tissues. Adaptive FoxP3⁺ Treg (iTreg) cells differentiate in secondary lymphoid organs and tissues. The peripheral population of FoxP3⁺ Treg cells comprises both nTreg and iTreg cells.’ (Curotto de Lafaille and Lafaille 2009).

It is known that activated human T-cells may transiently upregulate FOXP3 and that these FoxP3⁺ T-cells are not effective at suppressing inflammation.
It appears that only in circumstances in which human cells express FoxP3 at high and constitutive levels do they acquire suppressor function (Allan et al. 2008). It is, therefore, of note that not all CD25^+ cells are suppressive and not all FoxP3^+ cells express CD25 (Fontenot et al. 2005). It has been shown that inflammatory environments may induce T-cell plasticity (Murphy and Stockinger 2010). Furthermore, it is postulated that there may be a threshold of expression and/or activation of transcription factors by FoxP3^+ Tregs, which allows them to act either as lineage-specific regulators or contributors to Th1 or Th17 effector responses (Povoleri, et al. 2013). This potential for plasticity presents challenges for the identification of phenotypically stable T-reg lineages.

FoxP3 expression is important, but not sufficient, for the establishment of the Treg lineage because the development of a Treg-specific genome-wide methylation pattern is also critical (Morikawa, et al. 2014; Ohkura, et al. 2012). FoxP3 gene expression is controlled by four elements, containing conserved non-coding sequences (CNS), which are regulated by epigenetic modifications that determine chromatin structure and DNA methylation, altering the accessibility of the gene locus to transcription factors (Povoleri, et al. 2013) (Figure 41).
Four distinct regions of the FoxP3 gene are susceptible to epigenetic modification. These are the FoxP3 promoter and three other conserved non-coding sequences (CNS): CNS1 – the TGF-β sensor/enhancer, CNS2 – Treg-cell-specific demethylation region (TSDR), and CNS3 – a FoxP3 pioneer element. Epigenetic modifications include histone acetylation/deacetylation and CpG methylation/demethylation and are shown at each locus (Povoleri, et al. 2013).

The first site is in the FoxP3 promoter, a CNS site located 6 kb upstream of the translational start site of the FoxP3 gene. Activation occurs following TCR stimulation through binding of NFAT and AP1 (Mantel, et al. 2006). The FoxP3 promoter is thought to be is more accessible in Treg cells than Tconv (Baron, et al. 2007). Another CNS site, an evolutionarily conserved DNA hypomethylation pattern, the Treg-specific demethylated region (TSDR) is associated with enhancement and stabilization of FoxP3 expression (Floess, et al. 2007; Huehn, et al. 2009; Polansky, et al. 2008; Zheng, et al. 2010). The TSDR has been identified in both humans and mice and has been found to be fully demethylated in Treg cells and methylated in conventional T-cells (Tconv) (Baron, et al. 2007; Floess, et al. 2007; Polansky, et al. 2008). Activated Tconv cells and TGF-β-treated cells displayed no FoxP3 demethylation, although they expressed FoxP3, whereas ex vivo expanded Treg, remained demethylated even after extensive in vitro stimulation and expansion (Baron, et al. 2007; Floess, et al. 2007; Polansky, et al. 2008).
DNA methylation status of FoxP3 has been also shown to correlate with suppressive activity of nTreg (Huehn, et al. 2009). In a genome wide methylation analysis of human naive nTreg and conventional naive CD4+ T cells, it was found that FOXP3 binding coincided with hypomethylation in the Treg genome (Zhang, et al. 2013b). The TDSR, in combination with CD25 and FoxP3 expression, has, therefore, allowed identification of T-cells with a stable phenotype (Janson, et al. 2008).

Different subtypes of Tregs have been described, some of which are identified by the expression of their signature cytokines, IL-10 and TGF-β (Shevach and Thornton 2014; Zhang, et al. 2014) (Table 11). Helios, an Ikaros transcription factor, was proposed as a marker of nTreg (Thornton, et al. 2010) but this view has been subsequently challenged by the demonstration that Helios is induced during T cell activation and proliferation and then down-regulated (Akimova, et al. 2011; Elkord 2016).

Table 11: Some subtypes of regulatory T cell (Treg) and their actions (Zhang, et al. 2014)

<table>
<thead>
<tr>
<th>Subset</th>
<th>Specific marker</th>
<th>Secretory products</th>
<th>Actions</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>nTreg</td>
<td>CD4, CD25, Foxp3</td>
<td>IL-10, TGF-β</td>
<td>Block T cell proliferation, suppression of DCs, inhibition of effector Th1, Th2, and Th17 cells, elimination production of allergen-specific IgE, induce IgG4 secretion; suppress mast cells, basophils, and eosinophils; interact with resident tissue cells and participate tissue remodeling[13]</td>
<td>Thymus (9)</td>
</tr>
<tr>
<td>IκO(+) Treg</td>
<td>CD4, CD25, Foxp3, ICOS</td>
<td>IL-10, IL-17, IFN-γ</td>
<td>Suppress hapten-reactive CD69(+) T cells [15]</td>
<td>Generated from nTregs</td>
</tr>
<tr>
<td>Th1</td>
<td>CD4, Foxp3</td>
<td>IL-10, TGF-β</td>
<td>Similar to nTreg [16]</td>
<td>Periphery</td>
</tr>
<tr>
<td>CD8(+) Treg</td>
<td>CD8, Foxp3, CD25 (not for tonsil origin), CD28</td>
<td>IL-10, TNF-α, IFN-γ, Gr</td>
<td>Block activation of naive or effector T cells; suppress IgG3/γ antibody responses [9]; IL-4 expression and the proliferation of CD4(+) T cells [9];</td>
<td>Generated from CD8(+) T cells [9] and tonsils</td>
</tr>
<tr>
<td>IL-17-producing</td>
<td>CD4, Foxp3, ICOS, RORγT</td>
<td>IL-17</td>
<td>Inhibit the proliferation of CD4(+) effector T cells [10];</td>
<td>Differentiated from CD4(+)Foxp3(+)ICOS(+) Tregs in peripheral blood and lymphoid tissue [10]</td>
</tr>
</tbody>
</table>

nTreg = natural regulatory T cell; ICOS = inducible co-stimulator; Threg = inducible/adaptive regulatory T cell; T1 cell = IL-17-producing type 1 regulatory T cell; Gr = granzyme B; RORγT = RORgamma T transcription factor.
Cell surface markers for further characterising Treg have been described, which include glucocorticoid-induced tumour necrosis factor receptor (GITR), CTLA-4, Neuropilin-1, and PD-1, but there is no agreement amongst experts, as to which of these might constitute definitive Treg markers (Sakaguchi, et al. 2013). Furthermore, GITR and CTLA-4 are not exclusive to Treg and are also expressed by activated T cells.

Although T cells express IL-7Rα (CD127), it was found that lower levels of CD127 (CD127lo) were expressed by Treg (Liu, et al. 2006). Therefore, CD127lo in combination with high CD25 expression, has been proposed as a Treg phenotypic marker (Klein, et al. 2010; Liu, et al. 2006). It was demonstrated that FoxP3 interacted with the CD127 promoter and that the CD127lo marker could be used to selectively enrich human Treg cells for in vitro functional studies, resulting a purified CD4+CD25+CD127lo cells of which 85-95% were reported to express FoxP3+ (Liu, et al. 2006). However, CD127 may be also upregulated following T cell activation and higher levels than originally reported have been shown to be expressed by activated Treg (Simonetta, et al. 2010).

Thus, there remains a lack of consensus regarding immunophenotypic cell surface markers for functionally suppressive Treg, and, particularly, markers that might be helpful in identifying these Treg subsets in human autoimmune disease.

A recently discovered co-inhibitory molecule, T cell immunoreceptor with Ig and ITIM domains (TIGIT) has been proposed as a novel marker of Treg (Joller, et al. 2014). TIGIT is expressed by Tregs (Yu, et al. 2009) and suppresses a range of immune cells, including T-cells, dendritic cells and NK cells. Recent studies show that TIGIT ligation directly inhibits T cell proliferation and cytokine production in CD4+ T cells (Joller, et al. 2011). Furthermore, increased expression of TIGIT delineated Treg from activated effector T cells (Zhang, et al. 2013b). There is also evidence that
hypomethylation at the TIGIT locus is associated with FOXP3 binding and the nTreg phenotype (Zhang, et al. 2013b). Treg cells expressing TIGIT were found to selectively inhibit Th1 and Th17, but not Th2 responses (Joller, et al. 2014). Since, it is known that the Th1 and Th17 subsets are pivotal to the pathogenesis of autoimmune disease, TIGIT may be a biomarker of stable, functionally suppressive Tregs in these diseases.

A previous study, published by the supervisors of this PhD, characterised Treg in peripheral blood of subjects with ocular inflammatory disease, by flow cytometric immunophenotyping, using the previously described, well-established Treg markers: CD4, CD25 and FoxP3 (Lightman et al., 2014). In the current study, flow cytometry methods are also used to immunophenotype subjects’ bloods for Treg, however, the cell surface marker, TIGIT, was additionally used as a surrogate marker for capacity of Treg to selectively suppress Th1 and Th17 (Jager & Kuchroo, 2010; Joller et al., 2011; Joller et al., 2014; Kurtulus et al., 2015). Immunophenotypic markers were also added to the flow cytometry panel to characterise Th1 and Th17 populations, according to expression of their ‘signature’ transcription factors, T-bet and RORγt, respectively. However, whilst immunophenotypic studies may assess the presence or absence of cells or surface molecules, they do not evaluate the functional status of the cells. Relevant to this study was the assessment of Treg function, therefore, methods were included to assess the ability of CD127lo Treg to suppress proliferating T-cells.

The cytokine milieu is an important part of this ‘microenvironment’, which has been shown to regulate the Treg/Th17 balance and influence cell plasticity; disruption of this balance may lead to the development of inflammatory disease (Leung, et al. 2010; Lochner, et al. 2008; Omenetti and Pizarro 2015; Sefik, et al. 2015). In clinical trials of systemically-administered immunomodulating therapies, induction of disease remission in uveitis was
associated with modulation of cytokine production, in addition to systemic upregulation of Tregs (Calleja, et al. 2012; Lightman, et al. 2014). Levels of corticosteroids have been found to positively correlate with IL-10+FoxP3+CD25+CD4+ T regulatory cells but not with CD4+IL-17+ Th17 cells in the ocular form of Behçet’s Disease (Zhang, et al. 2016). In some clinical studies, pharmacological effects on cytokines were demonstrated without a significant increase in the proportion of systemic FoxP3+ Tregs levels (Molins, et al. 2015). These studies suggest that the mechanism of disease remission in uveitis, may be mediated through cytokines.

3.2 Methods

3.2.1 Ethical approvals
Research performed as part of this thesis adhered to the tenets of the Declaration of Helsinki and was carried out in accordance with the recommendations of the UK National Research Ethics Service (NRES) – London Harrow Committee and the Moorfields Eye Hospital National Health Service (NHS) Foundation Trust, Department of Research and Development (13/LO/1653; 16039). Written informed consent was obtained from recruited subjects.

3.2.2 Design, setting and study subjects

Design: Cross-sectional and prospective cohort observational study

Setting: Tertiary referral centre for clinical management of uveitis in the UK

Participants: A total of 50 patients prospectively recruited from a specialist uveitis clinic in tertiary care centre for ophthalmology in London UK and 10
volunteers recruited from a higher education institution (university) in London, UK.

3.2.3 Flow cytometry panel design, controls and standardisation
A multicolour flow cytometry panel for immunophenotyping Treg, Th1 and Th17 was designed taking into account: the required cell surface markers with the available fluorochrome conjugates, the expected frequency of expression in the sample, the excitation/ emission spectrum, the relative brightness of the chosen fluorochrome conjugate (Table 12) and the available laser channels in the flow cytometer used to acquire the data (Table 13) [The panel included phenotypic markers for B-cells, as described in the original aim of PhD thesis. However, the B-cell data was not analysed as part of this thesis.
Table 12: Template for flow cytometry panel design (markers shaded in grey were not analysed as part of this thesis)

<table>
<thead>
<tr>
<th>Cell markers (cell type)</th>
<th>Expected frequency of expression in PBMC</th>
<th>Human fluorochrome-conjugated antibodies</th>
<th>Excitation laser detector channel (nm)</th>
<th>Excitation/Emission spectrum (nm)</th>
<th>Relative brightness of fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>30-70%</td>
<td>VioBlue</td>
<td>405</td>
<td>400/455</td>
<td>1</td>
</tr>
<tr>
<td>CD8</td>
<td>5-30%</td>
<td>AlexaFluor700</td>
<td>633</td>
<td>633/719</td>
<td>5</td>
</tr>
<tr>
<td>CD4</td>
<td>25-60%</td>
<td>BUV395</td>
<td>355</td>
<td>348/395</td>
<td>5</td>
</tr>
<tr>
<td>CD25</td>
<td>2-40% depending on activation status</td>
<td>BV711</td>
<td>405</td>
<td>407/711</td>
<td>5</td>
</tr>
<tr>
<td>FoxP3</td>
<td>0.05-15%</td>
<td>AlexaFluor647</td>
<td>640</td>
<td>650/668</td>
<td>5</td>
</tr>
<tr>
<td>TIGIT</td>
<td>2-8%</td>
<td>PE</td>
<td>488</td>
<td>496/578</td>
<td>6</td>
</tr>
<tr>
<td>RORyt</td>
<td>0-5%</td>
<td>Alexa488</td>
<td>488</td>
<td>495/519</td>
<td>5</td>
</tr>
<tr>
<td>T-bet</td>
<td>10-40%</td>
<td>PerCp-Cy5.5</td>
<td>488</td>
<td>482/695</td>
<td>3</td>
</tr>
<tr>
<td>CD19</td>
<td>10-40%</td>
<td>BV605</td>
<td>405</td>
<td>405/603</td>
<td>6</td>
</tr>
<tr>
<td>CD24</td>
<td>Not known</td>
<td>PE-Cy7</td>
<td>488</td>
<td>496/775</td>
<td>6</td>
</tr>
<tr>
<td>CD38</td>
<td>Not known</td>
<td>PE-Dazzle594</td>
<td>488</td>
<td>496/612</td>
<td>6</td>
</tr>
<tr>
<td>Live/Dead fixable dye</td>
<td>Variable (depending on cell type)</td>
<td>Near Infra-red (IR)</td>
<td>640</td>
<td>750/775</td>
<td>NA</td>
</tr>
</tbody>
</table>
In order to ensure the validity of multicolour flow cytometry results, the following controls were incorporated into the flow cytometry methods:

- **Quality control**
  - This included monitoring instrument set-up and performance (with calibration beads being run weekly on the instrument), optimising sample preparation, standardising reagents, sample controls and data interpretation (Fleisher 2013)

- **Positive-negative discrimination**
  - This requires establishing criteria for the boundaries between stained (positive) cells and (unstained) negative cells. This may be achieved through the use of 1) isotype controls or 2) fluorescence-minus-one (FMO) controls.

---

### Table 13: BD LSRFortessa laser channels

<table>
<thead>
<tr>
<th>Laser (Excitation)</th>
<th>PMT (FL)</th>
<th>BP Filter (Emission)</th>
<th>LP</th>
<th>Fluorochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue Laser 488 nm</td>
<td>A FL1</td>
<td>690/50</td>
<td>655</td>
<td>PerCP-Cy5.5, PerCP eFluor 710, PerCP</td>
</tr>
<tr>
<td></td>
<td>B FL2</td>
<td>530/30</td>
<td>505</td>
<td>FITC, Alexa 488, eGFP, SYTOXGreen</td>
</tr>
<tr>
<td></td>
<td>C SSC</td>
<td>488/10</td>
<td></td>
<td>Venus, YFP</td>
</tr>
<tr>
<td>Red Laser 640 nm</td>
<td>A FL3</td>
<td>780/60</td>
<td>750</td>
<td>APC-Cy7, APC-H7, DRAQ5, Alexa 750</td>
</tr>
<tr>
<td></td>
<td>B FL4</td>
<td>710/50</td>
<td>685</td>
<td>Alexa 680, Alexa 700</td>
</tr>
<tr>
<td></td>
<td>C FL5</td>
<td>670/30</td>
<td></td>
<td>APC, Cy5, Alexa 647, Alexa 633, SYTOX Red</td>
</tr>
<tr>
<td>Violet Laser 405 nm</td>
<td>A FL6</td>
<td>780/60</td>
<td>735</td>
<td>BV 765</td>
</tr>
<tr>
<td></td>
<td>B FL7</td>
<td>710/50</td>
<td>690</td>
<td>BV 711</td>
</tr>
<tr>
<td></td>
<td>C FL8</td>
<td>676/29</td>
<td>630</td>
<td>BV 650</td>
</tr>
<tr>
<td></td>
<td>D FL9</td>
<td>610/20</td>
<td>570</td>
<td>7AAD, PI, BV 605</td>
</tr>
<tr>
<td></td>
<td>E FL10</td>
<td>529/24</td>
<td>505</td>
<td>Alexa 430, Zombie Aqua, BV 510, Krome Orange</td>
</tr>
<tr>
<td></td>
<td>F FL11</td>
<td>450/40</td>
<td></td>
<td>Pacific Blue, DAPI, CFP, Hoechst*, Alexa 405, BD HorizonV450, SYTOX Blue, Cascade blue, BV421</td>
</tr>
<tr>
<td>Yellow/Green Laser</td>
<td>A FL12</td>
<td>780/60</td>
<td>735</td>
<td>PE-Cy7</td>
</tr>
<tr>
<td>561 nm</td>
<td>B FL13</td>
<td>710/50</td>
<td>685</td>
<td>PE-Alexa700, PE-Cy5.5</td>
</tr>
<tr>
<td></td>
<td>C FL14</td>
<td>670/30</td>
<td>635</td>
<td>PE-Cy5, 7AAD, Alexa 594</td>
</tr>
<tr>
<td></td>
<td>D FL15</td>
<td>610/20</td>
<td>600</td>
<td>PE-Texas Red, mCherry, PI, Alexa 610, mRasberry, mStrawberry, Alexa 594, mRFP</td>
</tr>
<tr>
<td></td>
<td>E FL16</td>
<td>586/15</td>
<td>570</td>
<td>PE, DsRed, dTunato, Alexa 546</td>
</tr>
</tbody>
</table>

* Dyes possible but not optimal with this laser and filter
• Compensation controls

  o Multi-colour fluorochromatic dyes emit fluorescent signals, which may overlap in their emission spectrum. Flow cytometry panels are designed to minimise this type of overlap. However, in cases where it cannot be avoided, signal overlap can be corrected by electronic subtraction of the overlap in a process known as ‘compensation’.

Flow cytometry antibody concentrations for staining of stimulated and unstimulated cells were optimised by titration.

3.2.4 Patient recruitment for experimental assays

A total of 50 patients with a diagnosis of idiopathic non-infectious uveitis were consecutively recruited to the study from Moorfields Eye Hospital, London, UK between November 2014 and December 2016. Inclusion criteria for all patients were as follows: age 18 to 59 years; a current or previous diagnosis of noninfectious intermediate, posterior or pan-uveitis as per standardization of uveitis nomenclature (SUN) (Jabs et al., 2005); and the absence of known associated infectious disease or systemic inflammatory disease. Eligibility for recruitment was further determined by uveitic disease activity. To meet the clinical criteria for disease ‘in clinical remission’, patients were required to have no signs of disease activity at the time of recruitment; have a previous diagnosis of active non-infectious chronic relapsing and remitting uveitis which was treated with oral corticosteroids +/- second line corticosteroid-sparing treatments prior to the period of clinical remission; and have discontinued all immunosuppressive treatment and remained quiescent for at least 6 months. To meet the criteria for ‘active’ disease, patients were required to have new onset of uveitic disease occurrence at the time of recruitment (a new diagnosis or reactivation of
existing disease), which was regardless of treatment status. Disease activity was determined by clinical symptoms, examination with slit lamp biomicroscopy and clinical imaging (optical coherence tomography, fundus autofluorescence and fluorescein angiography). Patients who met the study inclusion criteria and who were clinically assessed to have active disease (i.e. anterior chamber cells ≥0.5+, vitreous cells ≥0.5+, inflammatory cystoid macular oedema, optic disc oedema, active vasculitis, or new/ active chorio-retinal lesions) were considered eligible as ‘active’ patients in the study. As controls, 10 age-matched subjects with no history of uveitis, systemic inflammatory disease or known infectious disease were enrolled onto the study.

3.2.5 Specimen collection for experimental assays
A 30ml sample of heparinized peripheral venous blood was obtained from all subjects using VACUETTE NH Sodium Heparin tubes and safety venupuncture equipment (Greiner BioOne, UK). All venous blood samples were obtained by a clinician experienced in phlebotomy (the thesis author) and stored at room temperature until processing for experimental assays and/ or crypreservation within a 4-hour period. For the active patient cohort, baseline (Time 0) blood samples were obtained before starting/ modifying the immunosuppressive treatment regime. Additional peripheral venous blood samples were prospectively obtained from four active patients at 2, 6 and 12 months after starting/ changing treatment.

3.2.6 PBMC isolation from peripheral blood samples
When peripheral whole blood is sampled for human studies of the immune system, it should be processed to remove erythrocytes (red blood cells) prior to staining for flow cytometric analysis or other \textit{in vitro} assays. Density
gradient centrifugation uses a solution of high molecular weight sucrose polymers, such as Histopaque-1077 (Sigma Aldrich, UK), which separates whole blood into two fractions above and below the density of 1.077g/ml. Peripheral blood mononuclear cells PBMC are the populations of immune cells that remain at the less dense, upper interface of the opaque layer, often referred to as the ‘buffy coat’. PBMC, including lymphocytes (T cells, B cells and NK cells), monocytes and dendritic cells are collected from this ‘buffy coat’ layer when the fractionation method is used. Erythrocytes and polymorphonuclear cells (PMNs), including granulocytes, such as neutrophils and eosinophils, are generally removed during this fractionation because they are denser than 1.077g/ml. Trace levels of basophils, however, might be present in the less dense PBMC fraction because they may be less dense than 1.077g/ml.

To optimize the yield of cells obtained from each blood sample, prior to processing samples from study subjects, the following tests were performed using volunteer peripheral blood and blood components, that is, non-clinical issue leukocyte blood cones for research use from NHS Blood and Transplant (Colindale, UK):

- Comparison of Leucosep (Greiner Bio-one) tubes (containing high density polyethylene barrier) with universal tubes (no barrier) for efficiency of density gradient centrifugation and cell viability.

- Optimization of time from venipuncture to PBMC isolation, using the following time points post blood sampling: 1 hour, 4 hours, 8 hours and 24 hours.

- Optimization of peripheral blood dilutions (undiluted, 1:2, 1:3, 1:4) prior to PBMC isolation.

- Optimization of ratio of Histopaque-1077 to blood volume (1:1, 1:2)
• Optimization of PBMC isolation protocol including centrifugation settings, spin-time and cell washes to create a stream-lined workflow from venipuncture to flow cytometric analysis.

The following protocol was used to obtain PBMC from fresh heparinized blood (≤ 4 hours following venipuncture) by Histopaque-1077 (Sigma-Aldrich, Gillingham, UK) density gradient centrifugation (Figure 42):

16ml of Histopaque-1077 solution was placed in a 50ml Leucosep tube (Greiner Bio-one, Germany) and centrifuged for 90 seconds at 1000 x g. This centrifugation step ensured that the majority of the Histopaque remained beneath the filters in the Leucosep tubes, thereby minimising contact with the blood samples. Venous blood samples were diluted 1:4 with RPMI 1640 before being gently pipetted onto the filters within the prefilled Leucosep tubes, under aseptic conditions. These tubes were then centrifuged for 15 minutes at 800 x g. Following centrifugation, PBMC were retrieved from the enriched cell fraction above the porous barrier (the barrier helped to avoid recontamination with pelleted erythrocytes and granulocytes) by means of a Pasteur pipette. Retrieved PBMC were gently pipetted into 20mL of prewarmed (37°C) sterile-filtered media RPMI-1640 supplemented with 10% human AB Serum (Sigma-Aldrich) in a polyethylene tube with a conical base. The tube was then gently inverted to mix any remaining Histopaque-1077 with the media, which helped to prevent it from forming a layer which would hinder isolated PBMC from being pelleted. The cell suspension was then centrifuged for 10 minutes at 300 x g. The pelleted cells were resuspended in supplemented RPMI-1640 and the centrifugation step was repeated at 5 minutes at 300 x g. The isolated PBMC were counted (see 'Cell counting'). 10 x 10^6 PBMC from each sample were reserved for multi-colour flow cytometric analysis and the remaining PBMC were cryopreserved (see 'Cryopreservation of PBMC').
3.2.7 Cryopreservation of PBMC

PBMC were cryopreserved in human AB serum with 10% Hybri-Max Sterile-filtered DMSO (Sigma-Aldrich) at -20°C for 1 hour prior to transfer to -70°C and then to liquid nitrogen for longer term storage, for subsequent DNA isolation and methylation analysis.
3.2.8 Optimisation of T-cell culture and stimulation conditions

Cell culture conditions were tested and optimised, by comparing the following:

- Cell medium (RPMI-1640 vs X-VIVO) for T-cell culture
- T-cell stimulation techniques:
  - PMA/ ionomycin stimulation
  - soluble anti-CD3 (HIT3a) NA/LE and anti-CD28 NA/LE
  - plate-bound anti-CD3 (HIT3a) NA/LE and anti-CD28 NA/LE
  - Dynabeads (bead mounted anti-CD3 and anti-CD28)

Following these tests, it was determined that experimental cell culture conditions requiring stimulated cells should use X-VIVO 15 medium with L-glutamine, gentamicin, and phenol red (Lonza, Cambridge, UK) for 5 days, with the addition of 5µg/ml soluble anti-CD3 (HIT3a) NA/LE, 1µg/ml anti-CD28 NA/LE (BD Biosciences) and 50IU IL-2 IS/ml (Miltenyi Biotec, Woking, UK) to the culture media, unless otherwise specified below.

3.2.9 Cell counting

Viable cell counts for all experiments, with the exception of high speed cell sorting, were determined using a haemocytometer with Trypan Blue dye (0.4%) exclusion, using cell counting methods described by Public Health England (PHE 2017).

Prior to cell sorting experiments, which required a high degree of counting accuracy, viable cell counts were determined using the Vi-CELL cell counter (Beckman Coulter UK Ltd, High Wycombe) for cell viability analysis by Trypan Blue exclusion.
3.2.10 Antibodies used for immunophenotyping by flow cytometry

For multi-colour immunophenotyping by flow cytometry, the following antibodies (Abs) were used: RORγt-Alexa Fluor 488, T-bet-PerCP-Cy5.5, CD3-VioBlue, CD25-Brilliant Violet 711, CD4-Brilliant Ultraviolet 395, FoxP3-Alexa Fluor 647, TIGIT-PE and Near Infrared live/dead fixable cell stain. For live cell sorting, the following Abs were used: CD4-PerCP-Cy5.5, CD127-Brilliant Violet 605, CD3-Brilliant Violet 785, CD25-PE-Dazzle594, CD14-APC-Fire and SYTOX blue dead cell stain. Abs were provided by BioLegend (London, UK), BD Biosciences (Oxford, UK), eBiosciences (Hatfield, UK), Miltenyi Biotec and ThermoFisher Scientific (Dartford, UK).

3.2.11 Treg and Th1/Th17 immunophenotyping by flow cytometry: staining protocol

10 x 10⁶ PBMC isolated from each subject sample by density gradient centrifugation were resuspended in 50 μL Flow Cytometry Staining Buffer (eBiosciences). The samples were then stained with directly conjugated Abs for cell surface molecules (CD3, CD4, CD25, TIGIT), as follows:

The appropriate volume of each primary antibody (Ab) was resuspended in 50 μL Flow Cytometry Staining Buffer and added to the 50 μL cell suspension, so that the final concentration of antibody was a 1:25 dilution and the final staining volume was 100 μL (i.e. 50 μL of cell sample + 50 μL of antibody mix) (the titration protocol, which determined the Ab concentration for staining, is included in the Appendix). The PBMC and Ab suspension was then gently vortexed to mix it. The PBMC were then protected from light and incubated for 40 minutes at 2-8°C to stain them for the required cell surface antigens. The stained cells were then washed by adding 1mL Flow Cytometry Staining Buffer and centrifuging at 350 x g for 5 minutes at room
temperature. This wash step was repeated. The supernatant was discarded prior to proceeding to the next stage of the staining protocol.

To eliminate potential artefacts due to dead cell contamination, the cell suspensions were then stained with a 1:1000 dilution of Zombie NIR (BioLegend) Fixable Viability Dye, a fixable dead cell stain. This was to allow dead cells, which existed prior to fixation/permeabilization process, to be excluded from the subsequent analysis.

Following staining of dead cells, PBMC were prepared for evaluation of intracellular proteins, using a technique which allowed the simultaneous analysis of cell surface molecules and intracellular antigens, including nuclear transcription factors, such as FoxP3, at the single-cell level. The following protocol, which combines fixation and permeabilization into a single step, was adapted from FoxP3 transcription factor Fix/Perm kit manufacturers’ protocol.

Following the last wash of the dead cell staining protocol, the supernatant was discarded and the sample was pulse vortexed to completely dissociate the pellet. 1 mL of Fixation/Permeabilization working solution was added to each tube and the tube was pulse vortexed. The samples were protected from light and incubate for 30 minutes at room temperature. 2 mL of 1X Permeabilization Buffer was added to each tube. The samples were centrifuged at 300-400 x g for 5 minutes at room temperature, then the supernatant was discarded. After decanting, the pellet was resuspended in the residual volume (approximately 100 μL) of 1X Permeabilization Buffer. Intracellular staining was performed using directed conjugated Abs recognizing transcription factors (FoxP3, RORyt, T-bet). Subject-specific fluorescence-minus-one (FMO) controls for CD25, TIGIT, FoxP3, RORyt and T-bet were included in each experiment. The samples were incubated with Abs for 30 minutes at room temperature, during which time they were protected from light. Following incubation, 2 mL of 1X Permeabilization Buffer was added to each tube.
Buffer to each tube. Samples were centrifuged at 300-400 x g for 5 minutes at room temperature, then the supernatant was discarded (this step was repeated for a further wash). The stained cells were then resuspended in approximately 300μL Flow Cytometry Staining Buffer prior to acquiring samples on a flow cytometer.

3.2.12 Comparison of multi-colour flow cytometry panel with a pre-manufactured Treg isolation kit

T-reg staining using titrated concentrations of the multi-colour panel Abs, designed for the study, was compared with staining using a Regulatory T Cell Staining Kit (CD4⁺CD25⁺FoxP3⁺) (eBiosciences, Thermofisher Scientific, UK), which had been pre-titrated and tested by the manufacturer (eBiosciences, UK) for staining and analysis of PBMC by flow cytometry. Staining was performed using the Treg kit manufacturers’ protocol (thermofisher.com, 2017), which specified the following Ab concentrations:

- The PCH101 antibody and the Rat IgG2a K Isotype Control: 5 μL (0.25 μg) per test.
- The OKT4 antibody: 5 μL (0.25 μg) per test.
- The BC96 antibody: 5 μL (0.125 μg) per test.

A test was defined as the amount (μg) of antibody that will stain a cell sample in a final volume of 100 μL.

3.2.13 Treg and Th1/Th17 immunophenotyping by flow cytometry: data acquisition

The peripheral blood levels of the following cell subsets were determined by flow cytometry: CD25⁺FoxP3⁺ Treg, CD25⁺FoxP3⁺TIGIT⁺ Treg, T-bet⁺ Th1, RORγt⁺ Th17, T-bet⁺ RORγt⁺ Th1/17, FoxP3⁺ T-bet⁺ Treg and FoxP3⁺
RORγt^+ T-reg in the CD3^+CD4^+ subset. Flow cytometric data was acquired using a LSRFortessa (BD Biosciences, San Jose, California) and BD FACSDiva version 6.1.3 software with a total of 50,000 events being recorded for each sample through a live, single cell CD3^+CD4^+ gate (workflow and gating strategy shown in Figures 43 & 44, respectively). Viable cells were identified by low uptake of the fixable dead cell stain. Single-stained OneComp and UltraComp beads (eBiosciences) were used to generate compensation matrices. FMO controls were used to identify gating boundaries where cell populations were ill-defined. Analysis of flow cytometry data was performed using FlowJo version 10 software (TreeStar Inc., Ashland, OR, USA).
Figure 43: Flow cytometry work-flow for immunophenotyping
Figure 44: Treg gating strategy illustrating that viable cells were identified by (1) low uptake of the fixable dead cell stain, followed by (2) identification of single cells, based on area (A) and height (H) of both forward scatter (FSC) and side scatter (SSC) profiles, then (3) lymphocyte phenotype based on their FSC-A against SSC-A profile and then finally (4) positive expression of CD3, CD4, FoxP3(high), CD25(high) +/- TIGIT markers.
3.2.14 Cell sorting for T-cell proliferation assays

Freshly isolated PBMCs from subject samples in each group were stained with CD3, CD4, CD25, CD127 and CD14 cell surface antibodies. CD3+CD4+CD25+CD127lo, CD3+CD4+CD25− and CD14+ populations representing Treg, putative T-effectors and monocyte subsets, respectively, were isolated directly from the stained PBMC by means of high speed Influx flow cytometric cell-sorting (BD Biosciences, San Jose, California) (process overview with gating strategy shown in Figures 45).

CD3+CD4+CD25− T-cells were washed and resuspended at 10 x 10⁶/ ml in sterile PBS (Sigma Aldrich) containing 1 μL of 1 mM Violet Proliferation Dye (VPD) 450 stock solution (BD Biosciences) for each 1 mL of cell suspension for a final VPD450 concentration of 1 μM, according to the manufacturer’s instructions. The cells were stained by incubating the dye-cell suspension in a 37ºC water bath for 10 minutes. The reaction was quenched by adding 9× the original volume of PBS to the cells, followed by centrifugation, discarding the supernatant, and resuspending the cells in 10 mL of RPMI 1640 medium with 10% FBS before washing again.

3.2.15 T-cell proliferation assay protocol to determine Treg function

The in vitro capacity of the Treg to suppress the proliferation of VPD450-labelled CD3+CD4+CD25− responding T-cells (Tresp) was assessed. The following tests were performed to optimise the assay:

- Comparison of T-cell culture in 6 well, 24 well, 48 well and 96-well plates (1 x 10⁶ per well density)
- Comparison of stimulation techniques
- Comparison of VPD450 with CFSE proliferation dye
VPD450-labelled CD3⁺CD4⁺CD25⁻ Tresp were co-cultured with sorted CD14⁺ monocytes (MC) at 1:1 ratio and sorted CD3⁺CD4⁺CD25hiCD127lo T-regs were co-cultured with Tresp and MC at a 1:3:3 ratio. Cell culture conditions were as previously described.

Figure 45: Process overview of cell sorting for T-cell proliferation assays using Treg sort gating strategy. Cell subsets were identified by expression of the following markers: Treg CD3⁺CD4⁺CD25hiCD127lo; Tresp CD3⁺CD4⁺CD25⁻ and MC CD14⁺
Data was acquired by flow cytometry and analysed using the cell tracking function of Modfit LT modelling software (Verity Software House, Maine, USA) to generate a statistic termed proliferation index (PI) (Figure 46). Dead cells were gated out using SYTOX Blue dead cell stain (ThermoFisher Scientific, UK) at the time of data acquisition. Percentage (%) suppression was determined for each subject sample, as per previously described methods, with adaptation for small numbers of isolated T-cells (Nguyen and Ehrenstein 2016; Ward, et al. 2014).

![Cell Tracking Wizard Basic Model](image)

**Figure 46:** Modelling of *in vitro* T-cell proliferation using Modfit LT modelling software

File: Stimulated Dynabeads VP450_Stainex
Date acquired: 25-FEB-2016
Date analyzed: 3-Jun-2017

Parent: 64.88% at 131.00
Generation 2: 15.59% at 116.13
Generation 3: 16.73% at 100.73
Generation 4: 3.75% at 84.47
Generation 5: 5.00% at 66.93
Generation 6: 1.85% at 48.22
Generation 7: 1.31% at 30.77
Generation 8: 0.00% at 17.35
Generation 9: 1.10% at 9.16
Generation 10: 0.00% at 4.69

Proliferation Index: 1.48
Nonproliferative Fraction: 0.81
Replication Index: 3.51
Calculated Original Cells: 65370

For cells at generation >= 3:
Upper Generation PI: 5.96
Precursor Frequency: 0.074466

Modeled Cells: 96536
Gated Events: 96808
Reduced Chi-Square: 15.683
3.2.16 Optimisation of cell culture conditions and T-cell cell culture for cytokine assays

Cell culture conditions were tested and optimised, by comparing the following:

- Cell medium (RPMI-1640 vs X-VIVO) for T-cell culture
- T-cell stimulation techniques:
  - PMA/ ionomycin stimulation
  - soluble anti-CD3 (HIT3a) NA/LE and anti-CD28 NA/LE
  - plate-bound anti-CD3 (HIT3a) NA/LE and anti-CD28 NA/LE
  - Dynabeads (bead mounted anti-CD3 and anti-CD28)

For experimental cell culture conditions requiring stimulated cells, freshly isolated PBMC were cultured in X-VIVO 15 medium with L-glutamine, gentamicin, and phenol red (Lonza, Cambridge, UK) for 5 days, with the addition of 5µg/ml soluble anti-CD3 (HIT3a) NA/LE, 1µg/ml anti-CD28 NA/LE (BD Biosciences) and 50IU IL-2 IS/ml (Miltenyi Biotec, Woking, UK) to the culture media, unless otherwise specified.
3.2.17 Cytokine analysis

Whole blood samples were centrifuged following collection to obtain serum supernatant samples, which were stored in 1.5ml aliquots at -70°C for subsequent multiplex cytokine analysis. Cryopreserved subject serum cytokine samples were thawed and immediately analysed for cytokines levels of TGF-β, IL-10, IFNγ, IL-17A and IL-22.

Acidified serum aliquots subject samples were analysed for TGF-β by the quantitative sandwich enzyme immunoassay technique (ELISA) (R&D Systems), according to the manufacturer’s instructions. The principles of the ELISA assay were as follows:

- Prior to analysis for TGF-β levels by ELISA, samples were prepared and acidified using a Sample Activation Kit according to the manufacturer’s instructions (R&D Systems).
- A monoclonal antibody specific for TGF-β1 was pre-coated onto a microplate.
- Standards, controls and samples were pipetted into the wells and any TGF-β1 present was bound by the immobilised antibody.
- After washing away unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 was added to the wells to sandwich the TGF-β1 immobilised during the previous step.
- Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, after which a colour developed in proportion to the amount of the TGF-β1 bound in the initial step.
- The colour development was stopped and the intensity of the colour was measured.
- The samples were read using the optical density OD) setting of the Modulus II Microplate Multimode Reader (Promega Corporation).

The remaining samples from the above subjects were analysed by multiplex bead arrays (R&D Systems) using a MAGPIX Luminex system (Luminex,
Texas, USA), according the manufacturer’s instructions. The Luminex assay was similar in principle to the ELISA assay, except it used colour-coded beads pre-coated with antibody, which were detected by a proprietary system, rather than a colorimetry system. The advantage of the Luminex system was that it allowed multiple cytokines to be simultaneously detected in the same sample. For this experiment, the assay protocol did not require the samples to be pre-acidified. The principle steps of the Luminex assay were, as follows:

- Colour-coded beads, pre-coated with analyte-specific capture antibody for IL-10, IFNγ, IL-17A and IL-22 were added to the samples. These analyte-specific antibodies captured the analytes of interest.

- Biotinylated detection antibodies specific to the above analytes were added to form an antibody-antigen sandwich.

- Phycoerythrin (PE)-conjugated Streptavidin was added.

- The beads were detected on the MAGPIX Luminex system (Luminex) analyser. This was a dual-laser flow-based detection instrument where one laser classified the bead and determined the analyte that was being detected and second laser determined the magnitude of the PE-derived signal, which was in direct proportion to the amount of bound analyte.

- The data were analysed using xPONENT software (Luminex).

Standard curves were plotted using the data obtained from the above assays (included in the Appendix). Minimum levels of detection based on these
standard curves for each array were: TGF-β (7.4pg/mL), IL-10 (7.30pg/mL), IFNγ (17.30pg/mL), IL-17A (2.65pg/mL) and IL-22 (6.10pg/mL).

For intracellular cytokine staining, freshly isolated PBMC from a patient with active disease (Patient 1) at 2 months and 6 months after baseline were stimulated with Dynabeads Human T-Activator CD3/CD28 (ThermoFisher Scientific) for 24 hours or a cell stimulation cocktail (containing PMA and ionomycin) 1:500 for 6 hours as a positive control, with the addition of protein transport inhibitor (containing Brefeldin A) to the culture medium for the last 4 hours. Staining and flow cytometric analyses were as in Chapter 3, with the following modifications: directly conjugated Abs for cell surface molecules (CD3 and CD4); fixation and permeabilization with an intracellular cytokine fixation/ permeabilization kit (BD Biosciences); and directly conjugated Abs recognizing intracellular cytokines (IL-10, IFNγ and IL-17A). Subject-specific fluorescence-minus-one (FMO) controls for IL-10, IFNγ and IL-17A were included in each experiment.

3.2.18 Thawing of cryopreserved PBMCs
RPMI 1640 medium with the addition of 10% human AB serum was brought to 37°C in a water bath until use. Cryopreserved PBMCs required for further experiments were identified from storage in liquid nitrogen tank, removed and placed in a 37°C water bath to facilitate rapid thawing. As the freezing medium liquified, prewarmed RPMI medium was added to the cryotubes under aseptic conditions and cells were gently resuspended in new tubes in a 20ml volume of prewarmed media. The cell suspension was then centrifuged for 10 minutes at 350 x g. The supernatants were decanted and cells were resuspended in media and re-washed to remove any remaining DMSO, before being used for further experiments.
3.2.19 Genomic DNA extraction
Genomic DNA extraction was performed directly on samples of thawed cryopreserved PBMC from each subject group using the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK). The genomic DNA extraction protocol was optimised for DNA yield and quality using leukocyte cone blood (NHS Blood and Tissue Bank, Colindale). The protocol was initially performed on cone blood-derived PBMC sorted into Treg and non-Treg T-cells, using the BD Influx high speed cell sorter, as described in Chapter 3. Unfortunately, cell losses occurred during the culturing of thawed cells and subsequent cell sorting, which meant that the samples did not yield the required DNA quantity (600ng-750ng) and quality (280/260) for bisulphite Amplicon Sequencing using an NGS approach on the Illumina sequencing platform. Therefore, the decision was made to perform DNA extraction on unsorted subject PBMC samples. The following DNA extraction protocol was used, adapted from the manufacturer’s instructions for extracting genomic DNA from cells:

A maximum of $5 \times 10^6$ PBMC were centrifuged for 5 min at $300 \times g$ (190 rpm). Pelleted cells were resuspended in $200 \mu l$ PBS. 20 μl proteinase K were added to the cell suspension. 200 μl Buffer AL was added and mixed thoroughly with the cell suspension by vortexing. Samples were incubated at 56°C for 10 min. Following this, 200 μl ethanol (96–100%) was added and the suspension was mixed thoroughly by vortexing. The resulting mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube. This was centrifuged at ≥ 6000 x g (8000 rpm) for 1 min. The flow-through in the collection tube was discarded. The spin column was placed in a new 2 ml collection tube and 500 μl of Buffer AW1 was added. This was centrifuged for 1 min at ≥6000 x g. The flow-through in the collection tube was discarded. The spin column was placed in a new 2 ml collection tube and 500 μl Buffer AW2 was added. This was centrifuged for 3
min at 20,000 x g (14,000 rpm). The flow-through in the collection tube was discarded. The spin column was transferred to a new 1.5 ml microcentrifuge tube. DNA was eluted by adding 200 μl Buffer AE to the centre of the spin column membrane. The sample was incubated for 1 min at room temperature (15–25°C). Following incubation, the sample was centrifuged for 1 min at ≥6000 x g to maximise the DNA yield. The spin column was discarded and the extracted DNA was resuspended in the microcentrifuge tube and stored at -70°C until methylation analysis.

3.2.20 DNA methylation analysis
DNA samples were transferred to Barts Genome Centre (London, UK) on ice, where they were analysed at five DNA sites of cytosine (CpG) methylation (FOXP3 TSDR, FOXP3 Promoter, TBX21, RORC2 and TIGIT) with bisulphite Amplicon Sequencing, using an NGS approach on the Illumina sequencing platform (Fluidigm, California, USA). A summary flow chart and bisulphite Amplicon target sites are shown in Figures 47 and 48, respectively. Further information on the primers used for the assays is included in the Appendix.
Figure 47: Flow diagram showing overview of DNA methylation analysis
3.2.21.1 Sample Quality

Prior to DNA bisulphite treatment, the integrity of the DNA was assessed using the Agilent 2100 Bioanalyser Tapestation (Agilent Technologies, Waldbronn, Germany) and the DNA concentration was measured using Qubit Fluorometric Quantification (Thermo Fisher Scientific).

3.2.21.2 Bisulfite conversion of DNA

500ng of normalised DNA was bisulphite converted using the EZ DNA Methylation kit (Cambridge BioSciences, Cambridge). The incubation conditions recommended by the manufacturers guide [95°C for 30 sec., 50°C for 60 min] x 16 cycles, 4°C hold were used for the conversion reaction. The yield of bisulphite converted DNA was estimated using the RNA-40 setting on the NanoDrop 8000 Spectrophotometer V2.0 (ThermoScientific, USA).
3.2.21.3 Targeted re-sequencing of methylation sites

Primers for the target sites were designed using Pyromark Assay Design 2.0 software (Qiagen) and synthesised with Fluidigm Universal CS1 and CS2 tags (Fluidigm, San Francisco, US) to allow for the construction of DNA libraries using Fluidigm proprietary indexes. Primer design was based on primers used in previously published research (Bending, et al. 2014; Zhang, et al. 2013b). In the case of the TSDR and Promoter regions of FOXP3, the primers were altered slightly so that the Amplicon size would be as close to 300bp as possible. Primer sequences were checked prior to synthesis for the assay by performing a BLAST sequence search.

MyTaq™ HS DNA Polymerase (Bioline, USA) was used to optimise the assays and amplify the DNAs. The PCR products were checked for amplification on an Agilent 2100 Bioanalyser D1K Tape (Agilent Technologies, Waldbronn, Germany) after which unique Fluidigm Barcodes were added to the PCR product (Table 2) under the following PCR cycling conditions: 95°C for 10 minutes; [95°C for 15 seconds, 60°C for 30 seconds, 72°C for 1 minute] cycle 14 times; 4°C hold.

To check that the barcodes had been successfully incorporated onto the PCR products, the barcoded PCR product was run on a Agilent D1K tape. If the barcodes had attached then a shift of ~60bp was observed between the PCR product and the barcoded products.

Equal volumes of barcoded products were pooled and primer dimer was removed using Agencourt Ampure XP beads (Beckman Coulter Life Sciences) at a volume of 1:1. The pool was quantified using High Sensitivity Qubit Reagents (Qiagen) and the library size was measured using the Agilent 2100 High Sensitivity D1K (Agilent Technologies, Waldbronn, Germany) to calculate the molarity of the library before sequencing.
150bp Paired-end sequencing was performed using the Miseq Illumina sequencer (Illumina, Inc) to a total of 400,000 reads passing filter per sample.

3.2.21.4 Analysis of variants
Bismark (Krueger & Andrews, 2011) was used to align bisulphite-treated reads to a human reference genome and calculate the cytosine methylation calls at the target sites. Biostatistical analysis generated beta (%) methylation values as a ratio of methylated to unmethylated DNA for each CpG site of interest.

3.2.21 Statistical analysis
Statistical analysis of final datasets was performed with SPSS version 24 (IBM Software) and GraphPad Prism version 7 (GraphPad Software). Data were plotted as histograms to assess normality and descriptive statistics were calculated. Differences between groups were determined by the Kruskal-Wallis test with post-hoc Bonferroni correction for multiple comparisons. Bivariate correlations between immunological variables were calculated using Spearman’s test. Relationships between selected variables, which had clinically relevant associations, were modelled using multiple linear regression and logistic regression, using ‘stepwise’ and ‘enter’ variable entry, respectively. Where possible, variables with a non-normal distribution were transformed to a normal distribution, using a log transformation, to include in the multiple regression model. All significance tests were 2-tailed. P values < 0.05 were considered significant. Results are expressed as frequency n (%) or median (IQR), unless otherwise stated.
Chapter 4: An Investigation into the Role of Treg in Clinical Remission of Sight-threatening Non-infectious Uveitis: Results

4.1 Subject characteristics
A total of 50 uveitis patients and 10 control subjects were recruited to the study (Table 14). Of the 50 patients recruited, 37 were in clinical remission and 13 had active disease at the time of recruitment. 22 (59%) of the patients in the clinical remission group received previous therapy with corticosteroids only, the remaining patients received additional second-line immunosuppressive treatment (Table 15).
Table 14: Subject demographics and clinical characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control n=10</th>
<th>Active n=13</th>
<th>Quiescent n=37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in years (IQR)</td>
<td>30 (29-33)</td>
<td>41 (27-46)</td>
<td>47 (35-55)</td>
</tr>
<tr>
<td>Female sex - no. (%)</td>
<td>6 (60)</td>
<td>6 (46)</td>
<td>19 (51)</td>
</tr>
<tr>
<td>Weight at baseline (kg)</td>
<td>-</td>
<td>75 (67-89)</td>
<td>73 (63-79)</td>
</tr>
<tr>
<td>Race or ethnic group - no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White/European</td>
<td>6 (60)</td>
<td>6 (46)</td>
<td>26 (70)</td>
</tr>
<tr>
<td>Asian/Middle Eastern</td>
<td>4 (40)</td>
<td>3 (23)</td>
<td>10 (27)</td>
</tr>
<tr>
<td>Black/Afro-Caribbean</td>
<td>0 (0)</td>
<td>4 (31)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Uveitis SUN classification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>-</td>
<td>6 (46)</td>
<td>16 (43)</td>
</tr>
<tr>
<td>Posterior</td>
<td>1 (8)</td>
<td>6 (16)</td>
<td></td>
</tr>
<tr>
<td>Panuveitis</td>
<td>6 (46)</td>
<td>15 (41)</td>
<td></td>
</tr>
<tr>
<td>Bilateral disease - no. (%)</td>
<td>-</td>
<td>10 (77)</td>
<td>30 (81)</td>
</tr>
<tr>
<td>Median visual acuity (logMAR)</td>
<td>-</td>
<td>0.2 (0.1-0.4)</td>
<td>0.2 (0.1-0.5)</td>
</tr>
<tr>
<td>Visually significant cataract (%)</td>
<td>-</td>
<td>2 (15)</td>
<td>9 (24)</td>
</tr>
<tr>
<td>Previous cataract surgery</td>
<td>-</td>
<td>2 (15)</td>
<td>15 (41)</td>
</tr>
<tr>
<td>Median disease duration since diagnosis – months (IQR)</td>
<td>- 33 (7-119)</td>
<td>147 (50-249)</td>
<td></td>
</tr>
<tr>
<td>Median time since last flare – months (IQR)</td>
<td>-</td>
<td>0 (14-114)</td>
<td>48 (14-114)</td>
</tr>
</tbody>
</table>
Table 15: Oral immunosuppression use by study subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control n=10</th>
<th>Active n=13</th>
<th>Quiescent n=37</th>
</tr>
</thead>
<tbody>
<tr>
<td>On oral immunosuppressive treatment at baseline- no. (%)</td>
<td></td>
<td>6 (46)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Previous oral immunosuppression received- no. (%)</td>
<td></td>
<td>10 (77)</td>
<td>37 (100)</td>
</tr>
<tr>
<td>Prednisolone only</td>
<td></td>
<td>4 (40)</td>
<td>22 (59)</td>
</tr>
<tr>
<td>Additional second line immunosuppression</td>
<td></td>
<td>6 (60)</td>
<td>15 (41)</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td></td>
<td>3 (23)</td>
<td>5 (14)</td>
</tr>
<tr>
<td>Ciclosporin</td>
<td></td>
<td>0 (0)</td>
<td>7 (20)</td>
</tr>
<tr>
<td>Azathioprine</td>
<td></td>
<td>1 (8)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td></td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Biologicals</td>
<td></td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Median duration of previous oral immunosuppression</td>
<td></td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>- months (IQR)</td>
<td></td>
<td>(4-12)</td>
<td>(7-72)</td>
</tr>
</tbody>
</table>

Of the active patients, who had intermediate uveitis, posterior uveitis or panuveitis, 11 (85%) had cells and/or haze in the vitreous, 7 (54%) had cells in anterior chamber, 6 (46%) had macular oedema, 3 (23%) had optic disc oedema, two (15%) had vasculitis and one (8%) had chorio-retinal lesions at baseline. Six (46%) of these patients with active disease were on systemic immunosuppression at the time of recruitment. A subgroup analysis comparing the active patients on therapy and active patients not on therapy at baseline did not reveal any differences between the groups. Four of the 13 active patients underwent additional immunophenotyping at 2, 6 and 12 months after starting or changing treatment.
4.2 Immunological results

The immunological biomarker levels and comparison across the three subject groups (active, remission and controls) with summary P-values are shown in Table 16, with the post-hoc pairwise comparisons and adjusted P-values shown in Table 17.
Table 16: Three-way comparison of differences in immunological markers between groups (important results highlighted in yellow)

<table>
<thead>
<tr>
<th>Immunological Marker</th>
<th>Subject Cohort</th>
<th>3-way Comparison</th>
<th>Summary P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cell subsets (%) and cell ratios in the</td>
<td>Control</td>
<td>Active</td>
<td>Remission</td>
</tr>
<tr>
<td>CD3+CD4+ compartment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD25+FoxP3+ (Treg)</td>
<td>5.6 (5.3-6.7)</td>
<td>4.4 (3.6-6.0)</td>
<td>7.1 (5.8-8.3)</td>
</tr>
<tr>
<td>CD25+FoxP3+ TIGIT+ (TIGIT+ Treg)</td>
<td>4.2 (3.3-4.4)</td>
<td>2.5 (2.1-4.3)</td>
<td>5.0 (4.2-5.9)</td>
</tr>
<tr>
<td>TIGIT+Treg to TIGIT+FoxP3+ (non-Treg)</td>
<td>0.6 (0.5-0.7)</td>
<td>0.4 (0.2-0.6)</td>
<td>0.6 (0.5-0.8)</td>
</tr>
<tr>
<td>Tbet+ (Th1)</td>
<td>57 (41-66)</td>
<td>59 (49-72)</td>
<td>49 (43-56)</td>
</tr>
<tr>
<td>RORyt+ (Th17)</td>
<td>2.4 (1.2-4.5)</td>
<td>1.5 (0.8-4.4)</td>
<td>1.7 (1.2-3.2)</td>
</tr>
<tr>
<td>Tbet to Th1</td>
<td>0.7 (0.6-0.8)</td>
<td>0.4 (0.3-0.6)</td>
<td>0.7 (0.5-0.9)</td>
</tr>
<tr>
<td>RORyt to Th17</td>
<td>0.4 (0.2-0.4)</td>
<td>2.0 (0.8-3.5)</td>
<td>2.8 (1.5-4.3)</td>
</tr>
<tr>
<td>Tbet+FoxP3+</td>
<td>0.7 (0.5-0.8)</td>
<td>0.7 (0.5-1.2)</td>
<td>1.1 (1.0-1.6)</td>
</tr>
<tr>
<td>RORyt+FoxP3+</td>
<td>0.6 (0.5-0.9)</td>
<td>0.5 (0.1-0.7)</td>
<td>0.6 (0.3-1.1)</td>
</tr>
<tr>
<td>RORyt+ Tbet</td>
<td>1.5 (0.6-2.5)</td>
<td>1.0 (0.4-2.0)</td>
<td>1.5 (0.9-2.3)</td>
</tr>
<tr>
<td>Serum cytokine levels (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>24 (23-25)</td>
<td>12 (10-12)</td>
<td>21.5 (20-23)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>37 (39-85)</td>
<td>62 (49-97)</td>
<td>161 (122-285)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>208(197-214)</td>
<td>409(405-419)</td>
<td>298 (296-300)</td>
</tr>
<tr>
<td>IL-17</td>
<td>14 (13-14)</td>
<td>15 (14-16)</td>
<td>13 (12-13)</td>
</tr>
<tr>
<td>IL-22</td>
<td>19 (17-20)</td>
<td>21 (20-22)</td>
<td>18 (17-19)</td>
</tr>
<tr>
<td>CpG site methylation levels (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXP3 Promoter</td>
<td>69 (52-75)</td>
<td>72 (66-77)</td>
<td>53 (46-68)</td>
</tr>
<tr>
<td>FOXP3 TSDR</td>
<td>69 (61-71)</td>
<td>76 (68-83)</td>
<td>63 (59-67)</td>
</tr>
<tr>
<td>TIGIT</td>
<td>54 (47-55)</td>
<td>59 (57-64)</td>
<td>48 (46-53)</td>
</tr>
<tr>
<td>TBX21/TBET</td>
<td>0.7 (0.6-0.8)</td>
<td>0.4 (0.3-0.5)</td>
<td>0.7 (0.5-0.8)</td>
</tr>
<tr>
<td>RORC2/RORγT</td>
<td>55 (52-61)</td>
<td>46 (44-51)</td>
<td>55 (51-58)</td>
</tr>
</tbody>
</table>
Table 17: Pairwise comparison of differences in immunological markers between groups

<table>
<thead>
<tr>
<th>Immunological Markers</th>
<th>Summary P-value (Kruskall-Wallis)</th>
<th>Adjusted P-values (Post-hoc Pairwise Comparison Between Remission, Active and Control Subjects)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Remission v. Active</td>
<td>Remission v. Control</td>
</tr>
<tr>
<td>T-cell subsets (%) and ratios in the CD3+CD4+ compartment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treg (CD25<em>FoxP3</em>)</td>
<td>0.000*** ↑0.000***</td>
<td>0.118</td>
</tr>
<tr>
<td>TIGIT*Treg</td>
<td>0.000*** ↑0.000***</td>
<td>0.661</td>
</tr>
<tr>
<td>TIGIT<em>Treg: TIGIT</em>FoxP3+</td>
<td>0.049↑↑0.049↑</td>
<td>1.000</td>
</tr>
<tr>
<td>Th1 (Tbet')</td>
<td>0.025↑↓0.024↑</td>
<td>0.755</td>
</tr>
<tr>
<td>Th17 (RORyT')</td>
<td>0.564 - - -</td>
<td>-</td>
</tr>
<tr>
<td>Treg: Th1</td>
<td>0.001↑↑0.001↑</td>
<td>1.000</td>
</tr>
<tr>
<td>Treg: Th17</td>
<td>0.000***↑0.005↑</td>
<td>↑0.000***↑0.014*</td>
</tr>
<tr>
<td>Tbet*Treg</td>
<td>0.000***↑0.005↑</td>
<td>↑0.000***</td>
</tr>
<tr>
<td>RORyT*Treg</td>
<td>0.363 - - -</td>
<td>-</td>
</tr>
<tr>
<td>Th1/17 (RORyT'Tbet')</td>
<td>0.183 - - -</td>
<td>-</td>
</tr>
<tr>
<td>Serum cytokine levels (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.000***↑0.011↑</td>
<td>0.082</td>
</tr>
<tr>
<td>TGF-β</td>
<td>0.001↑↑0.007↑</td>
<td>↑0.004↑</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.000***↓0.015↑</td>
<td>0.130</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.003↓↓0.002↑</td>
<td>0.460</td>
</tr>
<tr>
<td>IL-22</td>
<td>0.002↓↓0.001↑</td>
<td>0.693</td>
</tr>
<tr>
<td>CpG site methylation levels (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXP3 Promoter</td>
<td>0.037↓↓0.036↑</td>
<td>0.405</td>
</tr>
<tr>
<td>FOXP3 TSDDR</td>
<td>0.005↑↑0.003↑</td>
<td>0.709</td>
</tr>
<tr>
<td>TIGIT</td>
<td>0.004↑↑0.003↑</td>
<td>0.850</td>
</tr>
<tr>
<td>TBX21/ TBET</td>
<td>0.122 - - -</td>
<td>-</td>
</tr>
<tr>
<td>RORC2/ RORyT</td>
<td>0.006↑↑0.016↑</td>
<td>1.000</td>
</tr>
</tbody>
</table>
4.2.1 Clinical remission of uveitis is associated with higher levels of Treg polarised toward T-bet and TIGIT compared to active disease

To determine whether there was a difference in peripheral blood Treg levels between control, active and quiescent subjects in disease remission, their PBMC were analyzed by flow cytometry for levels of CD25^+FoxP3^+ Treg, FoxP3^+TIGIT^+ Treg, FoxP3^+TIGIT^+ T-cells and FoxP3^+T-bet^+ Treg in the CD3^+CD4^+ lymphocyte subset (Figures 49 and 51). Highly statistically significant differences were found between the active and remission groups in the levels of Treg (4.4±1.2 vs 7.1±1.3, P=0.0002) and TIGIT^+ Treg (2.5±1.3 vs 5.0±0.9, P=0.0002) and T-bet^+ Treg (0.7±0.4 vs 1.1±0.3, P=0.000) (Figures 50 and 55; Tables 16-17). The levels of CD4^+FoxP3^+TIGIT^+ Treg and CD4^+FOXP3^+TIGIT^+ T-cells in each subject sample were analysed as the ratio of CD4^+FoxP3^+TIGIT^+ Treg to CD4^+FOXP3^-TIGIT-. The ratio of CD4^+FoxP3^+TIGIT^+ Treg to CD4^+FOXP3^-TIGIT^+ T-cells was significantly higher in the remission group compared to the active group (0.6±0.2 vs 0.4±0.1, P=0.049) (Figure 52; Tables 16-17). CD25^+FoxP3^+ Treg, FoxP3^+TIGIT^+ Treg and FoxP3^+T-bet^+ Treg were also observed to increase as disease resolved in patients with active uveitis (Figure 56). There were no statistically significant differences between Treg levels in control and active uveitis patient groups (Tables 16-17).
Figure 49: Flow cytometry plots showing example % levels of CD25^+FoxP3^+ Treg (left column) and FoxP3^+TIGIT^+ Treg (right column) in the three subject groups

Figure 50: Dot plots showing comparative % levels of CD25^+FoxP3^+ Treg (left) and FoxP3^+TIGIT^+ Treg (right) in the three subject groups
Figure 51: Flow cytometry plots showing example % levels of CD4^FoxP3^TIGIT^ Treg and CD4^FOXP3^TIGIT^ T-cells in the three groups (analysed as the ratio of CD4^FoxP3^TIGIT^ Treg to CD4^FOXP3^TIGIT^).

Figure 52: Dot plots showing comparative ratios of CD4^FoxP3^TIGIT^ Treg to CD4^FOXP3^TIGIT^ T-cells in the three subject groups.
Figure 53: Flow cytometry plots showing example % levels of T-bet+ Treg in the three subject groups

Figure 54: Flow cytometry plots showing example % levels of FoxP3+RORγt+ Treg in the three subject groups
Figure 55: Dot plots showing comparative % levels of FoxP3⁺T-bet⁺Treg (left) and FoxP3⁺RORγ⁺Treg (right) in the three subject groups.

Figure 56: % levels of CD25⁺FoxP3⁺ Treg (top left), FoxP3⁺TIGIT⁺ Treg (top right), FoxP3⁺T-bet⁺ Treg (bottom left) and FoxP3⁺RORγ⁺ Treg (bottom right) in patients with active disease over 12 months after starting/ changing oral immunosuppression treatment.
4.2.2 FoxP3+RORγt+ Treg are not associated with clinical remission but may have a role in clinical resolution of non-infectious uveitis

Peripheral blood levels were analyzed for CD3+CD4+ FoxP3+RORγt+ T-reg (Figure 54). Although the remission and control groups had higher median levels of FoxP3+RORγt+ T-reg compared to the active group, these differences did not reach statistical significance (P=0.363) (Figure 55; Tables 16-17). However, an overall increase in FoxP3+ RORγt+ Treg levels was observed over 12 months in all four patients with active disease at baseline (Figure 56).

4.2.3 Clinical remission of uveitis is associated with overall lower levels of Th1 transcription factors and a higher ratio of Treg to Th1 compared to active disease

To determine whether there was a difference in Th1 (T-bet transcription factor expression) levels and ratios of Treg to Th1 between control, active and quiescent subjects in disease remission, their PBMC were analysed by flow cytometry for levels of CD25+FoxP3+ Treg and T-bet+ T-cells in the CD3+CD4+ lymphocyte subset (Figures 50 and 52). Significantly lower levels of T-bet transcription factor levels (48.5±6.8 vs. 58.6±11.7, P=0.024) and higher ratios of Treg:Th1 (0.7±0.2 vs. 0.4±0.1, P=0.001) were found in remission patients compared to active patients (Figure 59; Tables 16-17). Active patients also had significant lower ratios of Treg:Th1 compared to control subjects (0.4±0.1 vs. 0.7±0.1, P=0.013) (Tables 16-17). Disease resolution in active patients over the course of 12 months appeared to be associated with an increase in levels of Treg and decrease in levels of Th1.
4.2.4 Clinical remission of uveitis is not associated with overall lower levels of Th17 transcription factors or a higher ratio of Treg to Th17 compared to active disease

To determine whether there was a difference in Th17 (RORγt+ transcription factor expression) levels and Treg:Th17 ratios between control, active and quiescent subjects in disease remission, their PBMC were analysed by flow cytometry for levels of CD25+FoxP3+ Treg and RORγt+ T-cells in the CD3+CD4+ lymphocyte subset (Figure 57). No significant difference was found in Th17 levels between the three groups (P=0.564), although it was noted that both active (2.0±1.3) and remission (2.8±1.4) patients had significantly higher Treg:Th17 ratios in comparison to control subjects (0.4±0.3, P=0.014; P=0.0003 respectively) (Figure 58; Tables 16-17). Increased Th17 levels in three out of four patients with active disease were noted as the disease clinically resolved over 12 months (Figure 59).

![Flow cytometry plots showing example % levels of Th17 CD4*RORγt+ T-cells (above) and Th1 CD4*T-bet+ T-cells (below) in the 3 groups](image)

Figure 57: Flow cytometry plots showing example % levels of Th17 CD4*RORγt+ T-cells (above) and Th1 CD4*T-bet+ T-cells (below) in the 3 groups
4.2.5 Clinical remission of uveitis is not associated with decreased levels of ‘double positive’ effector RORγt+Tbet+ T-cells

Peripheral blood levels were analyzed for ‘double positive’ RORγt+Tbet+ T-cells in the CD3+CD4+ subset (Figure 60). There was no significant
difference in levels of RORγt+ Tbet+ T-cells between the three groups (P=0.183) (Figure 6; Tables 16-17).

Figure 60: Flow cytometry plots showing example % ‘double positive’ effector CD4+RORγt+ Tbet+ T-cells in the three subject groups

Figure 61: Dot plots showing % ‘double positive’ effector CD4+RORγt+ Tbet+ T-cells in the three subject groups

4.2.6 Clinical remission of uveitis is associated with Treg which demonstrate a high capacity to suppress proliferating T-effectors

Cell-sorted populations of Treg, putative T-effectors and macrophages were set up in in vitro culture and the T-cell proliferation index was modelled by Modfit software, which was used to calculate % suppression by Treg, as described above. Treg from active uveitis without treatment failed to suppress proliferation of T-cells in vitro (Figure 62). In contrast, Treg from
active uveitis treated with oral immunosuppression, Treg from control subjects and Treg from uveitis in clinical remission all showed *in vitro* functional suppression, with the highest suppressive capacity demonstrated by Treg from uveitis in clinical remission (Figure 62). Data is shown from the following subjects: Patients 1 and 50 (in active disease group), Patient 49 (in clinical remission group) and Volunteer 2 (in control group). Patient 50 had active disease and had not yet started oral immunosuppression, whereas Patient 1 had a diagnosis of active disease at baseline and had received 6 months of oral immunosuppression.

![Figure 62: Summary of T-cell and Treg co-culture assay proliferation data showing % suppression capacity of Treg from each subject group.](image)

Figure 63: Serum cytokine levels (pg/mL) in the three subject groups: A. IL-10 B. TGF-β C. IFN-γ D. IL-17A and E. IL-22

Figure 64: Intracellular cytokine levels (IL-10, IL-17A and IFN-γ) in a patient with active uveitis at 2 months and 12 months after changing oral immunosuppressive treatment.

4.2.7 Clinical remission of uveitis is associated with higher serum levels of TGF-β and IL-10 and lower serum levels of IFNγ, IL-17A and IL-22 compared to active disease

Cytokine levels of IL-10, TGF-β, IFNγ, IL-17A and IL-22 were assayed in serum from 10 remission patients, 8 active patients and 5 control subjects from the above groups. IL-10 levels were significantly higher in serum from
remission patients compared to active patients (21.5±2.4 vs 11.6±1.5, P=0.011), and lower in active patients compared to controls (11.6±1.5 vs 24.2±2.3, P=0.000) (Figure 63; Tables 16-17). TGF-β levels were higher in remission patients than in active (161.1±81.4 vs 62.7±24.0, P=0.007) and control (161.1±1.5 vs 56.9±24.0, P=0.004) subjects (Figure 63; Tables 16-17). Clinical remission was also associated with significantly lower serum levels of IFNγ (P=0.015), IL-17A (P=0.002) and IL-22 (P=0.001) compared to patients with active disease (Figure 63). Increased intracellular IL-10 and IL-17A and decreased intracellular IFNγ levels were found in CD4+ T-cells from active uveitis, 6 months after starting treatment, when the disease had clinically resolved (Figure 64).
Figure 65: DNA methylation levels at A. FoxP3 TSDR B. FoxP3 Promoter C. TIGIT D. RORC2 and E. TBX21
Figure 66: Change in DNA methylation levels at A. FoxP3 TSDR B. FoxP3 Promoter C. TIGIT D. RORC2 and E. TBX21 in patients with active treatment over a 12 month period after starting/changing oral immunosuppression.
4.2.8 Lower levels of methylation at key epigenetic CpG sites determining Treg function in PBMC were associated with clinical remission but were not observed with consistency in clinical resolution of uveitis

To determine whether there was a difference in methylation at epigenetic CpG sites associated with Treg function (FoxP3 and TIGIT), bisulphite Amplicon Sequencing using an NGS approach was performed using cryopreserved PBMC from 8 active patients, 8 control subjects and 12 remission patients. The sample consisted of 17 female subjects (61%) and 11 male subjects (39%). Cryopreserved served samples, at 12 months following treatment, were also obtained from 4 of the 8 active patients. Following biostatistical analysis of the epigenetic percentage (%) methylation data, a single CpG site demonstrating a high level of differential methylation between samples was selected for statistical comparison between the three groups at that site, as follows: FoxP3 TSDR (49117116), FoxP3 Promotor (49121204) and TIGIT (114012658). The median CpG site % methylation levels of the FoxP3 TSDR (48±3.6 vs 59±7.4, P=0.003) (Figure 65A), FoxP3 Promotor (53±11 vs 72±5.3, P=0.036) (Figure 65B) and TIGIT (48±3.3 vs 59±3.3, P=0.003) (Figure 65C; Tables 16-17). CpG sites were significantly lower in patients in clinical remission compared to active patients. When comparing % CpG site methylation within individual patients with active disease at 0 months and resolved disease at 12 months, a decrease in methylation at the FoxP3 Promotor was observed over time whereas the methylation of FoxP3 TSDR and TIGIT appeared more stable (Figure 66A, B & C).
4.2.9 Higher levels of methylation at RORC CpG sites, but not TBX21 CpG sites were associated with clinical remission, and increased levels RORC methylation may be a biomarker of disease response to treatment

To determine whether there was a difference in methylation at epigenetic CpG sites associated with the T-effector transcription expression levels (RORγt and T-bet) assessed by flow cytometry in this study, bisulphite Amplicon Sequencing using an NGS approach was performed using cryopreserved PBMC as previously described. Following biostatistical analysis of the epigenetic percentage (%) methylation data, a single CpG site demonstrating a high level of differential methylation between samples was selected for statistical comparison between the three groups at that site, as follows: RORC2/ RORγT (151798858) and TBX21/ T-BET (45810951). The median CpG site % methylation level of RORC2 was higher in patients in clinical remission (55±3.3 vs 46±3.4, P=0.016) and in control subjects compared to active (55±4.3 vs 46±3.4, P=0.014) patients (Figure 65D; Tables 16-17). Within the active patient cohort, higher RORC % CpG methylation levels were observed at 12 months compared to 0 months after starting/ changing immunosuppressive treatment (Figure 66D). TBX21 CpG sites had overall low levels of methylation compared to the other T-cell-associated epigenetic methylation sites and no significant difference in the median % methylation levels was found between the three cohorts (Figure 65E and 66E).
### 4.2.10 Bivariate correlations between immunological variables

#### Table 18: Correlation matrix of immunological variables (with statistically significant correlations in bold)

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Correlation Coefficient</th>
<th>Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Treg</td>
<td>% Methylation FoxP3 promoter</td>
<td>0.169</td>
<td>0.203</td>
</tr>
<tr>
<td>% Treg</td>
<td>% Methylation FoxP3 TSDR</td>
<td>0.330</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>Ratio of TIGIT Treg to TIGIT T-cells</td>
<td>0.207</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>IL-10 Levels</td>
<td>0.350</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>TGFβ Levels</td>
<td>0.297</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>% Methylation RORγt T-cells</td>
<td>0.355</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>Methylation RORC2</td>
<td>0.292</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>Methylation TBXT2</td>
<td>0.221</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>IL-22 Levels</td>
<td>0.367</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>IL-17 Levels</td>
<td>0.231</td>
<td>0.002</td>
</tr>
<tr>
<td>% Treg</td>
<td>IFNγ Levels</td>
<td>0.368</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>% Methylation FoxP3 promoter</td>
<td>0.189</td>
<td>0.107</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>% Methylation FoxP3 TSDR</td>
<td>0.330</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>Ratio of TIGIT Treg to TIGIT T-cells</td>
<td>0.207</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>IL-10 Levels</td>
<td>0.350</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>TGFβ Levels</td>
<td>0.297</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>% Methylation RORγt T-cells</td>
<td>0.355</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>Methylation RORC2</td>
<td>0.292</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>Methylation TBXT2</td>
<td>0.221</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>IL-22 Levels</td>
<td>0.367</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>IL-17 Levels</td>
<td>0.231</td>
<td>0.002</td>
</tr>
<tr>
<td>Previous Oral Treatment Duration</td>
<td>IFNγ Levels</td>
<td>0.368</td>
<td>0.002</td>
</tr>
</tbody>
</table>

**Note:** Correlation coefficients are given as Spearman's rho (ρ) with significant correlations highlighted in bold.
**Table 19: Bivariate correlation of Treg expressing T-bet with Treg expressing TIGIT (additional to data in correlation matrix)**

<table>
<thead>
<tr>
<th>Spearman's rho</th>
<th>Tbet_FoxP3 Correlation Coefficient</th>
<th>TIGIT_FoxP3 Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbet_FoxP3</td>
<td>1.000</td>
<td>.431**</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>.</td>
<td>.001</td>
</tr>
<tr>
<td>N</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>TIGIT_FoxP3</td>
<td>.431**</td>
<td>1.000</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>.001</td>
<td>.</td>
</tr>
<tr>
<td>N</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

**. Correlation is significant at the 0.01 level (2-tailed).

### 4.2.10.1 Summary of significant positive correlations (from Tables 18 & 19)

Previous oral immunosuppressive treatment duration positively correlated with the ratio of TIGIT Treg to TIGIT T-cells ($r=0.369$, $P<0.05$). Treg levels positively correlated with TIGIT levels ($r=0.683$, $P<0.01$) and the ratio of TIGIT Treg to TIGIT T-cells ($r=0.425$, $P<0.01$); serum TGF-β ($r=0.752$, $P<0.01$), serum IL-10 ($r=0.667$, $P<0.01$) methylation at the RORC locus ($r=0.419$, $P<0.05$) across all subjects. TIGIT levels positively correlated with serum TGF-β ($r=0.813$, $P<0.01$). Tbet levels positively correlated with IFN-γ
levels (r=0.463, P<0.05). IL-22 and IL-17 levels positively correlated with IFN-γ levels (r=0.567, P<0.05, r=435, P<0.05). IL-22 levels positively correlated with IL-17 levels (r=0.791, P<0.01). Methylation at the FOXP3 promoter positively correlated with methylation at the TIGIT locus (0.515, P<0.05) and serum IL-17 levels (0.564, P<0.05). The methylation at the FOXP3 TSDR locus positively correlated with methylation at TIGIT (0.392, P<0.05), serum IL-22 levels (0.623, P<0.05) and IL-17 levels (0.700, P<0.05). The methylation at the TIGIT locus positively correlated with serum IL-17 levels (r=0.590, P<0.05). Thet+FoxP3+ cell levels positively correlated with TIGIT+FoxP3+ cell levels (r=0.431, P<0.05).

4.2.10.2 Summary of significant negative correlations (from Table 18)
Previous oral immunosuppressive treatment negatively correlated with methylation of FoxP3 TSDR (r=-0.667, P<0.01), T-bet expression levels (r=-0.377, P<0.01) and IFN-γ levels (r=-0.544, P<0.05). Treg levels negatively correlated with methylation at the FoxP3 TSDR (r=-0.479, P<0.05), methylation at the TIGIT locus (r=-0.499, P<0.01), serum IL-22 levels (r=-0.524, P<0.05) and serum IL-17 levels (r=-0.421, P<0.05). Methylation at FoxP3 TSDR also negatively correlated with serum IL-10 levels (r=-0.561, P<0.01). Serum IL-10 levels negatively correlated with serum IL-22 levels (r=-0.465, P<0.05) and serum IFN-γ levels (r=-0.797, P<0.01). TGF-β levels negatively correlated with IL-22 levels (r=-0.422, P<0.05). Methylation at RORC2 negatively correlated with IL-22 (r=-0.668, P<0.05), IL-17 (r=-0.575, P<0.05) and IFN-γ levels (r=-0.620, P<0.05).
4.2.11 Linear regression modelling of previous oral immunosuppressive treatment (Ln_Prev_Rx, log transformed variable) as a predictor of FoxP3 TSDR methylation

\[ Y = -0.1868^X + 72.47, \ p = 0.0116 \]

Figure 67: Simple linear regression of the duration of the previous oral immunosuppression treatment (months) and DNA methylation of FoxP3 TSDR
Table 20: Summary of multiple linear regression model of methylation of FoxP3 TSDR

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>P-value</th>
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</thead>
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<tr>
<td>Previous duration of oral immunosuppressive treatment (years)</td>
<td>-5.919</td>
<td>0.002**</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.073</td>
<td>0.478</td>
</tr>
</tbody>
</table>
4.2.11.1 Model 1 with previous oral immunosuppression duration as a predictor and FoxP3 methylation as the outcome

### Model 1 Variables Entered/Removed\(^a\)

<table>
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<tr>
<th>Model</th>
<th>Variables Entered</th>
<th>Variables Removed</th>
<th>Method</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(Criteria:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Probability-of-F-to-enter</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;= .050,</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Probability-of-F-to-remove</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;= .100)</td>
</tr>
</tbody>
</table>

\(^a\) Dependent Variable: Meth_FOXP3_TSDR

### Model 1 Summary\(^b\)

<table>
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<tr>
<th>Model</th>
<th>R</th>
<th>R Square</th>
<th>Adjusted R Square</th>
<th>Std. Error of the Estimate</th>
<th>Durbin-Watson</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>.675(^a)</td>
<td>.456</td>
<td>.424</td>
<td>6.38633</td>
<td>1.528</td>
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</table>

\(^b\)
a. Predictors: (Constant), Ln_Prev_Rx

b. Dependent Variable: Meth_FOXP3_TSDR

### ANOVA

<table>
<thead>
<tr>
<th>Model</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Regression</td>
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<td>1</td>
<td>581.388</td>
<td>14.255</td>
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<tr>
<td></td>
<td>Residual</td>
<td>693.349</td>
<td>17</td>
<td>40.785</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1274.737</td>
<td>18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Dependent Variable: Meth_FOXP3_TSDR

b. Predictors: (Constant), Ln_Prev_Rx

### Coefficients

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
</tr>
<tr>
<td>1 (Constant)</td>
<td>83.673</td>
<td>4.534</td>
</tr>
<tr>
<td>Ln_Prev_Rx</td>
<td>-5.704</td>
<td>1.511</td>
</tr>
<tr>
<td>Model</td>
<td>Tolerance</td>
<td>VIF</td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>-----</td>
</tr>
<tr>
<td>1</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

a. Dependent Variable: Meth_FOXP3_TSDR
4.2.11.2 Model 2 with previous oral immunosuppression duration as a predictor and FoxP3 methylation as the outcome, with adjustment for subject age

Model 2 Variables Entered/Removed\textsuperscript{a}

<table>
<thead>
<tr>
<th>Model</th>
<th>Variables Entered</th>
<th>Variables Removed</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Ln_Prev_Rx, Age</td>
<td>.</td>
<td>Enter</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Dependent Variable: Meth\textunderscore FOXP3\textunderscore TSDR

\textsuperscript{b} All requested variables entered.

Model 2 Summary\textsuperscript{b}

<table>
<thead>
<tr>
<th>Model</th>
<th>R</th>
<th>R Square</th>
<th>Adjusted R Square</th>
<th>Std. Error of the Estimate</th>
<th>Durbin-Watson</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>.688\textsuperscript{a}</td>
<td>.473</td>
<td>.408</td>
<td>6.47712</td>
<td>1.488</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Predictors: (Constant), Ln\_Prev\_Rx, Age

\textsuperscript{b} Dependent Variable: Meth\textunderscore FOXP3\textunderscore TSDR
ANOVA\(^a\)

<table>
<thead>
<tr>
<th>Model</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Regression</td>
<td>603.487</td>
<td>2</td>
<td>301.744</td>
<td>7.192</td>
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<tr>
<td></td>
<td>Residual</td>
<td>671.250</td>
<td>16</td>
<td>41.953</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1274.737</td>
<td>18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Dependent Variable: Meth\_FOXP3\_TSDR

b. Predictors: (Constant), Ln\_Prev\_Rx, Age

Coefficients\(^a\)

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
</tr>
<tr>
<td>2</td>
<td>(Constant)</td>
<td>81.101</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>.073</td>
</tr>
<tr>
<td></td>
<td>Ln_Prev_Rx</td>
<td>-5.919</td>
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</table>
### Coefficients\(^a\)

<table>
<thead>
<tr>
<th>Model</th>
<th>Tolerance</th>
<th>VIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (Constant)</td>
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<tr>
<td>Age</td>
<td>.964</td>
<td>1.038</td>
</tr>
<tr>
<td>Ln_Prev_Rx</td>
<td>.964</td>
<td>1.038</td>
</tr>
</tbody>
</table>

\(^a\) Dependent Variable: Meth\_FOXP3\_TSDR

### Collinearity Diagnostics\(^a\)

<table>
<thead>
<tr>
<th>Model</th>
<th>Dimension</th>
<th>Eigenvalue</th>
<th>Condition Index</th>
<th>Variance Proportions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Constant) Age</td>
<td>Ln_Prev_Rx</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2.871</td>
<td>1.000</td>
<td>.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>.085</td>
<td>5.799</td>
<td>.00</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>.61</td>
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<tr>
<td>3</td>
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<td>.044</td>
<td>8.106</td>
<td>.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.38</td>
</tr>
</tbody>
</table>

\(^a\) Dependent Variable: Meth\_FOXP3\_TSDR
4.2.11.3 Distribution of residuals for Models 1 & 2

Figure 68: Distribution of residuals for multiple linear regression model of the methylation of FoxP3 TSDR

4.2.11.4 Summary of Results
A simple linear regression of the duration of the previous oral immunosuppression treatment (months) and DNA methylation of FoxP3 TSDR was significant (Figure 67). Model 1, a multivariate linear regression model of methylation of the FoxP3 TSDR, which included the duration of previous immunosuppressive treatment as a predictor, was also significant.
(P=0.002) and explained around 42% of the variance in FoxP3 TSDR. Additional predictive variables, including TIGIT\(^+\), FoxP3\(^+\), IL-10 and TGF-\(\beta\) levels, added stepwise to the model were not significant and were therefore removed from the final model. In Model 2, age was added as a co-variate to adjust for the effect of age on DNA methylation, methylation of the FoxP3 TSDR. The regression model remained significant, (P=0.006) despite adjustment for age, explaining around 41% of the variance in FoxP3 TSDR (Table 20). The residuals for the multiple regression model were checked for normal distribution (Figure 68).

4.2.12 Logistic regression modelling of clinical remission with TIGIT\(^+\) Treg levels as a predictor

Table 21: Summary of logistic regression model of clinical remission

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>95% C.I.</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIGIT (% expression)</td>
<td>3.1</td>
<td>1.5, 6.5</td>
<td>0.020*</td>
</tr>
<tr>
<td>Age</td>
<td>1.0</td>
<td>0.9, 1.1</td>
<td>0.365</td>
</tr>
</tbody>
</table>
4.2.10.1 Logistic regression model

**Omnibus Tests of Model Coefficients**

<table>
<thead>
<tr>
<th></th>
<th>Chi-square</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>18.402</td>
<td>2</td>
<td>.000</td>
</tr>
<tr>
<td>Block</td>
<td>18.402</td>
<td>2</td>
<td>.000</td>
</tr>
<tr>
<td>Model</td>
<td>18.402</td>
<td>2</td>
<td>.000</td>
</tr>
</tbody>
</table>

**Model Summary**

<table>
<thead>
<tr>
<th>Step</th>
<th>-2 Log likelihood</th>
<th>Cox &amp; Snell R Square</th>
<th>Nagelkerke R Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.904a</td>
<td>.308</td>
<td>.451</td>
</tr>
</tbody>
</table>

*a. Estimation terminated at iteration number 6 because parameter estimates changed by less than .001.*

**Hosmer and Lemeshow Test**

<table>
<thead>
<tr>
<th>Step</th>
<th>Chi-square</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.786</td>
<td>8</td>
<td>.671</td>
</tr>
</tbody>
</table>
### Classification Table

<table>
<thead>
<tr>
<th>Observed</th>
<th>Clinical Remission</th>
<th>Percentage Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.00</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>5</td>
</tr>
<tr>
<td>Step 1</td>
<td>Clinical Remission</td>
<td>.00</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>91.9</td>
</tr>
<tr>
<td>Overall</td>
<td>Percentage</td>
<td></td>
</tr>
</tbody>
</table>

a. The cut value is .500

### Variables in the Equation

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>S.E.</th>
<th>Wald</th>
<th>df</th>
<th>Sig.</th>
<th>Exp(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>TIGIT</td>
<td>1.145</td>
<td>.373</td>
<td>9.433</td>
<td>1</td>
<td>.002</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>.029</td>
<td>.032</td>
<td>.821</td>
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<td>.365</td>
</tr>
<tr>
<td></td>
<td>Constant</td>
<td>-4.826</td>
<td>1.875</td>
<td>6.624</td>
<td>1</td>
<td>.010</td>
</tr>
</tbody>
</table>
### Variables in the Equation

**(Confidence Intervals)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIGIT</td>
<td>1.513</td>
<td>6.524</td>
</tr>
<tr>
<td>Age</td>
<td>.967</td>
<td>1.095</td>
</tr>
<tr>
<td>Constant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

a. Variable(s) entered on step 1: TIGIT, Age.
4.2.10.3 Testing TIGIT as a biomarker of clinical remission

Table 22: TIGIT$^+$ Treg as a biomarker of clinical remission

<table>
<thead>
<tr>
<th>TIGIT</th>
<th>Observed - Clinical Remission</th>
<th>Observed - Active Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted - Clinical Remission</td>
<td>34 (TP)</td>
<td>5 (FP)</td>
</tr>
<tr>
<td>Predicted - Active Disease</td>
<td>3 (FN)</td>
<td>8 (TN)</td>
</tr>
</tbody>
</table>

where TP=true positive, FP= false positive, FN= false negative, TN=true negative

Sensitivity (%) = TP / (TP + FN) x 100 = 92

Specificity (%) = TN / (FP + TN) x 100 = 62

False positive rate (%) = FP/ (FP + TN) = 38

False negative rate (%) = FN/ (FN + TP) = 8

4.2.10.3 Summary of Results

A logistic regression model that included TIGIT levels and subject age (years) as predictors of clinical remission was significant ($X^2$ (2) = 18.402, p<0.001) and a good fit for the data (Hosmer & Lemeshow test; $X^2$ (8) = 5.786, p=0.671).
It correctly classified 84% subjects and explained around 45% of the variance in clinical remission of sight-threatening non-infectious uveitis. TIGIT was a significant predictor of clinical remission, whereas age was not significant (Table 21).

Peripheral blood TIGIT levels in the study population of patients with uveitis had a sensitivity of 92%, a specificity of 62%, a false positive rate of 38% and a false negative rate of 8% for detecting clinical remission (Table 22).

4.3 Discussion
In this study, the hypothesis that phenotypically stable Treg induced by immunosuppressive drugs are associated with sustained clinical remission of non-infectious uveitis was investigated. This was demonstrated by performing peripheral blood immunophenotyping of large group of patients, who had previously received immunosuppressive drug therapy and were in sustained clinical remission, and comparing these results with those from patients with active uveitis and control subjects. Our data show that the frequency of CD4+CD25+FoxP3+ Treg, TIGIT+ Treg, T-bet+ Treg and the ratio of CD4+CD25+FoxP3+ Treg to Th1 (T-bet+CD4+) T-cells are higher in patients in clinical remission compared to patients with active disease. Tbet+FoxP3+ cell levels showed significant positive correlation with TIGIT+FoxP3+ cell levels. The levels of CD4+FoxP3+TIGIT+ Treg and CD4+FOX3+TIGIT+ T-cells in each subject sample were analysed as the ratio of CD4+FoxP3+TIGIT+ Treg to CD4+FOX3+TIGIT+ T-cells and this ratio was significantly higher in the remission group compared to the active group. This suggested that TIGIT expression proportionally increased in the CD4+FoxP3+ compartment of T-cells in the clinical remission group compared to the active group, rather than increasing on all T-cells.
A novel approach of NGS bisulphate sequencing targeted towards T-cell genes of interest, was used to identify whether there was an epigenetic immune methylation pattern associated with clinical remission in uveitis. The addition of a methyl group (-CH3) to DNA is a common epigenetic mechanism that cells use to switch genes ‘off’. Extensive methylation of cytosine in DNA is known to correlate with reduced gene transcription. In this study, PBMCs from patients in clinical remission had lower levels of DNA methylation at CpG sites within the FOXP3 TSDR, FOXP3 Promotor and TIGIT loci compared to patients with active disease, supporting the higher levels of FoxP3 and TIGIT expression detected by flow cytometry in these patients. Through in vitro functional studies, it is demonstrated that Treg from patients in clinical remission, a population in which there were high levels of Treg and TIGIT expression, are effective at suppressing T-cell proliferation. Together, these findings suggest that Treg from patients in clinical remission are polarised towards Type 1 inflammation and have a stable highly suppressive phenotype, characterised by higher intracellular T-bet and extracellular TIGIT expression, respectively, in comparison to subjects with active disease.

The cytokine milieu in non-infectious uveitis has been previously been investigated by our group and others (Kramer, et al. 2007; Ooi, et al. 2006a; Ooi, et al. 2006b; Takase, et al. 2006; Torun, et al. 2005; Zhao, et al. 2017). The focus of this was on investigating serum levels of specific cytokines associated with the Treg, Th1 and Th17 subsets identified in the flow cytometric analysis. Intracellular cytokine levels of IL-10, IFN-γ and IL-17A were also evaluated in active disease at 0 months and 6 months after starting therapy, at which time, disease had resolved. It was found that patients in clinical remission had higher serum levels of IL-10 and TGF-β than patients with active disease. Whilst control subjects also had higher levels of IL-10 than active patients, the higher serum TGF-β differentiated
remission patients from controls and were perhaps related to high levels of Treg with suppressive function in this group. Tregs levels positively correlated with serum IL-10 and TGF-β levels, suggesting that Tregs may directly or indirectly influence the systemic cytokine milieu. Furthermore, increased intracellular expression of IL-10 by T-cells from active disease was increased 6 months after starting treatment, which corresponded with clinical resolution of disease.

Subjects’ levels of Th1 and Th17 were evaluated by flow cytometric detection of their respective transcription factors, T-bet and RORγt, along with the corresponding serum cytokine levels of IFN-γ, IL-17 and IL-22 from peripheral blood samples. Upregulated T-bet expression in association with significantly increased IFN-γ levels has been previously demonstrated in patients with active uveitis secondary to Vogt-Koyanagi-Harada (VKH) syndrome, a bilateral chronic granulomatous panuveitis, which tends to be sight-threatening (Li, et al. 2005). Th17 cells have also been shown to be involved in ocular inflammation (Amadi-Obi, et al. 2007) and, more recently, B27+ anterior uveitis (Zhuang, et al. 2017). Paradoxical expression of effector CD4 T-cell transcription factors, such as T-bet and RORγt, by Treg has, however, been suggested to enhance Treg suppressive capacity in murine models of inflammation (Koch, et al. 2009; Levine, et al. 2017; Ohnmacht, et al. 2015; Sefik, et al. 2015). These models demonstrate that intestinal RORγt+FoxP3+ Treg induced in vivo by the local microbiota display a stable suppressive phenotype and exist in dynamic balance with pathogenic Th17 (Ohnmacht, et al. 2015; Omenetti and Pizarro 2015). The local microenvironment, for example, the cytokine milieu and/or the microbiome, have been shown to regulate the Treg/Th17 balance and influence cell plasticity; disruption of this balance may lead to the development of inflammatory disease (Leung, et al. 2010; Lochner, et al. 2008; Omenetti and Pizarro 2015; Sefik, et al. 2015). Furthermore, during Type 1 inflammatory

In the current study, we show that in clinical remission and control groups, the levels of T-bet+ Treg and ratios of Treg to Th1 are higher and serum levels of IFN-γ levels are lower compared to active uveitis patients. We also found that Tbet+ Th1 levels in uveitis patients were overall lower in clinical remission compared to active and control subjects. Disease resolution in active patients over the course of 12 months appeared to be associated with an increase in levels of Treg and a decrease in levels of Th1. Intracellular expression levels of IFN-γ by CD4+ T-cells were also decreased at 6 months after starting treatment. However, RORγt+ Th17 and RORγt+ Treg levels did not significantly differ between the three subject groups. Treg are thought to regulate the expression of IL-22, a Th17 cytokine which facilitates inflammatory cell infiltration in uveitis (Kim, et al. 2016; Lin, et al. 2014). Although the ratio of Treg to Th17 was not significantly greater in clinical remission, it was noted that serum IL-17A and IL-22 levels were significantly lower in the patients in clinical remission, compared to active and control subjects. RORγt+ Treg levels were increased in all four active patients at 12 months compared to baseline, as the disease clinically resolved. However, unlike Th1 cytokine levels, Th17 cytokine levels did not appear to consistently decrease when disease resolved in active patients. Three out of four active patients had increased serum levels of IL-17A at 12 months after starting treatment, compared to baseline. Intracellular expression levels of IL-17A of by CD4+ T-cells in active disease also increased at 6 months after starting treatment. Our results show that Th17 express FoxP3+ during disease resolution, in response to immunosuppressive treatment, but suggest that Th17-mediated inflammation is not driving inflammation and that FoxP3+RORγt+ Treg are not significantly associated with clinical remission.
Previous studies have compared Treg levels between active uveitis, inactive uveitis and/or healthy control subjects but there has been variation in subject groups and clinical immunophenotyping. A study from Chen et al included 49 patients with VKH syndrome with uveitis and without uveitis, and showed a decreased percentage of CD4+CD25^{high} T-cells, a decreased frequency of FoxP3^{+} expression in CD4+CD25^{high} T-cells and reduced functionality of CD4+CD25^{high} ‘Treg’ in patients with active uveitis (Chen, et al. 2008). Yeh et al also demonstrated that patients with active uveitis (n=8) have lower percentages of CD4^{+}FoxP3^{+} lymphocytes than patients with inactive disease (n=12) (Yeh, et al. 2009). In contrast to the current study, several patients in their series demonstrated evidence of systemic autoimmune disease, such as sarcoidosis and multiple sclerosis, which are, of themselves, associated with abnormal Treg populations or deficits in Treg suppressive function. Another study compared Treg levels in active and inactive uveitis patients and found that patients in remission on treatment (n=25) had significantly increased levels of CD4^{+} CD25^{+}FoxP3^{+} Treg compared to active patients on treatment (n=6) (Ruggieri, et al. 2012). However, over half of the patients (53%) recruited to their study had a diagnosis of anterior uveitis, which is not usually sight-threatening and does not require systemic immunosuppression.

Regarding the comparison of Treg levels in active patients with control subjects, the results from the literature are mixed. Two of aforementioned studies show a higher level of Treg in control subjects compared to uveitis patients (Chen, et al. 2008; Ruggieri, et al. 2012), however this could be due to the inclusion of patients with systemic disease in the uveitis groups. In contrast, Yeh et al comment that they have previously observed that the percentages of CD4^{+}FoxP3^{+} lymphocytes do not differ between patients with uveitis and control subjects (unpublished data) (Yeh, et al. 2009). Molins et al compared Treg levels and cytokine production in 21 patients with active non-infectious uveitis with 18 controls (Molins, et al. 2015). They found that

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PBMCs from uveitis patients produced lower levels of IL-10 than those from controls but no differences were observed in Treg levels. TGF-β levels were not measured and functional assays were not performed in this study. Recently, Zhuang et al compared 20 patients with active B27+ anterior uveitis with healthy controls and observed an increase in CD4+IL-17+ T cells, a decrease of CD4+CD25+FoxP3+ Treg and a higher ratio of Th17/Treg in peripheral blood of patients compared to controls (Zhuang, et al. 2017). The authors conclude that the imbalance of Th17 and Treg cells may play a vital role in the pathogenesis of B27+ anterior uveitis. Of note, patients with B27+ uveitis and anterior uveitis were excluded from the present study due to the typically non-sight-threatening anterior anatomic localisation of inflammation and systemic disease associations.

There has been interest in glucocorticoid-resistant Th17 cells which are refractory to Treg mediated suppression (Basdeo, et al. 2015; Ramesh, et al. 2014), and these have been previously investigated in sight-threatening uveitis (Lee, et al. 2009). Furthermore, a subset of ‘non-classic’ Th17-derived Th1 have been described, which are demethylated at RORC, and are thought to play a pivotal role in the establishment and persistence of inflammatory disease (Mazzoni, et al. 2015). These non-classic Th1 express CD161, a marker of steroid-resistant human Th17, induced by RORC (Maggi, et al. 2010). In the clinical setting, however, most cases of uveitis show a good clinical response to high dose corticosteroids. Second-line immunosuppressive drugs are usually prescribed because the dose of corticosteroids required to suppress disease activity is very high and disease recurs on tapering the dose, rather than because the disease is refractory to corticosteroids. Only one patient in the current study received biological therapy (Adalimumab), as the rest were clinically responsive to corticosteroids, with or without addition of conventional steroid-sparing agents. Twenty percent of the clinical remission group received previous
therapy with cyclosporine, which has been shown to selectively attenuate steroid-resistant Th17 and to be efficacious in the treatment of steroid-refractory disease (Schewitz-Bowers, et al. 2015; Schewitz, et al. 2009). It is of note, in this study, that the clinical remission and control groups had significantly higher levels of RORC CpG methylation than the active group. Furthermore, within the active patient cohort, higher RORC % CpG methylation levels were observed at 12 months after starting/ changing immunosuppressive treatment, when disease had resolved. RORC CpG hypomethylation could, therefore, be a biomarker of clinical response to corticosteroid treatment and a potential predictor of clinical remission.

The findings of the current study are consistent with previous studies in uveitis demonstrating that Treg levels are higher and more functional in patients with inactive disease, compared to both patients with active and control subjects. We have previously observed that in ocular Bečhet’s Disease, peripheral blood Treg levels are increased by 3 months’ treatment with the immunomodulating therapy, pegylated interferon-α (pegIFNα), and that the increased levels of Tregs are still detectable at 12 months, six months after cessation of therapy (Lightman, et al. 2015). However, the expression of the co-inhibitory molecule TIGIT by Treg from patients in sustained clinical remission of disease, who are no longer on therapy, has not been previously investigated in uveitis. It is known that stable FoxP3 expression is ensured, at least partly, by DNA demethylation at the FOXP3 TSDR and that this infers commitment to the Treg lineage (Floess, et al. 2007; Huehn, et al. 2009; Polansky, et al. 2008; Zheng, et al. 2010). In a genome-wide methylation analysis of nTregs from healthy individuals, it was found that hypomethylation at the TIGIT locus was one of the most significantly differentially methylated regions that distinguished naïve T-cells and nTreg; that it was not altered by activation and is required for FoxP3 binding (Zhang, et al. 2013b). Other studies have demonstrated a key role
for TIGIT in Treg-mediated suppression of Th1 and Th17 subsets (Joller, et al. 2011; Joller, et al. 2014) and that TIGIT signalling in Treg directs their immunoregulatory phenotype in chronic disease settings (Kurtulus, et al. 2015). Our data show that hypomethylation at FOXP3 TSDR, FOXP3 promoter and TIGIT CpG sites, and higher levels of TIGIT expression by Treg, are associated with sustained clinical remission in relapsing and remitting sight-threatening non-infectious uveitis.

Many significant correlations were discovered between immunological variables investigated in this study. Previous oral immunosuppressive treatment duration positively correlated with the ratio of TIGIT Treg to TIGIT T-cells and negatively correlated with methylation of FoxP3 TSDR, T-bet expression levels and IFN-γ levels. Treg levels negatively correlated with methylation at the FoxP3 TSDR, methylation at the TIGIT locus, serum IL-22 levels and serum IL-17 levels. Treg levels positively correlated with TIGIT levels, the ratio of TIGIT Treg to TIGIT T-cells, serum TGF-β and serum IL-10 levels. Treg levels also positively correlated with methylation at the RORC2 locus across all subjects, which in turn, negatively correlated with IL-22, IL-17 and IFN-γ levels. TIGIT levels positively correlated with serum TGF-β. Methylation at the FOXP3 promoter positively correlated with methylation at the TIGIT locus and serum IL-17 levels. The methylation at the FOXP3 TSDR locus positively correlated with methylation at TIGIT, serum IL-22 levels and IL-17 levels and negatively correlated with serum IL-10 levels. Methylation at the TIGIT locus positively correlated with serum IL-17 levels. Tbet+FoxP3+ cell levels positively correlated with TIGIT+FoxP3+ cell levels. These results support previous evidence that Tregs and/or TIGIT levels, induced by immunosuppressive treatment, are associated low levels of DNA methylation at their loci and that they mediate some of their regulatory effect through the cytokine milieu.
It was further investigated whether clinical remission in non-infectious sight-threatening uveitis could be modelled by a linear relationship with predictive immunological variables, after adjusting for co-variates in the model. Multiple regression analysis showed that the duration of previous oral immunosuppressive treatment was the strongest predictor of methylation of the FoxP3 TSDR, which was significantly associated with clinical remission in sight-threatening uveitis and which relationship was remained significant after adjusting for age. Logistic regression showed that peripheral blood TIGIT levels, but not subject age, were a significant predictor of clinical remission in sight threatening non-infectious uveitis. TIGIT levels had high sensitivity (92%) but low specificity (62%) and, therefore, a high false positive rate when used as a predictor of clinical remission of sight-threatening non-infectious uveitis in the study population of uveitis patients. TIGIT levels explained almost half of the variance observed in the model of clinical remission, but there were clearly other factors, not accounted for in the model, which were also influencing clinical remission.

Our study addressed a need to define and investigate sustained clinical and biological disease remission in sight-threatening non-infectious uveitis. The total number of subjects recruited to this study (n=60) represents one of the largest sample sizes of published clinical immunology studies of sight-threatening non-infectious uveitis. To improve the specificity of this study towards detecting peripheral blood biomarkers associated with ocular inflammation, we excluded patients with known systemic diagnoses, for example, sarcoidosis or Behçet’s disease, and any other identifiable systemic inflammation. We were rigorous in our clinical phenotyping of the recruited subjects and undertook a comprehensive immunophenotypic analysis of CD4+ T-cells derived from these groups, including functional and epigenetic methylation studies, which has not been previously performed in uveitis patients. T-cell immunophenotyping by flow cytometry was performed.
on freshly isolated subject PBMCs (within 4 hours of venepuncture) but additional intraocular sampling was not undertaken as part of this study. The thirty-seven patients in clinical remission recruited to the study demonstrated a good clinical ocular response to immunosuppressive therapy given systemically, supporting the fact that systemic Treg and their associated microenvironment influence ocular immunity and that peripheral blood biomarkers have utility in monitoring ocular disease. Phenotypic analysis of Treg, Th1 and Th17 subtypes was based on ‘master transcription factor’ expression, with prospective intracellular cytokine analysis only in selected subjects with active disease, followed up over a 12-month period. An in-depth analysis of intracellular cytokine production and methylation at signature cytokine loci could be considered for a future study. The number of Treg functional assays that were performed as part of this study was limited by the remaining PBMC numbers after the immunophenotypic analysis, rendering firm conclusions from these results difficult. The assay findings were in keeping with those from previous studies, which have demonstrated that in the inflammatory setting of autoimmune disease, Treg have reduced suppressive function (Bending, et al. 2014; Ehrenstein, et al. 2004). However, they could be confirmed as part of a further study.

In summary, these study data show that CD4+ T-cells in chronic relapsing, remitting sight-threatening non-infectious uveitis deviate towards the Th1 type and that Treg with upregulated levels of T-bet and TIGIT expression are associated with disease remission. These data provide supporting evidence that Treg are part of the mechanism of clinical remission in non-infectious uveitis, which has been previously demonstrated in EAU model studies only (Ke, et al. 2008; Silver, et al. 2015; Sun, et al. 2010). The overall lower T-bet expression and IFN-γ levels detected in patients who achieve sustained clinical remission may be related to potent suppression of Type 1 inflammation by phenotypically stable Treg induced by systemic
immunosuppressive therapy. This is an important finding because a recent study in mice has shown that Treg expressing T-bet have a stable and functional phenotype, which potentiates suppression of Th1 autoimmunity (Levine, et al. 2017), but it has not yet been demonstrated in clinical studies. These results are also consistent with evidence from EAU that relapsing, remitting uveitic disease is closely associated with a Th1-like phenotype whereas monophasic inflammation is associated with a Th17-like phenotype (von Toerne, et al. 2010).

Finally, these data are relevant to the recent interest in quantitative identification and isolation of viable, functional Treg for downstream clinical purposes, which includes their generation ex vivo for immunomodulating therapies (Foussat, et al. 2017; Horwitz, et al. 2004). This requires reliable surface markers that are selectively expressed on functional Treg. Putative Treg surface markers include Helios (Thornton, et al. 2010) and low CD127 (Liu, et al. 2006) but there has been some debate in the literature about their reliability. The results of this study provide evidence that TIGIT has utility as a marker of functional Treg for immunomodulatory therapy, and that low levels of TIGIT could be a biomarker for increased risk of disease relapse in non-infectious uveitis.
Chapter 5 Patient Perspectives of Sight-threatening Retinal Disease and Pilot Health Behaviour Change Intervention

5.1 Background and study rationale

5.1.1 Study aim
To investigate perspectives and health behaviours of patients with sight-threatening non-infectious retinal disease (which does not have curative treatment) and identify suitable interventional approach(es) which could serve to increase sense of agency and/or self-efficacy of affected individuals, in order to potentially change the course of their disease and/or its consequences.

5.1.2 Objectives

- Conduct individual interviews with patients with sight-threatening non-infectious uveitis recruited from prospective study described in Chapters 3&4 to characterise their experience of uveitis and its treatment.

- Conduct a focus group of patients with dry age-related macular degeneration (AMD) to identify perspectives on treatment and an evidence-based lifestyle intervention.
• Analyse potential problems identified in terms of perceived problems/unmet needs and/or health behaviours, which could be further addressed.

• Identify a target need and/or behaviour, and design a pilot intervention.

• Test pilot intervention with focus group patients and perform a preliminary evaluation of effectiveness, in terms of engagement with intervention.

5.1.3 Study design, setting and participants

Design: Observational Case Series

Setting: NHS tertiary referral centre for ophthalmology, NIHR Biomedical Research Centre for Ophthalmology and local community locations in London, UK

Participants: 4 patients prospectively recruited from a specialist uveitis clinic in tertiary care centre for ophthalmology in the UK and 12 patients with a diagnosis of dry AMD.

5.1.4 Ethical approvals and consents

Research performed as part of this thesis adhered to the tenets of the Declaration of Helsinki and was carried out in accordance with the recommendations of the UK National Research Ethics Service (NRES) – London Harrow Committee and the Moorfields Eye Hospital National Health Service (NHS) Foundation Trust, Department of Research and Development.
A National Institute of Health Research (NIHR) Biomedical Research Centre (BRC) film and photography release consent form was signed by all subjects who attended the ‘Eating for Eye Health’ engagement focus group and cooking day.

5.1.5 Rationale for qualitative data collection

There is a paucity of opportunity for doctors, especially those in training, to learn about the psycho-social aspects of eye disease within routine clinical education and practice. To address this issue, some emic (from the perspective of the subject) methodologies were introduced into the research plan for the thesis. This research study was partly informed by a concurrent pilot clinical education project funded by a UCL ‘Liberating the Curriculum’ grant. This clinical education project was delivered in consultation with the eye research charity, Fight for Sight, in order to assist doctors in delivering patient-centred care to subjects with visual impairment and is summarised in the following short report [Gilbert, Rose] (Rose, et al. 2018):

‘Despite its importance in primary and emergency care, ophthalmology is felt by medical students and physicians to be under-represented in the medical undergraduate and foundation curriculum. Furthermore, there is a paucity of opportunity for students and junior doctors to interact with and learn from members of the visually impaired community. Focus groups of visually-impaired patients have highlighted the disparity between clinician and patient perspectives of eye disease. Successful medical education encourages clinicians to promote health whilst respecting individuals’ self-perceived needs and voluntary choices. However, the opposing forces of increased training expectations and reduced training resources could greatly impact on this. Junior medical trainees may struggle to administer patient-centred care if they have not previously encountered specific patient groups during their training. Theoretically constructed educational interventions which
incorporate patient perspectives have been previously described to address this.

Electronic virtual patients (e-patients) were incorporated into existing online and taught clinical ophthalmology courses at our institution, providing an opportunity for the learner to partake in fictional clinical ophthalmology scenarios without causing any harm to a real patient. The online scenarios were presented in both linear and interactive non-linear branching formats, the latter allowing users to determine their own path through the case, and to explore potential clinical consequences of their choices. These clinical programmes were further developed through an institutional ‘inclusive education’ grant in consultation with the eye research charity, Fight for Sight, to include the ‘patient perspective’ of eye disease. Following completion of online material and lecture attendance, course participants were invited to attend a tutorial and a half day seminar on the patient perspective of eye disease. During the tutorial, students were supported by teaching assistants in planning a written assignment and an oral presentation on a clinical ophthalmology topic of their choice. They were encouraged to consider the impact of eye disease on the patient. Following the tutorial, students presented their clinical topics to patients at a half day seminar and they, in turn, listened to four ‘expert’ patients talk about their experience of having an eye disease. Outcomes were evaluated through online and paper student satisfaction surveys (n=80).

Both linear and branching e-patient scenarios had ‘very good’ levels of satisfaction amongst students (83% vs 86%), however the branching scenario was reported as being ‘more authentic’ (91% vs 60%), since it simulated the real-life experience of managing a patient with eye disease. The ‘patient perspectives’ seminar helped students appreciate the impact of eye disease on patients. For example, patients described receiving their diagnosis as ‘the beginning’ of their clinical journey, rather than ‘the end’ as
some students perceived it, which encouraged students to consider their style of communication. All students reported increased levels of confidence in caring for real-life patients with visual impairment after course completion. Whether completion of this programme by clinicians positively impacts clinical communication, patient safety and patient satisfaction requires further investigation.’

The methodological approach to this study was informed by The Stereotype Content Model (SCM), which ‘aims to capture the texture of each group’s situation, at the same time identifying the fundamental underlying dimensions that help explain some shared experiences…[It] starts by analysing what people need to know about each other, in order to function in the same social space.’ (Fiske, et al. 2007; Fiske, et al. 2002; Fiske 2012)

Qualitative methodology, therefore, in the form of interviews and focus groups, was utilised in this study in order to allow study participants to express themselves in a less constrained way than would be the case with data collected via questionnaire (Wilkinson 2004). It was thought that the accounts elicited by interviews and focus groups would have the potential to provide unexpected insights into patient perceptions of treatment, including factors that may not have been previously considered relevant (Wilkinson 2004).

5.1.6 Rationale for targeting health behaviours in chronic disease
Health-related behaviours can contribute to major causes of mortality and morbidity. Chronic diseases, which include autoimmune diseases, in addition to more common diseases such as cardiovascular disease (CVD), are a major cause of death and disability (NICE 2007). Many of these diseases, for example, CVD, have well known associations with behaviours and lifestyle factors that can be changed (e.g. smoking tobacco and diet), so-called
‘modifiable risk factors’. Behaviour is known to be influenced by ‘a range of socio-economic, cultural and environmental conditions, social and community networks and individual factors, such as age’. Behaviour change interventions may be defined as ‘coordinated sets of activities that seek to change specific behaviours’ (NICE 2007)

It has been determined that ‘a combination of interventions that tackle population, community and individual-level factors are needed to help people change their behaviour in the longer term’ and that ‘the following approaches can help:

- Using evidence-based principles and behaviour change techniques (the smallest “active” component of an intervention designed to change behaviour).
- Having a theoretical basis for interventions. This also helps in understanding why an intervention is effective or not.’

(NICE 2007)

5.1.7 Using theoretical models to predict health behaviour and/or design interventions

Adapted from UCL MSc in Health Psychology Module 4 ‘Health Related Behaviours and Cognition’ Essay Assignment Submission (Gilbert 2017, unpublished):

‘Social cognition models (SCMs) (Conner 2005) were developed to examine the role of social cognitions in determining health-related behaviour. ‘Social cognitions’, in the health context, refer to an individuals’ thoughts and feelings associated with performing or not performing a particular health behaviour. (Conner 2005). It was hypothesised that individual differences in health behaviours, often attributed to socio-demographics factors, such as gender and socio-economic status (Blaxter 1990), might be predicted by the sets of social cognitions described by SCMs. Social cognitions represent a
useful focus for health research because psychological factors are potentially more amenable to change than socio-demographic factors (NICE 2007). Prominent examples of SCMs include: the Health Belief Model (HBM) (Abraham 2005; Janz and Becker 1984; Rosenstock 1974), Protection Motivation Theory (PMT) (Conner 2005; Maddux and Rogers 1983), the Extended Parallel Processing Model (EPPM) (Witte 1992), the Theory of Planned Behaviour (TPB) (Azjen 1991) and Social Cognitive Theory (Self Efficacy) (SCT) (Bandura 2000).

The HBM (Abraham 2005; Janz and Becker 1984; Rosenstock 1974), the oldest and most widely used SCM, focuses on two health cognitions: perception of threat (perceived severity and susceptibility) from illness and evaluation of behaviours to counteract the perceived threat (including beliefs about benefits and barriers to performing the behaviour). Cues to action, as triggers of the health behaviour, are also included in this model. The PMT (Conner 2005; Maddux and Rogers 1983) is considered an extended, revised version of the HBM, which includes an appraisal regarding the illness threat and the coping process of alternative behaviours which might diminish the threat. The EPPM (Witte 1992) is also concerned with aspects of an individual’s cognitions regarding health threat perception (perceived susceptibility and perceived severity) but was extended to involve ‘efficacy perception’. In the EPPM, efficacy perception describes the effects of response efficacy (beliefs regarding the utility of the behaviour in averting a threat) and self-efficacy (beliefs of one’s own ability to perform the behavior). The TPB (Azjen 1991) specifies the factors that determine an individuals’ conscious decision to perform a behaviour. This model posits that behavioural action can be predicted by intention to act, based on attitudes towards the behaviour, subjective norms and perceived behavioural control. Furthermore, attitude is determined by behavioural beliefs weighted by evaluation of behavioural outcomes; subjective norms are determined by
normative beliefs weighted by motivation to comply with these beliefs; and perceived behavioural control is determined by perceived internal and external resources weighted against perceived opportunities to perform the behaviour. In the SCT (Bandura 2000), three factors determine health behaviours: self-efficacy, proximal goals and outcome expectancies. Proximal goals regulate effort and guide action and are similar to the concept of intention. Outcome expectancies are similar to the behavioural beliefs of the TPB but can be further subdivided into physical, social and self-evaluative beliefs depending on the context.

In a meta-analysis of 18 studies (2,702 subjects) conducted to determine whether the HBM could longitudinally predict behaviour, only two of the constructs, perceived benefits and barriers, were found to be consistently strong predictors of behaviour (Carpenter 2010). The inconsistency in predictive power of the constructs within the HBM has also proved problematic in other SCMs. This issue could be related to the variability in measurement of the central constructs during the research (Glanz 2008). The method of data collection in research using SCMs is often a self-reported questionnaire, which can be subject to self-presentation/social desirability bias and recall bias. Furthermore, it has been suggested that questionnaire studies might change or create cognitions and/or behaviour, thereby introducing artefact into SCMs studies through ‘mere measurement effects’ (Godin, et al. 2008; Ogden 2003). However, an overlap in SCM variables has also been described (Conner 1996; Ogden 2003), particularly in the domains of intention, self-efficacy and outcome expectancies, which have strong relationships with health behaviours across several studies (Conner 2005). This suggests that the power of SCMs to predict behaviour might be attributed to these individual variables rather than ‘the set’ of cognitions described in each model.
It has been argued that studies of health research using SCMs have not strongly supported the specific model used in the study design (in that the variables were not predictive of health behaviours and/or that the variance explained was low) yet the models were not rejected (Ogden 2003). Therefore, the theory underlying the conceptual basis of SCMs is not being appropriately tested (Ogden 2003). Furthermore, the study design often used to test SCMs is cross-sectional or prospective with a focus on finding correlates of health behavior, rather identifying relationships. Experimental or quasi-experimental study designs using SCMs would assist in determining causation for repeated behaviours, where strong past-future relationship, rather than intention, might predict that the behaviour is likely to continue in the future (Weinstein 2007). Correlational studies can, therefore, lead to an overestimation in the strength of the relationship between the SCM constructs and health behaviour (Steptoe 2011). The prospective study design used to test SCMs also assumes ‘intention stability’, which has been shown to influence the intention-behaviour relationship (Sheeran and Abraham 2003). Further criticisms of SCMs include the lack of explicit inclusion of affective influences within the model and lack of focus on the volitional phase of action (Steptoe 2011). The Theory of Planned Behaviour (Conner 1998) and Protection Motivation Theory (Milne, et al. 2002) were subsequently extended include these respective domains.

Stage models, notably the Transtheoretical Model of Change (TTM) (Prochaska 1984), are a different form of SCM which describe health behaviours as encapsulating several discrete stages rather than linear. However, the actual ‘stages’ of the TTM have been criticised and, for this reason, it does not necessarily represent an improvement on the previously described SCMs, in terms of its ability to describe behaviour change (Steptoe 2011).
Using models, informed by theory, to explain individual behaviour in the context of health, is useful from the perspective of researchers and is consistent with an evidence-based approach to public health and medicine (Glanz 1997; Ogden 2003). Examples of applications of SCM in public health research include: the use of HBM in the prediction of individuals’ adherence to malaria prophylaxis regimens following return from travel (Abraham, et al. 1999) and in the investigation of factors influencing the decision to take a genetic test for colon cancer risk (Bunn, et al. 2002); and the application of the SCT model in determining the relationship between psychosocial factors and physical activity (Fisher, et al. 2011). Behavioural interventions involving more extensive use of theory have been shown to be associated with increases in treatment effect size and, in the case of TPB, more substantial effects on behaviour (Webb, et al. 2010).

However, a methodological approach which classifies behaviour change interventions (Abraham and Michie 2008) and uses models to understand individual behavior in the context of a wider system (Michie, et al. 2011) is likely to provide a more comprehensive explanation of behaviour change, than using individual social cognitions alone. A simple model, such as the COM-B model of behaviour in context (Michie, et al. 2011) is potentially amenable to use by health-care researcher without a specialist psychology background, and might assist in the generation of evidence to explain behaviour in varied healthcare settings (Figure 69).
Behaviour change taxonomy (Abraham and Michie 2008) might also help to identify which models are suitable for targeting specific types of health cognition and/or behaviour, to promote change.

5.1.8 Subject selection and recruitment
Patients with two forms of sight-threatening non-infectious retinal disease were selected for this study:

1. non-infectious uveitis
2. the dry form of age-related macular degeneration (AMD).

A total of 4 patients with a diagnosis of active idiopathic non-infectious uveitis were consecutively recruited to this study from Moorfields Eye Hospital, London, UK between November 2014 and December 2016. Patients with active uveitis only were included in this study because they would be started on treatment, whereas those in clinical remission would not, and they would be undergoing 3 further follow-up appointments over the course of 12 months, during which time they could be interviewed. Inclusion criteria for patients with uveitis were as follows: age 18 to 59 years; a current or previous diagnosis of non-infectious intermediate, posterior or pan-uveitis as per standardization of uveitis nomenclature (SUN) (Jabs et al., 2005); and the absence of known associated infectious disease or systemic inflammatory disease. Eligibility for recruitment was further determined by uveitic disease activity. To meet the criteria for ‘active’ disease, patients were required to have new onset of uveitic disease occurrence at the time of recruitment (a new diagnosis or reactivation of existing disease), which was regardless of treatment status. Disease activity was determined by clinical symptoms, examination with slit lamp biomicroscopy and clinical imaging (optical coherence tomography, fundus autofluorescence and fluorescein angiography). Patients who met the study inclusion criteria and who were clinically assessed to have active disease (i.e. anterior chamber cells ≥0.5+, vitreous cells ≥0.5+, inflammatory cystoid macular oedema, optic disc oedema, active vasculitis, or new/active chorioretinal lesions) were considered eligible as ‘active’ patients in the study.

Patients with dry AMD were recruited via the NIHR Moorfields Biomedical Research Centre, using an existing electronic database of patients who had registered their interest in involvement with research. An invitation email with the following text attachment was sent out to all patients with a diagnosis of dry AMD (approximately 40) in July 2015:

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“Dear ---

As part of our Research Community at the NIHR Moorfields BRC, we are always keen to inform you of any research engaging opportunities that you may be interested in. The NIHR Moorfields BRC has recently been asked to help organise a project called “Eating for Eye Health”.

“Eating for Eye Health” is a public engagement project initiated by research students and clinicians at UCL Institute of Ophthalmology and Moorfields Eye Hospital to raise awareness about nutrition and eye health, particularly for the older generation.

There is evidence that good nutrition can benefit eye health and we are keen to explore this! Through this project, we aim to explore simple ways of improving nutrition for the older generation, especially involving people who have received a diagnosis of "dry" age-related macular degeneration (AMD) for which there is currently no treatment.

Initially, we intend to hold a focus group event to discuss the relationship between nutrition and eye health, and to find out patients’ views on diet, in addition to a food tasting. Together we will compile a list of appealing antioxidant-rich recipes!

The focus group is a free event that is aimed at dry AMD patients and relatives. The focus group is to be held at----

As spaces are limited, they will be given on a first come first served basis. If you are interested in attending, please contact us via email at --- or by telephone on ---.

For more information on the project, please visit http://www.brcophthalmology.org/eating-eye-health. If you have any questions, please feel free to contact us via the telephone number above.

Thank you.”
5.1.9 Rationale for including patients with dry AMD in the focus group study on an evidence-based lifestyle intervention

The JLA PSP list, which described unanswered questions about ocular inflammatory disease, prioritised by patients, includes the following research question:

‘Can diet or lifestyle changes prevent uveitis from developing?’ (JLA 2017b)

Previous rodent model studies have investigated the use of anti-oxidants (vitamin E in the context of deficiency) (Cid, et al. 1992) and omega-3 fatty acids (Shoda, et al. 2015) in uveitic inflammatory disease and have found them to have an anti-inflammatory effect. Case studies of uveitis in association with coeliac disease have also shown an improvement in ocular inflammation on commencing a gluten-free diet (Klack, et al. 2011; Krifa, et al. 2010). However, there is currently no large scale epidemiological or clinical trial evidence to support dietary and/or lifestyle modification interventions in uveitis. Since uveitis is an uncommon disease, it is likely to be difficult to gather study evidence of sufficient power to support a lifestyle intervention for patients with uveitis. AMD, however, is a common disease affecting older people and there is some evidence from large scale epidemiological studies (Hogg, et al. 2017; Wu, et al. 2015) and clinical trials of carotenoid antioxidant supplementation (Age-Related Eye Disease Study Research 2001) to support that a lifestyle intervention, in the form of improved diet might help to protect against disease progression. AMD shares some similarity with uveitis in that it is sight-threatening retinal disease with an immunological and/or inflammatory component to disease pathogenesis (Ambati, et al. 2013). In contrast to non-infectious uveitis, where corticosteroids and other immunosuppressive drugs might be used to manage inflammation and prevent sight loss, there is no currently no licenced drug treatment available to prevent sight loss in patients with dry
AMD. Due to the existence of some clinical evidence supporting dietary intervention in AMD (but not in uveitis) and the lack of any approved drug therapy for dry AMD, the decision was made to invite patients with dry AMD to attend a focus group study to find out their views on a potential lifestyle intervention.

5.1.10 Interviews of patients with uveitis
Interviews with uveitis patients were conducted at baseline, 2 month, 4 month and 12 month patient visits. The format of the interview was unstructured and the duration of each interview was approximately 10 minutes at the end of the clinical consultation. The interviews took place in a semi-private clinical cubicle space in a hospital tertiary centre for ophthalmology in London, UK.

5.1.11 Focus group for patients with dry AMD
Patients with dry AMD were invited to a focus group event to discuss the relationship between nutrition and eye health, and to find out their views on diet, in addition to a food tasting. The focus group took place in a public venue: a reserved area in POD, London, UK, which is part of a chain of ‘healthy’ food outlets with a nutritionist-designed menu. The event began with an introduction to the focus group project followed by short presentations from NHS clinicians about the ‘AREDS study’ and about the evidence supporting the protective effects of the Mediterranean diet against the development of neurodegeneration and dementia.

Participants were offered a selection of ‘healthy fast food’, provided by the venue, including: falafel, tomato and Lebanese red pepper wraps, salmon, cream cheese and wasabi sandwiches, a vegetable dipping platter, ‘slow burner’ salad with lentils and Turkish cheese, blackcurrant superfood pots,
bee pollen bircher yoghurts and handmade ‘superfruit booster’ bars (Figure 70).

Figure 70: Healthy food selection provided for focus group participants at POD City Road

During the food tasting, there was a facilitated discussion to find out participants’ awareness of how diet and nutrition influence health and whether they would be interested in participating. This discussion was started with the following questions, informed by the ‘capability’, ‘opportunity’ and ‘motivation’ components of the COM-B questionnaire (COM-B-Q) (Michie 2014):
Capability:

- Did you know about the links between diet/lifestyle and AMD? Was this mentioned to you when you were diagnosed with your eye condition?
- What sort of diet or foods do you think might be beneficial for the eyes?
- Are you able to cook for yourself at home?
- Is there anything that makes it difficult for you to cook or eat some of the foods we have talked about?

Opportunity

Would it help you to change your diet or eat a greater variety of healthy food if you:

- Had more time to do it?
- It was more affordable?
- Had more people around you doing it?
- Had more encouragement and/or support?

Motivation:

If there was option of participating in a free community project about ‘eating for eye health’, would you:

- Feel that you wanted to do it?
- Believe that it would be a good thing to do?
• Feel that you would be able to participate regularly?

Participants were invited to share stories of how their eye problems have impacted their lifestyle and their thoughts on the changing trends in diet and nutrition. The focus group was concluded with a short survey, related to fruit and vegetable consumption habits, designed by an NHS dietician (Figure 71) and an evaluation of the event by the participants. The evaluation consisted brief questions displayed in large print a poster, on which participants were asked to stick a coloured post-it to indicate a ‘yes’ or ‘no’ response. The questions were as follows:

• Did you enjoy the event?
• Have you found the event useful?
• Have you learned something new?
• Did you like the food served?
• Has this event made you think about what you eat at home?
• Would you like to participate in a future community event to help you make changes your diet?

Over the past 12 months...

How often did you drink orange juice or grapefruit juice? Fresh or Bottled?

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How often did you drink tomato juice or vegetable juice? Fresh or Bottled?

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How often did you eat COOKED greens (such as spinach, kale, collard, mustard, chard)?

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How often did you eat RAW greens (such as spinach, kale, collard, mustard, chard)?

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<td>2 or more per day</td>
<td>other</td>
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How often did you eat carrots (fresh or frozen)?

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<th>1-6 times per year</th>
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How often did you eat peppers?

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How often did you eat salad (lettuce)?

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<td>1 time per day</td>
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<td>Other</td>
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### How often did you eat oily fish (mackerel, salmon, sardines)?

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<thead>
<tr>
<th>Frequency</th>
<th>1-6 times per year</th>
<th>7-11 times per year</th>
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### How often did you eat eggs?

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<tr>
<th>Frequency</th>
<th>1-6 times per year</th>
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### How often did you eat vegetable soup?

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*Figure 71: ‘Food propensity’ survey for dry AMD patients*

5.1.12 **Designing and piloting a complex behaviour change intervention**

A behaviour change intervention, with a focus on promoting a diet that could be beneficial to eye health, was designed according to the APEASE (Acceptability, Practicability, Effectiveness/cost-effectiveness, Affordability,
Safety/side-effects, Equity) criteria for designing and evaluating interventions and the COM-B (Capability, Opportunity, Motivation and Behaviour) model of behaviour change (Michie 2014; Michie, et al. 2011). This approach was used to inform the intervention because, in contrast to the social cognition models which focus on individual social cognitions, the COM-B model considers the individual within a system, involving the interacting model components (Capability, Opportunity, Motivation and Behaviour).

Furthermore, it was identified that this was a ‘complex intervention’ (Craig, et al. 2008) due to the following study attributes:

• The number and difficulty of behaviours required by those delivering or receiving the intervention
• The number and variability of outcomes
• The degree of flexibility or tailoring of the intervention permitted

Evaluations of complex interventions are often undermined by problems of acceptability, compliance, delivery of the intervention, recruitment and retention, therefore it is advised that pilot studies are conducted to ascertain feasibility of the intervention (Craig, et al. 2008). With these principles in mind, a pilot ‘Eating for Eye Health’ community kitchens project was designed, as follows:

**Aim:** To raise awareness of research that suggests nutrition may help protect against the progression of dry AMD and to encourage older people to regularly eat food that could support eye health.

**Method:** A community kitchens intervention model adapted from a previous NHS-funded community kitchens project, designed to target older people from lower socio-economic backgrounds, in the London Borough of Islington to include recipes with a higher antioxidant content to benefit eye health and relevant support for individuals with vision impairment.
**Evaluation/ outcome measure:** Event attendance/ participant engagement, rating of food taste acceptability prepared and served at the event (2-point scale: ‘thumbs up’ or ‘thumbs down’ after eating food served at each course) and self-rating cooking skills and ‘confidence’ scores (1-10) rated before and after the event.

### 5.2 Results

#### 5.2.1 Interviews with uveitis patients

**Patient 1, 23 year old female**

*Uveitis has changed my life completely, where do I even start?*

Patient 1 described a gradual deterioration in her vision over the few years since she had her diagnosis of uveitis, which more recently became ‘drastic’ due to the development of a secondary cataract. She said she can ‘barely make out anything from my left eye’, and said that it was causing her eye strain and headaches. She felt that the problems with her vision were affecting her work as a clerk at a local branch of a bank, and the cataract was making it difficult to focus and causing her making mistakes. Even though her line manager at work was aware of her eye condition, she felt anxious about asking for help and, even making eye contact with colleague, in case they could see that she had an eye problem. Although she was ‘not sure on the medical side of things’, she feels that being on immunosuppressive medication for 3 and a half years must have ‘impacted her body’ because she noticed weight gain, back pain and increased frequency of mood swings. She said that she also felt tired all the time and
lacked energy, 'Who knows what risks it may carry for my future, such as pregnancy or even my normal health in the long run?'

**Patient 7, 41 year old male**

*If I don’t work, I don’t get paid*

Patient 7 was a self-employed builder who had previously been on long-term high dose corticosteroids for uveitis (which were not tapered according to usual clinical practice) at his local hospital in East London. This experience had left him with ‘bad teeth’ and a distrust of doctors. He reported severe mood swings, including ‘angry outbursts’, mainly directed at his family, when he was taking high doses of corticosteroids. His job involved frequent travel to different building sites around the UK, so he found it difficult to attend his hospital appointments, especially if it meant loss of wages. He had difficulties remembering to take his medication and picking up repeat prescriptions, however, he described receiving good support for medication compliance from his wife, who was a registered nurse.

**Patient 12, 18 year old male**

*My mates went off and got jobs but [because of uveitis], I haven’t been able to get one.*

Patient 12 was a long-term patient of the uveitis clinic, who had, until recently, been attending the paediatric clinic. His parents were separated and he lived mostly with his father. When he stayed with his mother, he said there seemed to be more confusion over his medication dosage and he said that she would ‘sometimes forget to take him to hospital appointments’. He
said that he did not apply for work experience because he was worried about uveitis flare-ups and also because when ‘I’m on steroids, my face swells up like a chipmunk’. He said he would like a treatment that would make the uveitis ‘go away for good’, so he could apply for an apprenticeship as an electrician.

Patient 13, 32 year old female

‘Trying to balance everything is difficult but uveitis makes it worse.’

Patient 13 developed a post-partum flare up of uveitis. When she decided to return to her job as an estate agent she said that, ‘even with family support, I struggled’ to manage the demands of working, caring for a young baby and managing her uveitis. She disliked the weight gain she associated with oral corticosteroids but felt that they were more effective than the intraocular steroid treatment she had received in the past. After returning to work, she felt nervous about asking her employer for time off to attend hospital appointments as she felt this made ‘a bad impression’.

5.2.2 Focus group for AMD patients

The food intake questionnaire results indicated that the study participants had a fair intake of vegetables with 12 (100%) of focus participants reporting that they ate cooked greens, raw greens, carrots (raw and cooked), peppers and salad at least once per week (mean: twice a week, range: 1-6 times per week). The mean frequency of egg consumption was twice a week (range: twice a month to 3-4 times per week). However, the intake of fresh or bottled fruit juice, vegetable juice, vegetable soup and oily fish was generally lower, with 1-2 per month being the most frequent response. Analysis of the group discussion highlighted some of the ‘barriers’ that patients with visual impairment experienced towards making healthy dietary changes and,
particularly, cooking for themselves. These included: a lack of clear, practical information about nutrition from health professionals (fruit/vegetable juice was thought to be unhealthy due to its sugar content, whereas the bioavailability of micronutrients was not considered); confusing health information in the media; the difficulties of cooking with visual impairment; lack of confidence in preparing particular foods, such as fish, and using unfamiliar ingredients; fear of having to ask for help from others; and, significantly, the sense of isolation, hopelessness or even, shame, which can be brought up by the experience of having an untreatable progressive sight-threatening eye condition. Further qualitative analysis of the focus group discussion, using the COM-B framework, identified that behaviour intervention should target increasing participants’ capability and opportunity. The data suggested that the group was very motivated, so the motivation domain was not targeted in the intervention. The COM-B diagnostic tool (Michie 2014) was then used to identify what needed to change in order for focus group participants to make the behaviour change towards healthy cooking and eating. Targeting physical and psychological capability suggested that interventions which aimed to increase participants knowledge, skills and self-efficacy (confidence) regarding eating for eye health, in assistance to providing individualised physical assistance might be effective in changing behaviour. Targeting opportunity suggested that interventions that would increase social opportunity, provide the necessary materials at a low cost and would support participants in the behaviour change would be effective. A telephone consultation with the Macular Society, a patient charity which ‘supports those with macular degeneration, their families and their carers’ helped to guide how physical assistance might be offered to the project participants. Based on this advice, participants who had registered their interest in a further project event were contacted via email to ask them whether they would like to discuss any particular expectations or personal needs regarding their attendance of a further event,
for example, a cookery day. No registered participants indicated any special needs in this area. Therefore, the evidence from the focus group, the COM-B model and consultation with a patient charity informed the design of a community cookery day, to which the focus group participants were invited.

5.2.3 Community cookery day
Community Cookery Day was held at St Luke's Community Centre, near Moorfields Eye Hospital, London, UK. 8 out of 12 participants from the focus group attended, in addition to two further participants. A three-course menu of ‘Easy Recipes for Eye Health’ was devised, which included: butternut squash, turmeric and brazil nut soup; salmon fillet with red onion and peppers (Figure 74); baked sweet potatoes; spinach, orange and linseed salad; and apple and blackberry sponge pudding. The event was opened with a ‘food bingo’ icebreaker activity (Figure 72), an explanation about how the day would run, and a health and safety briefing about basic food hygiene.
Figure 72: Food bingo icebreaker activity

Participants were informed about the importance of consuming a variety of colourful fruits and vegetables, how to prepare food to optimise absorption of nutrients and about the importance of bowel health in the absorption of nutrients from food.
The project participants showed a high level of engagement with the cookery activity, which involved preparing the specific part of the menu assigned to them (Figure 73), for example, the soup or the salad. Whilst the main course (Figure 74) was cooking in the oven, there was a ‘food tasting’ activity, which featured a selection of so-called ‘superfoods’, for example sauerkraut, dark chocolate with raw cocoa nibs, and merlot grape juice. A dietician explained some of the science behind the ‘superfoods’ and answered the participants’ questions. When the food was ready, participants sat down together to enjoy the meal, with the dietician explaining the intended health benefits of the ingredients whilst each course was served. Participants were asked to rate each course of the meal by holding up a ‘thumbs up’ or ‘thumbs down’ sign to indicate taste acceptability of the food.
The post-event feedback was very positive and with all participants reporting an increase in confidence levels after the cookery session, and 8 out of 10 participants reporting a confidence level of 7 or above about cooking for themselves with the recipes provided. ‘Eat Colourful!’ was the project’s simple take home message. The attendees said that they particularly enjoyed the sense of community with others who shared their experiences of coping with dry AMD. Therefore, the pilot intervention appeared to be well received and successful in providing opportunity for healthy behaviour change and increasing participants’ self-efficacy and capability (confidence) in making the change. The event was recorded to create a promotional video for the project and a summary information booklet was created from the project findings and recipes for wider distribution.
5.3 Discussion

This study was undertaken to investigate the perspectives of patients with sight-threatening retinal disease. The qualitative analysis of perspectives of a small case series of patients with uveitis revealed some common themes, which included dissatisfaction with the side effects and long effects of oral immunosuppression, unpredictability of the disease flare ups, including the negative impact that this had on social relationships, childcare, work life and financial income and the impact of sight loss. These findings confirmed some of the themes of the JLA PSP on ocular inflammatory disease (JLA 2017b). It also confirms recent observations that,

‘Intermediate uveitis, posterior uveitis, and panuveitis require systemic therapies more than perhaps any other set of ophthalmic conditions, with a significant potential for medication adverse effects. The need for monitoring can itself be a significant burden on patients, which affects their functionality and sense of wellbeing… [and] noninfectious intermediate uveitis, posterior uveitis, and panuveitis differ from other common eye conditions, such as cataract, glaucoma, or macular degeneration, in that they affect a younger population that is often still of working or school age. These individuals are not merely worried about their vision, but also about remaining capable of providing for themselves and their families.’ (Burkholder and Ramulu 2017)

The pilot ‘Eating for Eye Health’ community kitchens described an approach to supporting dietary change for eye health and general wellbeing in participants with a diagnosis of dry AMD. Depth interviews with project participants indicated that the symptoms of dry AMD are insidious, and may start with difficulty in reading small print or writing. In the early phase, patients may attribute these difficulties to the wrong glasses prescription or even brush them off as a normal part of ‘getting older’. The diagnosis of an untreatable and progressive sight condition was described as ‘quite a shock’, which left affected individuals at a loss as to how to cope. In the clinical
setting of an NHS hospital, doctors are often limited to giving lifestyle advice about eye protection or explaining that dry AMD may further progress to the wet form of AMD. Treatment is available for the wet form of AMD (but not the dry form, as stated earlier) through regular drug injections to the eye, which often improve vision but are not curative. For many, this is appeared to be a frustrating situation. Clinicians are conscious of the adverse impact of AMD, but sometimes fall short of communicating advice that might help support patients through the aftermath of this diagnosis. Patients do not always feel confident in speaking up about their concerns, particularly in a busy clinic. Those with untreatable eye conditions, such as dry AMD, are often discharged from hospital follow-up, with advice to self-monitor their condition and to ‘come back if it gets worse’. This approach may leave patients feeling, at best, anxious and, at worst, literally and metaphorically ‘left in the dark’ by healthcare professionals. Raising awareness of the research suggesting that lifestyle modifications might benefit eye health is, therefore, important. However, simply passing on an information leaflet to patients is not an adequate solution; it is also crucial to offer support and encouragement to patients in implementing these changes into their lives, particularly in the context of an eye disease that causes progressive deterioration of vision.

Evidence from large clinical trials suggests that antioxidant vitamin supplements may help protect against the progression of AMD (Age-Related Eye Disease Study Research 2001) and it known that these key nutrients are found in a wealth of brightly coloured foods, such as red peppers, butternut squash, kale and summer berries, all of which have benefits that extend beyond AMD. Thus, following a successful bid for funding and support from UCL Culture, the ‘Eating for Eye Health’ engagement project was born. Through supporting patients in cooking and eating antioxidant-rich food via a community kitchens project, the project created an environment conducive to behaviour change for eye health.

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There is a tendency for health care professionals to engage with patients on their own terms, rather than the patients’ terms. The ‘Eating for Eye Health’ project highlighted the importance of team-work and communication in creating successful partnerships between healthcare professionals, patients and local communities. The scientific evidence to support nutritional approaches to eye health is emerging at a time when novel approaches to nutrition and diet are being brought to attention of the public, through popular media. Whilst information in newspapers, magazines or on the internet might be misleading or inaccurate, it is readily available and communicated in an understandable way to the general public. It was intended that ‘Eating for Eye Health’ would facilitate connection and two-way dialogue between health professionals with specialist knowledge and those who actually have experience of living with dry AMD.

It is well recognised that social engagement improves resilience and supports healthy aging (Mendes de Leon 2005; Ozbay, et al. 2007). The contemporary concept of ‘positive health’, which takes into account an individual’s psycho-social wellbeing, in addition to their physical health and body functioning, may be addressed through community engagement. The National Institute for Health and Care Excellence (NICE) has noted that, ‘Community engagement is a highly complex area with several important purposes. These include empowering people within communities to gain more control over their lives and to play a part in decisions that affect their health and wellbeing’ (NICE March 2016).

A practical way to operationalise these concepts within the NHS is through ‘social prescribing’ (Brandling and House 2009; Kimberlee 2016). This is sometimes referred to as community referral and is a means by which health professionals, in the primary care setting, may refer patients to a range of local, non-clinical services. Through recognising that peoples’ health is determined primarily by a range of social, economic and environmental
factors, social prescribing seeks to address peoples' wider health needs in a more integrated way. It may also support individuals in feeling a greater sense of agency regarding their health conditions. Holistic social prescribing has been shown to be beneficial to supporting patients' health and wellbeing, as well as reducing the demands on GPs and hospitals (Kimberlee 2016; Polley M.J. 2017). Evidence suggests that patients with AMD are under-consuming nutrients considered to be useful for their condition and that individuals, who are able to cook a hot meal, consume significantly more lutein and zeaxanthin than those who are not able to (Stevens, et al. 2015). It is therefore essential to design more effective dietary education and dissemination methods for people with, and at risk of, AMD (Stevens, et al. 2015). The community kitchens model, described here, is a specific example of how social prescribing might be applied in communities to address potential nutritional deficiencies, which may detrimentally influence clinical outcomes for eye disease. Older people with visual impairment are at increased risk of social isolation, loneliness and psychological distress (Matthews, et al. 2017), which are, in turn, associated with a greater risk of all-cause mortality (Russ, et al. 2012; Steptoe, et al. 2013). Study findings suggest that it is possible to reduce loneliness by using educational interventions focused on social networks maintenance and enhancement (Cohen-Mansfield and Perach 2015). Community kitchens have the potential to improve social interactions and nutritional intake of participants and their families (Herbert, et al. 2014; Iacovou, et al. 2013). Overall, evaluation findings from Eating for Eye Heath were in keeping with published evidence that community kitchens programmes have positive effects on participants' cooking skills confidence and reduce barriers of cost, waste, and knowledge (Garcia, et al. 2017).

Interestingly, ophthalmology patients from a previously published focus group study emphasized their expectations related to communication and
interpersonal relationships over and above technical medical interventions, for example, prescription medication and diagnostic testing (Dawn, et al. 2003). As a result, there is a growing appreciation amongst eye-care professionals that the patient is uniquely placed to understand the impact of disease and can use that position to transform ophthalmic care at the individual and collective level, from research to delivery of care (Dean, et al. 2017). Numerous initiatives exist to support patient and public involvement (PPI) in eye research, such as the JLA PSP which helped to inform the research in this thesis (JLA 2017a). A public engagement (PE) approach, however, simultaneously captures the patient and the public voice in its inherent appreciation that patients are part of the ‘public’ community. PE seeks to facilitate public conversations which are not wholly shaped by a ‘research agenda’, as is often the case in PPI. A combination of these approaches may be valuable in capturing patient perspectives of disease and developing patient-reported outcomes.

In summary, although the two groups with uveitis and AMD shared the experience of sight-threatening non-infectious retinal disease, they did differ considerably in subject age (the group with AMD being considerably older), disease characteristics and in how the disease impacted them. For the patients with uveitis, who were of working age, inflammatory flare-ups caused a significant disruption to working life and many of treatment side effects were unacceptable to them. The older patients with AMD, on the other hand, reported more symptoms of psychological distress related to the progressive, incurable nature of their condition and fear of isolation and/or loss of independence. However, the success of a non-clinical intervention in improving participant wellbeing and the observed positive effects of social engagement, suggest that there is value in investigating non-clinical interventions to support health and/or wellbeing of patients with sight-threatening uveitis.
Chapter 6 Summary Discussion, Study Limitations and Future Directions

The aims of this thesis were to individualise management of patients with non-infectious uveitis by investigating potential differences in clinical phenotypes of non-infectious uveitis, immunophenotyping of clinical remission of sight-threatening non-infectious uveitis and patient perspectives of sight-threatening retinal disease.

Chapter 2 was a clinical epidemiological survey to determine whether there were clinical phenotypic differences in multifocal choroiditis (MFC) and punctate inner choroidopathy (PIC), two subtypes of non-infectious uveitis with similar clinical characteristics, when classified using a standardised approach. Following classification as PIC or MFC, statistically significant differences were found in age, gender and refractive error (spherical equivalent). Qualitative analysis of OCT imaging did not reveal any significant differences in the anatomical involvement of lesions between PIC and MFC. It was concluded that the data supported some stable phenotypic differences, with PIC demonstrating a greater predilection for young, myopic women and CNV development than MFC. These could represent important differences in pathophysiology and have relevance for individualised management.

Despite efforts to standardise the classification, many of the clinical measurements retained a level of subjectivity. Although the sample size was the largest of previous studies of eyes with these conditions, it is still small compared to studies of more common eye conditions. The number of subjects/eyes imaged during an active phase of disease activity was low, compared to those imaged during inactive phases. These numbers were similar for both MFC and PIC, which is more likely to reflect infrequent
disease activity during the observation period, than challenges in the detection and imaging of disease activity (which would be more plausible for PIC than MFC). Mean lesion size in this study was based on AF images. Due to the clear delineation of lesion borders on AF images, a standardised image scale and a higher number of images available for analysis, this meant that the measurements taken had greater reliability and validity than measurements taken from colour fundus photos. However, given that areas of chorioretinal atrophy may enlarge over time in myopic eyes, retrospective clinical differentiation between these two diseases, based on lesion size, remains challenging. Evidence of clinical inflammation, required for our sub-classification of MFC and PIC, was determined from retrospective review of clinical documentation and imaging. Since inflammation (if it occurs in MFC) is episodic, it is possible that this may not have been captured during a clinic visit. Nevertheless, the fact that the MFC group had a higher proportion of subjects with ERMs supports a higher prevalence of intraocular inflammation in this group and suggests a reasonable level of accuracy in our disease classification according to inflammation. In our study, a standardised manual caliper measurement approach was used to measure choroidal thickness, although we did not adjust for age, myopia or CNV. Since we showed that PIC was associated with a significantly higher proportion of subjects with myopia, CNV and a higher degree of myopic refractive error than MFC, the influences of disease classification and inflammatory activity on choroidal thickness were likely underestimated.

In summary, the phenotypical differences that we identified between PIC and MFC should be further investigated, in conjunction with clinical imaging and pathophysiological data, in future studies. In particular, it would be useful to obtain more high resolution SD-OCT and EDI-OCT images and wide field fundus photos during active disease phases and to monitor disease progression over a longer follow-up period. Since we are entering an era of
'personalised' or 'stratified' approaches to disease management, it seems appropriate to sub-classify idiopathic non-infectious uveitis syndromes, where the evidence supports this, because it may have implications for disease management. Individualised treatment approaches are, in theory, much more favourable than standard “blockbuster” systemic immunosuppression with their associated morbidity. Going forward, it is suggested that in order to best utilise the increasing range of targeted immune modulating drugs coming to market, it is important to have non-infectious uveitis classifications, which are supported by clinical evidence, in addition to clinician opinion.

In the laboratory studies described in Chapters 3 and 4 laboratory studies, the aim was to improve the phenotyping of sight-threatening non-infectious uveitis to allow more individualised patient management, with a focus on immunophenotypic analysis of patients in clinical remission. It was investigated whether there is a peripheral blood immunophenotype associated with sustained clinical remission, using a prospective recruitment of 60 subjects (50 intermediate, posterior and panuveitis patients and 10 control subjects). The frequency of CD4+CD25+FoxP3+ Treg, TIGIT+ Treg, T-bet+ Treg and the ratio of Treg to Th1 was significantly higher in quiescent patients in long term drug-free remission compared to patients with active uveitic disease. Treg from a patient in clinical remission demonstrated a high level of in vitro suppressive function compared to Treg from control subject and Treg from a patient with untreated active disease. Targeted epigenetic CpG methylation analysis of PBMCs from selected patients and controls, using a Next Generation Sequencing (NGS) bisulphate sequencing approach showed significantly lower methylation levels at FOXP3 TSDR, FOXP3 Promoter and TIGIT sites and higher levels of RORC methylation in patients in clinical remission compared to those with active disease. Bivariate correlations demonstrated that DNA methylation was associated with the
observed levels of TIGIT$^+$ Treg and that this in turn influenced the cytokine microenvironment. Clinical remission was associated with significantly higher serum levels of TGF-$\beta$ and IL-10, which positively correlated with Treg levels, and lower serum levels of IFN$\gamma$, IL-17A and IL-22 compared to patients with active disease.

It was shown that duration of previous oral immunosuppressive treatment was the strongest predictor of methylation of the FoxP3 TSDR, which was significantly associated with clinical remission in sight-threatening uveitis and that that relationship was still significant after adjusting for subject age. Furthermore, peripheral blood TIGIT levels, but not subject age, were a significant predictor of clinical remission in sight threatening non-infectious uveitis. TIGIT levels had high sensitivity (92%) but low specificity (62%) and, therefore, a high false positive rate when used as a predictor of clinical remission of sight-threatening non-infectious uveitis in the study population of uveitis patients. Whereas a high false positive rate for a screening test for disease (where subjects without the disease may falsely test positive) may be acceptable in certain situations, it is a significant limitation in consideration of TIGIT$^+$ as a biomarker for clinical remission, because there is a high likelihood that it may indicate that a patient is clinical remission when they actually have active disease. Use of TIGIT$^+$ Treg, as a single surrogate marker for clinical remission of uveitis may not add much value to current clinical practice, wherein clinical remission is determined through clinical assessment of disease activity. It is also possible that the calculated test sensitivity, specificity, false positive and negative rates could have been skewed by the over weighting of the sample towards remission patients rather than active patients. TIGIT levels explained almost half of the variance observed in the model of clinical remission, but there may be other clinically relevant factors, not accounted for in the model, which were also influencing clinical remission. These would need to be further investigated in order to
develop better models for predicting clinical remission for individual patients, which could augment standard clinical assessment of patients.

The total number of subjects recruited to this study (n=60) represents one of the largest sample sizes of published clinical immunology studies of sight-threatening non-infectious uveitis. However, the group was weighted towards subjects in clinical remission, which limits the validity of the study findings. The thirty-seven patients in clinical remission recruited to the study, however, demonstrated a good clinical ocular response to immunosuppressive therapy given systemically, supporting the fact that systemic Treg and their associated microenvironment influence ocular immunity and that peripheral blood biomarkers have utility in monitoring ocular disease. T-cell immunophenotyping by flow cytometry was performed on freshly isolated subject PBMCs (within 4 hours of venepuncture) but additional intraocular sampling was not undertaken as part of this study. Although not easily feasible, a further study could validate these findings in uveitic ocular tissue and/or vitreous humour. Phenotypic analysis of Treg, Th1 and Th17 subtypes was based on ‘master transcription factor’ expression, with prospective intracellular cytokine analysis only in selected subjects with active disease, followed up over a 12-month period. An in-depth analysis of intracellular cytokine production and methylation at signature cytokine loci could be considered for a future study. The number of Treg functional assays that could be performed as part of this study was limited by the remaining PBMC numbers after the immunophenotypic analysis. Since Treg are a ‘rare’ cell population, further studies would require a larger blood sample (than 30ml) to allow functional assays to be performed alongside immunophenotypic analysis. Therefore, functional study findings should ideally be confirmed using larger blood samples in more subjects.

The results of this study provide evidence that TIGIT has utility as a marker of functional Treg for immunomodulatory therapy, and that low levels of
TIGIT may be a biomarker for increased risk of disease relapse. This could help to individualise patient treatment by assisting in determining whether systemic immunosuppressive drugs are inducing functionally suppressive Treg, and helping to inform clinical decision making. For example, to assist in determining whether the patient is in biological remission, in addition to having clinically inactive disease, and whether immunosuppressive treatment can be tapered. Finally, these data are relevant to the recent interest in quantitative identification and isolation of viable, functional Treg for downstream clinical purposes, which includes their generation \textit{ex vivo} for immunomodulating therapies (Foussat, et al. 2017; Horwitz, et al. 2004). TIGIT may prove to be a useful biomarker for identifying functional Treg for these therapies. Treg-based treatments may be a preferable option for patients with sight-threatening non-infectious uveitis because they aim to specifically enhance naturally-occurring mechanisms by which an individual regulates inflammation, at the immune cell level, and they potentially avoid the wide range of systemic side effects of conventional immunosuppression. However, further investigation of this is required.

The aim of the public engagement and qualitative analysis work described in Chapter 5 was to engage with patients who have sight-threatening non-infectious retinal disease (which does not have curative treatment) and identify suitable interventional approach(es) which could serve to increase sense of agency and/or self-efficacy of affected individuals, in order to potentially change the course of the disease. The qualitative analysis of perspectives of a small case series of patients with uveitis revealed some common themes, which included dissatisfaction with the side effects and long effects of oral immunosuppression, unpredictability of the disease flare ups, including the negative impact that this had on social relationships, childcare, work life and financial income and the impact of sight loss. These findings confirmed some of the themes of the JLA PSP on ocular
inflammatory disease (JLA 2017b) and could help to inform patient-reported outcomes, which measure treatment success in the patient’s terms rather than the clinician’s.

The pilot behaviour change intervention appeared to be well received and successful in providing opportunity for healthy behaviour change and increasing participants’ self-efficacy and capability (confidence) in making the change. Public engagement helps to foster support, within, and, between, communities, due to its preferential focus on the interface between research and wider society. Successful engagement with the dry AMD community meant Eating for Eye Health was not only a project about engaging participants with research on how to ‘eat right for their sight’ but it also opened a conversation regarding their unmet needs for support. Community kitchens methodology in PE is likely to contribute a unique perspective on the interplay between food, communities and the health of individuals, the project has the potential to contribute to the growing body of evidence suggesting that community engagement and social prescribing can positively impact health and wellbeing. Through the creation of a community, it is also hoped that awareness will be raised about AMD, which could attract public funding to this area and facilitate engaged research into improving clinical outcomes, including positive health.

Limitations to this study include the fact that it consisted of a small case series and that the findings were subjective and, therefore, not necessarily applicable to a wider population. Community kitchens interventions are potentially expensive and challenging to scale. This is relevant because the success of the project described in Chapter 5 appeared to be related, at least in part, to the small group size, which allowed participants to be receive more individual attention. Individuals who participate in studies and engagement projects tend to be of higher socio-economic status, more motivated to change behaviour and to improve their health than those who do not
participate in studies, which potentially perpetuates health inequalities. There is also the challenge of measuring the impact of the lifestyle intervention on altering the course of a disease, which requires precise surrogate endpoints, and are likely to require monitoring over a long period of time in order to see any effect (often longer than required with an drug intervention). The next stage of the work could involve a 'needs assessment' of different patient populations with visual impairment; an investigation into how to expand the community kitchens project and/or improve outreach to disadvantaged groups; or an evaluation of social, economic and health impacts of community kitchens and social prescribing on participants.

In summary, this thesis demonstrates that a mixed methods approach, where qualitative methodologies were explored alongside more conventional biomedical quantitative methods, could have value in developing individualised patient-centred therapeutic approaches for sight-threatening non-infectious uveitis.
References

Abraham, C. & Sheeran, P.

2005 Health belief model. In Predicting Health Behaviour: Research and Practice with Social Cognition Models
Abraham, C., S. Clift, and P. Grabowski

Abraham, C., and S. Michie

Agarwal, R. K., and R. R. Caspi

Agarwal, R. K., P. B. Silver, and R. R. Caspi

Age-Related Eye Disease Study Research, Group

Ahnood, D., et al.

Akbar, Arne N., et al.

Akimova, T., et al.

Allan, S. E., et al.

Allen, M., and J. Bellstedt

Amadi-Obi, A., et al.
2007 TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. Nat Med 13(6):711-8.
Ambati, Jayakrishna, John P. Atkinson, and Bradley D. Gelfand

Amer, R., and N. Lois

Armitage, C. J., and M. Conner

Atan, D., et al.

Azjen, I.

Baecher-Allan, C., et al.

Baltmr, A., S. Lightman, and O. Tomkins-Netzer

Bandura, A.

—

Baron, U., et al.

Barry, Robert J., et al.


Becker, C. M., et al.


Becker, Craig M., et al.

2015 The Salutogenic Wellness Promotion Scale for Older Adults. American Journal of Health Education 46(5):293-300.

Bending, David, et al.


Bennett, C. L., et al.


Benoist, C., and D. Mathis


Bhat, J., et al.


Blaxter, Mildred


Bloch-Michel, E., and R. B. Nussenblatt


Brandling, Janet, and William House


Brown, J., Jr., and J. C. Folk


Brown, J., Jr., et al.


Brunkow, Mary E., et al.
Bunn, J. Y., et al.

Burkholder, B. M., and P. Y. Ramulu

Calleja, S., et al.

Carpenter, C. J.

Caspi, R.

Caspi, R. R.

—

Castiblanco, Claudia, and C. Stephen Foster

Channa, R., et al.

Chatila, Talal A., et al.

Chavele, K. M., and M. R. Ehrenstein


Chen, L., et al.


Chen, S. N., and J. F. Hwang


Cid, L., et al.


Clare, Anthony


Clucas, C., and L. S. Claire


Cohen-Mansfield, J., and R. Perach


Conner, M. & Armitage, C. J.


Conner, M. and Norman, P. (Eds)


Conner, M., Norman, P. (Eds)


Cottrell, T. and Rosen, A.


Craig, Peter, et al.


Curotto de Lafaille, Maria A., and Juan J. Lafaille
Damsker, J. M., A. M. Hansen, and R. R. Caspi

Daniel, E., et al.

Davis, Janet L., et al.

Dawn, A. G., C. Santiago-Turla, and P. P. Lee

De Meyer, V., et al.

Dean, S., et al.


Denniston, A. K., et al.

Dick, Andrew D., et al.

Doria, A., et al.

Dreyer, R. F., and D. J. Gass

Dunlop, A. A., et al.

Durrani, O. M., et al.

Eldem, B., and C. Sener

Elkord, Eyad

2016  Helios Should Not Be Cited as a Marker of Human Thymus-Derived Tregs. Commentary: Helios(+) and Helios(−) Cells Coexist within the Natural FOXP3(+) T Regulatory Cell Subset in Humans. Frontiers in Immunology 7:276.
Engel, G. L.

Essex, R. W., et al.

Essex, R. W., et al.

Fairweather, D., et al.

2001  From infection to autoimmunity. J Autoimmun 16(3):175-86.
Fairweather, DeLisa, et al.

Fairweather, DeLisa, and Noel R. Rose

Fan, Q., Y. Y. Teo, and S. M. Saw


Fiske, S. T., A. J. Cuddy, and P. Glick

Fiske, S. T., et al.

276

Fiske, Susan T.


Flaxel, C. J., et al.

The use of corticosteroids for choroidal neovascularisation in young patients. Eye (Lond) 12 ( Pt 2):266-72.

Fleisher, T. A. & Oliveira, J. B.


Floess, S., et al.


Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky


Foster, C. S., et al.


Foster, C. Stephen, and Albert T. Vitale


Foussat, A., et al.


Fung, Adrian T. Mbbs MMed, et al.


Gass, J. D.


Gass, J. D., A. Agarwal, and I. U. Scott


Gass, J. D., and C. P. Wilkinson


Géhl, Zsuzsanna, et al.


Gilbert, Rose, et al.


Gilbert, Rose M., et al.


Gilbert, Rose, et al.


Gilbert, Rose, et al.


Glanz, K., Marcus Lewis, F. & Rimer, B.K.


Glanz, K., Rimer, B.K. & Viswanath, K. (Eds)

GMC

2017 Medical and Social Models of Disability: General Medical Council.

Godin, G., et al.

2008 Asking questions changes behavior: mere measurement effects on frequency of blood donation. Health Psychol 27(2):179-84.

Gregersen, Peter K., and Timothy W. Behrens


Gritz, D. C., and I. G. Wong

2004 Incidence and prevalence of uveitis in Northern California; the Northern California Epidemiology of Uveitis Study. Ophthalmology 111(3):491-500; discussion 500.

Herbert, J., et al.


Hirooka, K., et al.

2014 Increased macular choroidal blood flow velocity and decreased choroidal thickness with regression of punctate inner choroidopathy. BMC Ophthalmol 14:73.

Hirooka, K., et al.


Hogg, R. E., et al.


Hori, S., T. Nomura, and S. Sakaguchi


Hori, S., T. Takahashi, and S. Sakaguchi


Horwitz, D. A., et al.


Hu, Dan-Ning

2009 Photobiology of the Uvea.

Hua, R., L. Liu, and L. Chen
Huber, M., et al.

Huber, Machteld, et al.

2011  How should we define health? BMJ 343.
Huehn, J., J. K. Polansky, and A. Hamann

Iacovou, M., et al.

Jabs, D. A.

Jabs, D. A., R. B. Nussenblatt, and J. T. Rosenbaum


Jampol, L. M., and A. Wiredu

Janson, P. C., et al.

Janz, N. K., and M. H. Becker

JLA

2017a About Priority Setting Partnerships: Crown Copyright.

2017b Ocular Inflammatory Disease Top 10: Crown Copyright.
— 2017c  Sight Loss and Vision: Crown Copyright.
— 2017d  Top 10s of priorities for research: Crown Copyright.
Joller, N., et al.
Joller, N., et al.
Jones, N. P.
Kaiser, P. K., and E. S. Gragoudas
Ke, Yan, et al.
Keane, Pearse A., et al.
Kempen, J. H., et al.
Kempen, J. H., et al.
Khorram, K. D., L. M. Jampol, and M. A. Rosenberg

Kim, Y., et al.

Kimberlee, Richard

2016  What is the value of social prescribing? 2016 3(3).
Klack, K., R. M. Pereira, and J. F. de Carvalho

2011  Uveitis in celiac disease with an excellent response to gluten-free diet: third case described. Rheumatol Int 31(3):399-402.
Klein, S., et al.

Koch, M. A., et al.

Kramer, M., et al.

Kramer, M., and E. Priel

Krifa, F., et al.

Kung, Yung-Jen, et al.

Kurtulus, S., et al.

Lange, C., et al.

282
2009  Resolving the clinical acuity categories “hand motion” and “counting fingers” using the Freiburg Visual Acuity Test (FrACT). Graefe’s Archive for Clinical and Experimental Ophthalmology 247(1):137-142.
Lee, R. W., and A. D. Dick

Lee, R. W., et al.

Leung, S., et al.

Leung, T. G., et al.

Levine, Andrew G., et al.

Lewis, D. E., Harriman, G. R. & Blutt, S. E.


Li, Bin, et al.

Lightman, S., et al.


Lin, Shuman, et al.

Liu, W., et al.  

Lochner, M., et al.  

Lonergan, Mike, et al.  

Maddux, James E., and Ronald W. Rogers  

Maggi, L., et al.  
2010 CD161 is a marker of all human IL-17-producing T-cell subsets and is induced by RORC. Eur J Immunol 40(8):2174-81.

Mantel, P. Y., et al.  

Marques, Cintia, et al.  
2015 Genetic and epigenetic studies of FOXP3 in asthma and allergy. Volume 1.

Matthews, K., J. Nazroo, and J. Whillans  

Mazzoni, A., et al.  

McCluskey, Peter J, Hamish MA Towle, and Susan Lightman  

Medawar, P. B.  
1956 The immunology of transplantation. Harvey Lect (Series 52):144-76.

—  

Mendes de Leon, Carlos F.  

Michie, S., Atkins, L. & West R.
Michie, Susan, Maartje M. van Stralen, and Robert West

Milne, S., S. Orbell, and P. Sheeran

Molins, B., et al.

Morgan, C. M., and H. Schatz

Morikawa, H., et al.

Mosmann, T. R., and R. L. Coffman

Mrejen, S., et al.

Murphy, K. M., and B. Stockinger

Nature

Nguyen, D. X., and M. R. Ehrenstein

NICE

2007 Behaviour change: general approaches

National Insitute for Health and Social Care Excellence.

285

Nozik, R. A., and W. Dorsch

Nussenblatt, R. B., et al.

Ogden, Jane

Ohkura, N., et al.

2012  T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. Immunity 37(5):785-99.
Ohnmacht, Caspar, et al.

2015  The microbiota regulates type 2 immunity through RORyt<sup>+</sup> T cells. Science 349(6251):989-993.
Omenetti, Sara, and Theresa T. Pizarro

Ooi, K. G., et al.

Ouellette, J. A. & Wood, W.

Ozbay, F., et al.

Pachyda, S. I., et al.

PHE

2017 Cell Counting with a Haemocytometer.
Polansky, J. K., et al.
Polley M.J., Pilkening K.
Povoleri, Giovanni, et al.
Powrie, F., and D. Mason
Prochaska, J. O. & DiClemente, C. C.
Quartilho, A., et al.
Ramesh, R., et al.
Reddy, C. V., et al.
Restif, Olivier, and Andrea L. Graham
2015 Within-host dynamics of infection: from ecological insights to evolutionary predictions. Philosophical Transactions of the Royal Society B: Biological Sciences 370(1675).
Rich, Robert R.

Richardson, B.


Rose, Gilbert, et al.


Rosenblum, Michael D., Kelly A. Remedios, and Abul K. Abbas


Rosenstock, Irwin M.


Rowe, Fiona, et al.


RUBIN, GARY S., et al.


Ruggieri, S., et al.


Russ, Tom C, et al.


Saito, A., et al.


Sakaguchi, S, T Takahashi, and Y Nishizuka


Sakaguchi, S., et al.


Sangwan, V. S.

Scheider, A.


2015  Glucocorticoid-resistant Th17 cells are selectively attenuated by
Schewitz, L. P., et al.

2009  Glucocorticoids and the emerging importance of T cell subsets in steroid
Sefik, Esen, et al.

2015  Individual intestinal symbionts induce a distinct population of RORγ+ 
Serhan, Charles N., et al.

2007  Resolution of inflammation: state of the art, definitions and terms. FASEB 
journal : official publication of the Federation of American Societies for 
Shakoor, A., and A. T. Vitale

2012  Imaging in the diagnosis and management of multifocal choroiditis and 
Shantha, J. G., S. Yeh, and Q. D. Nguyen

Sharief, Lazha Ahmed Talat, Sue Lightman, and Oren Tomkins-Netzer

2018  Using Local Therapy to Control Noninfectious Uveitis. Ophthalmology 
125(3):329-331.
Sheeran, P., and C. Abraham

2003  Mediator of moderators: temporal stability of intention and the intention-
Shen, Z., et al.

2011  Comparison on clinical characteristics of multifocal choroiditis and 
Shevach, E. M., and A. M. Thornton

2014  tTregs, pTregs, and iTregs: similarities and differences. Immunol Rev 
Shimada, H., et al.

2008  Pathological findings of multifocal choroiditis with panuveitis and punctate 
Shoda, H., et al.

289
Dietary Omega-3 Fatty Acids Suppress Experimental Autoimmune Uveitis in Association with Inhibition of Th1 and Th17 Cell Function. PLoS One 10(9):e0138241.

Silva, L. M., et al.


Silva, P. S., et al.


Silva, Paolo S., et al.


Silver, Phyllis, et al.

Retina-Specific T Regulatory Cells Bring About Resolution and Maintain Remission of Autoimmune Uveitis. The Journal of Immunology 194(7):3011.

Simonetta, F., et al.


Smith, H, et al.

Effector and regulatory cells in autoimmune oophoritis elicited by neonatal thymectomy. The Journal of Immunology 147(9):2928-2933.

Spaide, R. F., N. Goldberg, and K. B. Freund


St Claire, Lindsay and He, Yuequn


Steele, C. M., and J. Aronson


Stein-Streilein, J., and J. W. Streilein


Steptoe, A. (Ed)

Handbook of Behavioral Medicine: Methods and Practice: Springer.
Steptoe, A., et al.  

Stevens, Rebekah, Hannah Bartlett, and Richard Cooke  

Streilein, J. W.  

Streilein, J. W., and S. W. Cousins  

Sun, M., et al.  

Suri-Payer, E., et al.  

Suttrop-Schulten, M. S., and A. Rothova  


Taira, K., et al.  

Takase, H., et al.  


Teshima, Takanori  
2011 Th1 and Th17 join forces for acute GVHD. Blood 118(18):4765-4767.

291
Tomkins-Netzer, O., et al.

Tomkins-Netzer, O., S. R. Taylor, and S. Lightman

Torun, N., et al.

Tran, D. Q., H. Ramsey, and E. M. Shevach

2007 Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 110(8):2983-90.
von Toerne, C., et al.

Vuong, V. S., et al.

Wachtlin, J., et al.

Wang, J., et al.

Ward, Stephen T., Ka-Kit Li, and Stuart M. Curbishley

Watzke, R. C., et al.


Webb, T. L., and P. Sheeran


Weinhold, Bob


Weinstein, Neil D.


West, R.


WHO


Wilkinson, S., Joffe, H. & Yardley, L.


Williams, G. J., et al.


Wilson, C. B., E. Rowell, and M. Sekimata


Witte, K.

1992  PUTTING THE FEAR BACK INTO FEAR APPEALS: THE EXTENDED PARALLEL PROCESS MODEL

COMMUNICATION MONOGRAPHS 59:329-349.

Wu, J., et al.

2015  Intakes of Lutein, Zeaxanthin, and Other Carotenoids and Age-Related Macular Degeneration During 2 Decades of Prospective Follow-up. JAMA Ophthalmol 133(12):1415-24.

Xu, H., et al.

2005  Differentiation to the CCR2+ inflammatory phenotype in vivo is a constitutive, time-limited property of blood monocytes and is independent of local inflammatory mediators. J Immunol 175(10):6915-23.

Xu, H., et al.

Yeh, S., et al.


Yeo, T. K., et al.


Yu, X., et al.


Zarranz-Ventura, J., et al.


Zhang, Huiyun, et al.


Zhang, X., et al.

2013a Spectral-domain optical coherence tomographic findings at each stage of punctate inner choroidopathy. Ophthalmology 120(12):2678-83.

Zhang, Xiaozhe, et al.

2016 Levels of corticosteroids positively correlate with IL-10+FoxP3+CD25+CD4+ T regulatory cells but not with CD4+IL-17+ Th17 cells in Behçet’s Disease. Investigative Ophthalmology & Visual Science 57(12):494-494.

Zhang, Y., et al.


Zhao, Jun, et al.


Zhuang, Z., et al.

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Zirm, E. K.

Appendix: Supplementary Material for Chapter 3
Experimental Laboratory and Clinical Study Methods

Materials for experimental lab studies

Table 23: List of materials for lab work, with product code and supplier (excluding antibodies)

<table>
<thead>
<tr>
<th>Product</th>
<th>Product Code</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Stimulation Cocktail (containing PMA and Ionomycin)</td>
<td>00-4970-03</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td>Cell Strainer 35µm in 12x75mm Polystyrene Tube</td>
<td>352235</td>
<td>BD Falcon/ SLS UK</td>
</tr>
<tr>
<td>Cytofix/ Cytoperm kit Fixation/Permeabilization Solution Kit</td>
<td>554714</td>
<td>BD Biosciences UK</td>
</tr>
<tr>
<td>DMSO, Hybri-Max, Sterile filtered, Bioreagent</td>
<td>D2650</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>DNeasy Blood and tissue kit (50)</td>
<td>69504</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Item</td>
<td>Code</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Dulbecco’s PBS – modified, without sodium chloride and magnesium chloride, liquid, sterile filtered</td>
<td>D8537</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>DRAK7 dead cell dye, for flow cytometry</td>
<td>424001</td>
<td>BioLegend</td>
</tr>
<tr>
<td>DynaMag 96 Side-skirted Magnet</td>
<td>12027</td>
<td>Thermofisher Scientific</td>
</tr>
<tr>
<td>EZ DNA Methylation Kit</td>
<td>D5002</td>
<td>Zymo Research/Cambridge Biosciences</td>
</tr>
<tr>
<td>Flow Cytometry Staining Buffer</td>
<td>00-4222-26</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td>FoxP3/ transcription factor fix/perm concentrate and diluent</td>
<td>00-5521-00</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td>Heat inactivated foetal calf serum</td>
<td>4-101-500</td>
<td>LabTech International UK</td>
</tr>
<tr>
<td>Histopaque-1077, sterile-filtered, density: 1.077 g/mL</td>
<td>10771</td>
<td>Sigma Aldrich UK</td>
</tr>
<tr>
<td>Item</td>
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</tr>
<tr>
<td>Human Serum, from human male AB plasma, USA origin, sterile-filtered</td>
<td>H4522</td>
<td>Sigma Aldrich UK</td>
</tr>
<tr>
<td>Regulatory T Cell Isolation Kit II, CD4+CD25+CD127dim/neg, human</td>
<td>130-094-775</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Regulatory T Cell Staining Kit #1 (CD4+CD25+FoxP3+), human/primate</td>
<td>88-4999</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td>IL-2 IS, human, research grade</td>
<td>130-097-742</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Legendplex Human Th Cytokine Panel</td>
<td>740001</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Luminex Assays, human magnetic, premixed multiplex</td>
<td>L120101</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Non-filled 50ml LeucoSep tubes, sterile</td>
<td>227290</td>
<td>Greiner Bio-one Ltd.</td>
</tr>
<tr>
<td>OneComp ebeads, compensation beads for flow cytometry</td>
<td>01-1111-42</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td>Protein transport inhibitor cocktail (containing brefeldin)</td>
<td>00-4950-03</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td>Item</td>
<td>Code</td>
<td>Supplier</td>
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</tr>
<tr>
<td><strong>Quantikine ELISA human TGF-β1</strong></td>
<td>DB100B</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td><strong>RPMI-1640 with sodium bicarbonate, without L-glutamine, liquid, sterile-filtered, suitable for cell culture</strong></td>
<td>R0883</td>
<td>Sigma Aldrich UK</td>
</tr>
<tr>
<td><strong>SAFETY Blood Collection Set + Luer Adapter 23G x 3/4” tubing length 7 1/2” (19cm), single-packed, sterile, latex-free</strong></td>
<td>450082</td>
<td>Greiner Bio-one Ltd</td>
</tr>
<tr>
<td><strong>Sample Activation kit 1 (acidification of samples for ELISA)</strong></td>
<td>DY010</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td><strong>SYTOX Blue dead cell stain, for flow cytometry</strong></td>
<td>S34857</td>
<td>ThermoFisher Scientific</td>
</tr>
<tr>
<td><strong>UltraComp eBeads, compensation beads for flow cytometry (compatible with UV and violet laser)</strong></td>
<td>01-2222-42</td>
<td>eBiosciences UK</td>
</tr>
<tr>
<td><strong>VACUETTE 9ml NH Sodium Heparin NR GRN/GRN</strong></td>
<td>455051</td>
<td>Greiner Bio-one Ltd</td>
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<tr>
<td>Antibody</td>
<td>Clone</td>
<td>Host</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>Violet proliferation dye 450</td>
<td>562158</td>
<td>BD Biosciences UK</td>
</tr>
<tr>
<td>X-VIVO 15 with Gentamicin and Phenol Red</td>
<td>L2BE02-060F</td>
<td>Lonza Cambridge/SLS</td>
</tr>
<tr>
<td>Zombie Near Infra Red Fixable Viability Dye, for flow cytometry</td>
<td>423106</td>
<td>BioLegend</td>
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Table 24: List of primary human antibodies used for lab studies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Host</th>
<th>Isotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>CD127 BV605</td>
<td>A019D5</td>
<td>Mouse</td>
<td>IgG1</td>
<td>BioLegend</td>
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<tr>
<td>CD14 APC-Fire</td>
<td>63D3</td>
<td>Mouse</td>
<td>IgG1</td>
<td>BioLegend</td>
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<tr>
<td>CD3, purified NA/LE</td>
<td>HIT3a</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>BD</td>
</tr>
<tr>
<td>CD3 Vioblue</td>
<td>BW264/56</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>CD3 BV785</td>
<td>OKT3</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD4 BUV395</td>
<td>RPA-T4</td>
<td>Mouse</td>
<td>IgG1</td>
<td>BD</td>
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<tr>
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<td>Rat</td>
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<td>eBiosciences</td>
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<td>CD4 PerCP-Cy5.5</td>
<td>OKT4</td>
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<td>BioLegend</td>
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<td>CD8a AF700</td>
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<td>eBiosciences</td>
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<td>Species</td>
<td>Isotype</td>
<td>Supplier</td>
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<td>IgG2a</td>
<td>BD</td>
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<td>FoxP3 PE</td>
<td>PCH101</td>
<td>Rat</td>
<td>IgG2a</td>
<td>eBiosciences</td>
</tr>
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<td>B27</td>
<td>Mouse</td>
<td>IgG1</td>
<td>BD</td>
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<tr>
<td>IL-10 BV605</td>
<td>JES3-9D7</td>
<td>Rat</td>
<td>IgG1</td>
<td>BD</td>
</tr>
<tr>
<td>IL-10 CF594</td>
<td>JES3-19F1</td>
<td>Rat</td>
<td>IgG2a</td>
<td>BD</td>
</tr>
<tr>
<td>IL-17A PE-Vio770</td>
<td>CZ8-23G1</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Miltenyi Biotech</td>
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<td>Mouse</td>
<td>IgG2b</td>
<td>BD</td>
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<td>IgG2a</td>
<td>BioLegend</td>
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<td>4B10</td>
<td>Mouse</td>
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<td>BioLegend</td>
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Titration and optimisation of antibody concentrations for flow cytometry

Below is an example of the staining protocol (shown for an anti-CD4 FITC antibody) used to titrate and optimise flow cytometry antibody concentrations for both stimulated and unstimulated cells (PBMC):

Number of samples required for titration experiment:

1) Stimulated - Unstained
2) Stimulated – Live/Dead
3) Stimulated – Live/Dead + CD4-FITC @ 1:12.5
4) Stimulated – Live/Dead + CD4-FITC @ 1:25
5) Stimulated – Live/Dead + CD4-FITC @ 1:50
6) Stimulated – Live/Dead + CD4-FITC @ 1:100
7) Stimulated – Live/Dead + CD4-FITC @ 1:200
8) Stimulated – Live/Dead + CD4-FITC @ 1:400
9) Stimulated – Live/Dead + CD4-FITC @ 1:800
Staining Calculations

Antibodies:

- Fixable Live/Dead Dye 1:1000
- CD4-FITC To be titrated

1. Stimulated - Unstained Control

Sample: 1

2. Stimulated – Live/Dead (post - CD4 stain)

Samples: 2 → 16

200 uL of Live/Dead dye dilution per sample of 1.0 x10^6 cells

15 samples require the Live/Dead dye

∴ 3000 uL of Live/Dead @ (1:1000)

→ 3000 uL = 3.0 uL of Live/Dead (1:1000) + 2997.0 uL of PBS (no protein)***

3. Stimulated – Live/Dead + CD4-FITC @ (1:12.5)

Sample: 3

→ 100 uL = 8.0 uL of CD4-FITC (1:12.5) + 92.0 uL of Staining Media

200 uL needed for serial dilution (2x)

→ 200 uL = 16.0 uL of CD4-FITC (1:12.5) + 184.0 uL of Staining Media

303
4. Stimulated – Live/Dead + CD4-FITC @ (1:25)
   Sample: 4
   →200 uL = 100.0 uL of CD4-FITC (1:12.5) + 100.0 uL of Staining Media

5. Stimulated – Live/Dead + CD4-FITC @ (1:50)
   Sample: 5
   →200 uL = 100.0 uL of CD4-FITC (1:25) + 100.0 uL of Staining Media

6. Stimulated – Live/Dead + CD4-FITC @ (1:100)
   Sample: 6
   →200 uL = 100.0 uL of CD4-FITC (1:50) + 100.0 uL of Staining Media

7. Stimulated – Live/Dead + CD4-FITC @ (1:200)
   Sample: 7
   →200 uL = 100.0 uL of CD4-FITC (1:100) + 100.0 uL of Staining Media

8. Stimulated – Live/Dead + CD4-FITC @ (1:400)
   Sample: 8
   →200 uL = 100.0 uL of CD4-FITC (1:200) + 100.0 uL of Staining Media
9. Stimulated – Live/Dead + CD4-FITC @ (1:800)

Sample: 9

→ 200 μL = 100.0 μL of CD4-FITC (1:400) + 100.0 μL of Staining Media

Calculations:

Dilution: _________________

Human PBMCs: Total # of cells:

% Viable ________________________________

x = _______________ cells/mL x ________________ mL

Viable cells/mL ________________________________

x = ______________________ cells

Total cells/mL ______________ = 1.0 x10^6 cells/mL x ______________ mL

x = _________________ mL or

x = _______________________ ul
NHS R&D-approved proforma for collection of clinical study data

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<thead>
<tr>
<th>Patient Details</th>
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<tbody>
<tr>
<td>Date of Birth</td>
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<tr>
<td>(dd mm yyyy)</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Weight</td>
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<table>
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<td>Best Corrected VA</td>
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<td>Uveitis activity status</td>
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<td>Vitreous cells</td>
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<td>Disc oedema</td>
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<tr>
<td>Ischaemic vasculitis</td>
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<tr>
<td>Retinitis/choroiditis</td>
</tr>
<tr>
<td>If active, prednisolone commenced?</td>
</tr>
<tr>
<td><strong>If Quiescent, list previous medications</strong></td>
</tr>
<tr>
<td>Prednisolone</td>
</tr>
<tr>
<td>Drug</td>
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<tr>
<td>------------------</td>
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<td>Mycophenolate</td>
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<td>Ciclosporin</td>
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<td>Biological Agents</td>
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<td>Other 2nd Line Agents</td>
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<tr>
<td>If Yes Specify (one or more)</td>
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Standard curves for ELISA and Luminex Assays
Primer sequences used in the NGS sequencing

Genomic FOX3 TSDR sequence

CAGAAGCTGGGGAGAGGCCTGGAGCAGCTTCTCCCTTATGCCCCCAGA
AGGACACAACCTGCGACACCTTGATCTTCGGACTCTGGCCTCCAGAAGCT
GTGAGATCGATCAGCTTCTGGGATGATCTTCCGGCAATGGATTTTTG
TATGACCCAGACAAACCTATATACGGATTTCGGTGCAATGGATTTTTG
CCACCACTCTCGGCCCATGTGGCTGGCTGGTCACTTCTGAAGCT
TGGCTGACCTCGACAAAGCATAGTACATCTCAGACGGACTGGAGAG
TCATCTGGTGGGGGTAGAGGACCTAGAGGGCCGGCTGGGCAGCCGGCT
TCCTGCACTGTCTTTGGGACGTTCCCTTTCTGACTGGTTTTCTCAAGAAGC
TGAATGGGGGATGTTTCTGGGACACAGATTATGTTTTCATATCGGGGTCT
GCATCTGGGCCCTGTGTGTCACAGCCCCACTTGCCAGATTTTTCCGCC
ATTAGCTCATGGCGCCGCCGATGCGCCGGGCTTCATCGACACCAGGGG
AAGAGAAGAGGGCGAGATACCCACACCAACAGGTTTCTCGAGAGAATCTG
CTGCCCTGTGCTCCAGCCAGCTCTGCCCACAGTGCTGAGGTGCAGCTGCTG
GCCCAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
TGGATGTGGTAGGTGATGTCCATCTGGCCACTATGACAAGCCCCTAGCT
CTGAAGACCTGGCCCTTCTTGGGTTGTGGAGAGGACCCAGGTTTGAAGCT
CTGAGAGTGCCAGGCCAGCTCCACAGAAGCTGGACCCCTGGGGTCTTCA
AATAATAACAACCAAGAGCTCAAGACATAGAAGACATTTCTATAGC
TAAAGGATACTTAAATCTCAAAAACACAGACTGTGAGGTCTACGTCGCA
AAGTCTGAGTATGTTGAGCCAGTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CCATCTGGACAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
GCCCTGAAACACAGCCACACTTCTAGGGCCCTAGTCTCCCTGTCCTCCA
CTGACACCAACCTGCTCCCAATATAGGCGACCTGCCGAGATGGCAACAG
GAGAGCTGAGGAGGTGGTTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
GGAGCTGGAAAAAGCCTACGCTTCCCAATACAGACGCCATCATGACAGCT
CCTCTAGGGGCCCACACAAATCAAGGTTTTCGCGGAGCCAGCCACGCTTCTG
ACAAATGGTGTCTGGAGATAATTTAGGGGAGATAAGATTATTTGCTAGTTG
GTACAGTTCATATTCCCAACCACCTCCTGCCCAGATTGAAATGTACC

Figure 75: Example process of primer design and sequence check showing the genomic FoxP3 TSDR sequence with the FoxP3 TSDR primer sequences highlighted in yellow.

310
Bisulfite converted **FOXP3 TDSR** sequence

GTGAGATGATTAATTTTTGTTGTTGAAGTTATTTAGTTGTGGTATTTTGT
TTATTAATTATGATTTGTTGTTTATTTGTTGTTGTTGTTGTTTATTGAAGTT
TTTGTGTAATTTTATGAGTAAAGGAATTTTTTTTTAAATATATTGAAGTT
TTTGTGTAATTTTATGAGTAAAGGAATTTTTTTTTAAATATATTGAAGTT
TTTGTGTAATTTTATGAGTAAAGGAATTTTTTTTTAAATATATTGAAGTT
TTTGTGTAATTTTATGAGTAAAGGAATTTTTTTTTAAATATATTGAAGTT
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TTTGTGTAATTTTATGAGTAAAGGAATTTTTTTTTAAATATATTGAAGTT
TTTGTGTAATTTTATGAGTAAAGGAATTTTTTTTTAAATATATTGAAGTT
Figures 76 and 77: Examples of primer design and sequence checking, showing the annotated bisulfite-converted genomic FoxP3 sequence with the primer sequence highlighted in yellow.

**Figure 76:** Example process of primer design and sequence checking, showing the annotated bisulfite-converted genomic FoxP3 sequence with the primer sequence highlighted in yellow.

**Figure 77:** Example screenshot of BLAST sequence search used to check primer sequence for FoxP3 TSDR
Table 25: Primer sequences used in the assay (the Fluidigm CS1 and CS2 tags are highlighted in bold).

<table>
<thead>
<tr>
<th>Target name</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse Primer (5’3’)</th>
<th>Annealing temperature*</th>
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</thead>
<tbody>
<tr>
<td>TBX21 down</td>
<td>ACACTGACGAC ATGGTTCTACA GGGAGGTTGTT AGTT</td>
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</tr>
<tr>
<td>RORC2 up</td>
<td>ACACTGACGAC ATGGTTCTACA TTTGTTTTTGAA GTATTTTGTTAA AGAG</td>
<td>TACGGTAGCAGA GACTTGGTCTCCC ACCCCTAAAAAA TACAAT</td>
<td>60°C</td>
</tr>
<tr>
<td>RORC2 down</td>
<td>ACACTGACGAC ATGGTTCTACA GGGGGGTTGTT ATTTGGTTATT</td>
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<td>60°C</td>
</tr>
<tr>
<td>TIGIT</td>
<td>ACACTGACGAC ATGGTTCTACA GAGGATGTTA GAGGATTTAG TTAATAG</td>
<td>TACGGTAGCAGA GACTTGGTCTACA CAACTACATCAA ATAAACCACAAAA</td>
<td>53°C</td>
</tr>
<tr>
<td>FOXP3 TDSR</td>
<td>ACACTGACGAC ATGGTTCTACA GTAGAGGATT TAAATAG</td>
<td>TACGGTAGCAGA GACTTGGTCTAAA CCTACTACAAAA AAAAC</td>
<td>53°C</td>
</tr>
<tr>
<td>FOXP3 Promotor</td>
<td>ACACTGACGAC ATGGTTCTACA TGGTGAAGTGG ATTGATAGAAAA GG</td>
<td>TACGGTAGCAGA GACTTGGTCTTAT AAAAAACCCCCCCC CACC</td>
<td>60°C</td>
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Table 26: Unique Fluidigm barcodes added to the PCR products in the assay

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<td>GAAGTGCCTG</td>
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