Genetics of Chemokines and Cytokines in Non-Infectious Posterior Segment Uveitis

A thesis submitted to the University College London for the degree of Doctor of philosophy

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April 2012

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I, Muhammad Ali Ahad confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Background and Aims:

Non-infectious posterior segment uveitis is a potentially blinding disease that usually affects people of working age group. Like other immune mediated diseases, uveitis is a complex polygenic disease. Several cytokines have been identified as important regulators of the immune system during, induction, progression and remission of ocular inflammation in uveitis. The work described in this thesis is based on the hypothesis that polymorphisms in chemokine and cytokine genes can predict clinical outcome in non-infectious uveitis.

Methods:

Functional polymorphisms in sixteen chemokine and cytokine genes were genotyped and there associations were studied in a cohort of British Caucasians suffering with non-infectious posterior segment uveitis.

Results:

This study has shown that polymorphisms in *IL-18*, *IL-10* & *CCR2* genes can influence the susceptibility to certain phenotypes of non-infectious uveitis.

Polymorphisms in many genes particularly, *IL-1β*, *IL-6*, *CCR5* & *IL-18* are found to affect the visual outcome and severity of the disease.

Conclusion:

The identification of these genetic variants that add susceptibility or resistance to uveitis has provided us further insights into the pathogenesis of uveitis. This work will help us identify patients who are at a greater risk of losing sight with this disease. This, in turn would allow tailored aggressive therapy to be given at presentation when vision is still good with a precise aim to prevent significant amount of blindness.

Acknowledgement

Firstly I would like to express my gratitude to all the patients, controls and their families for their understanding and their participation in this research, without their generous support this work would not have been possible.

I give my deepest thanks to my supervisor Prof Susan Lightman for her encouragement and for providing an exciting research opportunity. This work would not have been possible without her generous support, kind patience and round the clock help. I wish to thank all my colleagues in the labs and clinics particularly, Dr Atiyah Abdalla, Dr Tom Missotten, Dr Paolo Spagnolo, Dr Sri Goverdhan, Dr Victor Menezo and Mathew Rose-Zerilli. Their help was tremendous through the various parts of this journey and with whom I shared many moments of hope and despair during these years.

I am grateful to Dr Penny Lympany (Institute of Heart and lung Disease, Imperial College London), Prof Andrew Lotery (University of Southampton) and Dr Virginia Calder (Institute of Ophthalmology, UCL) for giving me the opportunity to do my research work in their laboratories.

I express my warmest gratitude to my Dad and Mom whose support and prayers are the key of my success. Finally to my wife Maria, for being here from beginning till end.

Table of Contents

List of Tables

List of Figures

Abbreviations

3`UTR	Three prime untranslated region
A	Adenosine
ANOVA	analysis of variance
APC	antigen presenting cell
AMPPE	Acute multifocal posterior placoid epitheliopathy
AU	Anterior uveitis
BEVA	Better eye visual acuity
BD	Behcet’s disease
BE	Both eyes
BLAST	basic local alignment search tool
C	Cytidine
CCL	CC-ligand
CCR2	CC-chemokine receptor 2
CCR5	CC- chemokine receptor 5
CDCV	common disease common variant
CMO	Cystoid macular oedema
dH_2O	deionised water
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
dsDNA	double strand DNA
EAU	Experimental autoimmune uveitis
ECM	Extracellular Matrix
EM	expectation maximization (algorithm)
EMSA	electrophoretic mobility-shift assay
EtBr	Ethidium Bromide

G	Guanosin
HH	Human haplotype
HLA	human leukocyte antigen
HWE	Hardy Weinberg equilibrium
ICAM	Intercellular adhesion molecule
IFN	interferon gamma
Ig	immunoglobulin
IL	interleukin
IOP	Intra ocular pressure
IRBP	Interphotoreceptor Retinoid Binding Protein
IU	Intermediate uveitis
l	litre
kb	kilo base
LD	linkage disequilibrium
MHC	major histocompatibility complex
µg	microgram
µl	micro litre
µM	micro molar
MCP	Monocyte chemotactic protein
MEWDS	Multiple evanescent white dot syndrome
MFC	Multifocal choroiditis
mg	milligram
MICA	MHC- class I chain-related gene A
MIP	Macrophage inflammatory protein
ml	millilitre
mM	mill molar

NF-κB	nuclear factor kappa-B
NCBI	National Centre for Biotechnology Information
ng	nanogram
OB	Ocular Behcet's
OR	odds ratio
P	probability
PCR	polymerase chain reaction
PIC	Punctate inner chorioretinopathy
PBMC	peripheral blood mononuclear cells
POHS	presumed ocular histoplasmosis syndrome
PSU	Posterior segment uveitis
PU	Posterior uveitis
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RBH	Royal Brompton Hospital
RPE	Retinal pigment epithelium
SNP	single nucleotide polymorphism
SDF	stromal cell-derived factor-1
SPSS	Statistical Package for the Social Sciences
SSP	sequence specific primer
T	Thymidin
TGF-β	Transforming growth factor-beta
Th	T-helper cell
TFBS	transcription factor binding site
TNF	tumour necrosis factor
*TNF*R	tumour necrosis factor receptor

TNFS	tumour necrosis factor superfamily
Treg	Regulatory T cells
UV	ultra violet
VA	Visual acuity
VKH	Vogt-Koyanagi-Harada Syndrome
WGA	whole-genome amplification
WT	wild type

Chapter one

1 Introduction

1.1 Overview

Uveitis is traditionally defined as inflammation of the uveal tract however most of the time the surrounding tissues are also affected. This inflammation could be either due to a direct insult by infectious agents or through an immune mediated process where infectious pathogens, environment or genes could be a trigger.

Uveitis is a relatively common inflammatory eye condition with reported annual incidence between 11.4 and 52.4 per 100,000 and prevalence between 38 and 730 per 100,000. (Paivonsalo-Hietanen et al. 1997) (Tran et al. 1994) (Mercanti et al. 2001) (Gritz and Wong 2004) It predominantly affects patients in the working age group (Suttorp-Schulten and Rothova 1996) and despite current advances in diagnosis and management, visual loss occurs in 35-50% of patients with this condition. (Durrani et al. 2004) (Rothova et al. 1996)

The most widely used classification of uveitis is the one devised by the International Uveitis Study Group, (Bloch-Michel and Nussenblatt 1987) and is based on the anatomic location of the inflammation. (Table 1-1) It broadly divides uveitis into anterior, intermediate, posterior and pan uveitis. Nevertheless, there are ambiguities in its use, and it does not provide criteria for the diagnosis of specific uveitic entities.

Table 1-1: The SUN Working Group anatomic classification of uveitis

Type	Primary Site of Inflammation	Includes
Anterior uveitis	Anterior chamber	Iritis Iridocyclitis Anterior cyclitis
Intermediate uveitis	Vitreous	Pars planitis Posterior cyclitis Hyalitis
Posterior uveitis	Retina or choroid	Focal, multifocal, or diffuse choroiditis Chorioretinitis Retinochoroiditis Retinitis Neuroretinitis
Pan uveitis	Anterior chamber, vitreous, and retina or choroid	

SUN: Standardization of Uveitis Nomenclature

Uveitis can also be classified on the basis of presence of obvious infectious entity.

a) Infectious uveitis, if it is present and

b) Non-infectious, with no discernible cause.

However, immune responses to infectious or non-infectious causes are not too dissimilar; furthermore, infections can play a major role in both the initiation and perpetuation of “autoimmune” diseases. Thus, it is often difficult to clinically identify infectious versus non-infectious uveitis. Therefore, in many cases of uveitis, the exact contribution of infectious agents in the cause of non-infectious uveitis remains unclear. For example, there is conflicting evidence that acute anterior uveitis is associated with Yersinia and chlamydial infections. (Wakefield et al. 1986) (Wakefield et al. 1990)

However once the diagnosis of infectious uveitis is made the treatment is primarily anti-microbial, (though steroids are needed to limit the inflammation) and the prognosis depends upon the sensitivity of the organism to medical therapy. Conversely the treatment of non-infectious uveitis is mainly immuno-suppression. This treatment is largely topical in anterior segment non-infectious

uveitis and predominantly systemic if the inflammation is in the posterior segment. This posterior segment uveitis is the most common cause of loss of vision in uveitis and is hall marked with the presence of inflammation in the vitreous. It may include inflammation of the choroid, retina, retinal vessels, vitreous and ciliary body either in isolation or as a part of pan uveitis. Anterior uveitis and posterior segment uveitis are two distinct pathogenic entities demonstrating a spectrum of clinical signs that reflect both the predominant anatomical site and pathological features of either anterior uveitis or posterior segment uveitis. (Forrester 1991) For this posterior group the term posterior segment intraocular inflammation (PSII) has been adopted. (Forrester JV et al. 1998) This inflammation can be an isolated phenomenon in the eye like pars planitis or it may be associated with systemic disease like sarcoidosis or Behcet's disease as shown in Table 1-2. Whatever the clinical phenotype is, histopathologically and immunologically they are all similar characterized by lymphocytic infiltration, with many macrophages and few plasma cells. (Lightman 2001) (Boyd, Young, and Lightman 2001) In this thesis this non-infectious posterior segment uveitis will be referred as PSU

Table 1-2 Main causes of non-infectious posterior segment uveitis

Immune-mediated ocular *	Immune-mediated systemic
Intermediate Uveitis	Sarcoidosis
White dot syndromes	Behcet's disease
Multifocal choroiditis	Vogt-Koyanagi-Harada
Birdshot chorioretinopathy	Multiple sclerosis
Serpiginous choroiditis	Sympathetic ophthalmia

* may have associated systemic involvement but insufficient to cause clinical disease

.

Although there is a clear lack of uveal tissue available for histology in PSU an animal model has been developed known as experimental autoimmune uveitis (EAU) (Nussenblatt 1991) (Nussenblatt and Gery 1996) The clinicopathological features of EAU parallel well the clinicopathological features of PSU in humans in that experimental models can be modulated to display the cardinal signs of iridocyclitis, vitritis, retinal vasculitis, and choroidal granulomata.(Forrester et al. 1992) The histological pattern of EAU clearly varies to some degree depending on the species, the antigen and the amount injected. However, in all cases the dominant feature of the disease in the posterior segment is the infiltration of T cells.

1.2 Clinical features and epidemiology

PSU comprises of heterogeneous phenotypes of uveitis ranging from isolated intermediate uveitis to uveitis associated with Behcet's disease. The clinical phenotype varies and thus the prognosis, but the underlying pathogenesis is the same. (Boyd, Young, and Lightman 2001) The hallmark of PSU is inflammation in the vitreous cavity either in isolation or as a part of pan or posterior uveitis. Very often the adjacent structures like the retina, optic nerve and retinal blood vessel are also involved. Broadly PSU is divided into two categories. (Forrester JV et al. 1998)

1) PSU predominantly involving the eye alone (with no clinically detectable other organ involvement)
2) PSU associated with systemic disease.

Epidemiological data suggest considerable variation in the frequency of PSU throughout the world depending upon the population studied. In general, the non-infectious uveitis syndromes are more common in

developed countries, mainly because of lower prevalence rates of the various infectious forms of uveitis. The most common type of non-infectious PSU is idiopathic. (Wakefield and Chang 2005), (Chang and Wakefield 2002) (Rathinam and Namperumalsamy 2007)The most common associated disease with PSU is sarcoidosis which accounts for 5-18.1% of all the cases in the US, Netherlands and Japan, (Wakabayashi et al. 2003) (Merrill et al. 1997), (Smit, Baarsma, and de 1993) (Rothova et al. 1992) Sarcoidosis, however, appears to be rare in Italy, Israel and china where its prevalence among uveitis population is less than 1%. (Mercanti et al. 2001) (Weiner and BenEzra 1991) (Yang et al. 2005) The Behcet's syndrome is the leading cause in Turkey, Saudi Arabia, Israel, China and Iran where it accounts for 6.5-28% of all cases of uveitis. (Sengun et al. 2005) (Islam and Tabbara 2002) (Weiner and BenEzra 1991) (Yang et al. 2005) (Soheilian et al. 2004) A study from North India highlights serpiginous choroidopathy as a leading cause of posterior uveitis with Vogt-Koyanagi-Harada Syndrome (VKH) and sympathetic ophthalmia as common causes of non infectious pan uveitis. (Singh, Gupta, and Gupta 2004) Study from China also reveals a high proportion of VKH syndrome (15.9%) besides Behcet's syndrome (16.5%). (Yang et al. 2005)

The clinical features and course of disease vary considerably between the different types of PSU, primarily because of the anatomical sites involved.

Outlined below are the salient clinical details of various sub types of PSU.

1.2.1 PSU predominantly involving the eye alone

1.2.1.1 Idiopathic intermediate uveitis

Intermediate uveitis is a clinical term that describes inflammation located primarily in the peripheral retina, vitreous base, and pars plana region of the ciliary body. (Bonfioli et al. 2005) It is characterized by a mild bilateral anterior chamber reaction with vitreous cells, retinal vasculitis, and macular oedema. Some cases demonstrate a unique opacified ridge in the peripheral retina, especially inferiorly, known as a snow bank and when present the disease is called pars planitis. (Henderly et al. 1987b) Intermediate uveitis represents 4–16% of uveitis patients in general ophthalmology practice (Lai and Pulido 2002) (Perkins and Folk 1984) and 6%- 23% in specialized uveitis clinics (Jakob et al. 2009) (Al-Mezaine, Kangave, and bu El-Asrar 2010) (Cimino et al. 2010)

Intermediate uveitis often presents with blurred vision and complaints of floaters. 80% of cases are bilateral, (Henderly et al. 1987a) (Smith, Godfrey, and Kimura 1973) but many years may elapse before the second eye is involved. Along with consistent vitreous inflammation, venous periphlebitis (vessel sheathing) may be found. Neovascularisation may occur at the optic nerve head and within peripheral snow banks. (Felder and Brockhurst 1982) Not infrequently, complications like posterior subcapsular cataract, secondary glaucoma, and cystoid macular oedema (CMO) can develop. Severe inflammation is not rare particularly if young, and visual loss is most commonly caused by chronic macular oedema or secondary glaucoma.

1.2.1.2 Posterior uveitis

Clinically, posterior uveitis can present as white dots at RPE or choroidal level, vasculitis, choroiditis (focal or multifocal) either in isolation or as a combination of these.

The white dot syndromes are a group of chorioretinal disorders of unknown aetiology that manifest multiple white dots in the deep layers of the retina, retinal pigment epithelium (RPE), or choroid. (Nussenblatt et al. 1995b) Small white dots are typically found early in disease and in some cases are discrete and evanescent; in others they are large with poorly defined borders and tend to coalesce over time. Fluorescein angiography is similar in most cases with patchy choroidal filling, early hypo fluorescence, and late hyper fluorescence, with more spots seen angiographically than clinically. The type and site of spots, along with angiographical findings and clinical course help us to differentiate white spots into punctate inner chorioretinopathy (PIC), presumed ocular histoplasmosis syndrome (POHS), acute multifocal posterior placoid epitheliopathy (AMPPE), multifocal choroiditis (MFC) with pan uveitis, and multiple evanescent white dot syndrome (MEWDS). Development of choroidal neovascular membrane (CNV) is a feared complication in all white dot syndromes

Punctate inner chorioretinopathy

Punctate inner chorioretinopathy (PIC) typically occurs in moderately myopic young women who present with blurred vision, light flashes, or paracentral scotomas associated with small yellow-white lesions of the inner choroid and RPE.(Watzke et al. 1984) Acute lesions develop into pigmented atrophic scars; peripapillary chorioretinal atrophy may appear along with linear equatorial atrophic and hypertrophic scars. The disorder is often bilateral, and has no

associated vitritis or anterior segment inflammation, and choroidal neovascularisation may occur. PIC and POHS are indistinguishable based on clinical signs, but the latter is associated with infection with H. capsulatum in the Ohio–Mississippi valleys, where histoplasmosis is endemic, although no such association is found with clinically indistinguishable POHS outside these histoplasma-associated areas. No such association is described for PIC.

Acute multifocal posterior placoid epitheliopathy

AMPPE is characterised with multiple large plaque-like lesions occur transiently at the level of the RPE.(Gass 1968) It typically presents with visual loss in young patients, without sex predilection, and following a viral prodrome. (Ryan and Maumenee 1972) It is bilateral in up to 90% of cases, with the second eye becoming involved within 1 month. (Polk and Goldman 1999) Spontaneous resolution typically occurs over days or weeks, with the dots becoming progressively pigmented, leaving a mottled RPE. Isolated areas of choroidal no perfusion may be noted underlying the placoid lesions and suggest choriocapillary occlusion. (Young, Bird, and Sehmi 1980)

Multiple evanescent white dots syndrome

MEWDS is characterized by the appearance of small white spots at the level of the RPE and is associated with a marked drop in vision, scotoma, and shimmering photopsia.(Jampol et al. 1984) It typically occurs in young women, and, as with AMPPE, it usually follows a viral illness. Small, pale spots in the mid peripheral retina may be accompanied by a granular appearance of the macula and rare cells in the vitreous. Later clinical findings include multiple atrophic choroidal scars with peripapillary pigmentary changes, choroidal neovascularisation and an absence of vitreous inflammation. The disorder

typically resolves in 6–7 weeks with return to normal visual acuity and fading of the fundus changes.

Multifocal choroiditis with panuveitis

MFC with panuveitis is also associated with multiple choroidal lesions but additionally has anterior uveitis and vitritis. (Palestine et al. 1985)

Birdshot retinochoroidopathy is an uncommon PSU characterized by vitritis and multiple ovoid, frequently ill-defined, cream-colored subretinal lesions that may be found in a centripetal pattern in the retinal posterior and mid periphery.(Kaplan and Aaberg 1980), (Ryan and Maumenee 1980) It typically occurs in midlife and is more common in females. Patients often present with floaters and decreased visual acuity associated with macular oedema. Macular oedema, vascular leakage, and CNV lead to significant visual impairment, with loss of useful vision in one or both eyes in 40% of patients. (Ryan and Maumenee 1980), (Cassoux and LeHoang 2000)

Sympathetic Ophthalmia

Sympathetic ophthalmia (SO) is a bilateral inflammatory disease that occurs following penetrating trauma to one eye. (Nussenblatt et al. 1995a) Incidence rates of 0.19% and 0.07% have been reported for accidental or surgical trauma, respectively. The onset may occur between 5 days and 66 years following trauma, although 65% of cases occur between 2 and 8 weeks. (Lubin, Albert, and Weinstein 1980), (Zaharia, Lamarche, and Laurin 1984) and 90% are noted within 1 year. (Green et al. 1972) (Kilmartin, Dick, and Forrester 2000)

On clinical examination, sympathetic ophthalmia is characterized by mutton-fat keratic precipitates, anterior uveitis, vitreous cells, prominent choroidal thickening, and papillitis. (Nussenblatt et al. 1995a) Retinal detachment, subretinal neovascularization, and vasculitis may occur. Visual prognosis in

sympathetic ophthalmia is variable, and the final acuity may correlate with the degree of inflammation. (Kilmartin, Dick, and Forrester 2000)

1.2.2 PSU associated with systemic disease

1.2.2.1 Sarcoidosis

Sarcoidosis is a chronic granulomatous disease of unknown aetiology. It is a systemic disorder that can involve many organs, including lungs, eyes, skin, lymph nodes, liver, spleen, and the central nervous system. (Bonfioli and Orefice 2005) In American and European studies, sarcoidosis has an incidence of 6–10 per 100,000, (Henke et al. 1986), (Karma 1979), (Sartwell and Edwards 1974) but this varies considerably with race, geography, diagnostic criteria and referral patterns. There is an increased incidence among African-Americans, with estimates ranging between 8 and 15 times that of the Caucasian American population. (Bresnitz and Strom 1983)

Sarcoidosis is characterized by formation of large granulomas within involved organs, such as the lung, where hilar lymphadenopathy is characteristic. Sarcoidosis is best diagnosed by the characteristic histological appearance of affected tissue on biopsy.

About one fourth of the patients with systemic sarcoidosis have ocular involvement, (Lee et al. 2009a) (Heiligenhaus et al. 2011) with reports as high as 78.6%. (Iwata et al. 1976) The most common ocular manifestation is iritis or iridocyclitis. (Lee et al. 2009a) (Heiligenhaus et al. 2011) The posterior segment is involved in 25%-60% of patients (Hassenstein et al. 2003) (Obenauf et al. 1978) and can include the optic nerve. Anterior segment examination may reveal conjunctival granulomata, aqueous cell

and flare, iris nodules, and posterior synechiae. Yellowish-gray nodular lesions may appear in the choroid or outer retina and can be confluent, and vitreous cells or "snow-ball" opacities may be present. (Papadia, Herbort, and Mochizuki 2010) Patchy retinal phlebitis with branch vein occlusion and retinal or disc neovascularization may be found.

1.2.2.2 Behcet's disease

Behcet's disease (BD) is a chronic, relapsing, multisystem disorder that particularly affects the eye, skin, and the oral and genital mucosa. However quite often patient develop arthritis, intestinal ulceration, and abnormalities of the cardiovascular and central nervous systems. (Sakane et al. 1999) Behcet's disease is characterized by an occlusive vasculitis of unknown aetiology with suggestions of genetic, infectious, and immune influence. Ocular findings in Behcet's disease, known as ocular Behcet's (OB) occur in a high proportion of patients and had been associated with blindness in 50–90% of the cases. (Chung, Liu, and Tsi 1986), (BenEzra and Cohen 1986) Recent data suggests that up to 48% of patients with OB experience severe visual loss. (Davatchi et al. 2010) The main cause of irreversible visual loss appears to be ischemic maculopathy, and use of biologic agents has significantly reduced the risk of severe visual loss. (Taylor et al. 2011)

Although Behcet's disease is found worldwide, it has a predilection for the area corresponding to the old silk trading routes extending from Japan, across the Far and Middle East, and to the Mediterranean. (Verity et al. 1999)

Patients with Behcet's disease may present with ocular manifestations 2–3 years after their initial systemic symptoms and carry a guarded visual prognosis.

(Saadoun et al. 2010) (Davatchi et al. 2010) The inflammation is recurrent, explosive, and lasts several weeks, followed by quiescent periods. Signs are common in the anterior segment and include non-granulomatous anterior uveitis distinguished by hypopyon in approximately one-third of patients. (Altenburg et al. 2006) (Chavis and Tabbara 1995) Posterior segment findings include vitritis, retinitis, optic disc oedema, retinal vasculitis and CMO. (Deuter et al. 2008) (Cassoux, Fardeau, and LeHoang 1999) Vaso-occlusive events occur, and may be followed by retinal neovascularization.

1.2.2.3 Vogt Koyanagi Harada

Vogt–Koyanagi–Harada disease (VKH) is a systemic disorder characterized by bilateral chronic uveitis associated with cutaneous and central nervous system signs. (Moorthy, Inomata, and Rao 1995) Darkly pigmented races are more commonly affected, and it is especially prevalent in Asia, representing up to 6-9% of all uveitis in Japan, (Goto et al. 2007) (Shimizu 1973) and up to 19% of all in Saudi Arabia. (Al-Mezaine, Kangave, and bu El-Asrar 2010) VKH is also well described in people with Hispanic and native North American ancestry and occurs at lower rates in Caucasians.

In 1978, the American Uveitis Society established diagnostic criteria for VKH based on clinical findings. (Snyder and Tessler 1980) The criteria, however, failed to consider different manifestations of VKH at varying stages of this disease, including evaluation by fluorescein angiography and ultrasonography. This problem has been rectified by the new diagnostic criteria, suggested by Rao *et al* (Rao, Sukavatcharin, and Tsai 2007) as shown in Table 1-3

Table 1-3 Revised criteria for diagnosis of VKH *

Complete VKH: Criteria 1–5 must be present
Incomplete VKH: Criteria 1–3 and either 4 or 5 must be present
Probable VKH (isolated ocular disease): Criteria 1–3 must be present

1. No history of penetrating ocular trauma or surgery preceding the initial onset of uveitis
2. No clinical or laboratory evidence suggestive of other ocular disease entities
3. Bilateral ocular involvement (a or b must be met, depending on the stage of disease when the patient is examined)
    a) Early manifestations of disease
        (1) Evidence of diffuse choroiditis (with or without anterior uveitis, vitreous inflammatory reaction, or optic disk hyperaemia) which may manifest as (a) focal areas of subretinal fluid, or (b) bullous serous retinal detachments
    b) Late manifestations of disease
        (1) History suggestive of prior presence of early findings noted in 3a and either (2) or (3) below, or multiple signs from 3.
        (2) Ocular depigmentation: either (a) sunset glow fundus or (b) Sugiura's sign
        (3) Other ocular signs including (a) nummular chorioretinal depigmented scars, or (b) retinal pigment epithelium clumping and/or migration, or c) recurrent or chronic anterior uveitis
4. Neurological/auditory findings (may resolve by time of evaluation)
    a) Meningismus (malaise, fever, headache, nausea, abdominal pain, stiffness of the neck and back, or a combination of these factors); note that headache alone is not sufficient to meet the definition of meningismus
    b) Tinnitus
    c) Cerebrospinal fluid pleocytosis
5. Integumentory finding (not preceding onset of central nervous system or ocular disease)
    a) Alopecia, or
    b) Poliosis, or
    c) Vitiligo

*Rao NA, Sukavatcharin S, Tsai JH. Vogt-Koyanagi-Harada disease diagnostic criteria. Int Ophthalmol. 2007 Apr-Jun;27(2-3):195-9

VKH typically presents with a prodrome of headache or meningism followed by an acute uveitic phase. The ocular signs are bilateral in approximately 95% of cases and may be delayed in the second eye by up to 2 weeks. Many patients progress to a phase of chronicity characterized by repeated attacks of uveitis, most commonly of the anterior segment. In the posterior pole, chronicity may be associated with neovascularization, subretinal fluid or fibrosis, and frank chorioretinal atrophy. (Kuo et al. 2000) (Sonoda, Nakao, and Ohba 1999) Visual prognosis is variable with 60% of patients achieving vision of 20/30 or better. (Moorthy, Inomata, and Rao 1995)

1.3 Immunopathology of Posterior segment uveitis

1.3.1 Overview

The development of inflammation in eye is multifactorial, including genetic susceptibility, nutritional status, innate stimuli such as injury or concomitant ocular and systemic infection, and the presence of autoreactive T cells. When no overt infectious agent is identified in uveitic patients, we presume that autoimmunity is the underlying pathogenesis. (Forrester 1992) Patients with non-infectious uveitis frequently exhibit immune responses targeted to ocular antigens such as uveal melanin, retinal arrestin (formerly known as the 48-kDa retinal soluble antigen [S-Ag]), interphotoreceptor retinoid–binding protein (IRBP), and recoverin (Gocho, Kondo, and Yamaki 2001) (Gery, Mochizuki, and Nussenblatt 1986). Although it is unclear whether these immune responses represent the etiological cause or an epiphenomenon of autoimmunization to the products of tissue breakdown they are believed to fuel progression of the disease. (Caspi 2010) Diseases in this group tend to have strong MHC associations (Pennesi and Caspi 2002), which is believed to indicate an autoimmune aetiology, as MHC molecules select and present antigens for recognition by T cells.

Antigen is the initiator of all acquired immune responses, whereupon the primary function of the immune system is to recognise exogenous antigens and eliminate pathogens. Additionally, the maintenance of immune regulation is brought about by tolerance to self-antigens acquired during development (central tolerance) or actively during adult life (peripheral tolerance) and in the case of exogenous antigens (viral and bacterial) immune responses are, in part, kept in check by inhibitory cytokines and soluble cytokine receptors. The eye

has evolved to limit intraocular inflammation so as to protect the delicate visual elements from damage that would be detrimental to visual acuity. This ability of the eye to curb and control immune responses is known as ocular immune privilege. (Caspi 2010) While it is generally accepted that immune privilege protects the eye from day-to-day inflammatory insults and contributes to the extraordinary success of corneal grafts, its role in protection from ocular autoimmunity has been disputative.

It has been demonstrated that elimination in the thymus of self-reactive T cells, (central tolerance), applies to retinal antigens (Avichezer et al. 2003). The mechanisms underlying central tolerance rely on an immature T cell interacting with its cognate tissue antigen through its specific T cell receptor (TCR). Tissue antigens, including the retinal antigens IRBP and arrestin, are ectopically expressed in the thymus under the control of the gene-regulatory protein autoimmune regulator (AIRE) (Gotter et al. 2004).

T cells, which react to tissue antigens that escape control in the thymus are usually subjected to regulation by peripheral tolerance mechanisms that include clonal deletion, clonal anergy, and active suppression. (Miller and Basten 1996) This induces T cells to become tolerant to their specific antigen when they encounter that antigen in healthy tissues; however retinal antigens residing in the eye are relatively inaccessible. Thus, circulating retinal antigen–specific T cells are likely to be “ignorant” of their cognate antigen rather than tolerant and can be activated by a chance encounter with an antigen, possibly in the form of a microbial component that structurally mimics their cognate tissue antigen (Wildner and edrichs-Mohring 2003). Infection may break this regulation because it is also well recognized that autoimmune diseases may be triggered by infectious agents. (Hausmann and Wucherpfennig 1997) Thus the induction

of autoimmunity represents a failure of peripheral (extrathymic) regulatory mechanisms to control autoreactivity.(Read et al. 1998) Therefore, by sequestering retinal antigens within the eye and hindering peripheral tolerance, immune privilege may actually predispose to ocular autoimmunity. Figure 1-1 explains this phenomenon diagrammatically.

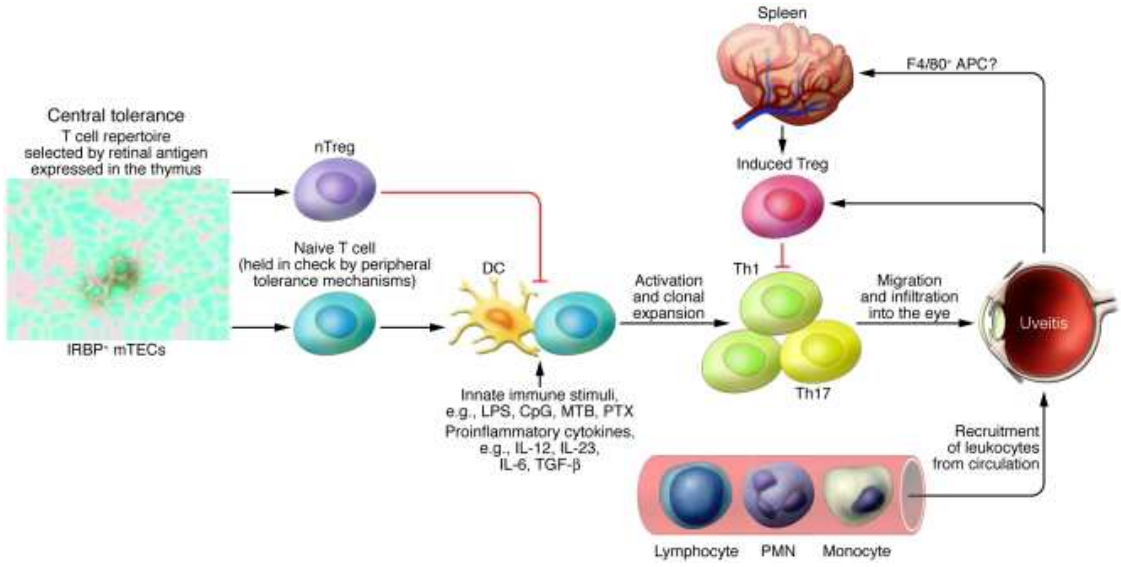


Figure 1-1 Central and peripheral tolerance for eye antigen and role in autoimmune uveitis.

Retinal antigen–specific T cells that have not been eliminated in the thymus encounter an activating stimulus in the context of costimulatory "danger" signals, escape from the control of nTregs, and differentiate into pathogenic effector T cells. These undergo clonal expansion, migrate to the eye, break down the blood-retinal barrier, and recruit inflammatory leukocytes from the circulation. The resulting inflammation results in damage to the tissue and release of ocular antigens, which triggers eye-specific regulatory mechanisms that terminate the disease and limit pathology. mTECs: medullary thymic epithelial cells, MTB: Mycobacterium tuberculosis Bacillus, PTX: pertussis toxin
Courtesy Caspi 2010, Journal of clinical investigation, 120 vol 9

This dynamic and continual regulation of immune responses is orchestrated by cytokines, controlling leukocyte behaviour and activation including antigen-presenting cells (APC) like, dendritic cells (DC), antigen-specific T cells, and non-specific leukocytes such as macrophages and granulocytes.

If CD4+ T cells mediate uveitis, then cells will recognize antigen (be it autoantigen or foreign antigen) only after the antigen has been processed and presented on the cell surface of antigen-presenting cells (APCs) as peptides within the peptide groove of major histocompatibility complex (MHC) class II antigens. APCs include DCs or macrophages. Antigen-specific T cells recognize peptide/MHC class II complex via their own unique T cell receptor (TCR), although the TCR can recognize several antigenic determinants (cross-reactivity) and each T cell can express a range of TCR. T cells will, however, not undergo activation and clonal expansion unless, in addition to TCR-MHC/peptide interaction, co-stimulation also takes place. To ensure T cell activation, resulting in receptor activation and cell signalling, there are key receptor-ligand interactions between T cell and APC in addition to CD4 antigen recognition. These include CD28-B7, CD40-CD40L, and CTLA-4- B7. Without co-stimulation T cells are rendered unresponsive (anergic). Figure 1-2 below explains this process schematically.

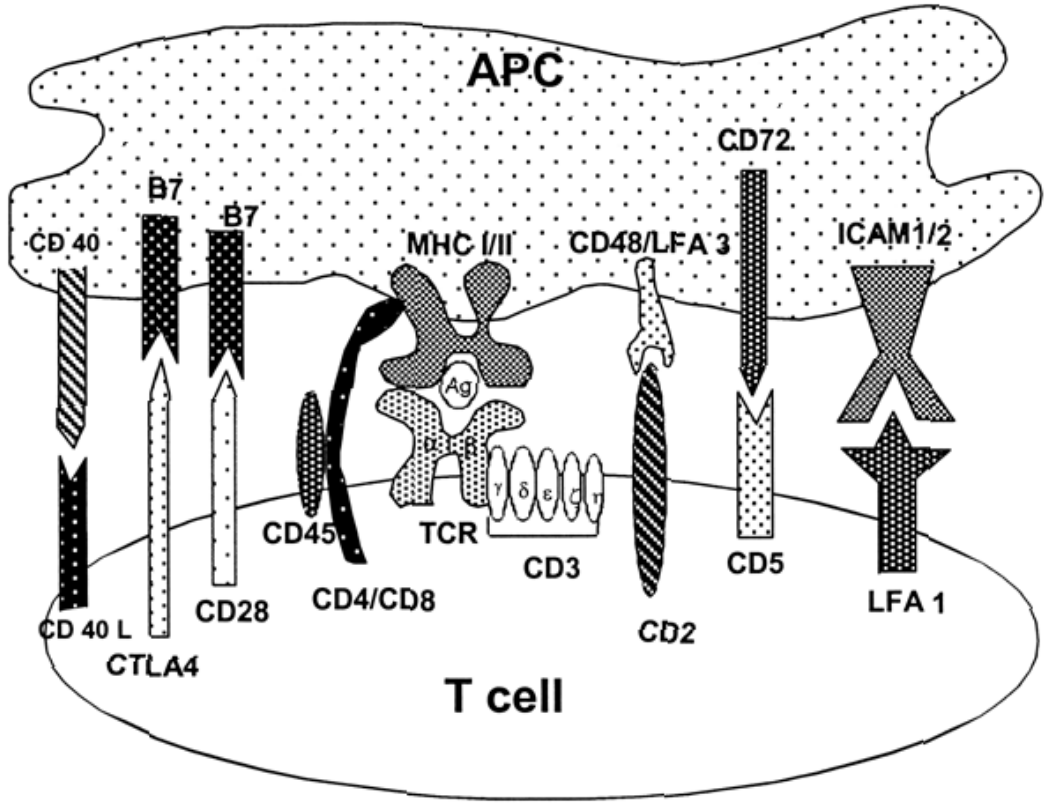


Figure 1-2: T-cell activation.

T-cell activation is initiated by the presentation of antigen, in the context of the major histocompatibility complex (MHC) of the antigen-presenting cell (APC), to CD3-TCR. This interaction between the APC and the T cell provides signal 1. Signal 2, a costimulatory signal also required for T-cell activation, is provided by the interaction between T-cell surface receptors and their respective ligands on the APC (e.g., the interaction between B7 and CTLA4 or CD28, CD40 to the CD40 ligand). Interaction between accessory molecules, such as intercellular adhesion molecule (ICAM) 1 and ICAM2 with lymphocyte function-associated antigen (LFA)-1, increases the association between the APC and the T cell. Additional signaling pathways (e.g., CD48 and LFA-3 to CD2, CD72 to CD5) also participate in T-cell activation

Irrespective of which triggering event breaks tolerance, antigen specific CD4+ T cells can only initiate autoimmune disease after peripheral clonal expansion and on further presentation of antigen at the target tissue, both of which serve to activate the T cell and initiate a proinflammatory cascade resulting ultimately in tissue damage. Naive T cells once activated differentiate into one of many types depending on cytokine environment present. (Figure 1-3)

The first are the Th1 cells, which show a cytokine profile of IFN-γ production. The cytokine profile of Th2 cells comprises IL-4, IL-5, IL-13 and perhaps TGF-β, and IL-10. In many animal models of human disease Th1 cells are associated

with the initiation of disease, whereas Th2 cells are related to disease downregulation and allergy initiation, or are involved in parasitic diseases.

Another subset of cells that has been the centre of great interest recently is that of the Th17 cells. (Chen and O'Shea 2008) These cells produce proinflammatory cytokines including IL-17 (hence the name), IL-21 and 22. These cells develop in different environments depending on whether we look in the mouse or the human. In humans, IL-1, IL-6, and IL-23 appear to promote these cells. The cells play a role in host defence mechanisms against fungi and bacteria, and also in autoimmune disease.

One theory is that Th1 cells may initiate an immune response but the Th17 cells are involved in more chronic activity. (Shi et al. 2009) (Yoshimura et al. 2008) It would be interesting to find whether Th1 cells and Th17 are distinct cells, or they are merely a function of the immune environment, so that under certain circumstances they produce IL-17 and under others a Th1 repertoire. In fact under experimental conditions it has been seen that Th17 cells may switch to a Th1 character, but that Th1 cells maintain that phenotype and do not change. (Shi et al. 2008) Also under experimental conditions in animals, when comparing these cell the nature of the intraocular inflammatory response was seen to be different. (Cox et al. 2008) Th17 did not induce a large lymphoid expansion and splenomegaly, as Th1 cells did; Th1 cells infiltrating the eye dissipate rapidly, whereas Th17 cells remain; and markers on the surface of these infiltrating cells were different. (Cox et al. 2008) There is another type of cells that suppress or modify an immune response and these are known as T-regulatory (Treg) cells. (Piccirillo 2008) It is hypothesized that these derive from a naive T cell under the influence of cytokines different from those of either Th1 or Th2 cells. The characteristic feature of these cells is their ability to produce

IL-10 and TGF-β. They are capable of down regulating both Th1 and Th2 cells but interesting by secreting TGF-β and in the presence of IL-6 Tregs can induce TH-17 proliferation. (Kimura and Kishimoto 2010) The diagram below (Figure 1-3) summarizes differentiation of T helper cells into sub categories depending upon cytokine environment.

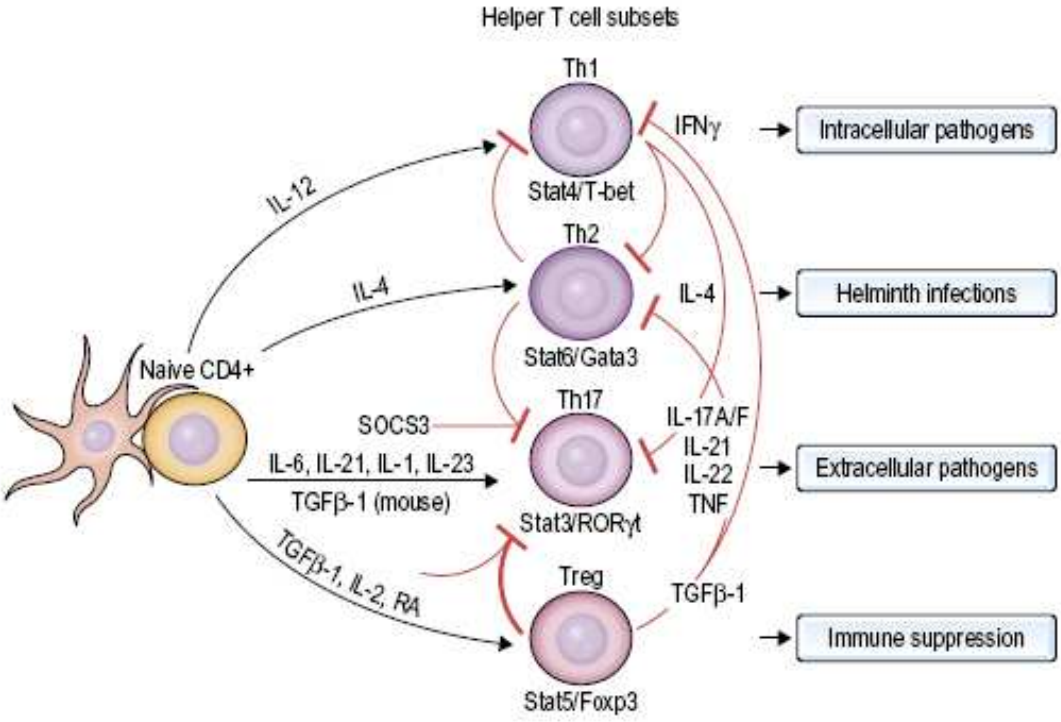


Figure 1-3: The CD4 T cell repertoire in inflammation and infection.

Once activated by occupancy of the T cell receptor (TCR), naïve CD4+ T cells in the presence of IL-12 differentiate to become Th1 cells, which produce IFN-γ. This process is regulated by Stat4 and T-bet. IFN- γ has important functions in host defence against intracellular pathogens. In response to IL-4, naïve T cells differentiate to Th2 cells through the activation of Stat6 and GATA3. The signature cytokine made by Th2 cells is IL-4, which plays a key role in host defence against helminth infections. In conjunction with TCR stimulation, naïve T cells cultured with TGFβ-1 can become CD4+CD25+Foxp3+ cells with suppressive effects. Although TGFβ-1 can upregulate Foxp3 expression, in the presence of IL-6, mouse naïve T cells develop to become IL-17-producing Th17 cells. In human T cells, IL-1 synergizes with IL-6 and IL-23 inducing Th17 differentiation. Stat3 and RORγt are the key transcription factors regulating this process. Th17 differentiation is inhibited by IFN-γ, IL-4, IL-2, retinoic acid, and SOCS3 (Suppressor of cytokine signalling 3).
(Modified from Chen & O'Shea Immunol Res (2008) Vol:41

Activation, of Th1 increases production of interleukin- 2 (IL-2) and subsequently other proinflammatory cytokines such as IFN-γ and tumour necrosis factor (TNF-α). Activated T cells express certain cell surface markers such as IL-2 receptor (IL-2r), CD69, and an isoform of common leukocyte antigen, CD45Ro,

intercellular adhesion molecule-1 (ICAM-1) and secrete IL-2 and IFN-Ɣ, all which have been reported within the peripheral circulation and ocular fluids of uveitis patients.(rocker-Mettinger et al. 1990) (Feron, Calder, and Lightman 1992) (Dick et al. 1992) (Muhaya et al. 1999) (Whitcup et al. 1992).

However T cells, including activated antigen-specific T cells, cannot cross the blood-retinal barrier unless there has been some systemic signal, specific or nonspecific, which renders the retinal endothelial cells susceptible to T cell transmigration (Xu et al. 2003). Entry of cells into the retina requires prior upregulation of adhesion molecules on the retinal endothelium and specific interactions between ligand receptor pairs such as intercellular adhesion molecule-1 and lymphocyte function-associated antigen (Xu et al. 2004)

As the disease progresses, many additional cells are recruited to the retina, including non-specifically activated T cells, granulocytes, macrophages and dendritic cells. Although EAU is CD4-T cell-mediated, macrophages play a central role in tissue damage. This has been demonstrated in macrophage depletion studies and also in studies in which T cells continue to infiltrate the tissue but the macrophage is disabled and tissue damage is attenuated (Dick et al. 2004). Recent studies have revealed the heterogeneity of macrophage populations, one set of which are pro-inflammatory while another, the alternatively activated macrophage, may have a role in modifying the inflammatory response. Other macrophages, particularly the resident macrophage, may have a scavenging role in clearing dead and dying cells in the absence of a marked inflammatory response (Taylor et al. 2005).

In EAU, infiltrating myeloid cells consist of DCs and activated monocytes, many of which express major histocompatibility complex class II antigen. In the later

stages of the disease, macrophages lose their major histocompatibility complex class II.

Factors regulating macrophage activity are not known though gamma Interferon (IFN-γ) and tumour necrosis factor alpha (TNF-α), both pro-inflammatory cytokines, are known to be involved in macrophage activation in inflammation generally.

However, while transgenic expression of IFN-γ in the rodent eye is associated with increased inflammation (Zhang et al. 2001), IFN-γ-deficient mice also develop EAU through a deviated immune response (Jones et al. 1997). Recent studies on accessory molecules involved in antigen presentation, such as CD40 and CD137, indicate a definitive requirement (Shao et al. 2005) (Bagenstose et al. 2005), while molecules involved in monocytes adhesion and trafficking, for example the chemokine macrophage inflammatory protein-1, are required at least for T cell entry into the retina (Crane et al. 2003)

The understanding of immunopathologic mechanisms is hindered by the genetic and clinical heterogeneity of patients, and limited access to clinical material from the site of disease. So most significant advances in both our understanding of immune mechanisms and the development of immunotherapy have arisen from experimental models of PSU.

1.3.2 Animal model

The most commonly used model is experimental autoimmune uveoretinitis (EAU), which is a retinal antigen-specific Th1 CD4+ T cell mediated posterior segment inflammation resulting in destruction of the retinal photoreceptors. (Forrester et al. 1990) (Caspi et al. 1986) T cells play a central role in EAU. The disease cannot be induced in T cell-deficient animals even with repeated immunizations, is ameliorated by T cell targeting agents such as cyclosporin A,

and can be transferred to genetically compatible, naive recipients with immune T cells. (Caspi et al. 1986)

Moreover, when varying the strain of animal (genetic susceptibility) or dose and type of immunizing antigen, more acute or chronic features of posterior segment inflammatory disorders can be elicited. The two most commonly studied uveitogenic retinal antigens are S-ag (also called S-Arrestin) and interphotoreceptor retinoid binding protein (IRBP). These models demonstrate a pivotal role for antigen-specific CD4+ (Th1) T cells, (Smith et al. 1999) (Caspi et al. 1986) nonspecific leukocyte infiltration, (Caspi et al. 1993) and proinflammatory soluble cytokines, including IFN-γ , IL-2, TNF-α, and IL-12.

EAU can be elicited not only by immunization with retinal antigens (Ags) but with their fragments (Agarwal and Caspi 2004), or by adoptive transfer of retinal Ag-specific CD4 + T cells between syngeneic rodents (Rizzo et al. 1996). Published data till 2007 provided evidence that a Th1-dominant response and the Th1 effector cell were critical for EAU development and that endogenous IL-12 was needed for EAU induction and its full expression (Caspi 2002) (Tarrant et al. 1998). However, susceptibility to EAU of IFN- γ deficient (GKO) mice, exacerbation of EAU by neutralization of endogenous IFN-γ, and the protective effects of high systemic IFN-γ in WT mice (Caspi et al. 1994) (Jones et al. 1997) (Tarrant et al. 1999) were an apparent contradiction with the notion that “EAU is purely Th1 mediated” . This requirement for IL-12 mediated IFN-γ and Th1 responses in autoimmune inflammation were recently questioned by several studies in other disease models. Mice deficient in IFN-γ, IFN-γR, IL-12Rb2, and the IL-12p35 chain were highly susceptible to experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). (Cua et al. 2003) (Gran et al. 2002) (Becher, Durell, and Noelle 2002) In

contrast, IL-23 and the IL-17 producing effector T cell were found to be necessary for induction of these diseases (Cua et al. 2003) (Murphy et al. 2003). The activity of the IL-17 producing effector T cells (Th17) was associated with induction of proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8, as well as with enhanced proliferation, maturation, and chemotaxis of neutrophils. These results led to the notion that the pathogenic effects previously attributed to the IL-12 – IFN-γ pathway are in fact largely if not solely mediated by IL-23 and the IL-23 driven Th17 effectors. (Cua et al. 2003) (Langrish et al. 2005).

In context with uveitis, Luger *et al.* then showed that lL-23 rather IL-12 is necessary for the induction of EAU. (Luger et al. 2008) They demonstrated that IL-17 plays a role in the pathogenesis of EAU induced by immunization in CFA, and that targeting IL-17 even late in the disease process can ameliorate pathology, indicating an effector role for this cytokine in pathogenesis of this type of EAU. Notably, however, severe EAU could be induced by uveitogenic Th1 cells without participation of host IL-17, and pathology of EAU induced with uveitogenic Ag-pulsed DCs required induction of an IFN- y producing effector T cell response. Finally, genetically *IL-17* deficient mice were able to develop substantial disease. Thus, in some situations Ag-specific IL-17 –producing effector T cells appear to be dispensable for pathogenesis. This means that EUA can be either Th1 or Th2 driven depending upon the initial exposure of antigen. Following Figure 1-4, explains their findings

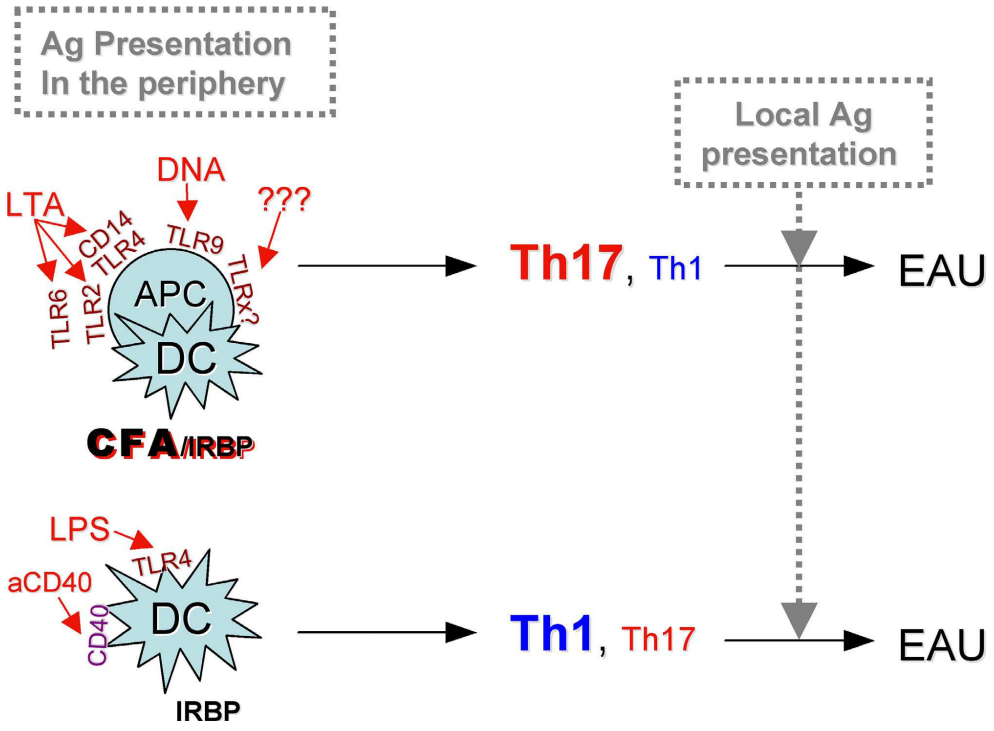


Figure 1-4: Schematic representation of the patterns of effector T cell dominance in the different EAU models.

Conditions of initial exposure to Ag that may determine effector dominance are the quality/ quantity of TLR signals and the type/variety of cells participating as APCs.
IL-17 plays a dominant role in EUA induced by immunization with the retinal antigen (Ag) interphotoreceptor retinoid-binding protein (IRBP) in complete Freund's adjuvant; its neutralization prevents or reverses disease, and Th17 effector cells induces EAU in the absence of interferon (IFN)-γ. In a transfer model, however, a polarized Th1 line can induce severe EAU independently of host IL-17. Furthermore, induction of EAU with IRBP-pulsed mature dendritic cells requies generation of an IFN-γ–producing effector response, and an IL-17 response by itself is insufficient to elicit pathology. Courtesy (Luger *et al.* Journal of Exp. Medicine Vol. 205, No. 4, April 14, 2008)

The widely held view now is that autoimmunity to retina can be either Th17 or Th1 driven. The IL-23 / IL-17 pathway plays an important role in EAU, and intensity of IL-17 response systemically and locally correlates with disease severity in mice immunized with IRBP/CFA. However, the role of the Th17 effector is redundant with Th1, and each effector phenotype by itself is sufficient to induce pathology in the absence of the reciprocal hallmark cytokine. It has also been noted that *IL-12* and *IFN-γ* deficient mice, typically exhibit more severe disease than their WT counterparts. This suggests that the Th17 effector may become more prominent in pathogenesis when the IL-12 / IFN-γ

pathway is reduced or eliminated. IFN-γ has been reported to inhibit commitment to the Th17 phenotype in vitro (Park et al. 2005). Also, congenital lack of IL-17 allows emergence of compensatory mechanisms involving Th1 and other proinflammatory cytokines including IL-22, (Luger et al. 2008) and the enhanced Th1 response in lymph nodes and in the eyes of IL-17 KO mice raises the possibility that IL-17 may have an antagonistic effect on the development of Th1 effectors, just as IFN- γ inhibits development of Th17 effectors. This has also raised the question of whether the Th17 effector response might be dominant particularly in situations where induction of disease occurs in the context of strong Toll-like receptor (TLR) signals, whereas in other conditions a Th1 response may predominate. (Luger et al. 2008)

Like the development of autoimmunity in animal models, current view in humans is that activation of autoaggressive antigen-specific T cells occurs systemically and that activated cells then “home” to the eye, where they are presented with antigen by local APCs. Once at tissue sites Th1 cytokine secretion is perpetuated via chemotaxis of nonspecific inflammatory cells and chemokine, TNF-α, and IFN-γ secretion by, activated retinal pigment epithelium. (Forrester et al. 1995) This results ultimately in immune-targeted tissue damage. In concurrence with such a theory, it has been shown experimentally that there are APCs within choroid (Forrester et al. 1994) and also within the retina, (Dick et al. 1995) and T cells can adhere to both the RPE and retinal vascular endothelium via ICAM-1. (Mesri, Liversidge, and Forrester 1994)

The recruitment of leukocytes is a critical feature of ocular inflammation. Chemokines and their G-protein-coupled surface membrane receptors mediate innate and adaptive immunity through mechanisms of selection and recruitment

of cells to sites of inflammation and disease. The expression of CCL3 [macrophage-inflammatory protein-1 (MIP-1)], MCP-1 [monocyte chemoattractant protein–1 (MCP-1)], and CCL5 [regulated on activation of normal T-cell-expressed and secreted (RANTES)] and CCR5 is increased in the choroid and retina during the course of uveitis. (Crane et al. 2000) (Elner et al. 1997b) (Crane and Liversidge 2008)

Though the inflammation pathways in immune mediate uveal inflammation are grossly similar in various sub groups of PSU, but there is considerable difference in the initiating event and type of cells that initiate and propagate the inflammation. Following section explain the histopathology and immune events specific to major sub types of PSU.

1.3.3 Immunopathology of Intermediate uveitis

Histological studies of the peripheral retina and ciliary body demonstrate condensed vitreous, fibroblasts, spindle cells, hyperplastic non-pigmented pars plana epithelium, lymphocytes, and blood vessels. (Eichenbaum, Friedman, and Mamelok 1988) (Pederson et al. 1978) Prominent lymphocyte cuffing with mural infiltration of retinal veins is also described histologically. (Pederson et al. 1978)

Vitreous biopsy at the time of active disease demonstrates lymphocytes, epithelioid cells, macrophages, and giant cells. (Nolle and Eckardt 1992) Vitreous lymphocytes are mostly T cells with variable numbers of macrophages and few B cells. (Davis et al. 1992) (Nolle and Eckardt 1992) A prevalence of CD4+ T lymphocytes has been found in the pars plana and snow bank, and these outnumber CD8+ T lymphocytes by 10:1. (Wetzig et al. 1988)

Cytokine analysis has shown elevated serum IL-8 (Klok et al. 1998) in active intermediate uveitis and seems to indicate a later predisposition to systemic

disease. Serum IL-1 receptor antagonist, which down regulates the immune response becomes elevated with successful treatment. (BenEzra, Maftzir, and Barak 1997) Circulating T lymphocytes have been identified that recognize several retinal antigens but are not specific to intermediate uveitis. (deSmet et al. 1990) In addition, as with the other uveitides antibodies against several retinal antigens have been found. (Nolle 1992)

It is tempting to speculate that an antecedent or subclinical infection could initiate disease through the process of molecular mimicry. The presence of glial elements in the pars plana, the glial targeting of MS and the potential for cross-reactivity of an antiviral response against myelin basic protein, together support this contention. The frequent association with infections such as Lyme disease (*Bartonella borgdorferi*), cat-scratch fever (*Bartonella henselae*), toxoplasmosis, and human lymphotrophic virus type I (HTLV-1) also points towards molecular mimicry. Some viral peptides can induce an auto reactive T lymphocyte response against myelin basic protein, (Wucherpfennig and Strominger 1995) but again, evidence for this proposed molecular mimicry in disease has not been demonstrated.

1.3.4 Immunopathology of Posterior uveitis

Post mortem studies of eyes with multifocal choroiditis, (Dunlop et al. 1998) (Charteris and Lee 1990) have shown a long-standing perivascular infiltrate consisting of B lymphocytes, CD3+ T cells (a pan-T marker which does not distinguish between CD4+ and CD8+), and scattered macrophages in the choroid. Early stage neovascular membranes and RPE hyperplasia were seen over the most intensely inflamed choroidal vessels, although Bruch's membrane remained intact. Scattered macrophages were found in the retinal pigment epithelium along with minor atrophy of the overlying photoreceptor

layer. Vitrectomy specimens also report a preponderance of T cells. (Nolle and Eckardt 1993) A single pathologic specimen of an enucleated phthisical eye showed focal lymphocyte and plasma cell infiltration of the iris and ciliary body (Nussenblatt et al. 1982). Diffuse granulomatous inflammation was noted in the outer retinal layers with epithelioid, giant, and plasma cells.

Elevated serum titres of circulating antibodies or reactive immune cells has suggested numerous infectious associations: Lyme disease with AMPPE, (Wolf et al. 1992) Epstein–Barr Virus (EBV) with MFC, (Tiedeman 1987) and histoplasmosis with POHS. (Schlaegel, Jr. 1979) The association of EBV with MFC has not been supported by other studies, (Nolle and Eckardt 1993) and even in POHS, the most consistent association, the relevance of anti-histoplasmosis titres, remain unknown. (Check et al. 1979) Several studies of patients with POHS failed to demonstrate evidence of *H. capsulatum* and attempts to identify intraocular constituents of the histoplasmosis cell wall have been negative. (Irvine et al. 1976), (Meredith et al. 1977) No antibacterial or antiviral titres have been found associated with MEWDS. Likewise for AMPPE, the prodromal illness suggests an infectious cause but none has been identified. (Deutman et al. 1972)

Antibody titres and studies of infectious agents in patients with birdshot retinochoroidopathy have not been informative. For example, prompted by the coexistence of seropositive ocular Lyme disease and HLA-A29 markers, (Suttorp-Schulten et al. 1993) a search for *Bartonella burgdorferi* was unsuccessful. Similarly, a postulated association with Q fever could not be established. (Kuhne et al. 1992)

The close association between HLA-A29 and birdshot retinochoroidopathy suggests that genetic susceptibility to disease is paramount in its pathogenesis.

It is now appreciated that the A29.2 mutation interferes with T cell T cell-receptor interactions, thus confirming the role of this function in disease pathogenesis. (Tabary et al. 1991) I

Immunopathology of sympathetic ophthalmia

The primary feature of sympathetic ophthalmia is a diffuse non-necrotizing granulomatous inflammation of the uvea, with prominent lymphocytic infiltration of the choroid. Within this infiltrate there are focal collections of epithelioid cells with occasional giant cells and rare plasma cells. (bu El-Asrar et al. 2007) These Dalen–Fuchs nodules are located between Bruch's membrane and the RPE. (Chan et al. 1986) Immunopathologic studies support the role of the T lymphocyte in the pathogenesis of sympathetic ophthalmia. (bu El-Asrar et al. 2007) (Furusato et al. 2011) Whereas CD4+ T lymphocytes dominate early in disease, the CD8+ T lymphocytes are more common later. (Chan et al. 1986), (Towler and Lightman 1995) Post enucleation immunocytochemical studies have demonstrated activated T lymphocytes expressing IL-2 receptors (Towler and Lightman 1995) and secreting IL-2 and IFN-Ɣ. Significantly, elevated ocular and systemic levels of IL-1 and TNF-α have also been found. (Chan et al. 1985b), (Palexas, Sussman, and Welsh 1992) B lymphocytes make up less than 20% of the cellular infiltrate [(Chan et al. 1985a), (Jakobiec et al. 1983) and may correlate with prolonged disease. Recent studies have shown that Non-granulomatous lymphocytes are predominantly CD3-positive and express more IFN-γ than cells within granulomas, consistent with Th1 cells. In contrast, granulomas and Dalen Fuchs' contain mainly CD68+, CD163+/- and express more IL-17, IL-18, IL-23, CCL19, and CXCL11 than non-granulomatous cells. Macrophages are the predominant inflammatory cells within granulomas and Dalen Fuchs' nodules. (Furusato et al. 2011)

The monocyte/macrophage markers CD68 are expressed in scattered inflammatory mononuclear cells and within granulomas and Dalen-Fuchs nodules. (bu El-Asrar et al. 2007) Most of the inflammatory cells are HLA-DR (+). Immunoreactivity for gelatinase B, MCP-1, and SDF-1 are observed in cells within granulomas and in scattered epithelioid cells. Immunoreactivity for MCP-1 are also noted in retinal pigment epithelial cells (bu El-Asrar et al. 2007)

The favoured theory is one of autoimmunity generated by exposure of intraocular constituents, possibly the choroidal melanocyte, to an intact immune system. Simultaneous contamination by pathogens may enhance this response. (Gasch et al. 2000)

1.3.5 Immunopathology of Sarcoidosis

Histopathological examination shows non-caseating granulomata in the absence of foreign material or infection. In the posterior segment, these granuloma may be found in retina and optic nerve and typically do not involve the choroid. They are composed of monocytes, macrophages, and epithelioid cells, the latter of which can fuse to form multinucleate giant cells. (Usui et al. 2002) (Chan et al. 1985a) Some investigators additionally identify a surrounding rim of lymphocytes, monocytes, and fibroblasts. Epithelioid cells are found in the vitreous and retina, and a sarcoid nodule has been found within an excised preretinal fibrovascular membrane. (Ogawa et al. 2000)

Immunohistochemical study of a globe with active ocular sarcoidosis showed a predominance of CD4+ T lymphocytes in the uvea and retina with only rare CD8+ T cells. (Angi et al. 1990) HLA class II antigens are present diffusely in granuloma and resident ocular immune cells. (Angi et al. 1990)

(Chan et al. 1987) Macrophages, epithelioid cells, and T lymphocytes within the granulomata express IL-2 receptors and IFN-γ. (Chan et al. 1987)

Studies of pulmonary sarcoidosis provide considerable insight into the mechanism of disease. Numerous studies have demonstrated the activation of T cells in sarcoid tissues; analysis of bronchial fluid have shown T-cell antigen receptor (TCR) activation in alveolar T cells and a normal transcription of the *IL-2* gene. T cells within the sarcoid granuloma also demonstrate expression of IL2, IL6, and INF-γ messenger ribonucleic acid (mRNA), indicating activation. (du Bois et al. 1992) In addition, these activated T cells secrete potent Th1 cytokines such as IL2 and IFN-γ (Agostini and Semenzato 1998) (Katchar, Eklund, and Grunewald 2003).

In addition to the T cell, macrophages are also fundamental to the formation of the sarcoid granuloma. Not only do they process the antigen before presenting it to the T lymphocytes, they also produce a cascade of cytokines that are chemoattractants and thus indirectly assist in the recruitment of additional immunocompetent cells. (Chan, Sharma, and Rao 2010) TNF-α is largely secreted by macrophages and has been implicated in the pathogenesis of granulomas in sarcoidosis (Chan, Sharma, and Rao 2010) Chemokine regulation on activation normal T-cell expression and secretion (RANTES or CCL5), macrophage inflammatory protein 1β (MIP-1β or CCL4), monocyte chemoattractant protein 1 (MCP-1 or CCL2), and IFN-γ–inducible protein 10 (CXCL10) are expressed in sarcoid granulomas and enhance the migration of effector cells. (Co et al. 2004) (Iannuzzi and Fontana 2011)

Similar to observations in Behcet's disease, T lymphocytes that are reactive against heat-shock proteins have been associated with sarcoidosis.

(Wilsher, Hallowes, and Birchall 1995) It was therefore postulated that the heat-shock proteins from various infectious pathogens may induce disease through molecular mimicry. Because of the predilection for sarcoidosis to the lung, several studies have focused on the role of inhaled pathogens and have found evidence for the presence of typical and atypical mycobacteria. (Kon and du Bois 1997)

The apparent predilection for the lung has suggested a possible airborne pathogen or an abnormal response to inhaled antigen, (Milburn et al. 1997) whereas the observed HLA associations support the notion that the host response is relevant to the course of disease. Although bacterial, viral, and environmental antigens have all been studied, none have been proven to be the cause for sarcoidosis. Mycobacterial antigens are probably on the top of the list. Mycobacterial tuberculosis catalase-peroxidase protein was identified in approximately 50% of sarcoidosis tissues sampled in a 2005 study. (Song et al. 2005) Based on the current understanding, it appears that sarcoidosis may result not from a single agent, but rather from multiple inciting agents that are capable of generating this Th1-mediated response in a genetically susceptible individual. (Chan, Sharma, and Rao 2010)

1.3.6 Immunopathology of Behcet's disease

Ocular Behcet's is a non granulomatous vasculitis that primarily affects the retinal and anterior segment vasculature with little RPE or choroidal destruction. (Sakane, Suzuki, and Nagafuchi 1997) Other major observations in Behcet's disease are neutrophil hyperactivity, endothelial injury, (Pivetti-Pezzi et al. 1992) and autoantigenicity, together with enhanced T and B cell responses to heat-shock proteins. (Sakane, Suzuki, and Nagafuchi 1997) Systemic Behcet's

disease is a systemic perivasculitis in which early neutrophils infiltration, endothelial cell swelling, and fibrinoid necrosis are described. Significant neutrophil infiltration is seen in all early lesions including eyes. (Mendoza-Pinto et al. 2010) Unlike many of the PSU, the choroid does not appear to be the major target of disease, and inflammatory cells are found in the anterior chamber, corneal endothelium, iris, and ciliary body. Occlusion of iris vessels and iris atrophy is found. The retina shows oedema with focal areas of infarction and necrosis while retinal vessels have thickened, hyalinized walls infiltrated by lymphocytes. (George et al. 1997) (Green and Bon 1967) In the anterior chamber, evidence of clonal proliferation of T cells has been found. (Keino et al. 2000) There may be late loss of photoreceptors and retinal ganglion cells but the choroid is only rarely involved.

Immunopathologic analysis shows that the CD4+ T lymphocyte is the major infiltrating cell in blood vessel walls and in the immediate perivascular area.(Charteris et al. 1992a) (Charteris et al. 1992b) A proportion of these retinovascular lymphocytes are activated, expressing IL-2 receptors. CD8+ T lymphocytes are less common with a reported CD4+/CD8+ ratio of 1.5:1 or less. (Charteris et al. 1992b), (George et al. 1997) Aqueous fluid also contains a preponderance of T lymphocytes. (Jakobiec, Lefkowitch, and Knowles 1984) Macrophages have been found in virtually all studies, and polymorphonuclear cells have been in most. (Charteris et al. 1992a) Along with the altered T lymphocytic populations, there is a notable influx of granulocytes, particularly neutrophils, into active lesions. Neutrophil hyperactivity has been repeatedly noted, (Takeno et al. 1995) (Yamashita 1997)] unlike in other PSU entities. Elevated levels of granulocytes in Behcet's disease may be consistent with the uniquely high incidence of acute hypopyon in this disorder and partially explain

the effective use of colchicine, an antigranulocytic drug, used to treat ocular and non-ocular manifestations in some centres.

Expansion of circulating CD4+ T cell clones that recognize retinal antigens (Yamamoto et al. 1993) has been reported, as have anti-HLA-B lymphocyte responses which can cross-react with retinal S-antigen. (Kurhan-Yavuz et al. 2000) A recent study has determined that the number of circulating lymphocyte precursor cells that recognize retinal S-antigen can be predictive of susceptibility to ocular disease. (deSmet and Dayan 2000)

Analysis of peripheral blood has identified various abnormalities of cytokine profiles, including the spontaneous production of IFN-γ by circulating lymphocytes. (Ohno et al. 1982a) Several studies have reported increased cytokine levels in serum and body fluids from patients with BD. Elevated levels of IL-1β (Pay et al. 2006) IL-2 (Akdeniz et al. 2004), IFN-γ, (Hamzaoui et al. 2003) IL-6 (Akdeniz et al. 2004) IL-8 (Borhani et al. 2009), IL-12 (Turan et al. 1997), IL-15 (Hamzaoui et al. 2006) IL-18 (Musabak et al. 2006) (Oztas et al. 2005) TNF-α (Akdeniz et al. 2004) (Kotter et al. 2005) (Oztas et al. 2005), and IFN-α (Kotter et al. 2005) suggest a hyper activated inflammatory response in patients with BD. Similarly, the serum IL-8 levels seem to correlate well with disease activity (Gur-Toy et al. 2005). Individual CD4+ lymphocytes can produce IL-2 and IFN-γ and may be a marker of disease activity. These Th1 cytokines are reduced in patients treated successfully; whereas in patients with unresponsive disease, they remained high.

Aqueous humour analysis have shown that a Th1 polarization and the presence of natural killer (NK) or CD8+ T cell-activating cytokines is a unique feature in ocular Behcet's. Aqueous TNF-α, IFN-γ and IL-15 levels are higher compared to other uveitis entities. (Ahn et al. 2006) CD8+ cell population are

higher in the aqueous humour of Behcet's patients with increase expression of CXCR3 and Intraocular CXCL8 and CXCL10 levels are higher in Behcet's than in non-Behcet's patients with uveitis (Kim, Chung, and Yu 2011)

Antibodies against vascular endothelium and several ocular components such as retina and cornea have been detected. [(Aydintug et al. 1993), (Kasp et al. 1989), (Chan et al. 1985b) Notably, in experimental systems, antibodies raised against bacterial or human heat-shock proteins are capable of cross-reacting with retinal antigens. (Tanaka et al. 1996) It therefore follows that exposure to exogenous or endogenous heat-shock proteins may stimulate a T lymphocyte response directed against both the target and retinal antigens. (Tanaka et al. 1996) This form of molecular mimicry could contribute directly to autoimmune disease.

The causative antigen in BD is unclear, and microbial, viral, and auto-antigens have been suggested as candidates. Several microbial antigens have been shown to stimulate T cells in BD patients, e.g., staphylococcal antigens, streptococcal antigens (Lehner 1997), Escherichia coli-derived peptides and Chlamydia pneumoniae (Ayaslioglu et al. 2004). Yanagihori *et al*. recently provided evidence for an anti-bacterial (streptococcal) host response toward Th1-immunity mediated by IL-12 (Yanagihori et al. 2006). HSP of various microbes could be involved in the pathogenesis of BD. A cross-reactive response to human HSP60 was suggested as causative in BD (Direskeneli and Saruhan-Direskeneli 2003) Herpes simplex virus had also been implicated in the pathogenesis of Behcet's disease due to similarity of the oral and genital ulcers, (Hamza and Slim 1991) (Hamzaoui and Ben Ammar 1992). Interestingly, the only studied animal model of Behcet's is that induced by inoculation with herpes simplex virus in which animals show several symptoms similar to

BD.(Sohn 1997) In summary, possibly triggered by a heat-shock protein, phosophoantigen, or superantigen, an abnormal T cell repertoire, may induce disease in susceptible individuals.

1.3.7 Immunopathology of VKH

Histopathologically, VKH disease shares many similarities with sympathetic ophthalmia and includes extensive granulomatous infiltration of the choroid and anterior uvea. (Lubin, Ni, and Albert 1982) (Rao 1997) The predominant cells are the T lymphocytes, (Matsuda 1970) (Sakamoto, Murata, and Inomata 1991) which are accompanied by collections of melanin-containing epithelioid cells and occasional multinucleate giant cells. There is little apparent necrosis, and the RPE and retina are largely intact with only focal disruption of morphology at sites of Dalen–Fuchs nodules. (Rao 1997)

Various investigators have proposed an underlying T-cell-mediated autoimmune process directed against melanocytes. It has also been postulated that such an autoimmune response might be triggered by an infectious agent in a genetically susceptible individual. (Sugita et al. 2006)

As VKH syndrome occurs most commonly in pigmented individuals, it has been hypothesized that VKH represents a T-cell-mediated autoimmune disorder against melanocytes of all organ systems. (Sheu 2005)

It is unclear what triggers the immune system of an individual with the genetic background to induce an autoimmune response against the target cells and antigens. Viruses, such as the Epstein-Barr virus, have been hypothesized as a possible triggering factor. (Bassili et al. 1996) The manifestations in the prodromal stage of VKH syndrome, such as meningismus and tinnitus, are perhaps consistent with this opinion. Epstein-Barr (EB) virus DNA has been

isolated from the vitreous of VKH patients. (Bassili et al. 1996) Sugita *et al*. (Sugita et al. 2007) studied the cross-reaction between tyrosinase peptides and cytomegalovirus (CMV) antigen by T cells in VKH patients. They found that CMV infection could stimulate the production of T cells that cross-react with tyrosinase by a mechanism of molecular mimicry. They suggested that these events may be responsible for the onset of VKH syndrome. During

the past few years, several cases of VKH-like disease have been described in patients treated with IFN-α for chronic viral hepatitis C. (Touitou et al. 2005) It has been supposed that molecular mimicry between viral antigens and melanocyte-related antigens could lead to the development of auto reactive antibodies causing VKH like manifestations.

Previous studies have shown that peripheral blood mononuclear cells (PBMC) from patients with VKH syndrome displayed a Th1 cytokine profile, such as IL-2, IFN-γ and IL-6, (Imai et al. 2001) (Norose and Yano 1996)

Recent studies have shown that IL-23 plays a pivotal role in the development and maintenance of autoimmune inflammation by inducing the differentiation of IL-17-producing CD4+ T cells. Increased expression of IL-23 p19 mRNA in PBMCs, higher IL-23 protein in the serum and supernatants of PBMCs, and an increased production of IL-17 by polyclonally stimulated PBMCs and CD4+ T cells in patients with active uveitis have been noted. (Chi et al. 2007) These results suggest that IL-23-stimulated production of IL-17 by CD4+ T cells may be responsible for the development of VKH syndrome.

Other researchers have confirmed these findings. Li *et al*. showed a significantly increased serum IL-21 level, as well as higher IL-21 mRNA expression by PBMCs, in patients having chronic or recurrent active VKH disease compared with patients having inactive VKH disease and with controls.

In vitro experiments showed that recombinant IL-21 significantly increased IL-17 production by PBMCs and by CD4 (+) T cells from patients and from controls. They concluded that IL-21 may be involved in the pathogenesis of chronic or recurrent VKH disease, possibly by promoting IL-17 secretion (Li et al. 2010)

Aqueous humour samples from patients with VKH show significantly higher levels of IL-15, IL-17, IFN-γ and TNF-α compared to healthy controls(El-Asrar et al. 2011)

In short various entities in PSU share an underlying immune aetiology; however, they can be clinically and immunopathologically distinguished. Although the initiating stimuli are not known, it is believed that an exogenous agent, such as a bacterium or a virus or an endogenous molecule may induce disease. Sympathetic ophthalmia, Vogt–Koyanagi–Harada disease and sarcoidosis are the three granulomatous disorders characterized by diffuse infiltration of CD4+ T lymphocytes and localized macrophagic aggregates in the choroid. Behcet's uveitis besides having typical findings of PSU, have abundant neutrophils and increased IL-8 cytokine production. However in all of them Th1 and at least in VKH and sympathetic ophthalmia Th17 cells in conjunction with human leukocyte antigens are likely to be involved.

1.4 Genetics of uveitis

1.4.1 Overview

Although we know that uveitis is an immune mediated disease but exactly what triggers uveitis and why some people are more susceptible than other is not known clearly. The association with HLA serotypes clearly points towards genetic predisposition.

Identifying genes that influence disease susceptibility can be done by studying a large number of unrelated, affected individuals for a specific association, or by studying a group of related individuals for an area of linkage.

The PSU are complex genetic disorders, meaning that multiple genes contribute to the susceptibility. An important conceptual consideration is that disease manifests in individuals with a genetic predisposition coupled with an environmental trigger. This is well illustrated by the example of reactive arthritis, where an inflammatory synovitis occurs approximately 1 to 4 weeks following an intestinal infection. However, this only occurs in a minority of individuals, as illustrated by documented outbreaks of food-borne illness. Furthermore, an individual's genetic risk will likely be due to specific alleles of different genes, and one can envision the interplay of multiple genes having various consequences on disease predisposition, severity, or outcome. Within the scope of this interplay, it is feasible that some genes will have a greater influence on inflammation in the uvea compared to others that may affect skin or joint inflammation. It is precisely this notion that fuels the current interest in searching for uveitis-specific genetic factors.

1.4.2 Major Histocompatibility complex

Associations between HLA serotypes with uveitis were among the first such HLA associations with human disease described. In the more than three decades since the association of the HLA-B27 serotype and acute anterior uveitis was first made, both class I and class II HLA genes have been implicated in conferring risk for uveitis . (Table 1-4)

Table 1-4 Human leukocyte antigen (HLA) associations with uveitis

Disease/syndrome	HLA	Relative risk *
Acute anterior uveitis	B27	26
Behcet's disease	B51, B52	5-10
Birdshot chorioretinopathy	A29	50-220
Idiopathic intermediate uveitis/pars planitis	DR15	3-7
Tubulointerstitial nephritis and uveitis	DRB1*0102, DQA*01	167
Vogt-Koyanagi-Harada syndrome	DR1, DR4 (DRB1*0405 in Asians)	4.2-17.4
Sympathetic Ophthalmia	DRB1*04	5.6

* see text for references

The HLA associations with human disease are strongest in uveitis among all diseases. In birdshot chorioretinopathy HLA-A29 confers a relative risk of about 50–220 (Shah et al. 2005) and *HLA-DRB1*0102*, in tubulointerstitial nephritis and uveitis [TINU] syndrome increases the risk by 167 times. (Levinson et al. 2003)

Various observations have been made regarding the HLA associations of intermediate uveitis. (rocker-Mettinger et al. 1992), (Davis, Mittal, and Nussenblatt 1992), (Martin et al. 1995), (Tang et al. 1997) HLA associations include HLA-DR, B8, and B51, the most significant relation being that with HLA-DR, which occurs in 67–72% of patients. (Davis, Mittal, and Nussenblatt 1992), (Malinowski et al. 1993) Among HLA-DR, the most striking association is with

DR15, which is also associated with multiple sclerosis. (Malinowski et al. 1993) (Tang et al. 1997) Association between pars planitis and HLA-DR2 has also been reported in 3 separate studies. (Malinowski et al. 1993) (Oruc et al. 2001) (Raja et al. 1999) and this association is actually due to HLA-DR15, 1 of the 2 sub alleles of HLA-DR2. (Oruc et al. 2001) (Raja et al. 1999) However, the HLA-DR15 association could not be confirmed in a Scottish population (Greiner et al. 2003) or Mexican Mestizo patients. (Alaez et al. 1999) The latter study found the *DRB1*0802/DQA1*0401/DQB1*0402* haplotype to be significantly increased in patients with classic pars planitis.

Other forms of PSU with known HLA associations include VKH, BD, sympathetic ophthalmia, presumed ocular histoplasmosis, and sarcoidosis. (Kilmartin et al. 2001) (Davey and Rosenbaum 2000) (Yabuki, Inoko, and Ohno 2000) (Levinson et al. 2003)

HLA associations in the white dot syndromes have been variably reported (Desarnaulds et al. 1996) and are largely anecdotal. HLA-B51 was found with increased frequency in patients with MEWDS, (Desarnaulds et al. 1996) (Borruat et al. 1998) and HLA- DRw2 and B7 with POHS. (Meredith, Smith, and Duquesnoy 1980) There is increased prevalence of HLA-B7 and HLA-DR2 antigens reported in patients with AMPPE. (Wolf et al. 1990) Multifocal choroiditis with panuveitis is associated with HLA-B7 but not DR2. (Spaide et al. 1990)

There is high correlation between birdshot retinochoroidopathy and HLA-A29, (Baarsma et al. 1986), (Nussenblatt et al. 1982) 271] which is found in less than 7% of the normal population, but in 80–98% of patients with BR. Its presence confers a relative risk of disease ranging from 50:1 to 224:1. (Nussenblatt et al. 1982) In particular, the *HLA-A29.2* subtype is exclusively

associated with disease, whereas the similar *HLA-A29.1*, which differs only at a single amino acid, is not associated. (de Waal et al. 1992)

BD is associated with the *HLA-B*51* and *HLA-B*52* alleles. (Al-Mutawa and Hegab 2004) (Zierhut et al. 2003) Stronger associations are noted between HLA-B51 and Behcet's disease in Japanese, Italian, and Greek pedigrees. (Balboni et al. 1992), (Ohno et al. 1982b) and (Yabuki et al. 1999a) (Yabuki et al. 1999b) *HLA-B5101*, in particular, and *HLA-B5102*, to a lesser extent, are associated with ocular Behcet's, as is the extended haplotype *B51-DR5-DQW3*. (Balboni et al. 1992) and (Mizuki and Ohno 1996)

There are strong HLA associations with VKH disease including HLA-DR4 and HLA-DRw53. (Ohno 1981) (Ohta 1996), (Sakamoto, Murata, and Inomata 1991) (Zhao, Jiang, and Abrahams 1991) HLA-DRB1 has also been found in Korean and Mexican populations. (Alaez et al. 1999) (Ohta 1996) and (Kim et al. 2000b) Some of these are similar to the HLA associations of sympathetic ophthalmia. (Davis et al. 1990) (Ohta 1996) Familial cases of VKH have been described as well. (Davis et al. 1990) (Ohta 1996)

Sympathetic ophthalmia occurs more commonly in association with HLA-DR4, DRw53, and Bw54 antigens. (Chan et al. 1986) (Davis et al. 1990) (Shindo et al. 1997) Kilmartin *et al* noted that *HLA-DRB1*0404* allele could increase the risk of development of sympathetic ophthalmia by 5.6% and *HLA-DRB1*0404-DQA1*0301* haplotype by up to 11% (Kilmartin et al. 2001)

The HLA associations in sarcoidosis are not clear cut. Association with HLA-DR has been reported in the Scandinavian and African-American populations, but in Caucasian Americans, HLA-B8 was associated with spontaneous resolution. (Berlin et al. 1997), (Maliarik et al. 1998), (Smith et al. 1981) Yet others report that the incidence, disease expression, and prognosis may be

epidemiologically associated with HLA-B1, B8, B13, and DR3, DR14, DR15 and DR17. (Bresnitz and Strom 1983), (Ishihara and Ohno 1997), (Rybicki et al. 1997)

HLA and genotype phenotype associations

Researchers have also asked whether HLA associations correlate with ocular disease phenotype. This is not the case, at least for most entities. Class I associations confer risk for anterior uveitis (HLA-B27–associated acute anterior uveitis), chronic posterior uveitis (HLA-A29 and birdshot chorioretinopathy), and posterior or anterior, acute or chronic uveitis as in Behcet's disease. Class II associations similarly can be associated with panuveitis (*HLA-DRB1*01* and *04* with VKH disease), intermediate uveitis (HLA-DR15 and pars planitis), or anterior uveitis (HLA-DR and DQ and TINU syndrome, *HLA-DPB1*0202* in juvenile rheumatoid arthritis). There is evidence, on the other hand, that the disease phenotype may differ between HLA-B27–positive and HLA-B27–negative patients with acute anterior uveitis, with worse ocular manifestations and greater prevalence of systemic disease in HLA-B27–positive patients (Rothova et al. 1987).

On the other hand, sympathetic ophthalmia is clinically and pathologically very similar to VKH disease, although the former is believed to be due to antigens released from immunologically privileged sites after surgery or injury and the latter has yet no established inciting event. Both are associated with *HLA-DR1*0405* in Asians and other HLA-DR4 subtypes in Caucasians ((Levinson et al. 2004b), (Kilmartin et al. 2001), (Shindo et al. 1997)), implying that diseases with similar phenotypes but differing precipitating events can have the same HLA genotypes that confer risks for developing disease.

HLA associations in different populations and HLA subtypes

Different populations can have different HLA associations with the same forms of uveitis depending on the prevalence of the relevant genes in the population. In Asian, the *HLA-DRB1*0405* allele is very strongly associated with VKH disease ((Shindo et al. 1994b) (Shindo et al. 1994a), but this is not true for Mestizo patients. ((Levinson et al. 2004b) (Alaez et al. 1999) Mestizo patients in fact have quite weak associations with HLA-DR1 and DR4.

The *HLA-B*5101* subtype was found in 56 of 57 Japanese patients with Behcet's disease ((Mizuki et al. 2001a) and 33 of 36 Iranian patients ((Mizuki et al. 2001b), but 18 Japanese control subjects who were HLA-B*51 positive had the *B*0501* allele, and in the Iranian population, no particular subtype was believed to predominate compared with controls. In Greek patients with Behcet's disease, *HLA-B*5101* was found, but so was *HLA-B*5108* (Mizuki et al. 2002).

Similarly, while some reports suggested that *HLA-29.1* was less common than *HLA-A29.2* in patients with birdshot chorioretinopathy, when examining patients genotype with DNA-based techniques, neither *HLA-A*2901* nor *A*2902* predominated (Levinson et al. 2004a).

1.4.3 Non-classical HLA genes, class III MHC genes, and non-MHC genes

It is not clear how HLA genes or their products play a role in the pathogenesis of ocular inflammatory disease. There is some evidence for a direct role of HLA molecules in the pathogenesis of disease, but indeed, such associations could be due to linkage disequilibrium with other genes in the MHC. Animals transgenic for HLA-B27 do not get inflammatory disease until they are taken from germ-free facilities, and it is believed that gut colonization with bacteria plays a role, a favoured theory being through "molecular mimicry". This is

consistent with the evidence that mucosal bacteria may play a role in human diseases, albeit often with subclinical mucosal inflammation. Nonetheless, just as with systemic HLA-B27 disease, the role of antigen presentation and molecular mimicry remains unproven. A mouse transgenic for *HLA-A*29* did develop spontaneous uveitis (Szpak et al. 2001) that did have features similar birdshot chorioretinopathy, which is very rare in animal models. Even in HLA-B27 transgenic animals that develop spondylosis, skin lesions, or arthritis, uveitis is uncommon, and when present, often mild. It did not appear that surface expression of the HLA-A29 molecule was necessary in the *HLA-A*29* transgenic animal for disease to develop, implying that antigen presentation by the HLA-A29 molecule was not a critical step in disease pathogenesis. Although the *HLA-A*29* transgenic mice should not have had any other human genomic material, it does remain possible that the *HLA-A29* gene or its product interact with other genes on the MHC (or are in linkage equilibrium with them), resulting in the strong association with disease.

Clearly, specific HLA genotypes are not necessary or sufficient for any form of uveitis. Investigators have searched for additional genes that may confer risk

The immunogenetics of MHC class I chain-related gene A and gene *B (MICA and MICB)* in Behcet's disease has also been explored. Recent refinements in genetic mapping suggest that some cases of Behcet's disease in Japan and Greece are associated with the *MICA* gene, located near, but not within, B51. (Kimura et al. 1998), (Ota et al. 1999) While evidence of both positive and negative associations have been described, these again may have been due to linkage disequilibrium with *HLA-B51* (Hughes et al. 2005). In addition, many of these studies have involved a very small number of patients. While a consistent picture has not emerged, the tantalizing suggestion that there may be

subgroups with particular disease phenotypes with specific *MIC* alleles remains an interesting observation, and several groups continue to examine this issue. (Levinson 2007)

1.4.4 Cytokine Gene Polymorphisms

Cytokines and cytokine receptors are major mediators of the immune response. Not surprisingly, polymorphisms in genes for cytokines are increasingly being associated with immune-mediated disease.

1.4.4.1 Tumour Necrosis Factor Alpha

The *TNF-α* gene resides in the MHC class III gene cluster, which is adjacent to *HLA-B*. Numerous studies have documented the major role that TNF plays during inflammation. Patients with inflammatory disease often have increased levels of TNF in their serum. Patients with uveitis have demonstrated higher concentrations of TNF in aqueous humour, (Santos et al. 2001b) and this difference is even more pronounced in HLA-B27–positive patients with uveitis compared to HLA-B27–negative patients. (Perez-Guijo et al. 2004) Furthermore, the newer TNF inhibitors have been efficacious in several inflammatory diseases, including Behcet's disease, psoriatic arthritis, and Crohn's disease. (Kalden 2002) (Tutuncu, Morgan, Jr., and Kavanaugh 2002). Anti-TNF treatment is now also available for uveitis associated with different immune-mediated conditions. (Neri et al. 2010) (Pleyer et al. 2011) (Neri et al. 2011)

From a genetic perspective, polymorphisms in the promoter region of *TNF-α* have been shown to alter expression of *TNF-α*. (Abraham and Kroeger 1999) (Kroeger, Carville, and Abraham 1997) Hence, there is much interest in characterizing the *TNF* genotype of patients with inflammatory disease.

However, the problem in doing so is the fact that *TNF-α* is coded very close to the *HLA* genes, making it difficult to determine whether an association with disease is independent of linkage disequilibrium with *HLA* genes. A recent study by Ahmad *et al* was designed to detect *TNF* promoter polymorphisms independent of *HLA-B* by conducting linkage disequilibrium analysis across 6 genes in a cohort of patients with Behcet's disease. (Ahmad et al. 2003) The authors reported a *TNF* promoter allele *(-1031C)* that was associated with Behcet's independently of HLA-B alleles. Other studies have also highlighted the role of *TNF-α* polymorphisms in Behcet's disease. (Kamoun et al. 2007)

A very interesting recent article reported an increase in a single-nucleotide polymorphism (SNP) *(TNF-857T)* in acute anterior uveitis. (Kuo et al. 2005) There was also a trend toward increased complications in HLAB27–positive subjects who were carriers of the *TNFRSF1A 201T or TNFRSF1A -1135T* alleles. (Kuo et al. 2005)

1.4.4.2 The Interferon Genes

The interferons are a group of cytokines that play a pivotal role in antiviral responses and polarization of T-cells. Type I interferons include interferon alpha (IFN-α) and interferon beta (IFN-β) and bind the same cell surface receptor. Type I interferons are produced in response to viral infection in a wide array of cell types. In contrast, interferon gamma (IFN-γ), the type II interferon, is primarily produced in activated T-cells and NK cells.

The type I *IFN* genes are clustered on the short arm of chromosome 9. Linkage analysis on a cohort of patients with multiple sclerosis suggested a role for *IFN-α* gene in the genetic predisposition to this disease. (Epplen et al. 1997) Multiple sclerosis is not the only disease with a uveitis component that has

been linked to *IFN-α* gene. Recently, a cohort of Japanese patients with sarcoidosis was studied by comparing various *IFN* SNPs in a case-control analysis. (Akahoshi et al. 2004) A variant allele (made up of 2 SNPs in linkage disequilibrium, *IFNA10, 60A* and *IFNA17, 551G* was found to be significantly associated with sarcoidosis compared to a healthy control population.

IFN-γ production is the hallmark of a Th1 response and polymorphisms in its gene that result in higher production for a given stimulus might be expected to produce more severe inflammation and tissue destruction. In one study of patients with intermediate uveitis, (Stanford et al. 2005) the *IFN-γ A874T* polymorphism, which reflects a constitutively higher production of IFN, was significantly associated with disease and, although not statistically significant, there was a trend towards an association with disease outcome.

1.4.4.3 Interleukin-10

IL-10 is an anti-inflammatory cytokine that was shown to suppress IFN-γ production, inhibit the Th1 response and promote the Th2 response. (de Vries 1995). In a recent study in patients with sympathetic ophthalmitis (Atan et al. 2005) significant associations were found between the *IL-10 -1082* SNP and disease recurrence from previously stable disease and the level of steroids required for maintenance therapy. In addition, the *GCC IL-10* promoter haplotype (*IL-10 -1082G, -819C, -592C*) was found to be protective against disease recurrence. The authors concluded that polymorphisms were markers for the severity of disease in sympathetic ophthalmitis. In another study on intermediate uveitis, analysis of disease outcome showed an association between *IL-10–1082 AA* homozygosity and bad outcome. (Stanford et al. 2005)

1.4.5 Chemokine and Chemokine Receptor Genes

The hallmark of uveitis is the infiltration of leukocytes into the eye, especially the uveal tissues. The specificity of this process is dependent on various chemokines and their receptors, many of which have been studied in animal models of uveitis. In addition to studies in animals, chemokines or chemokine receptors have been shown to be expressed in human uveal tissues (Silverman et al. 2003) and upregulated in peripheral blood or plasma of patients with uveitis compared to healthy controls. (Kaburaki et al. 2003), (Klitgaard, Ogard, and Krogh 2004)

Research directed at the examination of polymorphisms in chemokine and chemokine receptor genes, which may alter chemokine expression or function, in patients with uveitis is emerging. Chen *et al* compared cohorts of patients with Behcet's disease, idiopathic retinal vasculitis, and healthy controls for the frequencies of selected polymorphisms in the promoter regions of 2 chemokine genes, *MCP-1* and *RANTES*. (Chen et al. 2004) They found evidence for gender-specific disease association with polymorphisms in both genes in the patients with Behcet's disease. In another study by the same group, a particular haplotype of the fractalkine (*CX3CL1*) receptor, *CX3CR1* (*I249/M280*), was found to be associated with uveoretinitis in a cohort of patients with retinal vasculitis. (Wallace et al. 2006) This particular *CX3CR1* mutant exhibits a lower binding affinity for fractalkine and is associated with an increased risk of acquired immune deficiency syndrome (AIDS) progression in human immunodeficiency virus (HIV)-positive individuals. (Faure et al. 2000)

In another study an odds ratio of 2.1 was found for the *MCP-2518G* in HLA-B27–positive patients with acute anterior uveitis compared with HLA-B27

positive control subjects (Wegscheider et al. 2005). Table 1-5 summarizes the various genetic associations noted in uveitic syndromes.

Table 1-5: Non–Human Leukocyte Antigen immunogenetic associations with uveitis

Disease/syndrome	Suspected genes/loci
Acute anterior uveitis	*MIC; D95137 (9p21p24), 1q23-1-q31, TNF-857T; TNFSRF1A-201T, TNFSRF 1A-1135T;CCL2/MCP-2518G*
Behcet's disease	*MIC, interleukin-1, tumour necrosis factor promoter region*
Birdshot	*Myelin oligodendrocyte glycoprotein*
Blau syndrome	*CARD/NOD*
Intermediate uveitis	*Cytokine gene polymorphisms*

1.4.6 Genome-Wide Scans

Family-based studies searching for regions of linkage across the genome have been conducted for several inflammatory or autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, autoimmune thyroiditis, and psoriasis. This approach has also proven informative for diseases that have a uveitis component, including inflammatory bowel disease, (Hampe et al. 1999) Blau syndrome, (Tromp et al. 1996) multiple sclerosis, (Ebers et al. 1996) sarcoidosis, (Schurmann et al. 2001) ankylosing spondylitis (Laval et al. 2001) and Behcet's disease. (Mizuki et al. 2010) For diseases in which uveitis occurs in only a small minority of affected individuals, such as inflammatory bowel disease or multiple sclerosis, the genetic contribution of each susceptibility locus to uveitis will be difficult to ascertain due to the small size of these patient populations. However, diseases such as ankylosing spondylitis,

sarcoidosis and BD which have a high frequency of uveitis, offer the ability to conduct genetic studies with relatively larger cohorts, even when stratifying the analysis for uveitis. Recently GWAS have been done on subjects with Behcet's disease. (Mizuki et al. 2010) (Remmers et al. 2010)

The first genome-wide scan to specifically focus on the uveitis phenotype predominant in ankylosing spondylitis (AS) patients has recently been performed. (Martin et al. 2005) This study analyzed 76 affected sibling pairs with uveitis and compared the results to an analysis of 245 affected sibling pairs with AS. In both groups, strong linkage to the *MHC* region on chromosome 6 was observed, undoubtedly due to the association of spondyloarthritis and anterior uveitis with HLA-B27. Two other genetic regions showed significant linkage to anterior uveitis, areas at chromosome 1q25-31 and chromosome 9p21-24. Of these, *1q25-31* overlaps with a locus previously identified for ankylosing spondylitis. (Laval et al. 2001), (Zhang et al. 2004) In contrast, the other region (*9p21-24*) did not reveal a significant result in the companion ankylosing spondylitis scan, (Zhang et al. 2004), (Martin et al. 2005) but did overlap with an area of weak linkage in previous studies. (Laval et al. 2001) Therefore, it is likely that this region contains a uveitis-specific candidate gene. The locus includes a number of genes with known roles in inflammation including the type I interferon genes. However, additional studies will be needed to fully delineate the role of this region in predisposition to uveitis.

The GWAS in Behcet's disease did not specifically looked into association with uveitis, however loci have been noted on *IL-10* and *IL-23R* genes. (Mizuki et al. 2010) (Remmers et al. 2010)

In short, immunogenetic studies on uveitis patients have revealed loci other than HLA gene that may play crucial role in inflammation. Immunogenetic

studies hold promise for revealing additional pathologic mechanisms, monitoring or predicting the response to treatment, and diagnosis of uveitis.

1.5 Genetics of complex traits

1.5.1 Overview

Complex diseases are caused by many, possibly interacting, genes and environmental factors. This is in contrast to Mendelian diseases, such as Huntington's disease and cystic fibrosis, which are the results of some specific and rare mutations. These mutations alter or destroy the function of a single gene. There are many disorders caused by single genes, but these are rare in the population, often occurring in less than one in five thousand individuals (Botstein and Risch 2003).

Diseases inherited as complex traits are often much more common in the population. Examples of complex diseases include asthma, heart disease, chronic inflammatory diseases, and diabetes. There is a lot of interest in understanding these diseases better and in particular determining the extent to which genetics play a role in predisposing individuals to disease.

Complex diseases may result from genetic variants that are relatively common in the general population and are involved in the normal human health and development (Lander et al. 2001). In addition, some of these variants may have major effects, but many of them have minor effects (Thornton-Wells, Moore, and Haines 2004). Factors that influence these traits are:

a) an incomplete penetrance, i.e. not all susceptible individuals are affected,
b) the interactive effects between genetic loci and environmental factors,

c) heterogeneity, so that many genes participate in the development of disease phenotypes (Khoury et al. 2004) (Thornton-Wells, Moore, and Haines 2004).

These genes usually interact in complex networks whose expression is tightly regulated and coordinated. This gene-gene interaction is called epistasis or modifier genes, which is defined as the interaction between two or more genes to control a single phenotype. Epistasis can be antagonistic or synergistic depending on whether variational effects overlap or reinforce each other (Sanjuan and Elena 2006). These factors also complicate the efforts to identify genetic regions involved.

The relative contribution of both genes and environment to a disease varies in different diseases. Many diseases cluster in families demonstrating that genetics may play a role in determining disease susceptibility, but it is difficult to determine the true genetic association in complex traits since families share environmental factors and exposed to similar antigens and have similar activities (Wandstrat and Wakeland 2001).

1.5.2 Approaches to Gene Mapping in Complex Traits

An important step towards understanding a genetic disease is to identify the gene, or genes, that play a role in the disease aetiology. The most commonly used approaches for mapping genes associated with disease are linkage analysis and case-control association studies. In a linkage analysis, large numbers of DNA markers, that are closely spaced, are typed in families with multiple affected relatives. Markers that segregate with affected relatives, more often than expected, are used to localize the disease locus. This approach has the advantage of being an unbiased and comprehensive and has been

successfully applied to diseases with simple Mendelian inheritance such as cystic fibrosis (Salvatore, Scudiero, and Castaldo 2002).

However, linkage analysis has been less successful with regards to complex genetic disorders, mainly because of a limited power to detect the effect of common alleles with modest effects on the disease (Risch and Merikangas 1996) (Hirschhorn and Daly 2005). Another disadvantage of linkage studies is the preferable need for more than one generation: DNA needs to be collected from grandparents, parents and children. Moreover, when the disease occurs late in life, parental information is often lacking.

Therefore, for analysing the complex human disorders; such as uveitis, candidate gene-case control association studies are commonly used (Vink and Boomsma 2002). These studies look for evidence for a significant association between an allele/haplotype of a candidate gene and disease/phenotype characteristics (Newton-Cheh and Hirschhorn 2005). In these studies, there is no need for information derived from grandparents or children. Therefore, this strategy is often the preferred route for late-onset diseases (Hirschhorn and Daly 2005).

Candidate gene-case control studies are relatively easy to perform in a short period, which is one of the reasons why they have increasingly become popular. Genes rarely act alone, therefore, interactions between different genetic variants in a pathway or across different pathways is important (candidate pathway approach) (Suh and Vijg 2005).

However, there are several important statistical issues in the use of case-control association studies. Population stratification is the biggest criticism of case-control association studies (Hirschhorn et al. 2002). The control group must genetically be representative of the population under the study. False

association may arise in a case-control study when allelic frequencies vary across subpopulations. In a review of literature, and excluding perhaps the most important complex disease locus of all, the MHC where replication is high, only six of 166 positive associations were replicated by subsequent studies (Hirschhorn et al. 2002).

By typing several dozens of random panels of genetic markers, it is possible to detect and correct the control stratification (Pritchard and Rosenberg 1999). Moreover, a positive finding in a case–control study always needs confirmation in another group of subjects, which are not related to the first group (Hirschhorn et al. 2002).

1.5.3 SNPs and the Pathogenetics of Complex Traits

Genetic markers scattered throughout the genome can be used as a means to understand the pathogenetics of complex diseases. These markers include, variable number tandem repeats, short sequence repeat polymorphisms and Single Nucleotide Polymorphisms. SNPs are the simplest type of genetic variants. SNPs may have no significant function, however, depending on their position they may alter protein's structure and function through a single nucleotide base substitution in a gene's coding region. In addition, they may increase or decrease gene expression by altering transcription factors binding site when occurring in the promoter region or by affecting mRNA stability when occurring in 3` UTR region.

SNPs are important tool for complex genetic disorders studies. The studies look for a significant statistical association between SNP alleles and a disease, in order to pinpoint candidate causative genes (Newton-Cheh and Hirschhorn 2005). The Complex Trait Consortium suggested that a susceptible gene should meet more than one of these seven criteria (Abiola et al. 2003):

1. Polymorphisms in either coding or regulatory regions have been found.
2. Its function has been linked to the quantitative trait being analyzed.
3. Its function has been tested *in vitro*.
4. Its function has been tested in transgenic animals.
5. Its function has been tested in knock-in animals.
6. Its function has been tested by mutational analysis.
7. A homologous quantitative traits loci for the same phenotype in another species has been found.

In comparison with different types of genetic variants, such as microsatellites, there are several advantages of using SNPs as a mean to study the pathogenetics of complex diseases (Collins et al. 1998) (Xiong and Jin 1999). SNPs are plentiful throughout the human genome and alleles at some of these polymorphisms may be functional (Hoogendoorn et al. 2004). In addition, groups of SNPs might exhibit patterns of linkage disequilibrium called haplotypes which could be used to enhance gene mapping.

Since the identification of functionally relevant polymorphic sites is the ultimate goal of any genetic study, the more polymorphic loci one can type on a set of individuals, the more likely to identify the causal allele. Large SNP collections can therefore provide the necessarily means from which the putative SNPs can be obtained. However studying SNPs without prior knowledge of their functions or studying non-functional SNPs adds some difficulties. If a significant association with a SNP is noted, it is difficult to assert whether this association is true or it is a result of linkage with some other unknown SNP that may be functional.

1.5.3.1 Linkage Disequilibrium

When a particular allele is found together with a second allele more often than expected by chance, the loci are in disequilibrium. This non-random association of alleles at different loci is called Linkage Disequilibrium (LD). Recent studies have proposed that the underlying structure of LD in the human genome can be described by using a relative simple framework in which the data is dissected into series of discrete haplotype blocks (Daly and Day 2001) (Wall and Pritchard 2003). Patterns of LD are well known for being unpredictable. For example, pairs of sites that are tens of kilo bases apart might be in complete LD, whereas nearby pairs of sites from the same region might be in weak LD (Ardlie, Kruglyak, and Seielstad 2002).

Many different methods have been proposed for measuring the strength of LD. The most commonly used measures are *D′* and r^2. (See chapter 2 for calculations) (Devlin and Risch 1995) Both measures range from zero (no disequilibrium) to one (complete disequilibrium), but their interpretation is slightly different. *D′* is equal to one if just two or three of the four possible two-locus haplotypes are observed in the sample, and it is <1 if all four possible haplotypes are present. Therefore, the statistical significant values of *D′* that are near 1.0 provide a useful indication of minimal historical recombination (Hudson and Kaplan 1985). However, intermediate values are more difficult to interpret. Therefore, they should not be used for comparisons of the strength of LD between studies or measuring the extent of LD (Ardlie, Kruglyak, and Seielstad 2002).

The measure r^2 is the correlation of alleles at the two sites. $r^2 = 1$ if, and only if, the markers have not been separated by recombination and have the same allele frequency. In this case, exactly two out of the four possible two-locus

haplotypes are observed in the sample. So, observations at one marker provide complete information about the other marker, making the two redundant. It is also the most relevant measure for associational mapping, because there is a simple inverse relationship between r^2 and the sample size required for the study (Wall and Pritchard 2003). For example, suppose that SNP1 is involved in disease susceptibility, and the other is a nearby marker (SNP2) in LD with the SNP1. To have the same power to detect the association between the disease and the marker locus, the sample size must be roughly increased by $1/r^2$ when compared with the sample size for detecting association with the susceptible locus itself (SNP1) (Pritchard and Przeworski 2001). This also makes intermediate values of r^2 are easy to be interpreted (Ardlie, Kruglyak, and Seielstad 2002).

LD patterns in human populations are influenced by many factors (Ardlie, Kruglyak, and Seielstad 2002).

1) ***Genetic Drift*** is "the random fluctuations in gene and haplotype frequencies from one generation to the next due to the random sampling of gametes that occurs during the production of a finite number of offspring" (Tishkoff and Verrelli 2003). The effect of genetic drift on variation is a function of effective population size. It is particularly severe in a population that has undergone a bottleneck event which can be defined as a severe reduction in population size or a founding event that happens when a small group splits off from a larger group and colonizes a new region (Pritchard 2001). In contrast, genetic drift has a smaller effect on growing populations, and preserves any LD that existed when the populations started to expand (Tishkoff and Verrelli 2003).

2) ***Gene Conversions*** are short exchanges between chromosomes that are not accompanied by crossing over during meiosis. The effect can break down the LD in a manner similar to recombination or recurrent mutation (Ardlie et al. 2001).

3) ***Admixture or Migration*** can change LD between populations. Population admixture may mask, change or reverse genetic effects of genes underlying complex diseases (Deng, Chen, and Recker 2001). This may lead to the inconsistent results from association studies in admix populations (Deng, Chen, and Recker 2001).

1.5.3.2 Haplotypes

A haplotype is simply a unique combination of alleles found at neighbouring loci on a single chromosome or haploid DNA molecule. The original application of the haplotype method was to HLA allele frequency calculations, where groups of epitopes are distributed in a non-random manner. This distribution is due to certain alleles of different HLA genes, which are found in a linkage more often than what would be expected by chance.

However, it is important to identify the exact sequence variance that contributes to disease risk (Hafler and De Jager 2005). For example, the association of the MHC region with Multiple Sclerosis has been known since early 1970s, but it still unknown whether the association with the *HLADQB1*0602, HLA-DRB1*1501* haplotype is mainly due to a risk allele in the *HLA-DQB1 or HLA-DRB1* gene (Hafler and De Jager 2005). Therefore, the extent of the haplotype association in the studied population must be investigated for further variation discovery (Crawford and Nickerson 2005).

Role of Haplotype in Association Studies

Most candidate gene-association studies rely on the one-SNP at-a-time approach. However, the fact that these SNPs are not independent of one another emphasizes the necessity of studying the variations in these genes as phased haplotypes, *i.e.* co-segregation of alleles in the same chromosome (Clark 2004).

Phased haplotyping plays an important role in the candidate gene-association studies for many reasons. Firstly, haplotypes directly correspond to the unit of biological function, the protein. The protein products of the candidate genes occur in polypeptide chains whose properties may depend on particular combinations of a group of amino acids. The properties of the protein may depend on interactions between pairs or combinations of amino-acid sites. If these interactions are important, then haplotypes are of direct biological relevance. Secondly, the genomic variation in a population is structured into haplotypes. Finally, haplotypes can have statistical advantages by reducing the dimension of statistical tests for association studies (Clark 2004).

Unfortunately, haplotypes are costly to measure directly (Crawford and Nickerson 2005). Therefore most investigators rely on computerised statistical methods to either infer the most likely pair of haplotypes per subject, or to account for all possible haplotypes that are consistent with the observed un-phased marker data (Clayton, Chapman, and Cooper 2004).

1.5.3.3 Assessing the Function of Genetic Variants

Knowledge of SNP's function is crucial to direct the appropriate design and interpretation of the candidate gene-association studies. Assessing the function of a SNP can be done by using experimental systems, such as the effect of

SNP on transcription factor binding affinity, mRNA expression and stability, and the effect on protein structure and expression (Knight 2003). However, variants in multifactorial diseases may often have a moderate effect rather than a crucial one on the gene's function, making it difficult to find such functional relevance. For example, the functional effect of a SNP in an experimental system may be minute, but it might become more important in a specific human tissue. Therefore, the functional effects of the SNP cannot be detected in experimental systems (Knight 2003).

DNA regulatory regions are widely dispersed in the genome. They may be located in the 5′ or 3′ of the gene, or in the introns of a neighbouring irrelevant gene (Arnone and Davidson 1997) (Mancini-DiNardo et al. 2003). Variations in the regulatory regions may affect Transcription Factor Binding Site (TFBS) thereby it may affect gene expression. Defects in binding of these factors have been implicated in human diseases such as cancer and inflammation (Shaulian and Karin 2001).

Assessing SNPs that affect transcriptional regulation is commonly done by protein-DNA interaction assays and plasmid reporter gene expression. (Fried 1989) (Hoogendoorn et al. 2004) Transcription factors show a high degree of specificity in their DNA-binding domains for the DNA sequence to which they will bind. This DNA sequence is typically 5–8 nucleotides in length and specific to a given family of transcription factors.

The ability to screen the transcription factor activity is, therefore, important in gene regulation studies. A number of computational algorithms have been developed to predict the impact of nucleotide variations on the gene expression (Nardone et al. 2004). For example, by using online transcription factor databases, such as the TRANSFAC database, (http://www.gene-

regulation.com/pub/databases/transfac/doc/toc.html) researchers can find transcription factors binding sites in a given sequence (Wingender et al. 2000). Transcription Element Search System (TESS) is a web tool uses TRANSFAC database to predict transcription factor binding sites in the given DNA sequences. http://www.cbil.upenn.edu/cgi-bin/tess/tess

1.6 Objectives of the study

Genetic studies in medicine are often pursued in order to develop diagnostic tests or to allow the identification of individuals at risk for disease. In fact, HLA testing can play an important role in the diagnosis of ocular inflammatory disease. However, the predictive positivity of genetic testing in such rare diseases that are likely to be polygenic (and are also likely to have environmental contributions to disease pathogenesis) is too low to be useful in any practical way for screening populations at risk.

Immunogenetic studies have been used to better understand the pathogenesis and nosology of ocular inflammatory disease, as well as the interactions of nature and nurture in ocular inflammation. There are large gaps in our understanding of the pathophysiology of ocular inflammation, in part because it is very difficult to obtain tissue to study. While we can often observe the results of intraocular inflammation by clinical examination and adjunctive clinical testing, the risks to the eye of obtaining intraocular tissue for research purposes are considered too great to pursue. Intraocular specimens are sometimes available for research purposes, but these are often from eyes that have received treatment, and may be obtained late in the course of the disease, limiting the ability to perform systematic studies. Animal models have been helpful, but they involve artificial experimental protocols such as sensitizing a susceptible animal with retinal antigens and adjuvant so may not be directly applicable to human disease. In addition, some aspects of the immune response differ between animals commonly used in the laboratory and humans. Most of the literature on the immunogenetics of uveitis consists of studies with relatively small numbers of subjects, looking for associations of disease with specific genes. More sophisticated genome scans and population and family

studies are difficult mostly because these diseases are so rare and involvement of multiple family members is very rare for most of these diseases.

The most feasible way to identify the genes involved in the pathogenesis of uveitis is the candidate gene approach.

Association-based studies, in which the frequency of alleles at polymorphic loci are compared between an affected and a control population, are since long considered to be more effective tools for studying complex traits because they have greater statistical power to detect genes with small effect (Risch and Merikangas 1996) (Long and Langley 1999). However, the small number of known SNPs and the lack of high-throughput genotyping methods have, in the past, severely constrained this approach with respect to its systematic application to the unravelling of the genetic component of human complex disease phenotypes. The identification of large numbers of SNPs in the human genome and the wealth of novel opportunities for genotyping at low cost has now brought association analysis into the realm of the feasible. By comparing the frequency of SNPs in unrelated cases and controls, association studies can detect genes that are in linkage disequilibrium with one of such SNPs. When they occur in a gene, SNPs could actually be the causative genetic variant that changes the protein function or its expression, directly contributing to variation in phenotype. But rather than applying this principle to whole genome, much more power is obtained by using candidate genes. Candidate gene approaches focus on genes that are selected because of a prior hypothesis about their role in the phenotype, rather than rely on markers that are evenly spaced throughout the genome without regard of their function or context in a specific gene (Tabor, Risch, and Myers 2002). Such studies, which may now involve hundreds to thousands of genes, participating in the same and different

pathways in interaction with environmental factors, would be the first step in unravelling the genetic determinants of complex disease. At present, direct association studies of functional variants are limited by incomplete knowledge about functional variants. However, progress in genomics information technology is rapid and may soon provide a strong basis for such an approach. Subsequent functional genomics analyses of the discovered gene variants, in relation to the observed phenotypic endpoints, would then ultimately reveal the causal factors of complex diseases.

A major challenge in undertaking candidate gene–disease association studies is to choose Target SNPs that are most likely to affect the phenotype and that ultimately contribute to disease development. Variants in biologically plausible candidate genes are usually selected for study on the basis of both variant allele frequency and the functional effect of the variant on relevant traits. Although there is often sufficient information to assess the allele frequency of a candidate variant, understanding the functional significance of genetic variants is usually more difficult. Knowledge of gene and SNP function is crucial to direct the appropriate design and interpretation of candidate gene association studies.

A logical starting point for candidate gene study is to restrict analysis to known putative functional SNPs in the candidate pathway. Since PSU is predominantly Th1 mediated disease the focus of this study has been on the genetic role of Th1 mediating cytokine and chemokine genes in the predisposition to PSU. It must be pointed out here that when this research was started the role of Th17 cells in pathogenesis of PSU was not known.

Given the position of these cytokine and chemokine molecules in the pathway of inflammation, even minor variation in these genes could be critical for the

inflammatory response. This is relevant to the current clinical trails of uveitis since treatments in these trials are targeted at these inflammation stages of the disease. (Accorinti et al. 2007) (El-Shabrawi and Hermann 2002) (Joseph et al. 2003) (Nussenblatt 2005)

In the present work, I have studied the contribution of the cytokine and chemokine genes in susceptibility and severity of PSU using genetically homogeneous Caucasian populations. I employed the candidate pathway approach, taking advantage of its high power of detection of weak associations. I mainly studied functional SNPs so that the relation between the phenotype and genotype could be correlated more logically. Gene haplotypes were re-constructed when more than one SNP on a gene were studied. To increase the likelihood that a candidate gene would harbour the variations relevant to traits, I studied a wide variety of information, including previous genetic studies, animal models, and knowledge of biologic pathways.

The aims of the study were

1) To identify genes that may be involved in the pathogenesis of PSU
2) To identify SNPs that may predispose subjects to PSU
3) To identify SNPs that can predict the phenotype and act as indicator of visual outcome and response to treatment

Chapter Two

2 Materials and Methods

2.1 Populations and Study Design

This is a retrospective study of a cohort of patients suffering from non-infectious posterior segment uveitis. 205 Caucasian patients who attended the uveitis clinic at Moorfield's Eye Hospital were recruited for this study. (During the last part of research, 24 samples were ruined; hence 176 patient samples were available for cytokine genes analysis [Chapter5]).

There were two main cohorts of subjects who served as control populations. (N=283)

1) 169 healthy subjects who were admitted to Whipps Cross Hospital (WXH) for cataract surgery and
2) 114 healthy subjects who were seen at King's College Hospital (KCH) eye department for age related macular degeneration.
3) However, 142 Caucasian healthy subjects whose DNA were stored and used in other studies, (Spagnolo et al. 2005) were also available for *CCR2* & *CCR5* SNPs analysis in idiopathic PSU and OB. (Chapter 4)

(Again, during the last part of research, 3 samples from WXH and 9 samples from KCH were ruined; and I had total of 271 control samples for cytokine gene analysis [Chapter 5]).

Besides this there were 29 Caucasian patients with Behcet's disease with no ocular involvement, 30 non Caucasian patients with OB, and 56 non Caucasian controls used for *CCR2* gene analysis in OB. (Chapter 4)

2.1.1 Patients:

After approval from the Ethics Committee, patients with PSU were recruited from the Uveitis Clinic in Moorfield's Eye Hospital. PSU was defined as any non-infectious inflammation of choroid, retina, retinal vessels, vitreous or cilliary

body either in isolation or as a part of pan uveitis. Since non-infectious posterior segment uveitis includes a variety of conditions, inclusion in the study was restricted to idiopathic PSU and uveitis secondary to sarcoidosis and Behcet's. By definition this excluded patients suffering from the VKH syndrome, sympathetic ophthalmia, and Birdshort choroidopathy, uveitis related to multiple sclerosis or any other auto-immune condition. The reason for including sarcoidosis and Behcet's uveitis was that these two are the most common identifiable causes of PSU.

Thus depending upon cause and site of inflammation the patients were divided into four main groups.

1) Idiopathic intermediate uveitis,

2) Idiopathic posterior uveitis

3) Sarcoid related uveitis, and

4) Behcet's related uveitis.

All the Caucasian patients were UK resident patients whose ancestry was traceable back to the UK.

The non Caucasian patients with non ocular Behcet's disease were from Middle East.

Clinical Details:

Informed verbal and written consent was obtained from each patient and they then underwent complete ophthalmic examination. This included visual acuity, intra-ocular pressures, anterior segment slit-lamp examination, and dilated fundus exam. Where necessary fundus flourescein angiography, optical coherent tomography, B scan, visual field tests and electro diagnostic tests were performed.

Clinical details were obtained for each patient's notes and included: Age of onset, follow up period, laterality of disease, fluctuation of visual acuity throughout the progress of disease, minimum visual acuity during an episode of active inflammation, visual acuity in each eye at the end of follow up and final visual acuity with both eyes. The pattern of disease e.g., single event, recurrent with remissions or chronic, and if recurrent, the number of attacks during the follow up period were also noted. Also noted was the presence of complications like, cystoid macular oedema, glaucoma, choroidal neovascularization, cataract retinal detachment and phthisis bulbi.

The treatment given to the patient was recorded giving particular emphasis to use of systemic steroids, duration of steroids (whether or not on long term steroids of 10mg/day or more), number of second line immunosuppressive drugs used, and number of times intra-vitreal or periocular steroids were given.

Although most of the information was gathered from the patient notes, on their day of recruitment to study, The SUN Working Group Anatomic Classification of Uveitis (Jabs, Nussenblatt, and Rosenbaum 2005) was used to determine the course and activity of the disease. The National Eye Institute system for grading vitreous haze was adopted (Nussenblatt RB 1985) Since this was a retrospective study, particular attention was paid on reporting the visual acuity particularly final visual outcome.

2.1.2 Definition of disease characteristics

2.1.2.1 Sarcoidosis:

In all patients sarcoidosis was diagnosed histologically and according to the criteria defined in the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other

Granulomatous Disorders (WASOG) statement on Sarcoidosis (Costabel and Hunninghake 1999)

The diagnosis of systemic disease and biopsy were performed at Department of Respiratory Medicine, Royal Brompton Hospital.

Recently an updated criterion for the diagnosis of sarcoidosis has been recommended (Herbort, Rao, and Mochizuki 2009). (Table- 2-1) The criterion is shown as follows. Since all of my cases were biopsy proven sarcoidosis, the new criterion did not affect the suggested diagnosis of my cohort

Table- 2-1 Diagnostic criterion for Ocular Sarcoidosis

Table A Clinical signs suggestive of ocular sarcoidosis
1. Mutton-fat keratic precipitates and/or iris nodules at papillary margin or on stroma 2. Trabecular meshwork nodules and/or tent-shaped peripheral anterior synechiae 3. Snowballs/strings of pearls vitreous opacities 4. Multifocal peripheral chorioretinal lesions (active and atrophic) 5. Nodular and/or segmental periphlebitis (with or without candle-wax exudate) and/or macroaneurysm 6. Optic disc nodules/granuloma and/or solitary choroidal nodule 7. Bilateral inflammation (evident on clinical examination or on investigational imaging

Table B Laboratory investigations in suspected ocular sarcoidosis
1. Negative tuberculin test in a patient who either had BCG vaccination or previously had a positive tuberculin test 2. Elevated serum angiotensin converting enzyme and/or elevated serum lysozyme* 3. Chest X-ray: bilateral hilar lymphadenopathy 4. Abnormal liver enzyme tests (any two of: alkaline phosphatase; aspartate transaminase; alanine transaminase) 5. Chest CT scan in patients with normal chest X-ray * Lysozyme is required in patients treated with ACE inhibitors.

Table C Diagnostic criteria for ocular sarcoidosis
1. Biopsy-supported diagnosis with compatible uveitis: Definite ocular sarcoidosis
2. Biopsy not done; bilateral hilar lymphadenopathy with compatible uveitis Presumed ocular sarcoidosis
3. Biopsy not done; chest X-ray normal; 3 suggestive ocular signs and 2 positive investigational tests Probable ocular sarcoidosis
4. Biopsy negative; 4 suggestive ocular signs and 2 positive investigations Possible ocular sarcoidosis

Herbort CP, Mochizuki M, Rao NA, and Members of Scientific Committee of 1st IWOS. International criteria for the diagnosis of ocular sarcoidosis: results of the first international workshop on ocular sarcoidosis (IWOS). *Ocul Immunol Inflamm*. 2009; 17: 160–169.

2.1.2.2 Behcet's uveitis:

Behcet's was diagnosed according to the Criteria described by "International Study Group for Behcet's Disease", (Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease 1990) which is shown in Table 2-2

Table 2-2: Diagnostic criteria for Behcet's Disease

In the absence of other clinical explanations, patients must have:
(1): Recurrent Oral Ulcerations (aphthous or herpetiform) recurring at least three times in one 12-month period;
(2): and two of the following: a) Recurrent Genital Ulceration. b) Eye Lesions: anterior or posterior uveitis, cells in the vitreous by slit lamp examination or retinal vasculitis observed by an ophthalmologist c) Skin Lesions: erythema nodosum, pseudofolliculitis, papulopustular lesions or acneiform nodules in post adolescent patients not on corticosteroids. d) positive Pathergy

2.1.2.3 Glaucoma

Glaucoma was defined as consistent intraocular pressure above the 24 mm Hg, as the risk of glaucoma appears to increase substantially as the intraocular pressure increases beyond this level. (Sommer et al. 1991) The threshold for considering a rise in intraocular pressure substantial (for example, as in a rise in intraocular pressure attributable to corticosteroid use) was 6 mm Hg or greater.

2.1.2.4 Cataract

Cataract was defined as lenticular opacity that was a direct result of inflammation rather than age or any other cause. For unilateral eyes, it was quite easy as other eye usually served as control. In case of bilateral disease logistic regression analysis was used to correct for age and other causes of cataracts particularly diabetes and steroid use. The type was cataract also helped in the diagnosis as cataract associated with uveitis is usually posterior sub-capsular.

2.1.2.5 Cystoid macular oedema:

The clinical criterion was deemed sufficient for the diagnosis of cystoid macular oedema and epiretinal membrane. However OCT scan or Fundus flourescein angiography was utilized for diagnosis where needed.

2.1.2.6 Choroidal Neovascular membrane:

The presence of choroidal neovascular membrane was always confirmed with FFA.

2.1.2.7 Permanent Visual Loss:

The presence of permanent visual loss was defined as any visual acuity of 6/15 or less caused by macular scar, optic atrophy, macular ischemia, refractory macular oedema or choroidal neovascular membrane. This did not include loss of visual acuity secondary to cataract, vitreous debris, or transient CMO.

2.1.2.8 Visual acuity measurement and conversion to LogMAR

Since this was a retrospective study so visual acuity was measured exclusively as Snellen acuity. However for statistical purpose reporting 1 or 2 lines of loss of visual acuity on a standard Snellen chart is problematic because of the inconstant relationship of “lines” with changes in the visual angle. For example, a decrease of 1 line of visual acuity from 6/9 to 6/12 represents a small decrement (33%) in the angle of resolution, whereas loss of 1 line from 6/36 to 6/60 represents almost doubling of the visual angle. For this precise reason it is recommended (Jabs, Nussenblatt, and Rosenbaum 2005) to use logarthrimic charts. (first devised by Bailey and Lovie) (Bailey and Lovie 1976) The major advantage of this notation especially for research purposes is the ability to measure and score low VA accurately, which can then be included in statistical analysis. (Lovie-Kitchin 1988) Another advantage is the regular progression of the optotype sizes from one line to the other in geometric format as each optotype on one line is 1.2589 times larger than the optotype on the next. (Bailey and Lovie 1976; Ferris, et al. 1982) These features permit inter-row interpolation of VA values. When each line has five optotypes such as EDTRS chart, an interpolated logMAR (logarithm of the minimal angle of resolution) score can be created by assigning 0.02 logMAR units for each optotype read correctly(Ferris, III et al. 1982)

Since logarithmic visual acuities were not available, Snellen visual acuities were converted to LogMAR and a doubling of the visual angle analyzed. This was calculated by finding the Logarithm of the reciprocal of decimal visual acuity

$$\text{Log MAR} = -\log_{10}(\text{visual acuity fraction})$$

Although this logMAR reporting is superior to the number of lines on a Snellen chart approach, there remained problems introduced by the limitations of Snellen charts, particularly in the poorer ranges of visual acuity (that is 6/36 or worse), where a line was represented by one or two letters. This increased the likelihood of skewing the data towards very poor vision. So before interpreting the visual acuity data it should be kept in mind that this data is logarithmic conversion of Snellen acuity and not actually recorded from logarithmic format charts

Although calculations were done in log MAR visual acuity, the results were described in Snellen acuity because it is clinical easy to comprehend. For visions described as hand movement or perception of light LogMAR vision was converted as suggested by Johnson *et al*. (Johnson et al. 2003) The table was slightly modified to accommodate counting fingers and hand movement visual acuity, which is not mentioned in their table. Counting fingers was given a value of 2 and hand movement a value of 2.5. (Table 2-3)

Table 2-3: Visual acuity notions (conversion table)

Snellen 6m	Snellen 20ft	Decimal	LogMAR
6/6	20/20	1.00	0.0
6/9	20/32	0.63	0.2
6/12	20/40	0.50	0.3
6/18	20/60	0.33	0.5
6/24	20/80	0.25	0.6
6/36	20/120	0.17	0.8
6/60	20/200	0.10	1.0
3/60	20/400	0.05	1.3
1/60	20/1200	0.02	1.8
CF	CF	CF	2.0
HM	HM	HM	2.5
PL+	PL+	PL+	3
NPL	NPL	NPL	4

Modified from: The Epidemiology of Eye Diseases. Johnson GJ. London 2003, Arnold (Johnson et al. 2003)

The standardization of uveitis nomenclature (SUN) working group recommendations were used for reporting visual loss. (Jabs, Nussenblatt, and Rosenbaum 2005) Moderate visual loss was defined as VA of 6/15 or less and severe visual loss as VA of 6/60 or less.

2.1.2.9 Event rate

Although there was a minimum follow up of 24 months, the follow up period varied between patients. Since uveitis is a chronic disease and a complication or flare up can develop anytime during the course of the disease event rates were used to analyze the results. “Rate is the number of events in those at risk for the event divided by total follow-up person time” It reports the proportion at presentation (or study entry) and the rate during follow-up (incidence). It is calculated by dividing the number of events (e.g., patients with elevated intraocular pressure) by the sum of the patients’ follow-up time (typically expressed as person years).

2.1.2.10 Event rate to explain “Final Visual acuity”

Same problem is reporting of final visual acuity, because visual acuity outcomes, as a whole, are influenced by the duration of follow-up. “Variable follow-up” does not account for the effect of time. The reporting of rates can avoid the incorrect conclusion made from analysis of final visual acuity.

Final visual acuity results were reported as rates, and life table (Kaplan-Meier) were generated. The most appropriate ways to report visual acuity outcomes are rates falling below given thresholds, such as 6/15 or worse or (6/60) or worse, or as a doubling of the visual angle. However survival analyses also

lead to overestimating of event rate because the event rate (ie end point) was recoverable. For example, loss of four lines of visual acuity due to macular oedema is recoverable, in contrast to an event from which one could not recover, such as death. In my study I only used survival analysis when there was evidence of permanent visual loss. This condiserably decreased the number of subjects and hence power to detect significant difference.

2.1.2.11 Bilateral eye disease

Another problem in reporting is that the two eyes of a single patient often are linked (i.e. behave more similarly than chance alone), so reporting all eyes is statistically problematic because it violates the basic principle of statistical analysis that events are independent. The problem can be addressed by appropriate analyses that correct for linked events such as generalized estimating equations. Simpler solutions were used, however. Firstly I analyzed the results of “involved eye/s” (worst eye) and the results of the “better eye” (e.g., the eye with better acuity). This approach gave an estimate of what happened to each eye with disease. Secondly I analyzed what happened to patient because of the disease or genotype and this included analysis like best corrected visual acuity with both eyes open or in other words visual acuity of better eye. (BEVA)

2.1.2.12 Outcomes Reporting

To report the anterior chamber or vitreous activity, improvement in the inflammation was defined as either a two-step decrease in the level of inflammation or a decrease to “inactive,” and worsening of the inflammation

was defined as either a two-step increase in the level of inflammation or an increase to the maximum grade. This reporting system is based on the recommendations by International Uveitis Study Groups. (Jabs, Nussenblatt, and Rosenbaum 2005)

2.1.2.12.1 Remission:

Term remission was reserved for inactive disease for at least 3 months after discontinuing all treatments for eye disease

2.1.2.12.2 Steroid Sparing:

Reduction in the dose of prednisone to 10 mg per day or less (or its equivalent for other corticosteroids) while maintaining inactive uveitis was considered successful corticosteroid sparing.

2.1.2.12.3 Intensity of Uveitis:

Amount of visual impairment was used to report intensity of the disease. It was defined as amount of visual impairment during the episode of inflammation and attributed directly to the inflammation or macular edema. Since it was a retrospective study this VA assessment gave a crude indirect estimate of intensity of inflammation. It was graded into

a) mild to moderate (doubling of visual angle from base line
b) and severe (quadrupling of visual angle from base line)

2.1.2.12.4 Response to treatment

The long term use of systemic steroids or use of second line immuno-suppressants, are indirect measure of severity of disease. It also means failure of treatment to control the inflammation. However it should be noted that some times second line of immuno-suppressants are used as steroid sparing agents

(to prevent side effects of steroids). While considering the use of second line immuno-suppressants as an indirect measure of severity or failed response to steroids, it was made sure not to include their steroid sparing role. This was done by proper history taking from patients and from entries in the clinical notes. Response to treatment was graded depending upon how quickly and effective the initiation of treatment controlled the acute episode of inflammation

2.1.3 SNP selection

In the present work, I have studied the contribution of the cytokine and chemokine genes in susceptibility and severity of PSU using genetically homogeneous Caucasian populations. I employed the candidate pathway approach, taking advantage of its high power of detection of weak associations. I mainly studied functional SNPs so that the relation between the phenotype and genotype could be correlated more logically. Gene haplotypes were re-constructed as wells. To increase the likelihood that a candidate gene would harbour the variations relevant to traits, I studied a wide variety of information, including previous genetic studies, animal models, and knowledge of biologic pathways.

As suggested by the Complex Trait Consortium and discussed in chapter 1, a candidate gene should meet more than one of these seven criteria (Abiola et al. 2003):

1. Polymorphisms in either coding or regulatory regions have been found.
2. Its function has been linked to the quantitative trait being analyzed.
3. Its function has been tested *in vitro*.
4. Its function has been tested in transgenic animals.

5. Its function has been tested in knock-in animals.
6. Its function has been tested by mutational analysis.
7. A homologous quantitative traits loci for the same phenotype in another species has been found.

Since candidate gene approaches focus on genes that are selected because of a priori hypotheses about their role in the phenotype, rather than rely on markers prior knowledge is foremost important (Tabor, Risch, and Myers 2002). First of all extensive literature search was done to get, up to date knowledge of biological pathways involved in the pathogenesis of immune mediated uveitis. Since the main focus was on cytokine and chemokine genes, involvement of these molecules in uveitis was studied by gathering original research work from pubmed, and ImmPort (The Immunology Database and Analysis Portal). This included studies in transgenic and knock-out animals, and human in-vivo and in-vitro studies. Once the candidate genes were selected online databases were looked to identify functional polymorphisms in these genes. The following Table 2-4 lists some available public resources from where I gathered the main information about the functional SNPs. Researchers should use different sources because limiting their resources to one database would miss a great deal of information. (Marsh, Kwok, and McLeod 2002)

Table 2-4: Publically available internet sources for SNP studies

Name	URL	Description
SNPper	http://snpper.chip.org/bio/snpper-enter	The Children's Hospital Informatics Program (CHIP) at Children's Hospital in Boston serves as the bioinformatics core for several national genomics investigations. Primarily interests in genes of the innate immunity pathway in humans.
GeneSNPs	http://www.genome.utah.edu/genesnps	Operated by the University of Utah, this site contains information about SNPs resulting from the US National Institute of Environmental Health's initiative to understand the relationship between genetic variants and response to environmental stimuli.
The genetic annotation initiative	http://cgap.nci.nih.gov/GAI/	A National Institutes of Health-operated site that contains information on candidate genes related to cancer and tumour genesis.
dbSNP	http://www.ncbi.nlm.nih.gov/SNP	A more comprehensive NIH-operated database containing information on SNPs with broad applicability in research.
HUGO mutation database initiative	http://www.hgvs.org	Maintained by the Human Genome Organization (HUGO), this database provides access to information about human mutations.
Human SNP database	http://www-genome.wi.mit.edu/SNP/human/index.html	Managed by the Whitehead Institute for Biomedical, this site contains information about SNPs collected by the Whitehead projects.
Gene Canvas	http://www.ecgene.net/genecanvas/	A database of cardiovascular candidate genes and their polymorphisms managed at INSERM, France.
ALFRED	http://alfred.med.yale.edu/alfred/index.asp	Supported by the US National Science Foundation, this site provides data on different populations in order to enhance their differences in frequency and to facilitate the study of haplotypes

I also studied few non- functional SNP. There were two main reasons to study the non-functional SNPs

1) Either non-functional SNPs in candidate genes were found to be associated with immune mediated disease in the medical literature

2) Or Non-functional SNPs were in a well defined haplotypes that has been associated with immune mediated diseases.

2.2 Laboratory Methods

2.2.1 DNA Extraction

DNA was extracted from peripheral blood using modified high salt method (Bunce, Fanning, and Welsh 1995) or with commercial kits (Qiagen UK Ltd, UK) according to the manufacture's instructions

2.2.1.1 Modified high Salt method

Eight mls of peripheral blood, collected in vacutainers containing tri-sodium citrate as anticoagulant, were transferred to a 15 ml polypropylene tube (Beckton Dickenson, UK). Blood samples were centrifuged at 2500 rpm for 20 minutes at room temperature to separate blood components. The buffy coat of white blood cells was aspirated from the top of the red blood cells and was placed into a fresh 15 ml tube. Ten mls of red cell lysis buffer were added to remove erythrocytes contaminating the white blood cells .Tubes were inverted several times and were left at room temperature for 15 minutes or until complete haemolysis was evident. Samples were then centrifuged at 2500 rpm for 20 minutes to pellet the WBC. The supernatant was discarded into Hycolin and white cell pellets were washed with red cell lysis buffer. Pellets, at this stage, were either stored at –20 ℃ for future DNA extraction or the DNA was immediately extracted. The DNA extraction involved re-suspension of the WBC pellet in three mls of nuclei lysis buffer. The proteins were then precipitated by adding of 1 ml of 6 M NaCl and separated from the DNA by adding 2 mls of 24:1 Chloroform-Iso-amyl alcohol. Samples were agitated until a homogenous milky solution was formed and were centrifuged at 3000 rpm for 30 minutes. After the centrifugation, three visible phases were evident: A lower phase

containing chloroform and dissolved lipids and lipoproteins, an interface of precipitated proteins and cell debris, and an upper phase containing DNA from the lysed WBC. The upper phase, approximately 3 mls, was transferred into a new tube and 10 mls of an absolute ethanol were added. The tubes were inverted several times resulting in the precipitation of DNA. The DNA was transferred into a sterile 1.5 ml tube and left to air dry for 30 minutes to allow evaporation of the excess ethanol. The DNA was then re-suspended in an appropriate amount of sterile distilled water and was stored frozen at –20 ℃ in aliquots until required.

2.2.1.2 Qiagen Kit DNA extraction

Ten mLs of peripheral venous blood were collected for DNA extraction in all patients and controls. Genomic DNA was extracted from EDTA-chelated peripheral whole blood, with commercial kits (Qiagen UK Ltd, UK) according to the manufacture's instructions. Briefly, 500 µL of QIAGEN Protease was pipetted into the bottom of a 50 mL centrifuge tube and 10 mL of blood was added and mixed briefly. A further 6 mL Buffer AL® (red cell lysis buffer) was added and mixed thoroughly by vortexing. The mixture was then incubated at 70^{o}C for 10 min. 10 mL of ethanol (96-100%) was added to the sample and mixed again by vortexing. Solution was then transferred onto the QIAamp Maxi column® placed in a 50 mL centrifugation tube and centrifuged at 3000 rpm for 3 min. The filtrate was discarded and 5mL of Buffer AW1® was then added to the QIAamp Maxi column® and centrifuged at 5000 rpm for 1 min. A further 5 mL of Buffer AW2® was added and the mixture centrifuged at 5000 rpm for 15 min. The QIAamp Maxi column® was then placed in a clean 50 mL centrifugation tube and 1mL of Buffer AE® or distilled water was then added by

pipetting directly onto the membrane of the QIAamp Maxi column®. This was incubated at room temperature for 5 min and centrifuged at 5000 rpm for 5 min. The filtrate which then contained the extracted DNA was stored in tubes at -20^{o}C.

2.2.1.3 PicoGreen Assay for DNA Quantitation

The methodology for genotyping used in this study is highly dependent on the DNA concentration. Therefore, a reliable method for DNA quantification is required. PicoGreen (PG) (Molecular Probes, NL) is a fluorochrome that selectively binds to double strand DNA (dsDNA). It has an excitation maximum at 480 nm and an emission peak at 520 nm. There is a little background interference as the free dye does not fluoresce, but upon binding to dsDNA it exhibits a more than1000-fold fluorescent enhancement. This method has been found to be more accurate than the optical density method and it is not affected by ssDNA or RNA (Blotta et al. 2005) (Ahn, Costa, and Emanuel 1996)

The PG lambda dsDNA stock solution, stored in darkness, was used to generate a standard curve as outlined in Table 2-5. The working solution was prepared by diluting PG stock 1:200 with 2 x TE (Tris-HCL EDTA) by mixing 100 µL 20 x TE, 895 µL dH2O and 5 µL PG.

Table 2-5 Preparation of standards for PG quantification

Final Standard Concentration	Volume of known standard	Volume of dH_2O
(A) 2000 ng/ml	40µl of stock (100 µg/ml)	1960 µl
(B) 1000 ng/ml	500µl of A (2000 ng/ml)	500 µl
(C) 500 ng/ml	500µl of B (1000 ng/ml)	500 µl
(D) 200 ng/ml	50µl of A (2000 ng/ml)	450 µl
(E) 50 ng/ml	50µl of C (500 ng/ml)	450 µl
(F) 5 ng/ml	50µl of E (50 ng/ml)	450 µl
(G) 0 ng/ml	0	500 µl
(H) Blank		

DNA samples that were needed to be quantified were diluted 1:250 in distilled water (i.e. 2 µL DNA in 498 µL dH_2O). Ten µL of DNA standards were added to wells A1 to A8 in a 96 well PCR plate (Corning International, NL), as set out in Figure 2-1, and 10 µL of each diluted DNA sample were added to the remaining wells. A further 10 µL of PG solution was then added to each well and the plate was briefly centrifuged.

Plates were analysed by the Lambda FLUORO 320 plus fluorimeter, with kinetiCalc4 (KC4) analysis software (Bio-Tek Instruments Inc., Vermont USA). The KC4 software, from the average Fluorescent Unit Count (AFU) of the DNA standards, constructed the standard curves (Figure 2-2). The DNA concentration of the unknown samples was determined by reading off the standard curve using KC4. The concentration was then multiplied by the dilution factor, which was 500.

Extracted DNAs were re-checked for quality by gel. Figure 2-3 shows an example for SSc DNA run in gel and the concentration of PG.

Figure 2-1 96 well plate layout

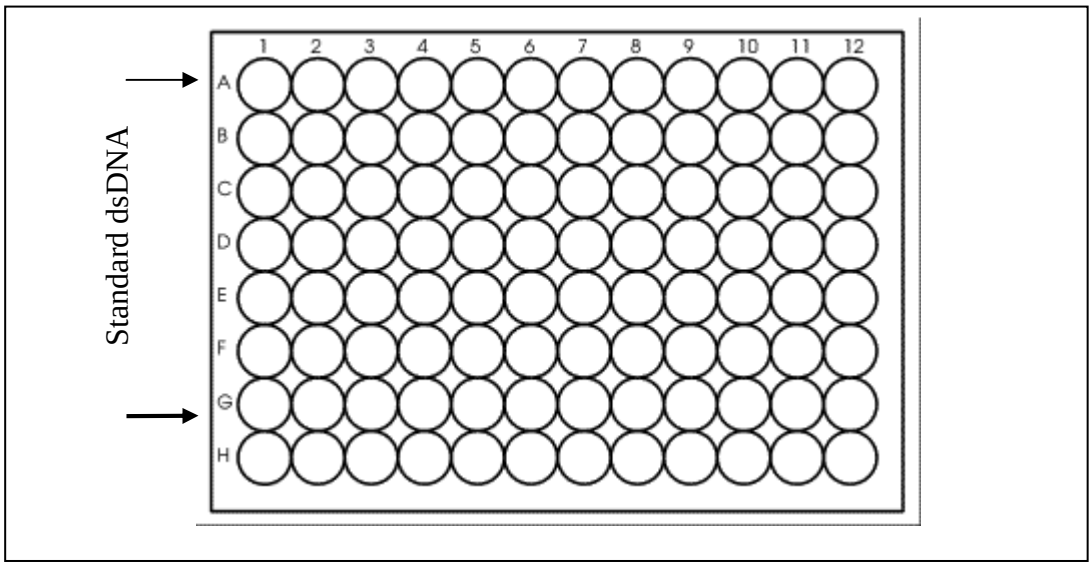


Figure 2-2 Standard fluorescence-DNA concentration curve generated by lambda DNA quantification using PicoGreen

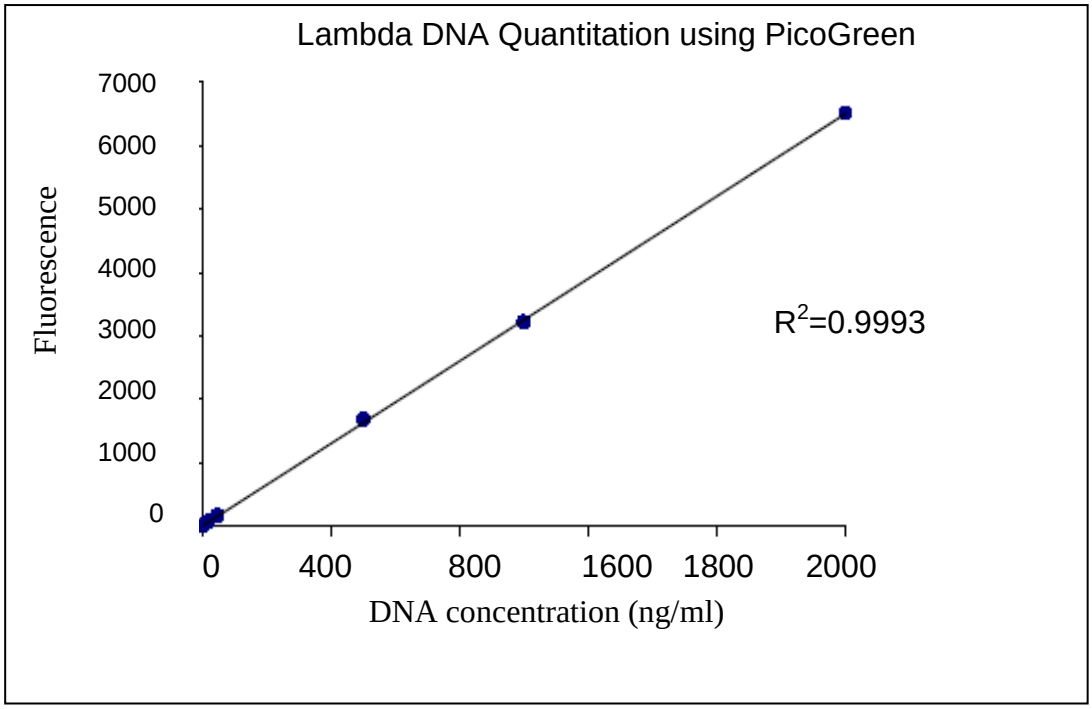
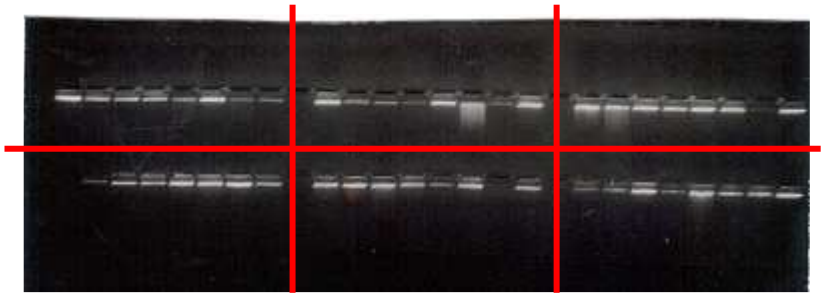


Figure 2-3: DNA quality evaluation.

An example of using gel to test DNA, after extraction and PicoGreen measurement. 48 DNA samples from control population



DNA quantification for sample in the 1st row of the

NO	ng/uL	Volume µL	Gel Quality
1135	274	250	Good
1193	73	200	Good
1200	113	350	Good
1205	282	500	Good
1221	72	300	Weak
1230	252	50	Good
1233	100	400	Weak
1241	70	400	Weak
1242	125	500	Good
1262	44	1000	Weak
1263	90	200	Weak
1289	27	1000	Weak
1314	476	350	Good
1363	136	400	Bad
1400	69	450	Weak
1478	261	450	Good
1537	196	400	Good
1580	550	300	Bad
1593	550	500	Good
1605	311	450	Good
1645	508	250	Good
1677	550	500	Good
1693	19	500	Bad
17520	550	400	Good

DNA quantification for sample in the 2nd row of the

NO	ng/uL	Volume µL	Gel Quality
1805	2.7	250	Bad
1814	449	400	Weak
1840	470	500	Good
1874	248	400	Good
1879	259	200	Good
1918	468	300	Good
1953	418	150	Good
28	129	200	Good
134	193	200	Good
510	393	150	Good
904	366	400	Good
983	306	100	Good
17	70	100	Weak
123	186	750	Good
34	0	100	Bad
58	390	100	Good
69	337	100	Weak
101	85	400	Weak
101d	435	500	Good
110	72	500	Weak
29	223	400	Good
116	273	500	Good
118	198	500	Good
118d	447	300	Good

2.2.2 SNP Genotyping Methods

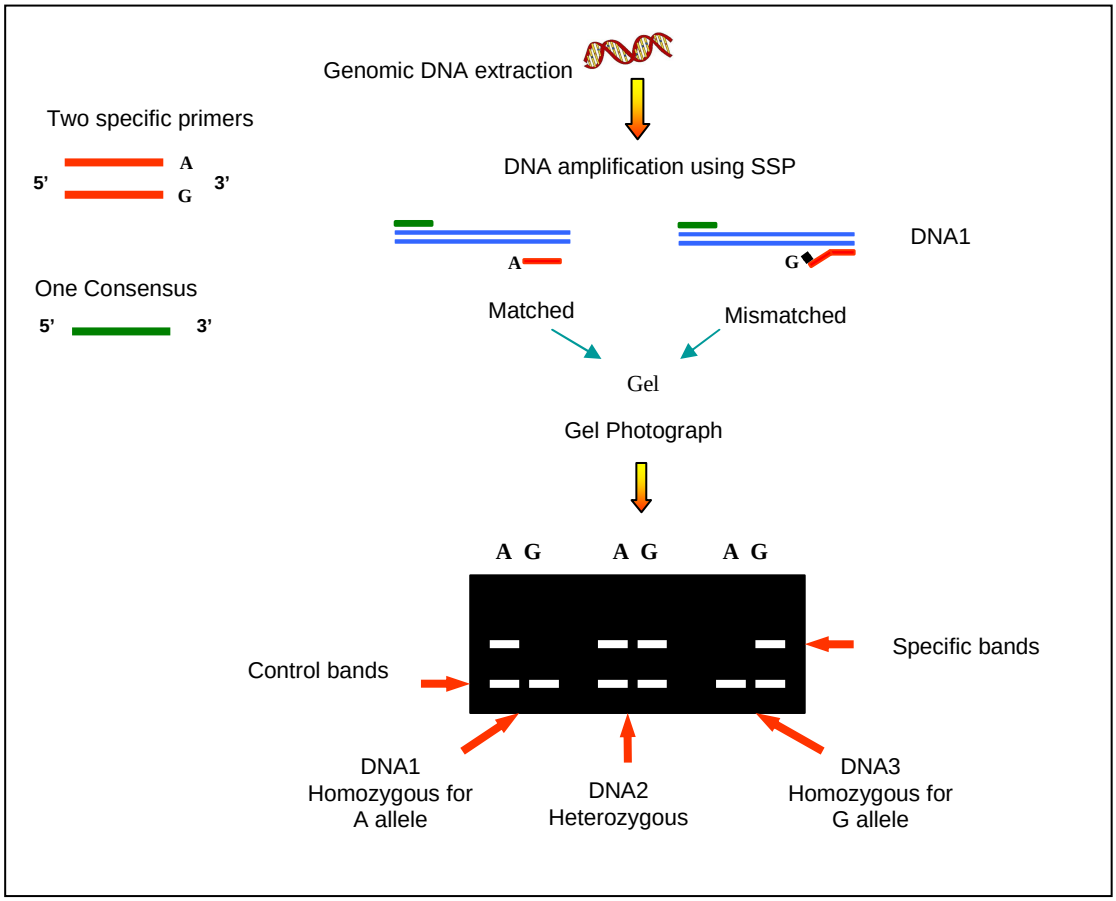
2.2.2.1 SNP Analysis Using SSP-PCR

SNP is a wide range term that includes the nucleotide substitutions, the insertions and the deletions. SSP-PCR can be used to genotype most SNP types with two SSP reactions giving an accurate representation of the genotype at a given locus within three hours. Also, large deletions can be genotyped sometimes by using SSP-PCR (Collins et al. 2002) (Spagnolo et al. 2003).

SSP-PCR involves designing specific primers that will or will not allow amplification using the 3'-mismatch principle. For a single locus, two different PCR reactions detect two allelic variants: One containing a specific primer complementary to one variant, and the other containing a specific primer complementary to the other variant in conjunction with a consensus primer. The amplification of DNA will only occur if the 3' nucleotide of the primer matches the 3' nucleotide of the DNA target, allowing the DNA polymerase to bind to the target and amplify the desired sequence. (Figure 2-4) Any amplified DNA segments can be visualized by electrophoresis

Figure 2-4 Diagram shows SSP-PCR steps

Adding A & G specific primer into a single reaction will lead to three possible outcomes depending upon which allele matches, as shown in gel photograph. The subject may be either homozygous for A allele DNA 1, homozygous for G allele DNA 3 or heterozygous DNA 2.



2.2.2.1.1 Primer Design for SSP-PCR

I designed all chemokine SNPs primers reported in this thesis for this method. The sequences and variations of the genes of interest were found through a variety of sources, including journals and on-line genomic databases. On line databases such as the SNPper database http://snpper.chip.org/bio/snpper-enter and the university of Utah Gene SNPs database http://www.genome.utah.edu/genesnps/ were used in this study. All primers were designed to work under the same amplification procedures. For this reason, a set of primer design rules had to be established. Criteria of primer

design were; a length of 19 to 25 bases, a salt adjusted melting temperature (Tm) of 58 to 61 ˚C, a GC content of 45 to 55 % and with no secondary structures, for example, hairpin loops (Table 2-6). The specificity of primer product were tested against ladder control with known product size and in some cases the primer were used to genotype DNA sample which has known genotype.

Table 2-6 General criteria for primer design

Allele Specific and Consensus Primers Criteria

- Tm ~ 60 ˚C
- GC content 45 - 55 %
- Primer length = 19 - 25 bp
- No hairpins
- No primer dimmer
- Can be either forward or reverse

1 General Considerations

- If possible avoid long GC stretches and primers longer than 21 bases.
- 3` mismatches are the critical ones. A mismatch within the first three nucleotides of a primer (from 3` end) is normally enough to ensure no amplification. Mismatches in the second half of the primer will under stringent conditions ensure no amplification, but may weakly amplify so it is better to avoid these.
- Download the genomic DNA sequence (not mRNA) that contain the polymorphic site and highlight your primers.
- If the specific primer is a sense primer then your consensus for that primer should be anti-sense and vice versa.

The FastPCR programme by Ruslan Kalendar, University of Helsinki, Finland, (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) was used to calculate the primers Tm and GC content and to check for primer dimers and hairpin

formation. In addition, the online program Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to confirm the estimated Tm and GC content. Once designed, primer sequences used in SSP-PCR genotyping were screened against BLAST program from the NCBI http://www.ncbi.nlm.nih.gov/BLAST/. The BLAST program has the capacity to compare the primer sequences with a comprehensive catalogue of all the known human DNA sequences. This process minimizes the possibility of the primers annealing to sites of non-specific interest or mis-priming during PCR amplification.

Control primers were added to the reaction and amplified simultaneously with each PCR in order to confirm that PCR reaction was successful. For allele specific amplicons between 250 – 400 bp, the control primers termed 63/64 (which give two bands sizes on a gel of approximately 650 and 1300 bp each of HLA-DRB1 intron 3) were used at a concentration of 2 µg/mL:

63: 5'- TGC CAA GTG GGA CAC CCA A- 3'

64: 5'- GCA TCT TGC TCT GTG CAG AT - 3'

For amplicons size between 400 – 950 bp, control primers termed 210/211, which gives a 249bp amplicon from the Adenomatosis Polyposis Coli gene, at a concentration of 5 µg/ml:

210: 5'- ATG ATG TTG ACC TTT CCA GGG - 3'

211: 5' - TTC TGT AAC TTT TCA TCA GTT GC - 3'

2.2.2.1.2 Primer Titration

Generally, PCR reactions are optimised by adjusting cycling conditions and magnesium concentration. However, the SSP-PCR setup used in this study

includes several PCR reactions run simultaneously in the same plates using the same PCR conditions including the magnesium concentration. Therefore, the only parameters that can be altered are the primer concentrations. Specific and consensus primers were, therefore, experimentally titrated to obtain the optimal working concentrations to avoid false positive and false negatives.

Depending on the quantity of PCR end product produced in the initial tests (shown by the strength of the bands seen on the gel), either an upward or a downward primer titration is performed. Altering specific and consensus primers have different effects. Increasing the concentration of specific primers will increase the amount of specific product, whereas increasing the concentration of consensus primers may increase both the amount of specific product and the amount of mis-primed product. Therefore, the ratio of the concentration of specific to consensus primers is important.

To optimise primer concentrations, titration on 10 DNA samples were performed. Five µL stock specific and consensus primers (at 2000 µg/ml) were added to one ml primer mix (10 µg/ml) to prepare the primer master mix. PCR amplification was performed using this primer master mix. Based on end product results further primer mixes of higher or lower concentrations were made. Probable homozygous and heterozygous samples were identified for use in the subsequent optimisation experiments.

For example, Table 2-7 shows a downward primer titration. 2.5 µL of each allele-specific primer were then added into five rows of 96-well plate, followed by adding 2.5 µL of consensus primers. The final primer volume is five µL. Figure 2-5 shows the primer titration for *MCP-1* 2518 polymorphisms and the optimum concentration used for genotyping.

Table 2-7 Grid showing layout of 96-well plate for downward primer titration

2.5 µL of each allele-specific primer were added into five rows of 96-well plate, followed by adding 2.5 µL of consensus primers. The final primer volume is five µL. Concentrations used here is an example. Depending on the strength of the bands seen on the first primer test, concentrations can be increased or decreased.

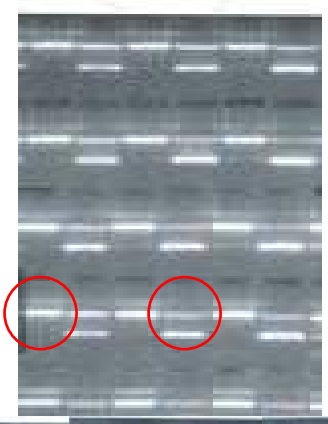
Specific / Cons		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
Volume used to prepare primer mix →		10	10	8	8	6	6	4	4	2	2
↓	Final volume (µL) in 1 ml primer mix →	5	5	4	4	3	3	2	2	1	1
10	5										
8	4										
6	3										
4	2										
2	1										

Figure 2-5 SSP-PCR primer titration experiment

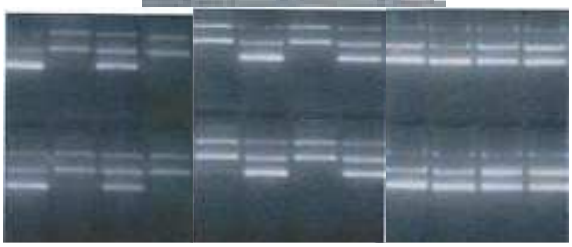
Figures in the table are volume of allele specific primer/ consensus primers in uL per 1 mL primer mix. Panel A shows an upward primer titration using increasing specific primers. Columns represent downward titration using decreasing consensus primers. Note the false positive reactions on the right hand side of the gel as the primer concentration increase. The highlighted concentrations were chosen as the optimum concentration. Panel B shows the optimized primer concentrations tested on six samples: two heterozygotes, two homozygotes for the first allele and two homozygotes for the second allele. There is clear discrimination between all three genotypes.

Specific / Cons		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
Volume used to prepare primer mix		8	8	10	10	12	12
	Final volume (µL) in 1 ml primer mix	4	4	5	5	6	6
14	7	4/7	4/7	5/7	5/7	6/7	6/7
12	6	4/6	4/6	5/6	5/6	6/6	6/6
10	5	4/5	4/5	5/5	5/5	6/5	6/5
8	4	4/4	4/4/	5/4	5/4	6/4	6/4
6	3	4/3	4/3	5/3	5/3	6/3	6/3

Panel A



Panel B



2.2.2.1.3 SSP-PCR Reactions

Supplied primers (Sigma Genosys, UK) at concentration of 0.2 μM were re-suspended in a sterile distilled water (not deionised) (Baxter, UK) to a concentration of 2000 μg/ml. Each primer mix comprised of appropriate amount of each specific and consensus primers, control primers (see primer sections 2.2.4.1 and 2.2.4.2 for volumes) and cresol red indicator (Sigma, Poole, UK) (Appendix A), prepared in a sterile distilled water (Baxter, UK). Cresol red undergoes a pH related colour change from orange to purple when adding the acidic DNA.

PCR-SSP amplifications were carried out using 96, 192 or 384-well thermal cyclers MJ Research 96V or PTC200 machines (GRI, Braintree, UK). The DNA was amplified in 13 μl reaction mixtures consisting of 5 μl of primer mix and 8μl PCR reaction mix: 1X PCR Buffer (Bioline) (Appendix A); 2 mM $MgCl_2$; 200 μM of each dNTP (dATP, dGTP, dTTP, dCTP); approximately 0.08 μg of genomic DNA; and 0.175 units of Taq polymerase (Bioline, UK)). Reaction mixtures were dispensed under 10 μl of mineral oil (Sigma, Poole, UK) to prevent evaporation. PCR-SSP thermal cycling parameters are shown in Table 2-8 Table. After PCR amplification, ten mls of orange G loading buffer (Appendix A) was added to each sample. PCR amplicons were identified by gel electrophoresis.

Table 2-8 Thermal cycling parameters for SSP-PCR

No. of cycles	Temperature (°C)	Duration (seconds)
1	96	60
5	96 70 72	20 45 25
21	96 65 72	25 50 30
4	96 55 72	30 60 120
1	20	30

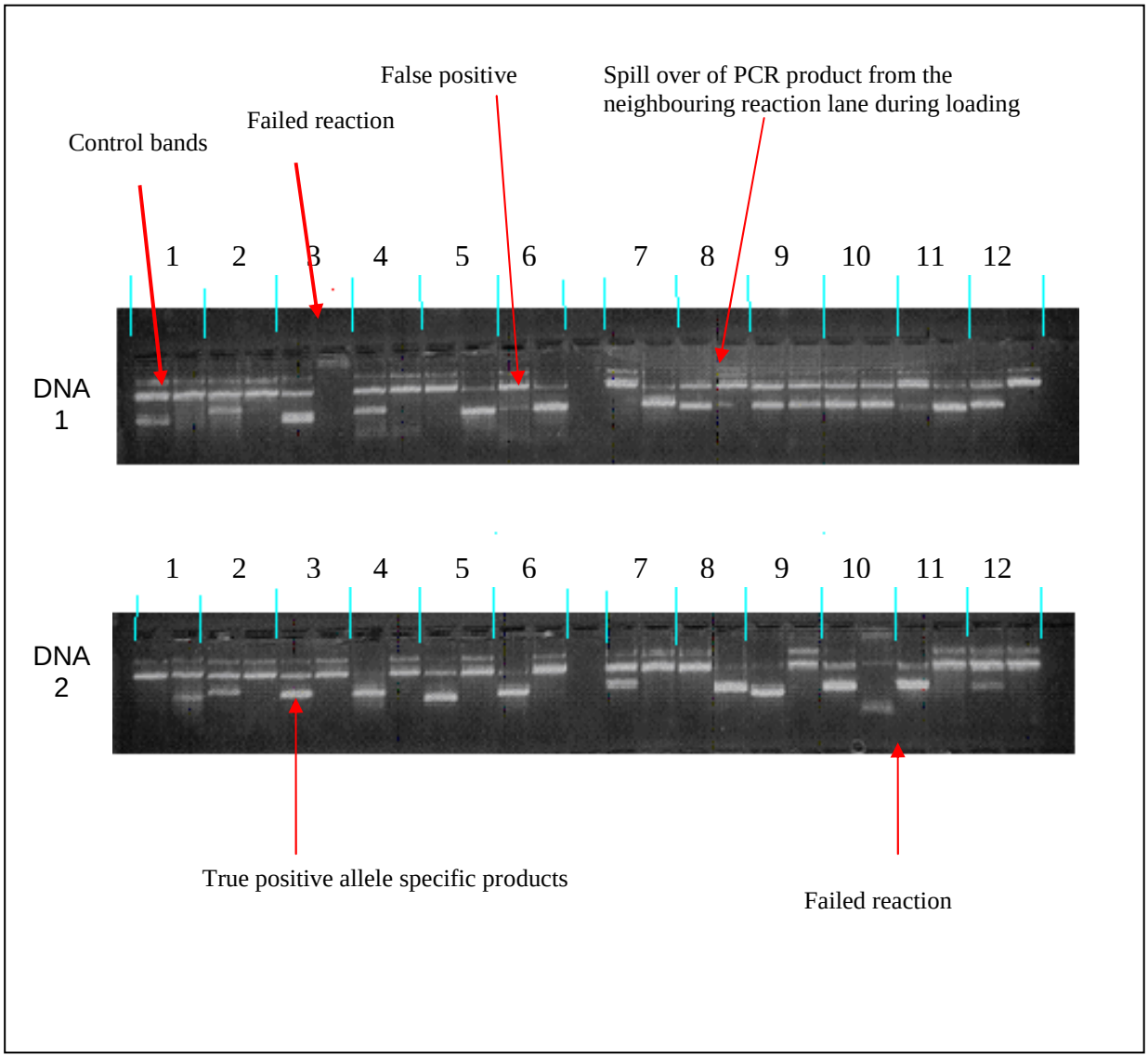
2.2.2.1.4 Agarose Gel Electrophoresis

Amplicons were loaded into the wells of 2% agarose gels (Appendix A)(Bioline, UK) with 0.5X Tris Borate EDTA (TBE) buffer (Sigma, Poole, UK) and were stained with 0.14 µg/ml of ethidium bromide (Sigma, Poole, UK). Band separation was done by electrophoresis, which was carried out at 200 Volts for 20 minutes. Gels were transferred onto a transilluminator and photographed on Polaroid film using the Polaroid MP4 Camera system over ultraviolet light (320 nm).

The stringent conditions outlined in this section ensured amplification using SSP-PCR occurred only with an exact primer match. Therefore, the presence of a specific allele was scored by observing an amplicon of the expected size in

association with a control PCR product. The absence of a specific allele was scored by observing only a control PCR product (Figure 2-6.).

Figure 2-6 A typical gel photograph of SSP-PCR results



All reactions in this photo were run with control primers 63/64. The allele specific product is formed only when that allele is present in the DNA sample. If the product is formed in both reactions, the individual is heterozygous, and if only in one reaction, the individual is homozygous for that allele. False positive reactions can occur for a number of reasons, such as mis-priming due to presence of excess primer or DNA contamination.

2.2.2.2 SNP Analysis using 5’Nuclease Activity Assay

I used TaqMan® 5’Nuclease Activity Assay, to study the SNPs in cytokine SNPs. 5’Nuclease Activity Assay was first described by Holland *et al*. in 1991. (Holland et al. 1991) The chief attributes of 5’Nuclease assay is that it is completely homogenous. After mixing the sample and the reaction components, the assay is run in a closed compartment with no post PCR processing step. Results are obtained by simply measuring the fluorescence of the completed reaction. By eliminating post PCR processing, allelic discrimination with flourogenic probes reduces the time of analysis, labour , supply cost of post-PCR steps, reduces the risk of cross-over contamination and minimizes source of error. The assay has a sensitivity of PCR so that a minimum amount of genomic DNA is required. The use of endpoint fluorescence measurements maximize throughput.

In the 5' nuclease PCR array, a hybridization probe included in the PCR is cleaved by the 5' nuclease activity of Taq DNA polymerase only if the probe target is being amplified. By using flourogenic probe, first synthesized by Lee *et al*. in 1999 (Lee, Connell, and Bloch 1993) cleavage of the probe can be detected without post PCR processing. The flourogenic probe consists of an oligonucleotide labelled with both a fluorescent reporter dye and a quencher dye. In the intact probe, proximity of the quencher reduces the fluorescence signal observed from the reporter dye due to Foster resonance energy transfer (FRET). Cleavage of the flourogenic probe during the PCR array liberates the reporter dye, causing an increase in fluorescence intensity. A Detection System measures this increase in fluorescence during the thermal cycling of PCR, providing "real time" detection of PCR product accumulation.

For a Bi-allelic system, probes specific for each allele are included in the PCR array. The probes can be distinguished because they are labelled with different fluorescent dyes (FAM™ and VIC™). A mismatch between probe and target greatly reduces the efficiency of probe hybridization and cleavage. Thus, substantial increase in the FAM™ or VIC™ fluorescent signal indicates homozygosity for the FAM™ or VIC™ specific alleles. An increase in both signals indicates heterozygosity.

ABI TaqMan® Allelic discrimination assay

The TaqMan® Allelic Discrimination assay employs this probe technology that exploits the 5´-3´ nuclease activity of AmpliTaq Gold® DNA Polymerase to allow direct detection of the PCR product by the release of a fluorescent reporter as a result of PCR. This PCR system is optimized for yield. AmpErase® UNG is required for the prevention of PCR product carryover (Longo, Berninger, and Hartley 1990).

The AmpliTaq Gold enzyme cleaves the TaqMan® probe with its 5´–3´ nuclease activity. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter (Figure 2-7). The 3´ end of the TaqMan® probe is blocked to prevent extension of the probe during PCR. Below is the brief account of all the reagents used in TaqMan® assay

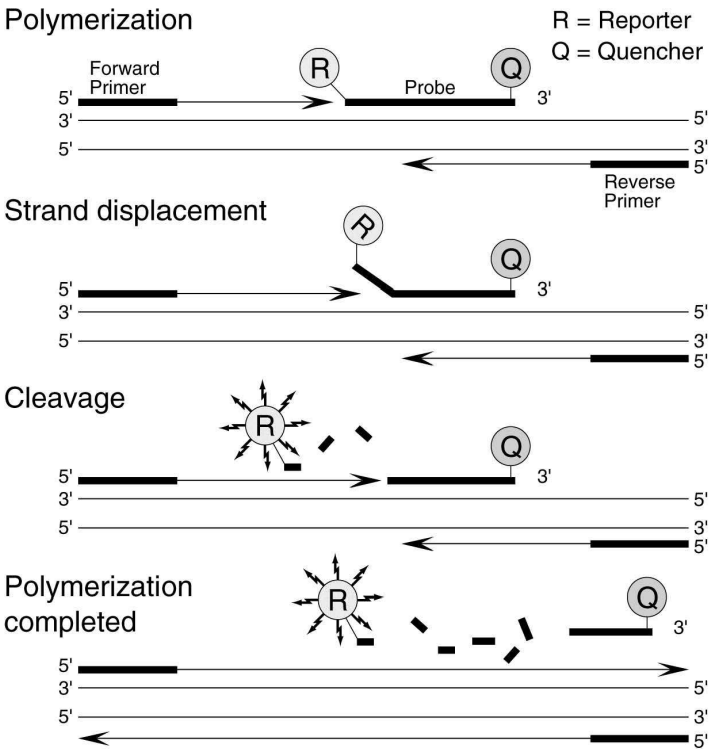


Figure 2-7: 5´–3´ nuclease activity of DNA Polymerase during one phase of PCR

The allele-specific labelled probe is located directly over the single nucleotide polymorphism of interest. During polymerase chain reaction, the reporter is cleaved from the probe and, at the endpoint, the genotype is determined according to the ratio of fluorescence attributable to each fluorophore

2.2.2.2.1 PROBES

The ABI probes have an exclusive MGB (minor groove binder) moiety added to the probe to increase the stability and specificity of probe hybridisation. Incorporating an MGB molecule allows TaqMan® Probes to be much shorter. Without the MGB, AT-rich probes often need to be over 30 and sometimes over 40 bases long to satisfy amplicon design guidelines. Longer probes perform relatively poorly compared to shorter MGB equivalents. TaqMan® MGB probes are typically between about 13 and 20 bases long; approximately half the length of standard probes.

MGB is a small crescent-shaped molecule that fits snugly into the minor groove of duplex DNA. In TaqMan® probes, the MGB group is attached at the 3' end along with the quencher dye. When the TaqMan® probe hybridizes, the MGB

stabilizes annealing by folding into the minor groove of the DNA duplex created between the probe and the target sequence. Stabilization is much more effective when the duplexes are perfectly matched (i.e. when there are no sequence mismatches).

2.2.2.2.2 Dyes

The quencher in TaqMan® probe is non-fluorescent quencher (NFQ) dye (sometimes called a “dark” quencher). A non-fluorescent quencher is essentially a chromophore that acts as the energy transfer acceptor from the reporter molecule that does not emit a detectable fluorescent signal of its own. The advantage is that a less complicated signal with lower fluorescent background is emitted from the reaction. This improves spectral discrimination and makes data interpretation easier. The NFQ used is a proprietary molecule that is effective over a broad wavelength range.

The reporter dyes used in TaqMan® system are FAM™ and VIC™. FAM™ is 6-carboxyfluorescein dye and emits blue light. VIC™ is 4-dichloro-6-carboxyfluorescein dye and emits green light. NFQ quenches the current range of reporter dyes (FAM™, VIC™ and TET™ dyes) effectively.

The process of release of fluorescent dye occurs in every cycle and results in increase in fluorescence for each of the FAM™ and VIC™ reporters. The increase in fluorescence is measured, and is a direct consequence of target amplification during PCR.

Both primer and probe must hybridize to their targets for amplification and cleavage to occur. The fluorescence signals are generated only if the target sequences for the probes are amplified during PCR. Because of these

requirements, non-specific amplification is not detected. (Lee, Connell, and Bloch 1993)

The Sequence Detection Systems from Applied Biosystems are used to Detection measure the increase of reporter fluorescence following PCR. Reporter signals are normalized to the emission of a passive reference:

$$Rn\ (Allele1) = \frac{\text{Emission Intensity of Allele 1 Reporter}}{\text{Emission Intensity of Passive Reference}}$$

$$Rn\ (Allele2) = \frac{\text{Emission Intensity of Allele 2 Reporter}}{\text{Emission Intensity of Passive Reference}}$$

2.2.2.2.3 Primers and probes designing.

Primers and TaqMan® probes were designed using Primer Express software (version 2.0; sequences) and synthesised and supplied by Applied Biosystems UK. The reporter dyes chosen were 6-FAM™ and VIC™. Applied Biosystems recommends the general use of TaqMan® MGB probes for allelic discrimination assays, especially when conventional TaqMan® probes exceed 30 nucleotides.

2.2.2.2.3.1 TaqMan® MGB Probe Design Guidelines

The manufacturer guidelines were followed to design the probes:

1) Probes with a guanine residue at the 5´ end of the probe were avoided. A guanine residue adjacent to the reporter dye will quench the reporter fluorescence, even after cleavage.
2) Probes with a Primer Express software–estimated Tm of 65–67 ℃ were selected.

3) MGB probes were made as short as possible without being shorter than13 nucleotides.

4) Runs of an identical nucleotides were avoided. This is especially true for guanine, where runs of four or more should be avoided.

5) Polymorphic site was positioned in the central third of the probe. The polymorphic site can be shifted toward the 3′ end to meet the above guidelines; however, the site must be located more than two nucleotides upstream from the 3′ terminus.

2.2.2.2.3.2 Primer Design Guidelines

After selecting probes for the assay, primers were chosen based on the guidelines below.

1) Runs of an identical nucleotides were avoided. This is especially true for guanine, where runs of four or more should be avoided.

2) The Primer Express software–estimated Tm for the primers was set 58–60 ℃.

3) Guanine + cytosine content were kept within 20–80%.

4) It was made sure the last five nucleotides at the 3′ end contain no more than two guanine + cytosine residues.

5) The forward and reverse primers were placed as close as possible to the probe without overlapping it.

2.2.2.2.4 Premixes

Though custom made Probes and primers were made for most of the cytokine SNPs, some of the SNPs were detected by using pre-made assays supplied by Applied Biosystems. TaqMan® SNP Genotyping Assays are validated primer

and TaqMan® MGB probe sets for the detection of human SNPs. Each product is delivered as pre-mixed primers and TaqMan® MGB probe(s) at a 20X concentration. (Proprietary formulation)

2.2.2.2.5 Master Mix

The TaqMan® Universal PCR Master Mix was used for real-time or plate read (endpoint) detection of DNA. The mix is optimized for TaqMan® reactions and contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, (uridine, uracil-N-glycosylase) dNTPs with dUTP, Passive Reference dye ROX, and optimized buffer Components (proprietary formulation). The AmpliTaq Gold enzyme is a thermal stable DNA polymerase. The enzyme has a 5´ to 3´ nuclease activity, but lacks a 3´ to 5´ exonuclease activity (Holland et al. 1991).

Prevention of PCR Product Carryover

Treatment with uracil-N-glycosylase (UNG) can prevent the reamplification of carryover PCR products. This method involves substituting dUTP for dTTP in the Reagent Master Mix and adding AmpErase UNG to the mix prior to amplification (Longo, Berninger, and Hartley 1990). Products from PCR amplification contain uracil, and are readily distinguishable from native thymidine-containing DNA templates. UNG eliminate products from previous PCR amplifications by excising uracil residues and degrading the resulting a basic polynucleotide with heat. Therefore, only native nucleic acid templates are being PCR amplified. PCR products from previous amplifications are not reamplified. Misprimed, nonspecific PCR products created before thermal cycling are degraded, but native DNA template is not The 2-minute hold cycle at 50 ℃ is necessary for optimum AmpErase UNG clea vage of the uracil-deoxyribose linkage. The 10-minute hold cycle at 95 ℃ necessary to activate

AmpliTaq Gold DNA Polymerase also cleaves the phosphate ester backbone of the PCR products that contained uracil nucleotides and reduces the AmpErase UNG activity substantially. Because UNG is not completely deactivated during the 95 ℃ incubation, it is important to keep the r eaction temperatures greater than 55 ℃ to prevent amplicon degradation.

2.2.2.2.6 TaqMan® SNP Genotyping Assay Protocol

Materials

Optical 384-Well Reaction Plate

Optical Adhesive Films

TaqMan® Universal PCR Master Mix (stored at 4C)

TaqMan® 10X Assay Mix (labelled probes and unlabeled primers) (stored at -20 or -30 C) or Custom made primers and probes

Method

1 uL of 20ng/ul DNA template was pipetted into Optical 384-Well Reaction Plate for 384-well format. Reagents were added according each reaction well in the plate according to tables below per reaction. (Table 2-9)

Table 2-9 Concentrations of probes, primers and reagents for TaqMan® assay

Reagent	Volume Per Reaction	Final Concentration
DNA Template	20 ng -- 1uL of 20ng/ul	
Mol. Biol. Grade Water	1.0	-
TaqMan® Universal PCR Master Mix	2.5	1X
TaqMan® 10X Assay Mix	0.5	1X
OR 0.9 mM primers, and 0.2 mM probes	Variable (if more than 0.5 then decrease water to make final volume of 5uL	
Final Reaction Volume (384)	5.0	-

Layout of 384 wells plate

Because sample protein and fluorescent contaminants may interfere with this assay and give false positive results, it may be necessary to include a No Amplification Control well that contains the sample and no enzyme. If the absolute fluorescence of the No Amplification Control is greater than that of the No Template Control after PCR, fluorescent contaminants may be present in the sample. In the 384 well plate, 8 wells contained template DNA but no enzyme, 8 wells just plain water, 8 wells with known Allele 1 (with enzymes and master mix) and 8 wells with known allele 2. (Figure 2-8) This will make allele calls at a 99.7% confidence level using the automated allele calling routine. (Manufacturer guidelines)

Figure 2-8: Layout of 384 wells plate.

Shaded wells acting as reference.

Plate was centrifuged and then sealed with optical Adhesive seal or optical heat seal.

Thermal Cycling conditions are given below: (Table 2-10)

Table 2-10 Parameters for thermal cycles in TaqMan® assay

Step	Temperature (C)	Time (minutes)
1	50^{o}	2:00
2	95^{o}	10:00
3	60^{o}	1:00
4	Go to Step 3	41 more cycles (total of 42)
5	4^{o}	Hold

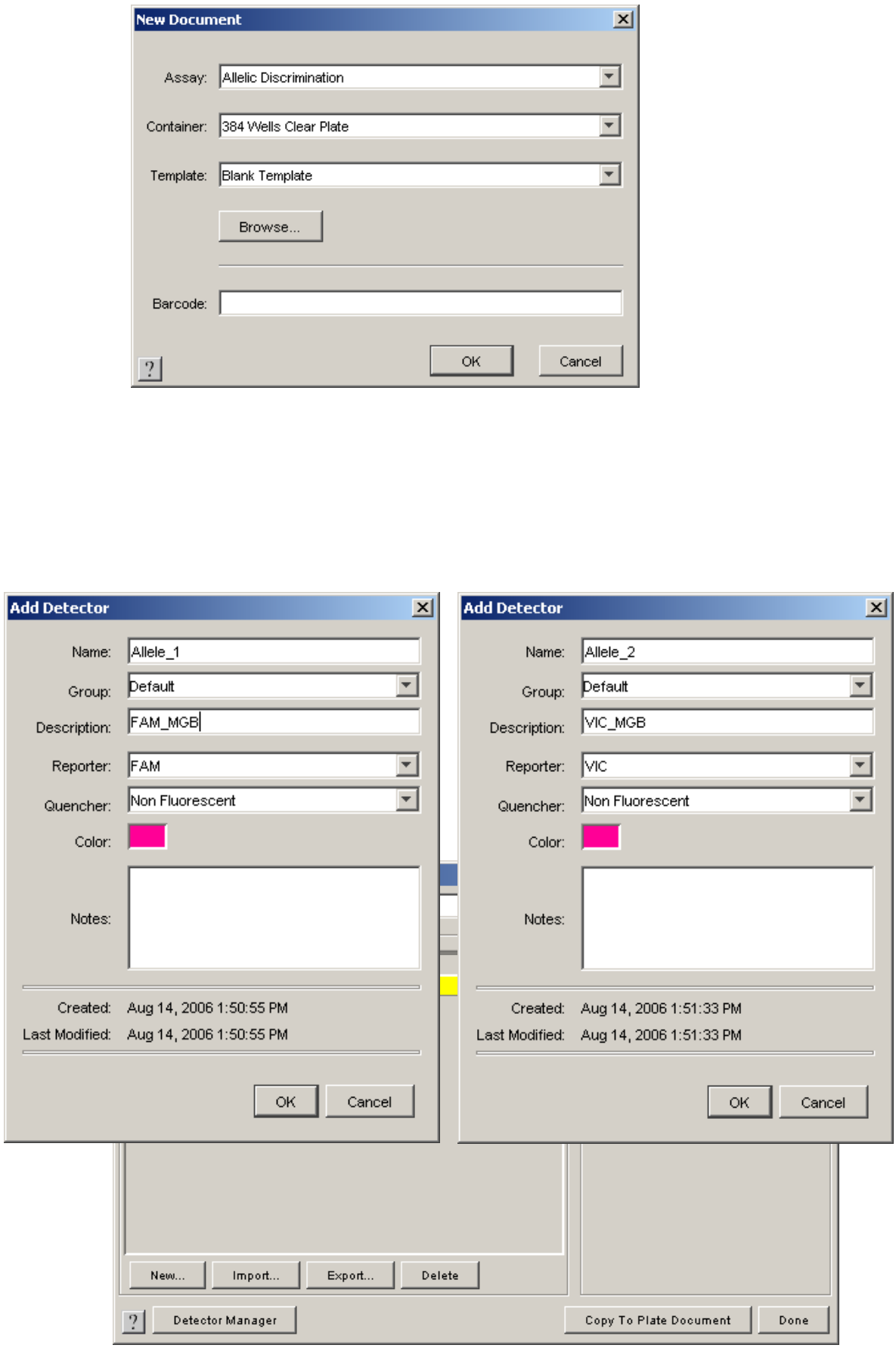
Post-PCR plates were either analyzed straight away or stored for up to 48 hours prior to reading. Plates were stored in a fridge with temperature of 4^{o} and protected from light.

2.2.2.2.7 Creating SDT Document for End-Point Read on ABI 7900

The software that controls the ABI 7900 instrument and used for analysis is called “SDS 2.X”. In order to read the fluorescence from the TaqMan® plate, the software needs to know what dye and dye channels to look for. Since for all TaqMan® assays I used FAM™ and VIC™ dyes, a template was set up to read all TaqMan® plates.

In SDS software “Allelic Discrimination” programme was used. FAM™ & VIC™ dyes were added using “detector manager” tool. A file was thus created for a specific SNP. (Figure 2-9.)

Figure 2-9 Steps used in making of TaqMan® assay file and allelic discrimination analysis



2.2.2.2.8 End Point Plate Read

Once a new desired file was created, plate was placed in the instrument block. Using plate read tool the system analysed the fluorescence in less than 2 minutes to finish whole process.

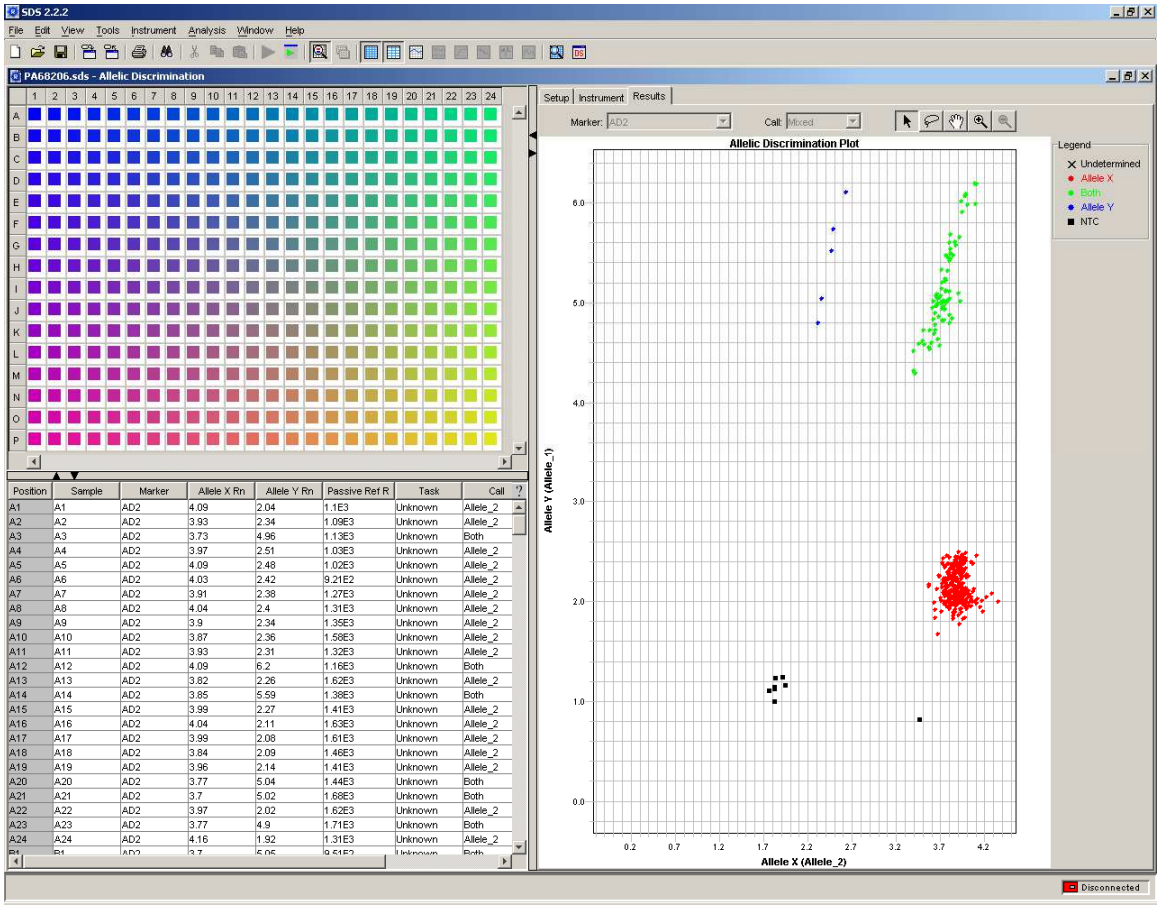
Analysis of TaqMan® SNP Genotyping Assay Data

By using “analyse data” tab the analysis was shown as a graph

The “Results” tab shows the FAM™ and VIC™ fluorescence for each well that was marked “Use”. The “Y” axis is the normalized amount of FAM™ signal (Allele_1); the “X-axis” is the normalized amount of “VIC™” signal (Allele_2) Figure 2-10.

Figure 2-10: A typical example of allelic discrimination analysis.

Homozygous samples for Allele 1 (Red Cluster) are along the “X-axis” with little VIC™ fluorescence. Homozygous samples for Allele 2 (Blue Cluster) are along the “Y-axis” with little FAM™ fluorescence. Heterozygous samples (Green Cluster) will be in between the two homozygous clusters. Negative Controls should be around the point of origin with low FAM/VIC fluorescence.



If any samples are in between any two clusters, these samples were marked as “Undetermined” and whole experiment was repeated for those samples.

Examples of various genotype plots are given below:

Figure 2-11 TaqMan® SNP Assay with Excellent Clustering

Homozygous samples for Allele 1 (Red Cluster) are along the “X-axis” with little VIC™ fluorescence. Homozygous samples for Allele 2 (Blue Cluster) are along the “Y-axis” with little FAM™ fluorescence. Heterozygous samples (Green Cluster) are in between the two homozygous clusters. Negative Controls are around the point of origin with low FAM/VIC fluorescence. Note excellent clustering of alleles.

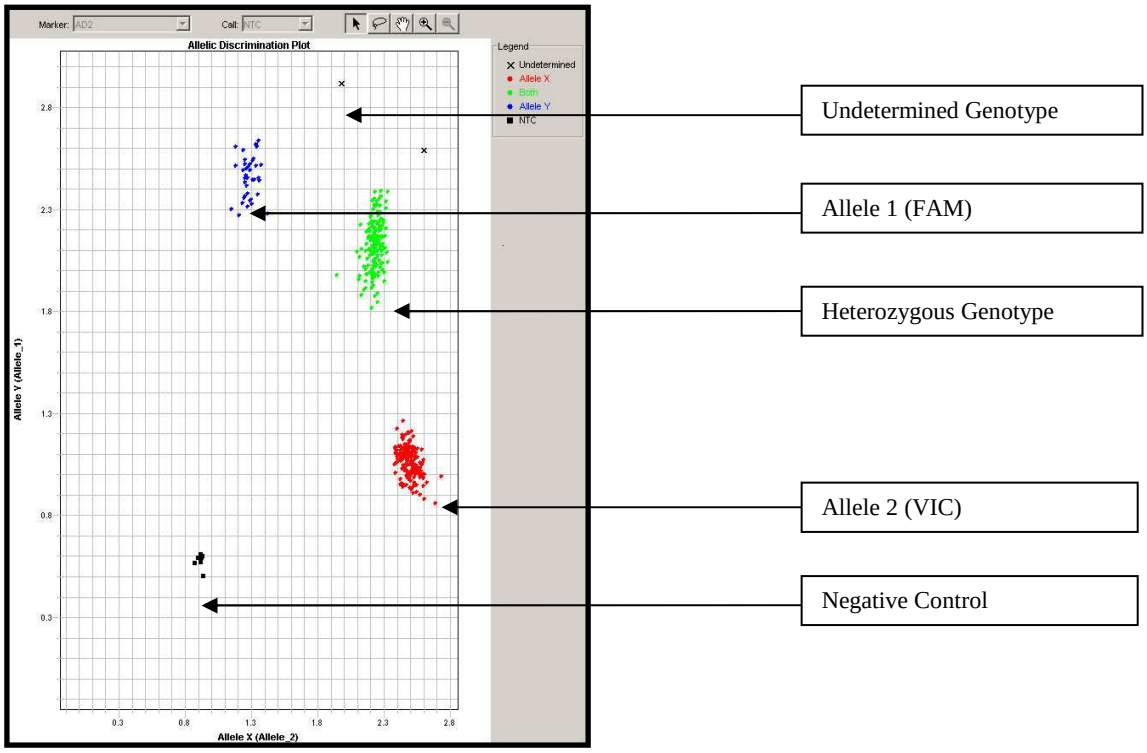


Fig 2.12 TaqMan® SNP Assay with Good Clustering (Dispersed but Genotype-able clusters)

Homozygous samples for Allele 1 (Red Cluster) are along the “X-axis” with little VIC™ fluorescence. Homozygous samples for Allele 2 (Blue Cluster) are along the “Y-axis” with little FAM™ fluorescence. Heterozygous samples (Green Cluster) are in between the two homozygous clusters. Negative Controls are around the point of origin with low FAM/VIC fluorescence.

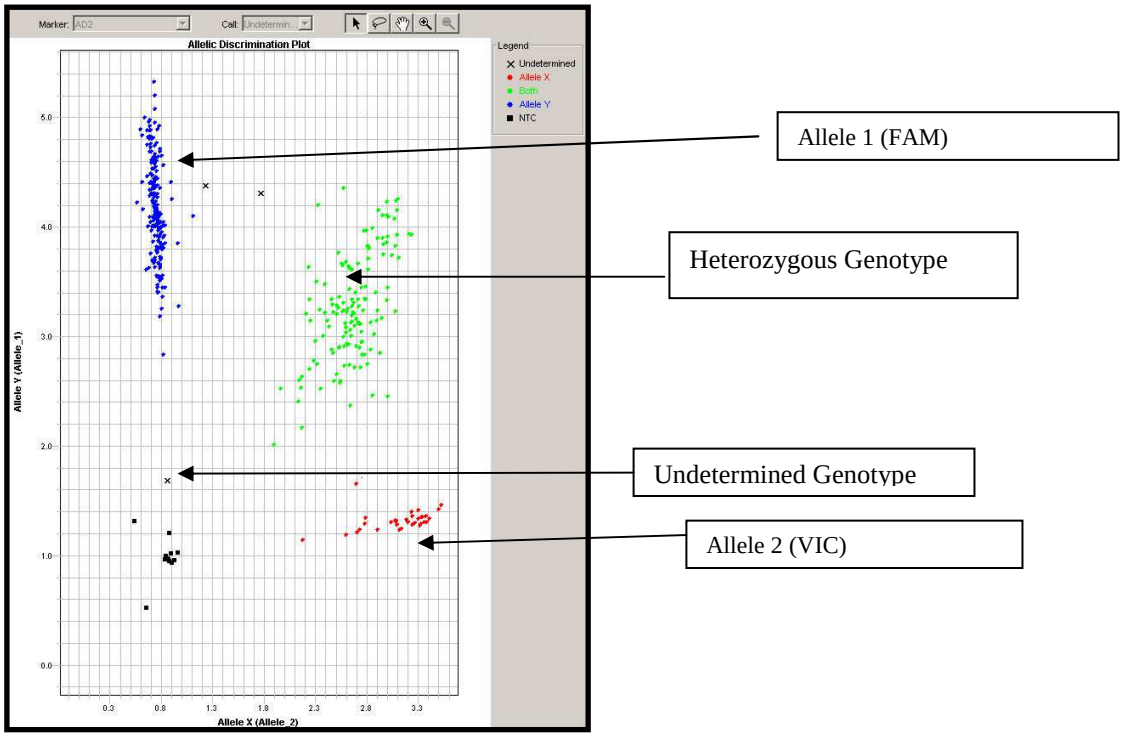
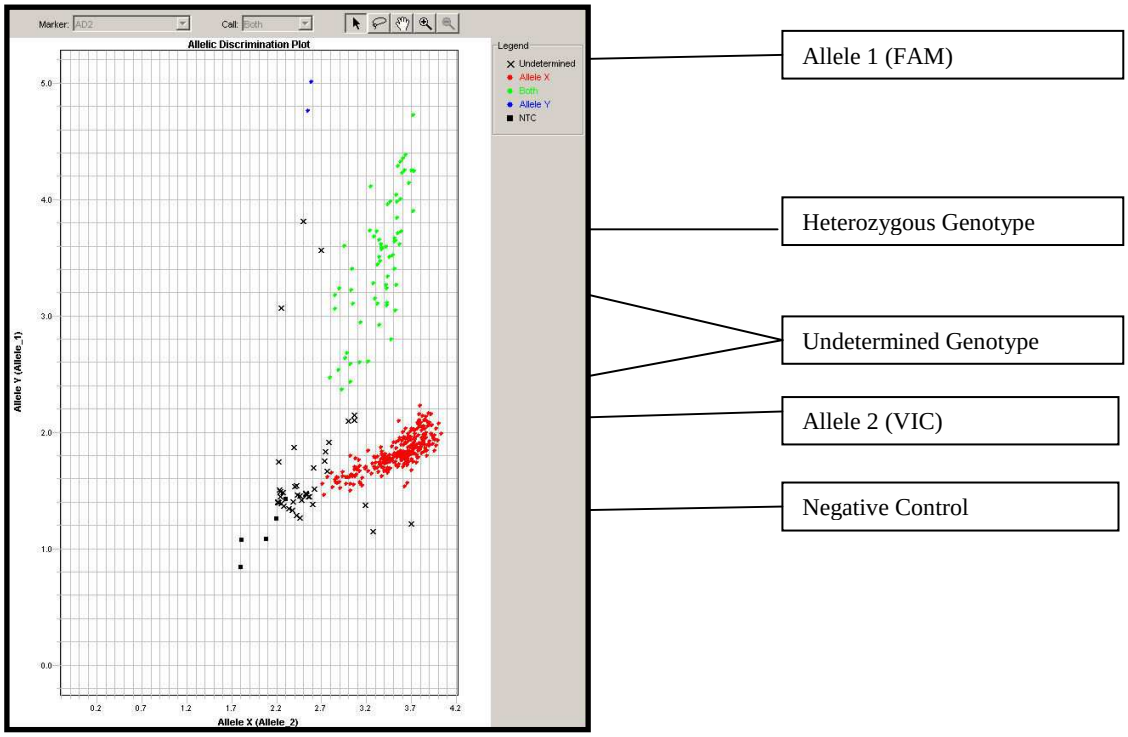


Figure 2-12 TaqMan® SNP Assay with Poor Separation between VIC™ and NTC Clusters (Un-optimized Assay)

Homozygous samples for Allele 1 (Red Cluster) are along the “X-axis” with little VIC™ fluorescence. Homozygous samples for Allele 2 (Blue Cluster) are along the “Y-axis” with little FAM™ fluorescence. Heterozygous samples (Green Cluster) are in between the two homozygous clusters. Negative Controls are around the point of origin with low FAM/VIC fluorescence.



2.3 Statistical Analysis

All the statistical methods used and hypothesis tested in the thesis are described below. Significance testing, confidence intervals, regression models were all calculated by Statistical Package for Social Sciences (SPSS Version 13.0) Chicago Illinois. It is among the most widely used programs for statistical analysis in social science. It is used by market researchers, health researchers, survey companies, government, education researchers, and others. In addition to statistical analysis, data management (case selection, file reshaping, creating derived data) and data documentation (a metadata dictionary is stored with the data) are features of the base software.

2.3.1 Hypothesis Testing

The most common ways to make an inference for the population from which the analyzed sample has been drawn are significance probability calculation or confidence interval (CI) estimation. A CI contains the result of a significance test, but a significance test cannot provide the confidence limit. The statistical tests are essentially a test of whether the confidence limits include unity.

Each experiment starts with a null hypothesis which simply states that there is no difference between the two samples. A test statistics generally relies on the comparison with the observed distribution of what is expected if the null hypothesis is true. If the null hypothesis is rejected by statistical testing, the alternative hypothesis (the effect is not zero; there is a difference, etc.) is accepted. The general test statistics is as follows:

Test statistics = (observed value - hypothesized value) / standard error of the observed value

Test statistics yield a probability of observing the value we found (or an even more extreme value) when the null hypothesis is true. If the statistics gives a P (probability of error) value of <0.05, this means that the observed value would have a probability of occurrence somewhere in the extreme 5% of the relevant distribution curve for the data and imply an unusual finding.

In significance testing, a type I error (a false-positive finding) is considered to be more serious, and therefore more important to avoid, than a type II error (missing a positive finding). If 0.05 is the pre-determined level of statistical significance, this equals to reaching one incorrect conclusion (false positive) with no biological relevance in twenty tests. The value 0.05 is the probability of getting an erroneous result every time one compares two groups, which are in fact equivalent. This level of error can be accepted in the context of a single test of a prespecified hypothesis

2.3.2 Design

Population stratification can be thought of as confounding by ethnicity. If ethnicity of cases and controls are reliably known, a stratified analysis would eliminate this problem. However, it is the unknown stratification within the population that causes this undesirable effect. Both my controls and patients were white Caucasians of United kingdom origin. This was simply determined by asking the patients about their ancestry.

In case-control studies, ideally there should be at least one control per case. If the number of cases is limited and cannot be increased easily, it may be an idea to increase the number of control to increase statistical confidence. The law of diminishing returns, however, dictates that a maximum of five controls per one case is the limit (Coggon Rose Barker 1997). A higher control to case ratio will not provide further benefit and may even result in type I errors. Since

PSU itself is a rare disease and recruiting ideal number of cases is always difficult. Hence I increased my control population as much as possible to increase the statistical confidence.

2.3.3 Statistical Power Estimates

Power calculations and expectation should be realistic in a genetic association study with a complex (multifactorial) disease. Genetic susceptibility to such diseases involves a large number of alleles, each conferring only a small genotypic risk (like odd ratio = 1.2 to 2.0), that combine additively or multiplicatively to confer a range of susceptibilities in interaction with environmental factors. Underpowered studies are one of the main reasons for failure to replicate an initial association study. (Hirschhorn et al. 2002) Therefore, if the study is adequately powered, the chance of drawing the wrong conclusion is reduced.

There are many factors that determine the power of a study:

1. The sample size: The larger the sample size, the greater power for the study. However, it is important to determine the optimal sample size, because further increases in sample size may not increase the power, but only increase the cost and time of the study.
2. The variability of the observation: The power increases as the variability decreases.
3. The effect of the interest (frequency of the allele): The power is greater for alleles that are more common.
4. The P value

Recently, more factors have been reported to influence the power of association studies; the effect size of the susceptibility locus and the extent and

distribution of linkage disequilibrium in the region (Zondervan and Cardon 2004).

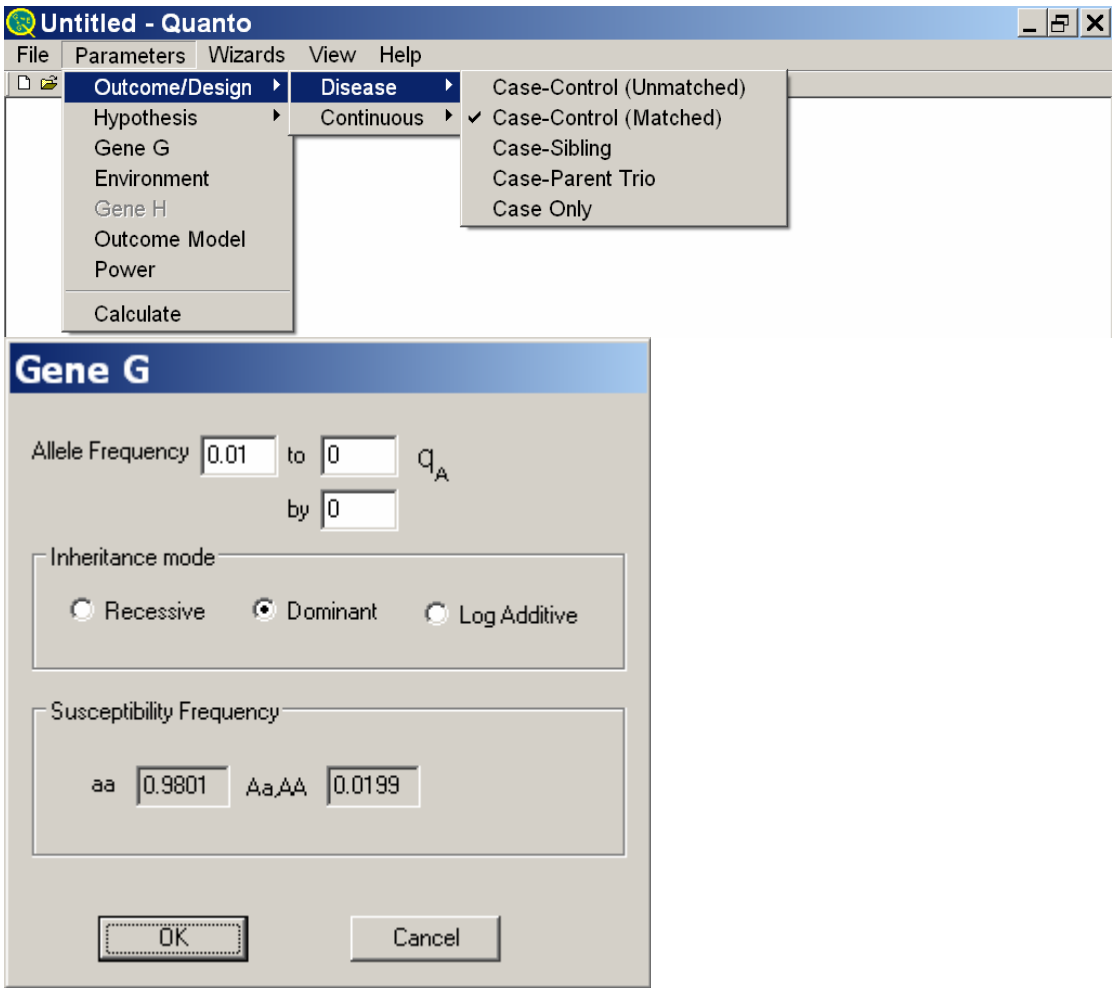
In prospective studies, the sample size calculations are performed to determine the number of subjects that will give the adequate power. However, in the retrospective studies, where the number of subjects is already given, the power to detect a certain difference afforded by the fixed sample size is determined. Since this was a study with fixed sample size, the determination of power was done retrospectively. Power calculations were performed using Quanto® Version 1.0, which is specifically designed for use in genetic studies

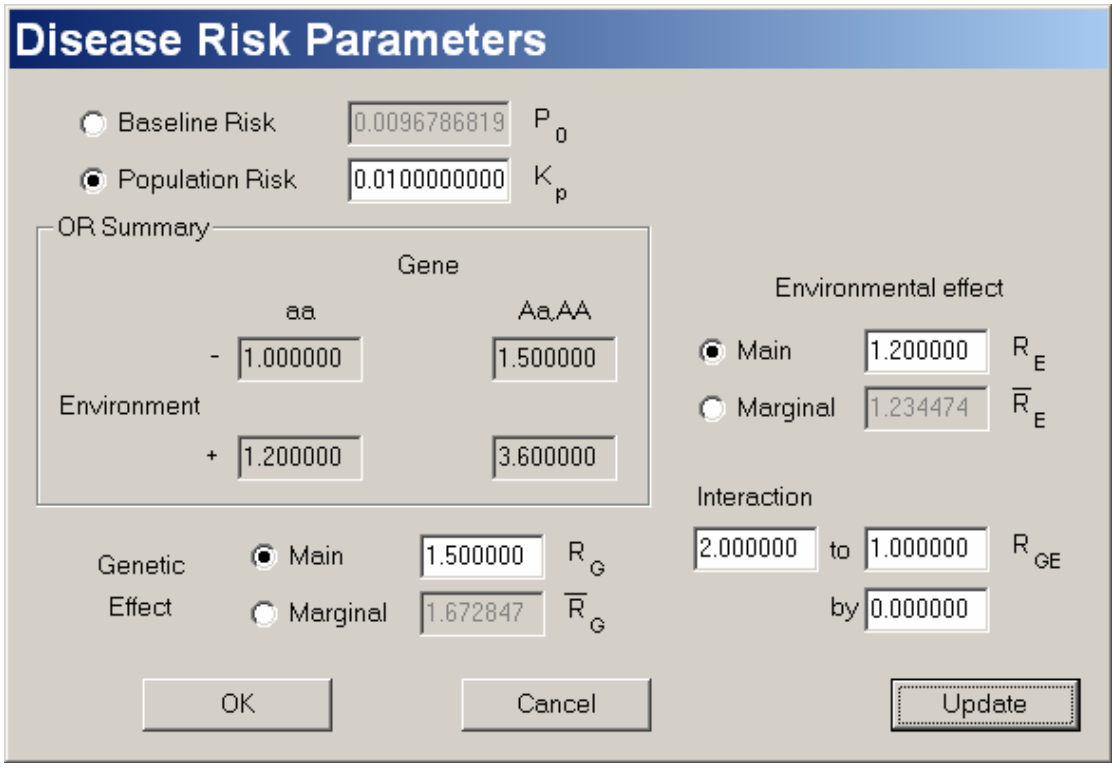
Quanto® is a program for computing either power or required sample size for association studies of genes, environmental factors, gene-environment (G×E) interaction, or gene-gene (G×G) interaction. The program is written in C++ and is designed to run under Windows. (Figure 2-13)Two types of outcomes are considered, a disease (binary) outcome and a quantitative (continuous) outcome.

Quanto® uses logistic or log-linear model to calculate the effect of gene/genes on the disease trait. There are three genetic models to select from namely, dominant, recessive and log additive. Depending upon the effect of genes, model, and whether we are testing the association with disease or its traits and assuming a case control study the software was used to calculate the power of association. Quanto® is freely available from the following website (Hydra). http://hydra.usc.edu/gxe/

Figure 2-13 Quanto® programme showing gene model and effect

Note there are three options for inheritance model selection that is Recessive, Dominant and Log Additive.





2.3.4 Multiple Testing

The P value 0.05 is the probability of getting an erroneous result every time one compares two groups, which are in fact equivalent. This level of error can be accepted in the context of a single test of a prespecified hypothesis. The probability of getting at least one statistically significant result as a result of type I error in multiple comparisons is, however, much higher than this. This probability can be calculated with the formula:

$$1-(1-a)^{n}$$

Where n is the number of comparisons and a is the arbitrarily chosen significance level which corresponds to the accepted failure rate for each comparison. If the probability of getting the right result is 0.95 (P = 0.05) in a single test, it would be $(0.95)^{20}$ = 0.36 in 20 tests. Thus, the probability of getting it wrong is 1 - 0.36 = 0.64. This is to say that, for 20 comparisons and the significance level of 0.05, the probability of getting at least one erroneous result is 0.64 (which is 0.92 for 50 comparisons). This probability is smaller when the significance level is lower (0.18 for 20 comparisons and the significance level of 0.01). This argument applies to situations when the multiple comparison tests are independent tests.

To avoid a type I error, when independent multiple comparisons are carried out, and all genotypes examined have the same chance of being increased or decreased, a statistical safeguard should be applied. There is no definite limit for the number of comparisons that makes this necessary but it is a must if it is greater than 20. This is usually done by multiplying the P value by the number of comparisons (Bonferroni inequality method). (Bland JM, 1995) This is basically lowering the statistical significance level and making the statistical test more conservative. For small numbers of comparisons (say up to five) its use is

considered to be reasonable, but for larger numbers and for the multiple tests that are not independent (highly correlated), it is highly conservative. The precise correction is obtained by the formula:

$P_{corrected} = 1 - (1-P)^{n}$

Where *P* is the uncorrected *P* value and n is the number of comparisons. The number to use for correction is not (as frequently done) the number of alleles or genotypes detected in the study but the number of comparisons one or more of which shows a significant result. For *P* values of less than 0.01, this formula gives almost the same result as simple multiplication. If the 'corrected' *P* value is still less than the pre-determined significance level (such as 0.05), then the result is significant.

There has always been a debate about what number to use in the multiplication. In contrast to the most common practice, this is not supposed to be just the number of alleles in the locus analyzed. If the allele frequencies are compared between patients and controls, the number of alleles is important as well as the number of comparisons in terms of age groups, sex groups, clinical subgroups, etc. In my work, if the SNP was not functional Bonferroni correction was applied by multiplying the p value with number of SNPs studied in that gene. In case of phenotypic traits, correction was done using multiple logistic regression.

2.3.5 Data Mining

Data mining is extracting the previous unknown, valid and actionable information from large databases and using that information to make critical decisions. KnowledgeSEEKER (KS) software (Angoss Software Corporation, Toronto, Canada) is a Windows based software application. Data mining is a key audit procedure. Genetic data and patients' clinical data were combined in

an Excel database. This database was loaded in and mined using KS to determine whether the apparent independent variables were associated.

There are many data tools to use in KS. For example, decision trees which were used to explain the relationship between several independent variables and a dependant variable and were used for analysis of the datasets in this thesis. The tree algorithm generates, for any selected node, a list of correlated variables from which a split can be chosen. The decision tree algorithms try to maximize the information gained in each split thus, KS will find the most significant split(s). Additionally, a split can be forced in order to test a particular hypothesis. Trees were grown and variables were identified in splits using a stepwise linear regression until no improvement in the association was found. However, KS was used as a preliminary step in the analysis of databases and to select variables for further classical statistical analysis.

2.3.6 Significance testing

2.3.6.1 Constructing a 2x2 contingency table

When two groups are to be compared in a case-control study, it is necessary to have a 2x2 contingency table cross-tabulating the frequencies. This table is required for significance testing, relative risk (RR) or odds ratio (OR) estimation and CI calculation. A contingency table may have more than two rows and columns (Table 2-11) but the 2x2 table approach stems from classic case-control studies in epidemiology as the most elemental data structure leading to ideas of association.

Table 2-11: Typical 2x2 table used in the thesis, showing the association and P value

Patients IN H-W	Controls IN H-W	GENE ID	*CCR2*	Locus ID	V64I

	Patients		Control		Genotype Differences		Analysis of individual positions		
GENOTYPE	Count	FREQ	Count	FREQ			Position ID	Chi2x2	p
AA	7	0.04	30	0.12	Chi2x3	15.57	11	7.98	0.00472
GA	59	0.34	110	0.43	p=	0.000417	12	3.91	0.04807
GG	110	0.63	116	0.45			22	12.35	0.00040
Total	176		256						
Allelic Carriage / phenotype									
A	66	0.38	140	0.55			1-Carriage	12.35	0.000409
G	169	0.96	226	0.88			2-Carriage	7.98	0.004725
Allelic Frequency									
A	73	0.21	170	0.33			Allele Freq	16.03	6.29E-05
G	279	0.79	342	0.67				-	
Total	352		512						

2.3.7 Choice of a statistical test

To check the statistical significance variety of tests were used depending size and type of variables.

2.3.7.1 Chi Square

The chi square test (χ^2), which is based on a large sample approximation, is a statistical test that can be used to determine whether the observed frequencies are significantly different from the expected frequencies, i.e., to determine the differences or deviations in genotype frequencies between cases and controls. Results were typically compared using a chi-square with 2x3 or 2x2

contingency tables. Below is an example of a 2x2 contingency table. (Figure 2-14)

Figure 2-14: 2 X 2 contingency table

Characteristic	Cases	Controls
Present	a	b
Absent	c	d

The numbers are entered into a table and the chi square test is concerned whether the columns (disease states) are contingent on the rows (characteristics). The chi square test statistic was calculated with the formula:

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

Where O is the observed frequency and E is the expected frequency.

2.3.7.2 Yates correction

When the Chi-squared test is used for small samples, Yates's correction may need to be used to make adjustments for continuity. The Chi-squared distribution is continuous. That means that the curve of the x^2 distribution model is continuous without any breaks. The values we calculate in x^2 are, however, discrete values. This is because observed frequencies vary in discrete units (the number of occurrences of an allele -the entries in the 2x2 table- may be 4 or 5 but not 4.6). With degrees of freedom greater than 1 and with expected frequencies of at least 5 in each cell, this is not a problem as the difference between the statistics and the true sampling distribution is so small. For example, the difference between 100 and 101 is negligible (1%) compared to

the difference between 4 and 5 (25%). Any difference between observed and expected frequencies will appear large when cell frequencies are small and may result in a type I error.

The Yates's correction helps make the discrete data generated by the test statistics [$\sum$ $(O-E)^2/E$] more closely approximate to the continuous Chi-squared distribution. This is achieved by changing the above formula to

$$[\sum (|O-E| -0.5)^2/E]$$

By doing so, the discrete data distribution and continuous data distribution are approximated better. This will result in a smaller calculated value of x^2 and will reduce the risk of a type I error. In relatively large samples, this would not make an important difference. Most textbooks recommend that the Yates's continuity correction should be applied when the sample size is small or the contingency table contains any number less than 10.

2.3.7.3 Fisher's Exact Test

When the Chi-squared test is used for small samples, Yates's correction may need to be used to make adjustments for continuity. This correction, however, does not remove the requirement for the expected frequencies. The expected frequencies in each cell (of a 2x2 table) should be at least five for the Chi ^{2} test to be reliable (note that the observed frequencies may be less than five). The general belief is that when there is one or more expected frequency of less than five, the alternative approach for significance testing is the Fisher's exact test.

The Fisher's exact test also uses the frequencies in a 2x2 table but the calculations are different from that of the other significance tests. It is based on

the observed row and column totals. The method consists of constructing all possible 2x2 tables giving the same row and column totals as the observed data. For each table, probability for such data to arise if the null hypothesis is true is calculated. Then, the overall probability of getting the observed data is calculated. To get the probability, the probability of the observed data and all other probabilities for alternative 2x2 tables equal or more extreme than that of the observed data are added up. Calculations are mathematically very complex; include factorial calculations, which may be very cumbersome for big numbers. The Fisher's exact test can now be applied by means of computers (which can cope with the calculations of factorials for large numbers) even to large samples. This test is essentially one-sided. To get the two-sided *P* value, if required, the *P* value may be doubled.

2.3.7.4 Odds Ratio (OR)

The OR is the ratio of the odds of the risk factor in a diseased group and in a non-diseased (control) group. (Table 2-12) OR is more appropriate for retrospective case-control studies.

Table 2-12 Odds ratio table

Groups	Exposed to factor	
	Yes	No
Case	a	B
Control	c	D
Total	a+c	b+d

The odds ratio was calculated as the odds of being a case in the exposed group divided by the odds of being a case in the unexposed group and is simplified to the formula below:

$$\text{Odds Ratios (OR)} = \frac{a \times d}{b \times c}$$

2.3.7.5 Confidence interval for odds ratio

When a value (OR obtained from a sample) is the estimate of an unknown "true" value (within the population from where the sample has been drawn), confidence intervals CIs can be applied to them. In the case of comparing two groups, a CI enables the researcher to see how large the difference between two proportions may be, not simply whether it is different from zero.

CIs can be calculated for different confidence levels. If a CI is calculated at a 95% level (as usually done), 5% of the time the true population parameter will not be contained within the interval calculated from the sample statistics. More technically, it means that 95% of all samples drawn from the population will have the population parameter within this interval. In a way, this is the acknowledgement of the fact that a different sample from the same population may produce a different result.

The width of the CI also gives us an idea about how uncertain we are about the unknown population parameter (the mean, for example). A very wide interval may indicate that the sample size should be increased to be more confident about the parameter.

2.3.7.6 Logistic regression:

Regression models are used when there are multiple variable that can affect the outcome. The association between the outcome (phenotype/trait) and causative/associated variable has to be corrected for these confounding factors. Simple logistic regression is used when the outcome variable is binomial (such as visual loss/ no visual loss, glaucoma absent or present). It is ideally suited for the analysis of case-control studies where the outcome is either being a case or control. The explanatory variables may be binomial, categorical or continuous (such as glaucoma yes or no, mild moderate or sever inflammation, visual acuity, etc). When the model selection is done and computation is complete, the outcome is a logistic regression equation in the following format:

Logarithm of odds = $a + b_1x_1 + b_2x_2 + ... + b_nx_n$

Here, logarithm of odds is the natural logarithm of the overall odds ratio for all variables included in the model and by using different values of the explanatory variables in the formula, different odds ratios for any combination of the values the variables can take (for example, being male or female, age etc.) can be calculated. Each coefficient (b) provides a measure of the degree of association between each variable and the outcome. This coefficient is the logarithm of the odds ratio for that variable (OR = e^b) controlled (adjusted or corrected) for the other variables in the model. It is also possible to calculate confidence intervals for the estimated odds ratio as well as the statistical significance of each coefficient. This property of logistic regression, which enables the calculation of controlled odds ratios, makes it unique in the analysis of multivariable data (Katz 2003). Model selection should not be left to the computer, which would use statistical stepwise methods to choose the best

selection, but the hypothesis and biological plausibility should be considered too.

2.3.7.7 Statistical Calculations:

As mentioned above, significance testing, confidence intervals, regression analysis were all calculated by Statistical Package for Social Sciences (SPSS Version 13.0) Chicago Illinois.

2.3.8 Genetics specific statistical calculations

2.3.8.1 Hardy Weinberg Equilibrium

To make sure that the allele frequencies are an accurate representation of the given study population, it is important to assure that there is no biased selection in the chosen population. The Hardy Weinberg Equilibrium (HWE) model compares the difference between expected and observed frequencies in a non-evolving population. Consider a population whose gene pool contains the alleles *A* and *a* and assign the letter *p* for the frequency of the dominant allele *A* and the letter *q* for the frequency of the recessive allele *a*. Since the sum of all the alleles must equal 100%, then $p+q=1$. In a diploid organism with alleles *A* and *a* at a given locus, there are three possible genotypes *AA*, *Aa* and *aa.* According to the model, the genotype frequencies are p^2 for *AA*, q^2 for *aa*, and 2(p) (q) for *Aa*. Therefore, the equation for genotype frequencies is: $p^2+ 2pq + q^2=1$.

The frequencies of *A* and *a* will remain unchanged generation after generation if the basic assumptions of the HWE model are met: 1) the population is large; 2) there is no population admixture 3) mutations are negligible; 4) individuals

are randomly mating; and 5) the natural selection is not operating on the population (i.e., the population is not evolving).

Normal or control populations should fit the HWE, and the deviation from HWE could indicate a population bias or a genotyping error. By contrast, patients are a selected group and do not necessarily conform to all the conditions of HWE. Thus, it is possible for case populations to be out of HWE, if there is a strong genetic link with the disease. However, this is a rare occurrence, especially within complex genetic diseases. Therefore, it is important to check for HWE.

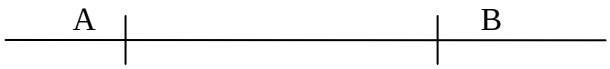
To determine whether a population is in HWE model or deviated from the model, allele frequencies are determined by counting the number of observations of an allele and dividing this by the total number of chromosomes sampled (i.e., two times the number of individual studied). The expected genotype frequencies (number of homozygotes and heterozygotes) are then calculated from these allele frequencies using the above equation. This expected genotype distribution is compared with the observed genotype using chi-square test. Non-significant ($p<0.05$) differences between expected and observed genotype frequencies indicate that the study population is in HWE, and vice versa. An in-house Microsoft Excel calculation (a kind gift from Dr P Panteledis) was used to calculate the chi-square and identify any population with significant deviation from HWE.

2.3.8.2 Linkage Disequilibrium (LD) Calculations

Various statistical methods can be used to measure the LD. The two main measures are the absolute value of *D`* and the $r^2$, both of which are based on the pair wise-disequilibrium coefficient *D (Lewontin 1988)*. The *D`* measure is

illustrated in Figure 2-15. If we assume that we have two markers A and B. *D* is the difference between the observed haplotype frequency A-B, and the frequency that would be expected to have if the alleles were randomly segregated [*f* (A1)*f* (B1)], where A and B have alleles labeled 1 and 2 and *f* is their frequency. *D*` is calculated by dividing D with D_{max} (the maximum D is possible for a given set of allelic frequencies at the two loci).

Figure 2-15 Illustration of *D*` measure



$$D = x1 - f(A1)\, f(B1)$$
$$D` = D/D_{max}$$

Assume we have two loci, A and B, which are located close to each other in the same chromosome. The term x1 is the observed haplotype frequency of the haplotype A-B. f (A1) is the frequency of allele A1 and f (B1) the frequency of allele B1.

The measure r^2 represents the statistical correlation between two sites and it is described as shown in Figure 2-16.

Figure 2-16 Formula for calculating r^2

$$r^2 = \frac{D^2}{f(A1)\, f(A2)\, f(B1)\, f(B2)}$$

2.3.8.3 Haplotyping Methods

Most current methods for obtaining genotyping data test each SNP independently. This means that the primary data will be an un-phased, not indicating how the alleles are associated in a heterozygous individual. Studying

phased data will give higher power for assigning a phenotype to a genetic region. Unfortunately, haplotypes are costly to be directly measured (Crawford and Nickerson 2005). Therefore, most investigators rely on the computerized statistical methods, using the knowledge of LD between markers, to re-construct maximum likelihood haplotypes from un-phased data.

Haplotyping Using Computerized Methods

During the course of this thesis, I used two computerized algorithms to re-construct un-phased data in order to predict the most probable haplotypes.

2.3.8.3.1.1 Expectation-Maximization Algorithm

The Expectation-Maximization (EM) algorithm is likelihood based method that assumes that alleles are in HWE. It then re-constructs and estimates haplotype frequencies using the genotype data (Excoffier and Slatkin 1995). It starts filling a preliminary list of haplotypes, which are inferred from a fixed starting position by examining the complete homozygotes and single-site heterozygotes. Then other individuals in the sample are screened for the possible occurrence of the previous recognized haplotypes (Excoffier and Slatkin 1995). The mathematics behind the EM is based on probability statistics and detailed discussion of its derivation is beyond the scope of this thesis. The accuracy of EM algorithm has been tested by Fallin and Schork who showed that haplotype frequency estimates via the EM algorithm do not deviate more than 5% compared with their true value for sample sizes more than 100 (Fallin and Schork 2000).

Departure from HWE may be a substantial source of error in EM algorithm because EM simply relies on HWE. In addition, the algorithm cannot handle missing genotypes and tends to assign new haplotypes (assuming nulls) when missing data is present. This was avoided by ensuring full genetic data was

generated on all samples. Finally, EM does not assign actual haplotype to individual. However, it was used to calculate intra-gene haplotypes and pair wise haplotype frequencies, which is required in the subsequent calculation of D'. (See calculation above)

A number of software packages perform the EM algorithm. Arlequin® was used to calculate haplotype frequencies and compute LD statistics. Arlequin® can be freely downloaded from http://anthro.unige.ch/arlequin/.

2.3.8.3.1.2 Bayesian Methods

The Bayesian methods for haplotype assignment treat the unknown haplotypes as random quantities and combine two aspects; the prior distributions (the patterns of haplotypes expected to be observed in population samples) with the information actually observed in the data (the likelihood). Then, the haplotype frequencies are calculated (Stephens and Donnelly 2003).

PHASE is a program that implements the Bayesian method for reconstructing haplotypes from un-phased population genotype data (Stephens, Smith, and Donnelly 2001). PHASE determines haplotypes by repeatedly selecting an un-phased genotype in a random way. Then it estimates individual's haplotype whilst assumes that all the other haplotypes are correctly re-constructed. The computational algorithm behind PHASE is Gibbs sampling, which is a type of Markov chain-Monte Carlo (MCMC) algorithm (details are not included in this thesis). It is similar to the EM algorithm but predicts the structure of the next sample haplotype by comparing it with the haplotypes already assigned rather than to the genotypes. In addition, PHASE assigns haplotypes to each individual and can handle missing data. However, PHASE assumes a complete

linkage between markers and so cannot be used in large genetic distances (greater than 100kb or less if recombination hotspots are present). PHASE is freely available to download from:

http://www.stat.washington.edu/stephens/software.html.

2.3.9 Prediction of Functional effects of SNPs

A major challenge in undertaking candidate gene disease association studies is to choose target SNPs that are most likely to affect disease phenotype and that ultimately contribute to disease development. Determining which polymorphisms are functional and concentrating on these offers great advantages and may be more fruitful than random disease associations on all SNPs, though this remains controversial (Risch 2000).

Since this work focused on the effects of functional SNPs on PSU phenotype, hence most of the SNPs tested in my research were functional. However few SNPs with no known functional effects were studied as well either because of their associations with different immune mediated diseases or they lie in close proximity to functional SNPs I studied. If any of the “non-functional” SNPs were noted to have any significant association, bioinformatics tools were used to detect any potential deleterious effects as a result of the SNP.

Two main online bioinformatics tools were used

1) Function Analysis and selection tool for single nucleotide polymorphisms (FASTSNP)
2) Transcription Element Search System (TESS)

2.3.9.1 FASTSNP

By accessing a variety of heterogeneous biological databases and analytical tools, FASTSNP identifies SNPs that are most likely to have functional effects. (Yuan et al. 2006) FASTSNP provides SNP prioritization services based on the information collected from the Internet at query time. The priorization results are based on the predicted functional effects and their estimated risk proposed by Tabor et al. (Tabor, Risch, and Myers 2002) For example the following things are taken into account as potential functional effects:

a) Premature translation termination (nonsense; stop codon gained)

b) Exons that cause an amino acid change (non-synonymous)

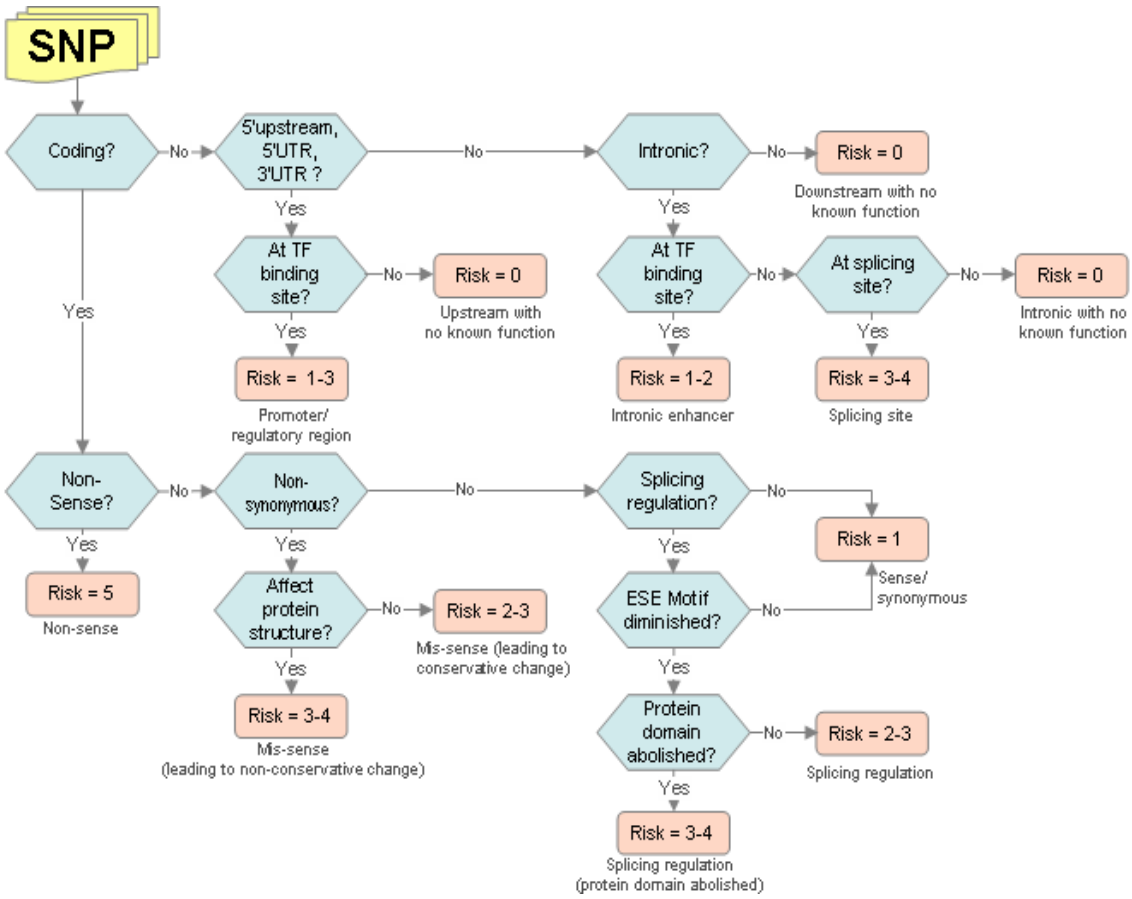
c) Splicing sites

d) Transcription factor binding sites at promoter regions.

e) Exonic splicing enhancer motifs

f) Exonic splicing silencer motifs and

g) 3utr post-transcriptional regulatory region.



Ranking	Risk definition
0	No effect
1	Very low
2	Low
3	Medium
4	High
5	Very High

Figure 2-17: The "FASTSNP" SNP prioritization decision tree

The SNP prioritization result is based on the predicted functional effects and their estimated risk proposed by Tabor *et al.* in Nat Rev Genet. (Tabor, Risch, and Myers 2002) (Copied from FASTSNP website http://fastsnp.ibms.sinica.edu.tw) Institute of Biomedical Sciences and Institute of Information Science, Academia Sinica. Taipei 115, Taiwan

FASTSNP accesses following online databases to gather and analyse the information before presenting the results.

---

1) NCBI dbSNP
http://www.ncbi.nlm.nih.gov/SNP
Provides the location of a SNP in a gene and its alleles, allele frequency, and context sequence.

2) Ensembl
http://www.ensembl.org
Provides a cross-reference/alternative data source for dbSNP. Also provides alternative transcripts and protein domain information.

3) TFSearch
http://www.cbrc.jp/research/db/ TFSEARCH.html
Predicts if a non-coding SNP alters the transcription factor binding site of a gene.

4) PolyPhen
http://www.bork.embl-heidelberg.de/PolyPhen
Predicts if a non-synonymous SNP alters an amino acid in a protein resulting in structural changes (damaged or benign) in a protein.

5) ESEfinder
http://rulai.cshl.edu/ESE
Predicts if a synonymous SNP is located in an exonic splicing enhancer motif, which would diminish the motif with a different allele.

6) Rescue-ESE
http://genes.mit.edu/burgelab/rescue-ese
Provides a cross-reference/alternative data source for ESEfinder.

7) NCBI GeneBank
http://www.ncbi.nlm.nih.gov/Genbank
Provides all spliced form mRNAs and their translated proteins of the gene sequence.

8) SwissProt
http://us.expasy.org/sprot
Provides the information about protein domains to determine if a SNP causes an alternative splicing that leads to a protein domain being abolished.

09) UCSC Golden Path
http://genome.ucsc.edu
Provides information about the final draft assembly of the genome sequence (i.e., Golden Path) for quality control of candidate SNPs.

10) NCBI Blast
http://www.ncbi.nlm.nih.gov/BLAST
Sequence comparison and search tool for quality control of candidate SNPs

11) HapMap
http://www.hapmap.org/
Provides information about the haplotype and linkage disequilibrium around a SNP.

12) FAS-ESS
http://genes.mit.edu/fas-ess/
Predicts whether a coding SNP will abolish exonic splicing silencer motifs

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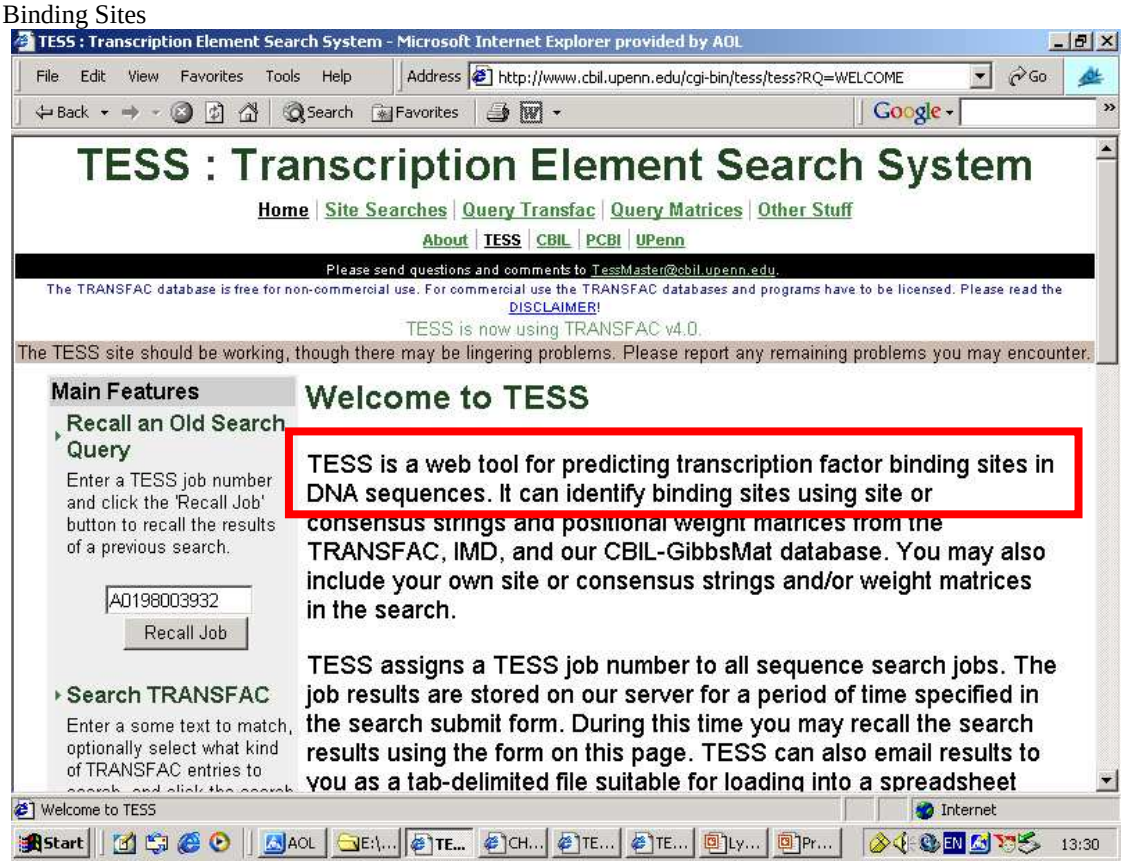
2.3.9.2 TESS

Advances in Bioinformatics give a great opportunity for researchers to select functional SNPs in the promoter region. TESS (Transcription element search system) software predicts transcription factor binding sites (TFBS) in a given DNA sequence (http://www.cbil.upenn.edu/tess/). It uses TRANSFAC ®, IMD, and CBIL-GibbsMat databases to identify these TFBS (Wingender 2004). With the increasing number of reported regulatory SNPs, our strategy should be to examine all known regulatory SNPs by TESS and select the putative functional SNPs. If any of these SNPs were found to have significant association TESS software was used to determine whether the change in allele had any putative effect on transcription of gene.

Figure 2-18: Transcription Element Search system website

TESS uses TRANSFAC, IMD, and CBIL-GibbsMat databases to identify these Transcription Factors Binding Sites



Chapter Three

3 Chemokine gene polymorphism in posterior segment uveitis

3.1 Introduction

3.1.1 Chemokines and their role in inflammation

The salient feature of inflammation is infiltration of leukocytes into the site. The maintenance of leukocyte recruitment during inflammation requires intercellular communication between infiltrating leukocytes and the endothelium, resident stromal and parenchymal cells. These events are mediated via the expression of cell-surface adhesion molecules, and the production of chemotactic molecules, known as chemokines.(Springer 1994) They are polypeptides that act as potent chemotactic factors for neutrophils, eosinophils, basophils, monocytes, mast cells, dendritic cells, NK cells, and T and B lymphocytes. (Sallusto, Mackay, and Lanzavecchia 2000) Chemokines are often differentially induced in response to different stimuli and it is though that this is important in recruiting appropriate effector cells to the site of inflammation, including the differential recruitment of Th1 and Th2 cells. (O'Garra, McEvoy, and Zlotnik 1998)

Chemically they are a group of low molecular weight (~ 8-14 kDa) structurally related proteins and so far 40 different types of chemokines have been discovered. Based on the configuration of cysteine residues near the NH_2-terminus of the proteins, they are divided into 4 types: CXC (α), CC (β), C (γ) and CX_3C (δ). (Rollins 1997)

CXC chemokines are, in general, clustered on human chromosome 4 and exhibit between 20% to 50% homology on the amino acid level. CC chemokines are, in general, clustered on human chromosome 17 and exhibit between 28% to 45% homology on the amino acid level. The one C chemokine, lymphotactin, is located on human chromosome 1, and the one CX_3C,

fractalkine, is located on human chromosome 16. Main chemokines and their groups are shown in Table 3-1

Table 3-1: **Common Chemokines according to their groups**

Subgroup	Chromosome	common Members
CXC	4q12-q13	GROα, GROβ, GROγ, IL-8, IP-10
CC	17q11.2	MCP-1, RANTES, MIP-1α, MIP-1β
C	1q23	Lymphotactin,
CX3C	16q13	Fractalkine

GRO: Growth related oncogene, MIP: Macrophage inhibitory protein, IP: Inducible protein.

Chemokines are produced by virtually all somatic cells including monocytes, macrophages, neutrophils, platelets, mast cells, T and B lymphocytes, NK cells, keratinocytes, mesangial cells, epithelial cells, fibroblasts and endothelial cells. These cells can produce chemokines in response to a variety of factors, including viruses, bacterial products, IL-1, TNF, Complement 5a (C5a), Leukotriene B4, and IFNs. (Baggiolini, Loetscher, and Moser 1995) They are not expressed in most resting cells but are rapidly up regulated on activation. The production of chemokines by both immune and non-immune cells shows that these molecules may play a pivotal role in organising chronic inflammation. Chemokines mediate their actions via specific cell-surface receptors that are member of rhodopsin superfamily of seven transmembrane-spanning G-protein-linked molecules. (Ben-Baruch, Michiel, and Oppenheim 1995) All of these receptors are comprised of approximately 350 amino acids and a molecular weight of around 40kDa. The receptors can also be grouped into four major families, CR, CCR, CXCR and CX3CR, which interact with C, CC, CXC and CX3C chemokines respectively.

Chemokine-receptor interactions can be either exclusive (e.g. high affinity binding of IL-8 to its receptor) or promiscuous. (Rajagopalan and Rajarathnam 2004) For instance, all four monocytes chemotactic proteins interact with *CCR2*, and at least MCP-2, MCP-3 and MCP-4 also recognize other receptors (CCR1 and CCR3).

Main chemokine receptors their ligands and expression on different leucocytes in shown in the Table 3-2

Table 3-2: Expression of chemokine receptors in leukocyte populations

Receptor	Main ligands	Main cells
CCR1	MCP-3, RANTES, MIP-1	Mo, T, NK, iDC, Neu
CCR2 B/A	MCP-1–4	Mo, T (act.) NK (act.)
CCR3	Eotaxin-1–3, MCP-3, RANTES	Eo, Ba, T (Th2)
CCR4	TARC, MDC	Th2, NK, iDC
CCR5	MIP-1b, MIP-1a, RANTES	Mo, Th1, iDC
CCR6	MIP-3a/LARC/Exodus	T, iDC (CD34)
CCR7	ELC/MIP-3	T, Mo, mDC
CCR8	I309, TARC	T cells, Mo
CXCR1	IL-8, GCP-2	Neutrophils
CXCR2	IL-8, GROs, NAP-2	Neutrophils
CXCR3	IP-10, MIG, ITAC	T h1
CXCR4	SDF-1	Widely expressed receptor
CXCR5	BCA-1	B
CX3CR1	Fractalkine	Mo, NK, T

Mo: Monocytes, NK: Natural killer cells, Neu: Neutrophils, Eo: Eosinophils, iDC: Immature Dendritic cells, mDC: Mature Dendritic cells, T: T cells, act: activated, BCA: B cell attracting, SDF: stromal cell derived factor.

In 2003, the international union of immunological societies (IUIS) in collaboration with WHO recommended the classification and nomenclature for chemokine and chemokine receptors. (Chemokine/chemokine receptor nomenclature 2003) This is shown in Table 8-1, Appendix B. The committee suggested systematic names for chemokine ligands and receptors starting from 1(e.g. CCL1, CCL2…..CCR1, CCR2…..etc)

As seen in the Table 8-1, systematic name for MCP-1 ligand is CCL2 and RANTES is labelled as CCL5. In my thesis I have continued using the name of

ligands rather than systematic name, as have many of the published studies in medical literature.

Chemokines can also be divided broadly into two categories: inducible chemokines that recruit leukocytes in response to physiological stress and constitutive chemokines that are responsible for basal leukocyte trafficking and forming the architecture of secondary lymphoid organs. Expression of inducible chemokines can be elicited by almost any stimulus that alters cellular homeostasis and mRNA encoding induced chemokines can increase over 300-fold within a few hours of activation. However, their inducibility and high expression also create the potential for persistent or inappropriately expression. In addition, collateral damage brought by activated leukocytes may not dampen the chemokine stimulus that initiated their recruitment. In fact, leukocyte-mediated injury may simply induce higher expression of these or even new chemokines, creating a feed forward scenario, that result in more extensive tissue damage. (Gerard and Rollins 2001) This role of chemokines is particularly seen in chronic immune mediated disease like multiple sclerosis, rheumatoid arthritis, graft rejection diseases and models. (Gerard and Rollins 2001) (MacDermott 1999) (Bodolay et al. 2002) (Dogan and Karpus 2004)

3.1.2 Role of chemokines in uveitis

In-vitro studies, animal experiments and studies on human subjects have provided considerable evidence to believe chemokines and their receptors play a key role in leukocyte recruitment into the eye in uveitis.

3.1.2.1 Evidence from in-vitro studies

The blood retinal barrier is maintained by RPE cells and retinal vascular endothelial cells. In the case of inflammation, the first chemotactic signals and

events should theoretically start at this barrier level. Various in-vitro studies have now shown that RPE cells are able to produce chemokines after being stimulated by cytokines. Elner *et al* demonstrated in 1990 that cytokine stimulated RPE cells were able to express IL-8 (Elner et al. 1990). The same group later showed that T-lymphocyte secretions induced *IL-8* and *MCP-1* gene expression and secretion in human RPE. They noted TNF and IFN-γ were necessary components of T-lymphocyte conditioned media for the induction of RPE *IL-8* and *MCP-1*. (Elner et al. 1997a) In another study the primary culture of RPE cells was stimulated with various cytokines. The mRNAs were isolated from the cells for Northern blot analysis. RPE showed increased *IL-8* and *MCP-1* gene expression. Gene expression and protein production of *IL-8* and *MCP-1* was also found to be stimulated by IL-1β and TNF-α. (Bian et al. 1999) Similarly Crane at el showed that RANTES was produced in vitro by RPE cultured cells in response to the pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ and proposed a role of RANTES in the development of the inflammatory eye disease endogenous posterior uveitis (Crane et al. 1998)

Cultured RPE cells were stimulated with IL-1β or TNF-α without and with dexamethasone or with cyclosporine-A. Secreted levels of RPE MCP-1 and IL-8 were measured in the media using (ELISA). Both *MCP-1* and *IL-8* mRNA were analyzed by Northern blot. Results showed that both TNF-α and IL-1β induced expression of *MCP-1* and *IL-8* mRNA. IL-1β-induced chemokine secretion was sensitive to dexamethasone, whereas *MCP-1* and *IL-8* induced by *TNF-α* were inhibited by cyclosporine-A. (Kurtz et al. 1997)

Along with RPE, retinal microvascular endothelial cells form the Blood retinal barrier. Studies have shown that these endothelial cells have the ability to

produce significant amounts of some of the major inflammatory chemokines, particularly MCP-1, RANTES, IL-8 and GROα. (Crane et al. 2000)

To attract the leucocytes, the chemokine ligands that are released by RPE cells should then bind to receptors on these inflammatory cells. In vitro studies have given us insight into how different chemokine receptors are up regulated on effector cells in uveitis.

Klitgaard *et al.* studied the expression of chemokine receptors on peripheral blood mononuclear cells using flow cytometry in 10 patients with acute anterior uveitis and in 10 healthy controls. They found a highly significant up-regulation of *CCR5* on CD4+ T-cells in the blood of patients with AU compared to in healthy controls. (Klitgaard, Ogard, and Krogh 2004) Qiao measured CXCR1(IL-8R1) and CXCR2 (IL-8R2) levels on the circulating leukocytes of patients suffering from ocular BD. CXCR2 expression in relapsing phase was significantly higher than in remission-phase patients or normal individuals. (Qiao et al. 2005)

3.1.2.2 Evidence from animal models of uveitis

Most of the evidence for the role of chemokines in uveitis comes from animal models. During inflammation IL-8 (CXCL8) is released from stimulated endothelial cells and facilitates transmigration of polymorphonuclear cell. In normal mice treated with anti-CXCL8 antibody, or in murine IL-8 receptor homologue deficient mice (mIL-8Rh-/-) challenged with endotoxin injected into the vitreous cavity, the number of infiltrating cells was greatly reduced compared to wild-type mice. (Becker et al. 2000) (Verma et al. 1999)

Recent studies in EAU have shown that *MCP-1*, *MIP-1*α and *RANTES* were most strongly expressed at the peak of disease in retinal tissue. (Crane et al. 2001)

Lewis rats injected with myelin basic protein (MBP) also develop anterior uveitis, (AU) which coincides with the onset of experimental autoimmune encephalomyelitis (EAE). This model is known as “anterior uveitis with EAE”. Initially, MCP-1 is detected pre-clinically in the iris and ciliary body as well as the spinal cord, and mRNA expression correlated with increasing disease severity. These results imply that MCP-1 contributes to the initial recruitment of inflammatory cells into both the eye and the CNS (Adamus et al. 1997)

Anterior uveitis can also occur in response to MBP in mice, and analysis of gene expression found increased levels of *RANTES, MCP-1* and *MIP-1α* at the onset of clinical AU. *MIP-1β* was highest at the peak of AU. *CCR2* was present at comparable levels throughout disease, while *CCR3* and *CCR5* were greatest at peak disease, and *CCR1* was highest at onset. Treatment with anti-MIP-1β or anti-MCP-1 delayed onset and reduced duration of AU, while anti-RANTES had no effect on disease. T cells appear to be the main source of these chemokines (Adamus, Manczak, and Machnicki 2001)

On recovery from EAE, animals are resistant to EAE but develop recurrent AU in response to a second challenge with MBP. *MCP-1* mRNA expression was raised in the iris/ ciliary body of rats at the pre-clinical stage of the recurrence of AU and remained high through to the recovery phase. *MIP-1*β and *RANTES* mRNA were also seen throughout the course of recurrent AU with *RANTES* peaking at the pre-clinical stage while *MIP-1*β was highest at the peak of disease. *MIP-1*α mRNA was first seen at the onset of recurrent AU, and decreased at peak disease. Anti- MIP-1α, and particularly -MIP-1β suppressed

the development of recurrent AU (Manczak et al. 2002). Anti- RANTES was not effective in protecting against recurrent AU. (Manczak et al. 2002)

3.1.2.3 Chemokines levels in patients with uveitis

Further confirmation of the roles of chemokines in uveitis can be derived from studies on Human subjects with active disease

Elevated levels of IL-8 have been found in the serum of patients with idiopathic AU (Klok et al. 1998). In a further study, aqueous humour was collected from patients with idiopathic AU and IL-8 was detected in 50% of patients with active untreated AU, but was not detected in quiescent disease. IP-10 levels were significantly greater in patients with active disease compared to non-active, as was MCP-1, which was undetected in non-active patients, and MIP-1β and RANTES were both higher in patients with ocular disease but remained at very low levels (Verma et al. 1997). A correlation between disease activity and levels of MCP-1 and MIP-1 β in serum has been demonstrated as both were controlled by treatment with prednisolone in patients with retinal vasculitis. (Wallace et al. 2003) Kramer *et al* measured serum levels of IL-8 in 25 patients during active uveitis and uveitis in remission and compared to age-matched controls. Levels of IL-8 were significantly elevated in patients with active disease and were decreased during remission. (Kramer et al. 2007). Similarly, Belguendouz *et al.* measured high levels of serum IL-8 in patients with Behcet's uveitis and idiopathic uveitis. In both groups corticosteroid therapy significantly reduced serum IL-8 levels. (Belguendouz et al. 2008) Serum levels of IL-8 were higher in active Behcet's disease compared to inactive disease and were noted to be indicator of early vascular involvement (Durmazlar et al. 2009). Sijssens

et al analysed aqueous levels of chemokines using multiplex immunoassay in children with uveitis and found high levels of RANTES, IL-8 and IP-10 (Sijssens et al. 2007). Yoshida et al measured aqueous MCP-1 levels by using ELISA technique in 31 patients with uveitis and found significantly higher levels of MCP-1 in patients with infectious and non-infectious uveitis compared to controls. (Yoshida et al. 2008) Similarly higher aqueous levels of MCP-1, RANTES and IL-8 were seen in active intermediate uveitis compared to levels in disease under remission. (Valentincic et al. 2011)

Takeuchi et al measured serum levels of chemokines by cytometric bead array using flow cytometry and found levels of CXCL9 and IP-10 were elevated markedly in the patients with ocular sarcoidosis and correlated with ocular disease activity and ACE level. (Takeuchi et al. 2006)

Klitgaard *et al*. studied the peripheral expression of chemokine receptors and early activation markers using flow cytometry in 10 patients with acute anterior uveitis and in 10 healthy controls. They found a highly significant up-regulation of *CCR5* on CD4+ T-cells in the blood of patients with AU compared to in healthy controls. (Klitgaard, Ogard, and Krogh 2004)

In another study, correlation between disease activity and levels of RANTES and MIP-1β in serum was demonstrated and levels were decreased by treatment with prednisolone in patients with retinal vasculitis. (Wallace et al. 2003)

These and similar studies have increased our understanding about the role of chemokines in uveitis, however the relationship is very complex and complicated by the fact that there is lot of redundancy and overlap in chemokines system. However it does appear that IL-8 MCP-1, RANTES, MIP-1

and IP-10 play significant role in recruiting monocytes and lymphocytes to the site of inflammation in uveitis.

Aims:

I hypothesized that genetic variability in genes encoding these chemokine molecules may play a role in the development and susceptibility of PSU. The aim of this study was to determine whether functional single nucleotide polymorphisms in these chemokine genes involved in uveitis would predict the disease severity and response to the treatment. As discussed in detail, (Risch and Merikangas 1996) studying functionally significant polymorphisms rather than random polymorphisms in the gene of interest offers considerable advantages in terms of detecting disease associated genes.

3.2 Methods

3.2.1 Experimental populations

3.2.1.1 Patients:

DNA of 205 Caucasian patients who attended the uveitis clinic at Moorfield's eye hospital were available for this study. Clinical phenotyping was done as discussed in detail in chapter 2. The patients were categorized into 2 main groups which were further sub divided into two.

A) Idiopathic posterior segment uveitis

1) Idiopathic intermediate uveitis
2) Idiopathic posterior uveitis

B) Uveitis associated with systemic diseases

3) Behcet's uveitis
4) Sarcoidosis uveitis

3.2.1.2 Controls

1) 169 healthy subjects who were admitted to WXH for cataract surgery and
2) 114 healthy subjects who were seen at KCH eye department for age related macular degeneration.

3.2.2 SNP Selection

After extensive literature search, functional polymorphisms in the chemokine genes involved in uveitis pathogenesis were gathered. Where the functional polymorphisms were not detected (in case of IL-8 receptors) any polymorphisms that had been associated with immune mediated disease was selected.

As a result six chemokines genes were selected because they have been shown to be significantly involved in the pathogenesis of uveitis and secondly SNPs in their genes have been found to be functional and associated with other autoimmune diseases. These were *MCP-1* and its receptor *CCR2*, *RANTES* and its receptor *CCR5* and *IL-8* and its receptor *CXCR-2 (IL-8Rb).*

3.2.2.1 MCP-1 SNPs

The gene encoding for *MCP-1* is located on chromosome 17q11.2-q21.1. The most commonly studied functional SNP in *MCP-1* gene is at position *-2518*.

3.2.2.1.1 MCP-1 -2518G/A

A biallelic polymorphism at the *−2518* position of the *MCP-1* gene promoter *(G→A)* was reported to influence the rate of *MCP-1* induction. (Rovin, Lu, and Saxena 1999) IL-1β induced luciferase activity was significantly greater from cells transfected with constructs containing *G* at position *-2518*. IL-1β treated peripheral blood mononuclear cells from individuals heterozygous or homozygous for *G* at *-2518* produced more MCP-1 than cells from individuals homozygous for *A* at *-2518*.

Gonzalez *et al* (Gonzalez et al. 2002) by utilising EMSA technique found that the mutant *MCP-1 -2518G* allele conferred greater transcriptional activity in

vitro. They also noted in-vivo, that this allele was associated with increased serum MCP-1 levels, as well as macrophages infiltration into interstitial tissues

A study done on Behcet's patients, (Cho et al. 2004) showed that when stimulated in vitro with IL-1βeta and LPS, the mononuclear cells from patients carrying the *G* allele showed a steeper increase in MCP-1 production than the boost observed in *AA* homozygotes. Similar results have been shown by other studies (Muhlbauer et al. 2003). (Tabara et al. 2003)

3.2.2.1.2 MCP-1-2076

-2076 SNP was selected on the basis that research done by our group noted association of this SNP with idiopathic anterior uveitis. (Yeo et al. 2006) Although Rovin *et al*. (Rovin, Lu, and Saxena 1999) showed that polymorphism at site *-2076* did not effect *MCP-1* expression; it is worth mentioning that in their study the maximum transcriptional activity of *MCP-1* promoter region was noted in subjects with the *AC* Haplotype (*A* at *-2076* and *G* at *-2518* in their study). In another study, the same *AC* haplotype was associated with accelerated progress to AIDS and death and a high risk of HIV associated dementia in HIV positive Caucasian Americans.(Gonzalez et al. 2002)

Sedlmeier *et al* recently studied *MCP-1* SNPs in 1630 subjects with metabolic syndrome and found that *T* allele at *-2076* was associated with decreased serum levels of IL-6. (Sedlmeier et al. 2007) Since MCP-1 is involved in IL-6 expression, an association seems conceivable. (Viedt et al. 2002) Using FASTSNP tool however did not predict that this SNP can have a functional significance.

3.2.2.2 RANTES SNPs

The gene encoding for RANTES is located on the same chromosome 17q11.2-q12 in the vicinity of other chemokines. Two functional SNPs in *RANTES* gene have been studied extensively for their role in immune mediated and other form of disease.

3.2.2.2.1 -403G/A and -28C/G

The two SNPs *-28C/G* and *-403G/A* in the promoter region of the *RANTES* gene have shown a possible association with *RANTES* gene expression. (Hajeer, al, and Ollier 1999) (al, Ollier, and Hajeer 1999) Liu *et al* did the functional analyses of *RANTES* promoter activity and demonstrated that the *RANTES-28G* mutation increases transcription of the *RANTES* gene (Liu et al. 1999). Another study also showed increase transcription of *RANTES* genes in subjects with *-28G* allele. (An et al. 2002)

Nickel *et al* transfected the human mast cell line HMC-1 and the T cell line Jurkat with reporter vectors driven by either the mutant (*A*) or wild-type (*G*). They found an up to 8-fold higher constitutive transcriptional activity of the mutant promoter *-403A* allele compared to *G* allele. (Nickel et al. 2000)

3.2.2.3 IL-8 & IL-8Rb SNPs

3.2.2.3.1 IL-8

IL-8 is the first chemokine discovered and is a potent chemoattractant of neutrophils and some lymphocytes. (Baggiolini, Walz, and Kunkel 1989) (Larsen et al. 1989) The gene encoding for IL-8 has a length of 5.1 kb and contains four exons. It maps to human chromosome 4q12-q21.

Two polymorphisms have been studied extensively for their functional effects *T>A* SNP at *-251* position and *C>T* SNP at *-845* position

3.2.2.3.1.1 -251 T/A

The functional effects of this SNP were first noted in 2000 when Hull *et al* noted that, *A* allele tended to be associated with increased IL-8 production by lipopolysaccharide stimulated whole blood. (Hull, Thomson, and Kwiatkowski 2000) The transcriptional promoter activity of the *IL-8* gene was assessed by luciferase assay. In the in-vitro assay, *IL-8 −251A* showed enhanced promoter activity in response to IL-1β or TNF-α. (Ohyauchi et al. 2005) In another study *T > A* change was associated with higher expression of IL-8 protein and more severe neutrophil infiltration (Taguchi et al. 2005). A Chinese study on patients with ulcerative colitis demonstrated higher serum levels of MCP-1 and severe clinical course in patients with *A* allele at *-251* position. (Li et al. 2009)

3.2.2.3.1.2 -845 C/T

The *C/T* polymorphism at the promoter site *-845* was discovered in *IL-8* gene. The *C* allele was noted to increase the expression of IL-8 and found to be associated with severe renal injury in SLE. (Rovin, Lu, and Zhang 2002). The

authors also noted that the frequency of mutant allele was very low in Caucasians compared to Africans.

3.2.2.3.2 IL-8Rb SNPs

Cellular activities of IL-8 are mediated by two related receptors, CXCR-1 (previously called IL8RA) and CXCR-2 (or IL8RB), which are encoded by 2 single-copy genes and located on chromosome 2q34–q35. In a murine animal model of endotoxin induced uveitis, genetically *IL-8Rh* (human *CXCR-2*) deficient mice showed decrease inflammation as compared to normal mice. (Brito et al. 1999)

3.2.2.3.2.1 CXCR2 (IL-8rb) +1208 T/C & +785 C/T

Recently two polymorphisms *on CXCR-2 gene, CXCR-2 +785 C/T and CXCR-2 +1208 T/C* have been shown to be associated with systemic sclerosis and cryptogenic alveolitis. (Renzoni et al. 2000) The *CXCR-2 +785 C/T* polymorphism is located in exon 11 and results in a silent codon change from *CTC* (leucine) to *CTT* (leucine) and is therefore unlikely to have functional significance., whereas the *CXCR-2 +1208* polymorphism is in the 3' untranslated region of exon 3, and has the potential of altering mRNA processing, stability, or translation. However, using FASTSNP tools, it was noted that *+785* SNP has a low to moderate chance to be functional. No such association was noted for *+1208* SNP. It is also possible that, both polymorphisms may be markers of a nearby polymorphism that is yet to be discovered, which in turn could have functional effects. Since there were no other functional SNPs in *CXCR-2* genes, these two polymorphisms were selected for the study.

3.2.2.4 CCR2 and CCR5 SNPs

CCR2 and CCR5 are mainly expressed by T cells and monocytes and they are receptors for MCP-1 and RANTES respectively. The gene encoding for *CCR5* has been mapped to chromosome 3p21-3 about 18 kb downstream of the *CCR2* gene.

3.2.2.4.1 CCR2 V64I

A SNP at nucleotide position *46295 G/A*, was identified in the open reading frame of *CCR2*. The *G→A* substitution leads to replacement of valine by isoleucine at amino acid 64 (*CCR2 V64I*) in the first transmembrane domain of *CCR2*. (Kostrikis et al. 1998)

Until recently this SNP was thought to be non-functional, however recent studies have shown that this *G* to *A* substitution may be functional.

Nakayama *et al*. observed up-regulation of *CCR2*A-64I compared to *CCR2*A without substitution. (Nakayama et al. 2004) Accordingly, a chemotaxis assay showed that cells expressing *CCR2*A-64I migrated more efficiently than those expressing *CCR2*A-64V. Pulse–chase experiments revealed that higher expression of *CCR2A-64I* was due to increased stability of *CCR2A-64I*. In parallel, the authors also measured *CCR5* surface expression that was more severely blocked by co-expression of *CCR2A-64I* than by *CCR2A-64V*. Furthermore, this negative effect of *CCR2A* on *CCR5* expression was shown to arise from the possibility of heterodimer formation between *CCR2A* and *CCR5*. Taken together, the authors concluded that *CCR2A-64I* polymorphism modulates *CCR5* surface expression.

Mellado *et al* also showed that valine to isoleucine substitution gives CCR2 receptor ability to heterodimerize with the CCR5 and/or CXCR4 receptor following MCP-1 binding. (Mellado et al. 1999)

Similarly the *CCR2 V64I* polymorphism was indicated to decrease *CCR5* re-expression after ligand induced internalization in CD4+ T cells (Sabbe et al. 2001)

3.2.2.4.2 CCR5 32bp deletion

CCR5 32bp Δ was first reported in 1996 and showed to confer near absolute resistance to HIV infection among HIV-exposed individuals homozygous for the Variant. (Dean et al. 1996) This genetic variant in the coding region of the *CCR5* structural gene involves a *32 base pair deletion* that shifts the open reading frame to create a truncated protein. This protein fails to reach the cell surface in individuals homozygous for the variant. (Samson et al. 1996a) (Liu et al. 1996) (Huang et al. 1996) *CCR5 32bp Δ* heterozygotes have reduced levels of quantifiable *CCR5* receptors on their cell surface, notably rather greater than the expected 50% reduction due to the gene dosage effect. The mean reduction to 20–30% of wild-type levels in *CCR5-+/-32 Δ* heterozygotes is perhaps because nascent *CCR5-/-32* polypeptides dimerize with their wild-type *CCR5* counterparts in the endoplasmic reticulum, retarding the transport of *CCR5* to the cell surface(Benkirane et al. 1997)

3.2.2.4.3 CCR5 -59029 G/A

The functional polymorphism *G/A* at nucleotide position *-59029* in the promoter region of *CCR5* was first noted by McDemott *et al* in 1998. (McDermott et al. 1998) The researchers measured In-vitro promoter activity by chloramphenicol acetyltransferase reporter gene. *59029-G* had 45% lower activity than *59029-A*

as a result of reduced *CCR5* mRNA production. Later another study showed that increased number of CD4 (+) cells expressing *CCR5* correlated with *CCR5-59029A* homozygosity without the interference of both the *CCR2-64* and the *CCR5 32 bp Δ* mutations. (Shieh et al. 2000)

Since all these eleven SNPs of six chemokine genes were either functional or if non-functional but associated with autoimmune diseases having pathogenesis similar to uveitis, it was planned to analyse these SNPs in patients with posterior segment non-infectious intra-ocular inflammation. Eight of SNPs alter the expression of genes on inflammatory cells which in turn affect the phenotype of the diseases. All of these chemokines are known to be involved in the pathogenesis of uveitis, so it is quite likely that similar effect occurs in posterior uveitis.

3.2.3 Genotyping

3.2.3.1 DNA extraction

The DNA extraction in patients and KCH controls was done with commercial kits (Qiagen UK Ltd, UK) according to the manufacture's instructions and as discussed in chapter 2

The DNA extraction of WXH controls was done by salt extraction method

3.2.3.2 Primer Design

To identify 11 SNPs multiple sequences for *MCP-1*, *RANTES*, IL-8 *CCR2*, *CCR5* and *IL-8Rb* deposited in GenBank were analysed. To verify the presence of these potential polymorphisms, sequence-specific primer–polymerase chain reaction (SSP-PCR) method was used in 10 SNPs. IL-8 -251 SNP was genotyped by 5' Nuclease Array by TaqMan®

The specific and control primer details for each polymorphism are shown in the Table 3-3 & Table 3-4. Table 3-5 shows the details of IL-8 -251 primers

Table 3-3: Details of primers designed for the ten chemokine polymorphisms (SSP-PCR)

Gene name	Chr	SNP ID	Locus	Headed	primer seq 5'-------3'	product size
MCP-1	17q11.2-q12	-2518	PROMOTOR	Sense	AAG TGG GAG GCA GAC AGC TA/G	252
				Antisense	CTG ATA AAG CCA CAA TCC AGA G	
MCP-1	17q11.2-q12	-2076	PROMOTOR	Antisense	TTCATGGTAAAGGATGCACTAACT/A	264
				Sense	TCCCAGAGCAGAGACTCTAT	
RANTES	17q11.2-q12	-403	PROMOTOR	Sense	CAT GGA TGA GGG AAA GGA GG/A	285
				Antisense	GAG TCT CTG TCT CTC CCT CA	
RANTES	17q11.2-q12	-28	PROMOTOR	Antisense	GCC CTT TAT AGG GCC AGT TG/C	314
				Sense	GTC CTA ACT GCC ACT CCT TG	
IL8	4q12-q13	-845	PROMOTOR	Antisense	GAA TAG ACA AGT GGT ACT AAG ACA/G	309
				Sense	AGC AAC AGT GGC TGA ACC AG	
*CCR2*A	3p21-3	V64I	EXON	Antisense	TTT TTG CAG TTT ATT AAG ATG AGG AC/T	808
				Sense	GAA GGC AGA AGG TGA ATA GTT C	
CCR5	3p21-3	-59029	PROMOTOR	Sense	GAG TGG TAG TTA CAT GCA TGT G/A	400
				Antisense	AAC CTG GGT GAC AGC AAG C	
CCR5	3p21-3	Δ 32 G/A	EXON	Sense	GCT CTC ATT TTC CAT ACA GTC AG/A	827/808
				Antisense	TAT ACA TAA GGA ACT TTC GGA GTG	
CXCL-2	2q35	1208	EXON	Sense	CCC ATT GTG GTC ACA GGA AGT/C	673
				Antisense	GGT CTT GTG AAT AAG CTG CTA TG	
CXCL-2	2q35	785	EXON	Sense	TCG TCC TCA TCT TCC TGC TC/T	280
				Antisense	ACA AAG GAA GGC CTG CTG TC	

Table 3-4: Control primers for SSP-PCR reaction

						Headed	Control Primers	Size
63	DRB exon 3					FARWARD	TGC CAA GTG GAG CAC CCA A	796
64	DRB exon 4					REVESRE	GCA TCT TGC TCT GTG CAG AT	
210	APC					FARWARD	ATG ATG TTG ACC TTT CCA GGG	256
211	APC					REVESRE	TTC TGT AAC TTT TCA TCA GTT GC	

Table 3-5: **Primer details of IL-8 251 SNP (TaqMan® Assay)**

SNPs	Headed	PRIMERS	ALLELES	PROBES	ANNEALING
					TEMERATURE
IL-8-251	Forward	GTCACATGGTCTATGATAAAGTTATCTAGAAATAA	A	6-FAM-AAGCATACAATTGATAATT-MGB	60
	Reverse	TACATTTAAAATACTGAAGCTCCACAATTT	T	VIC-AAGCATACATTTGATAATT-MGB	

3.2.4 Statistical Analysis

Statistical analysis was performed as mentioned in detail in chapter two. For each subject group, genotype frequencies were counted and the allelic and allele carriage frequencies calculated. All genotype frequencies in each population were tested for deviation from the Hardy-Weinberg equilibrium using the chi-square test. Data mining for significant associations was performed using knowledge seeker®, (Angoss softwares UK) and statistical calculations were performed with SPSS 12.0 (Chicago USA). Confidence intervals were calculated at the 95 % level and a value of $p < 0.05$ was considered as significant. Chi-square test was used to compare the genotypic and allelic frequencies in patients and controls. A correction for multiple comparisons was made using the Bonferroni method, adding the formula $pc = p \times n$ where pc represents the corrected value, p is the uncorrected value, and n is the numbers of comparisons performed. Haplotypes were constructed by inference and confirmed by using the PHASE algorithm

3.3 Results

205 patients were included in the study and were categorised into following groups shown in Table 3-6.

Table 3-6: The four sub groups of posterior segment uveitis

Idiopathic Intermediate uveitis	n=77
Sarcoid uveitis	n=33
Behcet's uveitis	n=31
Idiopathic Posterior uveitis	n=64
Total	n=205

The mean age was 36.52 years with SD + 14.15. The female to male ratio was 1.25: 1. Salient clinical features are shown in the Table 3-7.

Table 3-7: Clinical details of patients with PSU

Follow up in years	Mean 8.2 (2-42.6)
Sex	Males=94 females=111
Laterality of disease at final follow up	Bilateral=175, unilateral 30
Age of onset (years)	Mean=36.52 Range=(5-70.5)
Relapses (rate per year)	Mean=2.01 Range=(0-7)
Visual impairment during inflammation	Mean=doubling of Visual Angle: Range=(Nil---x8 of visual angle)
VA with both eyes open, after 24 months	Mean=6/9 Range=(6/5-6/60)
Permanent visual loss	n=85 Mean=6/24 Range=6/15-NPL
Need for systemic steroid	n=151
More than 10mg of steroids for long term control	n=83
On second line of immuno-suppressants	n=84
Cystoid macular oedema	n=125
Glaucoma	n=71
Cataract	n=95

3.3.1 Genotype frequencies in patients and controls

All genotype frequencies in case and control populations conformed to Hardy-Weinberg equilibrium. The genotype frequencies of patients versus Caucasian controls are shown in the Table 3-8.

Table 3-8: Genotypic & Allelic Frequencies of chemokine SNPs in Caucasian controls and Patients

GENOTYPE	CONTROLS	PATIENTS	P value	BEHCET	P value	INTERMEDIATE	P value	SARCOID	P Value	POST.	P Value
	283	**205**		**31**		**77**		**33**		**64**	
***MCP-1* -2518 A/G**											
AA	151(53%)	112(55%)		15 (48%)		41 (53%)		15 (45%)		41 (64%)	
AG	109(39%)	78(38%)		13 (42%)		33 (43%)		13 (39%)		19 (30%)	
GG	23(8%)	15(7%)		3 (10%)		3 (4%)		5 (15%)		4 (6%)	
ALLELE FREQ A/G	0.73/0.23	0.74/0.26		0.69/0.31		0.75/0.25		0.65/0.35		0.79/0.21	
***MCP-1* -2076 A/T**											
AA	150(53%)	137(67%)	P=0.002	24 (77%)	P=0.009	52 (68%)	P=0.02	21 (64%)		40 (62%)	
AT	110(39%)	60 (29%)	P=0.02	5 (16%)	P=0.01	20 (26%)		12 (36%)		23 (36%)	
TT	23 (8%)	8 (4%)		2 (6%)		5 (6%)		0 (0%)		1 (2%)	
ALLELE FREQ A/T	0.72/0.28	0.81/0.19	P=0.001	0.85/0.15	P=0.026	0.81/0.19	P=0.04	0.82/0.18	P=0.1	0.80/0.20	P=0.06
***RANTES* -403 G/A**											
GG	179(63%)	129(63%)		21 (68%)		45 (58.4%)		22 (67%)		41 (64%)	
GA	90(32%)	67(33%)		9 (29%)		31 (40.3%)		9 (27%)		18 (28%)	
AA	14(5%)	9 (5%)		1 (3%)		1 (1.3%)		2 (6%)		5 (8%)	
ALLELE FREQ G/A	0.79/.21	0.79/0.21		0.82/0.18		0.79/0.21		0.80/0.20		0.78/0.22	
***RANTES* -28 C/G**											
CC	267(94%)	196(96%)		31 (100%)		73 (95%)		32 (97%)		60 (94%)	
CG	15 (5%)	9 (4%)		0 (0%)		4 (5%)		1 (3%)		4 (6%)	
GG	1 (0.3%)	0 (0%)		0 (0%)		0 (0%)		0 (0%)		0 (0%)	
ALLELE FREQ C/G	0.97/0.3	0.98/0.02		1.00/0		0.97/0.03		0.98/0.02		0.97/0.03	
IL-8 -845 T/C											
TT	274(98%)	202(99%)		31 (100%)		76 (99%)		32 (97%)		63 (98%)	
TC	7 (2%)	3 (1%)		0 (0%)		1 (1%)		1 (3%)		1 (2%)	
CC	0 (0%)	0 (0%)		0 (0%)		0 (0%)		0 (0%)		0 (0%)	
ALLELE FREQ T/C	0.99/.01	0.99/0.01		1.00/0		0.99/0.01		0.98/0.02		0.99/0.01	

GENOTYPE	CONTROLS	PATIENTS	P value	BEHCET	P value	INTERMEDIATE	P value	SARCOID	P Value	POST.	P Value
	283	**205**		**31**		**77**		**33**		**64**	
IL-8 251T/A											
TT	69 (24%)	39 (19%)		8 (26%)		15 (19%)		6 (18%)		10 (16%)	
TA	145 (52%)	113(55%)		15 (48%)		40 (52%)		15 (46%)		43 (67%)	
AA	69 (24%)	53 (26%)		8 (26%)		22 (29%)		12 (36%)		11 (17%)	
ALLELE FREQ T/A	0.50/0.50	0.47/0.53		0.50/0.50		0.45/0.55		0.41/0.59		0.49/0.51	
***CCR2* V64I**					p=0.006						
GG	247(87%)	166(81%)		21 (68%)	p=0.003	61 (79%)		29 (88%)		55 (86%)	
GA	35 (13%)	35 (17%)		9 (29%)	p=0.012	14 (18%)		3 (9%)		9 (14%)	
AA	1 (0.3%)	4 (2%)		1 (3%)		2 (3%)		1 (3%)		0 (0%)	
ALLELE FREQ G/A	0.93/0.07	.90/.10	p=0.029	0.82/0.18	p=0.001	0.88/0.12	p=0.036	0.92/0.08	P=0.7	0.93/0.07	P=0.8
***CCR5* -59029A/G**											
AA	82 (29%)	75 (37%)		15 (48%)	p=0.027	27 (35%)		12 (36%)		21 (33%)	
AG	137(49%)	92 (45%)		12 (39%)		31 (40%)		17 (52%)		32 (50%)	
GG	63 (22%)	38 (19%)		4 (13%)		19 (25%)		4 (12%)		11 (17%)	
ALLELE FREQ A/G	0.53/0.47	0.59/0.41		0.68/0.32	p=0.030	0.55/0.45		0.62/0.38		0.58/0.42	
CCR5 32bp Δ											
wt/wt	216 77.6%)	162(79%)		26 (84%)		62 (81%)		27 (82%)		47 (73%)	
wt/Δ	61 (21.7%)	40 (20%)		5 (16%)		13 (17%)		6 (18%)		16 (25%)	
Δ/Δ	5 (1.7%)	3 (1%)		0 (0%)		2 (3%)		0 (0%)		1 (2%)	
ALLELE FREQ wt/Δ	0.87/0.13	0.89/0.11		0.92/0.08		0.89/0.11		0.91/0.09		0.86/0.14	
CXCR2 1208 T/C											
TT	65 (23%)	50 (24%)		10 (32%)		17 (22%)		10 (30%)		13 (20%)	
TC	152(54%)	106 (52%)		12 (39%)		46 (60%)		14 (42%)		34 (53%)	
CC	66 (23%)	49 (24%)		9 (29%)		14 (18%)		9 (27%)		17 (27%)	
ALLELE FREQ T/C	0.50/0.05	0.50/0.50		0.52/0.48		0.52/0.48		0.52/0.48		0.47/0.53	
CXCR2 785 T/C											
TT	67 (24%)	49 (24%)		8 (26%)		14 (18%)		6 (18%)		21 (33%)	
TC	141 (50%)	96 (47%)		13 (42%)		42 (55%)		14 (42%)		27 (42%)	
CC	74 (26%)	60 (29%)		10 (32%)		21 (27%)		13 (39%)		16 (25%)	
ALLELE FREQ T/C	0.49/0.51	0.47/0.53		0.47/0.53		0.45/0.55		0.39/0.61		0.54/0.46	

(Continued; Table 3-8: Genotype Frequencies chemokine SNPs in Caucasian controls and Patients)

3.3.2 Risk of Genetic Predesposition:

As seen in the previous Table 3-8 there were two SNPs that showed some association with the development of uveitis.

3.3.2.1 1): MCP-1 2076 and risk of developing uveitis:

As shown in detail in the Table 3-9 the wild type allele was more prevalent in the patients compared to the controls. Odds ratio 1.7 (95%CI: 1.2—2.5) the pc for allelic frequency difference was 0.002.

Table 3-9: Genotypic frequencies of MCP-2076 A/T in controls and patients.

Genotype *MCP-1* -2076A/T	Patients N= (205)	Controls N= (283)	Chi 2X2 (P value)
AA	137 (67%)	150 (53%)	0.00219
AT	60 (29%)	110 (39%)	0.02801
TT	8 (4%)	23 (8%)	NS
Allelic Carriage / phenotype			
A	197 (96%)	260 (92%)	NS
T	68 (33%)	133 (47%)	0.00219
Allelic Frequency			
A	334 (81%)	410 (72%)	0.00108
T	76 (19%)	156 (28%)	

As shown in the main Table 3-8, the frequency of *-2076AA* genotype was consistently high in all four groups but reached statistical significance in Intermediate uveitis [Odd ratio 1.8 (95% CI: 1.1—3.1) pc= 0.04] and Behcet's uveitis group [Odd ratio 3.0 (95% CI: 1.3—7.3) pc=0.018]. It appears that because of the number of subjects it did not reach statistical significance in posterior uveitis

The detailed genotypic frequencies of *-2076* SNP in Behcet's and intermediate groups are shown in Table 3-10 Table 3-11 respectively.

Table 3-10: Genotype frequencies in Behcet's patients and controls

GENOTYPE *MCP-1* -2076 A/T	Patients	Control	p
AA	24 (78%)	150 (53%)	0.00942
AT	5 (16%)	110 (39%)	0.0126
TT	2 (6%)	23 (8%)	NS
Total	31	283	
Allelic Carriage / phenotype			
A	29 (94%)	260 (92%)	NS
T	7 (23%)	133 (47%)	0.00942
Allelic Frequency			
A	53 (85%)	410 (72%)	0.02671
T	9 (15%)	156 (28%)	
Total	62	566	

Table 3-11: Genotype frequencies of *MCP-1* -2076 in Intermediate uveitis patients and controls

GENOTYPE *MCP-1* -2076 A/T	Patients	Control	p
AA	52 (68%)	150 (53%)	0.02274
AT	20 (26%)	110 (39%)	0.03673
TT	5 (6%)	23 (8%)	NS
Total	77	283	
Allelic Carriage / phenotype			
A	72 (94%)	260 (92%)	NS
T	25 (32%)	133 (47%)	0.02274
Allelic Frequency			
A	124 (81%)	410 (72%)	0.04222
T	30 (19%)	156 (28%)	
Total	154	566	

The two *MCP-1* SNPs showed quite strong linkage. Subjects who were homozygous *GG* at *-2518* were always homozygous for *AA* at *-2076*. Similarly subjects who were *TT* homozygous at *-2076* were always homozygous for *AA*

at position *-2518*. Using Phase® software three common haplotypes were constructed as shown in the Table 3-12.

Table 3-12: *MCP-1* haplotype frequencies in patients and controls

Haplotype	*MCP-1* Polymorphism		PSU	Control	Odds Ratio	
	2076	2518	(n = 205)	(n = 283)		p value
1	A	A	58.10%	47.00%	-	0.055
2	T	A	13.80%	25.50%	2.3 (1.4-3.8)	0.001
3	A	G	23.90%	24.30%	-	ns
Others			4.20%	3.20%	-	ns

. As we can see the table above the *TA* Haplotype was less frequent in patients when compared with the controls (p=0.001)

3.3.2.2 2): CCR-2 V64I and risk of development of Behcet's uveitis

CCR2 64I was significantly associated with the PSU, with an Odds ratio of 1.6 (95% CI0.9—2.6) (p=0.029). Sub group analysis showed that it was mainly due to Behcet's uveitis OR 3.2 (95% CI: 1.4—7.4) and to some degree intermediate uveitis, where allelic frequency of *A* allele was higher in patients (12%) compared to controls (7%).P=0.036 OR 1.7 (95% CI: 0.9—3.4) See Table 8-2, Appendix B

Genotypic frequencies of *CCR2-V64I* in Behcet's patients are shown in the Table 3-13

Table 3-13: Genotypic frequencies of *CCR2 V64I* SNP in Behcet's uveitis and controls

CCR2 V641 (*G/A*)	Patients	Controls	p
GG	21 (68%)	247 (87%)	0.00389
GA	9 (29%)	35 (13%)	0.01221
AA	1 (3%)	1 (0%)	NS
Total	31	283	
Allelic Carriage / phenotype			
G	30 (97%)	279 (100%)	NS
A	10 (32%)	36 (13%)	0.00389
Allelic Frequency			
G	51 (82%)	523 (93%)	0.00182
A	11 (18%)	37 (7%)	
Total	62	560	

In posterior uveitis and Sarcoid uveitis the frequencies were similar to the controls.

3.3.2.3 3): CCR-5 -59029 A/G association with Behcet's uveitis.

As shown in the main table 3.8 the *CCR5 -59029 A* allele was significantly more prevalent in Behcet's patients compared to controls OR 2.3 (95% CI: 1.1—4.8) p= 0.03). However this difference was modest and similarly in other three groups there was non-significant trend of higher prevalence of *A* allele.

3.3.3 Genotype phenotype correlation

3.3.3.1 MCP-1

3.3.3.1.1 MCP-1 2518 and early age of onset of disease

When the phenotypes of the patients were compared for different genotypes it was found that *MCP-1 -2518 G* allele had mild but significant association with

the age of onset of disease. The mean age of onset of disease in patients with A allele was 38 years compared to 34 years in patients with *G* allele (pc=0.04) as shown in Figure 3-1.

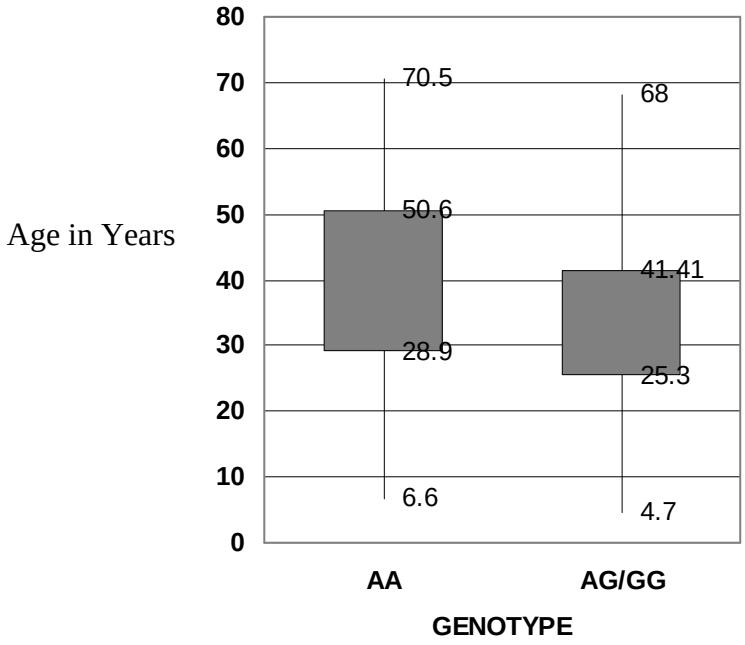


Figure 3-1: The mean age of onset in patients with *MCP-1* 2518 AA genotype versus patients with G allele carriers.

The mean age of onset in patients with AA genotype was 38 years compared to 32 years in patients harbouring G allele.

When patients were divided into 2 groups with 40 years as cut of, the *G* allele was more common in patient with age of onset before 40 years. (31% compared to 19% p=0.007 and pc=0.049). (See Table 8-3 Appendix B).

Sub group analysis showed that this effect was mainly seen in idiopathic PU & IU and to a lesser extent in sarcoid uveitis.

Intermediate uveitis

This trend of association of *G* allele with early onset of disease was seen in IU group as well. The mean age of onset of disease in patients with *AA* genotype

was 44 years compared to 31 years in the group carrying *G* allele. p=0.01 (Table 3-14)

Table 3-14: Age of onset in intermediate uveitis according to *MCP-1 -2518* genotype

Age AT ONSET				P value
MCP-2518 AG	Mean	N	Std. Deviation	
AA	44.34	41	10.62	
AG/GG	31.26	36	10.34	
Total	37.89	77	10.41	0.01

Posterior uveitis

As noted in intermediate uveitis, the *G* allele was again associated with early onset of disease. This is shown in Table 3-15

Table 3-15 Age of onset in posterior uveitis according to *MCP-1 -2518* genotype.

MCP-1 2518AG	Mean Age	(N)	Std. Deviation	P value
AA	41.64341	41	13.62393	
AG	34.16553	19	8.344877	
GG	32.61826	4	2.587735	
Total	38.59289	64	12.1757	0.02

In sarcoidosis patients the *A* allele of *MCP-1 -2518* showed a non-significant trend towards early age of onset of disease. The mean age of onset was 36 years in patients with *GG* genotype compared to 33 years among patients with *A* allele carriage p=0.06

In Behcet's group this association was not seen.

3.3.3.1.2 MCP-1 2518 and gender in Sarcoidosis

In sarcoidosis patients *MCP-1 -2518* showed association with gender. The wild allele *A* was significantly under represented among the male subjects as shown in the Table 3-16. The allelic frequency difference showed a significant p value of p 0.009, pc 0.03.

Table 3-16: *MCP-1 -2518* genotypes in sarcoidosis patients divided by gender

MCP-1 -2518	males	females	P	P
GENOTYPE	Count	Count	3 X 2	2 X 2
AA	2 (15%)	13 (65%)	7.83	0.00516
AG	8 (62%)	5 (25%)	0.01998	0.03582
GG	3 (23%)	2 (10%)		NS
Total	13	20		
Allelic Frequency				
A	12 (46%)	31 (78%)		0.00902
G	14 (54%)	9 (22%)		
Total	26	40		

3.3.3.2 RANTES

3.3.3.2.1 RANTES -403 and visual outcome

It was also noted that the *RANTES-403* polymorphism was associated with intensity of disease (VA impairment of 6 lines or more). (Figure 3-2)The *GG* genotype was more frequent in patients with more intense inflammation (n=74). The *GG* frequency was 76% as compared to 56% in patients with less severe inflammation (VA impairment of 3 lines) (n=131) (allele frequency difference p=0.006, pc=0.02)

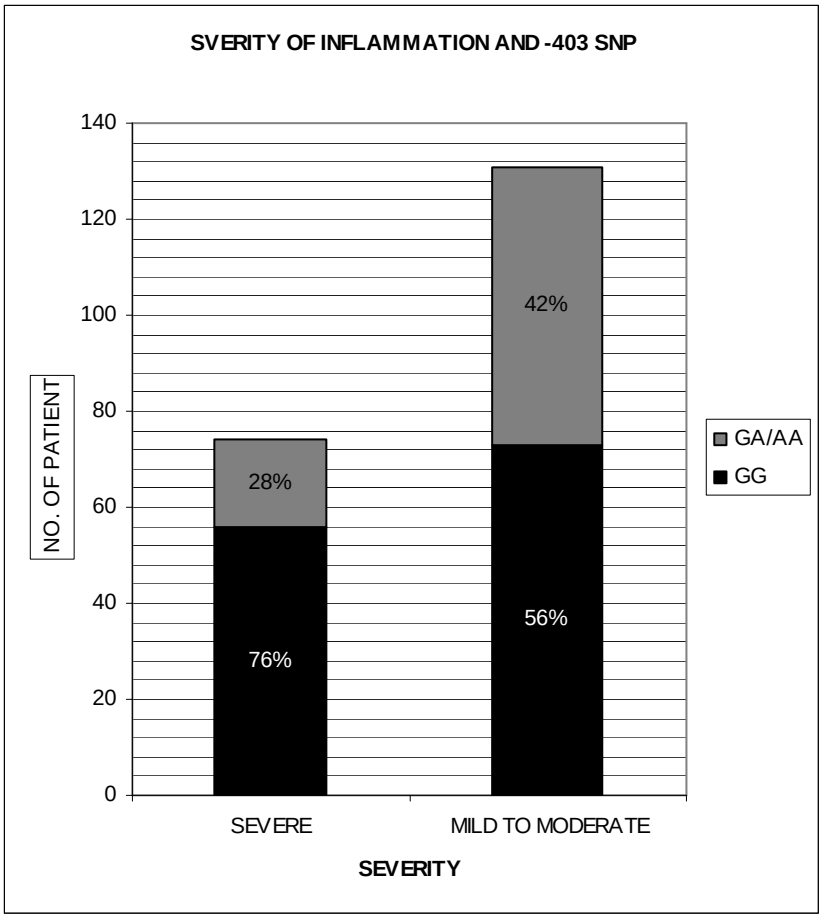


Figure 3-2: Severity of inflammation and *RANTES* genotypes in PSU.

The G allele was associated with severe inflammation during active episodes. Among patients who had at least one episode of severe inflammation (quadrupling of visual angle) 76% were homozygous for G allele.

It was also noted that patients carrying *A* allele had better visual acuity more or less through out the course of disease. Using multiple regression analysis and correcting for age, and type of uveitis, the difference was very significant after first year of disease but it stayed significant till 5th year and even at 10th year there was a non-significant trend of better vision in patients with *A* allele carriage. See Table 3-17.

Table 3-17: Difference in mean vision between patients with *RANTES -403 GG* phenotype and patients carrying *A* allele

Years from onset	RANTES GG		RANTES GA/AA		
	Mean VA	Range of VA	Mean VA	Range of VA	P value
First Year	6/24	(6/6--6/60)	6/9	(6/5--6/24)	0.001
Second Year	6/18	(6/9--6/60)	6/9	(6/6--6/24)	0.009
Third Year	6/24	(6/9--6/60)	6/15	(6/9--6/36)	0.02
Fifth Year	6/36	(6/12--4/60)	6/18	(6/9--6/60)	0.02
Tenth Year	6/36	(6/18--NPL)	6/24	(6/12--3/60)	0.1

Subgroup analysis showed that this association was seen in idiopathic IU and PU groups as discussed below

Intermediate uveitis

As noted in the entire group, the *RANTES -403 G/A* was predicting the visual outcome strongly in intermediate uveitis sub group. Patients carrying *-403A* allele had better visual acuity at year one, two, three, five and even at year ten there was a non significant trend of better vision with patients with *-403A* allele. See Table 3-18. This is shown graphically in the Figure 3-3. One can notice that after 5 years the difference between the two groups became insignificant.

Table 3-18: Visual acuities in Intermediate uveitis comparing *RANTES -403GG* versus – *403 GA/AA*

Years from onset	RANTES GG		RANTES GA/AA		
	Mean VA	Range of VA	Mean VA	Range of VA	P value
First Year	6/24	(6/6--6/36)	6/6	(6/5--6/24)	0.001
Second Year	6/18	(6/9--6/60)	6/9	(6/6--6/24)	0.004
Third Year	6/24	(6/9--6/60)	6/10	(6/9--6/24)	0.014
Fifth Year	6/36	(6/12--4/60)	6/12	(6/9--6/60)	0.034
Tenth Year	6/36	(6/18--1/60)	6/18	(6/12--5/60)	0.1

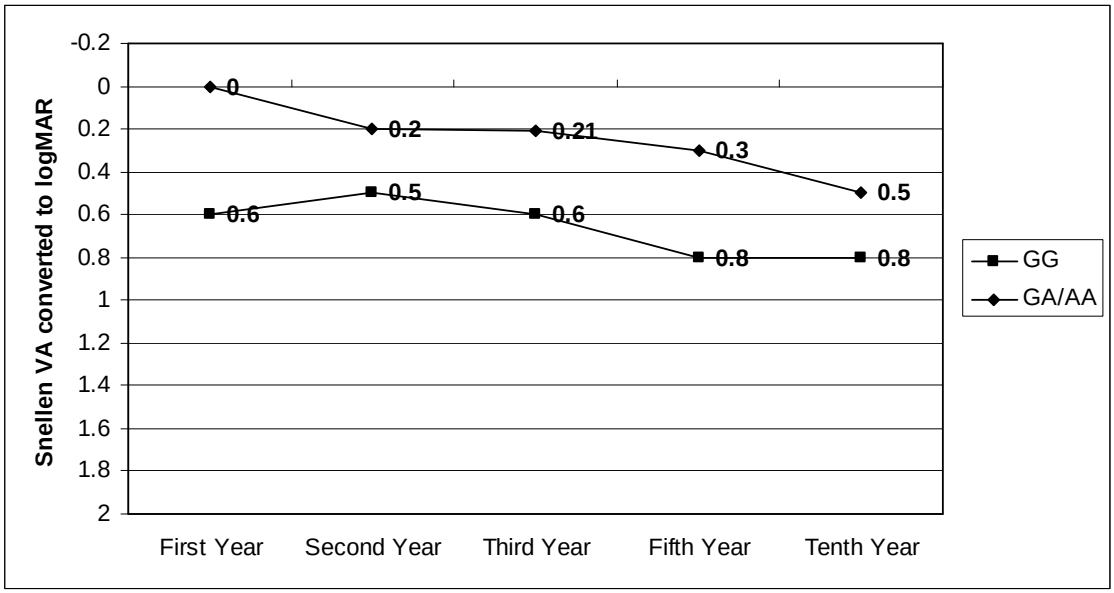


Figure 3-3: LogMAR Visual acuity trend between *RANTES GG* and *RANTES* GA/AA genotypes.

The IU patients with *A* allele carriage had a better visual acuity than *GG* genotype through out the course of the disease. However the difference in the visual acuities decreased as the disease progressed in time. . The values represent mean LogMAR VA (converted from Snellen) over period of times. For range of VA please refer to above Table 3-18

In posterior uveitis there was similar association noted. Patients with *GG* genotype at 3 years have mean vision of 6/36 compared to 6/12 in patients with *GA* or *AA* genotype. As seen in the Table 3-19 the difference became insignificant after 3 years but this was mainly because of the decrease in the number of subjects hence the statistical significance decreased. As seen in the graph (Figure 3-4) the difference between the Visual acuity at least remained the same if not worsened

Table 3-19 Mean VA between *RANTES GG* & *RANTES* GA/AA genotypes in posterior uveitis

Years from onset	RANTES GG		RANTES GA/AA		
	Mean VA	Range of VA	Mean VA	Range of VA	P value
First Year	6/24	(6/6--6/60)	6/9	(6/6--6/24)	0.005
Second Year	6/36	(6/12--5/60)	6/12	(6/9--6/24)	0.009
Third Year	6/36	(6/18--6/60)	6/12	(6/9--6/36)	0.01
Fifth Year	6/60	(6/18--1/60)	6/24	(6/12--6/60)	0.6
Tenth Year	2/60	(6/18--HM)	6/30	(6/12--3/60)	0.12

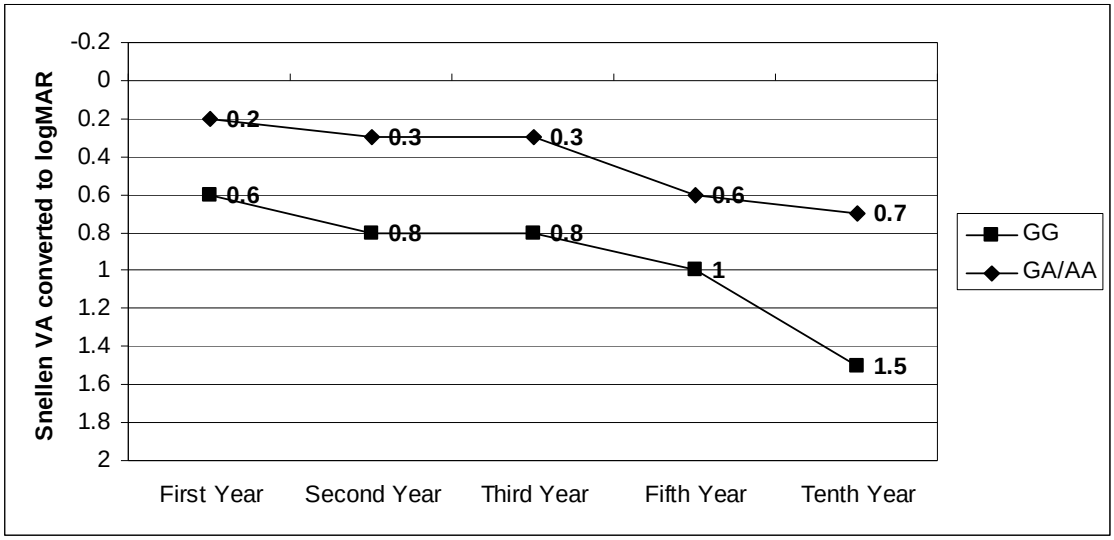


Figure 3-4 : Trend of logMARVA between *RANTES GG* & *RANTES* GA/AA genotypes in posterior uveitis.

Patients with *GG* genotype had consistently poorer VA throught the course of disease. The values represent mean LogMAR VA (converted from Snellen) over period of times. For range of VA please refer to above Table 3-19

Since there was no significant difference noted between the alleles in context of permanent visual loss the Kaplan-Meier analysis was not perfomed

Sarcoidosis

In case of sarcoidosis I did not notice the same association but *-403A* allele was associated with better visual outcome in another way. The mean visual acuity with both eyes open after the minimal follow up of 2 years (all sarcoidosis patients) was 6/9 in patients carrying *A* allele compared to 6/36 in patients with *GG* genotype. See Table 3-20.

Table 3-20: Better eye vision at 2 years, in sarcoidosis patients when divided into *RANTES -403GG* genotype and *RANTES -403GA* or *AA* genotype

*RANTES*403GA	N	Better eye VA	Std. Deviation	P value
GG	22	6/36 (6/12--2/60)	0.455601867	
GA/ & AA	11	6/9 (6/5—6/18)	0.206249329	0.001

I was unable to find association between *-403* SNP and visual outcome in Behcet's uveitis

As seen in Table 3-8, the *-28G* polymorphism was quite rare and there were very few subjects who carried *G* allele (*G* allelic frequency was less than 3% in 488 subjects tested). The two SNPs were in linkage disequilibrium. *RANTES -28 G/G* was not found in subjects with *-403 G* allele, and *RANTES -403G/G* was not observed in subjects carrying *-28 G* allele.

Since the *G* allele at *-28* position was so rare, no attempts were made to construct the *RANTES* haplotypes.

3.3.3.3 CCR2-V64I

3.3.3.3.1 CCR2 V64I and visual outcome in posterior uveitis

As seen in Table 3-21 below the *CCR2 64I* allele was associated with poor visual outcome in posterior uveitis. The difference in VA with both eyes open was more noticeable with long term follow up. In first three years there were no significant differences in the BEVA between two groups however by year 5 the mean BEVA in patients with *GG* allele was 6/12 compared to 6/60 in patients with *GA* genotype. Similar difference was noted at year 10 as well. It must be noted that the difference in vision was corrected for age and high intra ocular pressure (as association with IOP was noted as well)

Table 3-21: Better eye VA in posterior uveitis patients at 5 years when divided between *CCR2-64I* allele carriers and wild types

CCR2 V641(G/A)	N	Mean VA BE at 5 Years	P value
GG	50	6/12 (6/9—6/24)	
GA	7	6/60 (6/18---2/60)	
Total	57	6/18 (6/9---2/60)	0.001
	N	Mean VA BE at 10 Years	
GG	41	6/24 (6/18---6/60)	
GA	5	4/60 (6/24---1/60)	
Total	46	6/36 (6/18---1/60)	0.03

BE: Better eye

3.3.3.3.2 CCR2 V64I and visual outcome in Intermediate uveitis

Similar Results were noted in patients with intermediate uveitis, where patients with *A* allele carriage have poorer BEVA at third and fifth year. (See Table 3-22) However, this difference disappeared at 10th year.

Table 3-22: Better eye VA, in intermediate uveitis comparing the *CCR2 V64I* genotypes

Years from onset	CCR2 V (GG) Better eye VA		CCR2 VI (GA) Better eye VA		
	Mean	Range	Mean	Range	P value
Second Year	6/6	6/5---6/12	6/10	6/5---6/18	0.07
Third Year	6/6	6/5---6/12	6/24	6/6---6/36	0.001
Fifth Year	6/10	6/6---6/18	6/24	6/9---6/60	0.008
Tenth Year	6/18	6/6---6/36	6/36	6/9---3/60	0.09

3.3.3.3.3 CCR2 *V64I and high intra-ocular pressure in Intermediate and posterior uveitis*

It was also noted that among carriers of *A* allele (I) 60% (15/25) of the patients with idiopathic uveitis (both intermediate and posterior) developed raised intra-ocular pressure compared to 26.7% (31/116) of the patients with wild type genotype. P=0.001, OR 4.11; 95%CI: 1.67-10.11). (Figure 3-5) After correction for duration of disease, age of patient and treatment the pc was0.007

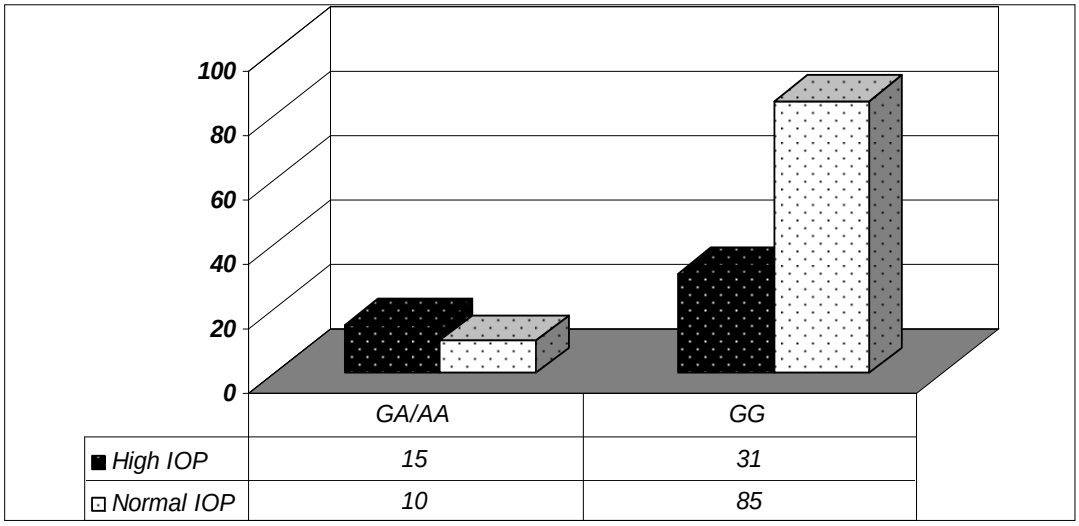


Figure 3-5: Association of high IOP with *CCR2 V64I* in idiopathic PSU.

More patients with high intraocular pressure carried A allele. 60% (15/25) of patients with A allele develop high intraocular pressure compared to 26.7% (31/116) in patients with *GG* genotype.

CCR2-V64I and association with gender in Behcet's

As shown in the table 3, *CCR-2 V64I* SNP also showed association with gender. Whether it carries any clinical significance or not, it was noted that *GG* genotype frequency was 82% in males and 33% in females. (p=0.008). See Table 8-4 in Appendix B

3.3.3.4 CCR5-32bp deletion

3.3.3.4.1 CCR5-32del and visual outcome in PSU:

The *CCR5 32bp Δ* was found to be associated with the visual outcome in the whole group. None of the patients who had VA of 6/12 or worse at minimum follow up of 2 years was *Δ/Δ*. The mean LogMAR VA with both eyes (i.e VA of better eye) after 2 years in both groups are shown in Figure 3-6. As seen in the figure, the mean VA (from better eye) after 2 years was 6/6 in patients harbouring the deletion allele compared to 6/12 with wild type genotype.

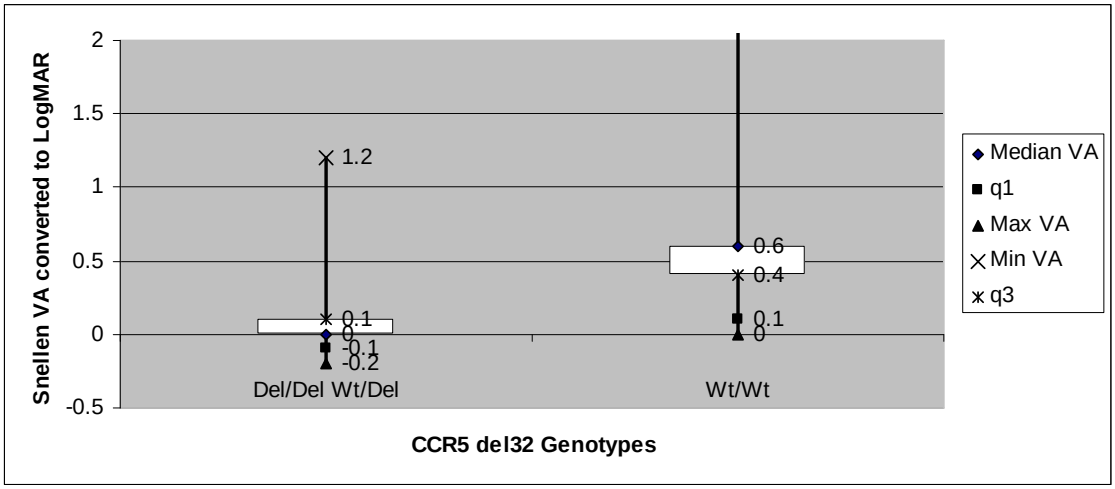


Figure 3-6: Mean LogMAR VA after 2 years in PSU when divided into *CCR5 32bp* carriers and wild types.

Mean VA in wild type is 6/12 (LogMAR=0) and is 6/6 (LogMAR=0.5) in Δ carrying subjects

When the sub group analysis was done significant differences in VA were achieved in idiopathic PU and IU. In Intermediate uveitis the p value was 0.002 and in posterior uveitis the p value was 0.004

It must be noted that in all the four groups the same association was noted but because of numbers in sarcoidosis and Behcet's groups, significant p value was not achieved. However collectively the association of *CCR2 32bp Δ* with visual outcome was significant

3.3.3.5 CCR5-59029

Some modest associations were noted between idiopathic PSU and steroid need.

3.3.3.5.1 CCR5-59029 and steroids use in intermediate uveitis

Many intermediate uveitis patients did not need steroids because the inflammation was not affecting the visual acuity. So the use of systemic steroids was the indicator of severity of disease.

It was noted that among intermediate uveitis the patients who received systemic steroid treatment the *G* allele carriage frequency was 51% compared to 87% among patients who did no receive steroids (p=0.001, pc 0.004) [95% CI: 6.2 (1.88-20.6)] (Table 3-23)

Table 3-23: CCR5 -59029 A allele and need for steroids in intermediate uveitis

CCR-5 59029 *G/A* GENOTYPE	steroids N=47	No steroids N=30	P value
GG	11 (23%)	8 (27%)	NS
GA	13(28%)	18 (60%)	0.004
AA	23 (49%)	4 (13%)	0.001
Allelic Carriage			
G	24 (51%)	26 (87%)	0.001
A	36 (77%)	22 (73%)	
Total			

3.3.3.5.2 CCR5-59029 and long term steroids in posterior uveitis

30 patients were on long term (12 months or more) low dose steroids to keep the disease under control. Wild genotype *GG* (that has low *CCR5* expression activity) was less prevalent in patients on long term steroids. P=0.03. Also *A* allele carriage was 93% in patients on long term steroids compared to 74% in patients with no long term steroids p=0.03 as shown in Table 8-5 in Appendix B

3.3.3.6 IL8-251

3.3.3.6.1 IL-8 251 and visual outcome in posterior uveitis

Patients with mutant allele *A* generally fared poorly compared to wild type *T* allele. 90% of the patients with *AA* genotype had permanent visual loss of some kind, p 0.002 pc 0.008. Frequencies are shown in following Table 3-24.

Table 3-24 Association of IL-8 251 genotype with permanent visual loss in posterior uveitis

IL-8 251 T/A GENOTYPE	Permanent Visual loss N=32	No Visual loss N=32	P value
TT	2 (6%)	8 (25%)	0.03887
TA	20 (63%)	23 (72%)	NS
AA	10 (31%)	1 (3%)	0.00286
Total	32	32	0.00375
Allelic Frequency			
T	24 (38%)	39 (61%)	
A	40 (62%)	25 (39%)	
Total	64	64	0.008

Among the patients with permanent visual loss, Kaplan-Meier analysis showed that patient with *AA* genotype had higher rate of moderate visual loss (VA 6/15 or worse) compared with the patients with *T* allele carriage. As seen in Figure 3-7, at two year of follow up, 50% of patients with *AA* genotype had moderate visual loss. On the other hand 80% patients with *T* allele carriage retained VA

of 6/15 or better at two years. (p=0.002) This difference however decreased with time and at five year and beyond it was non-significant.

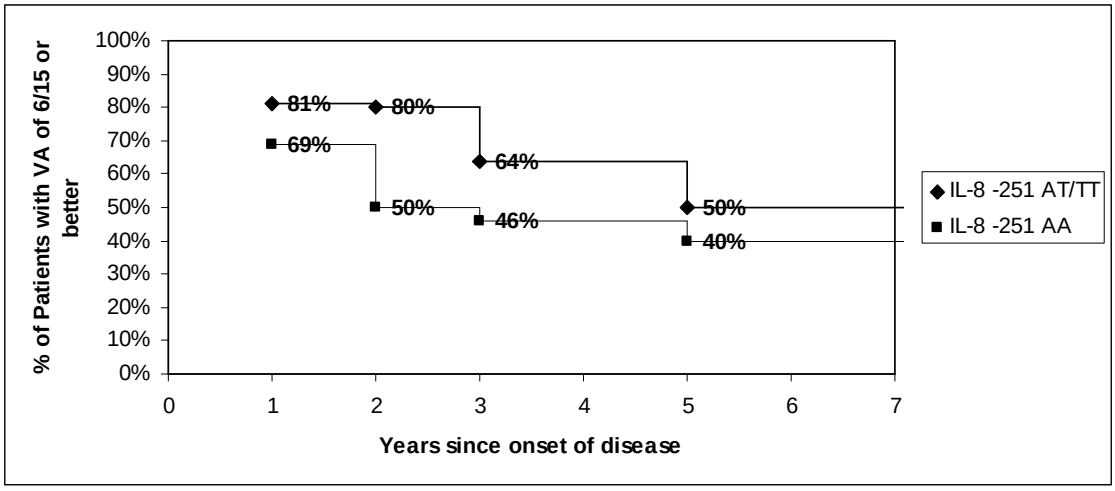


Figure 3-7: Rate of moderate visual loss between IL-8 -251 AA and IL-8 -251 AT/TT in patients with posterior uveitis.

Less proportion of patients with *T* allele developed permanent moderate visual loss compared to the patients with *AA* genotype.

It was also noted that VA with both eyes open was better in patients having T allele. The mean VA with better eye after 2 years in patients with *TT* genotype was 6/6 compared to 6/36 in patients with *AA* genotype. See Table 3-25 for details.

Table 3-25: Association of poor vision in posterior uveitis with IL-8 -251 A allele

IL8251TA	Mean Better eye VA	N	Std. Deviation	P value
TT	6/6	10	0.326777	
TA	6/12	43	0.293855	
AA	6/36	11	0.319978	
Total	6/12	64	0.323307	0.007

Better eye VA at 2 years with AA gentotype was worse compared to T allele carriage

3.3.3.7 IL-8Rb

3.3.3.7.1 IL-8Rb SNPs and development of cataract in Behcet's uveitis

As shown in the Table 3-26, *IL-8R* SNPs showed significant risk for cataract development. This association was very strong in case of *+1208* SNP. As mentioned in the methods chapter the presence of cataract was always corrected for age and steroid use (two factors that independently can increase the risk of cataract development.

Table 3-26: IL-8 +1208 & +785 SNP and risk of cataract in Behcet's patients

IL8R 1208 GENOTYPE	No Cataract (N=10)	Cataract (N=21)	Significance P value
CC	0 (0%)	9 (43%)	0.013994
TC	2 (20%)	10 (47%)	NS
TT	8 (80%)	2 (10%)	0.000008
Allelic Carriage / phenotype			
C	2 (20%)	19 (90%)	0.000008
T	10 (100%)	12 (57%)	0.013994
Allelic Frequency			
C	2 (10%)	28 (67%)	0.000003
T	18 (90%)	14 (33%)	
IL8Rb +785 GENOTYPE			
CC	7 (70%)	3 (15%)	0.001922
CT	2 (20%)	11 (52%)	NS
TT	1 (10%)	7 (33%)	NS
Allelic Carriage / phenotype			
C	9 (90%)	14 (66%)	NS
T	3 (30%)	18 (86%)	0.001922
Allelic Frequency			
C	16 (80%)	17 (40%)	0.003549
T	4 (20%)	25 (60%)	

As seen, the *T* allele at *+1208* position and *C* allele at *+785* position were conferring protection against the development of cataract in Behcet's uveitis. After correction using logistic regression the p values for allelic frequencies difference were still 0.00001 for *IL-8R +1208* and 0.001 for *IL-8R +785* SNPs

There was strong linkage disequilibrium between the two SNPs. When the haplotypes were constructed the association became more striking. All the patients with *CT* haplotype *(C at +1208 and T at +785*) developed cataract and all the *TC* haplotype homozygous did not develop cataract. P=9.1E-09 (0.000000009) pc 0.00000003

3.4 Discussion

This study was undertaken to investigate the effect of chemokine gene polymorphism on the susceptibility of PSU. Its aim was to analyse the gene polymorphism as a predictor of disease severity and response to the treatment in the patients with PSU

Patients with PSU were categorized into four groups depending on site and cause of inflammation.

The control group consisted of healthy Caucasians numbered 283. All genotype frequencies in case and control populations conformed to Hardy-Weinberg equilibrium.

3.4.1 MCP-1 -2518A/G

Two *MCP-1* SNPs have been studied widely for their associations with different diseases including immune mediated diseases, cancers and arthrosclerosis. As mentioned previously, the *-2518* is found to functional by many studies (Cho et al. 2004) (Gonzalez et al. 2002) (Rovin, Lu, and Saxena 1999). On the other hand the evidence that *-2076* SNP may have functional role is sparse. (Sedlmeier et al. 2007) *-2518* SNP has been shown to effect disease predisposition and outcome in Sarcoidosis, Crohn's disease, SLE and Behcet's, all diseases in which uveitis is part of the spectrum of disease. (Navratilova et al. 2007) (Hou et al. 2010) (Lee et al. 2009b) (Palmieri et al. 2010)

As far as *-2518* SNP is concerned the frequencies in both patients and controls were similar to other published studies. (Rovin, Lu, and Saxena 1999) (Steinmetz et al. 2004) Recently, studies (Wegscheider et al. 2005) (Lan et al. 2009) have shown that *MCP-1 -2518* may be associated with anterior uveitis; however I did not notice any association of this SNP with my posterior segment

uveitis patients. Similarly I was unable to confirm the findings from China and Korea where *-2518* allele was noted to be a risk factor for ocular Behcet's. (Hou et al. 2010) (Cho et al. 2004)

However with *-2076* SNP there was significant over representation of *A* allele and under representation of *T* allele in the patients. The genotypic frequencies in the control populations were similar to published studies (Rovin, Lu, and Saxena 1999) The frequency of *AA* genotype was consistently high in all four groups but reached statistical significance in Intermediate uveitis [Odd ratio 1.8 (95% CI: 1.1—3.1)] and Behcet's uveitis group [Odd ratio 3.0 (95% CI:1.3—7.3)]. Because of the number of subjects it did not reach statistical significance in the other two groups but the odd ratio was 1.5 95%CI (0.9—2.74)

In my study haplotype *TA* was more prevalent in controls as compared to PSU (p=0.001). This haplotype contains T allele at *-2076* (noted to be a protective allele in my study) and *A* allele at *-2518* (known to be associated with decreased *MCP-1* expression). It appears that the presence of these two protective alleles together on the same gene exert a combined protective effect against PSU. Although Rovin *et al*. (Rovin, Lu, and Saxena 1999) showed that polymorphism at site *-2076* did not effect *MCP-1* expression; it is worth mentioning that in their study the maximum transcriptional activity of *MCP-1* promoter region was noted in subjects with the *AG* Haplotype (*A at -2076 and G at -2518* in their study). In another study, the same *AG* haplotype was associated with accelerated progress to AIDS and death and a high risk of HIV associated dementia in HIV positive Caucasian Americans. (Gonzalez et al. 2002) In my study instead of the *AC* Haplotype being associated with the disease it was the *TA* haplotype (with both protective alleles), which was more common in the control group.

Alternatively, it is quite possible that the protective effective of *T* allele at *-2076* is independent of the *-2518* polymorphism, as also noted by Modi *et al.* in AIDS patients, where the *T* allele frequency was significantly higher in high risk exposed uninfected subjects as compared to sero-converters (Modi et al. 2003). I also used transcription element search system (TESS) to predict the transcription factors binding sites at -2076.

Using this web tool (http://www.cbil.upenn.edu/tess), it was noted that the *A to T* change at *-2076* promoter site forms a DNA sequence where c-Myb can transactivate. c-Myb is an important transcription factor which is involved in T cell development and maturation. (Allen, III, Bender, and Siu 1999) Elevated levels of c-Myb proteins have been noticed in T cells involved in certain immune mediated diseases like rheumatoid arthritis, systemic sclerosis and systemic lupus erythromatosis,(Kahan et al. 1989) (Boumpas et al. 1986) but the role of these in uveitis is unknown.

It has been proposed that the interaction of c-Myb transcription factors with the receptor gene leads to up regulation of expression of that gene by increasing DNA polymerase alpha expression and thereby DNA production. (Venturelli, Travali, and Calabretta 1990) The presence of the *A* allele in *MCP-1 -2076* may enhance c-Myb transactivation, whereas the T allele would prevent this c-Myb transactivation thereby conferring protection against uveitis. Whether *-2076* is a functional polymorphism or not, it appears that *MCP-1* promoter gene may harbour a genetic locus for uveitis particularly for Behcet's as noted by studies in Korea and china. (Hou et al. 2010) (Cho et al. 2004)

When I studied the effect of *MCP-1* polymorphisms I noted that patients with *G* allele at *-2518* (allele associated with increased *MCP-1* expression) developed the disease at earlier age compared to the patients with *A* allele. This is in

keeping with other studies that have also noted an association with the *MCP-1 -2518 G* allele and early onset of disease. (Nishimura et al. 2004) Sub group analysis replicated this effect in intermediate uveitis and posterior uveitis with very significant difference.

Based on these findings one could say that *G* allele can affect the age of onset in idiopathic posterior segment uveitis but not in uveitis secondary to systemic diseases.

I also noted that in sarcoidosis patients the *AA -2518* genotype was 15% in males compared to 65% in females. Though the difference was very significant, the clinical value is questionable particularly after I was unable to notice any other effect of this SNP on sarcoidosis phenotypes. However there is at least one study in the published literature that have shown association of *MCP-1* haplotypes (*-2518 & -2076*) with gender in patients with ocular Behcet's (Chen et al. 2004)

3.4.2 RANTES -403G/A and -28C/G

I studied two SNPs in the *RANTES* gene that are known to be functional. (al, Ollier, and Hajeer 1999) (Hajeer, al, and Ollier 1999) There was no significant difference in the genotypes between the patients and controls. The frequencies of both SNPs in both patients (and sub groups) and controls were similar to other published studies. (Hajeer, al, and Ollier 1999), (Gade-Andavolu et al. 2004), (Simeoni et al. 2004) As expected the *-28G* polymorphism was quite rare and there were very few subjects who carried *G* allele (*G* allelic frequency was less than 3% in 488 subjects tested). As mentioned in other studies (Nakajima et al. 2003) the two SNPs were in linkage disequilibrium. *RANTES* -

28 G/G was not found in subjects with *-403 G* allele, and *RANTES -403*G/G was not observed in subjects with *-28 G* allele.

Since the *G* allele at *-28* position was so rare, no attempts were made to construct the *RANTES* haplotypes.

The study showed that *G* allele at poition *-403* of *RANTES*, was a strong predictor of visual outcome in patients with PSU. Patients carrying *A* allele not only had better visual acuity during exacerbation of disease but compared to *GG* genotype exhibited good visual acuities through out the course of the disease. The difference was very significant up until 5 years of disease onset. Sub group analysis also showed that *A* allele was associated with better visual outcome in intermediate, posterior and sarcoid related uveitis. It must be noted that among sarcoidosis patients the difference was only significant until 18 months of onset of disease. I was unable to replicate this association in Behcet's uveitis group. Based on the results, we can infer that *-403* SNP is a predictor of visual outcome in patients with idiopathic posterior and intermediate uveitis. Its role in uveitis associated with systemic diseases need further studies.

It appears that *A* allele which is associated with increased *RANTES* expression is having a protective effect in patients with posterior uveitis. This is in contrast with the studies where they have found association of disease susceptibility and severity with the *A* allele. Recently evidence has accumulated that *RANTES* can actually help in controlling uveitis

Sonoda *et al.* (Sonoda et al. 2003) showed that in animal EAU which is the animal model for posterior uveitis, *RANTES* produced by ocular macrophages appears to suppress EAU. In another study (Adamus, Manczak, and Machnicki 2001) *RANTES* levels remained high during the recovery phase of

experimental autoimmune uveitis associated with EAE, and anti-*RANTES* antibodies treatment was not effective in suppressing the uveitis. A similar finding was noted in recurrent anterior uveitis in Lewis rats, where Anti-*RANTES* was not effective in protecting against the development of uveitis. (Manczak et al. 2002)

Recently Kim *et al*. measured levels on RANTES in aqueous humour and peripheral blood in patients with different types of uveitis. (Kim, Chung, and Yu 2010) They noticed that the RANTES levels were constantly lower in aqueous humour in all types of uveitis during active disease. These studies show that decrease expression of *RANTES* may be associated with prolonged and severe inflammation. And hence *A* allele which increases the *RANTES* expression helps in controlling the posterior uveitis.

3.4.3 IL-8 251T/A & -845T/C

In *IL-8* gene two SNPs are shown are shown to be functional, *-251 T/A* and *-845T/C* (Ohyauchi et al. 2005) (Rovin, Lu, and Zhang 2002).

The SNP at *-845* is very rare in Caucasians, and Asians, however more common in African races. (Rovin, Lu, and Zhang 2002). In my study the mutant allele *C* frequency was 1% in both patients and controls. In the patient group there were only three subjects who were heterozygous *TC* and no one was homozygous for *C* allele. Similarly there was no homozygous *CC* in control population and only 7 carried *C* allele.

As this SNP was so rare haplotype construction was not done as it would not have added any new information.

No association of this SNP was found with any of my patient sub groups and one of the reasons may be that because of the rarity of allele the sample size was too small to notice any associations

The *-251* SNP as shown in the results section was very polymorphic and the two alleles were more or less equally present in all the studied populations. I did not find any difference in the allelic frequencies between any of the populations and frequencies were similar to other published studies. (Howell et al. 2005)

The only significant association noted was in patients with posterior uveitis, where *A* allele was associated with permanent visual loss. It was also associated with poor VA in both eyes. (BEVA)

This functional SNP at *-251* position has been studied widely for disease associations and strong associations have been noted in gastric carcinomas and asthma. However there are studies showing that this SNP is also associated with immune mediated diseases like vasculitis and multiple sclerosis. (Amoli et al. 2002) (Kamali-Sarvestani et al. 2006)

There is no study in the literature that has noticed any association of *-251* SNP with uveitis phenotype except one. (Lee et al. 2007) Here the authors found that two haplotypes carrying *-251* alleles were common in patients with ocular Behcet's when compared to controls. However it is interesting to know that one haplotype carried the *A* allele and the other carried the *T* allele, meaning that this particular SNP did not have any independent role in disease predisposition.

3.4.4 CCR2 V64I

CCR2 V64I polymorphism which substitutes isoleucine for valine at position *64 (CCR2-64I),* in the first transmembrane domain of *CCR2* is correlated with

significantly delayed progression to acquired immunodeficiency syndrome (AIDS), especially in African populations. (Kostrikis et al. 1998) (Anzala et al. 1998) Since this polymorphism is common in Africans, it was proposed that *CCR2 64I* rather than *CCR5 32bp Δ* (which is very rare) confers protection against AIDS in Africans. Although this amino acid changed is conservative, various studies have shown that *64I* allele may be a protective factor in autoimmune diseases like sarcoidosis and multiple sclerosis. (Hizawa et al. 1999) (Petrek et al. 2000) (Miyagishi et al. 2003) A recent study has shown that the 64I allele could be a predictor of arthritis in psoriasis. (Soto-Sanchez et al. 2010) It is interesting to know that psoriatic arthritis but not psoriasis can be associated with uveitis. Initially thought to be a non-functional polymorphism, there is now increasing evidence that this SNP could be functional (Nakayama et al. 2004) (Sabbe et al. 2001) (Mellado et al. 1999)

All the allele frequencies in Caucasian patients and controls were in hardy Weinberg equilibrium. The *CCR2 V64I* was tightly linked with *CCR5 -59029* and 32bp Δ. *CCR2* 64I homozygosity as not found in subjects with the *CCR5 59029G* allele, while *CCR5 59029G* homozygosity was not observed in subjects with the *CCR2 64I* allele.

This is in accordance with other studies. (Nakajima et al. 2002) Also *CCR2 V64I* homozygous was never homozygous for *CCR5 32bp Δ.*

In my study there was a significant difference in the allelic frequencies between the Caucasian patients as a whole and controls. The *64I* mutation was 10% in patients as compared to 7% in controls (p=0.02). Subgroup analysis showed a striking association of 64I allele with Behcet's uveitis (n=31). In this group the *A* allele (*64I*) was present in 17% of patient p=0.001 and the genotype difference was very significant as well p=0.006. Since there is no published study on the

frequency of *64I* allele in Behcet's disease, it is difficult to tell whether this difference is due to uveitis in Behcet's disease or the disease itself. But I also noted increase frequency of *64I* allele in patients with intermediate uveitis (n=77) 12% compared to 7% in controls. (p=0.03)

It is interesting to note that the frequency of *64I* allele in populations where Behcet's disease is more common is between 13% [Turkish and Greek populations], (Karaali et al. 2010) (Apostolakis et al. 2005) to 27% in Korea. (Cheong et al. 2007) However this observation alone is not sufficient to assume that 64I can predispose to Behcet's disease.

Alternatively one can argue that my Behcet's patients may have a Lineage from above mentioned ethnicities. Although my patients were British Caucasians who's both parents were Ethnically British, the chances of gene admixture in older generations cannot be ruled out.

While studying the phenotype genotype associations of this SNP, *64I* allele was associated with poor visual acuity in patients with idiopathic intermediate and posterior uveitis. It was also associated with high intra-ocular pressure episodes during the inflammation in patients with intermediate and posterior uveitis. Since there is no known role of *CCR-2* gene in intra-ocular pressure, it is likely that this association is because of linkage of 64I allele with some other locus, yet to be identified

While this SNP did not confer any genetic disposition to idiopathic posterior segment uveitis, it was affecting the phenotype. On the other hand it was associated with Behcet's uveitis development but then not affecting the phenotype. No associations were noted with Sarcoid uveitis.

Another interesting finding was gender association of this allele in Behcet's disease. 67% of the females with Behcet's disease carried the *64I* allele.

However the numbers were too small to have any statistical power. Interesting a study from Iran (Mojtahedi et al. 2006) has shown a high prevalence of *32bp Δ* allele in female patients with Behcet's disease and as mentioned earlier the *V64I* SNP and *CCR-5 32bp Δ* are in strong linkage. I however did not notice any association of *32bp Δ* and female gender in Behcet's disease.

3.4.5 CCR5 -59029G/A and CCR5 32bp Δ

Two functional SNPs in *CCR5* gene were studied.

The *32bp Δ*, which is common in Caucasians compared to other ethnicities and play an important role against HIV infection and progress. However its role in autoimmune disease is not very clear. Individuals who are homozygous for a *32 bp deletion* in the *CCR5* gene comprise 1% of all whites, whereas individuals heterozygous for this deletion represent 20% of whites. (Liu et al. 1996)

The *Δ 32* allele frequency is 2-5% in Asian and 1% in Africans. The presence of deletion results in a truncated form of the functional receptor and homozygous carriers of this mutation fail to express *CCR5* on their cell surface.

The other SNP, *G/A* at nucleotide position *59029* in the promoter region of *CCR5* (*G59029A*), is also thought to be functional. (Shieh et al. 2000) (McDermott et al. 1998) Here the *A* allele is associated with increased expression of *CCR5* receptors on cell surface. Initial studies in patients with AIDS showed that *CCR5 -59029A* homozygous progressed more rapidly than those who were homozygous for the *G* allele. (An et al. 2000).

All genotype frequencies for the two SNPs were in H-W equilibrium in all populations and subsets. There was strong linkage between the two SNPs. All

patients with *Δ/Δ* phenotype were homozygous for *-59029* G allele. Similarly all *AA* homozygous for *-59029* were also homozygous for wt Allele

In my patients no significant difference was noted in the frequencies of *CCR5 32 bp Δ* between the patients and controls. In case of *-59029* SNP, the *AA* homozygous were more frequent in all the four patients groups compared to controls but only showed non significant trend except Behcet's uveitis. In this case the *AA* frequency was 48% compared to 29% in controls.

When the effects of these SNPs on the disease phenotype were studied, it became evident that the *32bp Δ* allele was associated with better visual outcome. Taking the whole group as one, none of the patients with a VA of 6/12 or worse (at the end of minimum follow up) carried the mutant allele. Similarly the mean vision of patients carrying the mutant allele at every time point was better than patients with wild type genotype. It must be noted that when the four groups were analysed the separately, the same association was evenly seen but statistical significance was not reached in sarcoidosis and Behcet's presumably because of the sample sizes. It is also possible that this SNP was exerting it effects only in idiopathic uveitis groups (i.e. IU and PU).

My findings that *32bp Δ* could have a protective effect on the progress of uveitis is consistent with other studies.

The *32bp Δ* allele was noted to be a protective factor against rheumatoid arthritis (Pokorny et al. 2005) and has been associated with less severe phenotype. (Zapico et al. 2000) (Garred et al. 1998) *CCR5 32bp Δ* has also been noted to contribute to protection from the development of primary Sjogren's syndrome. (Petrek et al. 2002)

By contrast, *32bp Δ* allele has found to be associated with increase susceptibility and severity of diseases like multiple sclerosis, (Pulkkinen et al. 2004) sarcoidosis. (Petrek et al. 2000)

The *-59029* showed association with the use of systemic steroids in IU and PU. It was also noted that among intermediate uveitis the patients who received systemic steroid had low carriage frequency of protective allele *G* and similarly among PU patients who stayed on long term steroids had low *G* allele prevalence. In case of IU the use of steroids is a measure of intensity of disease as many patients with IU may have 6/6 vision and many a times this is managed conservatively.

My results have shown that this SNP can be a predictor severity of the disease. Although association studies in immune mediated diseases have so far been unfruitful, recent studies have shown that *A* allele is associated with renal graft rejection. (Yigit et al. 2007) (Omrani et al. 2008) (Abdi et al. 2002)

Given the functional effects of these two polymorphisms and role of *CCR5* gene in PSU, it is plausible to state that these polymorphisms can affect the phenotype in idiopathic PU and IU.

3.4.6 IL-8Rb +1208T/C and +785C/T

Recently +1208T/C and +785C/T polymorphisms in the exon 3 of *CXCR2 (IL-8Rb)* have been shown to be associated with systemic sclerosis in Caucasians. (Renzoni et al. 2000)

The study showed that *T* allele of *+1208* and *C* allele of *+785* which were in linkage were associated with the disease

In my study I did find the linkage between *T* allele of *+1208* with *C* allele of *+785* but that was not 100%. I did not find any significant difference in the

genotypes between patients and controls. This is in line with association studies in Behcet's and Multiple sclerosis where genetic predisposition could not be accounted because of these SNPs. (Duymaz-Tozkir et al. 2005) (Kamali-Sarvestani et al. 2006)

One significant association was noted when the effect on disease phenotype was studied

In Behcet's patients the C allele at *+1208* and *T* allele at *+785* were strongly associated (p=9.1E-09) with cataract development.

The *+1208 T/C* is in the 3' untranslated region of exon *3*, and has the potential of altering mRNA processing, stability, or translation, but so far its functional effects are not known. Since association with cataract was very strong, further studies are needed to explore the role of IL-8Rb receptors in the development of cataracts in uveitis

3.5 Conclusion

This study was done to see whether SNPs is chemokine genes can predict genetic predisposition and outcome in PSU

Two SNPs showed that they can act as genetic risk markers

1) *MCP-1 -2076* for PSU as a whole but particularly for Intermediate and Behcet's uveitis

2) *CCR2 V64I* for Behcet's disease only

Following SNP associations were noted to predict the disease phenotype.

Table 3-27: The effect of chemokine SNPs on posterior segment uveitis phenotype

SNP & Allele	Effect of Allele on gene	Association	Group
MCP-1 2518G	***G* increases expression**	Early age of onset of disease	IU & PU
RANTES -403A	***A* increases expression**	Better visual outcome	IU & PU
CCR2 V64IA	***A* Increases expression**	Poor visual outcome / High IOP	IU & PU
CCR5 32bp Δ.	***Del*. decreases expression**	Better visual outcome	IU & PU
CCR5 -59029A	***A* increases expression**	Increased need for Steroids	IU & PU
IL-8 -251A	***A* increases expression**	Poor vision	PU
IL-8Rb +1208C	**Non-functional**	Increase incidence of Cataract	Behcet's

From the above table one can deduce that certain genes were involved in pathogenesis of a particular type of PSU but not other. However both idiopathic IU and PU shared the same associations and this further confirms that immunologically they are similar disease. The *RANTES* and its receptor *CCR-5* appear to play a role in the disease process in IU and PU together with *CCR-2* whose SNP is tightly linked. These effects are either direct results of these SNPs or indirect through linkage disequilibrium with other SNPs in close proximity. It will be worth checking SNPs in the proximity and constructing extended haplotypes in patients with idiopathic uveitis (Both IU and PU)

On the other hand effects of chemokine genes on Behcet's patients were different, and the role of *CCR2 V64I* in Behcet's needs to be further explored. No significant role of chemokine genes was noted in sarcoidosis group.

Chapter Four

4 *CCR5* & *CCR2* polymorphisms in idiopathic posterior segment uveitis and Ocular Behcet's

4.1 CCR5 & CCR2 polymorphisms in idiopathic posterior segment uveitis

4.1.1 Introduction

In the previous chapter I have shown that *CCR2 & CCR5* SNPs were associated with visual outcome in idiopathic intermediate and posterior uveitis. These associations were not noted in uveitides secondary to systemic diseases implicating that the inflammatory pathways may be different in these cases. The findings that genes involved showed similar association in both IU and PU groups, further confirms the notion that both disease entities share the same pathogenesis. (Forrester JV et al. 1998) The *CCR5 32bp Δ* was associated with better visual outcome and *CCR2 64I* mutation was associated with poor visual outcome. Also noted was that *CCR5 -59029 A* mutant allele was associated with more and continuous need of steroids to control the inflammation. As already mentioned in the previous chapter, the 3 SNPs were in strong linkage and the subject homozygous for *64I* allele or *-59029 A* allele (both noted to be pro-inflammatory) were never homozygous for *32bp Δ* (noted to be protective allele). Although my study showed that the protective effects of *64V* allele or *32bp Δ* were independent of each other it is very likely that these effects may be a result of linkage to another locus yet to be identified.

A large part of the information available on the functional consequences of *CCR5* gene polymorphisms in vivo stems from studies of HIV-1–infected individuals as *CCR5* is the major coreceptor for the HIV type-1 virus. Evidence is there that other SNPs in *CCR5* gene may be functional. The cells from individuals with the wild-type for *32bp Δ* genotype showed a wide range of surface expression of *CCR5*. (Wu et al. 1997) Other rarely occurring *CCR2* SNP, *m303A (V64I)* is a nonsense mutation of coding region that exerts effects similar to those of *CCR5Δ32*. (Quillent et al. 1998) An Asian-specific *CCR5* -

893 is a single nucleotide deletion in the *CCR5* coding region, and the levels of *CCR5* expression on the surface of CD4 positive cells are greatly reduced in individuals bearing the mutant allele (Shioda et al. 2001). The *CCR2* mutation, *CCR2 64I*, is in strong linkage disequilibrium with another mutation *CCR5 –1835T* in the second intron of the *CCR5* gene, is also associated with a delay in HIV-1 disease progression (Mummidi et al. 1998) (Kostrikis et al. 1998). With respect to the promoter region, Martin *et al*. identified 10 polymorphic nucleotide positions in the *517 bp* region of Caucasian *CCR5* promoters, and described 10 haplotypes in this region. (Martin et al. 1998) Of these, the P1 haplotype has *G*, *C*, and *A* at the *208th*, *627th*, and *676th* positions, respectively, from the transcription start site of the *CCR5* gene. Although both *CCR2 64I* and *CCR5Δ32* were consistently found in the *CCR5 P1* haplotype, the homozygotes for (*CCR2 64V*/*CCR5 P1*/lacking *CCR5*Δ32) haplotype exhibited an epidemiological association with rapid progression to AIDS. McDermott *et al*. (McDermott et al. 1998) reported that the presence of homozygous for *CCR5 59029G* lacking *CCR5Δ32* and *CCR2 64I* caused a delay by 3.8 years in the progression to AIDS compared to that seen in patients homozygous for *59029 A*. Position *59029* in McDermott's report corresponds to position *303* from the transcription start site. Since different reports have adopted different numbering systems for the *CCR5* gene Carrington *et al*. (Carrington et al. 1999) proposed a new numbering system starting from the translation start codon. Using this numbering system, Mummidi *et al*. reported six human haplogroups (*HHA, B, C*, *D*, *E*, *F* and *G*) involving *CCR2 V64I*, *CCR5Δ32*, and eight polymorphic positions (*–2733*, *–2554*, *–2459*, *–2135*, *–2132*, *–2086*, and *–1835*) in the *926 bp* region of Caucasian and African-American *CCR5* promoters. This is shown in detail in following figure.

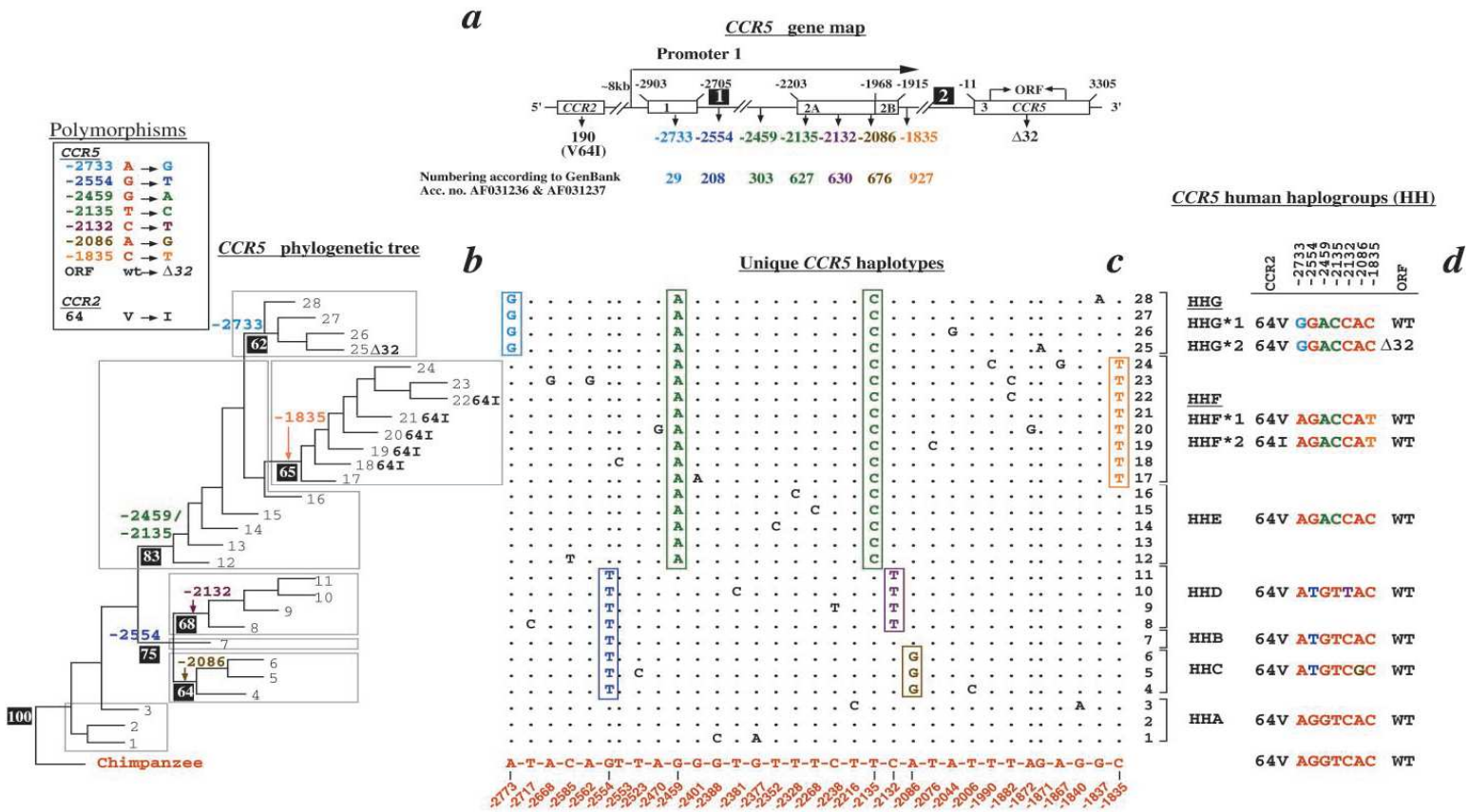


Figure 4-1: CCR5 SNPs & haplotypes as described by Mummidi et al. (Mummidi et al. 2000)
a) Scheme of *CCR2* and *CCR5* loci on chromosome 3 (not to scale; *hatched marks* denote gaps). ***b***) A phylogenetic tree depicting the relationships among the seven *CCR5* human haplogroups (HHA–HHG). A chimpanzee *CCR5* haplotype was used as an outgroup. ***c***) A schematic representation of the nucleotide sequences of the unique human *CCR5* haplotypes (22761 to 21835). The sequences of human *CCR5* haplotypes were compared with those found in the homologous region of chimpanzee *CCR5*. ***d)*** Classification of *CCR5* human haplogroups. Each haplotype within a haplogroup is characterized by the constellation of invariant polymorphisms indicated but differ from each other by additional SNPs. ***e)*** A model illustrating the potential evolution of human *CCR5* haplogroups

Recently Lui *et al*. (Liu et al. 2007) showed that construct lacking *-59029* site in the promoter region of *CCR5*, showed difference in the promoter activity, presumably due to *-2135* and *2086* SNPs. The study also showed that the effects of *CCR2 V64I* SNP were independent of *-1835* SNP to which it is tightly linked. The SNP *CCR5 -1835*C/T, located in the 50 cis-regulatory region of *CCR5* has been shown to result in loss of novel nuclear factor binding.(Mummidi et al. 2000) Transcriptional activity of different haplotypes in the cis-regulatory region of *CCR5* demonstrated that *CCR5*-haplotypes containing *1835T* (*HHF*) had significantly different promoter efficiency compared to other haplotypes. (Mummidi et al. 2000)

Aims and Objectives:

To further increase our insight into the role of *CCR5* gene in PSU, I hypothesized that theses *CCR5* haplotypes can affect the disease susceptibility and prognosis in idiopathic PSU. I only examined the effects of these haplotypes in idiopathic patients because of the previous results showing role of *CCR5* SNPs in IU and PU groups only. The aim of this study was to see whether *CCR5* haplotypes can affect the genotype of idiopathic PSU and whether the effects seen in previous studies can be attributed to other SNPs

4.1.2 Material and Methods

4.1.2.1 Subjects

There were 141 Caucasian patients with idiopathic posterior segment uveitis. 77 had intermediate uveitis and 64 had posterior uveitis.

4.1.2.2 Controls

Two groups of control subjects were used in this study: 169 healthy English Caucasian subjects who were admitted for cataract extraction and a cohort of 142 Caucasian healthy subjects whose DNA were stored and used in other studies. (Spagnolo et al. 2005) All the subjects were negative for any history of autoimmune disease.

4.1.2.3 Genotyping

4.1.2.3.1 DNA extraction

The DNA extraction in patients and was done with commercial kits (Qiagen UK Ltd, UK) according to the manufacture's instructions and as discussed in chapter 2

The DNA extraction of 169 controls was done by salt extraction method and other set of 142 controls already had DNA extraction.

4.1.2.3.2 Primer designing and genotyping:

The primers were designed as mentioned in chapter 2 in detail and the SNPs wee identified by using sequence specific primer-polymerase chain reaction (SSP-PCR) technique. All PCR reactions were run under identical conditions as mentioned previously in methods chapter. The Table 4-1 shows the details of the primers used to type *CCR5* SNPs in idiopathic PSU patients and controls.

Table 4-1 Sequences of primers used for PCR-SSP detection of *CCR5* polymorphisms

SNP Position	Locus	Accession No.	Position	Primer Sequence	Control Primers	Product Size (bp)
***CCR2*190G/A**	Exon,codingsequence	U95626	46276-295	F-TGTGGGCAACATGCTGGTCG/A	63 64*	297
			46554-573	R-AAGGTGACCGTCCTGGCTTT		
−5663A/G	Promoter	U95626	55830-852	R-GCTTGGTGAGTTTTGATATGCAT/C	63 64	290
			55563-582	F-AGCCCGACTGTCTACTGAAC		
−3900C/A	Promoter	U95626	57568-592	F-GCTATCTATCTTTCGAAAAACCAAC/A	63 64	305
			57852-872	R-CCTCCAGCTTCATTACTGCTA		
−3458T/G	Promoter	U95626	58030-049	R-AGCGATCAAGACACCCCCAA/C	210 211†	719
			57331-349	F-AGCAAGTCAGCAGCAACGC		
−2459A/G	Promoter	U95626	59029-050	R-GGACTTCACATTAACCCTGTGT/C	210 211	760
			58291-310	F-GCCGTGGATGCCTCATAGAA		
−2135T/C	Exon	U95626	59330-353	F-GTGAGAAAAGCCCGTAAATAAACT/C	210 211	951
			60272-292	R-TGCCACCACAGATGAATGTCA		
−2086A/G	Exon	U95626	59402-423	R-AGAATCAGAGAACAGTTCTTCT/C	63 64	267
			59137-158	F-AGCCTTACTGTTGAAAAGCCCT		
−1835T/C	Exon/Intronboundary	U95626	59653-674	R-AGCACTTGGTGTTTGCCAAATA/G	210 211	905
			58772-792	F-ATCTGGCATAGTGTGAGTCCT		
Delta32wt/ 32	Exon,codingsequence	U95626	62018-040	F-GCTCTCATTTTCCATACAGTCAG(wt)	63 64	244
			62015-070	F-GCAGCTCTCATTTTCCATACATTA(32)	215	

4.1.2.4 Statistical Analysis

For each subject group, genotype frequencies were counted and the allelic and allele carriage frequencies calculated. All genotype frequencies in each population were tested for deviation from the Hardy-Weinberg equilibrium using the chi-square test. Data mining for significant associations was performed using knowledge seeker® (Angoss software UK) and statistical calculations were performed with SPSS 12.0 (Chicago USA). A multiple logistic regression model was used to determine association between various genotype and the phenotypes. Power calculations were performed using QUANTO® ® Version 1.0, which is specific software for power calculations in genetic studies (http://hydra.usc.edu/gxe).

Haplotypes were identified by the computer program PHASE, version 2. (Stephens, Smith, and Donnelly 2001) Before generating haplotype, linkage between the SNPs was calculated by EM algorithm using Arlequin® population genetics software. Arlequin® is also freely available from http://cmpg.unibe.ch/software/arlequin3

4.1.3 Results

The clinical and demographic details are shown in the following Table 4-2.

Table 4-2: Characteristics of patients with Idiopathic posterior and intermediate uveitis

Follow up in years	Mean=6.8	Range=(1.5-42.6)
Sex	Males=59	Females=82
Laterality of disease	Bilateral=118	Unilateral=23
Age of onset (years)	Mean=37.82	Range=(5-70.5)
Recurrence (rate per year)	Mean=1.92	Range=(1-7)
Mean Visual impairment during inflammation	Doubling of visual angle	Range=(Nil-PL)
Better eye VA after 24 months	Mean=6/9	Range=(6/5-6/60)
Permanent visual loss	n=55	Mean=6/24, Range=6/15-HM
10 mg or more of long term steroids	n=51	
On second line of immuno-suppressants	n=51	
Cystoid macular oedema	n=85	
Raised intra-ocular pressure	n=46	
Cataract	n=55	
Idiopathic intermediate uveitis	n= 77	
Idiopathic posterior uveitis	n=64	

All the genotypic frequencies were within Hardy Weinberg equilibrium. As shown in the Table 4-3, there was no significant difference between the frequencies of SNPs in uveitis or any of the control groups.

Table 4-3: *CCR5* promoter polymorphisms genotype frequencies in idiopathic segment uveitis and controls

SNPs position	Uveitis	controls (A)	Controls (B)	Chi 2X2
	N=141 (%)	N=169 (%)	N=142 (%)	
CCR2 190				1.51
G/G	118 (84%)	139 (82%)	114 (80%)	
G/A	23 (16%)	30 (18%)	28 (20%)	
A/A	0 (0%)	0 (0%)	0 (0%)	
-5,663				0.5
A/A	45 (32%)	55 (32%)	38 (27%)	
A/G	63 (45%)	91 (54%)	78 (55%)	
G/G	33 (23%)	23 (1 3%)	26 (18%)	
-3,900				1.69
C/C	14 (10%)	21 (13%)	19 (13%)	
C/A	72 (51%)	92 (54%)	76 (54%)	
A/A	55 (39%)	56 (33%)	47 (33%)	
-3,458				1.47
T/T	12 (9%)	19 (11%)	19 (13%)	
T/G	72 (51%)	88 (52%)	74 (52%)	
G/G	57 (40%)	62 (37%)	49 (35%)	
-2,459				1.31
G/G	20 (14%)	32 (19%)	26 (18%)	
G/A	74 (52%)	87 (51%)	74 (52%)	
A/A	47 (33%)	50 (30%)	42 (30%)	
-2,135				1.31
T/T	20 (14%)	32 (19%)	26 (18%)	
T/C	74 (52%)	87 (51%)	74 (52%)	
C/C	47 (33%)	50 (30%)	42 (30%)	
-2,086				2.32
A/A	55 (39%)	56 (33%)	44 (31%)	
A/G	70 (50%)	89 (53%)	76 (54%)	
G/G	16 (11%)	24 (14%)	22 (15%)	
-1,835				1.15
T/T	0 (0%)	0 (0%)	0 (0%)	
T/C	24 (17%)	31 (18%)	26 (18%)	
C/C	117 (83%)	138 (82%)	116 (82%)	
Δ 32				1.98
wt/wt	109 (77%)	142 (85%)	111 (78%)	
wt/ Δ 32	27 (19%)	26 (15%)	30 (21%)	
Δ 32/ Δ 32	4 (3%)	1 (1%)	1 (1%)	

There was extensive linkage disequilibrium between the SNPs. The *-2459G/A* SNP was in complete linkage with *-2135T/C* similarly *CCR2 190G/A* was very tightly linked with *-1835C/T*. Using PHASE®, 12 haplotypes were constructed including 5 common haplotypes. As seen in Table 4-4, the prevalence of haplotype *HHC* was 50% in uveitis group compared to 64% in both control groups. (p=0.004). The frequencies of other haplotypes were quite similar between the groups. The frequency of *HHC* was 50% in both IU and PU groups.

Table 4-4: *CCR5* haplotype carriage frequencies in uveitis and controls

Haplotype	*CCR2* 190	-5663	-3900	-3458	-2459	-2135	-2086	-1835	Δ 32	Uveitis	Int. Uveitis	Post Uveitis	Controls	Chi 2X2
										n=(141)	n=(77)	n= (64)	n=(311)	P value
HHA	V	A	A	G	G	T	A	C	wt	14 (10%)	8 (10%)	6 (10%)	22 (7%)	NS
HHC	V	A	C	T	G	T	G	C	wt	70 (50%)	38 (50%)	32 (50%)	199 (64%)	0.004
HHC*1	I	A	C	T	G	T	G	C	wt	3 (2%)	2 (2%)	1 (2%)	6 (2%)	NS
HHC*2	V	A	C	T	G	T	G	C	Δ 32	2 (1%)	1 (1%)	1 (1%)	3 (1%)	NS
HHC*3	V	A	A	G	G	T	G	C	wt	2 (1%)	1 (1%)	1 (1%)	12 (4%)	NS
HHE	V	G	A	G	A	C	A	C	wt	72 (52%)	40 (52%)	32 (50%)	171 (55%)	NS
HHE*1	V	G	A	G	A	C	G	C	wt	1 (1%)	1 (1%)	0	3 (1%)	NS
HHE*2	V	G	A	G	A	C	G	C	Δ 32	2 (1%)	1 (1%)	1 (1%)	0 (0%)	NS
HHE*3	V	A	C	G	A	C	A	C	wt	4 (3%)	2 (2%)	2 (2%)	6 (2%)	NS
HHF*1	V	A	A	G	A	C	A	T	wt	0	0	0	6 (2%)	NS
HHF*2	I	A	A	G	A	C	A	T	wt	23 (16%)	12 (16%)	10 (15%)	53 (17%)	NS
HHG*2	V	G	A	G	A	C	A	C	Δ 32	27 (19%)	17 (22%)	10 (16%)	56 (18%)	NS

4.1.3.1 Effect on phenotype:

4.1.3.1.1 HHC and visual outcome

It was also noted that 63% (44/70) of the patients with *HHC* haplotype needed systemic steroids to control the inflammation compared to 81% (58/71) of the patients not carrying *HHC* haplotype. P=0.007 OR: 0.3758, 95% CI: 0.37 (0.18-0.78). (Figure 4-2)When subgroup analysis was done no significant difference were noted between IU and PU groups suggesting that effects of *HHC* haplotype were generalized to the whole idiopathic PSU group.

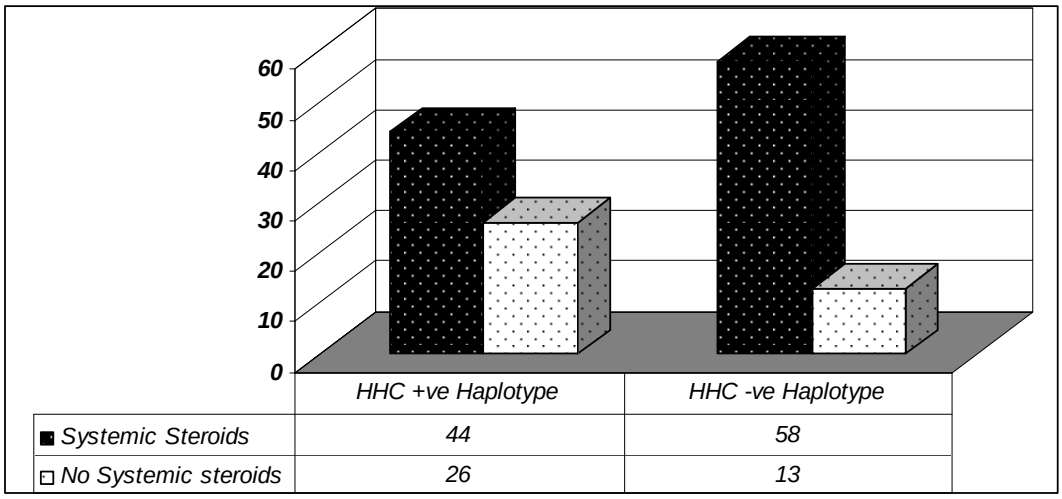


Figure 4-2: *CCR5* HHC haplotype and need for systemic steroids in idiopathic PSU.

Less patients with *HHC* haplotype needed systemic steroids (does not include peri-ocular steroids) to control the inflammation

Better eye visual acuity after 2 years (minimal follow up) was also noted to be better in patients carrying *HHC* haplotype in both IU and PU group. The difference remained significant after correcting for age, Table 4-5 shows this in detail.

Table 4-5: Better eye VA at 2 years in IU & PU patients carrying *HHC* haplotype.

	Intermediate Uveitis (N=77)		P value	Posterior Uveitis (N=64)		P value
HHC	HHC +ve N =(38)	HHC -ve N= (39)		HHC +ve N= (32)	HHC -ve N= (32)	
Better eye VA Range	6/9 (6/5---6/18)	6/18 (6/6---6/36)	0.01	6/12 (6/6---6/18)	6/30 (6/9---6/60)	0.008

This effect was however lost, as the time progressed and at third year and beyond and no significant difference in the visual acuities were found between the *HHC* and the rest of the haplotypes. So at least for a short term *HHC* carriers had better vision in both IU and PU groups

4.1.3.1.2 HHF2 and Visual acuity:

Patients carrying *HHF2* had worse VA in effected eye at year one, two, three and 5. The effect was seen in both IU and PU but it was more pronounced in IU. (Table 4-6)

Table 4-6: *HHF2* Haplotype and VA in IU & PU.

Intermediate Uveitis	Mean Visual acuity (Range)			
HHF2 Haplotypes	1st year	2and Year	3rd year	5 year
HHF2 +ve (12)	6/24 (6/6-6/36)	6/30 (6/9-6/60)	6/36 (6/9-6/60)	6/36 (6/12-4/60)
HHF2 -ve (65)	6/7.5 (6/5-6/18)	6/12 (6/6-6/24)	6/9 (6/9-6/18)	6/12(6/9-6/60)
P value	0.0001	0.009	0.003	0.01
Posterior Uveitis	Mean Visual acuity			
HHF2 Haplotypes	1st year	2and Year	3rd year	5 year
HHF2 +ve (15)	6/18 (6/5-6/60)	6/30 (6/9-5/60)	6/36 (6/12-6/60)	6/36 (6/18-1/60)
HHF2 -ve (49)	6/9 (6/5-6/24)	6/12 (6/9-6/60)	6/18 (6/9-6/60)	6/24 (6/12-6/60)
P value	0.008	0.005	0.02	0.1

Patients with *HHF-2* haplotype had worse mean visual acuities in both IU & PU group. The difference was more significant in early course of the disease.

This difference was more significant to start with but by year five it became less significant (at least in PU).

4.1.3.1.3 HHG2 and Visual Outcome

HHG2 was associated with better visual acuity. This association was not as strong as with *HHF2*, but was still quite significant in first two years of life. Again as seen in *HHF2* haplotype the association was stronger in IU group as compared to PU group and difference was only significant in the early years of the disease. (Table 4-7)

Table 4-7: Association of *HHG2* Haplotype with VA in patients with idiopathic PSU

Intermediate Uveitis		Mean Visual Acuity (Range)		
HHG2 haplotypes	1st year	2and Year	3rd year	5 year
HHG2 +ve (17)	6/6 (6/5-6/36)	6/9 (6/6-6/24)	6/12 (6/9-6/24)	6/12 (6/9-6/36)
HHG2 -ve (60)	6/12 (6/6-6/36)	6/18 (6/6-6/60)	6/18 (6/9-6/60)	6/36 (6/12-4/60)
P value	0.009	0.01	0.07	0.05

Posterior Uveitis		Mean Visual Acuity		
HHG2 haplotypes	1st year	2and Year	3rd year	5 year
HHG2 +ve (10)	6/9 (6/5-6/36)	6/12 (6/6-6/60)	6/18 (6/9-6/60)	6/24 (6/18-2/60)
HHG2 -ve (54)	6/18 (6/5-6/60)	6/24 (6/9-5/60)	6/30 (6/9-6/60)	6/36 (6/12-1/60)
P value	0.008	0.01	0.06	0.09

Patients with *HHF-2* haplotype had better mean visual acuities in both IU & PU group. The difference was more significant in early course of the disease.

The graphs below (Figure 4-3) show the trend of visual acuities in IU patients with *HHF2* and *HHG2* haplotypes.

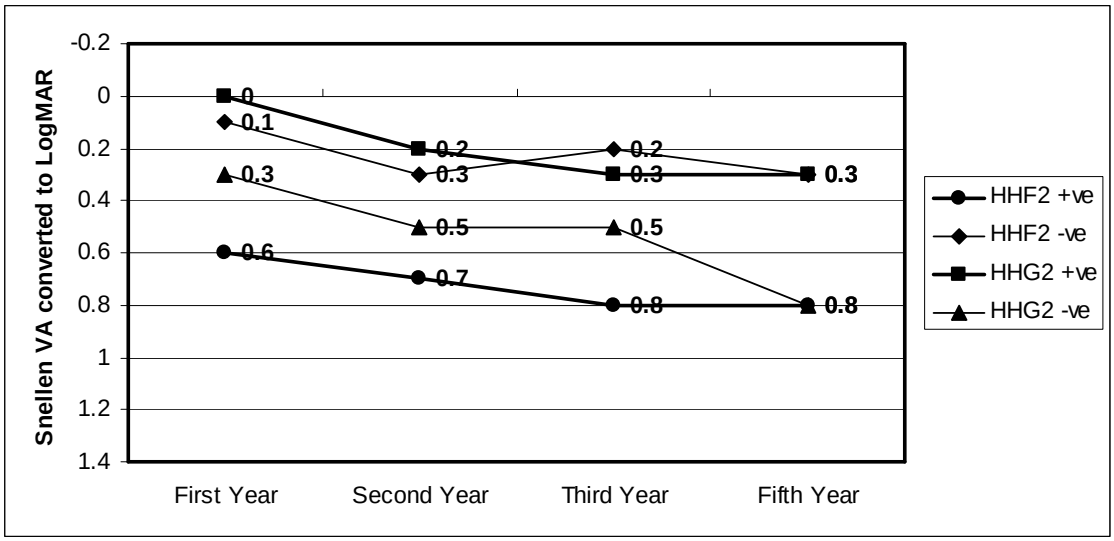


Figure 4-3: Visual acuity of patients carrying *HHF2* & *HHG2* haplotype in intermediate uveitis

IU patients carrying HHG-2 haplotype and patients not carrying *HHF-2* haplotype consistently had better visual acuities. . The values represent mean LogMAR VA (converted from Snellen) over period of times. For range of VA please refer to above tables 4-6 & 4-7

The Figure 4-4 below shows the trend in posterior uveitis patients

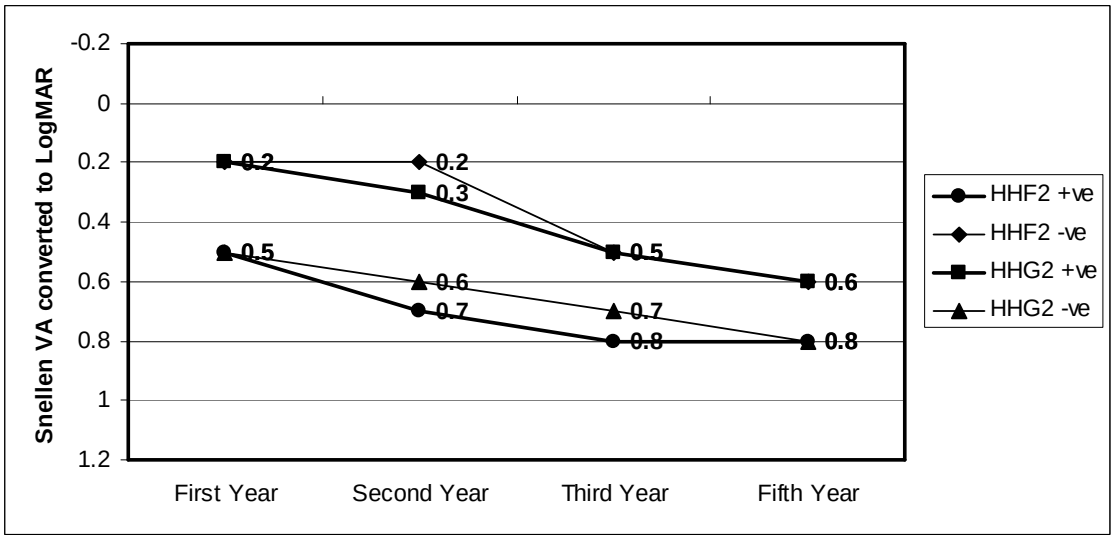


Figure 4-4: Visual acuity of patients carrying *HHF2* & *HHG2* haplotype in posterior uveitis.

IU patients carrying *HHG-2* haplotype and patients not carrying *HHF-2* haplotype consistently had better visual acuities. . The values represent mean LogMAR VA (converted from Snellen) over period of times. For range of VA please refer to above tables 4-6 & 4-7

In short, the *HHF2* haplotype was associated with poor visual acuity in both IU and PU groups. On the other hand, *HHG2* and *HHC* carriers had better visual acuity in both IU and PU groups.

When the individual SNPs (excluding the previously studied *V64I*, *32bp Δ* and *59029*) were analysed, no significant associations were noted except *-1835C/T* and *-2135T/C*. This was because of their strong linkage with previously studied SNPs. The *-1835C/T* which was tightly linked to *CCR2 V64I* showed similar associations with visual outcome and high intraocular pressures. The Table 4-8 below shows (and as noted in chapter 3 with *CCR2 V64I*) the *-1835* variant allele *T* was predictor of poor visual acuity in both groups. In case of intermediate uveitis the difference was more significant in early period of disease and in posterior uveitis it was more significant in later period of disease.

Table 4-8: Visual acuity in patients with intermediate and posterior uveitis, when categorised according to *-1835* genotype.

Intermediate Uveitis		Mean Visual Acuity (Range)		
CCR5 -1835 C/T	2and year	3rd Year	5th year	10th year
-1835CC	6/6 (6/6-6/60)	6/9 (6/9-6/36)	6/12 (6/9-6/12)	6/18 (6/12-6/36)
-1845CT	6/10 (6/6-6/60)	6/24 (6/9-6/60)	6/24 (6/9-60)	6/36 (6/18-1/60)
P value	0.06	0.001	0.008	0.09

Posterior Uveitis		Mean Visual Acuity		
	2and year	3rd Year	5th year	10th year
-1835CC	6/10 (6/9-6/18)	6/18 (6/9-6/36)	6/18 (6/12-5/60)	6/24 (6/12-4/60)
-1835CT	6/12 (6/9-6/60)	6/24 (6/12-6/60)	6/60 (6/18-1/60)	4/60 (6/18-HM)
P value	0.1	0.2	0.05	0.001

Similarly *-2135T/C* which was in 100% linkage with *CCR5* (*2459*) *59029* showed association with need of steroids in intermediate uveitis and need of long term steroids in posterior uveitis. Since the linkage was 100%, the results were exactly similar to *CCR5 59029*. The *C* allele of *-2135* SNP was associated with need of systemic steroids. (See Table 8-7 Appendix B)

It was also noted that *-3900* SNP was associated with presence of cataract. This was mainly seen in intermediate uveitis although there was a non-significant trend noted in posterior uveitis. Collectively patients who developed visually significant cataract after two years of onset of disease, (minimum follow up period) the *A* allele frequency was 82% compared to 58% of the patients who did not develop cataract. P=0.004, pc=0.02, 95% CI: 2.1 (1.2-3.5). Data shown in Appendix B, Table 8-6

4.1.4 Discussion

This study was done to demonstrate the role of polymorphisms in *CCR5* gene in idiopathic non-infectious posterior segment uveitis. The *CCR5* gene has been mapped to the short arm of chromosome 3 amongst a group of genes that encode multiple chemokine receptors including the *CCR2* gene. The ligands for CCR2 and CCR5 are MCP-1, MIP-1α, MIP-1β, MCP-2 and RANTES, which are known to play an important role in uveitis. (Crane et al. 2001) (Foxman et al. 2002) (Takase et al. 2006) (Verma et al. 1997) Recently Crane *et al.* (Crane et al. 2006) have shown that *CCR5* is markedly expressed on mononuclear cell infiltrates present in the inflamed retina in EAU. They also demonstrated that CCR5 plays a role in the recruitment of antigen-specific, Th1-polarized cells across the specialized BRB and into the retina during an inflammatory reaction. It is very likely that CCR5 mediates its inflammatory effects via MIP-a, MIP-b and MCP-2 rather than RANTES; as I noted that high *RANTES* producing genotypes were associated with better visual outcome. This finding was consistent with various animal and human studies. (Kim, Chung, and Yu 2010) (Sonoda et al. 2003)

All the SNPs were in Hardy Weinberg equilibrium and there was strong linkage disequilibrium between few SNPs. In my study the *HHC* was most frequent haplotype in patients and controls followed by *HHE*. The *HHG2* and *HHF2* were two other prominent haplotypes. These frequencies are similar to other studies in the literature. (Spagnolo et al. 2005) (Fischer et al. 2008) In my study, the frequency of *HHC* haplotype was significantly lower in IPSU groups compared to control groups. Not only this, among patients the frequency of this haplotype was lower in the group needing systemic steroids to control the inflammation. The need of steroid is the indirect measure of intensity of inflammation

particularly in case of IU. It was also noted that patients carrying *HHC* haplotype had better VA with both eyes open at two years time. This difference of visual acuity however lost it significant in long term follow up. It appears that *HHC* haplotype not only confers protection against the development of PSU, but also protects during the course of the disease. *HHC* is known to be associated with disease retardation and delayed progression to death in Caucasian patients with AIDS. (Gonzalez et al. 1999) Interestingly this *HHC* haplotype has also been found to reduce risk of developing Kawasaki disease. (Burns et al. 2005) Recently Mamtani *et al*. showed that subjects heterozygous for *HHC* may have a less risk of developing systemic lupus erythromatosis.(Mamtani et al. 2008) However the effect was not noted with subjects homozygous to *HHC* and they attributed this effect to *HHF2* haplotype. At least two of the SNPs defining this haplotype contain putative protective alleles i.e. *-2459* (*59029G*) and *190* (*V64*). Another SNP in *HHC* at *-2135* harbour *T* allele which was recently shown to cause low promoter activity compared to *C* allele.(Liu et al. 2007) Same study showed that *G* allele at *-2086* position lead to lower promoter activity and this allele is also part of *HHC* haplotype. Interestingly, *CCR5* haplotype *HHC* does not include the *32 deletion* variant, suggesting that decreased gene expression is not the key mechanism of protection against PSU and supporting the hypothesis that promoter variants might result in altered *CCR5* functions.

Presumably four of the alleles in *CCR5* gene promoter region that define *HHC* haplotype are in one way or other effect the functions of *CCR5* gene. This is very interesting to note that in IPSU none of the allelic frequencies were significantly different from controls but collectively as *HHC* haplotype they were significantly less in patients compared to controls. This is consistent with the

notion that complex diseases may result from genetic variants that are relatively common in the general population and many of them have minor effects (Wang et al. 2005) (Hirschhorn 2005) (Hirschhorn and Daly 2005).

Although I observed that *HHC* haplotype may have a protective effect in the development of uveitis, Spagnolo *et al*. (Spagnolo et al. 2005) noted that this same haplotype was associated with persistent lung disease in patients with sarcoidosis. They concluded that *CCR5 HHC* does not confer susceptibility to sarcoidosis per se but it only acts after "classic" sarcoid strikes. Recent study on 995 German Sarcoid patients however failed to replicate their findings. (Fischer et al. 2008)

There were two other significant associations. *HHF2* haplotype was associated with poor visual outcome and *HHG2* was the predictor of better visual outcome. As seen in the table below, this haplotype had 4 alleles (shown in *Italics*) that putatively increase *CCR5* gene expression or promoter activity and also contains *CCR2 64I* previously noted to be involved with poor visual outcome. The *HHF2* also had wild type at exon 4 (no 32bp deletion) and hence the association with the visual acuity were more significant with *HHF2* than *HHC* haplotypes.

Haplotype	***CCR2* 190**	**-5663**	**-3900**	**-3458**	**-2459**	**-2135**	**-2086**	**-1835**	**Δ 32**
HHF*2	***I***	**A**	**A**	**G**	***A***	***C***	***A***	**T**	***wt***

In case of *HHG2* haplotype the putatively functional SNPs in the promoter region (*2135*, *2086*) were similar to *HHF2* so one can infer that the effects are just because of *32 bp* deletion and *V* allele at *CCR2* position *64*. This is the reason that the visual acuity preservation noted in the presence of *HHG2* was not as strong as seen in the absence of *HHF2* haplotype.

Regarding *1835* SNP, where *C* to *T* change has some effect on the functional activity of *CCR5* promoter region (Mummidi et al. 2000) it is not known whether this change increases or decreases the effects of *CCR5* gene. However one must note that the protective haplotypes, *HHC* and *HHG2* had *C* allele and *HHF2* had *T* allele at site *-1835*. This does signify that *C* to *T* transition may have some proinflammatory effects; however the functional role of SNP *-1835* on *CCR5* gene needs to be further explored. FASTSNP tool also predicts that there are mild to moderate chances that this SNP can be fuctional.

My conclusions are based on *CCR5* haplotypes; therefore the reliability of the method used for haplotype reconstruction is critical. Although errors in haplotype assignment can originate from genotyping or calculation error as well as inherent marker ambiguity in the presence of SNP heterozygosity; however computational methods of haplotype inference such as PHASE (Stephens, Smith, and Donnelly 2001), the statistical method used in this study, have been shown to have high accuracy (Kraft et al. 2005) (Adkins 2004) (Stephens, Smith, and Donnelly 2001). Moreover, the frequencies of the deduced *CCR5* haplotypes in the cohort are consistent with those previously reported by Gonzalez *et al* (Gonzalez et al. 1999) (Fischer et al. 2008) strongly supporting the reliability of PHASE.

Conclusion:

This study has highlighted the role of *CCR5* gene in the prognosis of PSU. Few haplotypes have shown association with the susceptibility and phenotype of the disease. As *CCR5* gene is not the sole gene controlling the influx of activated T cells and macrophages to the site of uveitis hence the association these haplotypes seen here are modest. It should be noted that there is great redundancy in the immune system and this concept is further strengthened by a recent study where EAU developed in *CCR5* deficient mice. (Takeuchi et al. 2005) It is also quite possible that the association seen here could be due to a yet unidentified locus in the Chromosome 3p21 region where this gene lies along with other chemokine receptor genes *CCR1*, *CCR2* & CCR3.(Samson et al. 1996b) Chromosome 3 has been suggested as harbouring susceptibility loci for inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis. (Cornelis et al. 1998) (Duerr et al. 2002) (Pericak-Vance et al. 2004) These conditions having similar immuno-pathology like PSU. Linkage studies on chromosome 3 can be done in future to confirm these findings.

In short this study has shown that variations in *CCR5* genotype can affect the phenotype and outcome in patients with posterior segment uveitis but further studies are needed to replicate my results

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4.2 CCR2 & CCR5 polymorphisms in Ocular Behcet's

4.2.1 Introduction:

Behcet's disease (BD) is a relapsing multisystemic inflammatory disorder characterized by recurrent oral ulcers, genital ulcers and uveitis, but joints, skin, central nervous system, blood vessels and gastrointestinal tract are frequently involved and are the major cause of morbidity. The precise aetiology of the disease is unknown but it is widely believed that BD is a "profound inflammatory response triggered by an infectious agent in a genetically susceptible host." (Kaneko, Oyama, and Nishibu 1997) (Sohn et al. 2001) (Sohn, Lee, and Lee 2001) While rare in UK, BD is fairly common in countries lying at latitudes of between 30 and 45° N including the traditional silk route. (Table 4-9) Its prevalence in these countries has been reported as high as 420 per 100,000 (Azizlerli et al. 2003) compared to 0.5 per 100,000 in UK. (Chamberlain 1978) (Chamberlain 1980) Uveitis in BD often referred as ocular Behcet's (OB) occurs in about 30-70% of the patients with BD (Kural-Seyahi et al. 2003) (Tursen, Gurler, and Boyvat 2003) and leads to visual loss in approximately 25% of the patients.(Muhaya et al. 2000)

Table 4-9: Association between latitude and prevalence of Behcet's disease (adapted from Demirkesen et al.)

Latitude (°N)	Country/ethnic group	Prevalence of Behcet's disease per 100000
25	Saudi Arabia	20
31	Palestinian	>10
32	Iran	16.7
32	Morocco	>15
36	Japan	13.5
39	Portugal	1.53
39	Turkey	100
42	Italy	2.5
52	England	0.5
56	Scotland	0.27
60	Sweden	1.18

Geographical distribution of the disease & familial clustering points towards a genetic susceptibility. A well-documented association exists between the BD and *HLA-B51* (Muhaya et al. 2000) in a wide range of ethnic groups, with the *HLA-B*5101* subtype being the most frequently expressed and conferring a relative risk as high as 16. (Baricordi et al. 1986) The geographic distribution of *HLA-B51* closely mirrors that of BD, with low prevalence of *HLA-B51* and BD in the southern hemisphere and in Europe above 45^{o}N. (Middleton et al. 2003) (Verity et al. 1999)The exact role of the B51 molecule remains elusive, but it might be primarily involved in the disease development through specific antigen presentation or molecular mimicry with microbial antigens, as well as participation in linkage disequilibrium with a presently unknown susceptibility gene.

However, *HLA-B*51* cannot be the sole causative factor for the simple reason that roughly a third of patients, even in countries with a high disease prevalence, do not possess this allele. Furthermore, the risk, or odds ratio of Behcet's disease in an individual positive for *HLA-B*51* shows a wide variation across Europe and Asia. Also among the North American indigenous populations of Alaska and in Sub Saharan Africa the frequency of *HLA-B51* allele is quite high but the BD is virtually absent. (Verity et al. 1999) However it could be accounted for by the absence of an environmental trigger factor. It has been proposed that contribution of the HLA-B locus to the overall genetic susceptibility to BD is about 20%. (Gul et al. 2001)

SNPs in other genes in close proximity to HLA have also been noted as risk factors for BD, particularly Major histocompatibility complex class I-related chain A (*MICA*) and *TNF-α*. Ahmed *et al* showed that *C* allele at *-1031* site of *TNF-α* promoter site was associated with Behcet's disease independent of

HLA-b51 allele. (Ahmad et al. 2003) A recent meta-analysis has confirmed that Behcet's disease is associated with the *-1031C*, *-238A* and the *-857*T *TNF-α* promoter polymorphisms in various ethnic groups. (Touma et al. 2010)

Similarly various studies have shown that *MICA 006* and *009* loci are associated with BD susceptibility, however there is conflicting evidence whether this effect is independent or due to linkage with *HLA-B51* locus.(Marin et al. 2004) (Hughes et al. 2005) (Mizuki et al. 2007) (Park et al. 2002) Modest associations have also been found in coagulation factor V, endothelial nitric oxide synthase and intercellular adhesion molecule-1 genes.(Gurgey, Balta, and Boyvat 2003) (Kim et al. 2003)

Current understanding of the pathology of BD indicates that it is a Th1 generated immune response (Frassanito et al. 1999) (Kulaber et al. 2007) (Hamzaoui et al. 2002) (Pay et al. 2007). The immunopathology of BD has been discussed in detail in chapter one, however Figure 4-5 here explains our current understanding of the pathogenesis of Behcet’s disease.

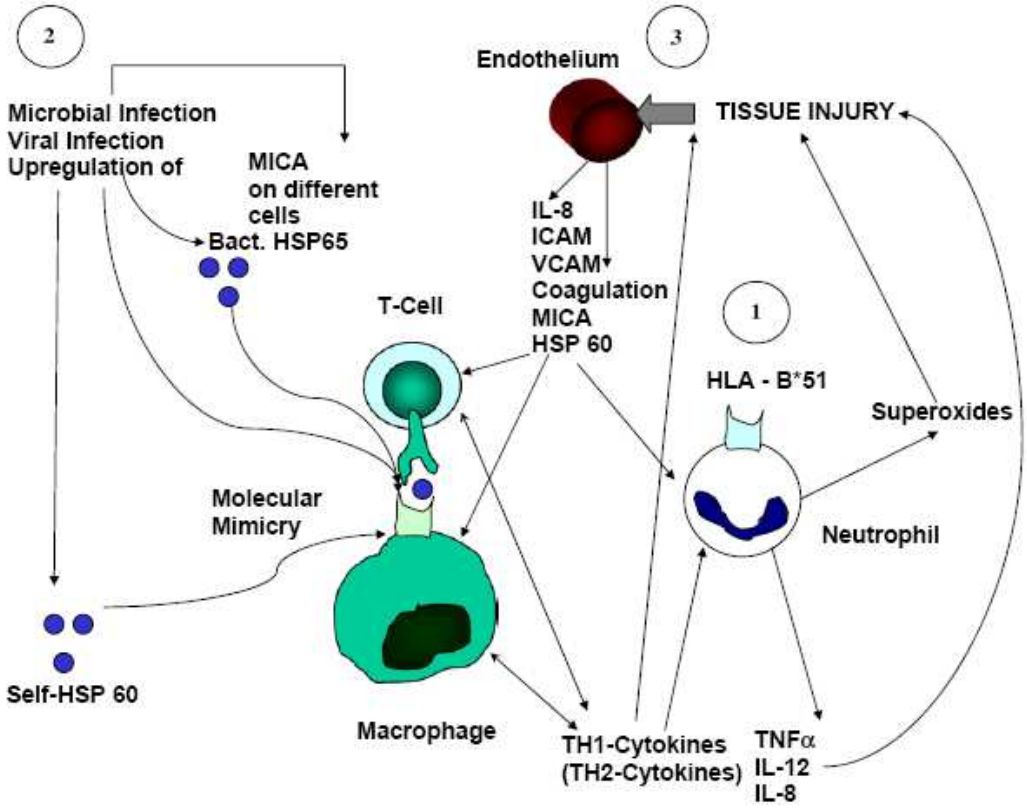


Figure 4-5: Hypothesis for the pathogenesis of BD.

(1) Genetic factors, e.g. *HLA-B*51*, induce a general hyperactivity of the immune system (TH1-response, granulocytes). (2) Bacterial or viral infection stimulate the expression of HSP60 (self) and MICA on different cells (e.g. endothelial cells), upregulation of adhesion molecules, activation of coagulation, stimulation of T cells (esp. gd and NK cells), continuing elevation of the cytokine production and finally induce (3) tissue damage by vasculitis. Additionally, a molecular mimicry with *HLA-B*51* may play a role. In parallel, B-cells will be stimulated polyclonally (not shown on the figure) and may produce more antibody (e.g. against HSP). Courtesy Deuter, CM 2008 Progress in Retinal and Eye Research

CC chemokine receptors play an important role in executing the immune response in Behcet's disease. (Dalghous, Freysdottir, and Fortune 2006) (Houman et al. 2004) Active BD is associated with increased activity of MCP-1, MIP-1β and RANTES (Bozkurt et al. 2003) (Kaburaki et al. 2003) (Ozer et al. 2005) which are ligands for CCR2 and CCR5 receptors

A recent study using flow cytometric analysis of lymphocytes from aqueous humour of OB showed, increased expression of CCR5 and *RANTES*, which were comparable with non Behcet's uveitis levels (Kim, Chung, and Yu 2010). Houman *et al* did flow cytometric analysis on peripheral blood lymphocytes that

showed increased expression of CCR5 in all forms of Behcet's diseases. (Houman et al. 2004)

In the previous chapter I showed that *CCR2 V64I* could be one of the genetic risk marker in patients with OB. Though this SNP is now thought to be functional but the role of *CCR2* in OB is not clear. So it is difficult to say whether this association seen is independent or secondary to linkage with other loci in *CCR2* or even in *CCR5* gene as these two genes are in close proximity. The aim of this study was to see whether SNPs in nearby *CCR2* and *CCR5* loci show any association with OB

4.2.2 Methods:

4.2.2.1 Subjects:

31 British Caucasian patients diagnosed with OB were recruited for this study. Besides this I had 29 Caucasian patients with Behcet's disease for more than 5 years but no uveitis.

I also had 30 non-Caucasian British patients with OB. This group included patients from Middle East.

4.2.2.2 Controls:

169 healthy subjects who were admitted to Whipps Cross hospital for cataract surgery and cohort of 142 Caucasian healthy subjects whose DNA were stored and used in other studies.(Spagnolo et al. 2005)

I also had 56 non Caucasian (Middle East and West Asia) healthy subjects who were admitted for cataract surgery at WXH.

4.2.2.3 DNA extraction and genotyping:

As previously described the patients Genomic DNA was extracted from EDTA anticoagulated peripheral venous blood by Qiagen Kits using standard protocol supplied by the supplier. The controls DNA was extracted by salt extraction method as described in detail in chapter 2. Genotyping was performed using polymerase chain reaction with sequence-specific primers (PCR-SSP) method as described previously. The *CCR2* and *CCR5* primer designed to detect these polymorphisms are shown Table 4 10 &Table 4 11. The locations of the SNPs on *CCR2* & *CCR5* gene are shown in

Figure 4 6 & Figure 4 7

Table 4-10: *CCR2* primers for SSP-PCR

SNP's position	Locus	Accession no	position	Primer sequence	primers*	bp
-6928 G/T	Promoter	U95626	39151-177	F-TGC ATA AAG AAT TCT AAG ATG TAC TG/T	210+211	799
			39930-950	R-AGG GTG AAC AAA GGT TCA CCA		
-6752 A/G	Promoter	U95626	39353-373	R-AAC CAG ATG GGA AGA GGG AAT/C	210+211	772
			38602-623	F-GGA AAT TGC TAA GGG CAT CGT T		
190 G/A	Exon coding	U95626	46295-321	R-TTT TTG CAG TTT ATT AAG ATG AGG AC/T	210+211	808
			45513-534	F-GAA GGC AGA AGG TGA ATA GTT C		
3000 A/G	Exon	U95626	49083-105	F-CCA GTG GGA ACT CCT AAA TCA AA/G	210+211	918
			49981-50000	R-ATA GGT AGA CCC TCC GGG AT		
3547 T/C	3'UTR	U95626	49633-652	F-TAC AGG CCA CAC AAC CCC AT/C	210+211	851
			50464-483	R-CCT GCT TAA CTC GAA CAG CC		
3610 A/G	3'UTR	U95626	49695-715	F-GAC ATC TGC CTC ATC CAA GCA/G	210+211	867
			50541-561	R-TAT GCC AAG ACC CTT CCT TAC		
3671 C/G	3'UTR	U95626	49776-796	R-CTG TGT CTT CTC ATT CAC CAG/C	210+211	870
			48927-946	F-TCG CTG TCA TCT CAG CTG GA		
4385 T/A	3'UTR	U95626	50470-490	F-TCG AGT TAA GCA GGT GGA AGT/A	210 +211	766
			51214-235	R-AAG GCC CCA TTG AAA CAA TGA C		

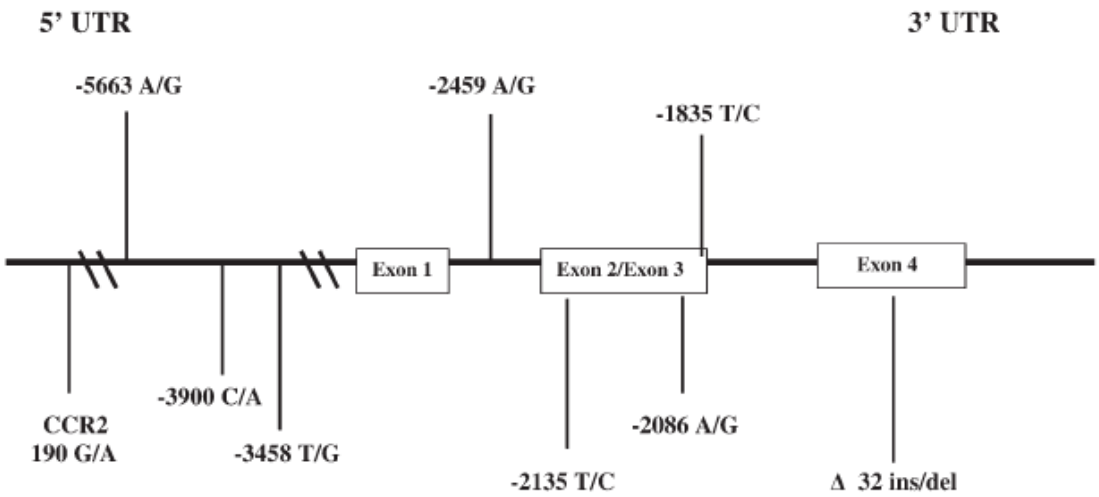


Figure 4-6 Map illustrating positions at which variations have been identified in the coding region of *CCR2*, *CCR5*, and 5_ upstream region of *CCR5*.

The numbering system used designates the first nucleotide of the translation start site of *CCR5* as position 1, and nucleotide immediately upstream of this as position _1.

Table 4-11: *CCR5* primers for SSP-PCR

SNP Position	Locus	Accession No.	Position	Primer Sequence	Control Primers	ProductSize (bp)
*CCR2*190G/A	Exon,codingsequence	U95626	46276-295	F-TGTGGGCAACATGCTGGTCG/A	63 64*	297
			46554-573	R-AAGGTGACCGTCCTGGCTTT		
− 5663A/G	Promoter	U95626	55830-852	R-GCTTGGTGAGTTTTGATATGCAT/C	63 64	290
			55563-582	F-AGCCCGACTGTCTACTGAAC		
− 3900C/A	Promoter	U95626	57568-592	F-GCTATCTATCTTTCGAAAAACCAAC/A	63 64	305
			57852-872	R-CCTCCAGCTTCATTACTGCTA		
− 3458T/G	Promoter	U95626	58030-049	R-AGCGATCAAGACACCCCCAA/C	210 211†	719
			57331-349	F-AGCAAGTCAGCAGCAACGC		
− 2459A/G	Promoter	U95626	59029-050	R-GGACTTCACATTAACCCTGTGT/C	210 211	760
			58291-310	F-GCCGTGGATGCCTCATAGAA		
− 2135T/C	Exon	U95626	59330-353	F-GTGAGAAAAGCCCGTAAATAAACT/C	210 211	951
			60272-292	R-TGCCACCACAGATGAATGTCA		
− 2086A/G	Exon	U95626	59402-423	R-AGAATCAGAGAACAGTTCTTCT/C	63 64	267
			59137-158	F-AGCCTTACTGTTGAAAAGCCCT		
− 1835T/C	Exon/Intronboundary	U95626	59653-674	R-AGCACTTGGTGTTTGCCAAATA/G	210 211	905
			58772-792	F-ATCTGGCATAGTGTGAGTCCT		
Delta32wt/ 32	Exon,codingsequence	U95626	62018-040	F-GCTCTCATTTTCCATACAGTCAG(wt)	63 64	244
			62015-070	F-GCAGCTCTCATTTTCCATACATTA(32)	215	

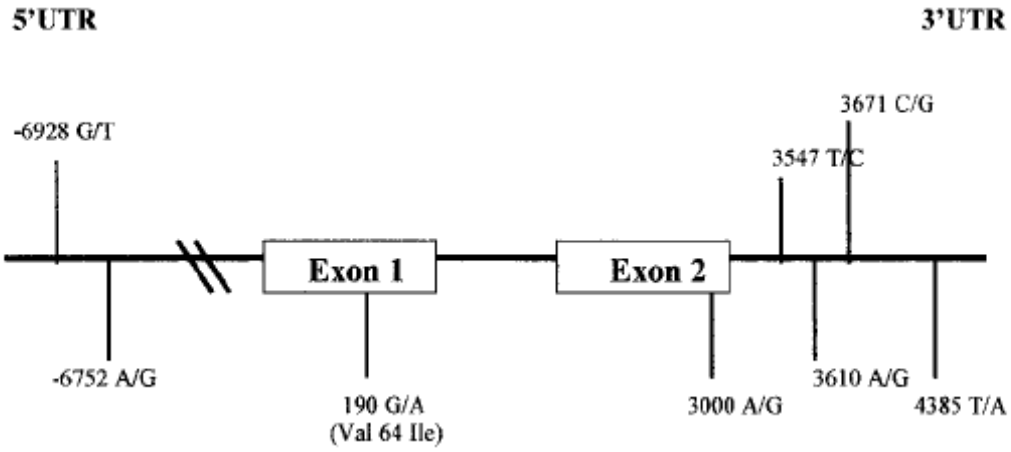


Figure 4-7: Positioning of the investigated single nucleotide polymorphisms across the C-C chemokine receptor 2 (*CCR2*) gene.

4.2.2.4 Phenotyping

Behcet's was diagnosed according to the Criteria described by "International Study Group for Behcet's Disease", (Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease 1990) (Evaluation of diagnostic ('classification') criteria in Behcet's disease--towards internationally agreed criteria. The International Study Group for Behcet's disease 1992) which is as follows:

Table 4-12: Diagnostic criteria for Behcet's disease

In the absence of other clinical explanations, patients must have:
(1): Recurrent Oral Ulcerations (aphthous or herpetiform) recurring at least three times in one 12-month period;
(2): and two of the following: a) Recurrent Genital Ulceration. b) Eye Lesions: anterior or posterior uveitis, cells in the vitreous by slit lamp examination or retinal vasculitis observed by an ophthalmologist c) Skin Lesions: erythema nodosum, pseudofolliculitis, papulopustular lesions or acneiform nodules in post adolescent patients not on corticosteroids. d) positive Pathergy

Though the aim of this study was not to check HLA-B51 serotypes or alleles, but where available the presence of HLA-B51 antigen was also recorded from the notes. This was done to reveal any association of this serotype/allele with *CCR2* & *CCR5* SNPs tested

4.2.2.5 Statistical methods:

The data were analyzed using the SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). It has been discussed in detail in chapter 2. Since most of the SNPs tested were non-functional, Bonferroni correction was particularly applied for multiple alleles testing if association was noted in a non-functional allele. The correction was not applied in case association was noted with a haplotype. The genotype and allele frequencies between patients with BD and controls were compared by the two-tailed chi 2 test. Multiple logistic regression was done to correct for confounding traits

4.2.3 Results:

The mean follow up period of the patients was 12.36 years and Table 4-13 presents the clinical features of the 31 patients. Of particular interest was that 74% developed some degree of permanent visual loss and 77% needed second line of immunosuppressive therapy.

Table 4-13: Clinical characteristic of patients with Ocular Behcet's.

Follow up in years (2.2-38.2)	mean=12.36
Age of onset (21-68years)	mean=32.5
laterality of disease Bilateral: Unilateral	4:1
Sex Ratio Males: Females	2.4:1
Anterior uveitis (n=1)	3%
Posterior Uveitis (n=5)	16%
Pan Uveitis (n=25)	81%
Vasculitis (n=22)	71%
Optic Atrophy (n=9)	29%
Ischemic Maculopathy (n=9)	29%
Glaucoma (n=12)	39%
Cystoid macular oedema (n=22)	71%
Visual Acuity after 24 months in good eye	6/5-HM (mean=6/18)
Permanent visual loss (n=23)	74%
>10mg of steroids for long term (n=20)	65%
second line of immuno-suppressant (n=24)	77%

4.2.3.1 CCR2 V64I in Caucasian patients with OB

As seen in Table 4-14 below, and as shown in chapter 3, *CCR2 64I* allele was more frequent in the Behcet's patients. Besides the 283 controls used in previous study this time I had another cohort of controls numbering 142. (Total 425) The genotypic frequencies were not significantly different between the control populations. The allelic frequency of *CCR2 64I* in the new control cohort was 10% compared to 7% in the cohort used in the study throughout. The pooled results are shown in the following table. With the addition of new control

group the *64I* frequency difference decreased a bit but still the difference was statistically significant.

Table 4-14: *CCR2 V64I* frequencies in OB and controls.

GENOTYPE *CCR2* 190 *G/A*	Patients	Control	Chi 2X3	Chi 2X2
	N (%)	N (%)		p
GG	21 (68%)	361 (85%)		0.0122
GA	9 29%)	63 (15%)		0.0362
AA	1 (3%)	1 (0%)		0.0150
Total	31	425	0.004	
Allelic Carriage / phenotype				
G	30 (97%)	424 (100%)		0.0150
A	10 (32%)	64 (15%)		0.0122
Allelic Frequency				
G	51 (82%)	785 (92%)		0.0055
A	11 (18%)	65 (8%)		
Total	62	850		

(pc=0.025 and OR of 3.16 95% CI; 1.55-6.44). *64I* was more common in females (n=9) where the allele carriage (*V/I or VV*) was 67% compared to 18% in males (n=22) (p=0.008). (Table 4-15) The allelic frequency of *64I* was 33% in females compared to 11% in males. This allelic frequency of 33% in females was very significant compared to 6% in female controls p=0.0000005.

Table 4-15: *CCR2 V64I* prevalence in both sexes in ocular Behcet's

CCR2 V64I (*G/A*)	Females N (%)	Males N (%)	Chi2x3= P value	Chi2x2 P Value
GG	3 (33%)	18 (82%)		0.008
GA	6 (67%)	3 (14%)	0.01	0.003
AA	0 (0%)	1 (5%)		NS
Total	9	22		
Allelic Carriage / phenotype				
G	9 (100%)	21 (95%)		NS
A	6 (67%0	4 (18%)		0.009
Allelic Frequency				
G	12 (67%)	39 (89%)		0.04
A	6 (33%)	5 (11%)		
Total	18	44		

4.2.3.2 CCR2 V64I in OB Vs BD

Since all the patients in my group had uveitis, in order to see whether this high frequency of *64I* allele is specific to Behcet's disease or uveitis in Behcet's, 29 British Caucasians with Behcet's disease but no ocular involvement (NOB) were typed for *CCR2 V64I*.

As shown in Table 4-16, 10% (n=3) of NOB were carriers of *64I* allele and when compared with OB group (32%) this difference was still significant despite low sample size. P=0.04 OR= 4.12 95% CI: 4.1 (1.00-16.94). Also the differences in the frequency between the sexes was also not significant in BD patients but when compared with OB the frequency of *64I* allele carriage in females in BD group (10%) was significantly low then from females in OB group (67%) p=0.001

Table 4-16: *CCR2-64I* frequency in ocular vs non-ocular Behcet's

Characteristics	OB (n=31)	NOB (n=29)	p value Chi 2x2
Gender ratio M/F	22 / 9	9 / 20	p=0.002
CCR2 VV	21 (68%)	26 (90%)	P=0.03
CCR2 V/I	9 (29%)	3 (10%)	
CCR2 II	1 (3%)	0 (0%)	
64 I allele carriage	10 (32%)	3 (10%)	p=0.04

	Male OB (n=22)	Male NOB (n=9)	
VV	18 (82%)	8 (89%)	
VI	3 (14%)	1 (11%)	
I	1 (5%)	0 (0)	
64I allele carriage in males	4 (18%)	1 (11%)	P=0.61

	Females OB (n=9)	Females NOB (n=20)	
VV	3 (33%)	18 (90%)	P=0.001
VI	6 (67%)	2 (10%)	P=0.001
II	0 (0%)	0 (0%)	
64I allele carriage in females	6 (67%)	2 (10%)	p=0.001

	OB	NOB	
HLA-B51% (OB Vs NOB)	21.4%	24.10%	P=0.9

4.2.3.3 CCR2 V64I in non Caucasians with OB

I than compared the frequency of 64I allele in 30 non-Caucasian patients with ocular Behcet's with 56 non-Caucasian controls. The frequency of *64I* allele was 22% in OB group compared to 13% in controls. This difference was not statistically significant, but the frequency of *64I* allele was higher in both non-Caucasian populations compared to Caucasians. (Table 4-17)

Table 4-17: *CCR2 V64I* genotypic frequencies in Non Caucasian OB and controls

CCR2 V64I Genotype	Non-Caucasian OB	Non- Caucasian Controls	P Value
GG	20 (67%)	44 (78%)	NS
GA	7 (23%)	10 (18%)	
AA	3 (10%)	2 (4%)	
Total	30	56	
Allelic Carriage / phenotype			
G	27 (90%)	54 (96%)	NS
A	10 (33%)	12 (21%)	
Allelic Frequency			
G	47 (78%)	98 (87%)	NS
A	13 (22%)	14 (13%)	
Total	60	112	

When the group was divided according to gender, again it was noted that the frequency of 64I allele in females was significantly higher than males. There were 20 males and 10 females with OB and the allelic frequency of *64I* was 50% in females compared to 8% in males. P=0.0001. Among the controls there were 22 females and the allelic frequency of *64I* was 14% compared to 50% with OB. P=0.001. In short in non- Caucasians there were significant differences in 64I allele frequencies between female OB and female controls Details are shown in Table 4-18.

Table 4-18: *CCR-2 V64I* frequencies in non-Caucasian OB patients and controls and association with sex

Non Caucasian OB CCR-2 *V64I*	Male OB N=20	Female OB N=10	P value Chi 2 X 3	P Value Chi 2x2
VV	17 (85%)	3 (30%)		0.002
VI	3 (15%)	4 (40%)	0.004	NS
II	0 (0%)	3 (30%)		0.009
Allelic Carriage / phenotype				
V	20 (100%)	7 (70%)		0.009
I	3 (15%)	7 (70%)		0.002
Allelic Frequency				
V	37 (92%)	10 (50%)		0.0001
I	3 (8%)	10 (50%)		

Non Caucasian Controls	Male Controls N=34	Female controls N=22		P
VV	27 (79%)	17 (77%)		NS
VI	6 (18%)	4 (18%)		NS
II	1 (3%)	1 (5%)		NS
Allelic Carriage / phenotype				
V	33 (97%)	21 (95%)		NS
I	7 (21%)	5 (23%)		NS
Allelic Frequency				
V	60 (88%)	38 (86%)		NS
I	8 (12%)	6 (14%)		

Female OB Vs Controls	Female OB N=10	Female Control N=22		P
VV	3 (30%)	17 (77%)	0.025	0.010
VI	4 (40%)	4 (18%)		NS
II	3 (30%)	1 (5%)		0.043
Allelic Carriage / phenotype				
V	7 (70%)	21 (95%)		0.043
I	7 (70%)	5 (23%)		0.010
Allelic Frequency				
V	10 (50%)	38 (86%)		0.001
I	10 (50%)	6 (14%)		

It must be noted that I did not have non-Caucasian patients with NOB so the comparison of *64I* frequencies between OB and NOB in non-Caucasian group could not be performed

HLA-B51 and *CCR2 V64I* relationship

28 patients out of total of 31 were serotyped for HLA-B51. Six (21.4%) were positive for HLA-B51. None of these six were carrier of *CCR2 64I* allele.

Among NOB patients, 24% percent (n=7/29) were HLA-B51+ and the frequency was quite similar to OB group. In NOB group only one out of these seven patients was heterozygous for 64I allele.

The results suggested that there was no obvious linkage between the two loci and also raised the possibility that *CCR2 64I* allele plays its role in the susceptibility of Behcet's disease in the absence of HLA-B51 serotype.

4.2.3.4 CCR2 Haplotypes in Behcet's Uveitis

The allelic frequencies of the 8 *CCR2* SNPs including *V64I* (*190 G/A*) are shown in the Table 4-19. As seen the table there were no significant differences of allele frequencies between the patients and controls. For each SNP the proportion of homozygous and heterozygous alleles were consistent with Hardy-Weinberg equilibrium.

Table 4-19: Allelic Frequencies of *CCR2* SNPs in OB and controls

SNPs Position	Allele	(n=62)	%	(n =338)	%	P value
		Ocular Behcet's		Healthy Controls		
-6,928	T	4	6%	27	8%	NS
	G	58	94%	311	92%	NS
-6,752	A	16	25%	71	21%	NS
	G	46	75%	267	79%	NS
190	A	11	18%	20	6%	0.001
	G	51	82%	318	94%	0.001
3,000	A	17	27%	71	21%	NS
	G	45	73%	267	79%	NS
3,547	T	19	30%	71	21%	NS
	C	43	70%	267	79%	NS
3,610	A	22	35%	95	28%	NS
	G	40	65%	243	72%	NS
3,671	G	7	12%	47	14%	NS
	C	55	88%	291	86%	NS
4,385	T	18	29%	78	23%	NS
	A	44	71%	260	77%	NS

From the investigated *CCR2* polymorphisms, I was able to deduce nine estimated *haplotypes* (Table 4-20). Haplotype *2* includes four SNPs (*T* at nucleotide position *4,385, T* at *3,547*, *A* at *3,000*, and *A* at *6,752*), which appear to be in 100% linkage, as they occur together in all subjects; these four SNPs were unique to this *haplotype* alone, being absent in all of the other haplotypes

As seen in the Table 4-20, haplotypes 1, 2 and 3 were quite prevalent in both populations. In controls I was able to deduct *9 haplotypes* but in patients *Haplotypes 6*, *7* & *9* were absent which is presumably because of the size of sample. The *haplotype 8* (containing the *I* allele at *64* position (*190 A*) was more frequent in the OB group (12% Vs 2%) p= 0.01. However because of the small sample size the 95% CI was very wide and the power was only 50%.

Genotype- phenotype studies failed to reveal any effect of *CCR2* haplotypes on the clinical course and outcome of the OB.

Table 4-20: *CCR2* Haplotypes in OB and controls

	SNPs								Behcet's	Controls	P Value
Haplotype	-6,928	-6,752	190	3,000	3,547	3,610	3,671	4,385	(n=31)	(n =169)	Chi 2x2
1	G	G	G	G	C	G	C	A	19 (60%)	110 (65%)	NS
2	G	A	G	A	T	A	C	T	14 (45%)	68 (40%)	NS
3	G	G	G	G	C	G	G	A	6 (20%)	42 (25%)	NS
4	T	G	G	G	C	A	C	A	1 (3%)	17 (10%)	NS
5	G	G	A	G	C	G	C	A	3 (9%)	11 (7%)	NS
6	T	G	G	G	C	G	C	A	0 (0%)	7 (4%)	NS
7	G	G	G	G	C	A	C	A	0 (0%)	5 (3%)	NS
8	G	G	A	G	C	G	G	A	4 (12%)	3 (2%)	0.011
9	T	G	G	G	C	G	G	A	0 (0%)	3 (2%)	NS

4.2.3.5 CCR5 haplotypes in Behcet's uveitis

CCR2 190 (*V64I*) is in close proximity with *CCR5* genes and it is tightly linked to many SNPs (particularly *-1835*). To see whether *CCR2 V64I* association seen in OB is due to linkage with SNPs in *CCR5* gene, I genotyped OB patients for *CCR5* promoter SNPs. The SNPs studied here were the same typed for IU & PU and discussed early.

All the SNPs were in consistent with Hardy-Weinberg equilibrium. As seen in the Table 4-21 apart from *V64I*, few SNPs showed moderate association with the OB group.

Firstly, at *-1835* position where *T* allele was more prevalent in OB group compared to the controls (3% Vs 0%) p=0.001, pc 0.009. This was expected as this allele is tightly linked with *V64I* SNP, but interesting the linkage in OB group was not as tight as seen in the two control groups where it was almost 100%.

Secondly, at *-5663* position where *AA* genotype was present in 52% of OB patients compared to 30% in the control group. (P=0.01, however after correction the significance was lost. pc 0.09)

Thirdly, as studied in chapter 3, SNP *-2459* (same as *-59029*) showed some week association p 0.03. This SNP was tightly linked to SNP at *-2135* but no significant differences were noted with this SNP.

Table 4-21: *CCR5* SNPs in OB and controls

SNP	Genotype	Behcet's		Controls 1		Controls 2		P value
		N= 31	%	N=169	%	N=142	%	
CCR 190	GG	21	68%	139	82%	114	80%	NS
	GA	9	29%	30	18%	28	20%	NS
	AA	1	3%	0	0%	0	0%	0.001
-5663	A/A	16	52%	55	32%	38	27%	0.01
	A/G	10	32%	91	54%	78	55%	0.01
	G/G	5	16%	23	13%	26	18%	NS
-3900	C/C	8	26%	21	13%	19	13%	NS
	C/A	14	45%	92	54%	76	54%	NS
	A/A	9	29%	56	33%	47	33%	NS
-3458	TT	6	19%	19	11%	19	13%	NS
	TG	15	48%	88	52%	74	52%	NS
	GG	10	32%	62	37%	49	35%	NS
-2459	GG	4	13%	32	19%	26	18%	NS
	GA	12	39%	87	51%	74	52%	NS
	AA	15	48%	50	30%	42	30%	0.03
-2135	TT	8	26%	32	19%	26	18%	NS
	TC	13	42%	87	51%	74	52%	NS
	CC	10	32%	50	30%	42	30%	NS
-2086	AA	14	45%	56	33%	44	31%	NS
	AG	13	42%	89	53%	76	54%	NS
	GG	4	13%	24	14%	22	15%	NS
-1835	TT	1	3%	0	0%	0	0%	0.001
	TC	6	19%	31	18%	26	18%	NS
	CC	24	77%	138	82%	116	82%	NS
Δ 32	wtwt	26	84%	142	85%	111	78%	NS
	wtΔ 32	5	16%	26	15%	30	21%	NS
	Δ 32 Δ 32	0	0%	1	1%	1	1%	NS

With these SNPs I was able to construct 11 haplotypes in control group including the common *HHC*, *HHE* haplotypes. (Table 4-22) In Behcet's group only 7 haplotypes were present as the following rare haplotypes were absent *HHC*2 HHE*1 HHF*1 & HHE*3*. This is presumably because of small sample size of OB group. No significant differences in haplotype frequencies were noted between the two groups.

Also I was unable to find any significant effect of theses haplotypes on the phenotype of uveitis in OB group.

Table 4-22: *CCR5* haplotypes in OB and controls

Haplotype	*CCR2*	-5663	-3900	-3458	-2459	-2135	-2086	-1835	Δ	Behcet's	Controls	Significance
	190								32	Uveitis		
										n= (31)	n=(311)	P value
HHA	V	A	A	G	G	T	A	C	wt	3 (10%)	22 (7%)	NS
HHC	V	A	C	T	G	T	G	C	wt	17 (55%)	199 (64%)	NS
HHC*1	I	A	C	T	G	T	G	C	wt	2 (6%)	6 (2%)	NS
HHC*2	V	A	C	T	G	T	G	C	Δ 32	0	3 (1%)	NS
HHC*3	V	A	A	G	G	T	G	C	wt	1 (3%)	12 (4%)	NS
HHE	V	G	A	G	A	C	A	C	wt	15 (48%)	171 (55%)	NS
HHE*1	V	G	A	G	A	C	G	C	wt	0	3 (1%)	NS
HHE*3	V	A	C	G	A	C	A	C	wt	0	6 (2%)	NS
HHF*1	V	A	A	G	A	C	A	T	wt	0	6 (2%)	NS
HHF*2	I	A	A	G	A	C	A	T	wt	8(25%)	53 (17%)	NS
HHG*2	V	G	A	G	A	C	A	C	Δ 32	4 (12%)	56 (18%)	NS

However when individual SNPs were studied the *CCR5 -5663* showed association with the need of second line of immunosuppressant. 24 patients out of total 31 patients needed second line of treatment and in this group the *GG* genotype was very infrequent (4%) compared to (57%) in the group not needing second line of treatment p 0.0008, pc 0.007. However the power of this significance was low because of small sample size. The genotypic frequencies are shown in detail in the following Table 4-23

Table 4-23: *CCR5 -5663* SNP association with need of second line immunosupression in OB

CCR5 -5663	On Second line of Immunosuppressant (n=24)	Not on Second line of Immunosuppressant (n=7)	Chi 3X2	Chi 2X2
GENOTYPE	Count	Count		p
AA	14 (58%)	2 (29%)		NS
AG	9 (38%)	1 (14%)	0.0036	NS
GG	1 (4%)	4 (57%)		0.0008
Total	24	7		
Allelic Carriage				
A	23 (96%)	3 (43%)		0.0008
G	10 (42%)	5 (71%)		NS

Since *-1835* and *CCR2 V64I* were linked, as expected I also noted the gender bias at *-1835* locus as well. As shown in the Table 8-8 Appendix B however, the association was not as strong as seen with *CCR2 V64I* SNP.

4.2.4 Discussion:

This study was done to further elucidate the role of *CCR2 V64I* SNP in the susceptibility of Behcet's induced uveitis. Initially thought to be non-functional polymorphism there is ever growing evidence that this valine to isoleucine change can have functional consequences. Nakayama *et al* (Nakayama et al. 2004) showed that cells expressing *CCR2A-64I* migrated more efficiently than those expressing *CCR2A-64V*. Pulse–chase experiments revealed that higher expression of *CCR2*A-*64I* was due to increased stability of *CCR2*A. Other studies have shown that this SNP can modulate the surface expression of *CCR5* (Lee et al. 1998) (Mellado et al. 1999) (Sabbe et al. 2001). This subject has been discussed in detail in chapter 3

CCR2 is the receptor for MCP-1 and the role of MCP-1 and CCR2 in BD is becoming clearer. Serum MCP-1 levels were noted to play a role in sustained low grade chronic inflammation in OB (Kaburaki et al. 2003) and high MCP-1 levels are found in aqueous of patients with OB. (Curnow et al. 2005) Like wise high expression of CCR2 has been noted on the circulating T cells of patients with BD. (Houman et al. 2004)

Both *CCR2* and *CCR5* genes have been mapped to chromosome 3p21.3-p24, and are clustered within a total distance of 350 kb on chromosome 3p21.3. (Daugherty and Springer 1997) (Samson et al. 1996b) *CCR2 V64I* is not only tightly linked to *CCR5* promoter loci particularly *-1835*, it is also noted to effect the expression of *CCR5* receptor. This has lead to believe that *CCR2* and *CCR5* work in close association and in vivo and in vitro studies have shown increased expression of *CCR5* on inflammatory cells during active Behcet's disease. (Dalghous, Freysdottir, and Fortune 2006) (Houman et al. 2004) (Imamura et al. 2005) (Onder et al. 2003)

The frequency of variant allele *64I* in OB was 18% in my patient population compared to 6%, 7% and 10% in three control groups. (Pooled frequency 8%) The addition of third control group further confirms that the *64I* frequency in controls is the true representation of population, rather than selection biased. This can be further confirmed by the fact that the frequency of 64I allele in UK normal populations is around 6-7%. (Hampe et al. 2001) (Martinson et al. 2000) (Spagnolo et al. 2003) (Spagnolo et al. 2005). When the frequency of 64I was compared with NOB it again showed that the frequency of 64I allele was 10% in NOB which is closer to controls rather than OB. Given that the frequency of HLA-B51 was quite the same in the OB and NOB groups it is quite possible that *CCR2 64I* allele could be a risk factor for uveitis in patients with Behcet's disease. It is interesting to note that frequency of *64I* allele is generally higher in countries where BD is more prevalent. For example in Korea the *64I* allelic frequency in healthy subjects is around 27% (Cheong et al. 2007), in Japan around 26% Hizawa, 1999 1475 /id} and around 13-14% in Turkey and Greece. (Apostolakis et al. 2007) (Yigit et al. 2007)

In my non-Caucasian patients (Middle East) the frequency in OB was 22% compared to 13% in non-Caucasian controls (Middle East & West Asia,). The frequency in the control population was similar to published studies, (Karaali et al. 2010) (Yigit et al. 2007) (Martinson et al. 2000)

Although the frequency in non Caucasians were higher in OB patients but the difference was not statistically significant. I did not have enough non-Caucasian patients with NOB to perform meaningful analysis but based on my findings in Caucasian patients I expect that frequency of *64I* allele in NOB would not be significantly different.

Another interesting finding was the high allele carriage frequency of *64I* among female patients (67%) with OB compared to NOB and controls. If only the male patients with OB were considered the frequency was 11% not significantly different from controls and NOB patients. This difference was also seen in Non-Caucasian OB patients where the *64I* frequency in females was 70% compared to 15 % in OB males and 22% in female healthy controls. It appears that the risk of OB associated with *64I* allele is higher for females compared to males. This is possible if we implicate virus exposure as an initiating event in Behcet's disease. We know that males and females differ in their susceptibility to variety of infections particularly Herpes Simplex. (Barna et al. 1996) (Burgos et al. 2005) (Suligoi et al. 2004) Herpes simplex not only is important in occurrence of Behcet's disease, (Suzuki and Suzuki 2004) but also its clearance may require CCR5 molecule. (Sorensen and Paludan 2004) Besides Herpes virus can cause persistent expression of CCR2 and other chemokines at primary and latent sites which can cause immune mediated diseases. (Cook et al. 2004) Very interesting Mojtahedi *et al* from Iran (Mojtahedi et al. 2006) recently showed that *CCR5 32bp Δ* could be a risk factor for Behcet's disease in Iranian women but not in men. This gender bias in Behcet's patients have been noted earlier by Chen *et al* (Chen et al. 2004), who found that genetic variation in *MCP-1* and *RANTES* (which are ligands for *CCR2* and *CCR5* respectively) can predispose to Behcet's disease depending on gender. Interestingly a similar association has been noted in another genetic study, where risk of myocardial infarction was only seen in female carriers of *64I* allele. (Petrkova et al. 2003)

Studying further 8 SNPs in *CCR2* gene did not reveal any effect on susceptibility to OB or on the phenotype of the disease. This may be a true finding, or it could be a false negative result because of relatively small sample

size. One important finding was that *CCR2 64I* allele though was conferring risk of OB development but it did not affect the severity or outcome of the disease. Increased stability of *CCR2* as a result of *64I* mutation theoretically should make more CCR2 available for MCP-1 and other ligands. Experimental animal studies have shown that while MCP-1 plays a role in the initiation of uveitis (Adamus et al. 1997) (Mo et al. 1999) (Tuaillon et al. 2002), the levels do not particularly follow the activity of uveitis. It has also been seen that the serum MCP-1 levels do not correlate with disease activity in BD but they are consistently higher than controls. (Kaburaki et al. 2003) Hence any polymorphism that would lead to *CCR2* over expression (in turn driving T cells/monocytes towards MCP-1) can increase the susceptibility to uveitis and may not affect the phenotype of disease. Hence in this study I noted that while *V64I* polymorphism acted as a risk factor for OB, it did not affect the severity, response to treatment or visual outcome.

On the other hand when SNPs in the promoter region of *CCR5* gene were studied, modest associations were noted with *-5663 A* allele, *-2459 A* allele and stronger association was seen with *-1835 T* allele. This is presumably because *CCR2 V64I* is strongly linked to these SNPs but one must not forget that *-2459 A* allele has shown to increase the expression of *CCR5* independent of *CCR2 64I* allele. (Shieh et al. 2000) Similarly, *C-1835-T* substitution, results in loss of novel nuclear factor binding. (Mummidi et al. 2000)Investigations of the transcriptional activity of different haplotypes in the cis-regulatory region of *CCR5* have demonstrated that *CCR5*-haplotypes containing *1835T (HHF)* have significantly different promoter efficiency compared to other haplotypes. The *-5663* SNP is up until now is thought to be non functional and its effects are probably as a result of linkage to other functional SNPs in the proximity.

However in-vitro or in-silico studies can be done to find out functional effects of this SNP.

Given that more associations were noted in *CCR5* SNPs rather than *CCR2* SNPs it is possible that *CCR2 64I* allele exert its effects by altering *CCR5* expression either indirectly through linkage or through direct functional effects. There is ever increasing body of evidence that *CCR2* 64I allele can directly alter the expression of *CCR5* receptor gene. Nakayama *et al* noted CCR5 surface expression was more severely blocked by co-expression of *CCR2*A-*64I* than by *CCR2*A-*64V*. (Nakayama et al. 2004) This negative effect of *CCR2*A on *CCR5* expression was shown to arise from the possibility of heterodimer formation between *CCR2*A and *CCR5*. Taken together, the authors concluded that *CCR2*A-*64I* polymorphism modulates *CCR5* surface expression.

Mellado *et al* showed that valine to isoleucine substitution gives CCR2 receptor ability to heterodimerize with the CCR5 and/or CXCR4 receptor following MCP-1 binding. (Mellado et al. 1999) Similarly the *CCR2V64I* polymorphism was indicated to decrease CCR5 re-expression after ligand induced internalization in CD4+ T cells (Sabbe et al. 2001)

This theory seems attractive particularly if we believe that a pathogen is involved in the aetiology of Behcet's disease and decreased *CCR5* expression can lead to all sort of immune mechanisms. *CCR5* deficient mice challenged with certain pathogens, e.g., HSV, influenza virus and mycobacterium tuberculosis, demonstrate symptoms ranging from increased inflammatory cytokine production and leukocyte infiltration to increased T cell clonal expansion (Nansen et al. 2002) (Sorensen and Paludan 2004) (Algood and Flynn 2004). These findings have frequently been reported in patients with BD (Gul 2001) (Suzuki and Suzuki 2004) (Houman et al. 2004). However this

theory should be interpreted with caution as I did not notice any association of *CCR5 32bp Δ* which is known to decrease the expression of *CCR5*. However as discussed earlier, a report from Iran shows association of *32bp Δ* genotype with Behcet's disease in females only. (Mojtahedi et al. 2006)

The other possible way that *64I* mutation can exert its effects is through reduce cell expression of CXCR4 by heterodimerisation of CCR2 with CXCR4. (Lee et al. 1998) (Mellado et al. 1999) This chemokine receptor has recently been reported to enhance the active retention of highly differentiated primed T cells at sites of chronic inflammation. Use of topical steroids leads to up regulation of CXCR4 in primed T cells in uveitis (Curnow et al. 2004) signifying the protected role of CXCR4 in uveitis but whether down regulation of CXCR4 on monocytes plays any role in the triggering or propagation of uveitis in OB is not known at all. Interestingly the aqueous levels of CXCL12 the ligand for CXCR4 are decreased in active Behcet's uveitis and idiopathic anterior uveitis. (Curnow et al. 2005)

The association of *64I* seen with OB is unlikely to be due to linkage *with HLA-B51* gene. In fact the opposite is true as none of the carrier of *64I* allele was positive for HLA-B51 antigen. The frequency of HLA-B51 (22.27%) was quite similar to 26.3% reported from Irish patients with OB. (Kilmartin, Finch, and Acheson 1997). It is quite likely that *CCR2 V64I* can exert its risk of developing uveitis in the absence of *HLA-B51* gene.

Conclusion:

In summary this study has shown that *64I* allele could be a genetic risk marker for uveitis in Behcet's disease. *64I* probably does not predispose to Behcet's disease, nor does it affect the severity of the uveitis. But once BD is there it can precipitate the onset of OB and this effect is particularly seen in females in both Caucasian and non-Caucasian populations.

64I SNP either exerts its effects directly through *CCR2* stability and or *CCR5*/CXCR4 expression or indirectly through linkage with loci in *CCR5* genes. Given the fact that among tested for HLA-B51, all the patients with *64I* allele were negative for HLA-B51 it seems quite likely that *CCR2 64I* imparts risk of OB in the absence of HLA-B51.

Chapter Five

5 Cytokine gene polymorphism in posterior segment uveitis

5.1 Introduction

5.1.1 Cytokines and their role in inflammation

Immune function depends on the biologic activities of numerous small glycoprotein messengers, termed cytokines. Typically, cytokines exhibit broad functional activities that mediate not only effector and regulatory immune functions, but also wider effects across a range of tissues and biologic systems. Most of the cytokines indeed manifest multiple biological activities on different target cells, and many cytokines are produced by a variety of cell types in response to different stimuli. Furthermore, the expression of a given cytokine gene is invariably influenced by other cytokines, forming a network of "cytokine cascades"(Kohase et al. 1987)

Historically cytokines were classified on the basis of their presumed function, cell of secretion, or target of action. They were categorized as follows

- Interferons: IFNs (IFN-α, IFN-β, IFN-γ)
- Tumour necrosis factors: TNFs (TNF-α, lymphotoxin-α, lymphotoxin-β)
- Interleukins: ILs
- Chemokines
- Transforming Growth Factors: TGFs
- Hematopoietic colony-stimulating factors (CSFs)

It were Mosmann and colleagues (Mosmann et al. 1986) (Mosmann and Coffman 1989) who revolutionized the field of immunology by categorising T-helper (Th) cells into two distinct populations with contrasting and cross-regulating cytokine profiles.

Th2 cells, which produce the cytokines IL-4, IL-5, and IL-13, are involved in allergic responses and the clearance of extracellular pathogens, such as worms.(Mosmann and Coffman 1989)

IFN-γ-producing Th1 cells, on the other hand, contribute to the elimination of intracellular pathogens and are involved in cell-mediated and delayed-type hypersensitivity responses.(Mosmann and Coffman 1989) This leads to the division of cytokines into Th1 mediating cytokines and Th2 mediating cytokines. In earlier studies, Th1 cells were implicated as the causal agents in the pathogenesis of autoimmunity as these cells possessed the ability to transfer disease and elevated levels of IFN-γ were detected in vivo in areas of inflammation. However, studies in several autoimmune disease models suggested a more complex story. Mice genetically deficient in *IFN-γ* gene not only were not protected but also exhibited enhanced susceptibility in many models of autoimmunity.(Ferber et al. 1996) (Jones et al. 1997) (Matthys et al. 1998) The identification of Th17 cells, a CD4+T-cell subset that produces IL-17, (Aggarwal et al. 2003) has helped to shed some light on this apparent paradox. These cells, like Th1 cells, have the capacity to cause T-cell-mediated inflammation and autoimmune disease. Moreover, the Th17 response is remarkably elevated in mice lacking *IFN-γ*. However, in experimental autoimmune uveitis (EAU), mice lacking *IL-17* are also not protected and display elevated numbers of IFN-γ-producing CD4+ T cells. (Haak et al. 2009) (Luger et al. 2008) This raises the possibility, that Th1 and Th17 cells might cooperate with one another to propagate the inflammatory response

The human genome project has facilitated discovery of numerous cytokines, posing considerable challenges in resolving their respective and synergistic

functions in complex tissues in health and disease. Such understanding is essential with the advent of cytokine-targeting therapies in the clinic

This all has lead to categorization of cytokines by structural homologies shared with related molecules. (Commins, Borish, and Steinke 2010) Table 5-1 shows in details the 7 superfamilies of cytokines. The Superfamilies share sequence similarity and exhibit homology and some promiscuity in their reciprocal receptor systems; they however do not exhibit functional similarity

Table 5-1: Structural classification of cytokines

FAMILY	MEMBERS
Hematopoietic	
Common g chain	IL-2, IL-4, IL-7, IL-9, IL-15, IL-21
Shared b chain (CD131)	IL-3, IL-5, GM-CSF
Shared IL-2b chain (CD122)	IL-2, IL-15
Other hematopoietic	IFN-γ, IL-7, IL-13, IL-21, IL-31, TSLP
IL-1 family	IL-1°, IL-1 β, IL-1ra, IL-18, IL-33
gp130-utilizing	IL-6, IL-11, IL-27, IL-31, ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), leukaemia inhibitory factor (LIF), oncostatin M (OSM), osteopontin
IL-12	IL-12, IL-23, IL-35
IL-10 superfamily	IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29
IL-17	IL-17A-F, IL-25 (IL-17E)
Interferons	
Type I interferons	IFN-a, IFN-b, IFN-v
Type II interferon	IFN-γ (also a hematopoietic cytokine)
Type III interferons	IFN-l1 (IL-29), IFN-l2 (IL-28A), IFN-l3 (IL-28B)
TNF superfamily	TNF-α, TNF-b, BAFF, APRIL

BAFF: B cell activating factor, APRIL: A proliferation-inducing ligand

From (Commins, Borish, and Steinke 2010)

However the classification that still proves more relevant in clinical and immunological practice still divides cytokines according to their functions and effects on inflammatory cells.

The Table 5-2 below shows one way of classifying cytokines

Table 5-2: Immunological classification of cytokines

TH Family	Cytokine repertoire	Cytokines involved in differentiation
TH1	IFN-γ, TNF-α, TNF-b, GM-CSF, IL-2, IL-3	IL-12, IL-18, IFN-γ, IL-27
TH2	IL-2, IL-3, IL-4, IL-5, IL-9, IL-13, IL-24, IL-25, IL-31, TNF-α, GM-CSF	Mainly IL-4
TH9	IL-4, IL-9	TGF-β
TH17	IL-17 (IL-17A), IL-17F, IL-21, IL-22	IL-6 & IL-23:
nTreg/iTreg	IL-10	TGF-β, IL-10 & IL-2
TH3	TGF-β, IL-10	IL-10, IL-4

GM-CSF: Granulocyte-macrophage colony-stimulating factor

(Commins, Borish, and Steinke 2010)

Cytokine receptors

In recent years, the cytokine receptors have come to demand the attention of more investigators than cytokines themselves, partly because of their remarkable characteristics, and partly because a deficiency of cytokine receptors has now been directly linked to certain debilitating immunodeficiency states. In this regard, and also because the redundancy and pleiomorphism of cytokines are, in fact, a consequence of their homologous receptors, many authorities think that a classification of cytokine receptors would be more clinically and experimentally useful.

A classification of cytokine receptors based on their three-dimensional structure has, therefore, been attempted. Such a classification, though seemingly cumbersome, provides several unique perspectives for attractive pharmacotherapeutic targets. (Table 5-3)

Table 5-3: Classification of cytokine receptors

Cytokine Receptors	Typical ligands
Type -1 IL-2R---IL-7R, IL-12R, GM-CSFR	IL-2, IL3, IL4, IL-5, IL-6, IL-7, IL-9, IL-12, GM-CSF
Type-2(immunoglobulin family) IFNR1, IFNR2, IL-10R, M-CSFR	IFN-α , IFN-β , IFN-γ, IL-10, M-CSF
Type-3(TNF receptors family) TNFR1, TNFR2,	TNF-α, TNF-β , CD40
Type-4 IL-1R1	IL-1α, IL-1β

GM-CSFR: Granulocyte-macrophage colony-stimulating factor Receptor, M-CSFR: macrophage colony-stimulating factor Receptor

Cytokines play a major role in orchestrating immune mediated inflammation. The proof not only comes from animal models, in-vitro or in-vivo studies, but also from the successful use of drugs targeted against cytokines in controlling immune mediated diseases. Similarly in uveitis, our knowledge about role of cytokines is ever expanding

5.1.2 Cytokines in Uveitis

Evidence for the role of cytokines in the pathogenesis of uveitis comes principally from:

(a) Detection of cytokines in ocular tissue or fluid

(b) Studies on experimental animal models and

(c) in-vitro studies such as cytokine arrays and gene expression.

5.1.2.1 Animal Studies

EAU serves as a model for several human ocular diseases of suspected autoimmune aetiology (Gregerson et al. 1986) (Caspi et al. 1986). EAU is elicited by immunization with retinal antigens (Ags) or their fragments (Agarwal and Caspi 2004), or by adoptive transfer of retinal Ag-specific CD4 + T cells between syngeneic rodents (Sanui et al. 1989) (Rizzo et al. 1996)

Indeed, a pivotal role for antigen-specific CD4+ Th1 cells and pro-inflammatory cytokine-mediated disease has been demonstrated in both rat and mouse. In fact nude rats devoid of T cells, are unable to develop experimental autoimmune uveitis.(Nussenblatt 1991) IFN-γ is also considered to be a major effector cytokine in the pathogenesis of autoimmunity.(Baccala, Kono, and Theofilopoulos 2005) Concentrations of IFN-γ have been found to predominate in experimental autoimmune uveitis at peak inflammation in Lewis rats, and as such, susceptibility to experimental autoimmune uveitis in Lewis rats is thought to be connected with a Th1-dominant response involving IFN-γ. (Okada et al. 1998), (Caspi et al. 1997) A slight peak in *IFN-γ* mRNA expression in the iris and ciliary body, again corresponding with maximal inflammation, has also

been observed in experimental autoimmune anterior uveitis. (de and Verwaerde 2002)

IL-12, produced by macrophages and dendritic cells, has been reported to be a dominant factor in the development of Th1 cells. (Yoshida et al. 1994) *IL-12*-deficient mice have shown resistance to induction of experimental autoimmune uveitis, but following IL-12 administration to cells from these mice, the cells are induced to produce large amounts of *IFN-γ* and can adoptively transfer experimental autoimmune uveitis to naïve recipients. (Tarrant et al. 1998) In addition, anti-IL-12 monoclonal antibodies administered prior to immunization prevented experimental autoimmune uveitis induction in mice. (Yokoi et al. 1997) Furthermore, experimental autoimmune uveitis-susceptible mice show a greater amount of IL-12 receptor expression on Th1 cells correlating with IFN-γ expression. (Wu et al. 2003)

TNF-α is synthesized by monocytes, macrophages, neutrophils, mast cells, NK cells and T cells. (Pfeffer 2003) (Grivennikov et al. 2006) During the inflammatory process, it orchestrates the initiation of further leukocytic infiltration via adhesion molecule upregulation, dendritic cell maturation and survival, macrophage activation, and driving Th1 T cell responses within tissues in experimental autoimmune uveitis.(Dick et al. 2004) Increased TNF-α expression in inflammatory cell infiltrates has been seen in experimental autoimmune uveitis near peak inflammation as well. (Okada et al. 1998) Increased *TNF-α* mRNA has also been detected in the iris and ciliary body at the peak of the disease in experimental autoimmune anterior uveitis.(de and Verwaerde 2002) Conversely, the neutralization of TNF-α activity with a p55 TNF receptor fusion protein after the induction of experimental autoimmune uveitis delays its onset and attenuates tissue damage. (Dick et al. 1998)

IL-2 is produced mainly by activated T lymphocytes and can activate T cells and NK cells. It has been detected in experimental autoimmune uveitis at concentrations approaching those of IFN-γ near maximal inflammation. (Okada et al. 1998) IL-2 has also been shown to induce anterior uveitis in a rabbit model after intravitreal injection. (Samples, Boney, and Rosenbaum 1993) Administration of IL-2 receptor mAb in efferent-stage experimental autoimmune uveitis in the Lewis rat has also achieved partial experimental autoimmune uveitis suppression. (Higuchi et al. 1991)

In Lewis rats, IL-1α injected intravitreally has been demonstrated to induce intraocular inflammation in agreement with the concept of IL-1, initiating a cascade of inflammatory mediators. (Ferrick et al. 1991) IL-1β is also a pro-inflammatory cytokine and is derived predominantly from activated macrophages but also from B cells and vascular endothelial cells. IL-1β injected intravitreally into Lewis rats is also associated with a breakdown in the blood-retinal barrier and induces an inflammatory response. (Claudio, Martiney, and Brosnan 1994) Conversely, IL-1β blockers have been shown to inhibit experimental uveitis (Xuan et al. 1998).

IL-6 is a macrophage-derived cytokine, also thought to be produced by endothelial and ocular parenchymal cells. (Ohta et al. 2000b) It is a pleiotropic cytokine that is able to function in a pro- and anti-inflammatory manner. Macrophage activation and the differentiation of B cells to plasma cells are other known key effector roles for IL-6. It is readily induced by TNF-α, IFN-γ and IL-1. Evidence exists to suggest that IL-6 is involved in the direct induction of IL-2 receptor expression, differentiation and proliferation of T cells. (La Flamme and Pearce 1999)It is even thought to be more active in this respect than both IL-1 and TNF-α. (Joseph, Miner, and Croft 1998) Increased levels of

IL-6 have been detected in the aqueous humour in a murine model of experimental autoimmune uveitis with serial sampling demonstrating an increased local production at disease onset followed by a decrease as the inflammation mounted. (Ohta et al. 2000a)

TGF-β describes a group of pleiotropic cytokine isoforms that includes TGF-β1, -β2 and -β3. All are structurally and functionally similar having effects on many cell types including macrophages, T cells and B cells. For T cells, TGF-β inhibits T cell proliferation and suppresses cytotoxic T cells. (Kehrl et al. 1986) In the eye, TGF-β is present at high levels in the aqueous humour and is thought to be implicated in the maintenance of immune privilege through the inhibition of antigen-driven T cell activation and proliferation. (Cousins et al. 1991) TGF-β2 levels have been found to be increased in experimental autoimmune uveitis and are thought to be antagonized by IL-6, such that TGF-β2 suppresses inflammation once IL-6 levels fall. (Ohta et al. 2000b) TGF-β2, in conjunction with α-melanocyte-stimulating hormone, has been demonstrated to reduce the incidence and severity of experimental autoimmune uveitis. (Namba et al. 2002)

IL-4 is a multifunctional cytokine that can be secreted by several cell types. The most important producers of IL-4 in T cell-mediated disease are CD4+ Th2 cells activated by antigen recognition through the T-cell receptor. Among the effector functions of IL-4 are induction of IgE production by B cells and direct differentiation of naïve CD4+ T cells into a Th2 population, (Gascan et al. 1991) thereby favouring the growth of cells which produce IL-4 and IL-5 while inhibiting the production of IFN-γ. (Seder 1994) IL-4 is also involved in the inhibition of TNF-α and IL-1 production by activated monocytes, (Te Velde et al. 1990) In experimental autoimmune uveitis, the addition of IL-4 has been

reported to block the production of IFN-γ, while anti-IL-4 mAb therapy has been shown to lead to enhanced IFN-γ production. In experimental models of uveitis, both IL-4 and IL-10 are thought to be important cytokines for the immunosuppressive effects of CD4+ T regulatory cells. (Yotsukura et al. 1997) The production of both IL-4 and IL-10 has been documented to correlate with a recession of disease, suggesting their possible importance in the spontaneous termination of experimental autoimmune uveitis.

IL-10 is an essential anti-inflammatory multifunctional cytokine produced primarily from T cells and activated macrophages. IL-10 plays a key role in the differentiation and function of T regulatory cells which figure prominently in control of immune responses and tolerance in vivo. (Moore et al. 2001) T regulatory cells, themselves, produce IL-10, as well as TGF-β. (Levings et al. 2002) In this scenario, IL-10 is thought to act as a positive autocrine factor for the development of T regulatory cells. (O'Garra and Barrat 2003) It has been proposed that the higher expression of the *IL-10* gene in some rat strains may confer a greater resistance to experimental autoimmune uveitis.(Sun et al. 2000) Furthermore *IL-10* mRNA expression has been shown to coincide with downregulation of *IFN-γ* and *TNF-α* coinciding with the resolution of experimental autoimmune uveitis. (Rizzo et al. 1998) More recently, the local administration of an adeno-associated viral vector expressing IL-10 significantly decreased experimental autoimmune uveitis disease severity. (Broderick et al. 2005)

TH17 role

The identification of Th17 cells, a CD4+T-cell subset that produces IL-17, (Aggarwal et al. 2003) subset has now broadened our understanding of

inflammatory processes in human disease and has helped to explain some of the anomalies seen in the Th1/Th2 axis.

Susceptibility to EAU of IFN-γ deficient (GKO) mice, exacerbation of EAU by neutralization of endogenous IFN-γ, and the protective effects of high systemic IFN-γ in WT mice (Caspi et al. 1994) (Jones et al. 1997) (Tarrant et al. 1999) are in apparent paradox with this notion that EAU is purely Th1 mediated. Mice deficient in *IFN-γ, IFNGR, IL-12Rb2*, and the *IL- 12p35 chain* were highly susceptible to experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) (Cua et al. 2003) (Gran et al. 2002) (Becher, Durell, and Noelle 2002). In contrast, IL-23 and the IL-17 –producing effector T cell whose differentiation and maintenance are promoted by IL-23 were found to be necessary for induction of these diseases (Cua et al. 2003) (Murphy et al. 2003). The activity of the IL-17 producing effector T cells (Th17) was associated with induction of proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8, as well as with enhanced proliferation, maturation, and chemotaxis of neutrophils. These results led to the notion that the pathogenic effects previously attributed to the IL-12/IFN- y pathway are in fact largely if not solely mediated by IL-23 and the IL-23 driven Th17 effector (Cua et al. 2003) (Langrish et al. 2005). However, in EAU, mice lacking *IL-17* are also not protected and display elevated numbers of IFN-γ-producing CD4+ T cells. (Haak et al. 2009) (Luger et al. 2008)

Luger *et al*. showed that either Th1 or Th17 cells can drive tissue damage depending on the methods used to initiate disease. (Luger et al. 2008) Kroenke *et al*. showed that adoptive transfer of either Th1 or Th17 cells can induce EAE and clinical paralysis in mice, but the pathology induced by Th17 cells differs from that induced by Th1 cells. (Kroenke et al. 2008) Thus Th17

cells are unlikely to be the sole players in driving tissue damage in these classical models of autoimmunity. Although Th17 is a welcome addition to our understanding of immune-mediated tissue damage, we still need the Th1/Th2 axis and other inflammatory mediators to explain many aspects of human autoimmune, allergic, and infectious diseases. The diagram below explains our current understanding of role of cytokines in the induction of posterior segment uveitis based on animal models.

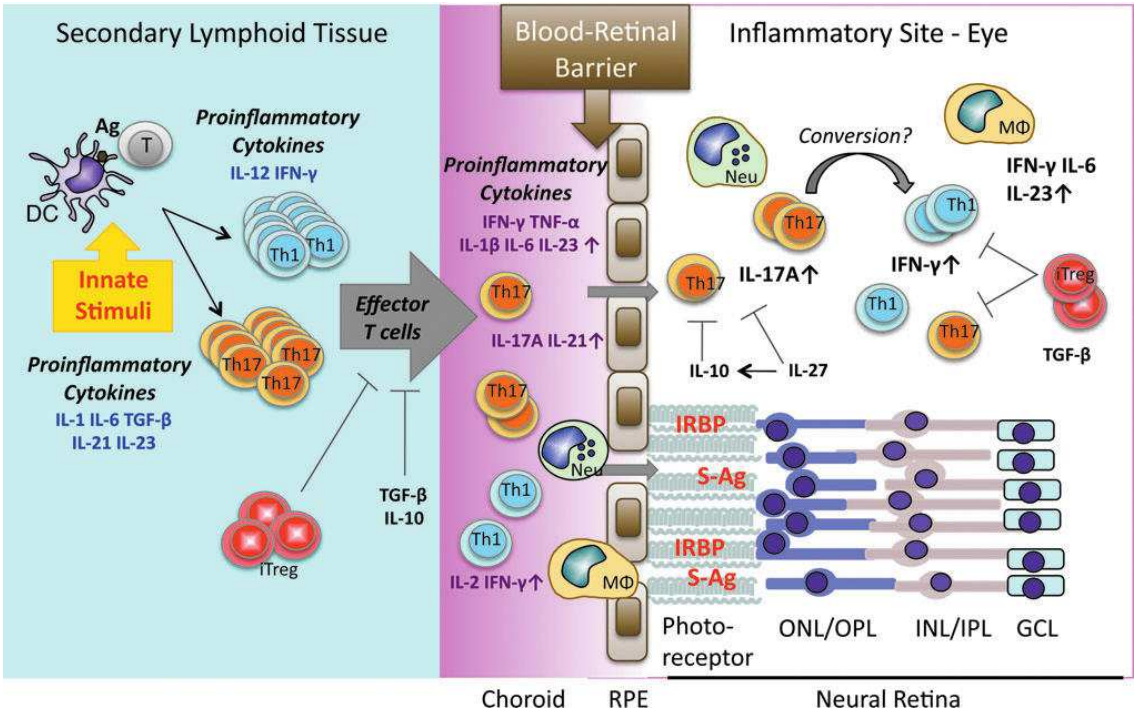


Figure 5-1: Cytokine networks in pathogenesis of uveitis.

Courtesy Caspi & Horai, 2011, Journal of interferon & cytokine research.
Presentation of Ag in the periphery in the presence of cytokines and innate (environmental) stimuli induces T cell activation, differentiation, and clonal expansion. Activated effector (uveitogenic) T cells then migrate and extravasate into the eye. Upon breakdown of blood–retinal barrier (BRB), leukocytes and lymphocytes (Th1 and Th17) that are recruited from circulation, as well as inflammatory cytokines, amplify the inflammation, resulting in uveitis. RPE, retinal pigment epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer

5.1.2.2 Role of cytokines in Human uveitis

Various pro-inflammatory cytokines have been investigated in patients with uveitis. IL-1β and IL-6 have been detected in the vitreous aspirates of patients with idiopathic panuveitis and may act as a local amplification signal in pathological processes associated with chronic eye inflammation. (Franks et al. 1992) IL-2 has been demonstrated in the choroid and aqueous humour of patients with idiopathic uveitis, and serum levels have also been found to positively correlate with the aqueous humour levels. (Hooks, Chan, and Detrick 1988) (Lacomba et al. 2000)

Adult patients with idiopathic uveitis have also been shown to have elevated aqueous humour and serum levels of TNF-α (Santos et al. 2001b) IFN-γ, as well as IL-2. (Lacomba et al. 2000)

Elevated serum TNF-α levels seem to be positively correlated with recurrent episodes of uveitis of idiopathic origin. (Santos et al. 2001b) Significantly increased levels of IFN-γ have also been detected in the aqueous humour of patients with chronic idiopathic anterior uveitis. (bi-Hanna, McCluskey, and Wakefield 1989)

Multiplex analysis has recently enabled the detection of significant increases in IL-6, IFN-γ in the aqueous humour of patients with idiopathic uveitis, as compared to non-inflammatory control aqueous humour. (Curnow et al. 2005)

TGF-β2 has been detected in patients with idiopathic uveitis at significantly lower levels than in control groups. (de Boer et al. 1994) (Curnow et al. 2005)

IL-6 has been detected in vitreous, as well as aqueous humour aspirates of patients with idiopathic uveitis. (Franks et al. 1992) More specifically, increases in vitreous levels of IL-6 have been detected in patients with active

intermediate and posterior uveitis, (Perez et al. 2004) reflective of on-going inflammation during disease hence, role in the propagation of uveitis.

In humans, IL-4 has only been detected in low levels and at levels not significantly different from controls in the aqueous humour and serum of patients with idiopathic uveitis. (Lacomba et al. 2000) It has also been found to be produced at low levels by T cells derived from the vitreous humour of patients with uveitis. (Muhaya et al. 1999)

A decrease in aqueous humour IL-10 has been demonstrated in conjunction with an increase in activated CD4+ T cells in anterior uveitis. (Calder et al. 1999)

5.1.2.2.1 Ocular Behcet's

Significantly raised IL-2, IL-6, IFN-γ and TNF-α levels have, been found in the aqueous humour and serum of patients with OB. (Lacomba et al. 2000) (Santos et al. 2001b) (Santos et al. 2001a). Increased serum levels of IL-2 receptors, IL-6, TNF-α, IFN-γ have also been found by others in Behcet's disease. (Bardak and Aridogan 2004) (BenEzra et al. 1993) (Hamzaoui et al. 2002)

Furthermore, significant positive correlations appear to exist between elevated serum TNF-α levels and recurrent episodes of uveitis associated with BD. (Santos et al. 2001b) IL-5 and IL-10 detection by multiplex analysis has revealed the aqueous humour levels in uveitis associated with patients with BD to be not significantly different from that of controls.(Curnow et al. 2005) Aqueous and vitreous humour levels of IL-10 were also reported as unchanged from controls in patients with OB.(Ongkosuwito et al. 1998) Significant reductions of TGF-β have, however, been detected as compared to controls. (Curnow et al. 2005)

5.1.2.2.2 Sarcoid Uveitis

Among patients with sarcoidosis, raised bronchoalveolar lavage levels of IFN-γ, TNF-α, (Wahlstrom et al. 2001) IL-12,(Kim et al. 2000a) IL-6 and IL-8 (Takizawa et al. 1997) have all been detected, while only a few cells positive for IL-4 have been observed, suggesting a polarization towards a Th1 cytokine profile. (Wahlstrom et al. 2001) T cell clones derived from the aqueous humour of a patient with sarcoidosis in one study were shown to produce large amounts of IL-1α, IL-6 .(Sakaguchi et al. 1998) Analysis of aqueous and vitreous humour samples of patients with sarcoidosis has also documented significantly increased levels of IL-6 as compared to controls.(Xu et al. 1997)

No significant differences in the levels of IL-4 have been found as compared to controls in the aqueous humour (Lacomba et al. 2000) and vitreous humour (Ongkosuwito et al. 1998) of patients with sarcoidosis. The expression of TNF-α on peripheral blood CD4+ lymphocytes has been documented to be significantly increased in patients with presumed sarcoid intermediate uveitis.(Murphy et al. 2004) HighIL-2/IL-5 and IFN-γ/IL-5 ratios were also found in the peripheral blood lymphocyte culture supernatants further illustrating the polarization of the systemic immune response towards Th1 in this type of uveitis. (Murphy et al. 2004)

5.1.2.2.3 IL-17 in Human uveitis

Since the discovery that Th17 cells also play a part in animal models of immune mediated diseases, researchers have been studying the role of IL-17 in human uveitis and related diseases. So far the studies are few and results not so convincing but results suggest that, IL-17 does play part in VKH and

Behcet's disease. Expression of *IL-23*p19 mRNA, *IL-23*, *IL-17*, and *IFN-γ* is found to markedly elevated in OB. (Chi et al. 2008) However the frequencies of both IL-17-producing and IFN-γ-producing T cells from PBMCs are significantly upregulated in BD patients with active uveitis concluded that the IL-23/IL-17 pathway together with IFN-γ is associated with the active intraocular inflammation in BD patients (Chi et al. 2008)

Recently a study showed that significantly higher levels of IL-17 and IFN-γ were observed in active BD patients as compared with controls. (Chi et al. 2010) Treatment with CsA inhibited the production of both cytokines in association with an amelioration of intraocular inflammation.

The patients with VKH disease with active uveitis showed an elevated level of *IL-23*p19 mRNA in PBMCs, higher IL-23 in the serum and increased production of IL-17 by polyclonally stimulated PBMCs and CD4(+) T cells. (Chi et al. 2007)

Another study showed, that *IL-17*, *IFN-γ*, were upregulated in patients with active uveitis in VKH. (Liu et al. 2009) Again CsA and corticosteroids were able to down regulate all these elevated levels which correlated with the clinical improvement of the uveitis.

These and similar studies on animal models and human subjects have increased our understanding about the role of cytokines in uveitis, however the relationship is very complex and complicated by the fact that there is lot of redundancy and overlap in cytokine system. However it does appear that IL-1, IL-4, IL-6, IL-10, IL-12 & IL-18, IL-17 & IL-23, together with TNF-α and IFN-γ play significant role in orchestrating immune response in uveitis.

Aims

I hypothesized that genetic variability in genes encoding these cytokine molecules may play a role in the development and susceptibility of PSU. The aim of this study was to determine whether SNPs in cytokine genes involved in uveitis would predict the disease severity and response to the treatment. As discussed in detail, (Risch and Merikangas 1996)studying functionally significant polymorphisms rather than random polymorphisms in the gene of interest offers considerable advantages in terms of detecting disease associated genes.

This study was done to further explore the role of cytokine gene polymorphism in PSU and see whether these polymorphisms can predict the visual outcome and prognosis in patients with non-infectious posterior uveitis

5.2 Material and Methods

5.2.1 Subjects

Samples of 176 Caucasian patients with PSU were available for genotyping. As mentioned in the chapter two, few samples were ruined before I could start my work on cytokine gene polymorphism. (I used 205 subjects in previous study)

5.2.2 Controls

Two groups of control were used in this study.

1) 166 healthy Caucasian subjects who were admitted for cataract extraction at WXH

2) And a cohort of 105 Caucasian patients attending the eye clinics for age related macular degeneration. All the subjects were negative for any history of autoimmune disease.

Again along with the PSU samples, some control samples were ruined as well. (I used 283 control subjects in previous study)

5.2.3 SNP selection

Cytokines and their receptors have been shown to be highly polymorphic. The vast majority of polymorphisms found in cytokine genes and their receptors are located in the promoter, intronic and 3′ untranslated regions. Some polymorphic loci appear to consistently alter cytokine production; however, the majority of cytokine polymorphisms appear to have little or no influence on cytokine production and expression. Extensive literature search was performed to identify functional SNPs in cytokines involved in pathogenesis of PSU. It must be noted that at the time of my research the role of IL-17 in uveitis was just emerging and no functional SNP had been discovered at that stage. Hence the *IL-17* gene did not form part of my research work. SNPs were selected on the basis of fact that

1) SNPs were functional (proved by in-vitro or in-vivo studies)
2) Cytokine genes, in which the SNPs were identified, had a proven role in the pathogenesis of uveitis
3) SNPs role had been studied in other immune mediated diseases.

Following SNPs were selected

5.2.3.1 IL-1β -511

IL-1β is a member of the *IL-1* super family. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2. A *C*

to *T* SNP at promoter position *-511* is shown to be functional. Earliest study showed that mononuclear cells from carriers of allele *T* had a slight, but non-significant, elevated capacity to produce IL-1β in vitro. (Santtila, Savinainen, and Hurme 1998). Hall *et al.* tested LPS-induced secretion of IL-1β protein as measured by an ex vivo blood stimulation assay. *T* allele at *-511* was significantly associated with a 2-3-fold increase in LPS-induced IL-1β protein secretion. (Hall et al. 2004) Another study demonstrated that carriers of *IL1β -511 T* show a significantly higher plasma level of IL1β than those of *IL1β -511 C*. (Hulkkonen, Laippala, and Hurme 2000)

5.2.3.2 IL-4 -590

IL-4 orchestrates its Th2 effects by inducing IgE production from B cells. The gene encoding is present on chromosome 5q31.1. A SNP at position *-590* was first discovered in 1995, when it was noted *C* to *T* change was associated with three fold increase in total serum IgE levels and three fold increase in *IL4* transcription activity. (Rosenwasser et al. 1995) Nakashima *et al.* using intracellular cytokine detection assay showed that peripheral Th cells in subjects carrying *T* allele expressed more *IL-4* compared to *C* allele carriers. (Nakashima et al. 2002)

Further studies showed that the *T* allele was associated with high serum IgE levels in African (Vafa et al. 2009) and as well as Caucasian subjects. (Basehore et al. 2004) (Kabesch et al. 2003)

5.2.3.3 IL-6 -174

IL-6 gene is present on chromosome 7p21 and *-174* SNP was first noted in 1998. (Fishman et al. 1998) Luciferase reporter vector assay showed that *-174*C construct had lower expression than the *-174*G construct. After

stimulation with LPS or IL-1, expression from the *-174*C construct did not significantly change after 24 h, whereas expression from the *-174*G construct increased by 2.35+/-0.10- and 3.60+/-0.26-fold, respectively, compared with the unstimulated level. Plasma levels of IL-6 were also measured in 102 of the healthy subjects, and the *C* allele was found to be associated with significantly lower levels of plasma IL-6. Using enzyme immunoassay, IL-6 concentrations were measured in serum samples of 65 subjects. (Belluco et al. 2003) The serum levels of IL-6 were more than double in the carriers of *GG* genotype compared to *C* allele carriage (wild type)

5.2.3.4 IL-10 SNPs

The functional polymorphisms in the promoter region of *IL-10* were first reported in late 1990s, (Turner et al. 1997) (Rosenwasser et al. 1995) where it was noted that variants in promoter region could affect the transcription and expression of *IL-10* gene and in turn lead to altered production of IL10 by PBMC. Crawley *et al.* used transient transfection studies to investigate the transcription of reporter genes driven by *IL-10* promoter allele. They also performed whole blood cultures to assess IL-10 production by each genotype. The *ATA* haplotype (containing *A* allele at *-1082 & -592*) was associated with lower transcriptional and lower *IL-10* production under lipopolysaccharide. (Crawley et al. 1999)

Yilmaz *et al*. measured IL-10 levels of stimulated PBMCs in vitro. After stimulation with purified protein derivative (PPD) and Staphylococcus aureus Cowan strain I SAC, *-1082* A allele carriers produced significantly lower levels of IL-10 than non-carriers (Yilmaz, Yentur, and Saruhan-Direskeneli 2005)

Recent studies have again confirmed that the *G* allele at *-592* and *G* allele at *-1082* lead to increased transcriptional activity, increased *IL-10* expression, and increased IL-10 production. (Donati et al. 2008) (Claudino et al. 2008) (Larsson, Rymo, and Berglundh 2010) The *IL-10* -592 is in complete linkage with other published SNP *IL-10 -819*, so only *IL-10 -592* & *-1082* SNPs were selected for the study.

5.2.3.5 IL-12 +1188

IL-12 is a heterodimeric cytokine composed of a p35 and p40 subunit, encoded by *IL12A* (chromosome 3q25.33) and *IL12B* (chromosome 5q33.3), respectively. A polymorphism was discovered at *+1188* 3' UTR site of *IL12B* gene in 2000. (Huang, Cancilla, and Morahan 2000) *IL-12* p40 and p70 secretion by monocytes, in relation to *+1188* genotype was determined in 63 healthy donors. After stimulation of monocytes with SAC and IFN-γ, there was 3 folds increase in IL-12 p70 secretion among the carriers of *CC* (variant) genotype (Seegers et al. 2002). Individuals *CC* homozygous at *IL12 +1188* had significantly higher IL-12 secretion levels from LPS and PPD stimulated PBMCs than *AC* heterozygotes or *AA* homozygotes (Yilmaz, Yentur, and Saruhan-Direskeneli 2005). IL-12p70 levels in culture supernatants after stimulation with PMA+Ionomycin were assessed by ELISA. Healthy individuals with *C* allele had an increased production of IL-12 p70 compared with non-carriers. (varez-Rodriguez et al. 2009)

5.2.3.6 IL-18 SNPs

Gene encoding IL-18 is located on chromosome 11q22.2-q22.3. Giedraitis et *al.* identified five SNPs: *-656, -607, -137, +113 and +127* (Giedraitis et al. 2001). Using luciferase construct, they found that haplotypes 1 (*G-C-G-TC*) and 3 (*T-*

A-G-T-C) resulted in increased expression in stimulated HeLa cells, compared to haplotype 2 (*T-A-C-GT*). The authors postulated that *-137 G>C* may be responsible for variation in gene expression. Latter studies showed the *G/C* change at *-137* position and *C/A* change at *-607* position leads to decrease expression of *IL-18* gene. Zhou *et al* examined promoter activities by a dual luciferase assay system in a cell line, THP-1. (Zhou et al. 2005) The promoter activity of the haplotype *-137G/-607C/-656G* was significantly higher than that of the other common haplotype, *-137G/-607A/-656T*, accounting for the association between serum IL-18 levels and the SNPs.

Serum IL-18 levels were determined using ELISA in patients with active untreated adult onset still's disease. (Chen et al. 2009) Median levels of serum IL-18 were significantly lower in patients with *-607 AA* genotype compared to those with *CA* genotype or *CC* genotype.

IL-18 -137 & IL-6 -607 SNPs were selected for my study

5.2.3.7 IFN-γ +874

The *IFN-γ* gene, *IFNG*, is located on 12q24.1, contains 4 exons that span 5.4 kb. As with most cytokine genes, there are no common variants located within coding regions. Several polymorphisms have received attention in disease-association studies, including the intron 1 *+874 A>T*

This SNP was first noted by Pravica *et al*. who found *T to A* polymorphism coincides with a putative NF-kappa B binding site. (Pravica et al. 2000) EMSA demonstrated reduced binding to the *T* allele using excess unlabelled NF-kB consensus sequence. Authors concluded that this SNP might have functional consequences for the transcription of the human *IFN-γ* gene and could directly influence the level of IFN-γ production.

A later study confirmed this finding. (Hoffmann et al. 2001) Cytokine protein production was assessed by enzyme-linked immunosorbent assay after costimulation of cultured peripheral blood lymphocytes in healthy subjects. *T* allele was associated with higher cytokine production. Another study showed *IFN-γ +874T-A* polymorphism was related to the IFN-γ response to PPD antigen and the magnitude of the response decreases during transition from *TT*- to *TA* and to *AA* genotypes (Sallakci et al. 2007)

5.2.3.8 IFNGR

*IFNGR*1 is located on chromosome 6q23.3 and encodes IFN-γ receptor. A functional SNP at promoter site *-56* was first noted by Juliger *et al*. who showed lower levels of luciferase reporter gene expression in the presence of the mutation, indicating the importance of this position for promoter activity, and suggesting that this SNP might negatively influence the expression level of *IFNGR*1 at the cell surface. (Juliger et al. 2003)

This has now been confirmed by recent studies. A reporter gene assay showed that, after stimulation with IFN-γ, the *IFNGR*1 gene promoter construct that contained the *-56T* allele, manifested higher transcriptional activity than did the construct with the mutant *-56C* allele (Matsuda et al. 2007). In another luciferase reporter assay a 10-fold increase ($p<0.001$) in luciferase expression was associated with the *IFNGR1-56*T* allele. (Canedo et al. 2008)

5.2.3.9 TNF-α

TNF is the most widely studied cytokine gene in relation with polymorphisms and disease. This is due to the location of *TNF-α* gene in the major histocompatibility complex (MHC). This has prompted speculation about the

role of *TNF*-α in the aetiology of MHC-linked diseases, particularly autoimmune diseases.

The most commonly studied *TNF*- α polymorphism is *-308 G/A*. A study in 1997 (Kroeger, Carville, and Abraham 1997) examined functionality of this SNP using EMSA analysis, and found increased binding of a factor to the *A* allele. In luciferase assays that contained the region *993 to +110* of the *TNF* promoter with each *-308* allele found that the *A* allele resulted in a 1.7-fold increase in luciferase expression 24 h after PMA-stimulation of Jurkat and U937 cells.

Further in-vitro studies showed that the *–308 A* allele is a more powerful transcriptional activator than the *–308G* allele. Louis *et al*. and Brinkman *et al*. found that the peripheral blood mononuclear cells of the individuals having *–308 A/A* genotype synthesized more *TNF*-α than the individuals having *G/G* genotype. (Louis et al. 1998) (Brinkman et al. 1995)

Another putative functional promoter polymorphism, *-238 G>A* occurs within the *TNF* repressor site (Fong, Siddiqui, and Mark 1994) but has shown contradicting functionality in a number of studies. A study by Kaluza *et al*. transfected a 691 bp fragment of *TNF*- α promoter in a luciferase vector into the Raji and Jurkat cell lines and demonstrated a reduction in gene expression from the rare allele. (Kaluza et al. 2000) They also found a non-significant decreased expression of *TNF*- α from PBMCs possessing the rare allele.

TNF-α production in whole blood cultures upon stimulation with LPS was determined in 179 individuals (Huizinga et al. 1997) LPS-stimulated blood cells of *−238G/A* individuals yielded significantly less TNF-α than those of *−238G/G* individuals

TNF-α *-857* is another SNP that has noted to be associated with anterior uveitis. (Kuo et al. 2005) The functional effects of this SNP are however not known, but

since strong associations of this SNP with noted, I selected this SNP, along with *-308* & *-238* SNPs.

LTA+252

LTA gene is present on chromosome 6p21.3 and it encodes lymphotoxin-alpha also known as *TNF*-b. A, *G to A* SNP at position *+252* intron -1 was first noted in 1991. The variant allele was noted to be more transcription activator than the wild type allele (Messer et al. 1991)

Whichelow *et al*. measured the secretion capacity of *LTA* of peripheral blood mononuclear cells (PBMC) from PHA stimulated healthy individuals and diabetic patients. (Whichelow et al. 1996) They showed that those with the variant allele secreted significantly higher levels of TNF-β than those with the wild type allele

Knight *et al*. developed upon the principle of chromatin immunoprecipitation (ChIP) to examine in vivo the effect of nontranscribed polymorphism on gene expression (Knight et al. 2003). The authors used this technique to examine the *TNF* region and showed that *LTA +252 G* allele is associated with increased *LTA* mRNA expression.

5.2.4 DNA extraction and genotyping

Qiagen commercial kit (Qiagen UK Ltd, UK) method was used to extract genomic DNA from 176 PSU patients and 105 controls from KCH.

Salt extraction method was used to extract DNA of 166 healthy controls from WXH.

Genotyping for 14 polymorphisms in 10 genes was performed using the 5'Nuclease Activity Assay first described by Holland *et al*. (Holland et al. 1991)

Primers and TaqMan® probes were designed using Primer Express software (version 2.0; sequences shown in Table-1) and synthesised and supplied by Applied Biosystems UK. The reporter dyes chosen were 6-FAM and VIC™. Using the Applied Biosystems Allelic Discrimination PCR protocol, 5 ml PCR reactions containing 20 ng of DNA, 0.9 mmol primers, and 0.2 mmol probes (final concentrations) were performed in 384 well plates and run on the Applied Biosystems 7900 HT sequence detection system. PCR annealing temperatures ranged from 60˚C to 62˚C. Thermal cycling consisted of 1 cycle of 50˚c for 2 minutes and 95˚c for 10 minutes and then 45 cycles of 95˚c for 15 seconds and 60˚c for 1 minute. SDS version 2.0 software was used to analyse real time and endpoint fluorescence. Table 5-4 describes the details of primers and probes used to type cytokine SNPs.

Table 5-4: Primers and probes used to detect cytokine SNPS

SNPs		PRIMERS	ALLELES	PROBES	ANNEALING
					TEMERATURE
IL-1β-511	Forward	GGTCTCTACCTTGGGTGGTGTT	T	6-FAM-TCTGCCTCAGGAGC-MGB	62
	Reverse	TCCTCAGAGGCTCCTGCAAT	C	VIC-TGCCTCGGGAGCT-MGB	
IL-4-590	Forward	CCT GTC CTT CTC AAA ACA CTA AAC TTG	C	VIC-AGA ACA TTG TCC CCC AGT-MGB	60
	Reverse	GCA GAA TAA CAG GCA GAC TCT CCT A	T	6-FAM-AGA ACA TTG TTC CCC AGTG -MGB	
IL-6-174	Forward	GCTGCACTTTTCCCCCTAGTT	C	6'FAM-CTTTAGCATGGCAAGAC-MGB	62
	Reverse	GCTGATTGGAAACCTTATTAAGATTGT	A	VIC-CTTTAGCATCGCAAGAC-MGB	
IL-10-592	Forward	GGT AAA GGA GCC TGG AAC ACA TC	C	VIC-CGC CTG TCC TGT AGG-MGB-NFQ	60
	Reverse	CCC TTC CAT TTT ACT TTC CAG AGA	A	6-FAM-CGC CTG TAC TGT AGG A-MGB-NFQ	
IL-10-1082	Forward	ACA CAC AAA TCC AAG ACA ACA CTA CTA A	G	VIC-ATC CCT ACT TCC CCC TCC CAA AGA A-TAMRA	62
	Reverse	GGA GGT CCC TTA CTT TCC TCT TAC C	A	6-FAM-CCC TAC TTC CCC TTC CCA AAG AAG C-TAMRA	
IL-12 1188	Forward	GGATCACAATGATATCTTTGCTGTATTT	A	VIC-CATTTAGCATCTAACTATAC-MGB	60
	Reverse	CTATACATAAATTAGCTGATTGTTTCAATGA	C	FAM-TTTAGCATCGAACTATAC-MGB	
IL-18-137.	Forward	GGCACAGAGCCCCAACTTT	C	6-FAM CGGAAGAAAACATTT-MGB	60
	Reverse	CGAGTACTTCTTTTAATGTAATATCACTATTTTCA	G	VIC CGGAAGAAAAGATTT-MGB	

Contd: Table 5-4: Primers and probes used to detect cytokine SNPS

IL-18-607	Forward	CCCAAGCTTACTTTCTGTTGCA	A	6-FAM AAGTGTAAAAATTATTAAATAAA-MGB	60
	Reverse	AAGCCACACGGATACCATCATT	C	VIC AAAAATTATTACATAAAATTCT-MGB	
IFNG+874	Forward	ACATTCACAATTGATTTTATTCTTACAACA	T	6-FAM TGTGTGTGAGATTTG -MGB	60
	Reverse	GTGCGAGTGTGTGTGTGTGTGT	A	VIC TGTGTGTGTGATTTGA -MGB	
*IFNGR*1-56	Forward	GGTGACGGAAGTGACGTAAGG	T	6-FAM CCAGCACTGCCCT -MGB	60
	Reverse	CGCCTGCGGGACCAG	C	VIC CCAGCGCTGCCCT -MGB	
*TNF*a-238	Forward	ATC AGT CAG TGG CCC AGA AGA	A	VIC-CTG CTC TGA TTC CGA-MGB	60
	Reverse	GCA TCA AGG ATA CCC CTC ACA	G	FAM-CTG CTC CGA TTC CGA-MGB	
TNF-308	Forward	CCAAAAGAAATGGAGGCAATAGGTT	A	6-FAM-CCCGTCCtCATGCC-MGB	60
	Reverse	GGACCCTGGAGGCTGAAC	G	VIC-CCCGTCCCCATGCC-MGB	
LTA+252	Forward	5'-CAGTCTCATTGTCTCTGTCACACATT-3'	G	5'-CCATGgTTCCTCTC-3' (FAM) MGB	60
	Reverse	5'-ACAGAGAGAGACAGGAAGGGAACA-3'	A	5'-CTGCCATGaTTCC-3' (VIC) MGB	

5.2.5 Statistical Analysis

For each subject group, genotype frequencies were counted and the allelic and allele carriage frequencies calculated. All genotype frequencies in each population were tested for deviation from the Hardy-Weinberg equilibrium using the chi-square test. Data mining for significant associations was performed using knowledge seeker®, (Angoss softwares UK) and statistical calculations were performed with SPSS 12.0 (Chicago USA). Confidence intervals were calculated at the 95 % level and a value of $p < 0.05$ was considered as significant. Chi-square test was used to compare the genotypic and allelic frequencies in patients and controls. A correction for multiple comparisons was made using the Bonferroni method. A multiple logistic regression model was used to determine association between various genotype and the phenotypes.

Power calculations were performed using Quanto® Version 1.0. Haplotypes were identified by the computer program PHASE, version 2.

Before generating haplotype, linkage between the SNPs was calculated by EM algorithm using Arlequin® population genetics software.

5.3 Results

. 176 Caucasians patients were recruited for this study out of which 81 were males and 95 females. The age of onset ranged from 4.6 to 70.5 years with a mean of 36.81 years. All the patients had a follow up of at least 2 years with a majority of patients had a follow up of 5 years. The follow up ranged from 2 years till 42.6 years with a mean of 8.34 years. Salient features of the patients are shown in Table 5-5.

Table 5-5: Clinical features of patients with PSU

Phenotype	Number	%
Idiopathic intermediate uveitis	67	38.0%
Idiopathic posterior uveitis	58	33.0%
Behcet's	27	15.4%
Sarcoidosis	24	13.6%
Total	176	100%
Need of systemic steroids	130	73.86%
On long term systemic steroids	69	39.20%
On second line of immunosuppressive agents	74	42.05%
Cystoid macular oedema	111	63.07%
Cataract	81	46.02%
Glaucoma	62	35.23%
Permanent visual loss	76	43.18%
Vasculitis	58	32.95%
Follow up in years	Mean=8.4	Range(2--42.6)
Sex	Males=79	females=92
Laterality of disease	Bilateral=148	Unilateral 28
Age of onset (years)	Mean=34.12	Range=(5-70.5)
Relapses (rate per year)	Mean=2.1	Range=(0-7)
Visual impairment during inflammation	Mean=x2 of visual angle	Range=(Nil-PL)
Better eye visual acuity after 24 months	Mean=6/9	Range=(6/5-HM)

Genotyping of the SNPs showed that all genotypic frequencies were in HWE. The Table 5-6, Table 5-7, & Table 5-8 show in detail the frequencies of all 14 cytokine SNPs typed.

Table 5-6: Frequencies of *IL-1*, *IL-4*, *IL-6* & *IL-10* SNPs in PSU and controls

SNP	Intermediate		Posterior		Behcet's		Sarcoidosis		Total PSU		Total Controls		P Value	
	N	%	N	%	N	%	N	%	N	%	N	%	PSU Vs Controls	Between PSU groups
IL-1β 511T/C													NS	NS
CC	30	44.80%	26	44.80%	9	33.30%	9	37.50%	74	42.00%	120	44.30%		
CT	23	34.30%	23	39.70%	18	66.70%	12	50.00%	76	43.20%	120	44.30%		
TT	14	20.90%	9	15.50%	0	0.00%	3	12.50%	26	14.80%	31	11.40%		0.01
IL-4 590T/C													NS	NS
CC	47	70.10%	46	79.30%	18	66.70%	17	70.80%	128	72.70%	195	72.10%		
CT	18	26.90%	10	17.20%	9	33.30%	6	25.00%	43	24.40%	65	23.90%		
TT	2	3.00%	2	3.40%	0	0.00%	1	4.20%	5	2.80%	11	4.00%		
IL-6 174G/C													NS	NS
GG	28	41.80%	23	39.70%	14	51.90%	10	41.70%	75	42.60%	106	39.10%		
GC	34	50.70%	23	39.70%	9	33.30%	9	37.50%	75	42.60%	132	48.70%		
CC	5	7.50%	12	20.70%	4	14.80%	5	20.80%	26	14.80%	33	12.20%		
IL-10 592C/A														
CC	44	65.70%	32	55.20%	9	33.30%	14	58.30%	99	56.30%	154	56.80%		
CA	21	31.30%	21	36.20%	14	51.90%	9	37.50%	65	36.90%	99	36.50%		
AA	2	3.00%	5	8.60%	4	14.80%	1	4.20%	12	6.80%	18	6.60%		0.0009
IL-10 1082G/A													NS	NS
GG	14	20.90%	14	24.10%	3	11.10%	6	25.00%	37	21.00%	62	22.90%		
GA	41	61.20%	30	51.70%	13	48.10%	11	45.80%	95	54.00%	148	54.60%		
AA	12	17.90%	14	24.10%	11	40.70%	7	29.20%	44	25.00%	61	22.50%		

Table 5-7: Frequencies of *IL-18*, IFN-γ & *IFNGR* SNPs in PSU & controls

SNP	Intermediate		Posterior		Behcet's		Sarcoidosis		Total PSU		Total Controls		P Value (Chi 2 X 2)	
	N	%	N	%	N	%	N	%	N	%	N	%	PSU Vs Controls	Between PSU groups
IL-12 1188A/C													NS	NS
AA	45	67.20%	41	70.70%	15	55.60%	13	54.20%	114	64.80%	176	64.90%		
AC	22	32.80%	15	25.90%	12	44.40%	10	41.70%	59	33.50%	83	30.60%		
CC	0	0.00%	2	3.40%	0	0.00%	1	4.20%	3	1.70%	12	4.40%		
IL-18 137G/C														
GG	37	55.20%	25	43.10%	11	40.70%	14	58.30%	87	49.40%	157	57.90%		
GC	25	37.30%	23	39.70%	11	40.70%	8	33.30%	67	38.10%	99	36.50%		
CC	5	7.50%	10	17.20%	5	18.50%	2	8.30%	22	12.50%	15	5.50%	0.009	
IL-18 607C/A														
CC	22	32.80%	14	24.10%	8	29.60%	12	50.00%	56	31.80%	110	40.60%	0.02	
CA	38	56.70%	29	50.00%	11	40.70%	8	33.30%	86	48.90%	125	46.10%		
AA	7	10.40%	15	25.90%	8	29.60%	4	16.70%	34	19.30%	36	13.30%		
IFNG 874 A/T														
AA	15	22.40%	18	31.00%	5	18.50%	11	45.80%	49	27.80%	87	32.10%		
AT	35	52.20%	26	44.80%	13	48.10%	12	50.00%	86	48.90%	131	48.30%		
TT	17	25.40%	14	24.10%	9	33.30%	1	4.20%	41	23.30%	53	19.60%		0.005
IFNGR 56C/T													NS	NS
TT	19	28.40%	22	37.90%	10	37.00%	9	37.50%	60	34.10%	93	34.30%		
TC	31	46.30%	31	53.40%	13	48.10%	13	54.20%	88	50.00%	134	49.40%		
CC	17	25.40%	5	8.60%	4	14.80%	2	8.30%	28	15.90%	44	16.20%		

Table 5-8: Frequencies of *TNF* & *LTA* SNPs in PSU and controls

SNP	Intermediate		Posterior		Behcet's		Sarcoidosis		Total PSU		Total Controls		P Value (Chi 2 X 2)	
	N	%	N	%	N	%	N	%	N	%	N	%	PSU Vs Controls	Between PSU groups
TNF 238G/A													NS	NS
GG	62	92.50%	50	86.20%	24	88.90%	20	83.30%	156	88.60%	239	88.20%		
GA	5	7.50%	7	12.10%	3	11.10%	4	16.70%	19	10.80%	31	11.40%		
AA	0	0.00%	1	1.70%	0	0.00%	0	0.00%	1	0.60%	1	0.40%		
TNF 308G/A														
GG	43	64.20%	41	70.70%	17	63.00%	18	75.00%	119	67.60%	192	70.80%	NS	NS
GA	21	31.30%	16	27.60%	8	29.60%	5	20.80%	50	28.40%	69	25.50%		
AA	3	4.50%	1	1.70%	2	7.40%	1	4.20%	7	4.00%	9	3.30%		
TNF 857C/T													NS	NS
CC	52	77.60%	49	84.50%	21	77.80%	21	87.50%	143	81.30%	226	83.40%		
CT	13	19.40%	8	13.80%	5	18.50%	3	12.50%	29	16.50%	44	16.20%		
TT	2	3.00%	1	1.70%	1	3.70%	0	0.00%	4	2.30%	1	0.40%		
LTA 252A/G													NS	NS
AA	26	38.80%	29	50.00%	10	37.00%	13	54.20%	78	44.30%	119	43.90%		
AG	33	49.30%	25	43.10%	14	51.90%	10	41.70%	82	46.60%	120	44.30%		
GG	8	11.90%	4	6.90%	3	11.10%	1	4.20%	16	9.10%	32	11.80%		

5.3.1 Effect of cytokine polymorphisms on genetic predisposition to uveitis

5.3.1.1 Association of IL-18 & IL-12 SNPs with retinal vasculitis

As shown in Table 5-7, the frequency of *IL-18 -137 CC* was significantly higher in PSU compared to controls p=0.009 pc 0.03. It was 12.5% in PSU more than double as compared to controls where it was 5.5%. The subgroup analysis in the table will show that *CC* phenotype was much common in Behcet's and posterior uveitis. When further analysis was done, it was found that this *IL-18 -137 C* allele was strongly associated with the development of retinal vasculitis. Though in sarcoidosis patient the over all frequency of *CC* was comparable to controls but in sarcoidosis patients who clinically exhibited retinal vasculitis it was 17%. There were 58 patients who developed vasculitis as a part of the disease including patients with Behcet's disease and sarcoidosis and the frequency of *-137CC* genotype was 19% compared to 6% among controls (p0.0001) and 9% among patients with no element of vasculitis pc>0.05. (Table 5-9)There was a subgroup of patients in posterior uveitis that had idiopathic vasculitis (IRV) only with no other ocular structures involvement. In this sub group of 16 patients the frequency of *-137CC* genotype was 38% compared to 6% in controls p=0.000001 pc=0.000008 and 10% in patients not including idiopathic retinal vasculitis p=0.001. (Table 5-9)

Table 5-9: *IL-18* & *IL-12* SNPs in patients with vasculitis

Genotype	IRV	PSU with Vasculitis	PSU	PSU excluding IRV	PSU without Vasculitis	Controls	P Value	
	n=(16)	n=(58)	n=(176)	n=(160)	n=(118)	n=(271)	IRV VS Controls	IRV Vs rest
IL-18 -137								
CC	38% (6)	19% (11)	13% (22)	10% (16)	9% (11)	5% (15)	0.0000001	0.001
CG	31% (5)	40% (23)	38% (67)	39% (62)	37% (44)	37% (99)	NS	NS
GG	31% (5)	41% (24)	49% (87)	51% (82)	53% (63)	58% (157)	0.03	NS
C allele	53% (17)	39% (45)	32% (111)	29% (94)	28% (66)	24% (129)		
G allele	47% (15)	61% (71)	68% (241)	71% (226)	72% (170)	76% (413)	0.0002	0.005
IL-18 -607								
AA	56% (9)	33% (19)	19% (34)	16% (25)	13% (15)	13% (36)	0.0000004	0.000008
CA	31% (5)	41% (24)	49% (86)	51% (81)	55% (65)	46% (125)	NS	NS
CC	13% (2)	26% (15)	32% (56)	34% (54)	32% (38)	41% (110)	002	NS
A allele	72% (23)	53% (62)	44% (154)	41% (131)	40% (95)	36% (197)		
C allele	28% (9)	46% (54)	56% (198)	59% (189)	60% (141)	64% (345)	0.000005	0.0007
IL-12 1188								
AA	69% (11)	66% (38)	65% (114)	64% (103	64% (76)	65% (176)	NS	NS
AC	19% (3)	29% (17)	34% (59)	35% (56)	36% (42)	31% (83)	NS	NS
CC	13% (2)	5% (3)	2% (3)	1% (1)	0% (0)	4% (12)	NS	0.0004
A	78% (25)	80% (93)	82% (287)	82% (262)	82% (194)	80% (435)		
C	22% (7)	20% (23)	18% (65)	18% (58)	18% (42)	20% (107)	NS	NS

IL-18 -137 CC & IL-18 -607 AA was more common in patients with retinal vasculitis particularly idiopathic retinal vasculitis
IRV: Idiopathic retinal vasculitis, PSU: Posterior segment uveitis

The other SNP in *IL-18* gene at position *-607* was in strong linkage with SNP at *-137*. Subjects homozygous for *-137CC* were always homozygous for *AA* at *-607* and also subjects homozygous for *CC* at *-607* were never homozygous for *CC* at *-137*.

This *IL-18 -607 A* allele also showed strong association with retinal vasculitis (see Table 5-9). The *AA* genotype was present in 56% of the patients with idiopathic retinal vasculitis compared to 13% in controls p=0.0000001, pc 0.000008. The *AA* genotype frequency was 33% among patients who developed vasculitis compared to 13% who did not p=0.001. Using PHASE® 4 haplotypes were deducted as shown in Table 5-10 and as expected patients with vasculitis had a frequency 69% of haplotype carrying *C* at *-137* and *A* at *-607* compared to 22% in patients not having vasculitis or 27% in controls. P=0.0001

Table 5-10: *IL-18* haplotypes in patients and controls

IL-18 Haplotypes	PSU n=(176)	Controls n=(271)	Retinal Vasculitis N= (16)	P Value
137G/607C	52.9%	56.5%	17.8%	
137G/607A	14.9%	16.1%	13.2%	
137C/607A	32.2%	26.7%	69%	0.0001
137C/607C	0.0%	0.7%	0%	

IL-12 another IFN-γ inducer and upon which *IL-18* ability to induce IFN-γ depends also showed moderate association with idiopathic vasculitis. Again as shown in Table 5-9 the *IL-12 -1188 CC* genotype was present in 13% of patients with idiopathic vasculitis compared to 1 % in patients with PSU excluding idiopathic vasculitis p=0.0004, pc 0.003

5.3.1.2 IL-1β -511 and Behcet's Disease

A glance at the Table 5-6 will show that in patients with Behcet's disease none of them were homozygous for *TT* at *IL-1β* 511 site. Though the numbers were small to reach to any statistical significance but the difference compared to other sub groups was quite substantial. The *TT* frequency was in the range from 12% in sarcoidosis and about 21% in Intermediate uveitis group as shown in the Table 5-6. It must be noted that there was no significant difference in the genotypic frequencies between Behcet's and control groups. Details are shown in the following Table 5-11

Table 5-11: *IL-1β -511* SNP frequency in OB.

GENOTYPE *IL-1β -511* C/T	PSU N= 149	OB N=27	Controls N= 271	P value OB Vs PSU	P value OB Vs Controls
CC	65 (44%)	9 (33%)	120 (44%)	NS	NS
CT	58 (39%)	18 (67%)	120 (44%)	0.007	0.02
TT	26 (17%)	0 (0%)	31 (11%)	0.018	NS
Allelic Carriage					
C	123 (83%)	27 (100%)		0.018	NS
T	84 (56%)	18 (67%)		NS	NS
Allelic Frequency					
C	188 (63%)	36 (67%)		NS	NS
T	110 (37%)	18 (33%)			
Total	298	54			

OB: ocular Behcet's

5.3.1.3 IL-10 -592 and Behcet's disease

At position *-592* of *IL-10* the wild type allele *C* was less prevalent in Behcet's disease compared to the rest of the groups and the controls. (Where the frequencies were comparable) As shown in the following Table 5-12 the allelic frequency of *C* allele was 59% in Behcet's compared to 80% in rest of the group. P=0.0009 pc 0.003.

Table 5-12: IL-*592* genotypic frequencies in OB

GENOTYPE *IL-10 -592* C/A	PSU N= 149	Behcet's N= 27	Controls N=271	P Value OB Vs PSU	P Value OB Vs Controls
CC	90 (60%)	9 (33%)	154 (57%)	0.009	0.01
CA	51 (34%)	14 (52%)	99 (37%)	NS	NS
AA	8 (5%)	4 (15%)	18 (7%)	NS	
Total	149	27	271		
Allelic Carriage					
C	141 (95%)	23 (85%)	253 (93%)		NS
A	59 (40%)	18 (67%)	117 (43%)	0.009	0.01
Allelic Frequency					
C	231 (78%)	32 (59%)	407 (75%)	0.004	0.01
A	67 (22%)	22 (41%)	135 (25%)		

5.3.1.4 Sarcoidosis and IFN+874 A/T

Compared to the rest of the group and the controls the *T* allele was less frequent among patients with sarcoidosis. As shown in the Table 5-13, the *TT* genotype was present in 4% of the sarcoidosis patients compared to 26% in rest of the group. P=0.01, the allelic frequency difference was quite significant as well p=0.005, pc 0.02.

Table 5-13: IFN-γ *+874* SNP frequencies in Sarcoidosis uveitis

GENOTYPE IFN+*874* A/T	Rest of PSU N= 152	sarcoidosis N=24	P Value Chi2x2
AA	38 (25%)	11 (46%)	0.034334
AT	74 (48%)	12 (50%)	NS
TT	40 (26%)	1 (4%)	0.017055
Allelic Carriage / phenotype			
A	112 (74%)	23 (96%)	0.017055
T	114 (75%)	13 (54%)	0.034334
Allelic Frequency			
A	150 (49%)	34 (71%)	0.005601
T	154 (51%)	14 (29%)	
Total	304	48	

5.3.2 Genotype phenotype correlation

5.3.2.1 IL-1β -511 SNP

5.3.2.1.1 IL- β -511 SNP and secondary glaucoma

Sub group analysis also revealed that the minor allele *T* at position *-511* of *IL-1β* was less frequent in patients with PSU who developed glaucoma. As shown in Table 5-14, the IL-*511*b *TT* genotype was present in 5% of the patients with glaucoma compared to 21% in patients who did not develop glaucoma. The allelic frequency difference was very significant with a p value of 0.0001. After correcting for age and duration of disease this was still significant p 0.0007

Table 5-14: *IL-1β* 511 SNP and risk of secondary glaucoma in PSU

IL-1β 511 C/T	PSU with Glaucoma n=(62)	PSU without glaucoma n=(114)	P value Chi 2 X 2
CC	36 (58%)	38 (33%)	0.001
CT	23 (37%)	45 (46%)	
TT	3 (5%)	23 (20%)	0.006
C allele	95 (77%)	129 (57%)	
T allele	29 (23%)	99 (43%)	0.0001

However correcting for sub type of PSU revealed that the effect was primarily seen in patients with idiopathic intermediate and posterior uveitis.(Table 5-15) In the idiopathic group (PU and IU) the *T* Allele frequency was 20% in patients with glaucoma compared to 46% in patients not suffering with secondary glaucoma. P=0.000005

Table 5-15: *IL-1β* 511 SNP and risk of secondary glaucoma in idiopathic PSU

IL-1β -511 C/T Genotype	Glaucoma N=43	No Glaucoma N=81	P Value Chi2x2
CC	29 (67%)	27 (33%)	0.000281
CT	11 (26%)	34 (42%)	NS
TT	3 (7%)	20 (25%)	0.015717
Allelic Carriage / phenotype			
C	40 (93%)	61 (75%)	0.015717
T	14 (33%)	54 (67%)	0.000281
Allelic Frequency			
C	69 (80%)	88 (54%)	0.000005
T	17 (20%)	74 (46%)	

5.3.2.1.2 IL-1β -511 and outcome in Behcet's uveitis

It must be noted that though this SNP showed some association with the development of Behcet's uveitis but no effect was seen on the development of secondary glaucoma in Behcet's patients. However there were some moderate effects of this SNP on prognosis and final vision of patients with Behcet's uveitis. Although there was no homozygous for *TT* allele in Behcet's group so the analysis was done between *CC* and *CT* genotype. It was noted that Behcet's patients carrying *CT* genotype had poor vision at 5 and 10 years compared to patients with *CC* genotype as shown in the Table 5-16. Despite a very low number of subjects for the analysis the difference in the vision was very significant between Behcet's patients with *CC* genotype and *CT* genotype.

Table 5-16: The association of CT genotype with poor vision in patients with Behcet's uveitis

	1st year	2nd year	3rd year	5th year	10th year
Asymp. Sig.	0.1	0.01	0.04	0.003	0.01
Mean VA CT	6/12	6/24	6/36	6/60	1/60
Range	6/6-6/24	6/9-6/60	6/12-3/60	6/12-1/60	6/18-NPL
Mean VA CC	6/12	6/9	6/18	6/18	6/36
Range	6/5-6/24	6/6-6/18	6/9-6/60	6/9-3/60	6/12-1/60

5.3.2.2 IL-6 -174 SNP and visual outcome

The mutant allele *C* was noted to be associated with poor visual outcome and severity of the disease in the whole PSU group. There were 64 patients who had severe episodes of inflammation (as defined in chapter 2). 25% (16/64) of the patient with severe disease were homozygous for *CC* allele compared to 9% (10/112) with mild to moderate disease. P=0.004.95% CI: 3.4 (1.4-8.0). (Figure 5-2) Sub group analysis showed that this effect was seen in posterior uveitis mainly and to certain extent in intermediate uveitis group.

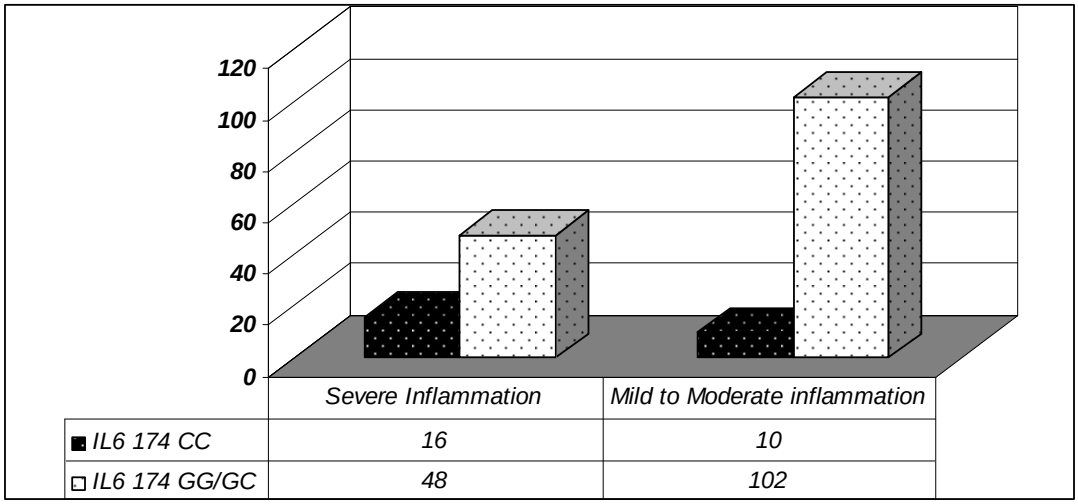


Figure 5-2: *IL-6 -174* SNP relation with intensity of inflammation in PSU.

Among patients with severe inflammation (quadrupling of visual angle) *IL-174 G* allele was more prevelant than *CC* genotype.

5.3.2.2.1 IL-6 -174 in Posterior uveitis

As we can see in the Table 5-17 below, the C allele is associated with permanent visual loss and severe inflammation is patients with idiopathic posterior uveitis. Patients with severe inflammation had a *CC* frequency of 43% compared to 6% in patients with less severe inflammation: p=0.0005, pc 0.002 95% CI: 12.69 (2.4-65.9) Details are shown in the table below (Table 5-17)

Table 5-17: *IL-6* 174 role in visual outcome PU.

IL-6 -174 G/C	PVL N=32	No PVL N=26	P VALUE Chi2x2
GG	9 (28%)	14 (54%)	0.046432
GC	13 (41%)	10 (38%)	NS
CC	10 (31%)	2 (8%)	0.027622
Allelic Carriage / phenotype			
G	22 (69%)	24 (92%)	0.027622
C	23 (72%)	12 (46%)	0.046432
Allelic Frequency			
G	31 (48%)	38 (73%)	0.007182
C	33 (52%)	14 (27%)	
IL-6 -174 G/C	Severe Disease N=23	Not severe N=35	
GG	7 (30%)	16 (46%)	NS
GC	6 (26%)	17 (49%)	NS
CC	10 (43%)	2 (6%)	0.000514
Allelic Carriage / phenotype			
G	13 (57%)	33 (94%)	0.000514
C	16 (70%)	19 (54%)	NS
Allelic Frequency			
G	20 (43%)	49 (70%)	0.004423
C	26 (57%)	21 (30%)	

PVL: Permanent visual loss

Among the patients with permanent visual loss, Kaplan-Meier analysis showed that patient with *CC* genotype had higher rate of moderate visual loss (VA 6/15 or worse) compared with the patients with *G* allele carriage. As seen in Figure 5-3, at two year of follow up, 81% of patients with *GG*/GC genotype retained VA of better than 6/18 compared to 48% with *CC* genotype. (p=0.001) This difference however decreased with time and at five year and beyond it was non-significant.

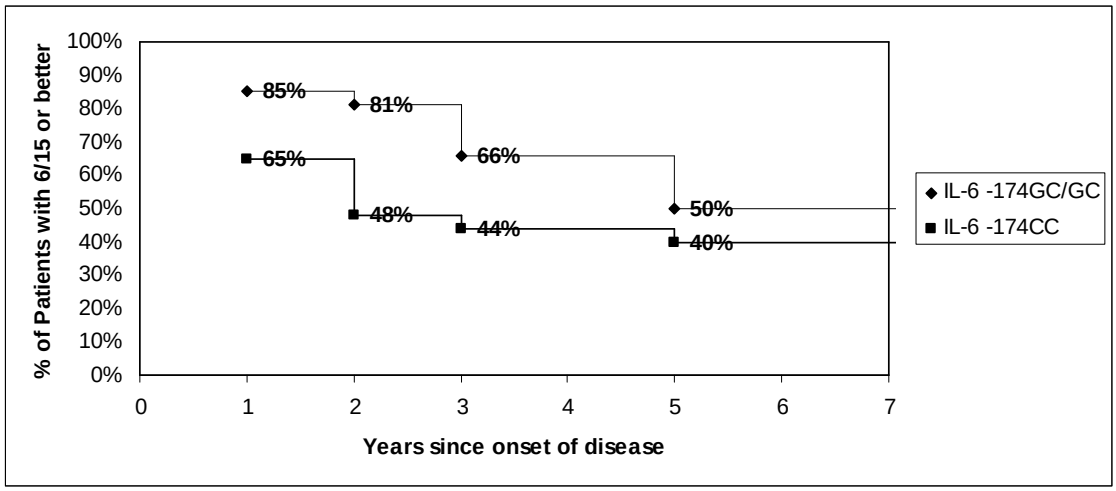


Figure 5-3: Kaplan-Meier analysis showing rate of moderate visual loss between *IL-6 -174 CC* & *IL-6 -174 CG/GG* genotypes in patients with posterior uveitis.

5.3.2.2.2 IL-6 –174 in Intermediate uveitis

Table 5-18 below shows the genotypic frequencies of *IL-6 -174* SNP in patients with intermediate uveitis. As the table shows patients with severe inflammation had *CC* frequency of 21% compared to 2% in rest of the group: p=0.007. However this association was not as strong as seen in posterior uveitis group

Table 5-18: *IL-6* 174 role in severity of uveitis in IU.

IL-6 174 Genotype	Mild to Mod. Int. Uveitis N=48	Severe Int. Uveitis N=19	P Value
GG	23 (48%)	5 (26%)	NS
GC	24 (50%)	10 (523%)	NS
CC	1 (2%)	4 (21%)	0.007
Allelic Carriage / phenotype			
G	47 (98%)	15 (79%)	0.007
C	25 (52%)	14 (74%)	
Allelic Frequency			
G	70 (73%)	20 (53%)	0.02
C	26 (27%)	18 (47%)	

Mild to moderate uveitis: Doubling of visual angle during inflammation. Severe uveitis: quadrupling of visual angle during inflammation

Among patients with intermediate uveitis this SNP was also affecting the response to treatment as well. The carriers of wild type allele were taking second line of immunosuppressants, and long term steroids less frequently than the carriers of the mutant allele. This is shown in the following Table 5-19

Table 5-19: *IL-6 -174* SNP and response to treatment in IU.

IL-6 -174 G/C	Long term steroid N=20	No Long term steroids N=47	P value Chi2x2
GG	3 (15%)	25 (53%)	0.003
GC	15 (75%)	19 (40%)	0.009
CC	2 (10%)	3 (6%)	NS
	Steroids N= 42	No steroids needed N=25	
GG	11 (26%)	17 (68%)	0.0007
GC	27 (64%)	7 (28%)	0.004
CC	4 (10%)	1 (4%)	NS
	Sec. line of treatment N=22	No sec. line of treatment N=45	
GG	4 (18%)	24 (53%)	0.006
GC	16 (73%)	18 (40%)	0.01
CC	2 (9%)	3 (7%)	NS

Not only this, it was also noted that the mean number of second line of treatment needed in intermediate uveitis group was significantly less between the genotypes. The mean number was 1.1 for *GG* genotype, 1.9 for *GC* and 2.5 for *CC* genotype, p=0.01. (Figure 5-4)

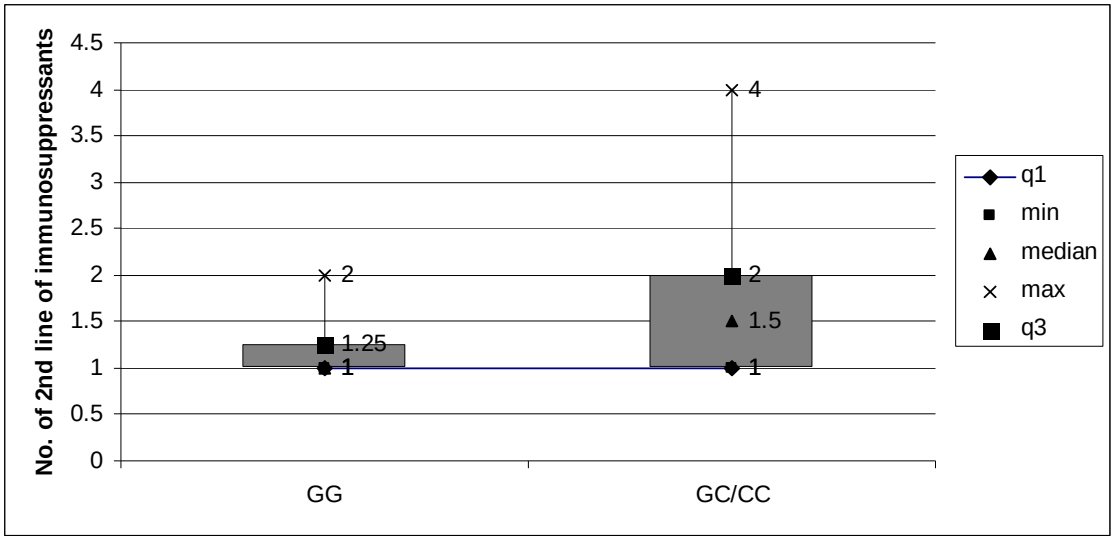


Figure 5-4: Comparison of number of second line of immunosuppressants needed to control inflammation between *IL-6 174 GG* or *GC/CC* carrying subjects in IU group.

The mean number of second line of immunosuppressants needed among patients with *GG* genotype in IU was 1.1 compared to 2.2 in rest of the patients

In posterior uveitis group the same association was noted between *IL-6 174* genotypes and need of second line of treatment. However the effect noted was modest. (Table 5-20)

Table 5-20: *IL-6 -174* SNP and need of second line of treatment in PU.

IL-6 174 G/C	Sec. line of treatment N=25	No Sec. line of treatment N=33	P value Chi2x2
GG	10 (40%)	13 (39%)	NS
GC	6 (24%)	17 (52%)	0.03
CC	9 (36%)	3 (9%)	0.02
Allelic Carriage			
G	16 (64%)	29 (91%)	0.01
C	15 (60%)	20 (61%)	NS

Again similar to intermediate uveitis mean number of second line treatment used was significantly less in carriers of wild type compared to mutant genotype. (1.6 Vs 2.9 p=0.03). (Data not shown)

5.3.2.3 Association of IL-10 592 with visual outcome and ischemic visual loss

The *A* allele at position *-592* was noted to be effecting the visual outcome. The carriers of *A* allele had poor visual acuity compared to *C* allele carriers. This effect was seen in all four groups but was more significant in posterior uveitis, Behcet's and sarcoidosis. (Table 5-21) Though the effect seen in intermediate uveitis was not as significant as other groups, but the same allele was associated with permanent visual loss in patients with intermediate uveitis. Similar trend was seen in other groups but presumably did not reach significance due to numbers. Among IU patients with PVL (N=22) the *A* allele frequency was 30% compared to 12% in IU with no PVL (N=45) p=0.02. See Table 8-9, Appendix B

Table 5-21: *IL-10* 592 SNP and visual outcome in PSU groups

	Disease	First Year	Second Year	Third Year	Fifth Year	Tenth Year
Asymp. Sig.		0.1	1	0.05	0.02	0.02
Mean VA CC vs CA/AA	Intermediate Uveitis	6/9 vs 6/12	6/12 vs 6/12	6/12 vs 6/24	6/12 vs 6/36	6/18 vs 6/36
Asymp. Sig.		0.04	0.02	0.001	0.002	0.02
Mean VA CC vs CA/AA	Posterior uveitis	6/9 vs 6/18	6/12 vs 6/36	6/12 vs 6/60	6/18 vs 1/60	6/36 vs 2/60
Asymp. Sig.		0.0002	0.0007	0.001	0.001	0.009
Mean VA CC vs CA/AA	Behcet's Uveitis	6/12 vs 6/36	6/18 vs 6/60	6/18 vs 1/60	6/24 vs 1/60	6/24 vs 1/60
Asymp. Sig.		0.09	0.2	0.03	0.009	0.01
Mean VA CC vs CA/AA	Sarcoid uveitis	6/9 vs 6/12	6/9 vs 6/12	6/12 vs 6/36	6/18 vs 6/60	6/18 vs 4/60
Asymp. Sig.		0.05	0.09	0.001	0.009	0.01
Mean VA CC vs CA/AA	Total	6/9 vs 6/15	6/12 vs 18	6/12 vs 6/60	6/18 vs 6/60	6/20 vs 1/60

The *A* allele was noted to be associated with vasculitis and ischemic visual loss as well. This was noted in the whole group and since number were too small (11) further subgroup analysis did not reveal any obvious significant association. The *AA* genotype was 27% in patients who developed ischemic visual loss compared to 5% in the rest of the patients p=0.002, pc=0.004. The allelic frequency difference between the two groups was p=0.0006, pc=0.001. (Table 5-22)

Table 5-22: *IL-10 -592* association with ischemic visual loss

IL-10 -592 Genotype	PSU with Ischemic visual loss n=(11)	PSU with no ischemic visual loss n=(165)	P Value
CC	2 (18.2%)	99 (60.0%)	0.008
CA	3 (54.5%)	58 (35.2%)	
AA	6 (27.3%)	8 (4.8%)	0.001
Allelic Frequency			
C allele	10 (45.5%)	256 77.6%)	
A allele	12 (54.5%)	74 (22.4%)	0.0006

Using PHASE, 3 common haplotypes were deduced as shown in Table 5-23.

Table 5-23: *IL-10* haplotypes in PSU and controls

IL-10 HAPLOTYEPS		Patients n=(176)	Controls n=(271)	P Value
H1	592C / 1082G	79 (44.7%)	118 (43.5%)	NS
H2	592A / 1082A	45 (25.8%)	65 (24.1%)	NS
H3	592C / 1082A	50 (28.2%)	82 (30.2%)	NS
H4	592A / 1082G	2 (1.4%)	6 (2.2%)	NS

The haplotype 2, *AA* haplotype was associated with poor vision, and compared to the rest of haplotypes patient carrying this haplotype consistently had poorer vision as shown in the table. This effect was seen in more or less all four groups. (Table 5-24)

Table 5-24: Association of *IL-10* haplotype AA (2) with visual outcome in PSU groups

	Disease	First Year	Second Year	Third Year	Fifth Year	Tenth Year
Asymp. Sig.		0.1	1	0.05	0.02	0.01
Mean VA Rest of Haplotypes Vs AA	Intermediate Uveitis	6/9 vs 6/12	6/12 vs 6/12	6/12 vs 6/24	6/12 vs 6/36	6/18 vs 6/48
Asymp. Sig.		0.03	0.01	0.0009	0.002	0.01
Mean VA Rest of Haplotypes Vs AA	Posterior uveitis	6/9 vs 6/18	6/12 vs 6/48	6/12 vs 1/60	6/18 vs 1/60	6/36 vs 2/60
Asymp. Sig.		0.0009	0.0007	0.001	0.001	0.009
Mean VA Rest of Haplotypes Vs AA	Behcet's Uveitis	6/12 vs 6/24	6/18 vs 6/60	6/18 vs 1/60	6/24 vs 1/60	6/36 vs 1/60
Asymp. Sig.		0.1	0.2	0.03	0.009	0.01
Mean VA Rest of Haplotypes Vs AA	Sarcoid uveitis	6/9 vs 6/15	6/9 vs 6/12	6/12 vs 6/36	6/18 vs 6/60	6/18 vs 5/60
Asymp. Sig.		0.04	0.06	0.001	0.001	0.01
Mean VA Rest of Haplotypes Vs AA	Total	6/9 vs 6/18	6/12 vs 6/18	6/12 vs 6/60	6/18 vs 6/60	6/24 vs 1/60

The haplotype *2*, due to presence of *A* allele at *-592* showed association with ischemic maculopathy. *AA* haplotype was present in 82% of the patient with ischemic maculopathy compared 39% in patients with no ischemic maculopathy p=0.008. It was also noted that patients carrying the common haplotype CG was associated with better visual prognosis. As shown in, Table 5-25, 77% of the Patients with better eye vision of 6/12 or better (at 24 months) carried the *GG* haplotype compared to 53% in patients not carrying *GG* haplotype p=0.006 pc 0.02. When group analysis was done a non-significant trend of better vision with *CG* haplotype was noted in all groups however significance was only achieved when they were all combined together.

Table 5-25: *IL-10* haplotype AA and ischemic visual loss

Haplotype	PSU with Ischemic visual loss n=(11)	PSU with no ischemic visual loss n=(165)	P value
592A / 1082A	10 (82%)	65 (39%)	0.008
	Patients with VA BE 6/12 or better n=(146)	Patients with VA BE 6/15 or worse n=(30)	
592C / 1082G	113 (77%)	16 (53%)	0.006

VA BE : VA from better eye at 24 months

5.3.2.4 IL-18 137 & 607 and Behcet's

The *IL-18* SNPs showed very significant associations with visual outcome and prognosis in patients with Behcet's uveitis. Patients carrying the mutant alleles i.e. *C* at *137* and *A* at *607* position had less severe inflammation, quicker response to treatment and less patients had permanent visual loss. (Table 5-26) However it must be noted that because of small number of patients these associations should be read with caution. As mentioned in the chapter two, whenever there were associations noted in case of permanent visual loss,

Kaplan-Meier analysis was performed. However, as the number of individuals with permanent visual loss in OB group were very low the survival analysis did not reveal any meaning full results.

Table 5-26: *IL-18 -137* SNP and prognosis in ocular Behcet's

IL18-*137* G/C GENOTYPE	Permanent visual loss N=19	No Permanent visual loss N=8	P Value Chi2x2
GG	8 (42%)	3 (38%)	NS
GC	10 (53%)	1 (13%)	NS
CC	1 (5%)	4 (50%)	0.006
Allelic Carriage			
G	18 (95%)	4 (50%)	0.006
C	11 (58%)	5 (63%)	NS
GENOTYPE	Good Response N=11	Bad Response N=16	
GG	2 (18%)	9 (56%)	0.047
GC	4 (36%)	7 (44%)	NS
CC	5 (45%)	0 (0%)	0.0028
Allelic Carriage			
G	6 (55%)	16 (100%)	0.0028
C	9 (82%)	7 (44%)	0.047
Allelic Frequency			
G	8 (36%)	25 (78%)	0.0019
C	14 (64%)	7 (22%)	
Total	22	32	
GENOTYPE	Severe Inflammation N=19	Not Severe Inflammation N=8	
GG	7 (37%)	4 (50%)	NS
GC	11 (58%)	0 (0%)	0.005
CC	1 (5%)	4 (50%)	0.006
Allelic Carriage			
G	18 (95%)	4 (50%)	0.006
C	12 (63%)	4 (50%)	NS

Table 5-27 shows the details of association between *IL-18 -607* SNP and prognosis in ocular Behcet's.

Table 5-27: *IL-18 -607* SNP and prognosis in ocular Behcet's

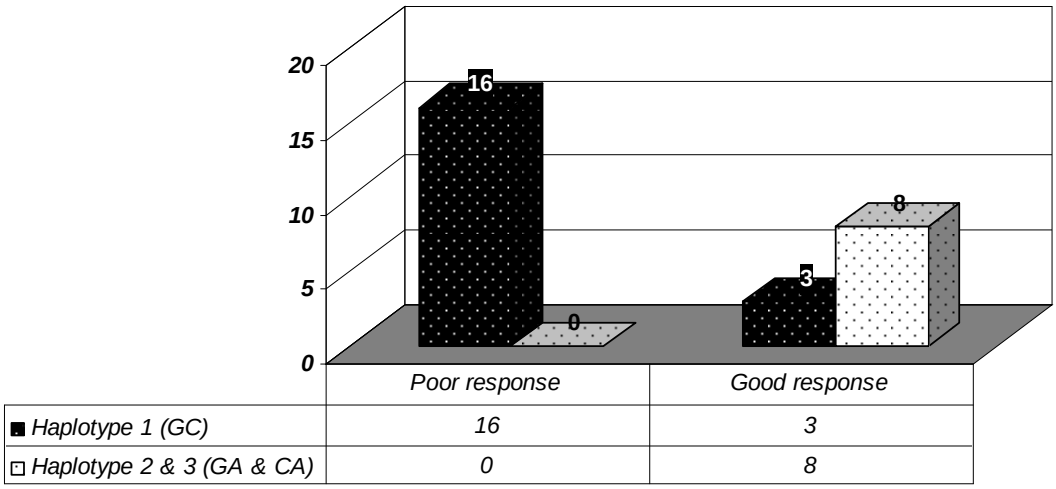
IL-18 -607 C/A Genotype	Good Response N=12	Poor Response N=16	P Value Chi2x2
CC	1 (8%)	8 (50%)	0.019
CA	3 (25%)	8 (50%)	NS
AA	8 (67%)	0 (0%)	0.0001
Allelic Carriage			
C	4 (33%)	16 (100%)	0.0001
A	11 (92%)	8 (50%)	0.019
Allelic Frequency			
C	5 (21%)	24 (75%)	0.000005
A	19 (79%)	8 (25%)	
Total	24	32	
Genotype	Severe Inflammation N=19	Mild Inflammation N=8	
CC	5 (26%)	3 (38&)	NS
CA	11 (58%)	0 (0%)	0.005
AA	3 (16%)	5 (63%)	0.015
Allelic Carriage			
C	16 (84%)	3 (38%)	0.015
A	14 (74%)	5 (63%)	NS

Also patients harbouring wild type genotypes at both *-137* and *-607* positions had worse visual acuity through out the course of the disease compared to the mutant alleles (Table 5-28)

Table 5-28: Association of *IL-18* SNPs with long term visual outcome in OB

SNP *IL-18 -607* C/A	1st year	2nd year	3rd year	5th year	10th year
Asymp. Sig.	**0.013**	**0.023**	**0.0276**	**0.009**	**0.009**
Mean VA CC	**6/18**	**6/24**	**6/36**	**6/60**	**1/60**
Range VA CC	6/6-6/24	6/9-6/60	6/9-3/60	6/12-2/60	6/24-NPL
Vs CA/AA	**6/9**	**6/12**	**6/18**	**6/18**	**6/36**
Range CA/AA	6/5-6/18	6/6-6/24	6/9-6/60	6/9-1/60	6/12-3/60
IL-18 -137 G/C					
Asymp. Sig.	**0.034**	**0.0177**	**0.0094**	**0.0204**	**0.009**
Mean VA GG	**6/18**	**6/30**	**6/60**	**6/60**	**1/60**
Range VA GG	6/5-6/24	6/9-6/60	6/12-3/60	6/9-1/60	6/18-NPL
Vs GC/CC	**6/10**	**6/10**	**6/12**	**6/24**	**6/36**
Range GC/CC	6/6-6/18	6/6-6/24	6/9-6/36	6/12-2/60	6/12-3/60

IL-18 haplotype *1 (GC)*, containing wild type alleles at both positions was associated with poor response and poor visual outcome. 84% of the patients with haplotype *1(GC)* had poor response and none with the patients with haplotype *2 (GA)* or 3 *(CA)* had poor response. P=0.0000004 as shown in Figure 5-5



	Poor response	Good response
■ *Haplotype 1 (GC)*	*16*	*3*
⊡ *Haplotype 2 & 3 (GA & CA)*	*0*	*8*

Figure 5-5: Association of *IL-18* haplotypes with response to treatment in OB.

Patients harbouring *IL-18 Haplotype-1*, had poorer response to steroids compared to rest of the patients with ocular Behcet's

Patients with *IL-18 Haplotype 1 (GC),* had poor visual acuity through out the course of disease compared with patients carrying the rest of the haplotypes as shown in Table 5-29

Table 5-29: Association of *IL-18* haplotype with visual outcome in OB

IL-18 Haplotypes	1st year	2nd year	3rd year	5th year	10th year
Asymp. Sig.	0.0013	0.01	0.00176	0.008	0.0009
Mean VA Haplotype 1	6/20	6/36	6/60	4/60	1/60
	6/12-6/24	6/12-6/60	6/18-3/60	6/18-1/60	6/12-NPL
Vs Haplotype 2 and 3	6/7.5	6/10	6/18	6/20	6/24
	6/5-6/12	6/6-6/18	6/9-6/60	6/9-6/60	6/12-5/60

5.3.2.5 IFNGR-56 C/T and sarcoidosis uveitis

The frequency of rare genotype *CC* was around 16% in both PSU and patient groups. It frequency varied considerable among the PSU groups ranging from 8.3% in Sarcoidosis to 25.4% in IU. Data mining did not reveal any obvious association of this SNP with phenotypic attributes except that the wild type allele was associated with poor response to treatment and worse visual acuity in patients with sarcoidosis. This effect was not seen in any other patients groups and even in sarcoidosis group the associations were modest. As seen in the Figure 5-6, the VA in patients after the minimal follow up period of 2 years was 6/24 in patients with wild type genotype compared to 6/9 in patients carrying the rare allele p =0.005. However no significant differences were noted in the visual acuities between the two alleles beyond two years.

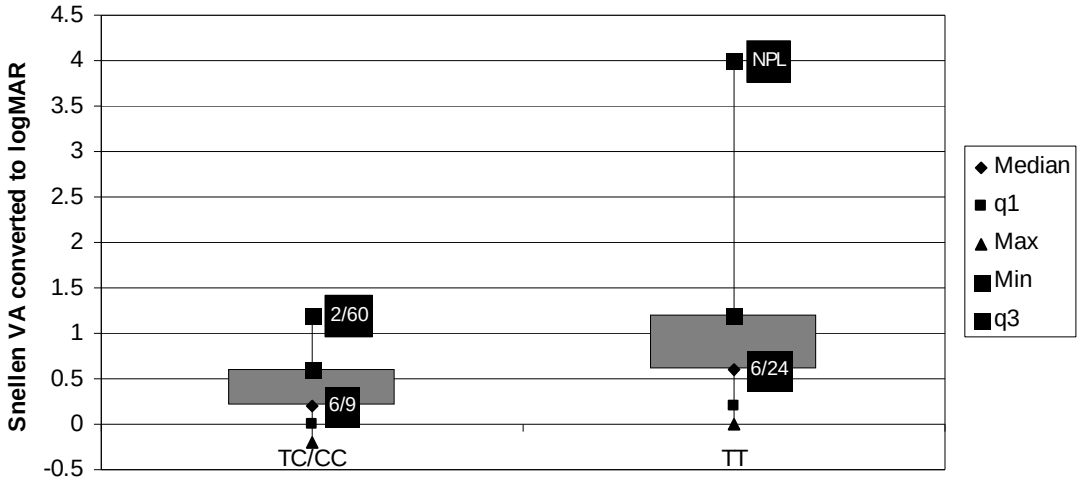


Figure 5-6: Association of *IFNGR* genotypes with VA in patient with ocular sarcoidosis

Mean Snellen VA in patients after the minimal follow up period of 2 years was 6/24 in patients with wild type genotype compared to 6/9 in patients carrying the rare allele

It was also noted that sarcoidosis patients who responded poorly to the treatment had a higher frequency of wild genotype *CC* (70%) compared to the ones who did not (15%). P=0.005 (Table 5-30)

Table 5-30: The association of *IFNGR -56* SNP with response to treatment in ocular sarcoidosis

IFNG-R *-56* C/T GENOTYPE	Bad Response N=10	Good Response N=14	P Value Chi2x2
TT	7 (70%)	2 (14%)	0.005
CT	2 (20%)	11 (79%)	0.004
CC	1 (10%)	1 (7%)	NS
Allelic Carriage			
T	9 (90%)	12 (93%)	NS
C	3 (30%)	11 (86%)	0.005
Allelic Frequencies			
TT	7 (70%)	2 (14%)	
CT/CC	3 (30%)	12 (86%)	0.005

5.3.2.6 TNF-α and CMO

The three *TNF-α* SNPs did not show any significant associations with the clinical features of PSU except that there were some moderate associations between *-238* SNP and CMO. Patients with rare allele *A* develop fewer episodes of CMO per year in and responded better to intra-vitreal injections of steroids in IU, PU and Sarcoid uveitis. (Table 5-31) However it should be noted that there was no difference in the genotypic frequencies between patient developing CMO and not developing CMO in general or at group levels.

Table 5-31: *TNF-α -238* SNP and association with CMO in PSU groups

TNF -238 G/A	CMO/ year	P Value	Mean Number of I/vitreals to control CMO	P Value
Intermediate Uveitis				
GG	3.4		3.1	
GA/AA	0.6	0.002	1	0.007
Posterior Uveitis				
GG	3.9		2.7	
GA/AA	1	0.008	1.1	0.01
Sarcoid Uveitis				
GG	2.8		2.9	
GA/AA	0.9	0.01	1.6	0.03

I/Vitreals: Intra-vitreal steroid injections, CMO: cystoid macular oedema

TNF SNPs *-308* and *-857* showed moderate associations with type of treatment but lost the significance after Bonferroni correction and multiple logistic regression. Four *TNF* haplotypes were constructed as shown in Table 5-32.

Table 5-32: *TNF* haplotypes in patients and controls

TNF HAPLOTYPES	Patients n=(176)	Controls n=(271)
238G/ 308G/ 857C	58.9%	61.1%
238G/ 308G/ 857T	12.5%	11.3%
238G/ 308A/ 857C	21.1%	19.6%
238A/ 308G/ 857C	7.5%	8.0%

Haplotype *GGT* containing *T* at *-857* showed modest association with the need of second line of immunosuppressive treatment. *GGT* haplotype was present in 25% (19/74) of the patients needing second line of treatment compared to 12% (13/102) in rest of the patients p value was 0.029. See Figure 5-7. Sub group analysis did not reveal any association with particular group and individual analysis did not reach to statistical significance because of numbers.

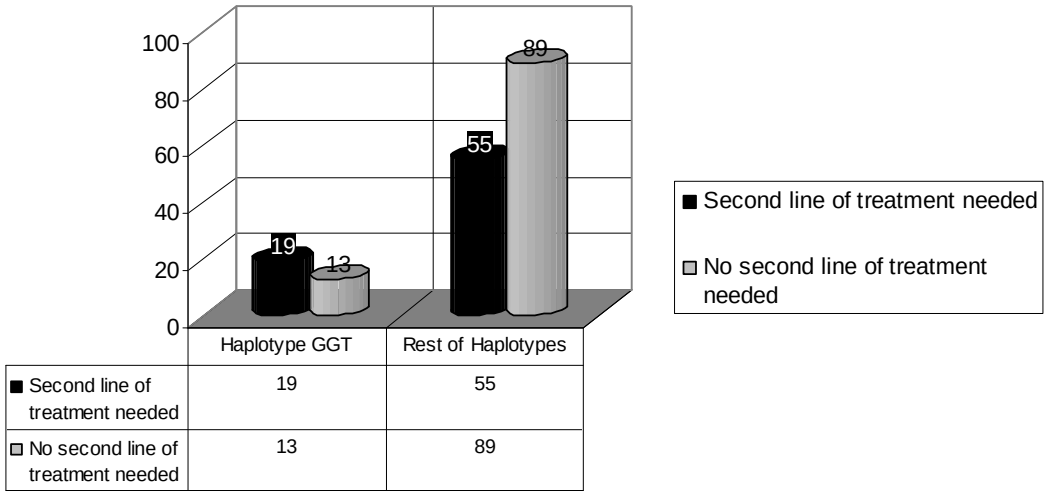


Figure 5-7: TNF haplotypes in PSU and their association with need of second line of immunosuppressive treatment.

5.4 Discussion

This study was conducted to determine role of various functional polymorphisms of cytokine genes in PSU. Quite a few of the SNPs were noted to affect the phenotype of PSU and act as predictor of complications. The study showed that *IL-18* polymorphisms were associated with retinal vasculitis and *IL-10* polymorphism exhibited genetic susceptibility to development of Behcet's uveitis. It was also noted that *IL-1β*, *IL-6*, *IL-10* and *IL-18* were affecting the genotypes of various sub groups of PSU.

5.4.1 IL-18 & IL-12

IL-18 is expressed in retinal pigment epithelial cells (RPE) (Jiang et al. 2001), although it does not play a significant role in EAU, *IL-18* deficient mice produce low level of IFN-γ and TNF-α in EAU (Jiang et al. 2001). IL-18 is able to active Th1, Th2 or Th17 cell responses. In the present of IL-12 and IL-2, it can induce IFN-γ leading to Th1 response. (Tarrant et al. 1998) On the other hand in the presence of IL-23 it can active Th17 cells. (Boraschi and Dinarello 2006). High aqueous levels of IL-12 have also been noted in various forms of immune mediated uveitides. (El-Shabrawi et al. 1998) Recently Qiao *et al* has shown that IL-18 plays an important regulatory role in retinal vascular development. (Qiao et al. 2004) Their results on *IL-18* knock out mice show that IL-18 plays an anti angiogenic role and absence of it may lead to up regulation of angiogenic factors and may lead to vascular leakage and non perfusion

The *G/C* change at *-137* position and *C/A* change at *-607* position leads to decrease expression of *IL-12* gene (Giedraitis et al. 2001) (Zhou et al. 2005). Association have been noted between these SNPs and susceptibility to inflammatory bowel disease, Behcet's and SLE (Lin et al. 2007) (Keskin et al.

2009) (Ben et al. 2011) (Tamura et al. 2002). Interestingly in Behcet's disease and SLE, vasculitis is a salient feature of the disease. However it should be noted that the functional affects of these SNPs are very moderate and not replicated by all studies. My results show that the alleles that decrease the expression of *IL-18* and *IL-12* genes were strongly associated with retinal vasculitis. In the conditions where vasculitis is the primary pathology the serum levels of IL-18 have noted to be quite high denoting the role of *IL-18* gene in vasculitis. (Hamzaoui et al. 2002) (Esfandiari et al. 2001) (Hultgren et al. 2007) (Novick et al. 2009) (Hamzaoui et al. 2002). Now these two statements seem contradictory, but as mentioned the functional effects of the SNPs are at the best moderate, and the associations seen here are more likely to be related to another allele with definite functional role. This SNP is yet to be identified; however these results indicated presence of genetic susceptibility marker for retinal vasculitis on *IL-18* gene. Recently Sanchez *et al*. tested the effect of the *IL18 -1297 T/C rs360719* polymorphism on the transcription of *IL18* by electrophoretic mobility shift assay and western blot. They found a significant increase in the relative expression of *IL18 mRNA* in individuals carrying the *rs360719 C*-risk allele; in addition they showed that the polymorphism creates a binding site for the transcriptional factor OCT-1. (Sanchez et al. 2009). They also suggested that this SNP may play an important role in determining the susceptibility to SLE which is one of the most common systemic vasculitis. Another recent study (Palomino-Morales et al. 2010) showed association of this SNP with biopsy proven giant cell arteritis in 212 Caucasian patients. The gene mapping of *IL-18* and particularly studying the *IL-18 -1297* SNP should be the aim of further study.

The *IL-18* SNPs showed moderate genetic susceptibility to Behcet's but were strongly associated with visual outcome in Behcet's uveitis. Patients with low producing alleles, develop less severe inflammation, better long term vision and less permanent visual loss. Studies have shown that not only serum IL-18 levels are higher in patient with Behcet's disease but the levels correspond to the disease activity (Musabak et al. 2006) (Hamzaoui et al. 2002) (Oztas et al. 2005). This does mean that lower levels of IL-18 will be associated with less severe disease. If one accepts that these two functional polymorphisms decrease the expression of *IL-18* gene than it is plausible that carriers of these allele among Behcet's uveitis will develop less severe disease. Interestingly a study showed that the *IL-18* gene polymorphisms were not associated with a susceptibility to BD in the Korean population, but the patients carrying the *GG* genotype at position *-137* had a higher risk of developing the ocular lesions. (Jang et al. 2005) Other studies have also noticed the genetic susceptibility to Behcet's disease with *IL-18* SNPs (Lee et al. 2006) (Keskin et al. 2009)

My results also showed association of *IL-12 +1188 C* allele with retinal vasculitis. The A/C change at *+1188* position of *IL-12* leads increase secretion of IL-12 from LPS stimulated PBMCs (Seegers et al. 2002) (Yilmaz, Yentur, and Saruhan-Direskeneli 2005) Compared to the functional affects of *IL-18*, these changes are more significant and replicable. IL-12 also play a more definite role in the pathogenesis of different autoimmune vasculitides, Frassanito *et al* showed that serum levels of IL-12 correlate with disease activity in Behcet's vasculitis, (Frassanito et al. 1999) . Similar findings were noted in patients with Takayasu vasculitis (Verma et al. 2005). High *+1188* C allele frequency was also noted in Turkish patients with Takayasu vasculitis

(Saruhan-Direskeneli et al. 2006). Another recent study has noted that *C* allele is more come in patients with Behcet's disease compared to controls (Alayli et al. 2007). I did not notice any over representation of *C* allele in my Behcet's population, but it must be noted that all OB patients who had clinical evidence of retinal vasculitis were carriers of *C* allele.

5.4.2 IL-1β

IL-1β being a potent inducer of IL-2 is though to play an important role in induction of uveitis. The *C/T* SNP at position *-511* leads to increase secretion of IL-1β (Hall et al. 2004), (Santtila, Savinainen, and Hurme 1998), (Hulkkonen, Laippala, and Hurme 2000) and associations of this polymorphisms have been noted with ankylosing spondylitis and Crohn's disease. (Nemetz et al. 2001) (Timms et al. 2004) both diseases in which uveitis can develop. The SNP did not affect susceptibility to PSU except in Behcet's uveitis where the association was modest. Recent studies on Turkish subjects with Behcet's disease have shown similar results where *T* allele was rare in patients compared to controls (Alayli et al. 2007). Other studies in Behcet's patients failed to notice this association but nearby SNPs was noted to be affecting susceptibility to the disease denoting that there may be a genetic susceptibility locus in *IL-1β* gene(Karasneh et al. 2003) (Coskun et al. 2005). The *-511* SNP also showed association with visual outcome but again the effects were modest, however needless to say other studies have also noted effect of *-511* SNP on severity of Behcet's disease. (Akman et al. 2008)

What was more significant was that the high secretor allele *T* was rare among patients who developed secondary glaucoma. Studies have shown that as a result of high IOP, IL-1 is secreted from trabecular meshwork endothelial cells which act either by remodelling of juxta canallicular extra cellular matrix (the

sight of out flow obstruction) (Bradley et al. 2000) or control IOP through activation of ELAM-1/IL-1/NF-kappaB response. (Wang et al. 2001) (Wang et al. 2003) Recently, Birke *et al* showed on porcine eyes that trabecular cells treated with IL-1β augmented the outflow by three folds by inducing ELAM-1. (Birke et al. 2011) Hence the uveitic patients who have high secretor allele *T* will produce more IL-1 in trabecular meshwork, so IOP is controlled and they do not progress to glaucoma, conversely uveitic patients with *C* allele will have a higher chance of developing glaucoma. Based on similar hypothesis some authors have studied this SNP in glaucoma and have noted association between glaucoma and SNPs in *IL-1β* genes. (Lin et al. 2003) (Mookherjee et al. 2010) In short this SNP could act as a predictor of development of glaucoma in uveitic patients.

5.4.3 IL-6 174

IL-6 has been detected in vitreous, as well as aqueous humour aspirates of patients with idiopathic uveitis. (Franks et al. 1992) More specifically, increased vitreous levels of IL-6 have been detected in patients with active intermediate and posterior uveitis, (Perez et al. 2004) reflective of on-going inflammation during disease hence, role in the propagation of uveitis. The medical literature lacks studies that have looked into association of *IL-6* polymorphisms and uveitis; however few studies in Behcet's patients are present. Most of these studies found no association between *IL-6* polymorphisms and Behcet's disease (Storz et al. 2008) (Chang et al. 2005). In my study I noted that *IL-6* polymorphism was associated with severity of the disease and response to treatment in patients with idiopathic PSU. Patients with rare allele *C* were noted to have more severe disease and permanent

visual loss. This is contradictory to the observations that *C* allele was associated with decreased IL-6 expression. (Fishman et al. 1998) (Belluco et al. 2003) Experiments however have shown that both *C* and *G* allele can be associated with increased IL-6 depending upon the cell lines used and initial stimuli. (Terry, Loukaci, and Green 2000). Also there is evidence now that some SNPs in distal promotor region may have more affect on the transcription of *IL-6* gene and *IL-6 174* SNP may be in linkage with these SNPs. (Samuel et al. 2008)

This could be the reason that studies have failed to notice association between *IL-6 -174* SNP and uveitis.

However more interesting and consistent finding was effect of this SNP to response of treatment. The wild type carriers in intermediate uveitis needed less steroids and less second line of immunosuppression compared to rare type allele carriers. One interpretation is they have less severe disease but it also meant they responded very quickly and effectively to steroids and hence needed less treatment. This is in consistent with various studies that have shown that *IL-6 174* genotypic differences can affect response to systemic immunosuppression. Pawlik *et al* treated peripheral blood monocytes with dexamethasone and noted that although patients with *C* allele produced less *IL-6* compared to wild type carriers, but dexamethasone was unable to decrease the production of *IL-6* in *CC* genotype compared to *GG* genotype. (Pawlik et al. 2006) They also showed that patients with rheumatoid arthritis responded variably to glucocorticoids and methotrexate according to their *IL-6 174* genotype (Pawlik et al. 2005). Fabris *et al* recently showed that among patient with rheumatoid arthritis, *C* carriers responded poorly to retuximab (anti CD20 antibody) compared to *G* carriers. (Benucci et al. 2010) (Fabris et al.

2010b) (Fabris et al. 2010a) My findings, further confirm these findings that *IL-6* genotypes can be a marker to drug response in idiopathic uveitis particularly intermediate uveitis.

5.4.4 IL-10

IL-10 is an anti-inflammatory cytokine that was shown to suppress IFN-γ production, inhibit the Th1 response and promote the Th2 response. (de Vries 1995) A number of studies described beneficial effects of IL-10 administration in different models of cell-mediated autoimmunity however the regulation of cellular responses by IL-10 is complex and may be influenced by many factors. Intra-ocular transfer of *IL-10* gene has shown to inhibit experimental autoimmune uveoretinitis (Verwaerde et al. 2003) (de et al. 2002) while injection of IL-10 can control EAU (Rizzo et al. 1998)

In human studies it has been shown that IL-10 expression on T cells correlate with visual recovery in PSII (Greiner et al. 2004) and levels of IL-10 increase in aqueous humour as the patients are treated with steroids (Ooi et al. 2006)

Among cytokines, *IL-10* gene is probably the most widely studied in uveitis gene association studies. Stanford *et al.* showed that in patients with intermediate uveitis *IL-10-1082* AA homozygosity was associated with bad outcome. (Stanford et al. 2005). Another studied on patients with sympathetic ophthalmitis noted associations between the *IL-10 -1082* SNP and disease recurrence from previously stable disease and the level of steroids required for maintenance therapy. In addition, the *GCC IL-10* promoter haplotype (*IL-10 -1082*G, *-819*C, *-592*C) was found to be protective against disease recurrence. (Atan et al. 2005) The same group recently showed that other SNPs in *IL-10* gene could predispose to PSU; (Atan et al. 2010) however they did not notice any association with *-1082* SNP

Although I did not notice any difference in the frequencies of *IL-10* SNPs between the patients and controls but at position *-592* of *IL-10* the wild type allele *C* was less prevalent in Behcet's disease compared to the rest of the groups and the controls. (Where the frequencies were comparable) Just recently, (two years after my study was done) genome wide association study on 1215 patients with BD noted about 5 SNPs in *IL-10* gene were strongly associated with BD, with one SNP, *rs1518111* to be very significant. (Remmers et al. 2010) Another recent study in Turkish Behcet's patients found that *IL-10 -1082 GA* genotype was more frequent whereas the *AA* genotype was less common in the BD group compared to the control group. (Dilek et al. 2009) Wallace *et al*. found that *-1082* and *-819* SNPs may be relevant in influencing the immune response in Behcet's disease in some patient groups (Wallace et al. 2007). It should be kept in mind that this *-819* SNP is in 100% linkage with -592 SNP I found to be associated with ocular Behcet's. My finding is just another confirmation that genetic susceptibility locus for Behcet's uveitis may lie in *IL-10* gene.

I also found that *A* alleles at both positions were associated with poor visual outcome in all the four sub groups of PSU. Similarly the *G* at *-1082* and *C* at *-592* were associated with better visual acuity. This is consistent with the findings that polymorphisms in *IL-10* gene can be the predictor of visual outcome in uveitis. Stanford *et al* genotyped 125 Caucasian patients with intermediate uveitis and found that *IL-10 -1082 AA* genotype was associated with poor visual outcome. (Stanford et al. 2005) Atan *et al* studied *IL-10* SNPs in British patients with sympathetic uveitis. They noted associations between the *IL-10 -1082* SNP and disease recurrence from previously stable disease and the level of steroids required for maintenance therapy. In addition, the *GCC*

IL-10 promoter haplotype (*IL-10 -1082*G, *-819*C, *-592*C) was found to be protective against disease recurrence (Atan et al. 2005). Compared to these studies, I showed the effect of *IL-10* SNPs on visual outcome is seen in most of the types of PSU.

A strong association was also noted between the *IL-10 -592 A* allele, *IL-10 AA* haplotype (*IL-10 -592A & -1082A*) with ischemic maculopathy. As far as the association with vasculitis is concerned, it is known that *IL-10* attenuates the injury and intimal thickness after inflammation. (Zimmerman et al. 2004). Giachini *et al* showed that *IL-10* confers vascular protection from ET-1-induced injury and vasoconstriction (Giachini et al. 2009) A recent study showed that endothelial nitric oxide synthase (eNOS) expression was reduced by TNF-α in in-vitro and in-vivo experiments, whereas IL-10 restored the eNOS expression (Zemse et al. 2010). This eNOS catalyzes NO production which is a potent vasodilator. These studies show that that *IL-10* is involved in prevention of vascular injury and its related damage and in fact the *AA* haplotype has associated with more lesions in Kawasaki disease (Yang et al. 2003). One can derive from these studies that local low expression of *IL-10* should lead to ischemia in retina/ macula and hence ischemic maculopathy. This is shown by my finding that low *IL-10* producer alleles were associated with ischemic maculopathy in PSU.

5.4.5 IFN-γ 874 & IFNR -56

IFN-γ is the primary cytokine which defines Th1 cells. IFN-γ is secreted by Th1 cells, cytotoxic T cells and NK cells, it promotes Th1 differentiation by upregulating the transcription factor T-bet, ultimately leading to cellular immunity.

Up until recently IFN-γ induced Th1 cell response in PSU was thought to be the main orchestrator of inflammation, (rocker-Mettinger et al. 1990) (Feron, Calder, and Lightman 1992) (Dick et al. 1992) (Muhaya et al. 1999) (Whitcup et al. 1992). However recent discovery of Th-17 cells has suggested that Th17 cells also play role driving tissue damage in posterior segment uveitis.

In my study I did not notice any significant associations between *IFN-γ* or *IFN-R* SNPs and uveitis except in Sarcoidosis. Here the *IFN-γ +874 T* allele was less prevalent compared to rest of the groups and controls. (4.2% in Sarcoidosis, Vs 19% in controls, and 28% in rest of PSU) Similarly *IFNR -56 SNP* showed some moderate association with visual outcome in Sarcoid uveitis group only. Here the *T* allele which increases the production of *IFNR* was associated with poor response and poor visual acuity after 2 years. Recent study have noted (Wysoczanska et al. 2004) association between *IFN-γ* polymorphisms and sarcoidosis however the association noted was very modest. It must be noted that *+874T* allele, which was rare in sarcoidosis patients, actually increases the IFN-γ production. This either means IFN-γ is decreased in sarcoidosis uveitis or it means that this association noted may be false positive (small sample size) or due to another SNP yet undetected that is in linkage with *+874* SNP. The second explanation is likely as the role of IFN-γ in sarcoidosis is quite significant. Studies have shown that IFN-γ is spontaneously released by activated alveolar macrophages in active sarcoidosis, and levels of IFN-γ are higher in sarcoidosis and correlate with disease activity (Robinson, McLemore, and Crystal 1985) (Moller 1999) (Tsiligianni et al. 2005) (Kopinski et al. 2007)

This will be consistent with my finding that *IFNGR -56T*, which increases expression of *IFNGR* is associated with poor outcome. There are not many

studies in medical literature about role of *IFNGR* SNPs in sarcoidosis and none have noted any significant association (Akahoshi et al. 2004)

5.4.6 TNF SNPs

During the inflammatory process, TNF orchestrates the initiation of further leukocytic infiltration via adhesion molecule upregulation, dendritic cell maturation and survival, macrophage activation, and driving Th1 T cell responses within tissues in experimental autoimmune uveitis.(Dick et al. 2004) Increased TNF-α expression in inflammatory cell infiltrates has been seen in experimental autoimmune uveitis near peak inflammation as well. (Okada et al. 1998) These studies denote that TNF plays role in propagation of uveitis rather than induction of uveitis. None of the SNPs studied in my patients showed any association with genetic susceptibility to PSU. This is consistent with study where authors did not find significant differences in the allelic frequencies of *TNF* gene between controls and PSU (Atan et al. 2010). On the other hand studies have noted the role of *TNF* alleles in genetic predisposition of anterior uveitis particularly HLA-B27 negative iritis, (El-Shabrawi et al. 2006) (Kuo et al. 2005)

In my study, patients with *-238 G* allele (high producer allele) were noted to have more episodes of CMO. This was mainly seen in intermediate and posterior uveitis group and to some extent in Sarcoid uveitis group as well. It must be noted that patients with *G* allele were not prone to develop CMO compared to *A* allele carriers, however once CMO was there it was more common and more prolonged in patients with *G* allele carriers. This shows that *TNF-α* allele does not increase the risk of CMO, but if CMO is there it leads to more severe and more frequent episodes. In fact anti TNF therapy has been used successfully to control CMO in uveitis and other collagen vascular

diseases. (Fukuda et al. 2008) (Kachi et al. 2010) (Markomichelakis et al. 2011) (Markomichelakis et al. 2004)

Moderate effect of *TNF* haplotypes was noted particularly *TNFH-2* which is defined by *C/T* SNP at *-857*. More patients with haplotype *2* needed second line of immunosuppression compared to rest of the haplotype. This effect was seen in all four groups to certain extent but only reached significance when the groups were combined together. The *-857 T* allele has already been noted to predispose to idiopathic anterior uveitis (Kuo et al. 2005).

Since the *TNF* genes lie in cross proximity to *HLA* gene complex, it is quite possible that associations seen between anterior uveitis and *TNF* SNPs may be simply due to linkage between *HLA* and *TNF* gene complexes.

5.5 Summary and Conclusion

The study showed that few functional SNPs in Th1 mediated cytokine pathways could increase the genetic susceptibility and can affect the prognosis of posterior segment uveitis.

Following were the notable findings:

Table 5-33: Summary of associations of cytokine SNPs in PSU

SNP & Allele	Effect of Allele on gene	Association	Disease Group
IL-18-607A & 137C	**Both decrease expression**	**Genetic susceptibility**	**Retinal vasculitis**
IL-10 -592A	***A* decreases expression**	**Genetic susceptibility**	**Behcet's**
IL-1β -511C	***C* decreases expression**	Secondary glaucoma	IU & PU
IL-6 -174C	***C* decrease expression**	Poor response to treatment	IU & PU
IL-10 1082A & 592A	***A* decrease expression**	Poor visual outcome	IU & PU
IL-18 137C & 607A	**Both decrease expression**	Better visual outcome	Behcet's
TNF- 238A	***A* decrease expression**	Less CMO	IU & PU
IFNR -56T	***T* increases expression**	Poor visual outcome	Sarcoidosis

The study also highlighted that intermediate and posterior uveitis shared same immunopathology as the gene pathway involves were more as less similar. On the other hand the SNPs associated with Behcet's and sarcoidosis were different from each other and from idiopathic uveitis group. The study has confirmed the association of IL10 gene with visual outcome in PSU and genetic predisposition to Behcet's uveitis. The study has shown that variations in *IL-18* gene can predispose to retinal vasculitis and *IL-1β* gene plays a role in the pathogenesis of secondary glaucoma. Further studies in different and bigger populations are needed to confirm these findings.

Chapter six

6 Conclusion and Future Directions

This study set out to test the hypothesis that genetic variants in cytokine and chemokine genes may affect the phenotype of PSU. Following significant associations were noted between the genotypes and clinical characteristics of the diseases:

1) SNPs in *CCR2 & IL-10* genes, increased genetic susceptibility to ocular Behcet's.
2) SNP in *MCP-1* gene was associated with increased risk of developing PSU.
3) SNPs in *IL-18* gene increased genetic susceptibility to vasculitis.
4) SNPs in *IL-10* gene were predictor of visual outcome in IU & PU.
5) *IL-1β* SNP was associated with risk of secondary glaucoma in IU & PU.
6) *IL-6* SNP was associated with visual outcome in IU & PU.
7) SNPs in *CCR5* gene were predictor of visual outcome in IU & PU.

6.1 Verification of recent association studies in PSU

This research has confirmed some of the recent findings in PSU immunogenetics. This is quite significant as PSU is a rare disease and it is very difficult to recruit enough number of patients for valid genetic studies. This is crucial as this decreases the power of the study. Repeating the experiments in different population and reproducing the results increase the power of the hypothesis. My work has verified following recent findings

a) I found that allelic variations in *IL-10* gene were associated with poor visual outcome in the four sub groups of PSU. This is confirmation of the findings that polymorphisms in *IL-10* gene can be the predictor of visual outcome in intermediate uveitis (Stanford et al. 2005) and sympathetic uveitis

(Atan et al. 2005). Although my sample population did not contain sympathetic uveitis patients (a type of PSU) but my study confirmed this effect of *IL-10* genotypes in intermediate, idiopathic posterior and PSU secondary to sarcoidosis and Behcet's.

b) I also showed that at position *-592* of *IL-10* gene the wild type allele *C* was less prevalent in Behcet's disease compared to the rest of the groups and the controls. (Where the frequencies were comparable) Recently, genome wide association study on 1215 patients with BD noted 5 SNPs in *IL-10* gene strongly associated with BD, with one SNP, *rs1518111* very significant. (Remmers et al. 2010) Recent studies on Turkish Behcet's patients (Dilek et al. 2009) and Middle Eastern patients (Wallace et al. 2007) have also confirmed that *IL-10* genotypes can be a genetic risk for the development of Behcet's disease.

6.2 Short falls of the study

As discussed earlier, an important issue in genetic studies is the sample size of the studied population. The bigger the population sample is, the more powerful the study is. Indeed, one of the main weaknesses of genetic epidemiology studies based on candidate gene approaches has been the lack of replication. This was particularly the case for Sarcoidosis and Behcet's groups in my study. Small sample size of OB and Sarcoid uveitis not only decreased the power of the study, but it lead to false negative results particularly in sarcoidosis group. Many a times associations were noted with good to moderate odd ratios, but because of the number of samples the confidence intervals were very wide and p value higher than significant.

There have been many studies exploring a previously published statistically significant finding for a genetic variant, which failed to reproduce those findings, suggesting a large number of false-positive reports (Ioannidis et al. 2001). The utilization of large validation studies as well as meta-analyses and pooled analyses to combine both statistically significant and non-significant results from individual studies has allowed for the validation of several candidate genes. Therefore, sufficient information of raw data should be published to facilitate future meta-analysis (Hirschhorn et al. 2002). However, results from different centres and studies might differ significantly because of genuine heterogeneity or publication bias, i.e. publication of only positive results (Ioannidis et al. 2003). Despite that, meta-analysis is widely accepted as the highest level of evidence in association studies and helps to minimise random error and identify publication bias (Ioannidis et al. 2006)

Another way to increase the reproducibility is to increase the power of the study. This can be achieved by multi centre studies and this is particularly important with rare diseases. During this research I faced the problem of recruiting patients with ocular Behcet's disease that is very rare in the UK unlike in Turkey and Far East. Studies involving rare diseases should be multicentre and in the context of posterior segment uveitis multi-centre study have been done in UK (Atan et al. 2005). Here the authors managed to collect 26 patients from the whole of the UK with sympathetic uveitis. Multicentre approach or repeating these experiments in different population set are needed if we want to replicate the findings of Behcet's disease in this thesis.

6.3 Future Directions

My work has explored the role of cytokine and chemokine genes in the pathogenesis of uveitis. The study has noted some novel associations that need to be confirmed

***IL-1β* glaucoma:** The role of *IL-1β* gene in secondary glaucoma should be further elucidated. There are quite a few studies that have noted an association between *IL-1β* gene and primary open angle glaucoma (Wang et al. 2006) (Lin et al. 2003) (Mookherjee et al. 2010). The study should be repeated in different population of PSU and also this association should be tested in patients with anterior uveitis, to determine whether secondary glaucoma is the most common reason for loss of sight.

***IL-18* vasculitis:** Another significant finding was the association between *IL-18* gene and the development of retinal vasculitis. This association was seen in isolated retinal vasculitis and vasculitis secondary to Behcet's and sarcoidosis. Since the sample size was relatively small it should be repeated in a bigger population sample. Further more the functional affects of *IL-18* SNPs are modest, and there is a need to study further SNPs in *IL-18* gene, to find a definite functional SNP or study the functional affects of these putative SNPs.

***CCR2 CCR5* and Visual outcome:** The role of *CCR5* in visual outcome should be further assessed. The tag-SNPs and whole gene typing can be carried out to look for the loci.

***CCR2* and Behcet's:** Although there are no reports in the literature about association between *CCR2* genotype and Behcet's uveitis but the role needs to be further elucidated, particularly the role of *CCR2* in the development of uveitis in female patients with Behcet's disease.

The work in this thesis has further increase our insight into the pathogenesis and role of cytokine and chemokine genes in posterior segment uveitis. This work can be used in the following three domains of genetic studies

6.3.1 Gene function identification (physiological genomics)

Our current knowledge on gene functions is mainly based on highly specialized model organism-based experiments such as knockout, transgenic and subsequent homology studies. These study designs can easily shed light on the physiologic role of a gene, but give little insight into human pathogenic processes for which interventions could be designed effectively. The fact is that most common chronic human diseases are due to naturally occurring variation in genes (possibly at multiple sites or within multiple genes) as opposed to complete or partial gene deletions. Thus, the gross interruption of a genome, as in a knockout experiment, may give erroneous impressions about not only the role of that gene in more natural settings but also about its potential as a therapeutic target. In addition, a host of redundancy, feedback and compensatory mechanisms present in physiologic systems has been shown to complicate the generalizability of current functional genomic study design results for this reason. (Schork, Fallin, and Lanchbury 2000) Thus, knockout, transgenic and related experiments are simply not sufficient to provide an adequate and comprehensive picture of human disease processes that could lead to the design of effective therapies and pharmacologically based prevention strategies. Alternate methods are therefore needed and results from studying the functional SNPs here can play an important role. This work has shown the role of *CCR2* and *MCP-1* in posterior segment uveitis. Similarly

significant association were noted between retinal vasculitis and *IL-18* genes. On the contrary experiments on knot out mice have failed to notice this association. Ultimately, the combination of SNP-based genetic technologies with genomic technologies (such as sequence structure analysis, expression profiling and protein level assays, etc., which assess molecular physiology and pathology) is the real future of medical and pharmaceutical research, rather than either one in isolation. (Schork, Fallin, and Lanchbury 2000) Knowledge gained can be used to develop drugs that specifically target against the cytokine molecules. There has been case reports of treatment refractory uveitis with anti IL-6 antibodies (Hirano et al. 2011) and IL-1R antagonist anakinra (Teoh et al. 2007). However, mixed results have been seen with phase 1 and 2 trials using anti CCR2 and anti CCR5 antibodies to control rheumatoid arthritis. (Vergunst et al. 2008) (van Kuijk et al. 2010)

6.3.2 Diagnostics/risk profiling

One of the most promising roles of SNP association studies is the potential to use the information to develop diagnostic or prognostic tools. In fact some of the HLA genotypes have been used to help with the diagnosis particularly HLA-A29 in Birdshort choroiditis. Although my research showed that alleles in *CCR2 V64I* and *IL-10 -592* acted as genetic marker for Behcet's disease, but the power of the study was to low to generalize these findings. On the other hand quite a few of the SNPs particularly in *IL-10*, *IL-6* and *CCR5* genes were a predictor of poor visual outcome and prognosis. My work not only reproduced previous findings but the associations were stronger as well. This research will play a stepping stone role in the future risk profiling studies in uveitis. However SNP-based risk assessment tool for

uveitis will require well designed large-scale epidemiological studies. This is because PSU is influenced by a number of genetic and non-genetic factors, each of which may make only a small contribution to disease risk in the population at large. Prediction of genetic risk will be useful in diagnosis, treatment, prognosis and prevention strategies, as family history is currently, but with greater power. (Schork, Fallin, and Lanchbury 2000)

6.3.3 Prediction of response to drugs (pharmacogenomics)

SNP association studies can help stratifying populations for the purposes of improving the effectiveness of interventions of one sort or another. This research demonstrated that *IL-6 174* SNP and *CCR5* 59029 SNP were influencing the response to steroids in idiopathic intermediate and posterior uveitis. This work can be used in future pharmacogenomics studies in uveitis. However, if a gene that influences responsiveness to a particular compound has been identified then one will need to assess the frequency or penetrance of that gene in other populations and in light of other factors that may influence response to the compound. This is particularly important to find the responsiveness in population with heterozygous alleles.

6.4 Future of Genetic Studies in PSU

6.4.1 In Silico analysis of SNP functions

Since the start of the human genome project, a great deal of effort has been directed towards finding disease-causing SNPs. However, the functional aspects of most of these SNPs remain unknown. Functional analysis for these markers is becoming much more of an interest. As there is a vast number of SNPs, it might not be feasible for researchers to carry out wet laboratory experiments on every SNP to determine their biological significance. Thus, bioinformatics tools can be used to first screen for potentially functional SNPs. before further investigations be carried out using wet laboratory techniques. This translates into reduced costs and saved time. If prediction scores were available, quantitative ranking of functionally significant SNPs could be done to further prioritize SNPs

For analysis different publicly available computational algorithms are available, such as:

1) Sorting Intolerant From Tolerant (SIFT) (Ng and Henikoff 2003),
2) Polymorphism Phenotyping (PolyPhen) (Sunyaev, Ramensky, and Bork 2000), and
3) Function Analysis and selection tool for single nucleotide polymorphisms (FASTSNP) t. (Yuan et al. 2006).

The SIFT algorithm predicts whether an amino acid substitution affects protein function based on sequence homology among related genes and domains over evolutionary time, and the physical-chemical properties of the amino acid residues (Ng and Henikoff 2006). Sequence conservation and the nature of the amino acid residues involved are also incorporated by PolyPhen, but it also

values the location of the substitution within known structures and structural features of the protein available in the annotated database SwissProt (Sunyaev, Lathe, III, and Bork 2001) [5, 27]. By accessing a variety of heterogeneous biological databases and analytical tools, FASTSNP is able to identify SNPs most likely to have functional effects, such as changes to the transcriptional level and pre-mRNA splicing (Yuan et al. 2006). These tools can be used to study the functional affects of SNPs in this thesis where roles were either not clear or in contradiction with the findings. In particular *CCR2 V64I*, *IL-18 607 & 137*, *MCP-1* -2076.

6.4.2 High Throughput Genotyping

As complex disorders, many other genes in the innate immune system and co-stimulatory pathways are involved in the pathogenesis of PSU. The ultimate aims of the genetic studies in uveitis are to identify all the genes involved so that a diagnostic and prognostic profile can be developed. In the candidate-gene approach with manual genotyping a few polymorphisms in a selected number of genes can be studied. A high throughput genotyping method and a whole genome approach are required for a comprehensive genetic analysis.

Another important development during the course of this thesis is the completion of the International HapMap Project by The United States National Human Genome Research Institute (http://www.hapmap.org). Genotypic data on ~4 million different SNPs for three major continental groups are available from the HapMap Project, with more coming. The HapMap data can be used to select Tag-SNPs.

The SSP-PCR technique, which was used in this study, has been proven robust and cost effective. However, the need for manual gel running and genotype reading reduces the productivity of this technique. In the later part of

my research I used TaqMan® 5'Nuclease Activity Assay. After mixing the sample and the reaction components, the assay is run in a closed compartment with no post PCR processing step. Results are obtained by simply measuring the fluorescence of the completed reaction. By eliminating post PCR processing, allelic discrimination with fluorogenic probes reduces the time of analysis, labour, supply cost of post-PCR steps, the risk of cross-over contamination and minimizes the source of error. TaqMan® is however a singleplex reaction: one tube, one SNP. It can be multiplexed to 3 or 4 maximum SNPs per reaction with additional fluorescent colours. Recent advances in high through put genotyping has made it possible to study multiple SNPs in one reaction

The SNaPshot Multiplex System® (Applied Biosystems) is a primer extension-based method using capillary electrophoresis. It allows multiplexing up to 10 SNPs starting from as little as 3 ng DNA per sample. (Edenberg and Liu 2009) The SNPlex® Genotyping System from Applied Biosystems (De, V et al. 2005) uses the oligonucleotide ligation assay to discriminate SNPs, followed by polymerase chain reaction and capillary electrophoresis. It allows genotyping of up to 48 SNPs at a time. The LightTyper® system (Roche Applied Science) uses melting curve analysis to discriminate individual SNPs. These methods use fluorescently labelled oligonucleotides for detection of the SNPs (Bennett et al. 2003).

These and similar genotyping techniques are good for testing candidate genes and small regions. However, the basis of using haplotype tag-SNP analysis in complex traits assumes that the disease causative SNP is a common SNP. This is the basis of Common Disease/Common Variant (CD/CV) hypothesis. This hypothesis proposes that genetic risk for common diseases will often be

due to high frequency variants (Zondervan and Cardon 2004). If the complex diseases were due to large numbers of rare variants at many loci, haplotype tag-SNP strategy might fail because no single haplotype would harbour these individual variants (Suh and Vijg 2005). On the other hand, Hirschhorn and Daly suggested that “most rare alleles with frequencies <5% are likely to have arisen relatively recently, so there will have been less time for recombination and mutation to disrupt the haplotype on which they arose. Therefore, rare variants are expected to be on single, long haplotypes, as has been observed” (Hirschhorn and Daly 2005).

Even samples that are large in terms of our ability to do sophisticated and reproducible phenotyping (1000-2000 cases and controls) are proving to be underpowered in studies of complex traits in which the contribution of any one genetic variant is small. Small samples can, however, be very useful in replicating results in candidate gene approach. In this case, the prior probability is much higher and the likelihood of confirming a finding is increased. It is however important to cover a larger fraction of the variation by genotyping multiple SNPs chosen based on LD in addition to hypothesised functional SNPs, although cost and time (and concerns about multiple testing) can preclude full coverage. It is useful first to visualize the LD structure in the region of interest, using Haploview (Barrett 2009) (which can be performed within the HapMap website). A program such as Tagger (de Bakker et al. 2005) can aid in the selection of SNPs; it can be run from a server (through HapMap or directly at http://www.broad.mit.edu/mpg/tagger). Parameters such as minimum allele frequency (MAF) of SNPs to be "tagged" and the degree of correlation (r2) to be accepted as adequate can be adjusted so that a set of SNPs reasonable for sample size, technology, and budget can be selected.

6.4.3 Genome-wide Association Studies

An alternative to candidate gene approach is a genome wide association study. The number of SNPs per array has dramatically increased in the past few years, from ~10,000 to 100,000; 300,000; 600,000; and now >1 million. (Edenberg and Liu 2009) The Affymetrix Genome Wide Human SNP® Array 6.0 has more than 906,600 SNPs, and the Illumina Human1M-Duo Bead-Chip® assesses >1.1 million loci per sample.

As the density of SNPs on arrays has increased and the costs have gone down, microarrays are increasingly used for GWAS on individual samples. This approach is much more powerful and allows analysis of multiple phenotypes and endophenotypes at once, as well as analysis of quantitative traits. This approach has been successful in studies of macular degeneration, and type 2 diabetes (Klein et al. 2005) (Zeggini et al. 2008).

GWAS must be followed up. One can either attempt replication of the leading SNPs in another population, using candidate gene approach or perform follow-up GWAS on another population.

6.4.4 Next Generation genome sequencing

SNPs can, obviously, be detected by direct sequencing at high accuracy. This is the primary method of SNP discovery, and it demands the sequencing of a sufficient number of individuals at an accuracy and coverage that distinguishes real SNPs from sequencing artefacts. Next-generation sequencing technology is capable of sequencing from hundreds of thousands to hundreds of millions of DNA (or cDNA) fragments in a single instrument run in a massively parallel fashion. So far, three major platforms are commercially available: the Roche GS FLX Sequencer®, the Illumina Genome Analyzer (Solexa®), and the

Applied Biosystems SOLiD sequencer (SOLiD 3 System®). However, sequencing is not at this time an efficient way to genotype SNPs, although as sequencing technology progresses toward the much sought "$1000 genome", it will become the method of choice and will replace GWAS. (Edenberg and Liu 2009)

Despite exhaustive efforts, progress in gene mapping of posterior segment uveitis has been very slow. In the last 15 or so years, our knowledge of the association of HLA alleles in PSU has improved, but the exact mechanism involved remains elusive. Results showed in this thesis indicate that in addition to the HLA, cytokine and chemokine molecules also play an important role in the pathogenesis of these disorders. In the coming few years, accessory molecules, i.e. co-stimulatory molecules, innate immunity genes and trafficking molecules, are likely to play an even more important role and the acceleration in disease gene discovery will shed new light on the biological pathways involved in the pathogenesis of posterior segment uveitis.

7 Appendix A: Reagents Compositions

7.1 DNA Extraction

7.1.1 Salt Extraction Method

7.1.1.1 a) Red Cell Lysis Buffer (RCLB)

(144 mM NH_4Cl, 1 mM $NaHCO_3$)
7.7 g Ammonium chloride (Sigma, UK)

10 mL 100 mM (4.2 g 50 mL of H_20) $NaHCO_3$ (Sigma, UK)

Made up to one litre with distilled H_2O, autoclaved and stored at room temperature

7.1.1.2 b) Nuclear Lysis Buffer (NLB)

(10 mM Tris-HCl pH 8.2, 400 mM NaCl, 2 mM Na_2EDTA pH 8.0)
10 mL 1M Tris-HCl (Sigma, UK)

80 mL 5M NaCl (BDH, UK)

4 mL 0.5M N_2EDTA (Sigma, UK)

Made up to one litre with distilled H_2O, autoclaved and stored at room temperature

7.1.1.3 c) 5M Sodium Chloride Solution

292.2 g NaCl in one litre distilled H_2O

7.1.1.4 d) Chloroform/Iso-Amyl alcohol (24:1)

480 mL Chloroform (BDH, UK)

20 mL Iso-Amyl alcohol (BDH, UK)

Stored wrapped in aluminium foil at room temperature

7.1.1.5 e) Other Solutions

Molecular biology grade absolute ethanol (BDH, UK)

Sterile H_2O (Baxter, UK)

7.1.1.6 f) Other Equipment

Sorvall Refrigerated Centrifuge (Du Pont, UK)

15 mL and 50 mL Falcon tubes (Becton Dickson, USA)

7.1.2 QIAGEN® kit DNA Extraction:

7.1.2.1 QIAGEN Protease

Lyophilized powder (20 mg/ml)

Activity: 45 mAU/mg protein One mAU is the activity that releases folin-positive amino acids and peptides corresponding to 1 µmol tyrosine per minute

7.1.2.2 Buffer AL (QIAGEN®)

Red cell lysis buffer, proprietary formulation but contains Guanidium Chloride (25-50%)

7.1.2.3 Buffer AE (QIAGEN®)

10 mM Tris-Cl

0.5 mM EDTA; pH 9.0.

7.1.2.4 Buffer AW1 (QIAGEN®)

Proprietary formulation but main ingredient is Guanidinium Chloride 50-100%.

7.1.2.5 Buffer AW2 (QIAGEN®)

Proprietary formulation but main ingredient is essentially 70% Ethanol

7.1.2.6 Other Equipment

50 mL centrifuge tube

QIAamp Maxi column

7.2 Pico-Green DNA Quantitation

7.2.1 Reagents Supplied in Kit

- 20 x TE solution

- 200 mM Tris-HCl
- 20 mM EDTA (pH 7.5)
- Pico-Green solution in Dimethyl Sulphoxide (DMSO)
- Distilled H_2O

7.2.2 Other Equipment

- Fluorometer (Lambda Fluro 320 plus - MWG Biotech)
- Fluorometer software (KC4 - supplied as standard)

7.3 PCR Amplification

7.3.1 SSP-PCR

7.3.1.1 a) PCR Reaction Reagents

- 67 mM Tris-Base pH 8.8. (Bioline, UK)
- 16.6 mM Ammonium sulphate (Bioline, UK)
- 2 mM $MgCl_2$ (Bioline , UK)
- 0.01% v/v Tween 20 (Bioline, UK)
- 0.2 mM each of dATP, dATP, dGTP, dCTP (Bioline, UK)
- Taq polymerase (Bioline, UK)
- Mineral Oil (Sigma, UK)
- Distilled H_2O (Baxter, UK)

7.3.1.2 b) Cresol Red Dye

62.5 mg/L Cresol Red dye was made by mixing 0.625 mg in 100 mL dH_2O. This was used at 10 µl/mL in working primer stock solutions

7.3.1.3 c) Other Equipment

- MJ PTC-200 Thermal Cyclers (MJ Technologies, USA)
- Thermowell plate sealers (Costar, Netherlands)

- Thermowell 96 and 192 well plates (Costar, Netherlands)

7.3.2 TaqMan® SNP allele discrimination Assay

7.3.2.1 TaqMan® Universal PCR Master Mix:

Contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, (uridine, uracil-N-glycosylase) dNTPs with dUTP, Passive Reference dye ROX, and optimized buffer Components (proprietary formulation).

7.3.2.2 TaqMan® Assay Mix (labelled probes and un labelled primers)

or

7.3.2.3 Custom made primers and probes

7.3.2.4 Other Materials

Distilled H2O (Baxter, UK)

Optical 384-Well Reaction Plate

Optical Adhesive Films

7.3.2.5 Other Equipment:

The ABI PRISM 7900HT Sequence Detection System

7.4 Agarose Gel Electrophoresis

a) Orange G Loading Dye

- Per 250 mL of Orange G loading dye
- 250 mL Glycerol (Sigma, UK)
- 100 mL 0.1 M EDTA (Sigma, UK)
- 60 mL 1% Orange G (Sigma, UK)
- 90 mL 1M Tris-HCl (pH 7.5)

b) Ethidium Bromide (EtBr)

10mg/mL

200 mg EtBr were dissolved in 20 mL dH_2O

c) 2% Agarose Gels

- 10 grams Molecular biology grade agarose
- 500 mL 0.5 x TBE solution
- 0.5 x TBE solution:
- 89 mM Tris base (ICN Biochemicals, USA)
- 89 mM Boric Acid (BDH, UK)
- 2 mM EDTA (pH 8.8) (Sigma UK)
- 5 μl 0.5 mg/mL Ethidium Bromide per 500 mL gel (Sigma, UK)

d) Other Equipment

- 0.5 X TBE buffer (as above)
- Horizontal gel electrophoresis tanks (Sigma, UK)
- Electrophoresis power units (Pharmacia, UK)
- Polaroid film (Sigma, UK)

8 Appendix B

Table 8-1: Classification and Nomenclature for chemokines and receptors

Systematic name	Human chromosome	Human ligand	Mouse ligand	Chemokine receptor(s)
CXC chemokine/receptor family				
CXCL1	4q21.1	GROα/MGSA-α	GRO/MIP-2/KC?	CXCR2 > CXCR1
CXCL2	4q21.1	GROβ/MGSA-β	GRO/MIP-2/KC?	CXCR2
CXCL3	4q21.1	GROγ/MGSA-γ	GRO/MIP-2/KC?	CXCR2
CXCL4	4q21.1	PF4	PF4	Unknown
CXCL5	4q21.1	ENA-78	GCP-2/LIX?	CXCR2
CXCL6	4q21.1	GCP-2	GCP-2/LIX?	CXCR1, CXCR2
CXCL7	4q21.1	NAP-2	Unknown	CXCR2
CXCL8	4q21.1	IL-8	Unknown	CXCR1, CXCR2
CXCL9	4q21.1	Mig	Mig	CXCR3[a]
CXCL10	4q21.1	IP-10	IP-10/CRG-2	CXCR3[a]
CXCL11	4q21.1	I-TAC	I-TAC	CXCR3[a]
CXCL12	10q11.21	SDF-1 α/β	SDF-1/PBSF	CXCR4[b]
CXCL13	4q21.1	BCA-1	BLC	CXCR5
CXCL14	5q31.1	BRAK/bolekine	BRAK	Unknown
(CXCL15)		Unknown	Lungkine/WECHE	Unknown
CXCL16	17p13			CXCR6
C chemokine/receptor family				
XCL1	1q24.2	Lymphotactin/SCM-1α/ ATAC	Lymphotactin	XCR1
XCL2	1q24.2	SCM-1β	Unknown	XCR1
CX_3C chemokine/receptor family				
CX3CL1	16q13	Fractalkine	Neurotactin/ABCD-3	CX3CR1
CC chemokine/receptor family				
CCL1	17q11.2	I-309	TCA-3/P500	CCR8
CCL2	17q11.2	MCP-1/MCAF/TDCF	JE?	CCR2
CCL3	17q12	MIP-1α/LD78α	MIP-1α	CCR1, CCR5
CCL3L1	17q12	LD78β	Unknown	CCR1, CCR5
CCL4	17q12	MIP-1β	MIP-1β	CCR5[3]
CCL5	17q12	RANTES	RANTES	CCR1, CCR3, CCR5[c]
(CCL6)		Unknown	C10/MRP-1	Unknown
CCL7	17q11.2	MCP-3	MARC?	CCR1, CCR2, CCR3
CCL8	17q11.2	MCP-2	MCP-2?	CCR3, CCR5[c]
(CCL9/10)		Unknown	MRP-2/CCF18/MIP-1γ	CCR1
CCL11	17q11.2	Eotaxin	Eotaxin	CCR3
(CCL12)		Unknown	MCP-5	CCR2
CCL13	17q11.2	MCP-4	Unknown	CCR2, CCR3
CCL14	17q12	HCC-1	Unknown	CCR1, CCR5
CCL15	17q12	HCC-2/Lkn-1/MIP-1	Unknown	CCR1, CCR3
CCL16	17q12	HCC-4/LEC/LCC-1	Unknown	CCR1, CCR2
CCL17	16q13	TARC	TARC/ABCD-2	CCR4
CCL18	17q12	DC-CK1/PARC/AMAC-1	Unknown	Unknown
CCL19	9p13.3	MIP-3β/ELC/exodus-3	MIP-3β/ELC/exodus-3	CCR7[d]
CCL20	2q36.3	MIP-3α/LARC/exodus-1	MIP-3α/LARC/exodus-1	CCR6
CCL21	9p13.3	6Ckine/SLC/exodus-2	6Ckine/SLC/exodus-2/ TCA-4	CCR7[d]
CCL22	16q13	MDC/STCP-1	ABCD-1	CCR4
CCL23	17q12	MPIF-1/CKβ8/CKβ8-1	Unknown	CCR1
CCL24	7q11.23	Eotaxin-2/MPIF-2	MPIF-2	CCR3
CCL25	19p13.3	TECK	TECK	CCR9
CCL26	7q11.23	Eotaxin-3	Unknown	CCR3
CCL27	9p13.3	CTACK/ILC	ALP/CTACK/ILC/ESkine	CCR10
CCL28	5p12	MEC		CCR3/CCR10

Copied from: Chemokine/chemokine receptor nomenclature. IUIS/WHO Subcommittee on Chemokine Nomenclature. Cytokine. 2003 Jan 7;21(1):48-9

CCR2 V64I (*G/A*)	Patients		Control			
GENOTYPE	Count	FREQ	Count	FREQ	Chi2x2	p
GG	61	0.79	247	0.87	3.18	NS
GA	14	0.18	35	0.12	1.74	NS
AA	2	0.03	1	0.00	3.69	NS
Total	77		283			
Allelic Carriage / phenotype						
G	75	0.97	282	1.00	3.69	NS
A	16	0.21	36	0.13	3.18	NS
Allelic Frequency						
G	136	0.88	529	0.93	4.55	0.03286
A	18	0.12	37	0.07		
Total	154		566			

Table 8-2: Genotypic frequency of CCR-2 *V64I* in intermediate uveitis and control

MCP-1 2518 *A/G*	Age less than 40		Age 40 or above		Genotype Differences		
GENOTYPE	Count	FREQ	Count	FREQ			p
AA	57	0.46	55	0.69	Chi2x3=	10.86	0.00116
AG	58	0.46	20	0.25	p=	0.004	0.00208
GG	10	0.08	5	0.06			NS
Total	125		80				
Allelic Carriage							
A	115	0.92	75	0.94			NS
G	68	0.54	25	0.31			0.00116
Allelic Frequency							
A	172	0.69	130	0.81			0.00524
G	78	0.31	30	0.19			
Total	250		160				

Table 8-3: Age of onset of PSU in patients with *MCP-1* -2518 genotypes

Table 8-4: *CCR2 V64I* frequencies in Behcet's patients comparing males versus female.

CCR2 V64I (V=G & I=A)	Males	females		P value
	Count	Count		
GG	18 (82%)	3 (33%)		**0.008761**
GA	3 (14%)	6 (67%)		**0.003151**
AA	1 (4%)	0 (0%)		NS
Total	22	9		
Allelic Carriage / phenotype				
GG/ GA	21 (95%)	9 (100%)		NS
AA/ GA	4 (18%)	6 (67%)		**0.008761**
Allelic Frequency				
G	39 (88%)	12 (67%)		**0.039838**
A	5 (12%)	6 (33%)		
Total	44	18		

Table 8-5: The association of *CCR5 -59029* A allele and need for long term steroids in posterior uveitis

CCR-5 59029 *G/A*	Long term steroids	No long term steroids	P value
GENOTYPE	Count	Count	
AA	11 (37%)	10 (29%)	NS
GA	17 (57%)	15 (45%)	NS
GG	2 (6%)	9 (26%)	0.03
Total	30	34	NS
Allelic Carriage			
A	28 (93%)	25 (74%)	0.03
G	19 (63%)	24 (71%)	
Total			

Table 8-6: Cataract in idiopathic PSU and its relation with *CCR5 -3900* SNP

	Cataract		No Cataract		P value
CCR-5 *3900*	Count	FREQ	Count	FREQ	Chi 2X2
AA	28	0.51	27	0.31	0.0205
AC	26	0.47	46	0.53	NS
CC	1	0.02	13	0.15	0.01001
Total	55		86		
Allelic Carriage / phenotype					
A	54	0.98	73	0.85	0.01001
C	27	0.49	59	0.69	0.0205
Allelic Frequency					
A	82	0.75	100	0.58	0.00497
C	28	0.25	72	0.42	
Total	110		172		

Table 8-7: *CCR5 -2135* C allele and need for steroids in intermediate uveitis

CCR-5 2135 T/C GENOTYPE	steroids Count	No steroids Count	P value
TT	11 (23%)	8 (27%)	NS
TC	13(28%)	18 (60%)	0.004
CC	23 (49%)	4 (13%)	0.001
Total	47	30	NS
Allelic Carriage			
T	24 (51%)	26 (87%)	0.001
C	36 (77%)	22 (73%)	
Total			

Table 8-8: CCR-5 *-1835* SNP and gender in Ocular Behcet's

	Males		Females		Chi 2X3	Chi 2X2
GENOTYPE	Count	FREQ	Count	FREQ	P value	P value
CC	20	91%	4	44%		0.004977
CT	1	5%	5	56%	0.004569	0.001102
TT	1	5%	0	0%		NS
Total	22		9			
Allelic Carriage						
C	21	95%	9	100%		NS
T	2	9%	5	56%		0.004977
Allelic Frequency						
C	41	93%	13	72%		0.025444
T	3	7%	5	28%		
Total	44		18			

Table 8-9: Association of *IL-10* 592 SNP with permanent visual loss in intermediate uveitis

IL-10 -592 C/A	PVL		No PVL		P Value	P value
	N=22	%	N=45	%	Chi2x3	Chi 2X2
CC	10	0.45	33	0.73		0.02541
CA	11	0.50	12	0.27	0.045	NS
AA	1	0.05	0	0.00		NS
Allelic Carriage						
C	21	0.95	45	1.00		NS
A	12	0.55	12	0.27		0.02541
Allelic Frequency						
C	31	0.70	78	0.87		0.02368
A	13	0.30	12	0.13		

9 List of Original Communications

1) Chemokine gene polymorphisms in immune mediated posterior segment uveitis. Ahad MA, Missotten T, Abdalla A, Lympany P and Lightman S. Molecular Vision 2007; 13:388-396

2) Association between Heat Shock Protein 70/Hom genetic polymorphisms and uveitis in patients with sarcoidosis. Spagnolo P, Marshall S, Ahad M, Lightman S, du Bois R, Welsh K. Invest Ophthalmol Vis Sci. 2007 Jul;48(7):3019-25

3) Chemokine gene polymorphisms in idiopathic anterior uveitis. Yeo TK, Ahad MA, Spagnolo P, Lympany P, Lightman S. Cytokine. 2006 Jul;35(1-2):29-35

4) The association of *IL-12* and *IL-18* gene polymorphisms with retinal vasculitis in patients with non-infectious posterior segment uveitis. M. A. Ahad, M. Rose-Zerilli, S. Goverdhan, A. Lotery and S. Lightman. Invest Ophthalmol Vis Sci 2007;48: E-Abstract 5161.

5) Role of CCR–5 Gene Haplotypes in Immune Mediated Posterior Segment Uveitis. Ahad M, Spagnolo P, Lympany P, Lightman S. Invest. Ophthalmol. Vis. Sci. 2006 47: E-Abstract 3272

6) CCRV64I polymorphism as a genetic risk marker of Behcet's related uveitis. Ahad M, Missotten T, Lympany P, Lightman S. Ophthalmic Research 2005; 37:S1 05 Page193

7) Chemokine Genes Polymorphisms in Posterior Non–Infectious Uveitis Ahad M, Missotten T, Abdullah A, Lympany P, du Bois R, Lightman S. Invest. Ophthalmol. Vis. Sci. 2005 46: E-Abstract 3805.

8) *CCL-5* and CCR-5 chemokine gene polymorphism in posterior non-infectious uveitis Ahad M, Missotten T, Lympany P, Lightman S. Ophthalmic Research. 2004; 36: S1 04 Page 37

9) *RANTES* Chemokine promoter gene Polymorphism and need for immunosuppressant agents in posterior uveitis Missotten T, Ahad M, Abdalla A, Lympany P, Lightman S Ophthalmic Research. 2004; 36: S1 04 Page 86

10) CCR-5 Chemokine receptor gene Polymorphism and severe outcome in posterior uveitis Missotten T Ahad M, Abdalla A, Menezo V, Kuo NW, Lympany P, Lightman S. Ophthalmic Research. 2004; 36: S1 04 Page 87

10 Glossary

1. ADMIXTURE. The mixture of two or more genetically distinct populations. This has implications for studies of genotype–disease associations if the component populations have different genotypic distributions.
2. ALLELE. Alternative forms of a gene found at the same locus on a particular chromosome.
3. ALLELE FREQUENCY. The number of particular allele divided by the number of chromosomes in the study population.
4. ASSOCIATION STUDY. A genetic variant is genotyped in a population for which phenotypic information is available (such as disease occurrence, or a range of different trait values). If a correlation is observed between genotype and phenotype, there is an association between the variant and the disease or trait.
5. BAYESIAN. A statistical approach that assesses the probability of a hypothesis being correct (for example, whether an association is valid) by incorporating the prior probability of the hypothesis and the experimental data supporting the hypothesis.
6. BONFERRONI CORRECTION. The simplest correction of individual *P*-values for multiple-hypothesis testing: p corrected = 1 - (1 - p uncorrected)n, where n is the number of hypotheses tested. This formula assumes that the hypotheses are all independent.
7. CANDIDATE GENE. A gene for which there is evidence of its possible role in the pathogenetic mechanism of a disease. It choice can be based on evidence from animal model, genetic mapping studies or knowledge of biological pathways.

8. CARRIER FREQUENCY (PHENOTYPE FREQUENCY). The number of individual carrying a particular allele divided by the total number of individuals studied.
9. DEACETYLATION The modification of histones by removal of acetyl groups. Acetylation of histones allows transcription to occur, and deacetylation inhibits transcription.
10. DISEASE PHENOTYPE. The manifestation of a disease in terms of clinical characteristics, e.g. disease severity, disease subtype, and disease prognosis.
11. EPISTASIS. The interaction between two or more genes to control a single phenotype.
12. FOUNDER POPULATIONS. Those have been derived from a limited pool of individuals within the last 100 or fewer generations.
13. GENE. A region of DNA that encodes a protein.
14. GENOTYPE FREQUENCY. The number of individuals with a particular genotype divided by the total number of individual studied.
15. GENOTYPE. The genetic constitution of an individual, usually at a particular locus.
16. HAPLOTYPE. The combination of alleles found at neighbouring loci on a single chromosome or haploid DNA molecule.
17. HAPLOTYPE PHASING. Alleles in linkage within a chromosomal segment and found to be inherited as a unit.
18. HARDY–WEINBERG EQUILIBRIUM. The binomial distribution of genotypes in a population, such that frequencies of genotypes *AA*, *Aa* and *aa* will be p2, 2pq, and q2, respectively, where p is the frequency of allele A, and q is the frequency of allele a. Hardy–Weinberg equilibrium

applies in a population when there are no factors such as migration or admixture that cause deviations from p2, 2pq and q2.

19. HERITABILITY. The proportion of the variation in a given characteristic or state that can be attributed to (additive) genetic factors.
20. HETEROZYGOUS. The situation in which an individual carries two different alleles at the same locus.
21. HOMOZYGOUS. The situation in which an individual carries the same allele in the same locus.
22. LINKAGE DISEQUILIBRIUM. Correlation between nearby variants such that the alleles at neighbouring markers (observed on the same chromosome) are associated within a population more often than if they were unlinked.
23. LINKAGE MAPPING. Where genes are mapped by typing genetic markers in families to identify regions that are associated with disease or trait values within pedigrees more often than are expected by chance. Such linked regions are more likely to contain a causal genetic variant.
24. MEGABASE. (Mb) Unit of length for DNA fragments equal to 1 million nucleotides and roughly equal to 1 cM.
25. MINOR ALLELE. The less frequent of two alleles at a locus.
26. MULTIPLE-HYPOTHESIS TESTING. More than one hypothesis within an experiment. As a result, the probability of an unusual result from within the entire experiment occurring by chance is higher than the individual p-value associated with that result.
27. ODDS RATIO. A measure of relative risk that is usually estimated from case-control studies.

28. PAIRWISE LINKAGE DISEQUILIBRIUM. (Pair wise LD). The strength of association between alleles at two different markers.
29. PENETRANCE The proportion of individuals with a specific genotype who manifest the genotype at the phenotypic level. For example, if all individuals with a specific disease genotype show the disease phenotype, then the genotype is said to be completely penetrate.
30. POLYMORPHISM. The occurrence in one population of two or more genetically determined forms (alleles), all of which are too frequent to be ascribed to mutation. Generally, a polymorphism is defined as a genetic variation occurring at least in 1% of the population.
31. PROMOTER. Region of DNA that promote transcription
32. QUANTITATIVE TRAIT. traits are affected by more than one gene, and by the environment.
33. TAG SNPs are SNPs that unambiguously define a particular haplotype in a block
34. TRANSCRIPTION. The process of creating a complementary RNA copy of a sequence of DNA
35. TRANSMISSION DISEQUILIBRIUM TEST. (TDT) A family-based test for association that is immune to population stratification. The transmission of alleles from heterozygous parents to affected offspring is compared to the expected 1:1 ratio
36. UNPHASED DATA. Sequence data in which the phase of double heterozygotes was not determined.

11 References

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