

**CTLA4 gene Polymorphisms in Autoimmune  
Hepatitis (AIH): Gene and Clinical Disease  
Correlations in South African Patients**

**By**

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**I dedicate this thesis to my (soon to be) husband, Richard Meldau**

# Abstract

Autoimmune hepatitis (AIH) is a chronic hepatitis characterised by the presence of liver associated autoantibodies, raised serum gamma globulin levels and interface hepatitis on liver histology. There is a strong association with particular human leukocyte antigen (HLA) haplotypes and several other non-HLA genes have been implicated in contributing to disease severity. The cytotoxic T lymphocyte antigen 4 (*CTLA-4*) gene is the best studied non-HLA gene in AIH and other autoimmune diseases. This study investigated those *CTLA-4* gene polymorphisms reported in other parts of the world, including the -318 C/T promoter polymorphism, +49 A/G polymorphism in exon 1 and the CT60 G/A polymorphism in the UTR 3' of the gene, in South African patients with AIH. A cohort of 118 AIH patients from the MRC-UCT Liver Research Centre clinic at Groote Schuur Hospital, Cape Town was studied for this purpose. In addition, a family-based cohort was selected and recruited from 12 AIH patients who had family members with either AIH or any other form of autoimmune disease, to investigate whether there may be any novel, disease-associated sequence variations in the *CTLA4* gene. To investigate a possible role for soluble CTLA4 (sCTLA-4) in the disease progression of AIH, the concentrations of sCTLA-4 during different stages of AIH disease activity were measured in patients, using a commercial ELISA kit.

No new polymorphisms were identified and sCTLA4 concentrations did not correlate with AIH disease activity. The known *CTLA4* gene polymorphisms, -318 C/T, +49 A/G and CT60 G/A, were not significantly associated with AIH in the South African population. At first presentation, females were on average 13.47 years older than their male counterparts. Black African patients were, at first presentation, on average 12.78 years younger than their caucasian counterparts, and had more severe disease, as assessed by biochemical parameters. Furthermore, there was a highly significant difference in the genotypic frequencies at the CT60 locus amongst ethnic groups. Black African patients had the highest frequency of the CT60 G/G genotype, while the CT60 A/A genotype was completely absent from this group. This was not obviously associated with their disease and needs to be further investigated.

# Abbreviations and Symbols

## Abbreviations

ABA	acrylamide-bisacrylamide
AgNO <sub>3</sub>	silver nitrate
AIH	autoimmune hepatitis
Ala	alanine
ALB	albumin
ALC-1	anti-liver cytosol 1
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANA	antinuclear antibody
Anti-LKM	anti-liver, kidney microsomal antibody
APC	antigen-presenting cell
APS	ammonium persulphate
AP-50	chlathrin pit adaptor complex 50
AST	aspartate aminotransferase
Bcl	B cell lymphoma
BLAST	Basic Local Alignment Search
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
CTLA4	cytotoxic T lymphocyte antigen 4
dH <sub>2</sub> O	deionised water
DHPLC	denaturing high performance liquid chromatography
DNA	deoxynucleic acid

DNTP	deoxynucleotide triphosphate
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratories
fICTLA4	full length cytotoxic T lymphocyte antigen 4
g	gram
G	centrifugal force
GTT	gamma glutamyl transpeptidase
h	hour
HBV	hepatitis B virus
HLA	human leukocyte antigen
HRP	horse radish peroxidase
ICOS	inducible T-cell co-stimulator
IDDM	insulin dependent diabetes mellitus
IFN- $\gamma$	interferon-gamma
Ig	immunoglobulin
IL	interleukin
INR	international normalised ratio
iu	international units
Kb	kilo bases
LP	liver-pancreas antigen
MHC	major histocompatibility complex
min	minute
MgCl <sub>2</sub>	magnesium chloride
ml	millilitre

mM	millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	nannograms
NOD	non-obese diabetic
OD	optical density
PAGE	polyacrylamide gel electrophoresis
pANCA	perinuclear antineutrophilic cytoplasmic antibody
PCR	polymerase chain reaction
PS-DVB	polysterene-divinylbenzene
RA	rheumatoid arthritis
RE	restriction enzyme
RNA	ribonucleic acid
s	second
sCTLA4	soluble cytotoxic T lymphocyte antigen 4
SLA	soluble liver antigen
SLE	systemic lupus erythematosus
SMA	smooth muscle antibody
SMS	Sequence Manipulation Suite
SNP	single nucleotide polymorphism
SSCP	single-strand conformational polymorphism
TBE	tris borate EDTA
Tc cells	cytotoxic T cells
TCR	T cell receptor

TEAA	triethylammonium acetate
Th cells	T helper cells
Thr	threonine
T <sub>m</sub>	melting temperature
TMB	tetramethyl-benzidine
TNF $\alpha$	tumour necrosis factor $\alpha$
Treg	regulatory T
UTR	untranslated region
V	volt
w/v	weight per volume

### **Symbols**

A	Adenine
T	Thymine
C	Cytocine
G	Guanine
q	long arm of a chromosome
$\mu$ l	microlitre
$\mu$ M	micromolar
$\alpha$	alpha
$\beta$	beta
%	percentage
$^{\circ}$ C	degrees celsius

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# Chapter 1

## The Immune System and Autoimmunity: Review

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### 1.1 Immune System

In order to protect itself against the disease causing effects of pathogens, the human body has developed a complex immune system for foreign antigen detection and elimination. The immune system is a highly integrated and effective biological system that determines the nature of attack on the body and initiates the appropriate response. The first line of defence against pathogens is the protective layer of skin and mucous membranes which constitute the ectoderm of the body. If a pathogen manages to breach these surfaces, the body has a need for more advanced defences against such infection. There are two types of immune responses: the innate or natural response, and the acquired or adaptive response (Delves and Roitt 2000a).

#### 1.1.1 Innate (Natural) Immune System

Innate immune responses are encoded in the host's germ-line genes and are characterised by a non-specific response to infection. Such responses are generic and characterised by their inability to change and adapt following repeated activation. Various cells and molecular components form part of the innate system. These cells (detailed below) are genetically programmed to recognise molecular patterns shared by a range of foreign antigens. The innate system constitutes the initial host response as it acts very rapidly upon contact with invading pathogens (Chaplin 2003; Delves and Roitt 2000a).

Phagocytic cells like monocytes, macrophages and neutrophils recognise antigens, such as microorganisms, and engulf them through phagocytosis. Inside these cells, the phagocytosed antigens are exposed to toxic molecules (Delves and Roitt 2000a). Apart from their phagocytic properties, neutrophils also produce reactive oxygen

species that are cytotoxic to bacteria. Monocytes and macrophages, including Langerhans cells, Kupffer cells, microglial cells and certain dendritic cells, have the added ability to present digested fragments of antigen to T cell receptors (TCR) thereby acting as antigen-presenting cells (APC) (Chaplin 2003). This is achieved with the help of class I and II major histocompatibility complex (MHC) molecules expressed on their surfaces.

Cells such as eosinophils, basophils and mast cells can act by releasing molecules such as cationic proteins and superoxides into extracellular fluids, and play a central role in the response to allergens and parasitic infections. These cells also release proinflammatory cytokines which attract leukocytes to sites of inflammation (Abraham and Arock 1998; Delves and Roitt 2000a).

Natural killer cells target immunoglobulin (Ig) G coated cells through antibody-dependent cytotoxicity, and are important in defence against viral infections. They also have the ability to recognise and destroy nucleated cells that lack MHC class I (MHC I) molecules as a result of microbial interference or malignancy (Chaplin 2003; Delves and Roitt 2000a).

### **1.1.2 Acquired (Adaptive) System**

The acquired immune response is thought to have evolved more recently than the innate response. Whereas innate responses remain unchanged, acquired responses adapt and strengthen with every pathogen encounter. It does so through the rearrangement of germline lymphocyte genes (somatic rearrangement) to establish exclusive specificity of lymphocytes to antigens (Chaplin 2003; Delves and Roitt 2000a).

Lymphocytes originate in the foetal liver and bone marrow haematopoietic stem cells. B-lymphocytes reach full maturity in the bone marrow, while T lymphocytes are relocated to the thymus, where they complete their development and undergo negative selection (see section 1.1.3; page 5). Both B and T lymphocytes have

receptors that recognise and bind to small epitopes on the surfaces of antigens, leading to their activation (Delves and Roitt 2000a). As T lymphocyte activation is relevant to this dissertation it is further described in section 1.2; page 7.

Immature B lymphocytes can recognise many different antigens, through receptors on their surface. Once these receptors recognise an antigen, the B cell undergoes rapid proliferation to form a vast number of lymphocytes that are highly specific for that antigen. During this period of proliferation, B cells mutate rapidly and thereby strengthen the specificity of their receptors, increasing the strength of binding to the specific antigens. This process is commonly referred to as clonal selection. The resultant antigen-specific B lymphocytes secrete high numbers of immunoglobulin or antibodies that resemble the B lymphocyte receptors in structure and antigen specificity (Delves and Roitt 2000a; Delves and Roitt 2000b; Edelman 1973; Chaplin 2003).

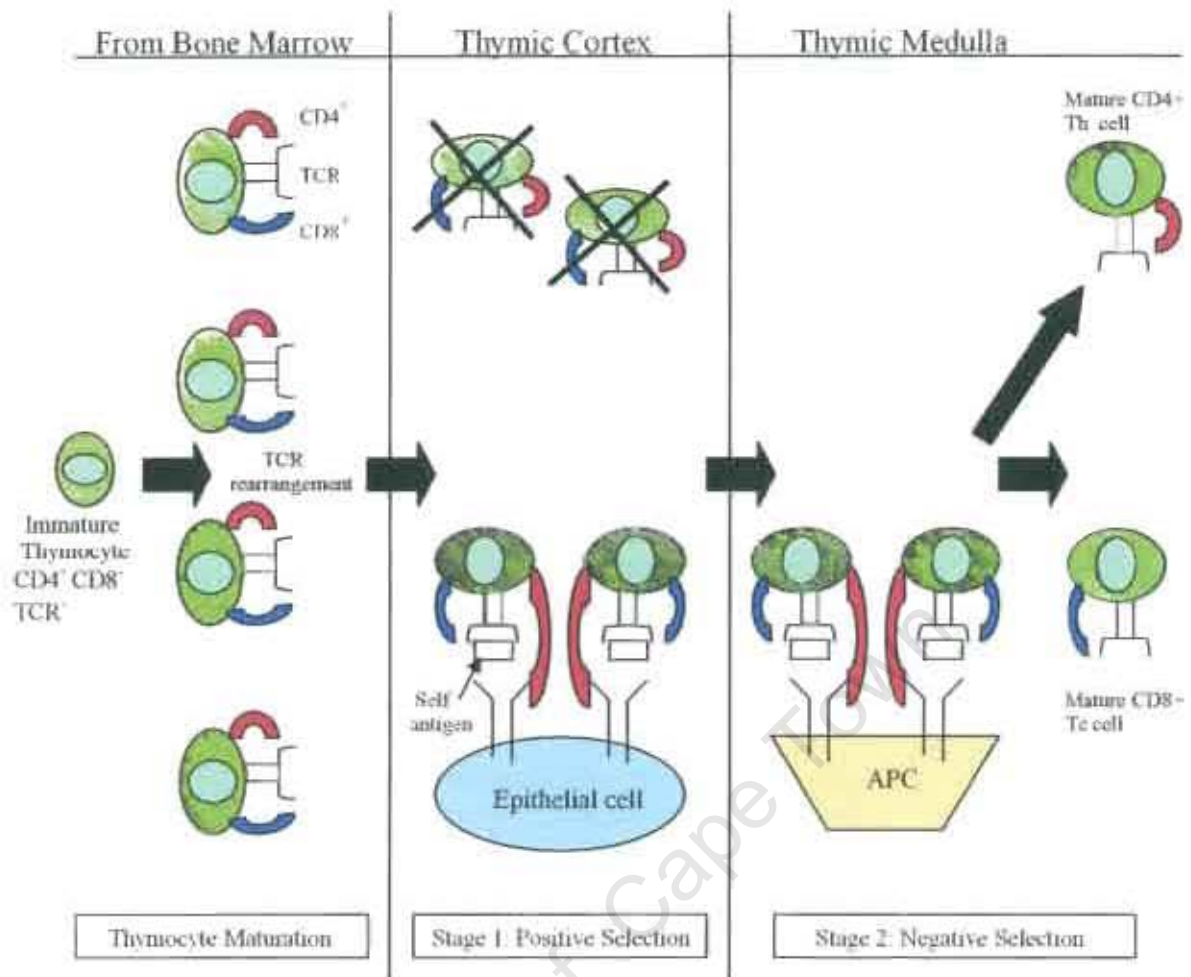
During the proliferation period a distinct group of T and B cells, called memory cells, are formed. These cells remain in the body after the infection has subsided. In the case of a subsequent infection by the same agent, these cells ensure a very rapid secondary response that is highly specific to the antigen (Delves and Roitt 2000a; Chaplin 2003).

### **1.1.3 Immune Tolerance**

The immune system needs a mechanism to distinguish foreign antigens from the body's own proteins, the recognition of self. Tolerance is critical in keeping the acquired immune system unresponsive to self-antigens. B cell tolerance is induced primarily in the bone marrow, while T cell tolerance is induced in one of two places: centrally, in the thymus, or peripherally, in the secondary lymphoid tissues (Abbas et al 2004; Ermann and Fathman 2001; Simmonds and Gough 2005; Delves and Roitt 2000a). A breakdown in central or peripheral tolerance results in immune responses that are directed against self. This may be organ-specific, as in autoimmune hepatitis (AIH) or systemic, leading to multi-systemic autoimmunity, as in systemic lupus

erythematosus (SLE) (Kristiansen et al 2000, Abbas et al 2004; Simmonds and Gough 2005).

Central tolerance occurs mainly in the foetal thymus via two steps (see figure 1.1). The first is when immature thymocytes expressing unique T-cell receptors undergo positive selection, resulting in a protective signal that prevents them from undergoing apoptosis (programmed cell death). This is achieved when cluster CD4 and CD8 positive thymocytes interact with MHC human leukocyte antigen (HLA) class I and II molecules on thymic cortical epithelial cells. In the second step, only surviving thymocytes bearing high affinity receptors for self-MHC molecules interact with APCs and thus undergo apoptosis. Through this process of negative selection, only T cells highly specific for foreign antigens are allowed to mature and be released into the immune system (Simmonds and Gough 2005; Mackay 2000). As most tissue-specific antigens are not present in the thymus or bone marrow, a second mechanism is needed to prevent autoimmunity (Shevach 2000). This is achieved through peripheral tolerance.

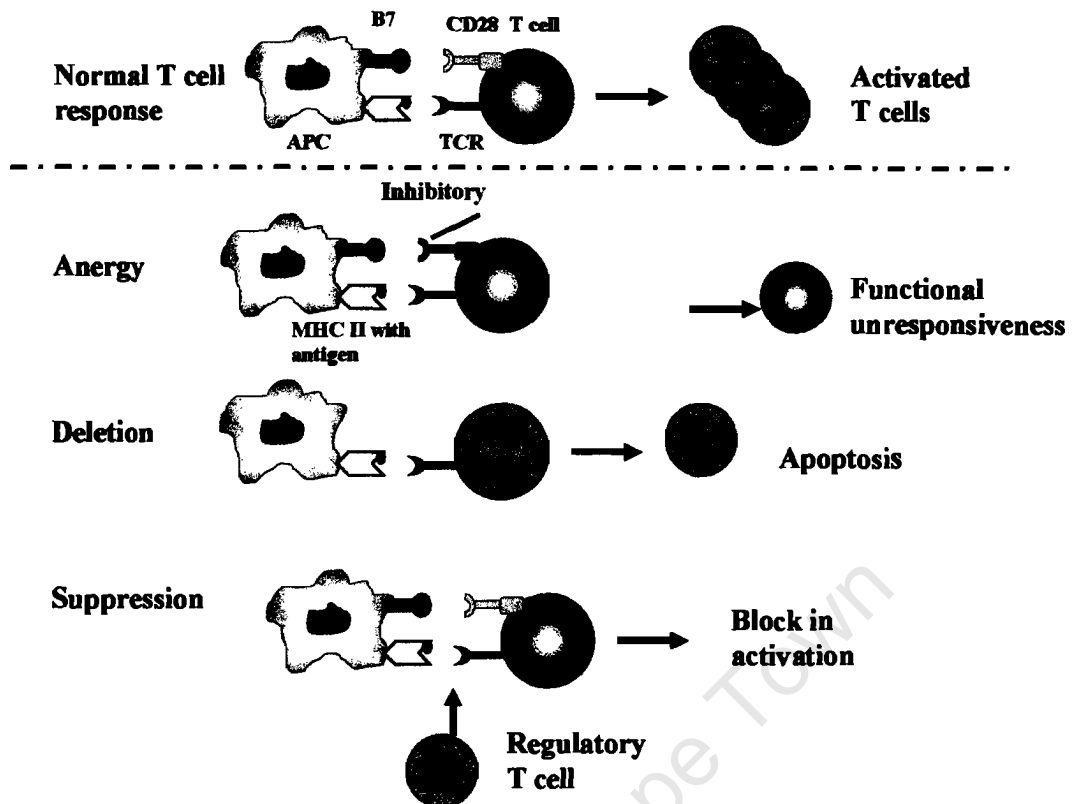


**Figure 1.1** Diagrammatic representation of central tolerance [Adapted from Simmonds and Gough 2005]. Immature,  $CD4^+ CD8^-$  thymocytes originate in the bone marrow and undergo TCR rearrangement to differentiate into double positive ( $CD4^+ CD8^+$ ) cells (thymocyte maturation). The  $CD4^+ CD8^+$  thymocytes then enter the thymus where only  $CD4^+ CD8^+$  thymocytes that interact with MHC-presented antigen on thymic epithelial cells receive a positive survival signal, while those that do not interact are deleted by apoptosis (positive selection). Thymocytes with too strong an association for self-MHC and self-antigens are deleted by apoptosis (negative selection), allowing the remaining thymocytes to mature into  $CD4^+$  T-helper (Th) cells or  $CD8^+$  cytotoxic (Tc) cells.

Peripheral tolerance is maintained through regulatory T cells, anergy (functional unresponsiveness) and apoptosis (see figure 1.3). The primary mechanism is through  $CD4^+ CD25^+$  regulatory T (Treg) cells, together with the co-stimulatory molecules cytotoxic T lymphocyte associated antigen 4 (CTLA4) and CD28. CTLA4 is constitutively expressed on Treg cells (Abbas et al 2004; Simmonds and Gough 2005; Sansom 2000). The role of Treg cells in peripheral tolerance has been demonstrated in various studies. For example, the removal of Treg cells in *nu/nu* mice caused severe autoimmune disease that was prevented when Treg cells from

normal Balb/c mice were given to these animals. The re-introduction of Treg cells restores self tolerance and prevents autoimmune disease (Sakaguchi et al 1985, Sakaguchi et al 1995, Suri-Payer et al 1999; Shevach 2000).

Other interrelated mechanisms of peripheral tolerance include anergy, a process that leaves T cells functionally unresponsive to the self antigens due to the absence of a co-stimulatory signal or suboptimal antigen receptor signalling, and deletion by apoptosis. Apoptosis of lymphocytes could occur through passive or activation-induced mechanisms. Passive cell death is thought to occur due to the loss of expression of antiapoptotic proteins such as B cell lymphoma (Bcl) family members, resulting from a lack of co-stimulation and cytokines. Activation-induced cell death results from feedback mechanisms triggered by lymphocyte activation resulting in the limitation of lymphocyte proliferation and differentiation (Abbas et al 2004; Van Parijs and Abbas 1998). As this activation is a critical part in peripheral tolerance and the prevention of autoimmunity, it is reviewed in detail in the following section.



**Figure 1.2** Mechanisms of peripheral T cell tolerance [Adapted from Abbas et al 2004]. In the normal T cell response, TCRs bind antigen on APCs, followed by the binding of CD28 co-stimulatory molecules to B7 receptors on the same APCs (see section 1.2.1; page 8). Anergy and deletion of T lymphocytes by apoptosis occur in the absence of the CD28-B7 signal. Suppression of activation is effected through Treg cells.

## 1.2 T Cell Activation

The activation of T cells requires two signals, the primary activation signal and a second, co-stimulatory signal. When the immune system encounters a foreign antigen, peptides from the antigen are presented by an APC to an uncommitted helper T cell ( $T_{H0}$ ) with the help of MHC class II molecules. This interaction constitutes the first activation signal of the  $T_{H0}$  cells which then differentiate into either  $T_{H1}$  or  $T_{H2}$  phenotypes. This is dependent on the effect of cytokines (small peptide signalling molecules) in the cellular environment, the nature of the antigen and the affinity of the binding between the TCR and MHC/peptide complex.  $T_{H1}$  cells produce interleukin 2 (IL-2) and interferon-gamma (IFN- $\gamma$ ) which promote cell-mediated immune responses through activation of macrophages and cytotoxic T

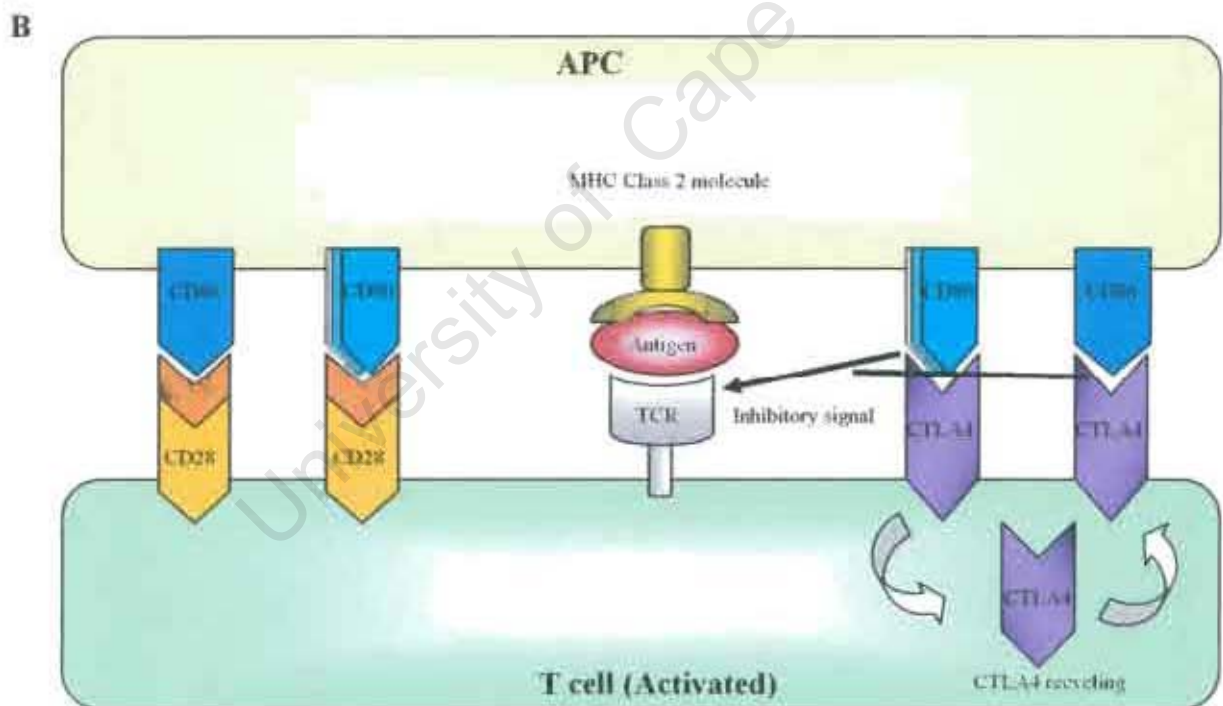
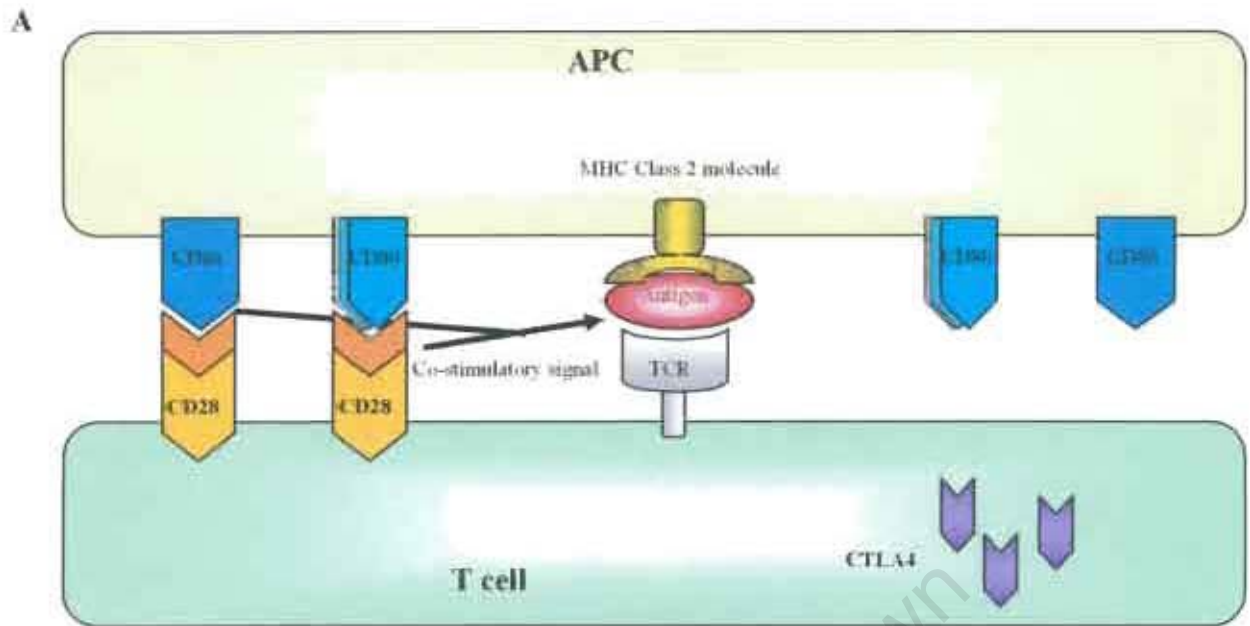
lymphocytes. T<sub>H</sub>2 cells, on the other hand, release IL-4, IL-5 and IL-10 which promote the production of antibodies by B cells (McFarlane and Heneghan 2004).

### **1.2.1 Co-stimulatory Pathway**

CD28, which is a member of the Ig gene superfamily on peripheral T cells, together with its ligands CD80 (B7-1) and CD86 (B7-2) on APCs, provide the second, antigen independent co-stimulatory signal (see figure 1.3) (Boise et al 1995; Chambers 2001; Simmonds and Gough 2005). Absence of this second signal results in the inability of the cells to respond to future activation signals (anergy) or even apoptosis (Boise et al. 1995; Sansom 2000).

Besides lowering the threshold for antigenic stimulation, CD28 co-stimulation also plays a critical role in maintaining T cell growth and proliferation through the upregulation of IL-2, significantly influencing T cell differentiation and augmenting T cell survival (Rulifson et al 1997; Boise et al 1995; Bluestone 1997)

This CD28 T cell co-stimulation is regulated by another member of the Ig gene superfamily, namely CTLA4. CTLA4, unlike CD28, is not found on resting T cells (Purohit et al 2005; Krummel and Allison 1995; Thompson and Allison 1997). However, upon T cell activation, CTLA4 is upregulated on the cell surface, where it functions as a T cell activation inhibitor by actively competing for the CD28 ligands (B7-1 and B7-2) and thereby increasing the threshold of activation for otherwise weak TCR engagements (Sansom 2000; Egen and Allison 2002). Therefore, in the absence of sufficient CTLA4, self-antigens can potentially initiate autoimmunity through uninterrupted and sustained co-stimulation (Abbas et al 2004). Indeed, CTLA4-deficient mice present with a severe lymphoproliferative disorder and autoimmune disease (Waterhouse et al 1995). Furthermore, CTLA4-Ig (a soluble fusion protein of human CTLA4 and the IgG1 constant region) treatment in mice has been shown to effectively block interaction of CD28 with its ligand, thereby inhibiting transplant rejection and inducing donor specific tolerance (Lenschow et al 1992; Turka et al 1992; Linsley et al 1992).



**Figure 1.3** Diagrammatic representation of the roles of CD28 and CTLA4 in the immunological synapse [Adapted from Sansom 2000, Simmonds and Gough 2005].

- A:** CD28 interaction with its ligands, CD80 and/or CD86, results in a co-stimulatory signal necessary for the complete activation of the T cell. CTLA4 is not found on the cell membranes of unstimulated T cells.
- B:** Upon T cell activation, CTLA4 is upregulated on the cell membrane, where it interacts with and competes for the same ligands as CD28, leading to T cell activation inhibition.

CTLA4 interacts with its ligands with a 10- to 50-fold higher affinity and 40- to 100-fold faster kinetics than CD28. Therefore, the amount of CTLA4 at the cell surface needs to be regulated. The cytoplasmic tail of CTLA4 initiates this regulation by facilitating the interaction of the CTLA4 protein with the clathrin pit adaptor complex (AP-50), causing it to be rapidly internalised (see figure 1.3) (Egen and Allison 2002; Sansom 2000; Kristiansen et al 2000; Van der Merwe et al 1997; Chambers 2001).

The CTLA4 protein has a reported half-life of about 2 h. Because of this relatively short turnover rate, the efficient transcription of the *CTLA4* gene and translation of its mRNA into protein is important for its optimal functioning as a regulatory ligand (Egen and Allison 2002).

CTLA4 is also thought to function by interrupting signal transduction. It may achieve this by recruiting and ordering intracellular signalling components to the T cell-APC synapse through a so-called YVKM motif in its cytoplasmic tail. This model is also partly based on the anticipated organisation of CTLA4/B7 complexes in the immunological synapse (Ostrov et al 2000; Sansom 2000; Chambers 2001).

The CD28/CTLA4 ligands, B7-1 and B7-2 share about 25% sequence homology and are thought to perform different functions. Although B7-2 is more abundant on resting APCs and is upregulated faster than B7-1 upon activation, B7-1 is thought to be a stronger ligand for CD28-mediated activation and has a higher affinity for both CD28 and CTLA4 (Sansom 2000).

Therefore it is thought that CTLA4 controls the rate of T cell activation even in low cell surface numbers, and is therefore considered a good candidate gene for autoimmune disease susceptibility studies (Simmonds and Gough 2005).

## **1.3 Autoimmunity**

It is estimated that approximately 1 in 31 people in the USA (3.2%) suffer from at least one autoimmune disease, the most prevalent forms of which include Graves' disease (1151.5/100,000), insulin dependent diabetes mellitus (IDDM) (192/100,000), rheumatoid arthritis (RA) (860/100,000), hypothyroidism (791.6/100,000) and vitiligo (400.2/100,000) (Jacobson et al 1997; Vyse and Todd 1996). Women have an estimated 2.7 times greater risk than men for developing an autoimmune disease although the reason for this is still unclear. Indeed, the effect of hormones and genes on the immune response is being investigated (Jacobson et al 1997; McFarlane and Henegan 2004; Whitacre 2001). In general it is probably true to state that autoimmune diseases result from complex and incompletely understood interactions between the environment and an individual's genetic susceptibility. Certainly, the complex phenotypes observed for autoimmune diseases, such as AIH, cannot be explained by a single genetic defect or environmental factor (Kristiansen et al 2000).

### **1.3.1 Environmental Factors**

Autoimmune disease is thought to be induced in susceptible individuals through environmental triggering factors such as infectious agents, toxins and drugs. Some drugs that have been described as possible triggering factors include the statins (atorvastatin and simvastatin), imatinib mesylate and IFN $\beta$  (Czaja 2000). Furthermore, it has been suggested that the triggering epitope in some autoimmune conditions is a short amino acid sequence shared by many antigens. An immune response directed against an epitope of a foreign antigen that is similar or identical to a self protein, could hypothetically result in the immune response being misdirected against self. This cross-reactivity is referred to as molecular mimicry and could lead to autoimmune disease (Ermann and Fathman 2001; Czaja 2005). The molecular mimicry between an epitope on the Epstein-Barr Virus (EBV) and one of the known SLE autoantigens is one such example. Thus it has been proposed that exposure to EBV could be a triggering factor for SLE (Parks et al 2005). Similar molecular mimicry between infectious agents, such as hepatitis A and EBV, and AIH has been suggested (Vento et al 1991; Vento et al 1995).

### 1.3.2 Genetic Factors

One of the greatest difficulties in studying “complex” genetic conditions is distinguishing between primary associations, and secondary associations that occur due to genetic linkage disequilibrium. It is difficult to know whether a genetic polymorphism (DNA sequence variation amongst individuals) that shows some association with disease is actually contributing to the susceptibility, or whether it is just in close linkage disequilibrium with the true causative gene, in which case it may not be affecting the aetiology of the disease (Barnette et al 2005).

The clustering of different autoimmune diseases in families indicates possible shared genetic backgrounds and mechanisms of disease (Ueda et al 2003). This is supported by the apparent sharing of autoimmune susceptibility loci amongst human autoimmune diseases and across species (Becker et al 1998). The degree of clustering is often estimated by the relative risk that the sibling of a patient has of developing the same disease, compared to the general population risk for that particular disease. It is estimated that for siblings of affected individuals with autoimmune disease, this relative risk ranges between 6 and 100 (depending on the specific condition) meaning that they have a 6- to 100-fold higher risk of developing autoimmune disease than the general population. However, these figures seem to differ substantially between population groups for each condition (Vyse and Todd 1996).

One of the difficulties in defining the exact genetic components involved in “complex” genetic diseases is the ability to correlate the various components of the clinical phenotype with the different factors that influence it. The factors influencing the clinical phenotype are also difficult to identify in the first place (Barnette et al 2005). The risk conferred by any individual susceptibility allele could be very low, but, when combined with other susceptibility loci and possible environmental factors, the ability to cause disease increases (Vyse and Todd 1996). For this reason, identifying disease genes or polymorphisms in autoimmune diseases has proved very difficult (Vyse and Todd 1996; Gregersen 2003). Where specific genetic associations with autoimmune disease are suspected, they may be validated through

the repetition of defined genetic studies by independent investigative groups using similarly defined cohorts (Barnette et al 2005).

### **1.3.2.1 HLA Haplotypes in Autoimmunity**

Genome-wide scans have repeatedly indicated an association of the chromosome region 6p21.3, containing the highly polymorphic MHC class II HLA region with several autoimmune conditions such as IDDM, SLE and AIH (Kristiansen et al 2000; Krawitt 2006). Certain HLA haplotypes may have either a disease susceptibility or protective effect in a given population. Finding these associations is often difficult due to the strong linkage disequilibrium that exists between genes of this region. Nevertheless, a number of associations have been identified (Undlien et al 2001). In SLE, HLA-DRB1\*0301 (DR3) is associated with disease in Canadian patients while the DR1, 6 and 7 alleles appear to have a protective effect. The HLA-DQA1, DQB1 genes, as well as the DR3, 4 and 9 alleles are important in susceptibility to IDDM, while DR3, 4, 7 and 13 is associated with disease in AIH (see section 2.3; page 24). DR4 is also the major susceptibility allele for RA in caucasians (white individuals of European ancestry) (Undlien et al 2001; Gladman et al 1999 Gebe et al 2002). The DQA1\*0501 and/or DQB\*0201 genes are also associated with celiac disease in 97% of cases, compared to about 40% of controls (Periolo and Cheriñavsky 2006).

The precise mechanisms through which the MHC class II region confers susceptibility to autoimmune diseases are constantly being investigated (see section 2.3; page 24). Also, as discussed earlier, the HLA region alone does not account for the entire genetic component for predisposition to autoimmune diseases (Kristiansen et al 2000).

Genome-wide scans of families with more than one affected individual, and affected sibling pairs have showed several, rather large loci, associated with different autoimmune conditions. The heterogeneity of phenotypes, population differences, use of different polymorphic markers and genetic maps, and imperfect statistical and analytical models have mostly led to a lack of replication of results between different research groups (Becker 1999). However, some loci that seem to overlap between

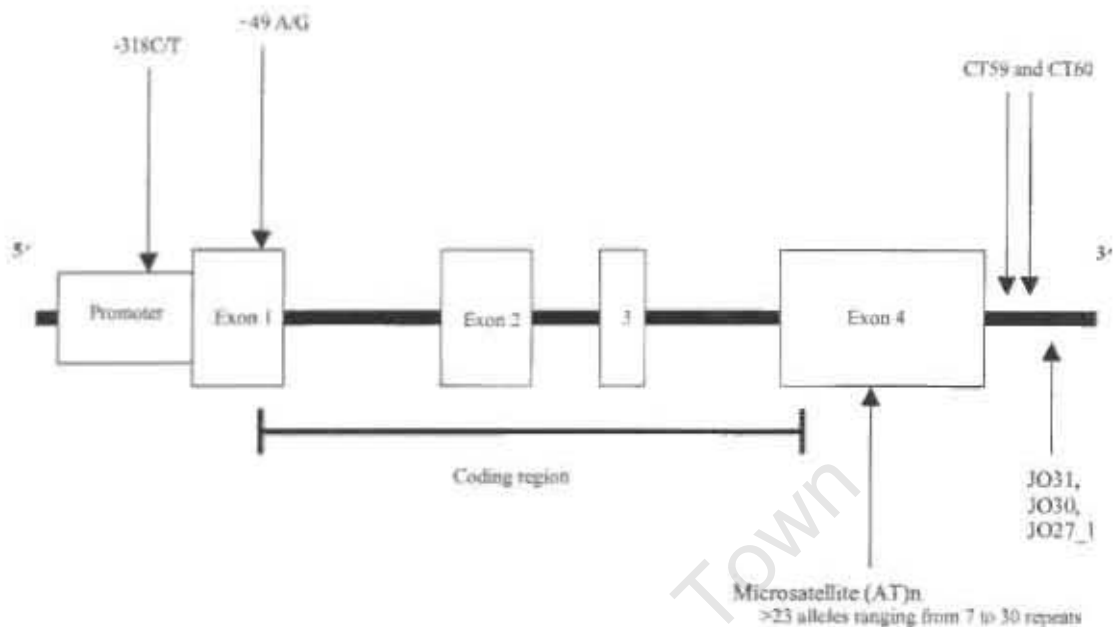
different autoimmune conditions have been identified, and these are being studied in greater detail.

### 1.3.2.2 *CTLA4* Gene in Autoimmune Disease

A region on chromosome 2 (2q33) appears to be a general autoimmune susceptibility locus. It contains three immunologically important genes that have been associated with autoimmune disease, namely *CTLA4*, *CD28* and inducible co-stimulator (*ICOS*) (Ueda et al 2003). These three genes are very similar in sequence and are all involved in immune regulation. The corresponding chromosomal region in the mouse is on chromosome 1 and is associated with the non-obese diabetic (NOD) mouse strain phenotype. In humans, associations have been investigated in, amongst others, Grave's disease. No associations were found in single nucleotide polymorphisms (SNPs) of *CD28* and *ICOS*. However, strong linkage was found for the region immediately 5' and 3' of *CTLA4* (Harper et al 1991; Ueda et al 2003).

The *CTLA4* gene shares 78% sequence homology with *CD28*, suggesting that these genes could have arisen through gene duplication. Similarity with the mouse genome is 76%, suggesting that the gene duplication event occurred before speciation. *CTLA4* encodes a 223 amino acid protein that consists of a cleavable leader peptide, an extracellular IgV-like ligand binding domain, a transmembrane region, and a cytoplasmic tail. The leader peptide is encoded on exon 1, the ligand binding domain on exon 2, the transmembrane region on exon 3 and the cytoplasmic tail on exon 4 of the *CTLA4* gene (Kristiansen et al 2000). The known polymorphisms of the *CTLA4* gene (see figure 1.4) include, amongst others:

- a -318 C/T SNP in the *CTLA4* promoter region (Deichmann et al 1996);
- a +49 A/G SNP in exon 1, encoding a Threonine (Thr) to Alanine (Ala) substitution at codon 17 (Agarwal et al 2000), and;
- a *CTLA4*(AT)<sub>n</sub> dinucleotide repeat microsatellite polymorphism in the 3' untranslated region (UTR) of exon 4. At least 23 different alleles have been identified, ranging from 7 to 30 repeats (Kristiansen et al 2000).



**Figure 1.4** Gene structure of *CTLA4*, indicating disease-associated polymorphisms previously identified in humans and associated with autoimmune disease. Relative positions of polymorphisms are indicated with arrows and blocks represent exons and the promoter region. Spaces between exons indicate intronic regions. This figure is not drawn to scale.

Several studies have tried to link these polymorphisms to various autoimmune conditions. Results seem to vary between different population groups and autoimmune conditions. In Chinese patients, the -318 C/T polymorphism has been associated with AIH (Fan et al 2004). The -318 C allele was also significantly decreased and the -318 C/T genotype significantly increased in patients with Wegener's granulomatosis, compared with controls (Giscombe et al 2002). On the other hand, the -318 T allele has been shown to be associated with a higher promoter activity in Swedish patients with Wegener's granulomatosis, and is therefore considered possibly protective for autoimmune disease (Wang et al 2002). To date there are no -318 C/T polymorphism associations with autoimmune thyroid disease, including Grave's disease and autoimmune hypothyroidism, or SLE in European caucasian, Hong Kong Chinese, Japanese, Spanish or Korean patients (Heward et al 1998; Ahmed et al 2001; Aguilar et al 2003; Hudson et al 2002).

The +49 A/G polymorphism has been shown to affect IL-2 regulation, cellular distribution of CTLA4 and T cell proliferation response under suboptimal T cell stimulation conditions such as limited ligand availability (Maurer et al 2002). The +49 G/G genotype has been found to be associated with AIH in northern European and Chinese patients, IDDM in Iranians and SLE in Japanese patients (Agarwal et al 2000; Fan et al 2004; Mojtahedi et al 2005; Ahmed et al 2001). However, no association was found between the +49 A/G polymorphism and SLE in Spanish and Korean patients (Aguilar et al 2003; Hudson et al 2002).

Three other possible disease-associated variants, CT60, JO31, JO30 and JO27\_1 have been identified within a 6.1 kb region within the 3' region of *CTLA4*. CT60 showed particularly strong association with autoimmune hypothyroidism and Graves' disease and to a lesser extent with IDDM (Ueda et al 2003). The CT60 G allele has since also been found to be associated with coeliac disease (Van Belzen et al 2004).

### 1.3.2.3 Soluble CTLA4

CTLA4 exists in two forms in humans: a full length CTLA4 (flCTLA4) molecule, encoded by exons 1, 2, 3 and 4, and an alternatively spliced, soluble form (sCTLA4) lacking the transmembrane domain encoded by exon 3 of the *CTLA4* gene. The frameshift caused by the alternative splicing leads to the inclusion of an extra 22 amino acids due to a new translational termination site 3' of the original site.

sCTLA4, unlike its full length counterpart, is constitutively expressed by unstimulated T cells (Purohit et al 2005) and is present in human serum. However, this expression of sCTLA4 is downregulated after T cell activation in normal cells, while flCTLA4 is upregulated. This suggests that sCTLA4 may play a more important role than flCTLA4 in the early stages of an immune response through its immunoregulatory properties (Magistrelli et al 1999; Wong et al 2005; Liu et al 2003). Mouse studies have shown that sCTLA4 binds to CD80/86 more avidly than full length CLTA4. This suggests that reduced levels of sCTLA4 may lead to

reduced blockade of CD80/CD86, leaving them available for T cell activation by CD28 (Oaks and Hallett 2000; Oaks et al 2000; Ueda et al 2003).

The possibility that specific polymorphisms within and around *CTLA4* might affect its splicing ability has been suggested by Oakes and Hallett (Oakes and Hallett 2000). Full length to sCTLA4 mRNA ratios have been shown to differ significantly between individuals with different genotypes of CT60, with a 50% lower ratio in individuals homozygous for the CT60 G allele. It is hypothesised that the CT60 G/A SNP may affect the efficiency of gene splicing in *CTLA4* (Ueda et al 2003). No correlations have been found between the -318 C/T promoter region polymorphism and sCTLA4 serum levels in patients with SLE (Liu et al 2003).

Serum sCTLA4 concentrations were increased in many SLE (Range: 28 to 78 ng/ml vs. 'virtually undetectable' in controls) and autoimmune thyroid disease ( $21.6 \pm 12.3$  ng/ml vs.  $5.9 \pm 5.4$  ng/ml) patients compared with healthy control individuals (Oakes and Hallett 2000; Liu et al 2003). These increases suggest that sCTLA4 inhibits CTLA4 function by binding to CD80/CD86 more readily than the latter, possibly interfering with the downregulation of T cell activation (Oakes and Hallett 2000). However, subsequent studies have shown conflicting data for other autoimmune conditions. For instance, no correlation was found between CT60 and sCTLA4 levels in serum of IDDM patients (Purohit et al 2005) and Liu et al (2003) envisioned a dual role for sCTLA4 whereby it also blocks CD28-mediated T cell activation by competing for its ligands. The exact function of sCTLA4 remains unclear.

One of the problems in elucidating a role for sCTLA4 is that studies on sCTLA4 have been in patients with active or inactive disease, some of whom were already on treatment. For example, Liu et al (2003) compared the serum sCTLA4 levels of SLE patients on different dosages of steroid therapy as well as patients on treatment and without treatment. Thus, there is a lack of data that compares serum sCTLA4 levels of patients during three important phases of disease, namely:

- At presentation with active disease (before treatment);

Genetic factors that appear to determine susceptibility to various autoimmune conditions, such as the HLA region and *CTLA4*, have been identified while other genetic factors are thought to determine susceptibility to specific autoimmune conditions. For most autoimmune diseases, genetic factors alone appear to be insufficient to trigger the autoimmune process and certain environmental factors such as pathogens, toxins and drugs are thought to be necessary in initiating autoimmunity (Mackay 2000).

Much work remains to be done to elucidate the complex genetic components of autoimmune disease. Autoimmune predisposing genetic components that have been identified need to be investigated further in different population groups and autoimmune conditions. Furthermore, the role of sCTLA4 in different autoimmune conditions needs to be investigated.

University of Cape Town

## Chapter 2

# Autoimmune Hepatitis and Autoimmune Hepatitis in Africa: Review

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### 2.1 Definition of Autoimmune Hepatitis and Introduction

Autoimmune hepatitis (AIH) is a chronic hepatitis characterised by the presence of liver associated autoantibodies, raised serum gamma globulin levels and interface hepatitis with a lymphoplasmacytic necroinflammatory infiltrate on liver histology (Alvarez et al 1999). AIH has a mean annual incidence of about 1.9 per 100,000 population in northern Europeans with a point prevalence of 16.9 per 100,000 individuals compared to 42.9 per 100,000 population in Alaskan natives. It accounts for about 5.9% of all liver transplants in the USA and 2.6% in Europe. Recurrence of disease after transplantation is estimated at about 27% in the UK (Boberg et al 1998; Milkiewicz et al 1999; Wiesner et al 1998; Hurlburt et al 2002; Czaja 2005). High ethnic variability in the occurrence, severity, onset of disease and response to therapy has been observed for AIH (Lim et al 2001; Zolfino et al 2002; Czaja 2005; Czaja et al 2002).

As is the case with most other autoimmune diseases, AIH shows a female preponderance of 4:1 in caucasoid populations. There is some evidence suggesting that hormonal differences, gender specific responses, and exposure to drugs and toxins contribute to the higher prevalence in women. Environmental agents, infections and pregnancy could also play a role in triggering disease. Despite the definite female preponderance, there seems to be no difference in the clinical expression of AIH between sexes (McFarlane and Heneghan 2004; Czaja 2005).

## **2.2 Diagnosis, Clinical Findings and Treatment**

Although not the focus of this dissertation, it is appropriate to consider some clinical aspects of AIH. It is a heterogeneous condition and presentation of disease can vary widely. There may be no visible clinical signs or non-specific symptoms such as fatigue, or acute severe hepatitis or decompensated chronic liver failure may be the presenting features. The diagnosis of AIH is therefore best based on the correlation of liver biopsy findings, positive liver associated autoantibodies, negative viral serology, a clinical history supportive of a diagnosis of AIH, response to immunosuppressive therapy and most importantly, the exclusion of other causes of chronic hepatitis. The diagnostic criteria for AIH that were put in place by the International Autoimmune Hepatitis Group in 1993 (Johnson et al 1993) and revised in 1999 (Alvarez et al 1999) give numerical scores to a number of biochemical, epidemiological, serological and clinical factors to determine the probability of a diagnosis of AIH in a given patient. The system is important in the reporting of research papers but is not practical in daily clinical work, except in the setting of specialised liver units (Zolfino et al 2002; Czaja 2005).

For clinical convenience, clinicians have separated the condition into types 1 and 2. However, this classification is not recognised by the International Autoimmune Hepatitis Group, because of the fact that it does not influence the nature of therapy. Type 1 is the most common form and occurs in 70-80% of AIH cases (Czaja 2005; Alvarez et al 1999). It is characterised by the presence of circulating antinuclear antibody (ANA) and/or smooth muscle antibody (SMA). Extended spectrum antibodies such as antiactin antibodies, atypical perinuclear antineutrophilic cytoplasmic antibody (pANCA), liver-pancreas antigen (LP) and soluble liver antigen (SLA) may also be observed.

Type 2 AIH is characterised by the presence of anti-liver, kidney microsomal antibody (anti-LKM-1) and/or anti-liver cytosol 1 (ALC-1) (Krawitt 2006; Alvarez et al 1999). In children particularly, type 2 AIH may be associated with partial serum immunoglobulin A deficiency (Vergani and Mielie-Vergani 2003).

Up to 25% of patients test negative for the standard liver-associated autoantibodies, but have disease that is indistinguishable from classic AIH. These patients constitute the cryptogenic steroid-responsive autoimmune hepatitis entity in the new classification of chronic hepatitis from the International Working Party on chronic hepatitis (Ludwig et al 1995). Diagnosis of these patients relies on a combination of serum biochemical abnormalities, hypergammaglobulinaemia, histological findings, history of autoimmunity in the patient or close family members and the exclusion of other causes of chronic liver disease (Ludwig et al 1995; Alvarez et al 1999).

It is not known whether AIH is attributable to a  $T_H1$  or  $T_H2$  response as the presence of liver autoantibodies appears to be a secondary effect of disease, rather than the cause. The current and generally accepted hypothesis on the pathogenesis of AIH is that of an unknown environmental agent, such as a drug or virus, triggering an autoimmune response through molecular mimicry (see Chapter 1). Epitopes from hepatitis A and EBV antigens that are similar to those of liver antigens in humans have been investigated as possible viral triggers of AIH. The subsequent injury is self-perpetuating and leads to disease (McFarlane and Heneghan 2004; Vento et al 1991; Vento et al 1995; Krawitt 2006).

### **2.2.1 Assessment of Liver Disease**

The assessment of the severity of liver disease in an individual patient relies on the use of a combination of tests that include:

1) The assessment of liver histopathology:

This includes the assessment of fibrosis and inflammation/ necrosis and also enables the exclusion of other causes of liver injury.

2) The assessment of liver synthetic function:

The ability of the liver to synthesise proteins is measured through serum albumin (ALB) levels, the production of clotting factors as reflected by the international normalised ratio (INR) and fibrinogen production.

3) The assessment of liver inflammation:

Hepatocellular injury is measured through histological assessment and biochemical assessment through aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in blood.

4) The assessment of bile duct injury:

Bile duct injury is reflected through histological assessment and the serum levels of alkaline phosphatase (ALP) and gamma glutamyl transpeptidase (GGT).

### **2.2.2 Treatment**

Standard treatment options available for AIH include anti-inflammatory or immunosuppressive therapy. Treatment with corticosteroids such as azathioprine and/or prednisone results in a 20-year life expectancy for over 80%, while untreated patients with interface hepatitis generally have a life expectancy of about 5 years. It is therefore important to be able to distinguish the disorder from other forms of chronic hepatitis (Krawitt 2006; Vergani and Mieli-Vergani 2003; Zolfino et al 2002). A combination of prednisone and azathioprine is currently the preferred treatment for AIH rather than prednisone or azathioprine alone, as it is associated with a lower rate of drug-related side effects. However, individual clinical assessments and regular follow-ups are necessary to determine the treatment of choice (Czaja and Freese 2002). Other treatment possibilities under investigation for severe disease include cyclosporine, tacrolimus, methotrexate and mycophenolate mofetil.

The use of corticosteroids in very mild AIH may be excessive and possible drug-related side effects need to be weighed against the benefits of treatment. Patients with cirrhosis, as well as menopausal and older patients may have an increased risk for drug-related complications. During pilot treatment studies, Budesonide and ursodeoxycholic acid have shown promise as agents for future treatment of mild AIH (Czaja et al 2000; Czaja and Freese 2002).

Treatment is generally considered inevitable in cases where serum AST levels are more than 10-fold the upper limit of normal, or more than 5-fold the upper limit of normal with gammaglobulin levels more than twice that of normal. Another indication for treatment necessity is the presence of bridging or multiacinar necrosis on histologic examination (Czaja and Freese 2002).

Conventional treatment regimens for adults start with about 50 mg azathioprine daily, together with 30 mg prednisone in the first week of treatment and reducing it weekly thereafter until a dose of about 10 mg prednisone per day is reached (Czaja 2005).

The treatment of children with AIH is not much different to that of adults, even though they are prone to much more severe disease. Initial treatment with daily doses of 2 mg/kg prednisone and early introduction of azathioprine is generally effective (Czaja and Freese 2002).

Treatment efficacy is affected by age, geographical regions, population groups and genetic predisposition. About 13% of AIH patients do not respond to conventional corticosteroid therapy. Upon azathioprine failure or toxicity, patients are treated with high prednisone or changed to alternative agents such as cyclosporine or tacrolimus. In prednisone failure or toxicity, alternative agents that have been used successfully include mycophenolate mofetil and azathioprine monotherapy. Where there is treatment failure with advanced liver disease, liver transplantation may be necessary (Czaja et al 2005; Czaja and Freese 2002).

## **2.3 Genetics of AIH**

Type I AIH is associated with the HLA-DRB1\*0301 (DR3) or the HLA-DRB1\*0401 (DR4) serotypes in northern European and North American patients. The HLA-DR3 haplotype is associated with early-onset, severe AIH, while HLA-DR4 associates with milder disease that has a better response to corticosteroid therapy (Krawitt 2006; Strettell et al 1997; Lim et al 2001). HLA-DRB1\*1301 occurs more

frequently in type I AIH in South America, and is associated with poor response to therapy, while the DRB1\*1302 appears to have a protective effect in the same population (Pando et al 1999; Goldberg et al 2001; Czaja et al 2002). Susceptibility in Japanese patients is linked to DRB1\*0405 (Ota et al 1992). In the light of this data, DRB1 has been implicated as the principle susceptibility locus for type I AIH (Czaja 2005).

On the other hand, type II AIH has been associated with the HLA-DQB1\*0201 alleles in Canadian and French paediatric patients, while HLA-DR2 seems to be protective in northern European caucasians (Djilali-Saiah 2004; Krawitt 2006). HLA-DRB1\*07 has also been associated with type II AIH in Brazil and Germany. German patients furthermore had higher frequencies of HLA-DRB1\*15 and HLA-DQB1\*06 (Bittencourt et al 1999; Czaja 1997). The lack of coherence in data from these different studies regarding susceptibility loci highlights the difficulty in linking a particular locus to the disease, and stresses the importance of defining homogenous study groups (Strettell et al 1997).

The  $\alpha$  and  $\beta$  chains of the MHC class II molecule form a peptide binding groove with 9 pockets into which antigen peptides fit, when bound to the molecule. Most of the polymorphisms that render the MHC such a variable region are found within these pockets, resulting in its unique binding patterns. HLA conferred susceptibility to AIH has been linked to the LLEQKR amino acid motif at positions 67-72 of the DR $\beta$  polypeptide. It is hypothesised that low binding affinity between self antigens and class II HLA molecules could lead to reduced tolerance. It is thought that the presence of a positively charged amino acid at position 71 of the DR $\beta$  polypeptide, such as Lysine in DRB1\*0301 and DRB1\*0401 and Arginine in DRB1\*0404 and DRB1\*0405 haplotypes, may be one of the main risk factors conferring disease susceptibility to AIH. The DRB1\*1501 allele, which protects against AIH in North Americans and northern Europeans, has a neutral, nonpolar Ala at position 71 of the DR $\beta$  polypeptide. The  $\beta$ 71 residue is situated at the tip of the  $\alpha$ -helix of the DR $\beta$  chain where it can possibly interact with both the presented antigen as well as the T cell receptor. It is therefore hypothesised that the more HLA molecules with

positively charged amino acids at position  $\beta 71$  that are expressed at the cell surface, the higher the susceptibility will be to the disease (Gebe et al 2002; Strettell et al 1997; Czaja 2005; Doherty et al 1994).

As with many other forms of autoimmune disease, HLA haplotypes alone do not account for the entire genetic component of susceptibility to AIH. Other autoimmune promoter genes, such as the tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) gene, the TCR constant  $\beta$  gene, the Gm immunoglobulin allotype and the *CTLA4*, gene have been associated with genetic susceptibility to AIH in various studies (Czaja 2005; Bittencourt et al 1999).

The *CTLA4* +49 G allele was found to be associated with type 1 AIH in northern European caucasians. In the same population, the *CTLA4* G/G genotype was found to be associated with higher mean serum AST levels and a higher frequency of antibodies directed against thyroid microsomal antigens. Work on French and Canadian children showed an association of type I AIH with the +49 A allele, while Bittencourt et al (2003) found no association with the *CTLA4* +49 A/G polymorphism and AIH in a Brazilian cohort (Agarwal et al 2000; Djilali-Saiah et al 2001).

This discrepancy in the *CTLA4* +49 data could indicate that either:

- there is a difference between the genetic predisposition of paediatric and adult forms of AIH, as was indicated for HLA association in Argentinean patients (Pando et al 1999); or
- genetic predisposition to AIH varies between population groups; or
- the *CTLA4* +49 A/G polymorphism is simply in linkage disequilibrium with the true disease-associated genetic factor; or
- the initial association of the *CTLA4* +49 G allele with type 1 AIH by Agarwal et al (2000) could have resulted from a co-occurrence of AIH and autoimmune thyroid disease, as was suggested by Djilali-Saiah et al (2001).

Thus, further work on the role of the *CTLA4* +49 A/G polymorphism in other population groups may help to shed light in this regard.

Djilali-Saiah et al (2001) also showed that there is an association between the *CTLA4* AT(n) microsatellite in the UTR 3' of exon 4, which may affect mRNA stability, and AIH. The -318 C allele was found to be significantly increased in Chinese patients with AIH (Fan et al 2004). There is no published data investigating the CT60 G/A polymorphism 3' of the *CTLA4* gene in AIH.

## 2.4 AIH in Africa

Although AIH clearly occurs in non-caucasian populations (Zolfino et al 2002; Hurlburt et al 2002; Lim et al 2001), the disease is poorly documented in Africa, giving rise to the impression in the literature that the disease is rare on the African continent. Most research on AIH to date has been on European caucasian or Japanese patients. However preliminary studies on small groups of non-caucasian patients in the UK (Zolfino et al 2002) and the USA (Lim et al 2001) have indicated possible differences between caucasian and non-caucasian patients with regard to AIH. Work done on a small cohort of non-caucasian patients in the UK suggested that disease in these patients tends to be more severe, occurs at a younger age and responds less well to standard immunosuppressive therapy, than in their caucasian counterparts. A high proportion of these patients also had underlying cholangiopathy, compared to the non-caucasian Europeans (Zolfino et al 2002). A study by Lim et al (2001) showed that 85% of African American AIH patients had cirrhosis at presentation, compared to 38% in a caucasian cohort. Their findings also suggested higher bilirubin levels in African American AIH patients and a need for higher prednisone doses in order to maintain remission (Lim et al 2001). It is important to note that these studies were done on small groups of patients living in the UK or America. There are no comprehensive studies of AIH in southern Africa that document its clinical, immunological and histopathological features.

Similarly, the genetic basis of AIH in southern African populations has not been studied. Genetic susceptibility seems to vary between population groups and it is

therefore important that genetic, environmental and cultural differences be investigated. Preliminary data from our Centre (the UCT-MRC Liver Research Centre at Groote Schuur, Cape Town) failed to show genetic association between the *CTLA4* exons 1, 2 and 3 with SLE or AIH (Hairwadzi et al 2002; Mda et al 2002). However, the promoter region, exon 4 and the UTR 3' of the gene was not investigated in these studies.

## 2.5 Summary

AIH is a heterogeneous condition in terms of clinical features, presence of autoantibodies, genetic predisposition, disease activity and response to treatment. There is a definite female preponderance in AIH and treatment currently relies mainly on immunosuppressant therapy. Although HLA haplotypes have been shown to play a role in genetic susceptibility to disease, other genetic factors such as *CTLA4* gene polymorphisms are currently of interest. There is discrepancy between results from different population groups, regarding the association of *CTLA4* polymorphisms with disease. This might imply a need to investigate the genetics of the condition in the context of ethnicity. Most studies on the genetics of AIH have focussed on American and northern European caucasoid populations.

## Chapter 3

### Research Questions, Aims and Objectives

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#### 3.1 AIH in the MRC-UCT Liver Clinic

AIH constitutes 34% of the chronic hepatitis patients that are seen at the MRC-UCT Liver Research Centre clinic at Groote Schuur Hospital, Cape Town, and a significant proportion of the clinical work load. Historical data (over 30 years) from this clinic shows that AIH affects all races and age groups with a female preponderance. Furthermore, our local experience shows that the disease occurs frequently in the non-caucasian populations (57%). Twelve percent of the clinic cohort of AIH patients are indigenous black Africans. Most of these black patients have advanced liver disease with 46% being cirrhotic at presentation (Hairwadzi et al 1999). It is difficult to separate late presentation, from a more aggressive disease with more severe injury, due to socioeconomic and health care access factors. Data from North America suggests that AIH in the non-caucasian populations is a more aggressive disease that requires higher steroid doses (Lim et al 2001).

There are a number of current studies focusing on AIH within the MRC-UCT Liver Research Centre. The work undertaken in this study is one such study, and focuses on the role of CTLA4 in AIH in defined ethnic groups.

#### 3.2 Background: Introduction and Previous Studies

As outlined in the preceding chapters, CTLA4 is important in the regulation of T cell activation by competing for the same ligands as the co-stimulatory molecule, CD28. Known polymorphisms within the *CTLA4* gene have been reported to be associated with several autoimmune conditions, including AIH (Kristiansen et al 2000; Fan et al 2004; Agarwal et al 2000; Mojtahedi et al 2005; Ahmed et al 2001; Ueda et al 2003; Van Belzen et al 2004; Djilali-Saiah et al 2001). However, there is conflicting data

regarding the role of these *CTLA4* polymorphisms in autoimmune disease and studies in some population groups have shown a lack of association of these polymorphisms with disease (Heward et al 1998; Ahmed et al 2001; Aguilar et al 2003; Hudson et al 2002; Bittencourt et al 2003).

A serum soluble form of CTLA4 (sCTLA4) exists and is thought to have a similar immunoregulatory function as the full length CTLA4 (fICTLA4). However, unlike fICTLA4, sCTLA4 is constitutively expressed on unstimulated T cells and may be of importance in the early regulation of T cell activation. It has been suggested that the efficiency of the splicing of the *CTLA4* gene to produce the soluble form of the protein is influenced by polymorphisms in or around this gene (Ueda et al 2003). Furthermore, Oakes and Hallet (2000) showed that patients with autoimmune thyroid disease had elevated levels of sCTLA4. The role (or lack thereof) of sCTLA4 in AIH remains to be elucidated.

Finally, results from preliminary studies on very small cohorts of non-caucasian northern European and American patients suggest that these patients present with more severe disease at a younger age, and require more aggressive therapy. However, further investigation in such groups needs to be performed.

### **3.3 Aims of the Study**

The aims of this study were:

- To investigate CTLA4 gene polymorphisms in AIH patients in South Africa and, if present, to examine whether such polymorphisms correlate with clinical disease.
- To evaluate possible differences in clinical disease and genetic susceptibility to AIH amongst various locally represented ethnic groups.
- To investigate a possible role for sCTLA4 in AIH and/or in the disease progression.

- To assess the severity of AIH and how this correlates with *CTLA4* genotypes, sex, ethnicity and age of onset.

### **3.4 Objectives of the Study**

The objectives of this study were:

- To assess the frequency of known polymorphisms in and around the *CTLA4* gene (-318 C/T, +49 A/G and CT60 G/A) in South African patients with AIH, and to compare this data to that of ethnically matched control groups.
- To screen the *CTLA4* gene for any novel DNA sequence variations in a South African cohort of AIH patients who had family members with either AIH or any other form of autoimmune disease. Both affected and unaffected family members who were available and gave consent were studied.
- To investigate possible associations or correlations of any polymorphisms in the *CTLA4* gene with clinical disease in the various ethnic groups of South African AIH patients.
- To investigate s*CTLA4* serum concentrations in South African AIH patients at presentation with AIH (before introduction of treatment) and during controlled disease (on treatment).
- To assess the severity of AIH by analysis of ALT, ALB and INR indices in order to investigate how disease severity correlates with *CTLA4* genotypes, sex, age of onset and ethnicity.

## Chapter 4

### Materials and Methods

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#### 4.1 Selection of Study Cohort and Isolation of DNA

##### 4.1.1 Study Subjects

A potential cohort of 122 Type 1 AIH patients from the UCT Groote Schuur Liver Clinic database was selected for use in screening the *CTLA4* gene for possible disease-associated polymorphisms. This cohort included all patients for whom DNA samples had been collected over a two year period prior to the study. Diagnosis of AIH in these patients was based on clinical data, autoimmune serology, liver biopsy and response to therapy, as has been described by the AIH working group (Johnson et al 1993). The severity of disease in these patients was determined, where possible, by measuring three important indices. Firstly, the degree of liver inflammation was assessed using the alanine aminotransferase (ALT) levels of the patient. Secondly, the ability of the liver to produce proteins was determined by measuring the albumin (ALB) concentration in the blood. Finally, the ability of the liver to maintain normal blood clotting was determined using the international normalised ratio (INR). The cohort used comprised 25 black African (6 male; 19 female), 34 caucasian (8 male; 26 female), 44 mixed ancestry (7 male; 37 female) individuals and 15 individuals of Asian (4 male; 11 female) descent, and was termed the “study cohort”. A description of the liver biochemistry, sex, ethnicity and age of onset of the patients that participated in this study cohort is presented in Appendix A.

A second, family-based cohort included a subset of 12 of the above female patients for whom DNA from consenting family members was available (see Appendix A\*), and was termed the “family-based cohort”. In all cases, family members were first degree relatives, with the exception of patient 974 where the family members were 2 uncles on the mother’s side. This cohort was chosen in order to have a basis for studying a potential disease-association of any novel sequence variations identified.

Thus, family members with AIH or any other form of autoimmune disease, as well as unaffected family members who gave informed consent, were included in this part of the study.

Furthermore a second, sub-cohort was assembled from the study cohort for use in sCTLA4 enzyme-linked immunosorbent assay (ELISA) analysis. This group comprised 18 patients who had presented with severe, acute fulminant AIH, with active disease at the time of blood collection. Two blood samples were collected from these patients. The first was taken at presentation with AIH, before commencement of treatment and the second was taken at a follow-up session, 3 months after treatment commenced.

A control cohort of 57 hepatitis B virus (HBV) infected patients representing all the ethnic groups of which the study cohort was comprised, was collected for background population studies during the time period of this study. These included 7 black African (3 male; 4 female), 2 caucasian (1 male; 1 female), 42 mixed ancestry (21 male; 21 female) individuals and 6 individuals of Asian (5 male; 1 female) descent. This group was chosen as the most suitable controls for our study, based on the fact that detailed clinical reports for these individuals were available that confirmed a lack of symptoms for AIH and/or any other form of autoimmune disease. However, the group represents the ethnic, age and sex demographics of the HBV patients at the UCT/MRC Liver clinic at Groote Schuur hospital, rather than the true population demographics of the region and therefore differs from the study cohort in this regard. This will be discussed further in chapter 6.

Informed, verbal consent for this and other related AIH studies was obtained from all participating individuals, prior to sample collection. Ethical approval for this study was obtained from the University Research Council Research Ethics Committee (Reference number: 090/99).

### 4.1.2 Isolation and Storage of DNA

On receipt of blood samples in the laboratory, each patient was coded and family information recorded prior to involvement of the researcher. These codes were used in the study, instead of patient information. In this way the study was blinded to the researcher, thereby preventing biased interpretation of results.

DNA was isolated from 2.5 ml whole blood, using the Wizard Genomic DNA Purification kit (Promega, Madison, USA) as per the manufacturer's protocol (see Appendix B) and quantified with the GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of DNA was assessed on the basis of the optical density (OD)<sub>260/280</sub> ratio obtained. Only DNA with an OD<sub>260/280</sub> ratio of 1.5 – 2 was utilised.

DNA samples for long-term storage were kept at –80°C. Aliquots of the DNA samples were diluted to 100 ng/μl and stored at 4°C for the duration of the project.

## 4.2 Primer Design and Polymerase Chain Reaction (PCR)

The genomic sequence of the *CTLA4* gene was obtained from both the National Centre for Biotechnology Information (NCBI) website (<http://www.NCBI.nlm.nih.gov>) and the ENSEMBL website (<http://www.ensembl.org/index.html>) and annotated using information from both these sites regarding SNPs, intron-exon boundaries, start and stop codons as well as dinucleotide repeats (see Appendix C).

PCR primers were designed based on the genomic sequence described above, using Primer3 ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) for the amplification of the first three exons of the *CTLA4* gene as well as the translated part of exon 4, the promoter region and an UTR downstream of exon 4. The latter amplicons (amplification fragments) included the site for the CT60 G/A

polymorphism (NCBI accession number: rs3087243). Primer3 allows for the specification of certain parameters such as primer length, GC content and melting temperature. Oligo Calculator was used to confirm the GC content and melting temperatures (<http://www.pitt.edu/~rsup/OligoCalc.html>). At least 60 bp of the flanking intronic regions were included in the amplicons, as splicing regulatory sites commonly occur within 40 bases from the exon splice sites. In addition, to assess primer specificity for the target sequences alone, the primer sequences were compared to the rest of the human genome, using the Basic Local Alignment Search Tool (BLAST) (<http://www.NCBI.nlm.nih.gov/BLAST>). Primers were manufactured by Integrated DNA Technologies, Inc (Coralville, USA). Further criteria for the design of primers, as well as primer sequences and information, are listed in Appendix D.

PCR reactions were performed (see Appendix E) and the products subjected to 6% polyacrylamide gel electrophoresis (PAGE) (see Appendix F) to assess the PCR products for purity.

### **4.3 Investigation of Previously Reported *CTLA4* Polymorphisms in AIH Patients**

Pure PCR products were obtained as described above (see section 4.2; page 34).

#### **4.3.1 Restriction Enzyme (RE) Digestion**

RE digestion is a cost-effective method commonly used for the detection of polymorphisms within DNA fragments. Commercially available REs recognise specific, short, palindromic sequences within DNA and cleave the DNA at the site of recognition. Where polymorphisms occur within a DNA fragment, RE recognition sites may be created or destroyed, enabling restriction map analysis to be performed. Once an enzyme has been identified for use in screening for a particular polymorphism or mutation (rare DNA sequence variations that are often disease-

causing), the DNA fragments to be screened are incubated with the enzyme and the resulting fragments analysed by electrophoresis.

Hence, in this study, assays were developed to screen all patient and control cohorts for previously reported polymorphisms (see Appendix G.1). RE maps for wild type and polymorphic DNA fragments were constructed using Carolina Webcutter software (<http://www.carolina.com/webcutter/carolina.asp>) and are shown in Appendix G.2.

The -318 C/T polymorphism destroys an *MnII* site in the *CTLA4*promA amplicon (see Appendix G.2.1), while the +49 A/G polymorphism creates an *ItaI* site in exon 1 (see Appendix G.2.2). In the UTR 3' of exon 4, the CT60 G/A polymorphism destroys an *HpyCH4IV* site (see Appendix G.2.3).

## **4.4 Polymorphism and Mutation Screening of the *CTLA4* Gene**

The *CTLA4* gene was screened for possible disease-associated polymorphisms and/or mutations in the 12 patients from the family-based cohort.

### **4.4.1 Polymorphism and Mutation Analysis**

#### **4.4.1.1 Denaturing High Performance Liquid Chromatography (DHPLC) Analysis**

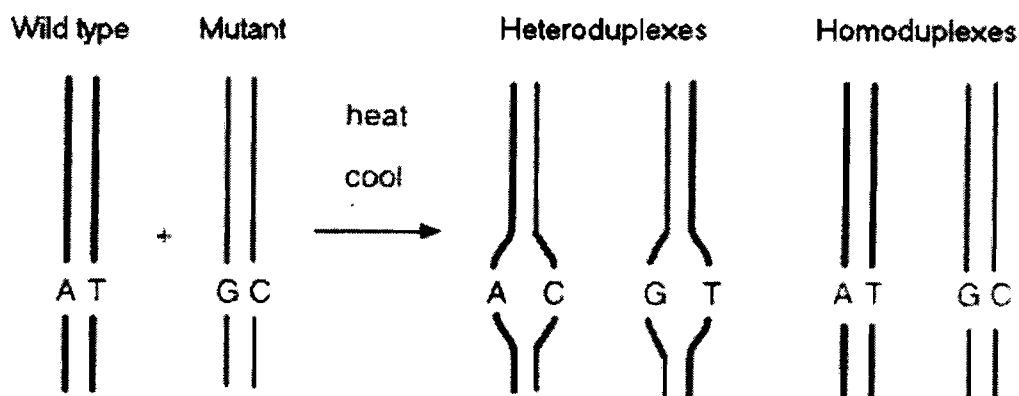
On successful amplification of target sequences of the *CTLA4* gene, DHPLC screening of exons 1, 2, 3 and 4a, as well as the promotor region (promA), was performed with the Transgenomic WAVE Nucleic Acid Fragment Analysis System (Transgenomic Inc., Omaha, USA) at the UCT Division of Human Genetics, Cape Town, South Africa (see Appendix H.1).

DHPLC under partially denaturing conditions is a highly sensitive and cost-effective method used for the separation of DNA based on size, sequence and temperature differences. This system relies on a form of reverse phase ion-pairing liquid chromatography to elute DNA at specific temperatures for the subsequent detection of heteroduplexes.

The stationary phase of the WAVE system consists of a separation cartridge containing a matrix made up of hydrophobic, electrostatically neutral, polystyrene-divinylbenzene (PS-DVB) co-polymer beads that are unable to bind DNA on their own. When treated with a positively charged ion-pairing reagent, 0.1M Triethylammonium Acetate (TEAA), the cartridge matrix is able to bind the negatively charged phosphate backbones of DNA molecules. TEAA acts as a bridging molecule for the indirect binding of DNA to the beads (Xiao and Oefner 2001).

The mobile phase of this system refers to the TEAA/acetonitrile buffer needed to elute the DNA from the cartridges (see Appendix H.1.1.2) by breaking the hydrophobic interactions between the PS-DVB beads and the TEAA/DNA (Taylor et al 1998).

DHPLC relies on a form of heteroduplex analysis for the detection of even single base changes in a DNA fragment. When a mutant DNA fragment, mixed with a corresponding wild type sample, is denatured and allowed to reanneal, the sample fragments form complexes called heteroduplexes. This heteroduplex formation is due to the non-complimentary nature of the mutant and wild type DNA at the site of the DNA sequence change (see figure 4.1). Where no sequence alterations occur in the sample DNA, only homoduplexes will form. The formation of heteroduplexes renders a double stranded DNA fragment less stable than homoduplex fragments under partially denaturing ion-pair reversed-phase liquid chromatographic conditions (Xiao and Oefner 2001), resulting in earlier elution with DHPLC.



**Figure 4.1 Schematic representation of heteroduplex formation during hybridisation**  
[Adapted from Taylor et al 1998].

DHPLC requires the use of wild type (polymorphism- and mutation-negative) DNA samples. These were obtained from individuals with no clinical presentation of AIH or any other known form of autoimmune disease. The relevant sections of DNA were amplified and the PCR products sequenced to ensure homozygosity. PCR products from each affected individual were mixed with wild type PCR product in a 1:1 ratio. This step is essential for the detection of homozygous changes in affected individuals, as it provides a wild type allele for the formation of heteroduplexes. Where polymorphisms were identified in amplicons through RE digests, wild type samples were substituted with controls that matched the sample at the particular allele, in order to prevent interference with detection of novel changes.

The mixtures were heteroduplexed and screened as described in Appendix H.1. Homoduplex controls were included in every experiment, consisting of only wild type DNA for comparison with samples and effective detection of all heteroduplexes generated.

DHPLC data were analysed using the WAVEMAKER<sup>TM</sup> Software (Transgenomic, Omaha, USA). Peaks that resulted from DHPLC analysis of sample DNA were compared to that of the wild type homoduplex control samples included in each experiment. Theoretically, samples that form homoduplexes will result in two peaks

(although only one is generally observed), while heteroduplexes can result in as many as four peaks. Where the resulting chromatogram patterns and/or number of peaks differed from that of the wild type control, samples were further analysed.

#### **4.4.1.2 Single Stranded Conformational Polymorphism (SSCP) Analysis**

Due to the small amplicon sizes of promA (372 bp) and exon 3 (367 bp), SSCP analysis could be performed in order to verify the DHPLC results obtained for these amplicons and/or detect any mutation or polymorphism not detected by WAVE (see Appendix H.2).

SSCP is an efficient method often used for the detection of single base substitutions in small fragments of DNA. The electrophoretic mobility of single stranded DNA is dependent on both size and sequence. This is based on the fact that single stranded DNA fragments form specific secondary structures while moving through a non-denaturing gel. Small DNA molecules differing by even as little as one base take on different conformations and migrate at different speeds in a non-denaturing polyacrylamide gel (Orita et al 1989; Sheffield et al 1993).

PCR products were subjected to non-denaturing MDE-SSCP in the presence and absence of glycerol. Glycerol acts by lowering the pH of the gel for better detection of larger fragments (Kukita et al 1997).

#### **4.4.2 Sequence Analysis**

Relevant PCR products of DNA used as wild type controls for DHPLC and SSCP analysis, as well as samples that showed aberrant mobility shifts on SSCP or DHPLC analysis were subjected to direct sequencing, to determine the precise sequence variations.

PCR products (50 - 100 µl) were purified for sequence analysis (see Appendix I) using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences,

Buckinghamshire, UK). Purified samples were then sequenced in both forward and reverse directions on an ABI 3130 XL DNA automatic sequencer using a Big Dye terminator cycle sequencing kit, by the University of Stellenbosch Genetics Department, Stellenbosch, South Africa. Sequence data were collected and analysed using BioEdit Sequence Alignment Editor Version 5.0.9.1 (Tom Hall, North Carolina State University, USA). To ensure that the software did not overlook any heterozygous changes, sequence electropherograms were also analysed for visible sequence alterations.

For the detection of homozygous sequence changes, the computer software Sequence Manipulation Suite (SMS) (Paul Stothard, Copyright © 2000) was used to reverse compliment all reverse sequences generated. These reversed sequences were then aligned with Genbank data (<http://www.NCBI.nlm.nih.gov>) and forward sequences generated, using BioEdit. BioEdit uses a form of ClustalW (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html>) for aligning sequences.

#### **4.5 Serum Soluble CTLA4 (sCTLA4) Quantification**

In order to compare the protein concentrations of sCTLA4 in the blood serum of patients at various stages of their disease, relevant blood serum samples were obtained (see section 4.1.1; page 33) and ELISAs performed.

Blood samples were collected and allowed to clot before centrifuging at 3,000 Xg for 5 min. All serum samples were stored in aliquots at -80°C and repeated freeze-thawing was avoided.

Samples that had a visible precipitate were clarified by centrifugation before analysis, and those that were grossly haemolysed were excluded from the study.

### **4.5.1 ELISA**

Concentrations of sCTLA4 protein in the blood serum of AIH patients at various stages of disease, were measured using the Human sCTLA-4 ELISA kit (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's protocol (see Appendix J).

Sandwich ELISAs are a form of immunosorbent assay commonly used for the quantitative detection of specific proteins in test samples such as blood serum. Microwell plates are coated with a monoclonal antibody directed against the desired protein (in this case, anti-sCTLA4 monoclonal antibody). Serum is then added to the wells and the protein of interest allowed to bind to the antibody in the wells. A second, biotin conjugated capture-antibody directed against the same protein is added, "sandwiching" the protein between two layers of antibodies. After washing the sample wells to remove any unbound biotin conjugate, streptavidin horse radish peroxidase (HRP) is added which binds to the biotin conjugated antibody. The HRP gives a colour product in the presence of a substrate that can be quantitated by measuring the absorbance at 450 nm. Protein concentrations are determined with the use of a standard curve produced using known protein concentrations.

### **4.6 Statistical analysis**

All data was sent to a Medical Research Council (MRC) statistician for statistical analysis. Numerical parameters, such as age at diagnosis, serum ALT activity, serum ALB and INR, were compared between groups using the Wilcoxon-Mann-Whitney test. Assessments of associations between pairs of categorical variables, such as ethnic group, sex and genotype, were performed with the Fisher's exact test. The T-test for paired observations was used to investigate possible trends in ELISA data. P-values of less than 0.05 were considered significant, while P-values of less than 0.01 were considered highly significant.

The control cohort was tested for Hardy Weinberg equilibrium at the -318, +49 and CT60 loci. This was done to assess the consistence of the cohort with Mendelian inheritance and to exclude the possibility of genotyping errors.

University of Cape Town

# Chapter 5

## Results

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### 5.1 Isolation of DNA

DNA was successfully isolated from 2.5 ml of blood from patients and controls, with an average DNA yield of  $\pm 300$  ng/ $\mu$ l. The OD<sub>260/280</sub> ratios of 118 of the 122 samples were between 1.5 and 2 rendering them suitable for inclusion in the study. The remaining four samples yielded DNA of low purity and concentration and were subsequently excluded from the study.

### 5.2 PCR of Samples for Restriction Enzyme Analysis and Polymorphism Screening

PCR products of the *CTLA4* gene promoter region (promA), exon 1 and the UTR 3' of exon 4 (*CTLA4*3' UTR) were successfully obtained from the DNA of the study and control cohorts, for use in the investigation of the known polymorphisms. Additionally, PCR products of the above regions, as well as exons 2, 3 and the translated part of exon 4, were obtained from the DNA of the patients in the family-based study cohort, as well as from randomly selected 'wild type' individuals, for detecting any novel DNA sequence variations (mutations or polymorphisms) (see section 5.3; page 44). Figure 5.1 illustrates PCR products obtained for the *CTLA4*3' UTR amplicon in 8 of the patients from the study cohort. Due to low DNA yield, PCR products could not be obtained for certain amplicons in 4 of the 118 samples from the study cohort and 1 of the 57 samples from the control cohort utilised in this study. This resulted in the variable numbers of patients and controls in the experiments described below.

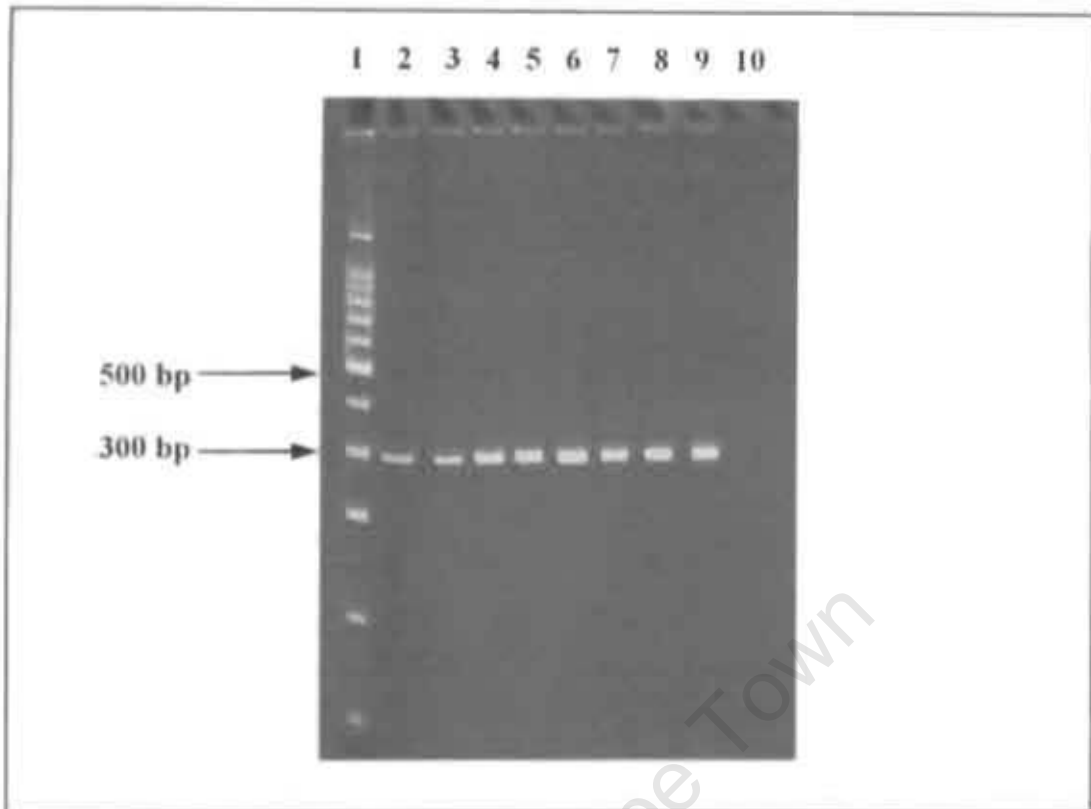


Figure 5.1 6% PAGE of the *CTLA4* 3' UTR PCR products. Lane 1 contains a 100 bp molecular weight marker and lane 10 a negative control (no DNA). Lanes 2-9 represent the PCR products of 8 samples from the study cohort. The sizes of all products obtained correspond to 296 bp.

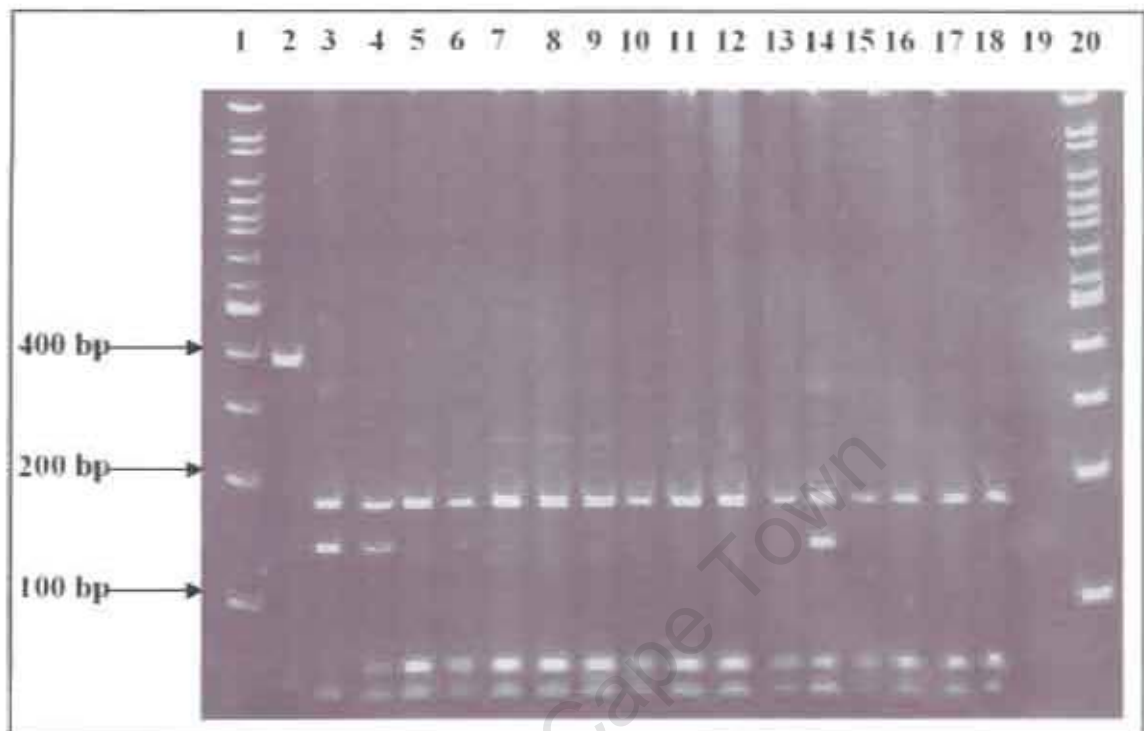
### 5.3 RE Analysis

The prevalence of known polymorphisms in our study and control cohorts was successfully determined using RE analysis.

#### 5.3.1 *MnlI* Digest

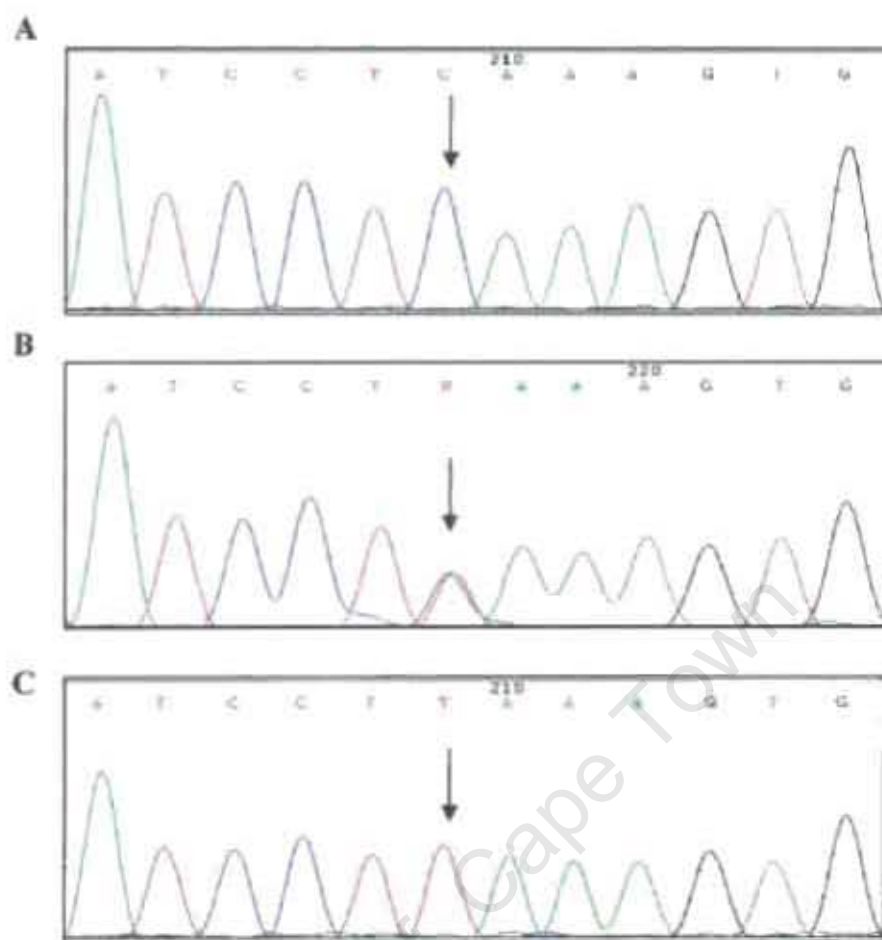
In wild type *CTLA4* promA amplicons, the RE *MnlI* cuts the 372 bp fragment at positions 188, 241 and 325, resulting in four fragments of various lengths (47 bp, 53 bp, 84 bp, 188 bp). However, the -318 C/T polymorphism results in the deletion of the *MnlI* restriction site (Deichmann et al 1996) at position 241 of the amplicons, leading to a loss of the 84 bp fragment. Figure 5.2 illustrates the *MnlI* digest products in 14 patients of the study cohort. Figure 5.3 shows the DNA sequences

representative of individuals that are wild type, heterozygous and homozygous, respectively, for the polymorphism at the -318 locus.

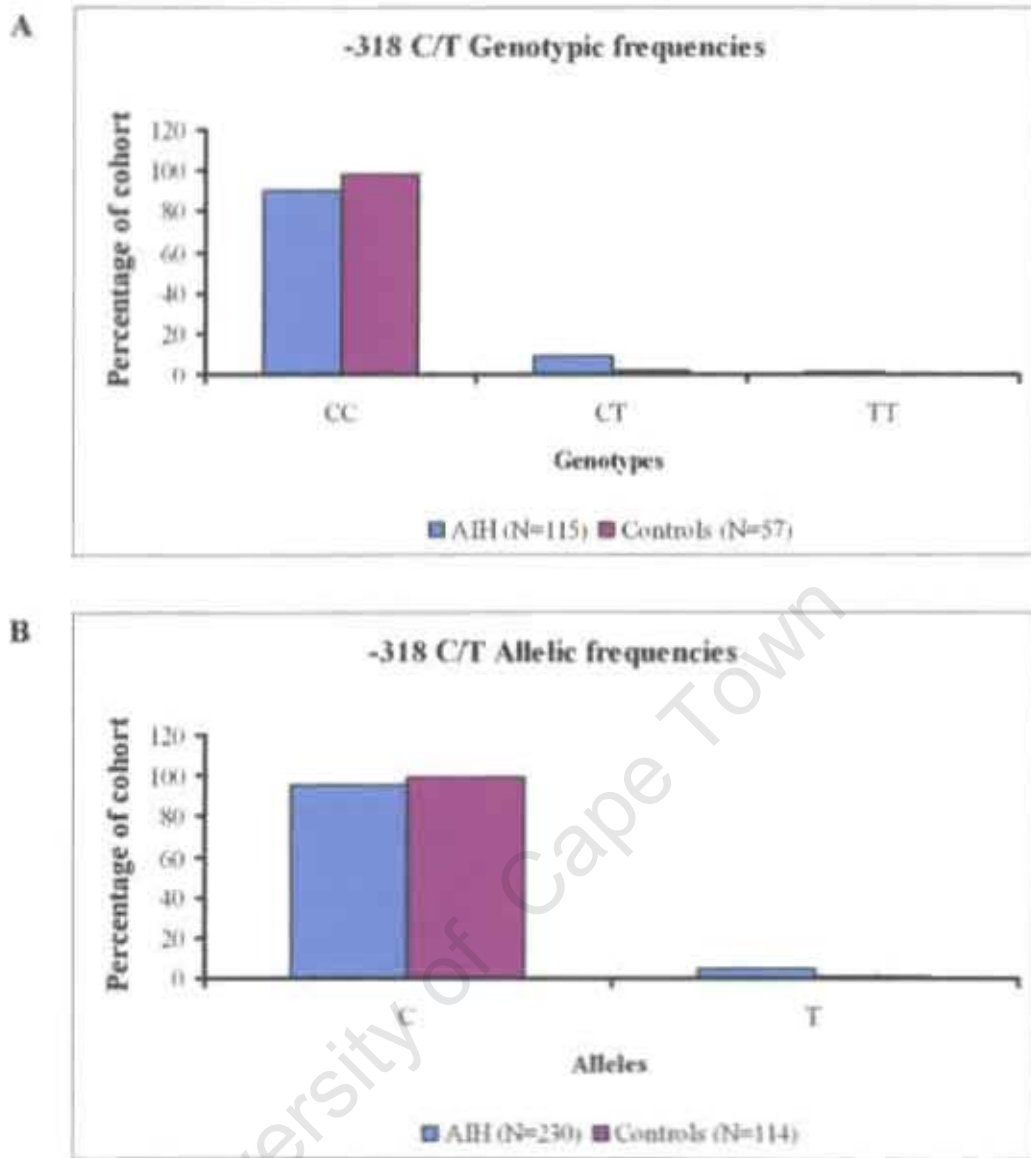


**Figure 5.2** 6% PAGE gel showing *MnlI* digest products in 14 DNA samples of the study cohort. Lane 1 and 20 contains 100bp molecular weight marker and lane 2 an uncut promA PCR product (372 bp). Lane 3 contains a homozygous positive control (-318 T/T) with three visible bands (47 bp, 137 bp and 188 bp). Lane 4 is a heterozygous control with the same three bands observed in lane 3 as well as two extra bands (53 bp and 84 bp). The 47 bp and 53 bp bands in the heterozygotes are indistinguishable from one another. The sample in lane 14 is heterozygous for the -318 C/T polymorphism that destroys a *MnlI* restriction site, while lanes 5-13 and 15-18 represents the wild type population.

A total of 115 samples from the AIH study cohort and 57 controls were screened with *MnlI*. Of the study cohort, 104 (90%) were C/C, 10 (9%) were C/T and 1 (1%) was T/T. Of the control cohort, 56 (98%) were C/C and 1 (2%) was C/T. There were no significant differences in the genotypic ( $p = 0.1281$ ) and allelic ( $p = 0.0679$ ) frequencies of AIH patients compared to that of the control cohort, as illustrated by the graphs in figure 5.4.



**Figure 5.3** Sequence electropherograms showing the forward sequences of promA. The position of the -318 polymorphism is indicated by arrows.  
**A:** The -318 wild type (-318 C/C) sample used as control  
**B:** Sample NH980, heterozygous (-318 C/T) for the -318 polymorphism  
**C:** Sample NH455, homozygous positive (-318 T/T) for the -318 polymorphism

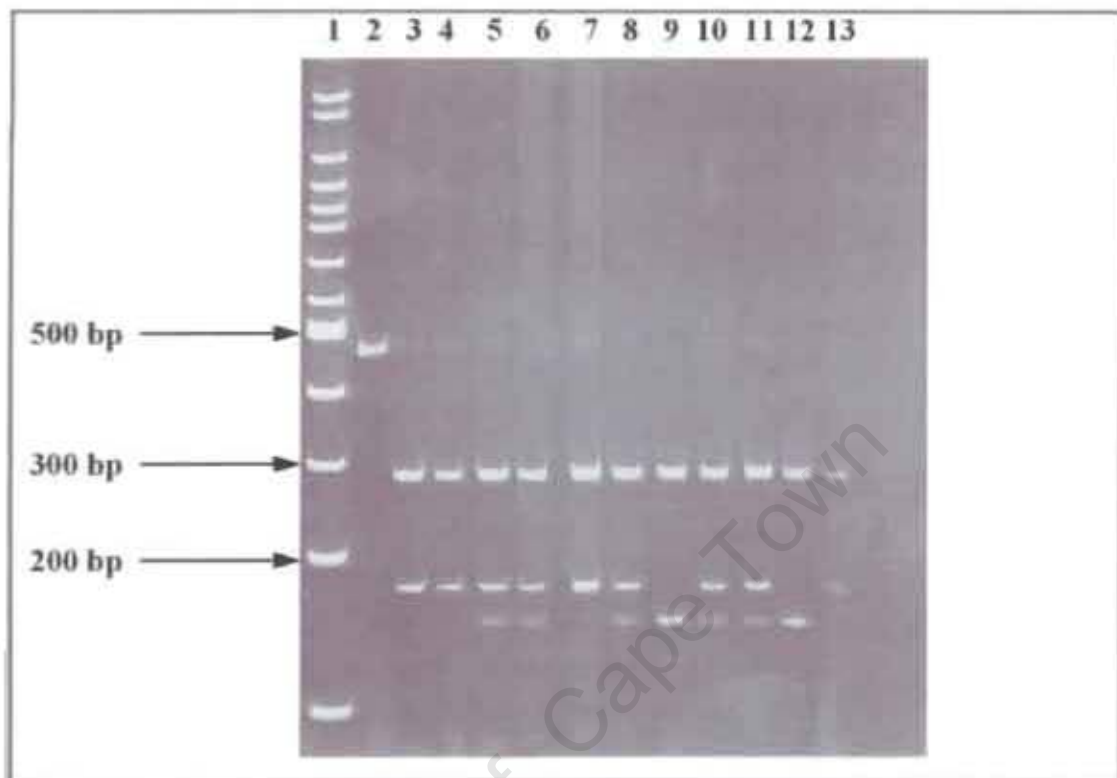


**Figure 5.4** Clustered column graphs of *MnlI* RE analysis data.  
**A:** Genotypic distribution of the -318 locus in patients and controls.  
**B:** Allelic distribution of the -318 locus in patients and controls

### 5.3.2 *Itai* digest

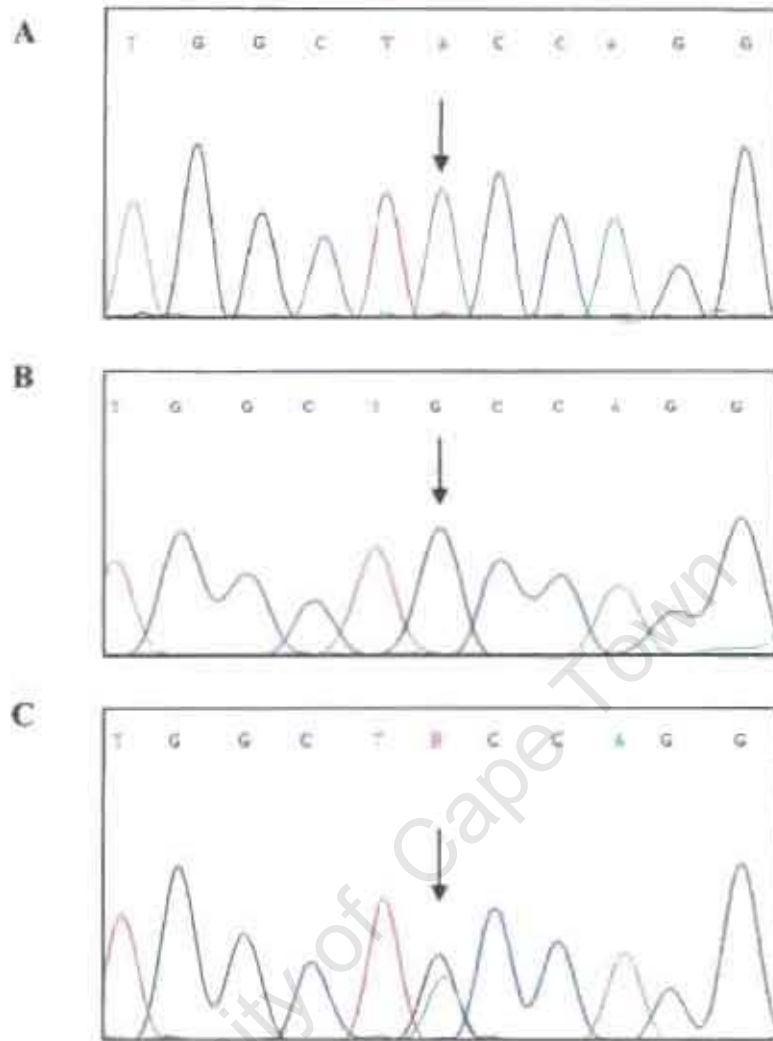
In wild type *CTLA4* exon 1 amplicons, the RE *Itai* cuts the 443 bp fragment at position 271, resulting in two fragments (172 bp and 271 bp). The +49 A/G polymorphism creates an *Itai* digestion site at position 296 of the exon 1 amplicon, in addition to the existing cutting site at position 271, resulting in three fragments (25 bp, 147 bp and 271 bp). Figure 5.5 illustrates the banding patterns observed after *Itai* digestion in 9 patients from the study cohort. Figure 5.6 shows the DNA

sequences representative of individuals that are wild type, heterozygous and homozygous, respectively, for the polymorphism at the +49 locus.



**Figure 5.5** 6% PAGE gel showing *ItaiI* digest products in 9 DNA samples of the control cohort. Lane 1 contains a 100bp molecular weight marker and lane 2 is an uncut exon 1 PCR product (443 bp). Lane 12 contains a homozygous positive control (+49 G/G) and two bands are observed: 147 bp and 271 bp. Another band of size 25 bp is expected. Due to the small size, it is not seen on this gel. Lane 13 contains a homozygous negative control (+49 A/A) with two visible bands of sizes 172 bp and 271 bp. The sample in Lane 9 is homozygous positive for the polymorphism. Lanes 5, 6, 8, 10 and 11 contain heterozygous (+49 A/G) samples, while lanes 3, 4 and 7 are wild type.

Of the 116 AIH patients screened with *ItaiI*, 48 were A/A (41%), 52 were A/G (45%) and 16 (14%) were G/G. Of the 56 controls screened, 22 were A/A (39%), 21 were A/G (38%) and 13 were G/G (23%). There were no significant differences in the genotypic ( $p = 0.3023$ ) and allelic ( $p = 0.3434$ ) distributions of AIH patients compared to that of the control cohort as illustrated by the graphs in figure 5.7.



**Figure 5.6** Sequence electropherograms showing the forward sequences of *CTLA4* exon 1. The position of the +49 polymorphism is indicated by arrows.  
**A:** The wild type (+49 A/A) sample used as control  
**B:** Sample PM, heterozygous (+49 A/G) for the +49 polymorphism  
**C:** Sample AC, homozygous positive (+49 G/G) for the +49 polymorphism

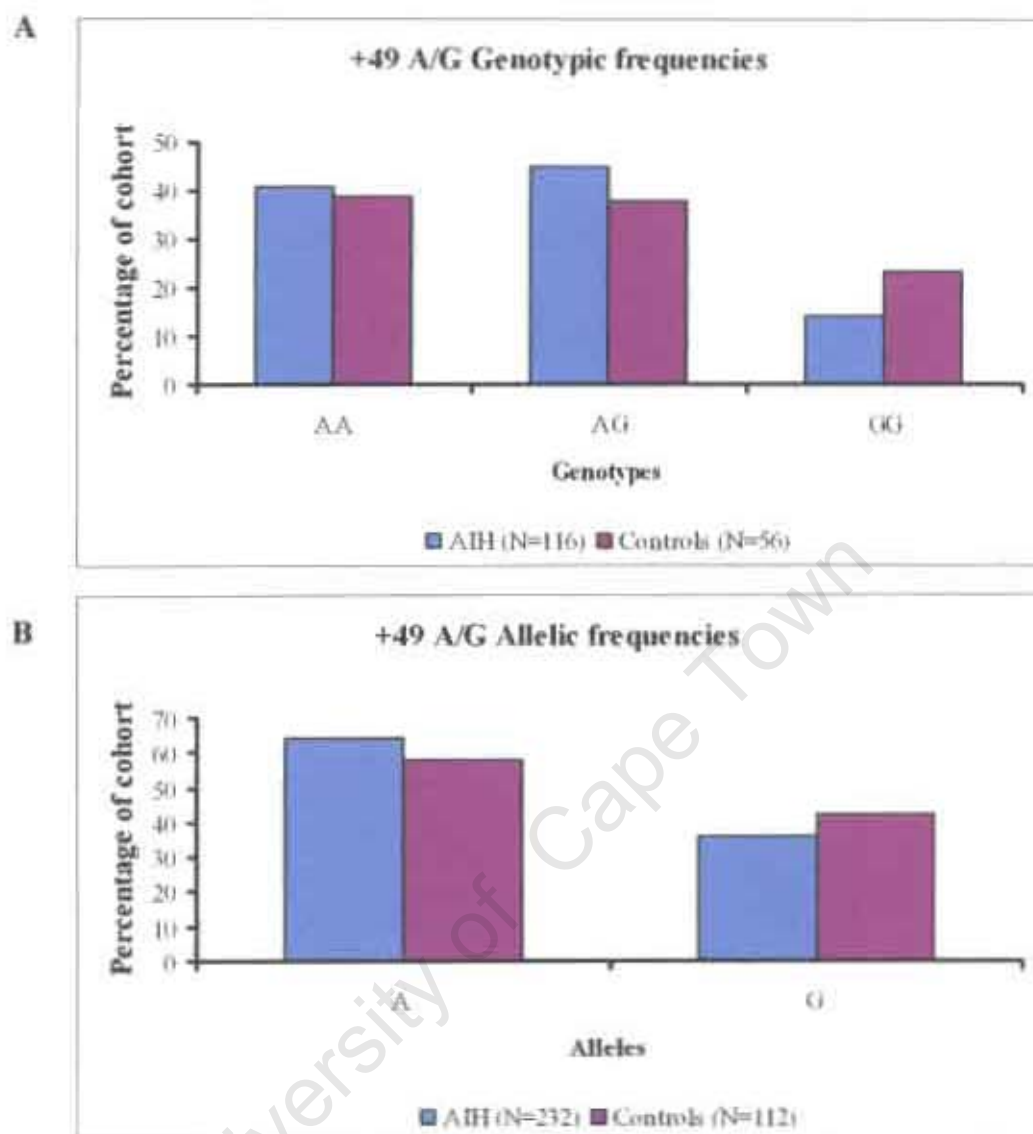
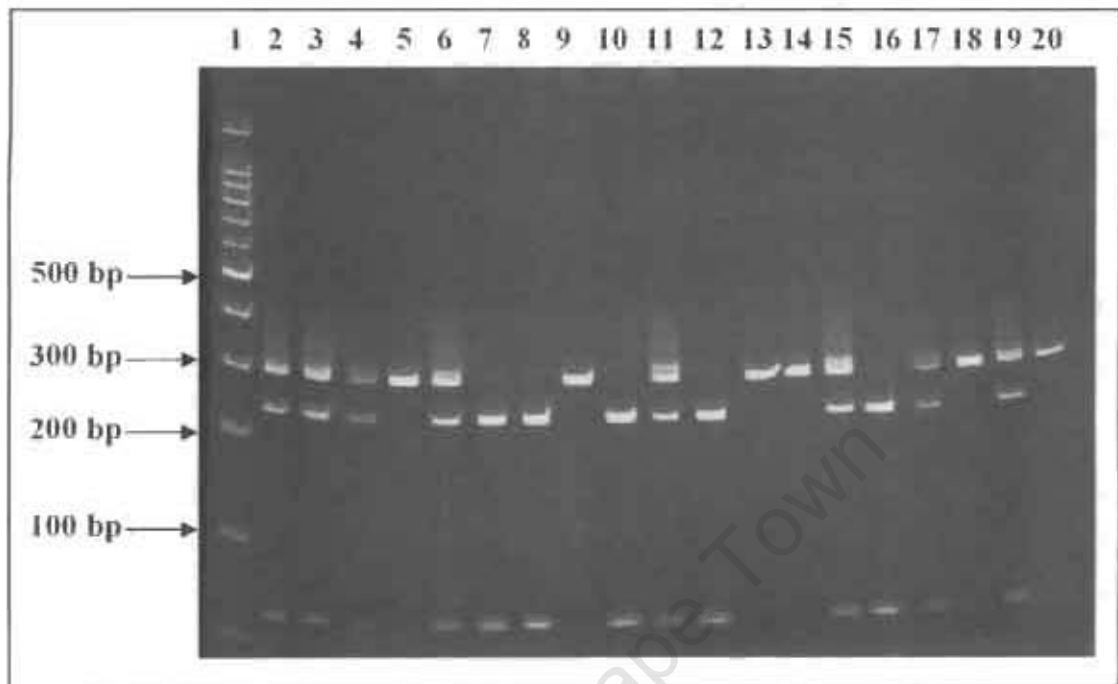


Figure 5.7 Clustered column graphs of *Itai* RE analysis data.  
 A: Genotypic distribution of the +49 locus in patients and controls.  
 B: Allelic distribution of the +49 locus in patients and controls

### 5.3.3 *HpyCH4IV* digest

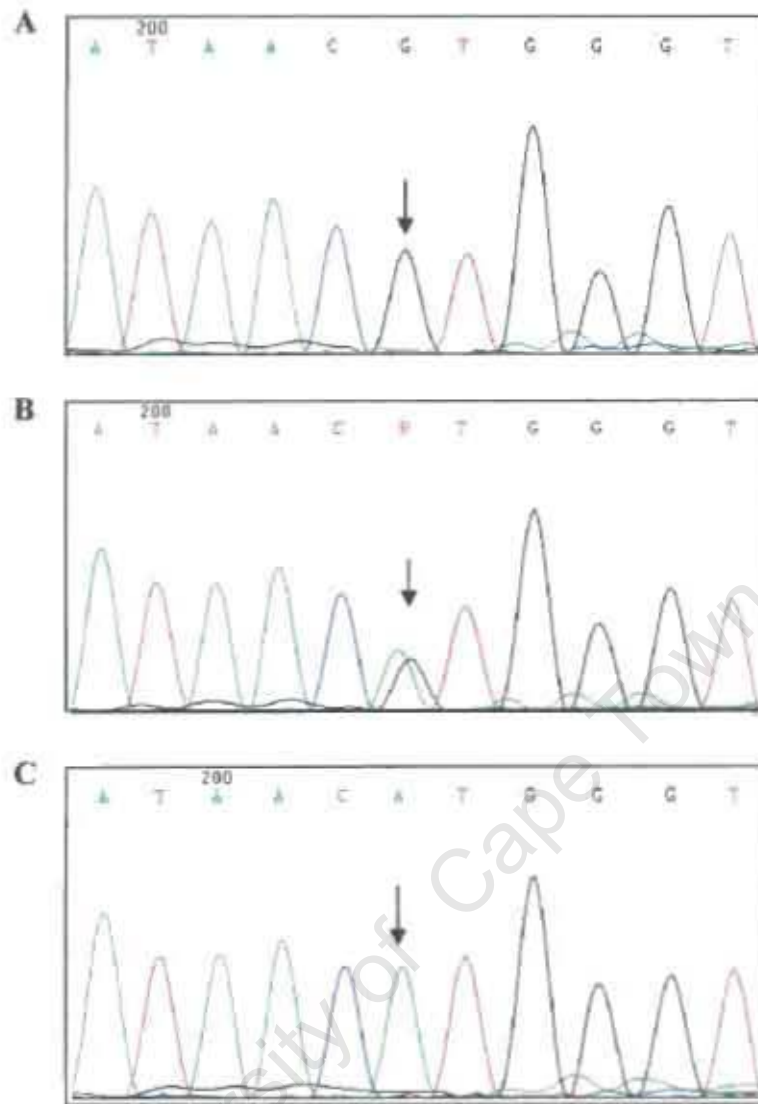
In wild type *CTLA4* 3' UTR amplicons, the RE *HpyCH4IV* cuts the 296 bp fragment at position 234, resulting in two fragments: 234 bp and 62 bp, respectively. The CT60 G/A polymorphism, however, results in the loss of the *HpyCH4IV* cutting site. Figure 5.8 illustrates the banding patterns observed after *HpyCH4IV* digestion in 18 individuals. Figure 5.9 shows the DNA sequences representative of individuals that

are wild type, heterozygous and homozygous, respectively, for the polymorphism at the CT60 locus.



**Figure 5.8** 6% PAGE gel showing *HpyCH4IV* digest products in 18 DNA samples of the patient cohort. Lane 1 contains a 100bp molecular weight marker and lane 20 an uncut 3' UTR PCR product (296 bp). Lanes 5, 9, 13, 14 and 18 contain samples that are homozygous positive for the polymorphism (CT60 A/A) and only one band is observed (296 bp). Lanes 2, 3, 4, 6, 11, 15, 17 and 19 contain heterozygous (CT60 G/A) samples and three bands of sizes 42 bp, 192 bp and 296 bp are observed, while lanes 7, 8, 10, 12 and 16 represents the homozygous negative (CT60 G/G) population with only the 42 bp and 192 bp bands present.

Of the 117 AIH patients screened with *HpyCH4IV*, 57 were G/G (49%), 45 were G/A (38%) and the remaining 15 were A/A (13%). Of the 57 controls screened, 27 were G/G (47%), 28 were G/A (49%) and 2 were A/A (4%). There were no significant differences in the genotypic ( $p = 0.1036$ ) and allelic ( $p = 0.5362$ ) distributions of AIH patients compared to that of the control cohort as illustrated by the graphs in figure 5.10.

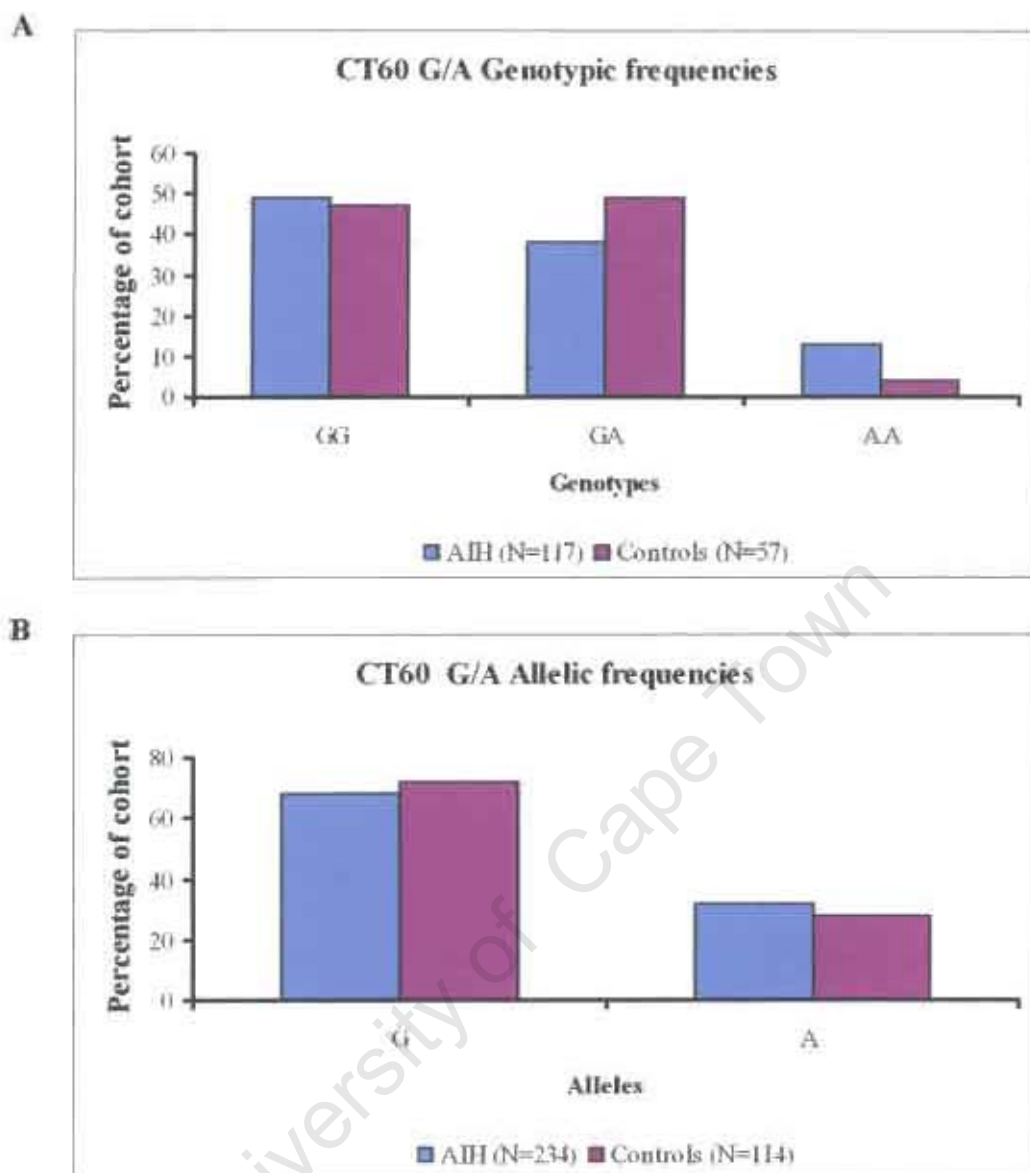


**Figure 5.9** Sequence electropherograms showing the forward sequences of the *CTLA4* 3' UTR amplicon. The position of the CT60 polymorphism is indicated with arrows.

A: Wild type sample (CT60 G/G)

B: Sample NH 286, heterozygous (CT60 G/A) for the CT60 polymorphism

C: Sample NH 326, homozygous positive (CT60 A/A) for the CT60 polymorphism



**Figure 5.10** Clustered column graphs of the *HpyCH4I*RE analysis data. **A:** Genotypic distribution of the CT60 locus in patients and controls. **B:** Allelic distribution of the CT60 locus in patients and controls

## 5.4 Genotypic and Allelic Correlations

Differences in genotypic and allelic frequencies amongst ethnic groups were investigated (see figure 5.11). The resultant p-values for the Fisher exact tests of equality of the proportions of genotypes over the groups were 0.2397 for -318 C/T,

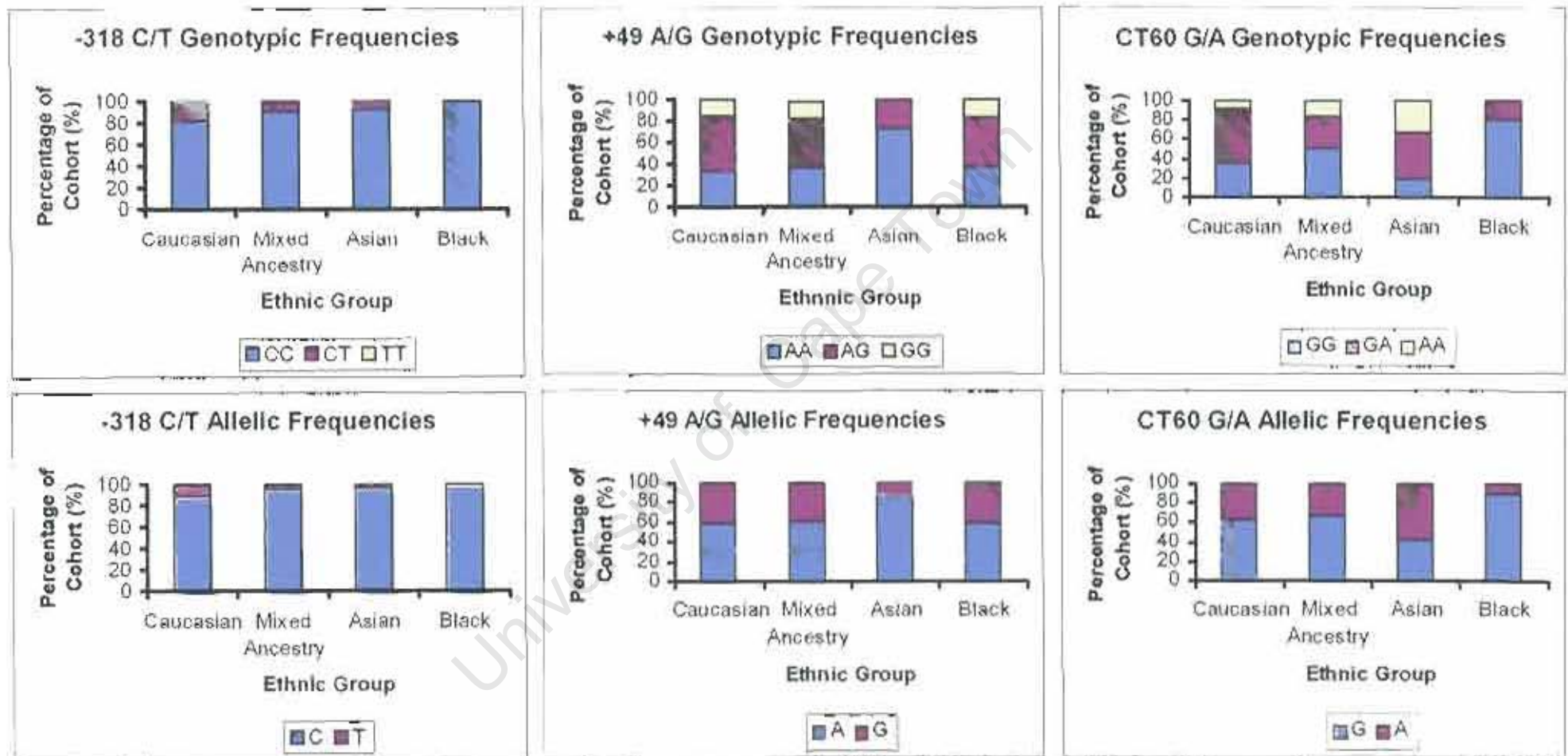


Figure 5.11 Genotypic (A) and allelic (B) frequencies of polymorphisms according to ethnic groups. Ethnic groups are indicated on the X axis, while the percentages of the respective groups are indicated on the Y-axis.

0.2512 for +49A/G and a significant 0.0006 for CT60 G/A. Similarly, there was a significant difference in allele frequencies at the CT60 G/A locus ( $p < 0.0001$ ).

We observed that the significant difference in the CT60 G/A polymorphism can probably be attributed to a relatively high prevalence of the polymorphism in the black group, compared to the other groups.

Possible genotypic frequency differences between sexes were also assessed. No significant differences were found. P-values for this are given in table 5.1. Similarly, there were no significant differences in allele frequencies for these polymorphisms between sexes.

**Table 5.1** Table of Fisher's exact test p-values for associations between genotypes and sex.

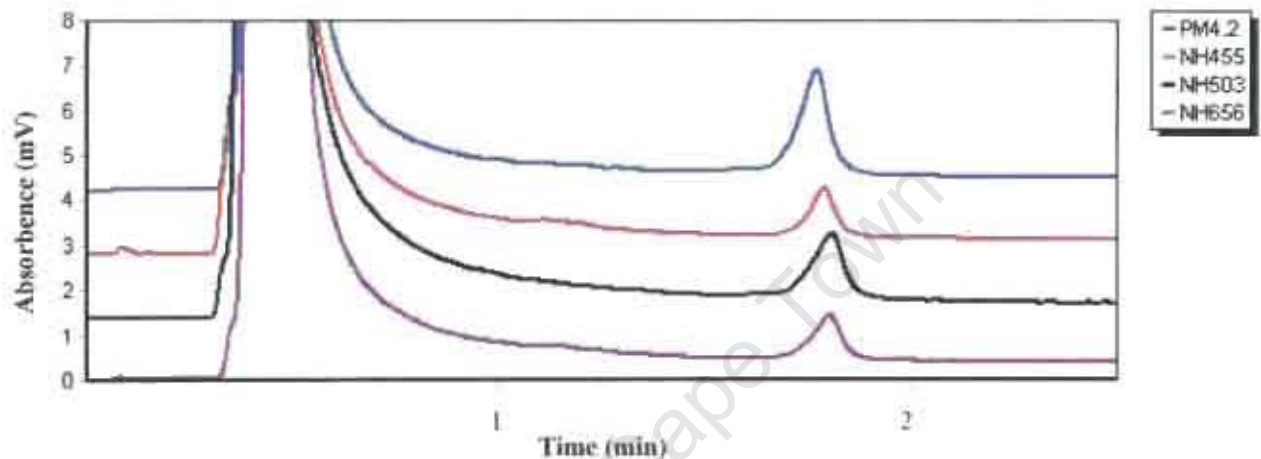
Polymorphism	Genotype-sex association p-value
-318 C/T	0.5354
+49 A/G	0.8564
CT60 G/A	0.2922

## 5.5 Screening for Novel Polymorphisms

PCR products from the twelve patients in the family based cohort were used in order to screen exons 1, 2 and 3, as well as the translated region of exon 4 and the promoter region of the *CTLA4* gene for novel DNA polymorphisms, using DHPLC. Furthermore, the relatively small product sizes of the exon 3 and the promA amplicons, made it possible to confirm the DHPLC results for these two fragments by SSCP.

### 5.5.1 DHPLC Screening (WAVE)

PCR products for which clear, single bands could be seen on PAGE, were used for DHPLC analysis. Figure 5.12 shows a chromatogram of the DHPLC analysis performed on 3 patient samples and a wild type control for *CTLA4* exon 2. Heteroduplex peaks were not observed for any of these samples. Overall, no novel DNA changes were identified in any of the amplicons screened with WAVE.

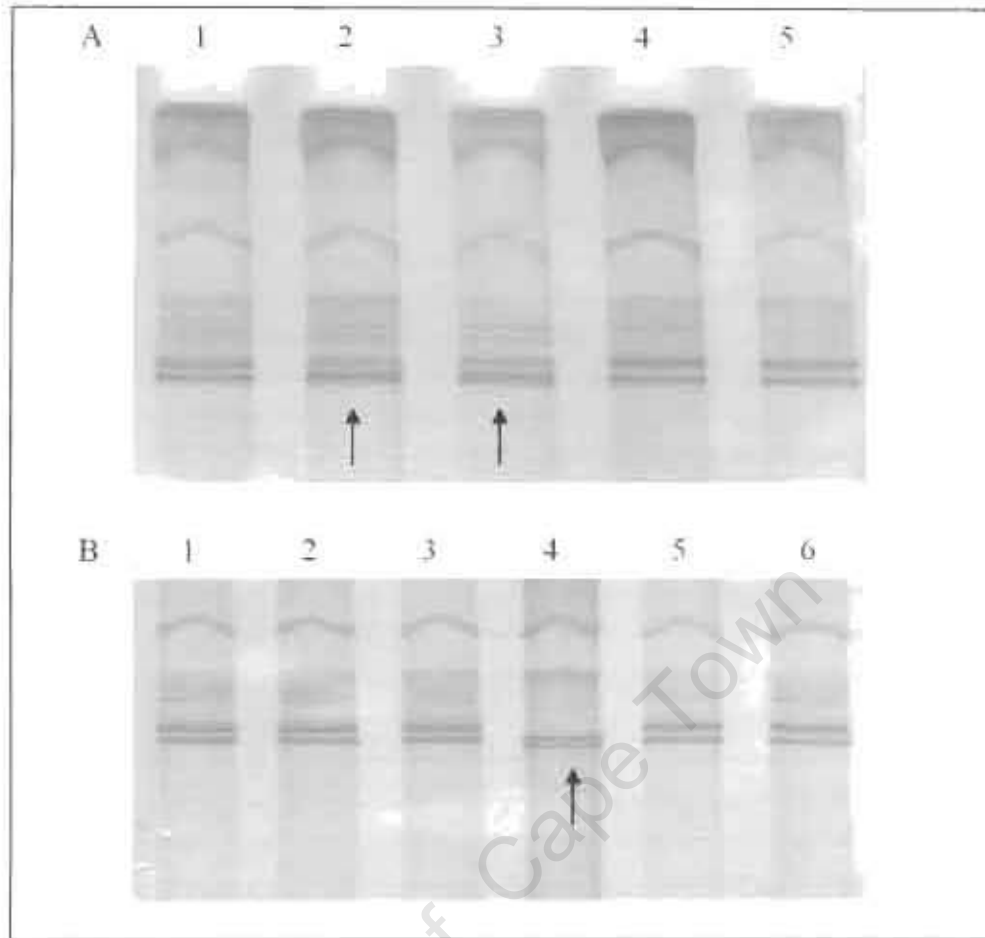


**Figure 5.12** Chromatogram results of the dHPLC analysis at 57.3°C for *CTLA4*, exon 2. The chromatograms for each of the three samples shown (NH455, NH503 and NH656) are not significantly different from each other or from the wild type sample (PM4.2) shown in blue.

### 5.5.2 SSCP

*CTLA4* promA and exon 3 PCR fragments were screened for possible sequence changes with SSCP. No DNA changes were identified with SSCP in exon 3 samples screened.

However, in the *CTLA4* promA amplicons, identical mobility shifts (changed patterns) were observed for samples NH659, NH980 and NH983, all of which were shown to be heterozygous for the -318 C/T polymorphism with RE analysis. Furthermore, two other samples (F6IIPA2 and NH455), showed identical patterns that differed from both the wild type and from the pattern mentioned above. These two samples were both shown to be homozygous for the -318 T allele with RE analysis. Figure 5.13 represents a selection of the *CTLA4* promA samples screened.



**Figure 5.13** SSCP results for *CTLA4* promA in patients from the family cohort. Mobility shifts were observed for samples NH980 (lane A2), NH983 (lane A3) and NH455 (lane B4) and are indicated by arrows. Lane B5 contains a negative control. The patterns observed for the samples in lanes A1, A4, A5, B1, B2, B3 and B6 were identical to that of the negative control.

One sample from each of the two patterns observed (NH980 and NH455) were sequenced in order to verify that there were no sequence variations, other than the ones mentioned above, present in these samples. No novel changes were identified.

## 5.6 ELISA analysis for sCTLA4 quantification

sCTLA4 concentrations for the 18 AIH patients selected from the study cohort were successfully determined using ELISA. Serum samples from these patients were collected at presentation with active disease, and again after three months of immunosuppressive treatment. The manufacturer's reference range indicates that there is no detectable sCTLA4 levels found in healthy donors.

A standard curve with a regression value (R) of 0.99 was drawn (see figure 5.14) from the OD<sub>450</sub> readings obtained for the sCTLA4 standards provided by the manufacturers. Using this curve, the sCTLA4 concentrations for each patient serum sample could be determined from their respective OD<sub>450</sub> readings (see table 5.2). The sCTLA4 concentrations were below 1.5 ng/ml at both stages of disease for all 18 patients serum samples quantified. Seven individuals showed an increase in sCTLA4 concentration, 5 a decrease, and the remaining 6 patients' were unchanged. Figure 5.15 plots the change in concentration of serum sCTLA in the 18 patients from presentation with active disease to three months after treatment. There was no significant change in sCTLA4 concentrations between the two time points of serum collection (p = 0.307).

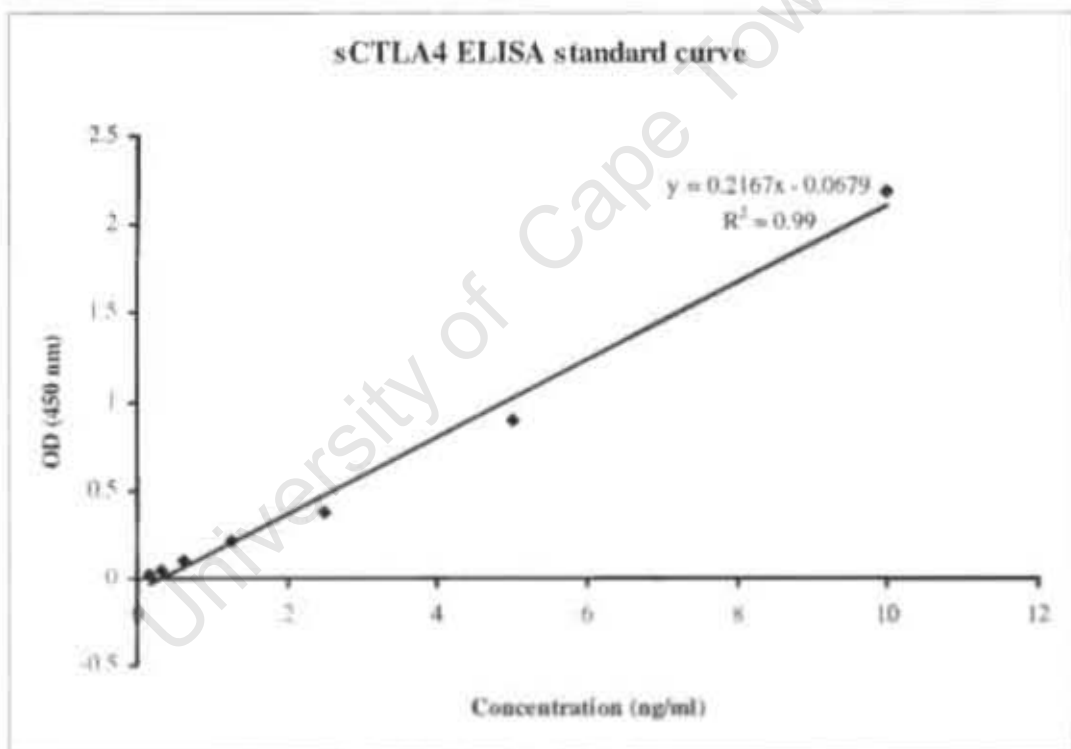
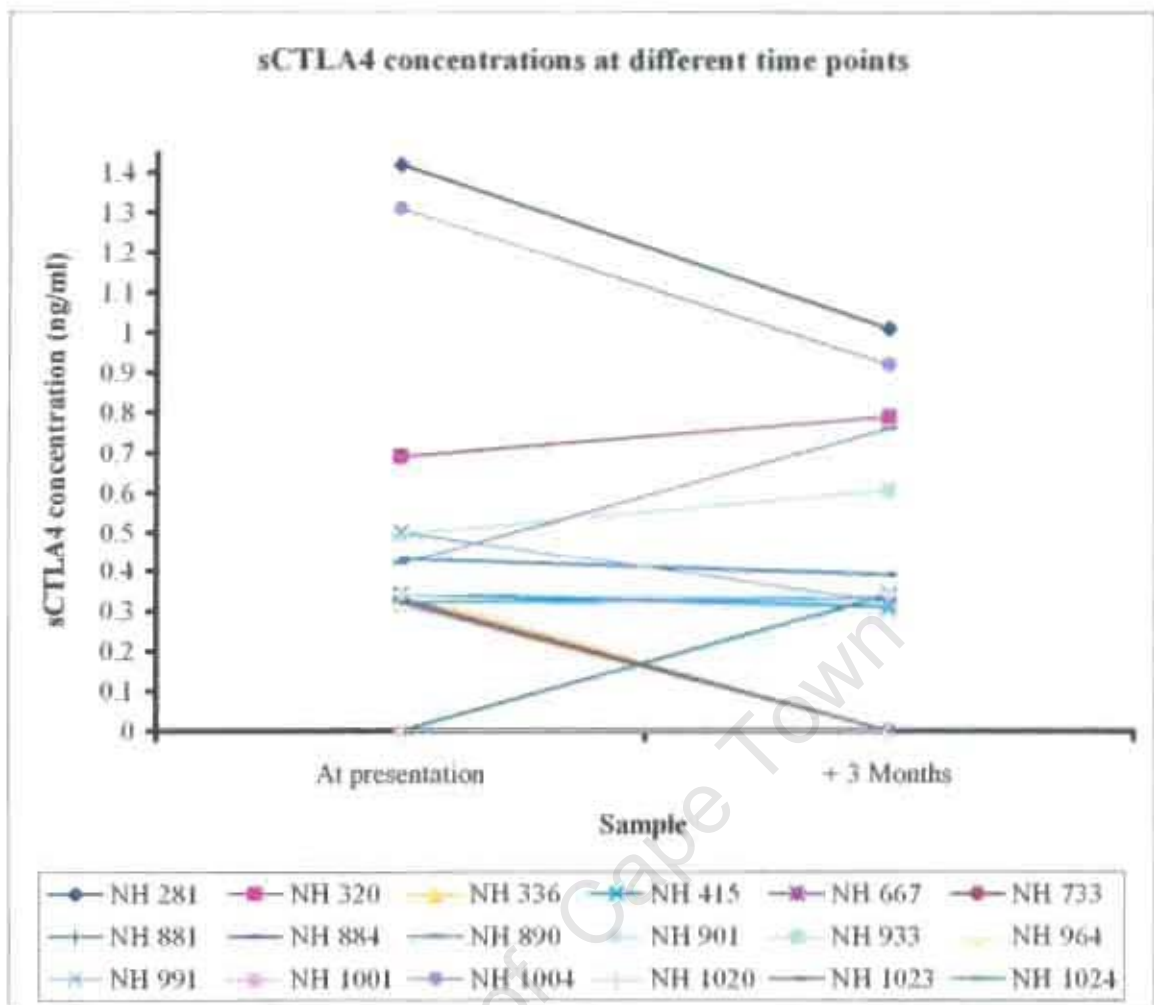


Figure 5.14 Standard curve determined using standardised concentrations of sCTLA4.

**Table 5.2 sCTLA4 concentrations of samples.** Concentrations were determined using the standard curve in figure 5.15. Samples that are labelled x.1 indicate serum samples collected from a patient at presentation with disease. Similarly, samples labelled x.2 were collected at a follow-up session three months after commencement of treatment.

Sample #	Concentration (ng/ml)	Sample #	Concentration (ng/ml)	Sample #	Concentration (ng/ml)	Sample #	Concentration (ng/ml)	Sample #	Concentration (ng/ml)
NH 667.1	0.00	NH 1020.1	0.00	NH 733.1	0.33	NH 415.1	0.34	NH 901.1	0.34
NH 667.2	0.34	NH 1020.2	0.00	NH 733.2	0.00	NH 415.2	0.31	NH 901.2	0.34
NH 1004.1	1.31	NH 281.1	1.42	NH 881.1	0.00	NH 1001.1	0.00	NH 933.1	0.50
NH 1004.2	0.92	NH 281.2	1.01	NH 881.2	0.34	NH 1001.2	0.00	NH 933.2	0.61
NH 1023.1	0.32	NH 991.1	0.50	NH 336.1	0.35	NH 320.1	0.69		
NH 1023.2	0.00	NH 991.2	0.32	NH 336.2	0.00	NH 320.2	0.79		
NH 1024.1	0.42	NH 964.1	0.32	NH 884.1	0.43	NH 890.1	0.32		
NH 1024.2	0.76	NH 964.2	0.00	NH 884.2	0.39	NH 890.2	0.33		



**Figure 5.15** Change in concentration of serum sCTLA4 in the 18 patients from presentation with active disease to three months after treatment.

## 5.7 Further Analysis Based on Patient Demographics

The patient cohort chosen for this study included all the patients from the UCT-MRC Liver Clinic at Groote Schuur hospital for whom DNA samples were available and of high enough quality for PCR amplification. It was thus appropriate (as defined in the study objectives) to analyse the groups further, based on sex, age, disease severity, age of onset, and ethnicity.

The sex distribution of the entire AIH cohort was 3.7:1 (Females:Males) and no significant differences were observed ( $p = 0.7186$ ) in the proportions of females versus males amongst ethnic groups (see table 5.3).

**Table 5.3 Gender, by ethnic distribution.**

	<b>Black African</b>	<b>Caucasian</b>	<b>Mixed Ancestry</b>	<b>Asian</b>	<b>All</b>
<b>Male</b>	6	8	7	4	25
<b>Female</b>	19	26	37	11	93
<b>All</b>	25	34	44	15	118

Analysis of data regarding the mean age of onset between sexes revealed that females present with AIH, on average, 13.47 years later than their male counterparts (see table 5.4) ( $p = 0.0002$ ). Furthermore, a significant difference was found in the mean ages of onset amongst ethnic groups ( $p = 0.0236$ ; when adjusted for sex,  $p = 0.0102$ ). This means that the mean of at least one group differs significantly from another. The significant difference observed was that caucasian patients from our study cohort presented with AIH, on average, 12.78 years later than black African, patients (see table 5.4).

**Table 5.4 Mean ages of onset, in years, between sexes and amongst ethnic groups.**

	<b>Black African (years)</b>	<b>Caucasian (years)</b>	<b>Mixed Ancestry (years)</b>	<b>Asian (years)</b>	<b>All (years)</b>
<b>Male</b>	20.3 *(7.3)	30.9 (16.1)	29.6 (15.2)	26.8 (12.1)	27.3 (13.4)
<b>Female</b>	34.3 (16.5)	47.7 (14.6)	37.2 (16.9)	44.1 (12.2)	40.4 (16.4)
<b>All</b>	30.9 (15.9)	43.8 (16.4)	36.0 (16.8)	39.5 (14.2)	37.6 (16.6)

\* Standard deviations are shown in brackets underneath the average ages

## 5.8 Disease Severity Markers

The ALT, ALB and INR data for all our patients were collected from the patient records at the UCT-MRC Liver Clinic at Groote Schuur hospital. These markers are commonly used as an indication of the severity of AIH (see section 2.2). The results are presented as medians in table 5.5 and table 5.6 and interquartile ranges (IQR) and p-values are given. There was a significant difference in the INR and ALB values between ethnic groups at a 5% confidence interval, but not for ALT. There were no statistically significant differences in these severity markers between sexes.

Table 5.5 Median liver biochemistry indices for the different ethnic groups.

	Black African	Caucasian	Mixed Ancestry	Asian	P-values
ALT (iu/ml)	148.5	184	415	214	0.0979
*( $<40$ )	(85.3; 332.3)	(85; 354)	(105; 876)	(96.5; 360)	
INR	1.55	1.00	1.30	1.30	0.0021
*( $<1.2$ )	(1.2; 1.8)	(1; 1.3)	(1.1; 1.9)	(1; 1.9)	
ALB (g/L)	30.5	38.0	35.0	35.0	0.0141
*(35-40)	(21.5; 35.3)	(33; 40.5)	(27.5; 40.5)	(25; 41)	

Liver biochemistry indices; alanine aminotransferase (ALT), international normalised ratio (INR) and albumin (ALB)

P-values compare liver biochemistry indexes amongst ethnic groups and were calculated using the Kruskal-Wallis test.

Interquartile ranges are indicated in brackets under each median

\* Normal ranges

**Table 5.6 Median liver biochemistry indices for the different sexes.**

	Male	Female	P-values
<b>ALT (iu/ml)</b>	172	216.5	0.7268
*(<40)	(89.5; 372.5)	(92; 524.25)	
<b>INR</b>	1.4	1.2	0.5153
*(<1.2)	(1.05; 1.7)	(1; 1.778)	
<b>ALB (g/L)</b>	34	35	0.7345
*(35-40)	(28.5; 39)	(28; 39)	

Liver biochemistry indices: alanine aminotransferase (ALT), international normalised ratio (INR) and albumin (ALB)

P-values compare liver biochemistry indexes between sexes and were calculated using the Wilcoxon-Mann-Whitney test.

Interquartile ranges are indicated in brackets under each median

\* Normal ranges

A possible association between age of onset (see Appendix A) and severity was investigated. Table 5.7 shows the p-values, as well as Kendal's coefficient of correlation between age and severity, as measured by the severity indices, ALT, INR and ALB. A significant negative correlation is seen for INR and a positive correlation for ALB values. The negative correlation for ALT was not significant.

**Table 5.7 Kendal's coefficient of correlation (Tau) between age of onset and severity, and associated p-values.**

Severity index	Tau	p-value
ALT	- 0.048	0.6150
INR	- 0.247	0.0002
ALB	0.185	0.0045

Disease severity indices were not significantly associated with any of the genotypes or alleles (not shown) of the polymorphisms investigated (see table 5.8). This was true with or without adjusting for age and/or ethnic group (data not shown).

Table 5.8 Median severity for each genotype investigated.

	-318 C/T			+49 A/G			CT60 G/A		
	CC	CT	TT	AA	AG	GG	GG	GA	AA
<b>ALT</b>	215 (94;628)	241 (119;366)	71 (71;71)	162 (86;506)	230 (113;428)	373 (83;718)	245 (102;474)	180 (87;399)	213 (91;859)
<b>INR</b>	1.3 (1;1.8)	1.05 (1;1.4)	5.2 (5.2;5.2)	1.4 (1;1.9)	1.2 (1;1.5)	1.6 (1;2.3)	1.35 (1;1.9)	1.1 (1;1.5)	1.4 (1.1;1.7)
<b>ALB</b>	35.5 (28;39)	33.5 (24.3;38)	27 (27;27)	35 (29.5;41)	36 (25;39)	35.5 (20.8;37.8)	35 (28;41.5)	35 (31;40)	35 (23.3;39)

Interquartile ranges are indicated in brackets under each median

# Chapter 6

## Discussion

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### 6.1 Subject Selection

Definitive diagnosis of AIH is sometimes difficult and may be complicated by the presence of overlapping features with other forms of chronic liver disease. Hence, all of the 122 patients in our study cohort had a diagnosis of AIH as defined by the International AIH group (Johnson et al 1993). Other forms of chronic liver disease, such as viral hepatitis, alcoholic liver disease, Wilson's disease and non-alcoholic fatty liver disease, were routinely excluded. All those patients who consented to the study during the recruitment phase were entered into a database.

The family based cohort used in the screening of the *CTLA4* gene for novel polymorphisms, consisted of a subset of female patients from the study cohort for whom DNA and consent from family members were available. These family members included individuals with and without AIH, or any other form of autoimmune disease. This was done to enable the investigation of a disease association of any possible DNA sequence variations found. We were unable to include male patients in this cohort due to the lack of availability of sufficient numbers of family members for any of the male patients in our cohort.

An inherent problem with complex genetic disease association studies is in finding the appropriate control cohorts. Control groups that are selected by phenotype or by age (hereafter referred to as "hypernormal" control groups) although controversial, are expected to improve the power of the study by increasing the difference in susceptibility alleles between the study and the control cohorts. Another option would be a control cohort of clear origin, such as all the individuals born in a certain

time period in a specified region (Hattersley and McCarthy 2005). However, we did not have access to any such groups that would be appropriate for this study.

A previously assembled control cohort that was recruited from the medical staff and students at Groote Schuur hospital in Cape Town, was not used in this study after 2.3% (4 of 171) were documented to have developed autoimmune conditions. Two cases of autoimmune hypothyroidism were noted from the medical staff group while 2 medical students developed inflammatory bowel disease with abnormal liver tests (possible primary sclerosing cholangitis) during the first 18 months following recruitment. This cohort had been recruited following the exclusion of autoimmune diseases using an oral interview that interrogated the participants regarding the presence of any symptoms suggestive of autoimmune diseases. No clinical examination or immunological or biochemical testing for possible autoimmune diseases had been performed. The occurrence of autoimmune disease in 2.3% of the cohort, picked up during a short period of time and without active surveillance of the cohort, was a major concern. It questioned the value of using such cohorts without active, objective screening for autoimmune disease.

Hence, we created an autoimmune disease hypernormal control group in whom AIH and other autoimmune diseases had been clinically and immunologically excluded. All patients presenting to the UCT Liver clinic with chronic hepatitis were routinely screened for AIH. Based on this, we managed to assemble a group of patients with confirmed viral hepatitis in whom other forms of chronic liver disease, including AIH, had been excluded, and who had no clinical or family history of any other form of autoimmune disease. However, the constitution and demographics of the HBV control group was affected by the following:

- The short time period of recruitment for the purposes of this study;
- The incidence of HBV infection in the different ethnic groups in South Africa affecting the representation of caucasian patients;
- The population demographics of the Western Cape Province of South Africa affecting the representation of black African patients;

- Possible referral bias to a tertiary specialist clinic. Caucasian patients tend to go to private hospitals or medical facilities, while rural black African patients attend clinics closer to home. Therefore, the majority of patients seen in our clinics were individuals from the mixed ancestry population group.

For these reasons, the control cohort used in this study could not be ethnically matched to the study cohort at the time of reporting these results. The result was that caucasian and black African patients were underrepresented in our control cohort, when compared to the study cohort. These limitations may be corrected by enlarging the control cohort for future studies. Where appropriate, during the statistical analyses, ethnic differences were taken into account when drawing conclusions. While mindful of the limitations, the current HBV cohort had major benefits in that it provided cases that were clinically and immunologically free of autoimmune disease. HBV infection is a non-immune disease that is endemic in southern Africa. The development of disease appears to be dependent on random clinical exposure to HBV rather than immunogenetics. HBV infection is entirely preventable with immunisations in all ethnic groups.

## **6.2 DNA Isolation and Amplification (PCR)**

Blood samples were collected from patients and the DNA isolated over a period of 2 years. All DNA samples were stored at -80°C. The DNA yield of 4 of these samples was very low, leading to problems with the PCRs. No fresh blood samples could be obtained from these patients. PCR products for these samples were obtained for some, but not all the amplicons of interest.

## **6.3 RE Analysis**

The RE analyses designed for the purpose of this study (see section 4.3.1; page 35) proved successful in genotyping patient and control DNA for the -318 C/T, +49 A/G and CT60 G/A *CTLA4* polymorphisms.

No significant differences were observed for genotypic or allele frequencies of the -318 C/T promoter polymorphism between the study and control cohorts. Power calculations (not shown) on our data indicated that, if our study cohort consisted of 200 individuals and our control cohort of 100 individuals, the observed genotypic frequencies at this locus would become significant ( $p = 0.0435$ ) at the 5% confidence interval. As mentioned in section 1.3.2.2, page 15, published data by Fan et al (2004) showed a significant association of the -318 C allele with AIH in Chinese patients. No associations have been found for the -318 C/T polymorphism with either autoimmune thyroid disease or SLE in European caucasian, Chinese, Japanese, Spanish or Korean patients (Heward et al 1998; Ahmed et al 2001; Aguilar et al 2003; Hudson et al 2002).

Only one of our patients and none of the controls were homozygous for the -318 T allele. Interestingly, the one patient that did have this genotype had very severe disease. This particular patient was a female caucasian, aged 20 years. She had high INR (5.2) and low ALB levels (27 g/L) and was deeply jaundiced at presentation. Submassive necrosis was found on the liver biopsy with features of bile duct injury suggestive of an overlap syndrome. This patient also had a strong family history of atopic eczema and other autoimmune diseases including psoriasis, IDDM and RA. No conclusions can yet be made on the basis of this. However, it would be interesting to investigate the possibility that the -318 T allele relates to severity of disease. Published data from two previous studies on the -318 C/T polymorphism indicated an association with Wegener's granulomatosis in Swedish patients, and a possible role for the C to T change in the regulation of *CTLA4* expression (Giscombe et al 2002; Wang et al 2002). Initial work by Giscombe et al (2002) showed that the -318 C allele was significantly decreased and the -318 C/T genotype significantly increased in patients with Wegener's granulomatosis compared with controls. No homozygous individuals in either Wegener's granulomatosis patients or healthy controls could be demonstrated. In a follow-up study, Wang et al showed an association of the -318 T allele with higher *CTLA4* promoter activity than the -318 C allele. The group therefore proposed that the -318 T allele is protective against autoimmune reactions in Swedish patients (Wang et al 2002). Our findings, although based on the data from only one patient, suggests that the -318 T allele, rather than

being protective, is associated with autoimmune disease and the presence the -318 T/T genotype results in a clinical phenotype of a more severe form of disease. In order to clarify the role of the -318 T allele in South African AIH patients, the effect of this polymorphism on promoter activity and expression of the CTLA4 protein needs to be investigated. In order to do this, more patients and controls with the -318 T/T genotype are required.

The frequency of the *CTLA4* +49 G/G genotype appeared slightly lower in the study cohort when compared with the controls (see figure 5.7; page 50). However, the differences observed between our study cohort and controls were not statistically significant. Power calculations (not shown) indicated that, if our study cohort consisted of 300 individuals and our control cohort of 200 individuals, the observed genotypic frequencies at the +49 A/G locus would become significant ( $p = 0.0238$ ) at the 5% confidence interval. Therefore, a larger study would increase the statistical power of our data.

If the observed decrease in the +49 G/G genotype frequency of our study cohort was found to be significant in a larger study, this would indicate a possible protective effect for the +49 G/G genotype. This would be in agreement with published data from Djilali-Saiah et al (2001). In previous pilot studies we have shown a lack of association between the +49 A/G polymorphism and AIH or SLE (Hairwadzi et al 2002). This finding, as well as the possibility of an observed decrease in the frequency of the +49 G/G genotype becoming significant with increased power for the study, contrasts with other published data. Indeed, previous studies have shown an association of the +49 G/G genotype with AIH in northern European and Chinese patients, IDDM in Iranians and SLE in Japanese patients (Agarwal et al 2000; Fan et al 2004; Mojtahedi et al 2005; Ahmed et al 2001). No association was found between the +49 A/G polymorphism and SLE in Spanish and Korean patients (Aguilar et al 2003; Hudson et al 2002).

Similarly, the frequency of the CT60 A/A genotype appeared slightly higher in our study cohort, when compared to the controls (see figure 5.10; page 53). Once again

this observed difference was not found to be statistically significant. A larger study and control cohort is needed in order to accept or reject this finding. Power calculations (not shown) showed that for a study cohort of 200 individuals and a control cohort of 100 individuals, the same observed frequency would become significant at the 5% confidence interval. Ueda et al (2003) described the CT60 G allele, and subsequently the CT60 G/G genotype, as disease-predisposing in autoimmune hypothyroidism and Graves' disease, while the effect was much weaker in IDDM. Furthermore, a study by Van Belzen et al (2004) showed an equivocal significant increase ( $p = 0.048$ ) in the CT60 G allele in Dutch coeliac disease patients. However, no significant difference was found by this group when they compared the genotypic frequencies of patients with that of controls.

Our study demonstrated a highly significant difference amongst ethnic groups at the *CTLA4* CT60 locus ( $p = 0.00056$ ). The observed frequencies showed a clear overrepresentation of the *CTLA4* CT60 G/G genotype in our black African patients, while the CT60 A/A genotype was completely absent from this group. Asian patients had the highest frequency of A/A genotype and the lowest frequency of G/G genotype. Similarly, the differences in allelic frequencies at this locus were highly significant (see section 5.4; page 53 and figure 5.11; page 54). This should be further investigated with larger numbers of individuals from black African and Asian patient groups, and the results would have to be compared to ethnically matched control cohorts with sufficient statistical power.

## **6.4 Mutation Screening**

No new polymorphisms or mutations were identified within our family-based cohort. As mentioned in section 6.1; page 65, these patients were chosen on the basis of the availability of affected and unaffected family members with autoimmune disease for follow-up studies. Therefore, the number of patients recruited for this section of the study was small and a bigger cohort is required to confirm / refute these findings.

## 6.5 ELISA

There is conflicting data on the normal range for sCTLA4 levels in human serum. Published data by Purohit et al (2005) and Wong et al (2005) found median concentrations of 1.69 ng/ml (range 0.0-11.5 ng/ml) and 1.61 ng/ml (range 1.32-1.85 ng/ml), respectively, for normal controls, while Magistrelli et al (1999) reported a median concentration of 36 ng/ml (range 3-90 ng/ml). Oaks and Hallet (2000) found the concentration of sCTLA4 in their healthy individuals to be “virtually undetectable”. We accepted the manufacturer’s analyses that there are no detectable levels of sCTLA4 in healthy patients.

To our knowledge, no previous studies have investigated the concentration of sCTLA4 patients with AIH. The serum sCTLA4 concentrations of our AIH patients were below 1.5 ng/ml regardless of the stage of disease activity. However, further studies are needed to investigate the normal range of sCTLA4 in healthy South African individuals and those with other autoimmune disease, in order to confirm that our patients’ results do not differ significantly from normal. Available funding for this project and the high cost of the sCTLA4 kits prohibited us from establishing a local control range, and we deemed it acceptable to follow the manufacturers findings. Our focus was on whether or not there is any detectable, significant correlation in sCTLA4 concentrations with disease severity.

The serum sCTLA4 concentrations of our AIH patients were very low to undetectable in all cases. Furthermore, there was no significant difference between serum sCTLA4 concentrations from serum samples taken at presentation of active AIH, and those taken after three months of immunosuppressive treatment and achievement of disease control. This suggests that the concentration of sCTLA4 is not associated with disease activity in South African patients with AIH. However, a more sensitive test, when available, might be valuable to confirm our results.

Further studies investigating the differences of sCTLA4 concentrations at these two time points should be performed in patients with other autoimmune conditions, such

as SLE and autoimmune thyroid disease. Published data in these diseases indicate higher concentrations of the soluble form of the protein at presentation with disease (see section 1.3.2.3; page 17). Such studies may clarify a role for sCTLA4 in various stages of autoimmune disease and allow us to further interpret our AIH results.

## 6.6 Patient Demographics

Black African patients from our study cohort were, on average, 12.78 years younger than caucasians at presentation with AIH. This is in agreement with preliminary studies described in section 2.4; page 27. This indicates that black African patients in South Africa present with disease at a younger age than caucasian patients. The reason for this is still unclear but could be due to a number of factors. For example, there could be further clinical factors, such as exposure to pathogens, or genetic or cultural factors, such as environment and living conditions. Further studies would need to investigate all these possibilities, to fully elucidate the reasons for our findings.

Female patients across all ethnic groups in our study cohort were, on average, 13.47 years older at presentation than their male counterparts. To our knowledge, no other studies have shown this before. Once again, the reasons for this need to be investigated in future studies. Possible determinants for this could include hormonal or genetic differences and/or cultural norms. Thus, studies correlating liver autoantibody profiles, HLA profiles and other factors affecting immune modulation with sex could be informative.

## 6.7 Disease Severity Markers

ALT, INR and ALB values were used as standard markers of disease severity in our study cohort. These indices are widely used as markers of liver function. In order to determine whether the *CTLA4* polymorphisms, -318 C/T, +49 A/G and CT60 G/A, played a significant role in disease severity, we investigated any possible association between these polymorphisms and these indices. The disease severity indices of the AIH patients were not significantly associated with any of the genotypes or alleles of

the polymorphisms investigated. This was true with or without adjusting for age and/or race.

Kruskal-Wallis test p-values indicated a significant difference in INR and ALB medians amongst ethnic groups. The biggest difference was observed between the black African and caucasian groups. The median ALB level in black Africans was lower by 7.5 g/L, compared to caucasians and the median INR was 0.55 higher in black Africans compared with caucasians. Since ALB and INR are both direct measures of liver function, these differences indicate that black African patients tend to have more severe disease than their caucasian counterparts. No significant difference was found for the ALT values amongst ethnic groups. High ALT concentrations only indicate the presence of liver injury and in no way correlates with disease severity. No significant difference was found in the median severity markers between sexes.

Previous studies have suggested that older patients with AIH tend to have more severe disease, histologically, at presentation compared to their younger counterparts (Newton et al 1997). It is not clear what the reasons for this are. It is also not clear whether such disease at an older age is *de novo* AIH or a clinical presentation of previously ongoing, asymptomatic AIH. Our study showed that INR correlated negatively with age, while ALB correlated positively with age. The ALT showed no significant correlation with age. In other words, INR appears lower, and ALB higher with advanced age of first presentation of AIH. This finding suggests that older South African patients have biochemically less severe disease at first diagnosis compared to their younger counterparts.

# Chapter 7

## Conclusions and Future Work

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This study is the first to investigate all the known *CTLA4* gene polymorphisms, with the exception of the (AT)<sub>n</sub> microsatellite repeat in the 3' UTR, in AIH, and attempt to correlate this with ethnicity, sex, disease activity and severity. It is also the first to investigate sCTLA4 protein in AIH during active (at presentation) and inactive disease (after 3 months of treatment).

### 7.1 Conclusions and Future studies

- No new polymorphisms or mutations were identified in those South African patients with AIH in our cohort. However, new polymorphisms or mutations may be revealed in AIH patients as yet not studied.
- The *CTLA4* -318 C/T promoter polymorphism, the +49 A/G polymorphism in exon 1, and the CT60 G/A polymorphism in the UTR 3' of *CTLA4* were not significantly associated with disease in South African AIH patients. However, a larger cohort may be needed in order to verify this.
- At presentation, black African patients from our AIH cohort tend to have more severe disease at a younger age than their caucasian counterparts. Although the reason for this remains unclear a significant difference in the frequency of the CT60 G/G genotype amongst ethnic groups was observed. Black African AIH patients in this study showed a higher frequency of this genotype compared to other ethnic groups, while the CT60 A/A genotype was completely absent. The possibility that this could contribute to disease susceptibility or severity in black African patients needs to be further investigated.

- South African female AIH patients tend to be older at presentation than their male counterparts. Once again, the reasons for this need to be investigated.
- The significance of the rarity of the -318 T/T genotype needs to be investigated. Our study suggests the possibility that the T allele associates with acute severe presentation of AIH in young patients. However, as this observation is based on data from a single patient, it is unwise to draw any conclusion at this stage. Thus, whether or not the -318 T allele relates to severity of disease and the possible reasons for the rarity of the -318 T/T genotype in our population are intriguing questions requiring further investigation.

To answer such questions a follow-up study prospectively investigating patients who present with acute, severe disease should be performed. To date such patients have been difficult to study as they often die before they can be legitimately entered into such a study.

- sCTLA4 concentration did not correlate with disease activity in AIH in South African patients. However, measurement of sCTLA4 in larger numbers of AIH patients, other autoimmune conditions and in a local, control population will confirm this and/or allow further interpretation.
- The influence of *CTLA4* polymorphisms on disease susceptibility and severity, if at all relevant, must be small, or as yet, not clear. Polymorphisms may work in a “facilitating” manner along with other genetic and/or external factors. Possibly a larger study cohort, which combines patients and data from various centres around the world, may shed light on a role for *CTLA4* in AIH.

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# Appendix A

## Clinical Description of Study Cohort

**Table A.1** Clinical description of the General AIH Study Cohort. Where possible, patients' sex, ethnicity and age are indicated together with their liver biochemistry indexes: alanine aminotransferase (ALT), international normalised ratio (INR) and ALB (albumin). The indexes ALT, INR and ALB are important measures of liver inflammation and are therefore important in assessing the severity of disease (see section 2.2.1; page 22).

Patient Code	Sex	Ethnicity	ALT	INR	ALB	Age
54	Male	Caucasian	83	1.4	49	30
75	Male	Caucasian	39	2.1	33	20
101	Male	Caucasian	1872	1	33	48
104	Female	Asian	180	1	22	37
239	Female	Black African	20	1	35	72
248	Female	Caucasian				45
272	Female	Mixed Ancestry	66	1.1	41	47
273	Male	Caucasian	240	1.1	35	24
274	Male	Asian	87	1	38	26
281*	Female	Mixed Ancestry	107	2.8	16	14
285	Male	Black African	1022	1.8	30	33
286	Female	Black African	118	0.9	27	62
296	Male	Black African				17
298	Female	Black African	339	1.3	35	36
299	Male	Caucasian				25

<b>Patient Code</b>	<b>Sex</b>	<b>Ethnicity</b>	<b>ALT</b>	<b>INR</b>	<b>ALB</b>	<b>Age</b>
317	Male	Black African	92	1.9	14	24
318	Female	Caucasian	55	1.3	39	37
320	Female	Black African	55	4.6	31	20
324	Female	Mixed Ancestry				17
326	Female	Asian	472	4.6	18	45
329	Female	Caucasian	79	1.2	28	52
334	Male	Asian	167	2	41	17
335	Female	Mixed Ancestry	635	4.1	20	27
336	Male	Black African	225	1.6	27	12
347*	Female	Mixed Ancestry	626	1.4	36	40
350	Male	Asian	230	1.5	33	44
351	Female	Mixed Ancestry	337	1	22	52
361*	Female	Mixed Ancestry	400	1	23	30
362	Female	Caucasian	118	1.1	44	40
382	Female	Mixed Ancestry	115	0.9	45	36
383	Male	Caucasian	151	0.9	39	50
389	Female	Mixed Ancestry	431	0.9	44	58
393*	Male	Black African	330	1.6	31	17
402	Female	Caucasian	1522	1	41	16
414	Male	Caucasian	172	0.9	41	46
415	Female	Mixed Ancestry	2167	1.4	32	34
416*	Female	Asian	98	0.9	40	34
424	Female	Black African	219	1.6	35	24

<b>Patient Code</b>	<b>Sex</b>	<b>Ethnicity</b>	<b>ALT</b>	<b>INR</b>	<b>ALB</b>	<b>Age</b>
450	Female	Asian	945	1.3	28	49
455*	Female	Caucasian	71	5.2	27	20
465	Female	Mixed Ancestry	419	1.8	31	47
503*	Female	Mixed Ancestry	379	1.1	34	46
504*	Female	Mixed Ancestry	719	2.4	14	29
509	Male	Mixed Ancestry	80	1.3	34	46
520	Female	Caucasian	47	1	44	53
534	Female	Caucasian	664	1	37	41
539	Female	Caucasian	184	1	35	66
614*	Female	Asian	214	0.8	43	20
641	Male	Caucasian	74	1	20	4
649	Female	Black African	812	1.1	36	37
655	Male	Black African	83	1.8	17	19
669	Female	Black African	30	1.5	17	16
695*	Female	Mixed Ancestry	40	1.3	36	50
699	Female	Mixed Ancestry	101	1.9	33	14
707	Female	Mixed Ancestry	418	1	22	41
720	Female	Caucasian	12	1	39	70
726*	Female	Mixed Ancestry	457	1	40	55
730	Female	Mixed Ancestry	162	1	35	6
732	Female	Black African	213	1	39	28
733	Female	Asian	902	1.5	41	65
739	Female	Asian	1920	3.1	28	35

<b>Patient Code</b>	<b>Sex</b>	<b>Ethnicity</b>	<b>ALT</b>	<b>INR</b>	<b>ALB</b>	<b>Age</b>
741	Female	Caucasian	405	1.4	23	61
875	Female	Black African	400	1.8	32	34
876	Female	Mixed Ancestry	213	1.2	45	32
877	Female	Black African	132	1.71	17	30
878	Female	Mixed Ancestry	27	1.9	50	57
880	Female	Asian	248	1.8	13	45
881	Female	Caucasian	808	1	40	34
884	Female	Mixed Ancestry	1216	1.2	31	49
888	Female	Asian	47	1	48	51
890	Male	Mixed Ancestry	1300	1.3	36	31
901	Female	Mixed Ancestry	1049	15	39	82
902	Female	Caucasian	539	0.8	36	58
904	Female	Caucasian	230	1	45	47
905	Female	Mixed Ancestry	18	1.2	42	64
906	Female	Black African	437	1.2	39	54
908	Female	Caucasian	1750	1.3	45	46
909	Female	Mixed Ancestry	716	3.3	37	54
917	Female	Caucasian	1.5	0.8	31	61
921	Female	Black African	144	1.4	30	38
926	Female	Caucasian	184	1	38	45
932	Male	Mixed Ancestry	125	1.6	12	12
933	Male	Mixed Ancestry	980	2.2	21	24
935	Male	Mixed Ancestry	1008	1.5	44	29

<b>Patient Code</b>	<b>Sex</b>	<b>Ethnicity</b>	<b>ALT</b>	<b>INR</b>	<b>ALB</b>	<b>Age</b>
938	Female	Caucasian	91	1	39	56
942	Female	Black African	64	1.4	16	7
944	Female	Caucasian				65
946	Female	Black African	115	1.4	39	23
948	Male	Asian	215	1.2	42	20
949	Female	Mixed Ancestry	1428	1.2	29	42
951	Female	Caucasian	105	1	32	64
952	Female	Asian	95	2.3	15	48
960	Female	Black African	86	1.2	38	52
961	Female	Mixed Ancestry	80	1.6	28	70
964	Female	Caucasian	213	1.4	38	55
966	Female	Black African	199	1.7	23	34
967	Female	Caucasian	1254	1.5	39	30
968	Female	Asian	84	1	35	56
970	Female	Mixed Ancestry	772	1.8	35	25
974*	Female	Mixed Ancestry	1729	2.3	37	32
984	Female	Mixed Ancestry	38	1.6	41	16
987	Female	Caucasian	303	0.8	41	43
994	Female	Black African	49	2.1	13	14
997	Female	Mixed Ancestry	85	3.9	45	56
999	Female	Mixed Ancestry	990	1.8	27	6
1001	Female	Mixed Ancestry	480	1.2	36	49
1002	Female	Caucasian	87	1.23	28	65

<b>Patient Code</b>	<b>Sex</b>	<b>Ethnicity</b>	<b>ALT</b>	<b>INR</b>	<b>ALB</b>	<b>Age</b>
1004	Female	Mixed Ancestry	242	1	21	16
1005	Female	Mixed Ancestry	1975	2	42	52
1008	Female	Mixed Ancestry	90	0.7	43	45
1009	Female	Black African	346	1	38	36
1010	Male	Mixed Ancestry	103	1.3	37	13
1016	Female	Caucasian	280	1	36	27
1017	Male	Mixed Ancestry	415	1	39	52
1020	Female	Mixed Ancestry	1248	2.1	27	22
1023	Female	Mixed Ancestry	365	1.2	32	33
1024	Female	Black African	153	2.28	24	34
1025	Female	Caucasian	278	1.2	36	44

\* Patients that were included in the family cohort

Normal ranges of liver biochemistry indexes: ALT <40; INR <1.2; ALB 35-40.

# Appendix B

## Isolation of Genomic DNA from Whole Blood

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### B.1 Materials

#### B.1.1 Equipment

- Sterile 15 ml centrifuge tubes (Axygen Scientific, Union City, USA)
- Super-Mixer 220V Vortex (Lab-Line Instruments, Melrose Park, USA)
- Techne Dri-Block DB • 2D Heating Block (Techne, Cambridge, UK)
- Jouan KR4 22 High Capacity Refrigerated Centrifuge (Jouan, Winchester, USA)
- Gilson Pipetman P1000 (Gilson Medical Electronics, Villiers-le-Bel, France)
- Sterile Pipette tips (Whitehead Scientific, Cape Town, South Africa)
- 5 and 10 ml Sterilin Sterile Disposable Plastic Pipettes (Bibby Sterilin, Staffordshire, UK)

#### B.1.2 Reagents

- Wizard Genomic DNA Purification Kit (Promega, Madison, USA)
- Reagents provided by the manufacturer (Promega, Madison, USA)
  - Cell lysis solution
  - Nuclei lysis solution
  - Protein precipitation solution
  - DNA rehydration solution
  - RNase solution

- Isopropanol, room temperature (Riedel-de Haën, Sigma-Aldrich GmbH, Steinheim, Germany)
- 70% Ethanol, room temperature (Sigma-Aldrich GmbH, Steinheim, Germany)

## B.2 Methods

- Add 7.5 ml of cell lysis solution to a sterile 15 ml centrifuge tube.
- Gently invert the tube of blood until thoroughly mixed. Transfer 2.5 ml of blood to the tube containing the cell lysis solution. Invert the tube 5-6 times to mix.
- Incubate the mixture for 10 min at room temperature (invert 2-3 times once during the incubation) to lyse the red blood cells. Centrifuge at 2,000 xG for 10 min at room temperature.
- Remove and discard as much supernatant as possible without disturbing the visible white pellet. Approximately 50-100  $\mu$ l of residual liquid will remain in the 15 ml tube. If blood sample has been frozen, repeat the above four steps until pellet is white. There may be some loss of DNA from frozen samples.
- Vortex the tube vigorously until the white blood cells are resuspended (10-15 s).
- Add nuclei lysis solution (2.5 ml) to the tube containing the resuspended cells. Pipet the solution 5-6 times to lyse the white blood cells. The solution should become very viscous. If clumps of cells are visible after mixing, incubate the solution at 37°C until the clumps are disrupted. If the clumps are still visible after 1 h, add additional nuclei lysis solution (825  $\mu$ l) and repeat the incubation.
- Optional: Add Rnase solution (825  $\mu$ l) to nuclear lysate and mix the sample by inverting the tube 2-5 times. Incubate the mixture at 37°C for 15 min, and then cool to room temperature.
- Add protein precipitation solution (825  $\mu$ l) to the nuclear lysate and vortex vigorously for 10-20 s. Small protein clumps may be visible after vortexing.

- Centrifuge at 2,000 xG for 10 min at room temperature. A dark brown protein pellet should be visible.
- Transfer the supernatant to a 15 ml centrifuge tube containing 2.5 ml room temperature isopropanol.
- Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.
- Centrifuge at 2,000 xG for 1 min at room temperature. The DNA will be visible as a small white pellet.
- Decant the supernatant and add one sample volume of room temperature 70% ethanol to the DNA. Gently invert the tube several times to wash the DNA pellet and the sides of the microcentrifuge tube. Centrifuge as above.
- Carefully aspirate the ethanol using either a pipette. The DNA pellet is very loose at this point and care must be taken to avoid aspirating the pellet into the pipette. Invert the tube on clean absorbent paper and air-dry the pellet for 10-15 min.
- Add DNA rehydration solution (200  $\mu$ l) to the tube and rehydrate the DNA by incubating at 65°C for 1 h. Periodically mix the solution by gently tapping the tube. Further rehydrate the DNA by incubating the solution overnight at room temperature.
- Store the DNA at 4°C.

## Appendix C

### Annotated Genomic Sequence for CTLA4

**Table C.1** Annotated genomic sequence of the *CTLA4* gene. Capital letters indicate translated regions of the gene. Primers are indicated with boxes around the text. Positions of published polymorphisms are indicated with a ^ or ^^ and explained in blue directly underneath the indicator. Start and stop codons are red and in bold. Where parts of the sequence are omitted, they are replaced with (...).

1	ccctttctga cttccacagg ctgaaccact ggcttctgct cctctacata atacttcaat
61	tccagcattg atctcactct atcatgatca tgggttttagc tgtctgtccc tgccactgct
121	gtgtgttctt cttgagggca ggaacatttg tttttcactt tttaaaaaac ctctgttgcc
181	cagtctggca ttaggaagtg cccattaggt tgttattgct tgttggcgct tgagctgggg
	. . .
961	atttaatctt cacttttgaa ttttctgctt gaaaaatttg tattagaaaa aaaagtctat
1021	ccttttatgg acggctctaa tctcttgaat catttggggtt ggcttttctt tggaccttct
1081	tcaactctgt tttgtctctg ttgagttaag gcttttaaga acacctgaat tctttccttc
1141	tgcaaaacca gaggcagctt cttttccgcc tattttcagt ttatttcttg tgattttaqt
1201	ttttttctct taaccaaagc ctaaattgat ttaggagaaa taaacttatt tgtaaagctg
1261	tcaag <b>ggacc attagaagga tgg</b> gtc <b>ttca</b> cagatagaat acagtttta ttaatgatgc
	<b>CTLA4promA</b>
1321	ctagacaaat cctgccatta gcccaagggc tcagaaagtt agcagcctag tagttttgca
1381	gatgtcaatg aatgaattg gactggatgg ttaaggatgc ccagaagatt gaataaaatt
1441	gggatttagg aggacccttg tactccagga aattctocaa gtctccactt agttatccag
1501	atcct^caaag tgaacatgaa gcttcagttt caaattgaat acattttcca tccatggatt
	^ C/T <a href="#">cr014435</a> (-318C/T)
1561	ggcttgTTTT gttca <b>gttga gtgcttgagg ttgt</b> cttttc gagctaacag ctaaac <b>cad</b>
	<b>CTLA4F1</b>
1621	<b>ggcttctttt ctcgtaa</b> aac caaaacaaaa aggctttcta ttcaagtg
	<b>CTLA4promA</b>
	<b>Exon 1</b> <span style="float: right;">cc ttctgtgtgt</span>
1681	gcacatgtgt aatacatatc tgggatcaaa gctatctata taaagtcctt gattctgtgt
1741	gggttcaaac acatttcaaa gcttcaggat cctgaaaggt tttgtcttac ttctgaaga
1801	cctgaacacc gctcccataa agcc <b>ATGGCT</b> TGCCTTGGAT TTCAGCGGCA CAAGGCTCAG
1861	CTGAACCTGG CT^GCCAGGAC CTGGCCCTGC ACTCTCCTGT TTTTCTTCT CTTCATCC^^CT

^ G/A rs231775 (+49A/G)

^^ G/C rs16840275

1921 GTCTTCTGCA AAG

**Intron 1** gtgagtg agacttttgg agcatgaaga tggaggaggt gtttctccta

1981 cctggggttc atttgtttca gcagtcaa^gg gcagtgattt atagcaaagc cagaagt-aa

^ a/g rs231776

**CTLA4R1**

2041 aggtaaaact ccaatctggc ttggctggct ctgtattcca gggccagcag ggagcagttg

2101 ggcggcagca aataaggcaa agagatagct cagaacagag cgccaggtat ttagtagggg

2161 cttcatgaat gcatgtgagt tggtttagta gagagacaca ggcaatttca gacccttcta

. . .

4201 tgtttgatcat atcagtggtc ttctgcccac aaccatcttg aagaatctat ttctcagtaa

4261 gaaaatatct ttatggagag tagctggaaa acagttgaga gatggagggg aggctggggg

4321 tgtggagagg ggaaggggta agtgatagat tcggtgaagg ggggagaaaa ggccgtggcg

4381 atgaagctag aaggcaglaag ggcttgccctg ggcttgcca tgaaggagca tgagttca-ct

**CTLA4F2**

4441 gagttccctt tggcttttcc atgctag

**Exon 2**

CAA TGCACGTGGC CCAGCCTGCT GTGGTACTEG

4501 CCAGCAGCCG AGGCATCGCC AGCTTTGTGT GTGAGTATGC ATCTCCAGGC AAAGCCACTG

4561 AGGTCCGGGT GACAGTGCTT CGGCAGGCTG ACAGCCAGGT GACTGAAGTC TGTGCGGCFA

4621 CCTACATGA^T GGGGAATGAG TTGACCTTCC TAGATGATTC CATCTGCACG GGCACCTCCA

^ T/C rs1065442

4681 GTGGAAATCA AGTGAACCTC ACTATCCAAG GACTGAGGGC CATGGACACG GGACTCTACA

4741 TCTGCAAGGT GGAGCTCATG TACCCACCGC CATACTACCT GGGCATAGGC AACGGAACCC

4801 AGATTTATGT AATTG

**Intron 2**

gtgag caaagccatt tcaactgagtt gacacctggt gcattgca-ct

4861 cttctatgca caaaaacagt tttgttccct aatttcagga ggtttacttt taggactgtg

4921 gacattctct ttaagagttc tgtaccacat ggtagccttg cttattgtgg gtggcaac-ct

**CTLA4R2**

4981 taatagcatt ctgactgtaa aataaaatga tttggggaag ttggggctct cgctctggag

5041 tgctaaccat catgacgttt gatctgtact tttgatatga tatgatgctc ctggggaagt

5101 agtcccaaat agccaaacct attggtgggc taccatgca atttaggggt ggacctcaag

5161 gcctggaagc tctaagtccc ttttttcacc aatggtggg agtagagccc tagagtttaa

**CTLA4F3**

5221 aactgtctca gggaggctct gctttgtttt ctggtgcag

**Exon 3**

A TCCAGAACCG TGCCCAGATT

5281 CTGACTTCCT CCTCTGGATC CTTGCAGCAG TTAGTTCGGG GTTGTTTTTT TATAGCTTTC

5341 TCCTCACAGC TGTTTCTTTG AGCAAAATG

**Intron 3**

g tgagtgtggt gctgatggtg caccatgtct

5401 gatggggata cctttagtgg tatcaactgg ccaaagatg atgttgagtt tagtgttctt  
5461 gagatgagat gaggcaataa atgaagagga aggacagtgg taaagaa **cgc actagaaccg**  
5521 **taggcat**tgg catttgaggt ttcagaatga ctaatatattt agatgaattt gtttgacatt

**CTLA4R3**

5581 gaatgttcat gtgcttctga gcagggttc aatttgagta accgttgcaa taacatgggg  
5641 cagctgtttt gctctttgtc ttcatgacaa ctgtacttaa gtaaacagcc ctgaaacatg  
5701 agattagget gggcagaatg ctgctagaga ggaccacttg gatggctctt attctccttc  
. . .  
6301 caattatcat aggctttctg ggtgttcttt ctgaagcttt ctcaaagtct ctttctccta  
6361 tcttccattc aaggcaaagc attgcccattt aacatcaaaa tcacagttat ttatctaaaa  
6421 taaattttaa tagctgaatc aagaaaatct cctgaggttt ataattctgt atgctgtga^a

^ a/g rs11571318

6481 cattcatttt ta **accagcta gggacceaat atg**tgttgag ttctattatg gttagaagtg

**CTLA4F4a**

6541 gcttccgtat tcctcagtag taattactgt ttctttttgt gtttgacag

**Exon 4**

C TAAAGAAAAG

6601 AAGCCCTCTT ACAACAGGGG TCTATGTGAA AATGCCCCCA ACAGAGCCAG AATGTGAAAA  
6661 GCAATTTTCAG CCTTATTTTA TTCCCATCAA **TGA**gaaacc attatgaaga agagagtcca  
6721 tatttcaatt tccaagagct gaggcaatc taactttttt gctatccagc tatttttatt  
6781 tgtttgatgca tttgggggga attcatctct cttaataata aagttggatg cggaacccaa  
6841 attacgtgta ctacaattta aagcaaagga gtgaaaagac agagctggga tgtttctgt**c**  
6901 **acatcagctc cactttcagt**t gaaagcatca cttgggatta atatggggat gcagcattat

**CTLA4R4a**

6961 gatgtgggtc aaggaattaa gttagggaaat ggcacagccc aaagaaggaa aaggcagga  
7021 gcgagggaga agactatatt gtacacacct tatattttacg tatgagac^gt ttatagccga

^ G/A rs13384548

7081 aatgatcttt tcaagttaaa ttttatgcct tttatttctt aaacaaatgt atgattacat  
7141 caaggcttca aaaatactca catggctatg ttttagccag tgatgctaaa ggttgattg  
7201 catatataca tatatatata tatatatata tatatatata ttttaatttg atagtattgt  
7261 gcatagagcc acgtatgttt ttgtgtattt gttaatgggt tgaatataaa cactatatgg  
7321 cagtgtcttt ccaccttggg tcccagggaa gttttgtgga ggagctcagg aactaatac  
7381 accaggtaga acacaaggtc atttgctaac tagcttgga actggatgag gtcatagcag  
7441 tgcttgattg cgtggaattg tgctgagttg gtgttgacat gtgctttggg gcttttacac  
7501 cagttccttt caatggtttg caaggaagcc acagctggtg gtatctgagt tgacttgaca  
7561 gaacactgtc ttgaagacaa tggcttactc caggagacc acaggtatga ccttctagga  
7621 agctccagtt cgatgggcc aattcttaca aacatgtggt taatgccatg gacagaagaa

7681 ggcagcaggt ggcagaatgg ggtgcatgaa ggtttctgaa aattaacact gcttgtgttt  
7741 ttaactcaat attttccatg aaaatgcaac aacatgtata atatttttaa ttaaataaaa  
7801 atctgtggtg gtcgtttt

**3' UTR**

**cc ggagttgtct ttatcatcct** tgcatattgaa tattgtgtt^t

^ t/c rs231721

**CTLA4F3UTR**

7861 aaatTTTTga ttgattcatt cagtatctgg tggagtctcc aatattagaa atactggaza  
7921 caaactgaaa aaccacaaaa ggacaaataa tgcttcatga gtcagctttg caccagccat  
7981 tacctgcaag tcattcttgg aaggatcca tcctctttcc ttttgatttc ttcaccacta  
8041 tttgggatat aac^gtgggtt aacacagaca ta^^gcagtcct ttataaatca

att**ggcatgc**

^ g/a rs3087243 (CT60)

^^ g/a rs11571319

8101 **tgtttaacac aggt**tcttca cctccccctt cttaccgcct gctttctcag ctcaacta=c

**CTLA4R3UTR**

8161 acaggcatta cagttgtcat ggcaaccca atgttggcaa ccacgtccct tgcagccatt  
8221 ttgatctgcc ttctgaaat atagagcttt tcctgtggc ttccaaatga actattttc  
8281 aaatgtgggg aaaacacaca cctgtggtcc tatgttgcta tcagctggca cacctaggcc  
8341 tggcacacta agccctctgt gattcttct taaccaatgt atagtctcag cacatttct  
8401 ttccacttaa ggtttcctat ttttgacctc cacatcctgt tattgtcttt tgcagtgatt  
8461 ctcagtgagg tcagggacca cctgtaactg aatccactgg gtcacttact aaaaacacca  
8521 attcacactt attcccagat gcgttgaatc agaatctttg agtgggtgggg ccaggagttt  
8581 gtatttccaa aaatcttccc agtgcttctt ataccatca agttttgaga atcactgctt  
8641 aggcaactct gtctgtgtct cttcctctgt ctctccccct tgctcattct cttgcttget  
8701 cttgctctct ttctctcttt cttgccccag caagtgctaa caaacacaca agacagcaca  
8761 tagtaagcat ttcaaaaggg gttgttaaata aaaaacgaat aggtctttca aagtaaggta  
8821 atgattatta ctagaatga gctccttttc tgtggaagac tgtgcttctt gctctctagg  
8881 ctttgatg gtagccatga agaaaaacac caatcgggag cctcagtgga tagtatatca  
8941 tttccactcc tctaaacgtc ttttagagaga ttactctttt tcatagttga tgggcagatc  
9001 tgagtgacag agagtgcctt acacaagtga tgccctccag gtacctttca gacaccctta  
9061 gataatggac tactttatat aagcttaata tttcctctgc tattgcctaa aattctacca  
9121 tttgttgctc actgagataa gacttgccct cttcattcag tctcaatctt ataaaaataa  
9181 aatacccttt agtcaaaaa agcttccccct atggcagaag aattgtacaa gaaagactat  
9241 gacccggggt tccgtgaaa ggggagaaaag caaatatgag ggtaagggag tgcactctgg  
9301 caccaaatga aagcctcttc taattcccat gtcatttatg agagaagatt ctatgtctgt

# Appendix D

## Primer Design

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### D.1 Primer Criteria

- Primer lengths of 18 to 22 bp were chosen to ensure specificity. Shorter primers generally tend to bind more non-specifically to the genome.
- Amplicon sizes of no more than 600 bp were chosen, to ensure compatibility with WAVE.
- Melting temperatures ( $T_m$ ) of 55 to 65°C were favoured.
- The  $T_m$  difference between primers of a pair was chosen to be ideally no more than 2°C (maximum 5°C).
- GC contents of 45 to 60% were favoured.
- Primer dimer formation due to complementarity between primers was avoided.
- The possibility of a primer folding back on itself and creating a hairpin loop due to self-complementarity was examined, and where possible avoided. If a loop forms at the 3' end of a primer, the 3' OH group is inaccessible to the Taq Polymerase for elongation of the strand. However, if this occurs at the 5' end, the primer can still be functional, but will in effect be shorter, resulting in possible non-specificity.
- Sequences containing three or more tandem repeats of a single nucleotide (e.g. GGG) were avoided, where possible.

## **D.2 Primer Information**

Table D.2.1 shows relevant primer information, such as size, melting temperature ( $T_m$ ), GC content, sequence and product size. The primer pair (promA) for the promotor region amplifies the section closest to the first exon. Likewise, the exon 4 fragment (4a) includes the coding part of the exon, and not the entire exon.

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**Table D.2.1 Primer sequences utilised and relevant information**

<b>Amplicon</b>	<b>Primer pair</b>	<b>Size (bp)</b>	<b>Product size (bp)</b>	<b>TM °C</b>	<b>% GC</b>	<b>Sequence</b>
promA	<i>CTLA4FPromA</i>	21	372	55.5	52.3	5' ggaccattagaaggatggtgc 3'
	<i>CTLA4RPromA</i>	20		54.9	50	5' ttacgagaaaggaagccctg 3'
Exon 1	<i>CTLA4F1</i>	20	443	53.9	50	5' gttgagtgcttgagggtgctc 3'
	<i>CTLA4R1</i>	20		55.5	50	5' atcactgccttgactgctg 3'
Exon 2	<i>CTLA4F2</i>	19	578	52.7	52.6	5' ggatgaagctagaaggcag 3'
	<i>CTLA4R2</i>	20		53.0	50	5' gctaccatgtgtacagaac 3'
Exon 3	<i>CTLA4F3</i>	20	367	55.4	55	5' gcctggaagctctaatgtcc 3'
	<i>CTLA4R3</i>	20		57.0	55	5' atgcctacggttctagtgcg 3'
Exon 4a	<i>CTLA4F4a</i>	21	427	56.3	52.3	5' accagctagggaccaatag 3'
	<i>CTLA4R4a</i>	20		53.5	50	5' ctgaaagtgagctgatgtg 3'
3' UTR	<i>CTLA4F3UTR</i>	21	296	52.9	47.6	5' ccggagttgtcttatcatcc 3'
	<i>CTLA4R3UTR</i>	21		56.1	47.6	5' acctgtgtaaacagcatgcc 3'

# Appendix E

## Polymerase Chain Reaction (PCR)

---

### E.1 Materials

#### E.1.1 Equipment

- Sterile 0.5 ml and 1.5 ml microcentrifuge tubes (Axygen Scientific, Union City, USA)
- Gilson Pipetman (P1000, P200, P20 and P2) (Gilson Medical Electronics, Villiers-le-Bel, France)
- Sterile filter pipette tips (Whitehead Scientific, Cape Town, South Africa)
- RoboCycler Gradient 40 Thermal Cycler with hot lid (Stratagene, Los Angeles, USA)
- Force 7 Microcentrifuge (Denver Instrument Company, Denver, USA)
- Super-Mixer 220V Vortex (Lab-Line Instruments, Melrose Park, USA)

#### E.1.2 Reagents

- Deoxynucleotide Triphosphates (dNTPs) (Promega, Madison, USA)
- Primers (forward and reverse) (See Appendix D)
- MgCl<sub>2</sub> 25mM (Promega, Madison, USA)
- Mg-free PCR Buffer (Promega, Madison, USA)
- Taq DNA polymerase (Promega, Madison, USA)
- dH<sub>2</sub>O

## E.2 Methods

- Work on ice.
- Use gloves at all times to prevent contamination.
- In a sterile 1.5 ml microcentrifuge tube, make up the following PCR cocktail:

**Table E.2.1 PCR reagent cocktail per PCR reaction**

Reagent	[Stock]	Volume ( $\mu$ l)	[Final]
Primers each (forward and reverse)	25 $\mu$ M or 10 $\mu$ M (refer to table E.2.2.)	1	0.2 to 0.5 $\mu$ M
MgCl <sub>2</sub>	25mM	5	2.5mM
Buffer	10X	5	1X
DNTPs (Each)	2.5 mM	1	50 $\mu$ M
Taq DNA polymerase	5 U/ $\mu$ l	0.2	1 Unit
dH <sub>2</sub> O		34.8	

- Aliquot the cocktail into 0.5 ml microcentrifuge tubes (48  $\mu$ l per tube).
- Add 2 $\mu$ l (100 to 200 ng) genomic DNA to each reaction (Add 2  $\mu$ l dH<sub>2</sub>O in stead of DNA to one of the tubes to serve as a negative control).
- Vortex tubes and centrifuge briefly.
- Perform the following PCR reactions in the thermocycler, ensuring that the hot lid is switched on to prevent evaporation of samples:
  - Denaturation at 95°C for 1 min.
  - 35 Cycles of denaturation at 95°C for 30 s, annealing at the specified temperatures (refer to table E.2.2) for 30 s and elongation at 72°C for 30 s.
  - Final elongation step at 72°C for 7 min.
- Store PCR products at 4°C until further use.

**Table E.2.2 Stock primer concentration and Ta for the PCRs performed.**

	<b>PromA</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4a</b>	<b>3'UTR</b>
<b>Annealing temperature</b>	53°C	53°C	59°C	55°C	55°C	55°C
<b>[Primer] (µM)</b>	25	10	25	25	10	10

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## Appendix F

### Polyacrylamide Gel Electrophoresis (PAGE)

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#### F.1 Solutions

##### F.1.1 Acrylamide-Bisacrylamide solution (ABA)

Acrylamide (Promega, Madison, UK) 30% (60 g)

Bisacrylamide (Promega, Madison, UK) 0.8% (1.6 g)

Make up to 200 ml with dH<sub>2</sub>O

##### F.1.2 10X TBE

Tris (MW = 121.14) 540 g

(Merck Chemicals, Wadeville, South Africa)

Boric Acid (Merck Chemicals, Wadeville, South Africa) 275 g

EDTA (Merck Chemicals, Wadeville, South Africa) 37 g

Make up to 5 L with dH<sub>2</sub>O and adjust pH to 8.3

Autoclave to sterilize for 30 min

##### F.1.3 10% Ammonium Persulphate (APS)

APS (Promega, Madison, USA) 1 g

Make up to 10 ml with dH<sub>2</sub>O

#### **F.1.4 Bromophenol Blue Loading Dye**

Bromophenol Blue 0.25% w/v (0.25 g)

(Associated Chemical Enterprises, Johannesburg, South Africa)

Sucrose (Saarchem, Krugersdorp, South Africa) 40% w/v (40 g)

0.5M EDTA (pH8) 4 ml

(Merck Chemicals, Wadeville, South Africa)

Make up to 100 ml with dH<sub>2</sub>O

#### **F.1.5 6% Acrylamide Gel**

10X TBE (F.1.2) 4 ml

ABA (F.1.1) 8 ml

Make up to 40 ml with dH<sub>2</sub>O

Add to this:

APS (F.1.3) 400 µl

TEMED (Merck Chemicals, Wadeville, South Africa) 40 µl

Mix well

#### **F.1.6 Ethidium Bromide Staining Solution**

Ethidium bromide (500 ng/ml) 200 µl

(Roche Diagnostics, Randburg, South Africa)

dH<sub>2</sub>O 200 ml

Mix by swirling gently

## **F.2 PAGE**

### **F.2.1 Materials**

#### **F.2.1.1 Equipment**

- Hoefler Vertical Slab Gel Unit, SE600 series with gel apparatus (Hoefler, GE Healthcare, Vienna, Austria)
- Hoefler PS1500 DC Power Supply (Hoefler, GE Healthcare, Vienna, Austria)
- Hamilton Microliter Syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland)
- Gel Documentation system, UVItech (UVItech Limited, Cambridge, UK)
- Mitsubishi P90E(B) Video copy processor (Mitsubishi Electric Corporation, Tokyo, Japan)

#### **F.2.1.2 Reagents**

- 6% Acrylamide Gel (F.1.5)
- 1X TBE running buffer (F.1.2)
- 500 ng/ml Ethidium bromide (F.1.6)
- Bromophenol blue loading dye (F.1.4)
- 100 bp molecular weight marker (Promega, Madison, USA)

### **F.2.2 Methods**

- Assemble gel apparatus by placing two glass plates, separated by 1.5 mm spacers and kept together with clamps, vertically onto a gel stand, and secure using cams.
- Make up a 6% Acrylamide solution (F.1.5) and pour between the glass plates in the gel setup.
- Ensure all air bubbles are removed.
- Insert a 20 well 1.5 mm gel spacer comb and allow to set for at least 45 min.

- **Carefully remove the comb from the gel.**
- **Assemble the upper buffer chamber on top of the gel and clamp into place.**
- **Pour 500 ml 1X TBE Buffer into the upper buffer tank.**
- **Mix 5  $\mu$ l DNA with an equal amount of PCR loading dye.**
- **Using the Hamilton syringe, carefully load samples into the wells.**
- **To one of the wells add 10  $\mu$ l 100 bp DNA step ladder (diluted 1:2 with bromophenol blue loading dye) to serve as a size standard.**
- **Run the samples in 1X TBE running buffer at 270 V for 1 to 1.5 h.**
- **Remove gel and stain in Ethidium Bromide solution for 10 min.**
- **Visualise PCR fragments using the gel documentation system and take a photograph for future reference.**

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# Appendix G

## Restriction Analysis

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### G.1 Restriction Enzyme Digests

#### G.1.1 Materials

##### G.1.1.1 Equipment

- Techne Dri-Block DB·2D Heating block (Techne, Cambridge, UK)
- Force 7 Microcentrifuge (Denver Instrument Company, Denver, USA)
- Super-Mixer 220V Vortex (Lab-Line Instruments Inc., Melrose Park, USA)
- Gilson Pipetman (P1000, P200, P20 and P2) (Gilson Medical Electronics, Villiers-le-Bel, France)
- Sterile pipette tips (Whitehead Scientific, Cape Town, South Africa)
- 0.5 ml and 1.5 ml sterile microcentrifuge tubes (Laboratory Scientific, Cape Town, South Africa)

##### G.1.1.2 Reagents

- Enzymes (see table G.1.2.1)
- Buffers (see table G.1.2.1)
- BSA (supplied by enzyme manufacturers)

#### G.1.2 Methods

- Working on ice, make up a reaction mixture of the appropriate enzyme and corresponding buffer (see table G.1.2.1) in a 1.5 ml microfuge tube to a final volume of 10  $\mu$ l per reaction with dH<sub>2</sub>O. For *MnII* digestions, include 2  $\mu$ g BSA according to the manufacturer's instructions.

**Table G.1.2.1 Individual reaction components for the 4 RE analyses.**

<b>Polym</b>	<b>Enzyme</b>	<b>[E] (U)</b>	<b>Buffer</b>	<b>Buffer 10X (µl)</b>	<b>Digestion time (h)</b>
-318 C/T	<i>MnlI</i> (New England BioLabs, Ipswich USA)	1	NEBuffer2	2	1
+49 A/G	<i>ItaI</i> (Roche, Mannheim, Germany)	1	Buffer H	2	3
CT60 G/A	<i>HpyCH4IV</i> (New England BioLabs, Ipswich, USA)	1	NEBuffer1	2	1.5

polym = polymorphism

[E] = enzyme concentration

U = Units

- Vortex well and spin down briefly in a microcentrifuge.
- Aliquot 10 µl of the above cocktail into 0.5 ml microcentrifuge tubes.
- Add 5 to 10 µl of PCR product (depending on the intensity of the PCR bands) to the enzymes in the tubes. Make up the volume in each tube to 20 µl with deionised water.
- Include a positive control with each set of reactions to confirm complete digestion of product.
- Vortex and spin down briefly in a microcentrifuge.
- Incubate at 37°C as indicated in table G.1.2.1.
- Analyse digestion products by electrophoresis on 6% PAGE (Appendix F). Include a predigestion PCR product. Stain with ethidium bromide and photograph the gel.

## G.2 Restriction Maps

### G.2.1 CTLA4 PromA

# CTLA4 PromA Wild type

372 base pairs

MseI  
TruI

ggaccattagaaggatggtgcttcacagatagaatacagtttttattaatgatgcctagacaaatcctgccatta base pairs  
cctggtaatcttcctaccacgaagtgtctatcttatgtcaaaaataattactacggatctgtttaggacggtaat 1 to 75  
Tru9I

MseI  
TruI

gccaagggctcagaaagttagcagcctagtagtttggagttgtcaatgaaatgaattggactggatggttaag base pairs  
cgggttcccagatctttcaatcgctcggatcatcaaaacctcaacagttactttacttaacctgacctaccaattc 76 to 150  
Tru9I

MnlI

gatgcccagaagattgaataaaattgggatttaggaggaccttgactccaggaaattctccaagtctccactt base pairs  
ctacgggtcttctaacttattttaaccctaaatcctcctgggaacatgaggtcctttaagaggttcagaggtgaa 151 to 225

MnlI

agttatccagatcctcaaagtgaacatgaagcttcagtttcaaattgaatacattttccatccatggattggctt base pairs  
tcaataggtctaggagtttcacttgtacttcgaagtc aaagtttaacttatgtaaaaggtaggtacctaaccgaa 226 to 300

MnlI

gttttggtcagttgagtgcttgaggttgctcttttcgacgtaacagctaaacccacggcttcctttctcgtaa base pairs  
caaaacaagtcaactcacgaactccaacagaaaagctgcattgtcgatttgggtgccgaaggaaagagcatt 301 to 372

Table by Enzyme Name

Enzyme name	No. cuts	Positions of sites	Recognition sequence
MnlI	3	188 241 325	cctc
MseI	2	46 146	t/taa
TruI	2	46 146	t/taa
Tru9I	2	46 146	t/taa

Every enzyme analyzed cuts this sequence.

# CTLA4 PromA -318 C/T

372 base pairs

MseI  
Tru1I

ggaccattagaaggatggtgcttcacagatagaatacagtttttattaatgatgcctagacaaatcctgccatta base pairs  
cctggtaatcttctaccacgaagtgtctatcttatgtcaaaaataattactacggatctgtttaggacggtaat 1 to 75

Tru9I

MseI  
Tru1I

gccaagggtcagaaagtttagcagcctagtagttttggagttgtcaatgaaatgaattggactggatgggtaag base pairs  
cgggtcccagtccttcaatcgtcggatcatcaaacctcaacagttactttacttaacctgacctaccaattc 76 to 150

Tru9I

MnlI

gatgccagaagattgaataaaattgggatttaggaggacctgtactccaggaaatttccaagtctccactt base pairs  
ctacgggtcttctaacttattttaaccctaaatcctcctgggaacatgaggtcctttaagaggttcagaggtgaa 151 to 225

MseI  
Tru1I

agttatccagatccttaaagtgaacatgaagcttcagtttcaaattgaatacattttccatccatggattggctt base pairs  
tcaataggtctaggaatttcacttgtacttcgaagtcaaagtttaacttatgtaaaaggttaggtacctaaccgaa 226 to 300

Tru9I

MnlI

gttttggtcagttgagtgcttgaggtgtcttttcgacgtaacagctaaaccacggcttcctttctcgtaa base pairs  
caaaacaagtcaactcacgaactccaacagaaaagctgcattgtcgatttgggtgccgaaggaaagagcatt 301 to 372

## Table by Enzyme Name

Enzyme name	No. cuts	Positions of sites	Recognition sequence
MnlI	2	188 325	cctc
MseI	3	46 146 240	t/taa
Tru1I	3	46 146 240	t/taa
Tru9I	3	46 146 240	t/taa

Every enzyme analyzed cuts this sequence.

## G.2.2 CTLA4 Exon 1

### CTLA4 Exon 1 Wild Type 443 base pairs

gttgagtgcttgaggtgtcttttcgacgtaacagctaaacccacggcttcctttctcgtaaaacccaaaacaaa base pairs  
caactcacgaaactccaacagaaaagctgcattgtcgatttgggtgccgaaggaaagagcattttggttttgtttt 1 to 75

aggctttctattcaagtgccttctgtgtgtgcacatgtgtaatacatatctgggatcaaagctatctatataaag base pairs  
tccgaaagataagttcacggaagacacacacgtgtacacattatgtatagaccctagtttcgatagatatatttc 76 to 150

tccttgattctgtgtgggttcaaacacatttcaaagcttcaggatcctgaaaggttttgctctacttctgaaga base pairs  
aggaactaagacacaccaagtttgtgtaaagtttcgaagtcctaggactttccaaaacgagatgaaggacttct 151 to 225

#### ItaI

cctgaacaccgctcccataaagccatggcttgcttggatttcagcggcacaaggctcagctgaacctggctacc base pairs  
ggacttgtggcgagggatatttcggtaccgaacggaacctaaagtcgccgtgttcgagtcgacttggaccgatgg 226 to 300

aggacctggcctgcactctcctgttttttcttctcttccatccctgtcttctgcaaagggtgagtgagacttttgg base pairs  
tcctggaccgggacgtgagaggacaaaaaagaagagaagtagggacagaagacgtttccactcactctgaaaacc 301 to 375

agcatgaagatggaggaggtgttctcctacctgggtttcatttgtttcagcagtc aaaggcagtgat base pairs  
tcgtacttctacctcctccacaaagaggatggaccacaaagtaacaaagtcgtcagtttccgtcacta 376 to 443

#### Table by Enzyme Name

Enzyme name	No. cuts	Positions of sites	Recognition sequence
ItaI	1	271	gc/ngc

Every enzyme analyzed cuts this sequence.



## G.2.3 CTLA4 3' UTR

### CTLA4 3'UTR Wild Type 296 base pairs

ccggagttgtctttatcatccttgcaatttgaatattgtgttcaaatttttgattgattcattcagtatctggtgg base pairs  
ggcctcaacagaaatagtaggaacgtaaacttataacacaagtttaaaaactaactaagtaagtcagaccacc 1 to 75

agtctccaatattagaaatactggaacaaactgaaaaaccacaaaaggacaaataatgcttcatgagtcagctt base pairs  
tcagaggttataatctttatgacctttgctttgactttttggtgttttctgtttattacgaagtaactcagtcgaa 76 to 150

tgcaccagccattacctgcaagtcatcttgggaaggtatccatcctctttccttttgatttcttcaccactattt base pairs  
acgtggtcggtaatggacgttcagtaagaaccttccataggtaggagaaaggaaaactaaagaagtggtgataaa 151 to 225

#### MaeII/HpyCH4IV

gggatataacgtgggttaacacagacatagcagtcctttataaatcaattggcatgctgtttaacacaggt base pairs  
ccctatattgcaccaattgtgtctgtatcgtcaggaaatatttagttaaccgtacgacaaattgtgtcca 226 to 296

#### Table by Enzyme Name

Enzyme name	No. cuts	Positions of sites	Recognition sequence
MaeII/HpyCH4IV	1	234	a/cgt

Every enzyme analysed cuts this sequence

# CTLA4 3'UTR CT60 G/A

296 base pairs

ccggagttgtctttatcatccttgcaatttgaatattgtggttaaaatgttattgattcattcagtatctgggtgg base pairs  
ggcctcaacagaaatagtaggaacgtaaacttataacacaaaatttaaaaactaactaagtaagtcataagaccacc 1 to 75

agtctccaatattagaaatactggaaacaaactgaaaaaccacaaaaggacaaataatgcttcatgagtcagctt base pairs  
tcagaggttataatctttatgacctttgtttgactttttgggtgttttctgtttattacgaagtactcagtcgaa 76 to 150

tgaccagccattacctgcaagtcattcttggaggatccatcctctttccttttgatttcttcaccactattt base pairs  
acgtggtcggtaaatggacgttcagtaagaaccttccataggtaggagaaaggaaaactaagaagtggtgataaa 151 to 225

gggatataacatgggttaacacagacatagcagtcctttataaatcaattggcatgctgtttaacacaggt base pairs  
ccctatattgtacccaattgtgtctgtatcgtcaggaaatatttagttaaccgtacgacaaattgtgtcca 226 to 296

The following endonucleases were selected but don't cut this sequence:

MaeII/ HpyCH4IV

# Appendix H

## Mutation Screening

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### H.1 Denaturing High Performance Liquid Chromatography (DHPLC)

#### H.1.1 Materials

##### H.1.1.1 Equipment

- Sterile 0.2 ml microcentrifuge tubes (Southern Cross Biotechnology, Cape Town, South Africa)
- Gilson Pipetman P20 (Gilson Medical Electronics, Villiers-le-Bel, France)
- Sterile pipette tips (Whitehead Scientific, Cape Town, South Africa)
- Force 7 Microcentrifuge (Denver Instrument Company, Denver, USA)
- Hybaid OmniGene Thermal Cycler (Hybaid, Teddington, Middlesex, UK)
- WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, USA)
- WAVEMAKER™ software (Transgenomic, Omaha, USA)
- Super-Mixer 220V Vortex (Lab-Line Instruments, Melrose, Park, USA)

##### H.1.1.2 Reagents

- WAVE High- and Low-Range Mutation Control Standards (Transgenomic, Omaha, USA)
- DHPLC Elution Buffer
- TEAA (Transgenomic, Omaha, USA)

- 25% Acetonitrile (Honeywell Burdick and Jackson, Morristown, USA) in water (Transgenomic, Omaha, USA)

### **H.1.1.3 PCR products**

- Wild type DNA PCR products
- PCR products to be screened

## **H.1.2 Methods**

- To determine the screening temperatures, enter the sequence of the DNA fragment to be screened into the WAVEMAKER™ Software (Transgenomic Inc., USA) and calculate the percentage helical fraction versus the melting temperature for the fragment. Thereafter, calculate the temperatures at which the entire fragment will be eluted from the column. More than one temperature may be needed for large fragments.
- To the PCR product from each affected individual to be tested add an equal volume of wild type PCR product of approximate same intensity (Allow 5 µl per screening temperature).
- Denature for 5 min at 95°C on the thermal cycler and allowed to cool slowly to room temperature on the block for at least 45 min for renaturing to take place.
- Place heteroduplex mixes in the WAVE autosampler and enter the appropriate methods into the sample spreadsheets provided by the WAVEMAKER™ Software (Transgenomic Inc., USA), according to the manufacturer's instructions.
- Screen the samples at the predetermined temperature for each exon (see table H.1.2.1.) alongside the WAVE High- and Low-Range Mutation Control Standards (Transgenomic Inc., USA).
- Analyse data with the WAVEMAKER™ Software by comparing the chromatograms of sample DNA to that of the negative control.

**Table H.1.2.1** Temperatures at which fragments for each exon were eluted during DHPLC screening.

Amplicon	Temperatures screened (°C)			
	1	2	3	4
Promotor	57	-	-	-
1	60.0	61.5	-	-
2	55.9	57.3	60.4	62.5
3	57.6	59.0	-	-
4a	65.0	-	-	-

## **H.2 Single Stranded Conformational Polymorphism (SSCP) Analysis**

### **H.2.1 Materials**

#### **H.2.1.1 Equipment**

- Hoefer Vertical Slab Gel Unit, SE600 series with gel apparatus (Hoefer, GE Healthcare, Vienna, Austria)
- PS 1500 DC Power Supply (Hoefer, GE Healthcare, Vienna, Austria)
- Hamilton Microliter Syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland)
- Gel Documentation system, UVItch (UVItch Limited, Cambridge, England)
- Mitsubishi P90E(B) Video copy processor (Mitsubishi, Tokyo, Japan)
- Magnetic Stirrer (LASEC, Cape Town, South Africa)
- 5 and 10 ml Sterilin Sterile Disposable Plastic Pipettes (Bibby Sterilin, Staffordshire, UK)
- Yihder TS-500 Orbital Shaker (LASEC, Cape Town, South Africa)

### **H.2.1.2 Reagents**

- **SSCP loading buffer**

Formamide (VWR International, Poole, UK) 95%

NaOH (Merck Chemicals, Wadeville, South Africa) 10 mM

EDTA pH 8 (0.5M) (Merck Chemicals, Wadeville, South Africa) 20 mM

Bromophenol Blue 0.02%

(Associated Chemical Enterprises, Johannesburg, South Africa)

Xylene Cyanol (Sigma-Aldrich, Steinheim, Germany) 0.02%

- **Non denaturing MDE-SSCP gel mixture**

2X MDE gel solution (Cambrex Bio Science, Rockland, USA) 15 ml

10 X TBE (Appendix F.1.2) 1.8 ml

Glycerol 3 ml

(Merck Chemicals, Wadeville, South Africa)

If glycerol was not added, the 3 ml were substituted with dH<sub>2</sub>O

Make up to 30 ml with dH<sub>2</sub>O

- **Silver staining solution I**

0.5 g AgNO<sub>3</sub> (Merck Chemicals, Wadeville, South Africa)

Make up to 500 ml with dH<sub>2</sub>O

- **Silver staining solution II**

7.5 g NaOH (Merck Chemicals, Wadeville, South Africa)

5 ml 15% Formaldehyde (BDH Chemicals, Poole, UK)

Make up to 500 ml with dH<sub>2</sub>O

- **10% APS (Appendix F.1.3)**

- **TEMED (Merck Chemicals, Wadeville, South Africa)**

- **0.6X TBE**

## H.2.2 Methods

- Assemble gel apparatus by placing two clamped glass plates, separated by 1.5 mm spacers, vertically onto a gel stand, and secure using cams.
- Pipette 5 ml of the MDE gel mixture into a small glass beaker. Add 20  $\mu$ l 10%APS and 20  $\mu$ l TEMED while stirring on a magnetic stirrer.
- Immediately pour the mixture between the glass plates of the gel apparatus and allow to set. This forms a gel plug.
- To the remaining MDE-SSCP gel mixture, add 175  $\mu$ l 10% APS and 17.5  $\mu$ l TEMED.
- Pour this on top of the gel plug between the glass plates.
- Ensure all air bubbles are removed.
- Insert a 20 well 1.5 mm gel comb at the top of the gel and allow to set for at least 45 min.
- Carefully remove the comb from the gel.
- Place the upper buffer tank on top of the gel and clamp into place using cams.
- Pour 500 ml 0.6X TBE Buffer into the upper buffer tank and fill the bottom tank with 2 L of 0.6X TBE.
- Mix 5  $\mu$ l PCR sample with an equal amount of SSCP loading buffer in 0.5 ml microcentrifuge tubes.
- Vortex and spin down briefly in a microcentrifuge.
- Denature samples at 95°C for 5 min on a heating block.
- Place samples on ice for 5 min to prevent renaturation from taking place.
- Using a Hamilton syringe, carefully load 10  $\mu$ l of the samples into the wells. Add a positive control to one of the lanes, if available.
- Include 10  $\mu$ l of a 100 bp DNA step ladder (diluted 1:2 with bromophenol blue loading dye) on the gel to serve as a size standard.
- Run the samples in the 0.6X TBE running buffer at 350V for 18 h.

- **Remove gel and rinse thoroughly in dH<sub>2</sub>O.**
- **Place gel in 500 ml silver staining solution I and shake gently for 10 min.**
- **Rinse thoroughly with dH<sub>2</sub>O.**
- **Place gel in 500 ml silver staining solution II and shake gently until bands are clearly visible.**
- **Rinse thoroughly with dH<sub>2</sub>O.**
- **Visualise PCR fragments using the gel documentation system with the use of a UV/white light converter screen and photograph for future reference.**

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# Appendix I

## Cleanup of PCR Products from Solution for Sequencing

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### I.1 Materials

#### I.1.1 Equipment

- Provided with kit (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences, Buckinghamshire, UK):
  - GFX MicroSpin™ columns
  - Collection tubes
- Gilson Pipetman P1000, P200 and P20 pipettes (Gilson Medical Electronics, Villiers-le-Bel, France)
- Sterile pipette tips (Whitehead Scientific, Cape Town, South Africa)
- Force 14 Microcentrifuge (Denver Instrument Company, Denver, USA)

#### I.1.2 Reagents

- GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK)
- Provided with kit:
  - Capture buffer
  - Wash buffer
- SABAX H2O (Adcock Ingram, Bryanston, South Africa)

## I.2 Methods

- Place one GFX column in a collection tube for each purification to be performed.
- Add 500  $\mu$ l of capture buffer to the GFX column.
- Transfer the DNA solution (up to 100  $\mu$ l) to the GFX column.
- Mix thoroughly by pipetting the sample up and down 4-6 times.
- Centrifuge in a microcentrifuge at 10,000 xG for 30 s.
- Discard the flow-through by emptying the collection tube. Place the GFX column back inside the collection tube.
- Add 500  $\mu$ l of wash buffer to the column. Centrifuge at full speed for 30 s.
- Discard the collection tube and transfer the GFX column to a clean 1.5 ml microcentrifuge tube.
- Apply about 30  $\mu$ l of SABAX H<sub>2</sub>O directly to the top of the glass fiber matrix in the GFX column.
- To elute DNA in a more concentrated form reduce the elution volume to no less than 10  $\mu$ l.
- Incubate the sample at room temperature for 1 min.
- Centrifuge at full speed for 1 min to recover the purified DNA.
- Note:
  - Using low elution volumes to concentrate the DNA sample will reduce recovery.
  - For 50  $\mu$ l elutions, the actual volume of sample recovered will range from 40 to 50  $\mu$ l. For a 10  $\mu$ l elution, the recovered volume will range from 5 to 7  $\mu$ l.

## Appendix J

# Serum sCTLA4 Quantification by Enzyme-linked Immunosorbent Assay (ELISA)

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### J.1 Materials

#### J.1.1 Equipment

- 5 and 10 ml Sterilin Sterile Disposable Plastic Pipettes (Bibby Sterilin, Staffordshire, UK)
- Gilson Pipetman P1000 and P10 (Gilson Medical Electronics, Villiers-le-Bel, France)
- Repeater pipette (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany)
- Eppendorf 12.5 ml Combitips® (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany)
- Beakers, flasks and cylinders for preparation of reagents
- Wash bottle (Laboratory Scientific, Cape Town, South Africa)
- Microwell strip reader (Anthos htII, Anthos Labtec Instruments, Salzburg, Austria)
- Deionized H<sub>2</sub>O

#### J.1.2 Reagents

- Human sCTLA4 ELISA kit (Bender MedSystems GmbH, Vienna, Austria)
- Reagents provided by the manufacturer:
  - Microwell plate coated with monoclonal antibody (murine) to human sCTLA4
  - Biotin conjugate anti-sCTLA4 monoclonal (murine) antibody

- Standard, lyophilised, 20 ng/ml upon reconstitution
- 20X Wash buffer concentrate (PBS with 1% Tween 20)
- Assay buffer concentrate (PBS with 1% Tween 20 and 10% BSA)
- Sample diluent (buffered protein matrix)
- Substrate solution I (tetramethyl-benzidine)
- Substrate solution II (0.02% buffered hydrogen peroxide)
- Stop solution (1M Phosphoric acid)
- Adhesive plate covers

## **J.2 Methods**

- Allow reagents to come to room temperature and mix thoroughly without foaming before use.
- Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and internal control sample\* should be assayed in duplicate. Remove extra microwell strips coated with monoclonal antibody (murine) to human sCTLA-4 from holder and store, tightly sealed, in foil bag with the desiccant provided, at 4°C.
- Wash the microwell strips twice with approximately 300µl wash buffer per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess wash buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 min. Do not allow wells to dry.
- Add 100 µl of sample diluent in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of reconstituted sCTLA-4 standard, in duplicate, into two adjacent wells. Mix the contents by repeated aspiration and ejection and transfer 100 µl to the two wells directly underneath, respectively.

Take care not to scratch the inner surface of the microwells. Continue doubly diluting five times, creating two rows of sCTLA-4 standard dilutions ranging from 10 to 0.16 ng/ml. Discard 100  $\mu$ l of the contents from the last microwells used.

- Add 100  $\mu$ l of sample diluent in duplicate to the blank wells.
- Add 90  $\mu$ l of sample diluent to the sample wells.
- Add 10  $\mu$ l of each sample, in duplicate, to the designated wells.
- Prepare biotin conjugate as per manufacturer's protocol.
- Add 50  $\mu$ l of diluted biotin conjugate to all wells, including the blank wells.
- Cover with a plate cover and incubate at room temperature (18° to 25°C) for 2 h with shaking at 100 rpm.
- Remove plate cover and empty wells. Wash microwell strips as above and proceed immediately to the next step.
- Prepare streptavidin-HRP as per manufacturer's protocol.
- Add 100  $\mu$ l of diluted streptavidin-HRP to all wells, including the blank wells.
- Cover with a plate cover and incubate at room temperature (18° to 25°C) for 1 h with shaking at 100 rpm.
- Prepare TMB substrate solution a few min prior to use as per manufacturer's protocol.
- Remove plate cover and empty wells. Wash microwell strips as above and proceed immediately to the next step.
- Pipette 100  $\mu$ l of mixed TMB substrate solution to all wells, including the blank wells.
- Incubate the microwell strips at room temperature (18° to 25°C) for approximately 10 min with shaking at 100 rpm. Avoid direct exposure to intense light. The colour development on the plate should be monitored and the substrate reaction stopped once an OD<sub>620 nm</sub> 0.6 – 0.65 is reached, before positive wells are no longer accurately recordable.

- Stop the enzyme reaction by quickly pipetting 100  $\mu$ l of stop solution into each well, including the blank wells. It is important that the stop solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme.
- Read absorbance of each microwell immediately on a plate reader at 450 nm. Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the sCTLA-4 standards.
- \* An internal control sample was included in each run to account for inter-assay variation.

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