The role of the HLA region in Hashimoto's Thyroiditis

by

Abigail Zeitlin

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SYNOPSIS

Hashimoto's Thyroiditis is an autoimmune disease of the thyroid gland characterised by thyrocyte death. Although it was previously thought that Hashimoto's thyroiditis shared common aetiology with Graves' disease, there is now significant evidence to suggest separate causal loci. The aims of this thesis were, i) to investigate HLA class II as a causal factor for Hashimoto's thyroiditis ii) to investigate the potential role played by HLA class I MICA and MICB in Hashimoto's thyroiditis. Several predisposing and protective loci within the HLA class II region were determined including the strongly associated DR4 haplotype and its component alleles. Of note DR4 is not associated with Graves' disease providing evidence for a difference in the aetiology of autoimmune thyroid disease. There were no strong associations in MICA and MICB in the context of Hashimoto's thyroiditis. Further work is required to screen the HLA gene region to determine additional casual loci for Hashimoto's thyroiditis. As the HLA gene region is an area of strong linkage disequilibrium this additional screening is also required to determine the primary associated loci. The data presented in this thesis provides further evidence for a difference in the aetiology of Hashimoto's thyroiditis compared to that of Graves' disease.

DEDICATION

For my father, who seems to be making a collection of these.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

1.1 Autoimmune disease

The immune system plays a key role in protecting the body against invading or foreign pathogens. The normal immune response is launched when immune cells recognise foreign pathogens in the host system. Upon recognition of the pathogen the immune cells attempt to neutralise the pathogens by various methods including recruitment of other immune cells, endocytosis/phagocytosis of small pathogens and initiation of necrosis of cellular pathogens. In autoimmune disease this response is directed not at a foreign pathogen but at the hosts own cells. The autoimmune response may differ in specificity, being directed at only one type of cell within one organ through to targeting several organs and systems within the body. The pathology of each disease will therefore be related to the cells, tissues and organs affected. Whilst originally autoimmune diseases were classified into two discrete groups (Goldsby and Goldsby 2003), 'organ' specific and 'non-organ' specific later findings have shown that a spectrum is more appropriate. This is due to the fact that even in 'organ' specific diseases although one tissue may be the major target of autoimmunity others tissues are also targeted.

1.2 Immune system regulatory mechanisms

To understand how immune system regulatory mechanisms break down to cause autoimmunity the normal function of this system must first be examined.

One of the key cells of the immune system are T lymphocytes. T lymphocytes begin as hemopoietic stem cells which are released from the bone marrow, at this stage showing no specific lineage. These early stem cells migrate to the thymus, changing into the T cell precursors, CD4-/CD8- T cells. To permit T cells to recognise a wide variety of antigens the T cell receptor (TCR) is encoded by a series of segmented genes which may be expressed in different combinations allowing for a large degree of diversity within the T cell population. At the point of entry into the thymus the CD4-/CD8- T cells have yet to rearrange the TCR gene segments. The CD4-/CD8- T cells remain in the thymus in this indeterminate state for around three weeks during which time they proliferate. After this stage the cells rearrange the TCR gene segments and begin to express TCRs on the cell surface. As the rearrangement of the TCR gene segments is a random process not all T cells will develop a combination which is functional and compatible with the host's immune system requirements. Due to this factor a double selection process is implemented to ensure that the eventual T cell population contains cells which express useful versions of the TCR; a process which is involves screening out self reactive T cells before they are released into the periphery.

Positive selection

The first stage of the double selection process includes positive selection, whereby thymic cortical epithelial cells present peptides bound by HLA class I and II molecules. Naive CD4+/CD8+ thymocytes which show an affinity to either the HLA class I or class II molecules receive a protective signal. Those which do not interact, due to low affinity for the individuals own HLA molecules, do not receive the protective signal and die through apoptosis. This first

stage eliminates thymocytes which are unreactive towards HLA bound peptides as this mimics the eventual process of antigen fragment presentation in a real immune response.

Negative selection

The initial stage of positive selection generates a mixture of pre-T cells which will react to a presented antigen. However due to the random TCR gene segment rearrangement, there will also be T cells which will react towards the host's own cells within this initial group. The process of negative selection is designed to eliminate these potentially harmful T cells. Medullary thymic epithelial cells, dendritic cells and macrophages act as antigen presenting cells by displaying peptide bound HLA class I and HLA class II molecules for recognition by the T cell population. Pre-T cells which react too strongly to the presented HLA molecules receive the signal for apoptosis (Simmonds and Gough 2004a, Alberts 2002). AIRE has been shown to be expressed in medullary epithelial cells and is thought to play a role in the presentation of tissue restricted antigens from the periphery that T cells may encounter (Giraud *et al.* 2007, Kyewski and Derbinski 2004). This process effectively removes any T cells which would view the hosts own cells as harmful antigens and as a result mount an autoimmune response.

This dual process of positive and negative selection should ensure that only T cells which are useful to the host's immune reaction remain and are released from the thymus after this process. However one of the major flaws in this is that not all self antigens are present in the thymus, hence they cannot be presented in the negative selection process. This results in the possibility that some of the T cells which survive the selection process may still be self-reactive.

1.3 The immune system as a causal factor for disease

The mechanisms mentioned in the previous section should prevent any self reactive T cells from remaining in the host system. These pathways are, however, subject to dysfunction for a variety of reasons, a factor which may lead to the onset of AID.

As part of the normal healthy immune system a small number self reactive T cells are released into the periphery due to the absence of various self antigens in the thymus. If a breakdown in the negative selection process occurs then an unusually high number of self reactive T cells may be released into the periphery. A mutation in *AIRE* is thought to be a causal factor in this breakdown as it induces a reduction in the transcription of the organ specific self antigens found in the thymus (Liston *et al.* 2007). *AIRE* has been the subject of much debate over recent years concerning its role in autoimmune disease. Mutations in the AIRE gene are known to cause autoimmune polyendocrinopathy-candidiases-ectodermal dystrophy (APECED). Although APECED is itself a multi-organ disease patients often show other autoimmune conditions such as thyroiditis and T1D indicating that *AIRE* may play a role as a general autoimmune susceptibility locus. It has been demonstrated that individuals who are homozygous for *AIRE* mutations or double *AIRE* knockout mice show a greater level of immune disfunction than those who are heterozygous (Mathis and Benoist 2009). As a result it has been postulated that whilst individuals who are homozygous for *AIRE* mutations develop APECED those who are heterozygous for mutations show characteristics of other autoimmune disorders. In argument to this theory however a study investigating the expression of common mutations in *AIRE* in a cohort

comprising of individuals with T1D, Addison's disease, GD or HT showed no association of the mutations (Meyer *et al.* 2001).

Conversely, a breakdown in the positive selection process may also lead to inappropriate autoimmune responses. During the positive selection stages only T cells with high affinity to the presented peptides receive the protective signal necessary to avoid apoptosis. However if an abnormally large amount of any given peptide is presented then T cells with only weak affinity will also react (McIver and Morris 1998). These cells when released into the periphery will react to even small amounts of antigen.

As has already been stated some self reactive T cells will be released into the periphery with the assumption that in a working system these will be indentified and removed by CD4+ regulatory T lymphocytes (Tregs). Tregs present in the peripheral immune system, detect the presence of the self-reactive T lymphocytes and send a destructive signal causing apoptosis preventing them causing autoimmune attack (Abbas *et al.* 2004). A number of studies have shown that either a reduction in the number of Tregs or a reduction in the suppressive abilities of the Tregs may cause self reactive T cells to remain and lead to autoimmune disease (Brusko *et al.* 2008, Curotto de Lafaille and Lafaille 2002, Paust and Cantor 2005, Tang *et al.* 2004).

1.4 Autoimmune Thyroid Disease

Two of the most common autoimmune diseases are GD and HT, both of which are centred around autoimmune attack against the thyroid gland. In total AITD represents 2-5% of the iodine

replete western population. To be able to understand how autoimmunity in HT and GD affects the normal function of the thyroid gland, the regulatory system of the hypothalamic-pituitary axis in a euthyroid (defined as normal thyroid function) individual must be explored.

1.4.1 Mechanisms of thyroid function

Thyroid hormone production by the thyroid gland is controlled by a cyclical mechanism relying on both positive and negative feedback. The following describes the normal process of the release of thyroid hormones within a euthyroid individual.

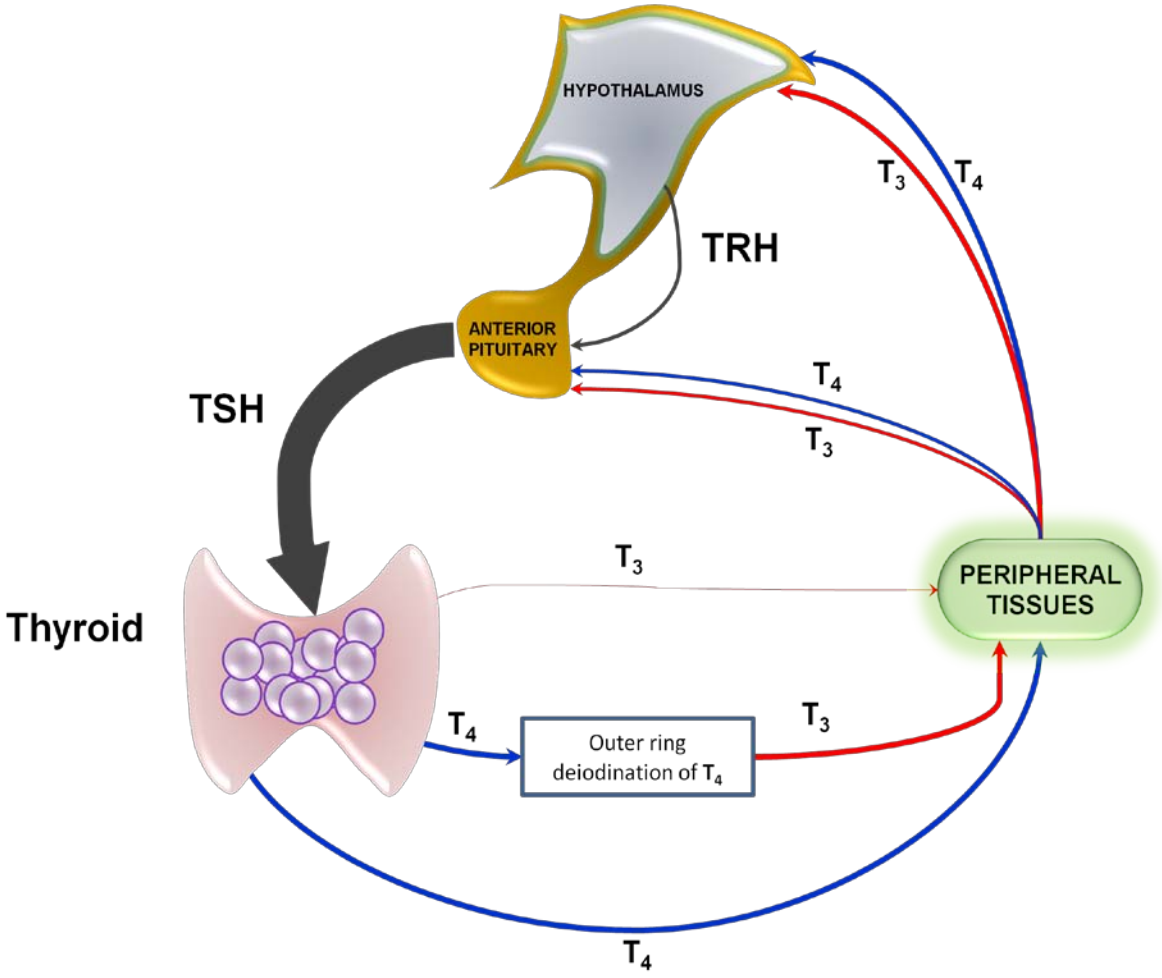
The Hypothalamus, located in the brain just below the thalamus, secretes thyrotropin releasing hormone (TRH) which acts as a stimulus to the anterior pituitary gland which is situated below the hypothalamus. This results in the release of thyroid stimulating hormone (TSH) from the anterior pituitary. The released TSH migrates to the thyroid gland where it binds to thyroid stimulating hormone receptors (TSHRs) (previously known as thyrotropin receptors). As the TSH binds to the TSHR it induces the catalyst thyroid peroxidase (TPO) to react with thyroglobulin (Tg) causing iodinisation (Deme *et al.* 1976, Chistiakov 2003), which results in the formation of thyroxine (T_4). T_4 is released from the thyroid and converted into its active form, triiodothyronine (T_3), by deiodination in the target tissues. Although the majority of T_3 is produced outside of the thyroid a small amount is produced within the thyroid by conversion of T_4 whilst still in the gland. The serum levels of T_4 and T_3 control the release of TSH from the anterior pituitary via negative feedback; as serum levels of T_4 and T_3 increase, this is detected by the hypothalamus and anterior pituitary gland and less TSH is released (Deneareaz and Lemarchand-Beraud 1995).

This mechanism therefore allows a constant level of serum T_4 and T_3 to be maintained. The schematics of this process are shown in *Figure 1.1*. It is disruptions within this pathway that can lead to the symptoms exhibited in AITD.

1.4.2 Graves' Disease

There are two main forms of disruption of the thyroid hormone system, hyperthyroidism and hypothyroidism which are key features of the common AITDs GD and HT, respectively. Although it is known that the thyroid and associated molecular pathways provide the basis for GD, the actual mechanisms by which the disease is caused still await clarification. The predominant feature of GD is hyperthyroidism. Atypical binding of TSHR autoantibodies (TSHRAb) to TSHR (McIver and Morris 1998) causes constant stimulation of the TSHR. This results in overactivity of the thyroid gland and hence the production of large quantities of thyroid hormones (Cooper 2003). Due to the presence of these antibodies the normal mechanism of negative feedback (see section 1.4.1) is disrupted. The TSHRAb bind to TSHR in place of TSH stimulating the thyroid to produce T_4 and T_3, an effect which replicates the mechanism of normal TSH binding. Whereas increasing serum levels of thyroid hormones would cause a reduction in the release of TSH due to negative feedback the TSHRAb continue to bind to TSHR independently of this negative feedback system. This causes a high concentration of T_4 and T_3 to be present in the serum.

Figure 1.1: The hypothalamic-pituitary axis



TSH: Thyroid stimulating hormone

TRH: Thyrotropin releasing hormone

T_4: Thyroxine

T_3: Triiodothyronine

The first few descriptions of the clinical manifestations of GD by Parry (Andrew 2004) and Graves (Weetman 2000) led the way for further investigation and the full range of symptoms associated with GD were soon uncovered. Early findings of hyperthyroidism, diffuse goitre and exophthalmos were validated and what had previously been described as 'palpitations' were attributed to tachycardia and arrhythmia; now recognized as a common symptom. In addition increased bowel frequency, heat intolerance and hyperhidrosis of the hands (excessive sweating), tremor and aspects of the ophthalmopathy described in earlier findings (also known as thyroid eye disease (TED)) were also attributed to the effects of GD (Simmonds and Gough 2004b). The onset of GD is typically in the 4th decade of life and a 5-10 fold increased female preponderance is seen (Tunbridge *et al.* 1977). From the beginning GD was described as a disorder of the thyroid, however it was not until the 1956 study by Adams and Purves (Adams and H.D. 1956) that the underlying mechanism was explored. This study concerning the assay of thyrotropins led to the discovery that autoantibodies directed at the thyroid stimulating hormone receptor (TSHR) may be the cause of the hyperthyroidism experienced in GD (Adams and H.D. 1956, Evans 2002). The involvement of TSHRAb is now accepted and it has been found that 90% of GD patients test positive for TSHR autoantibodies (Evans 2002). In addition the remaining 10% may also exhibit TSHRAb but in such small amounts that current assays cannot detect them.

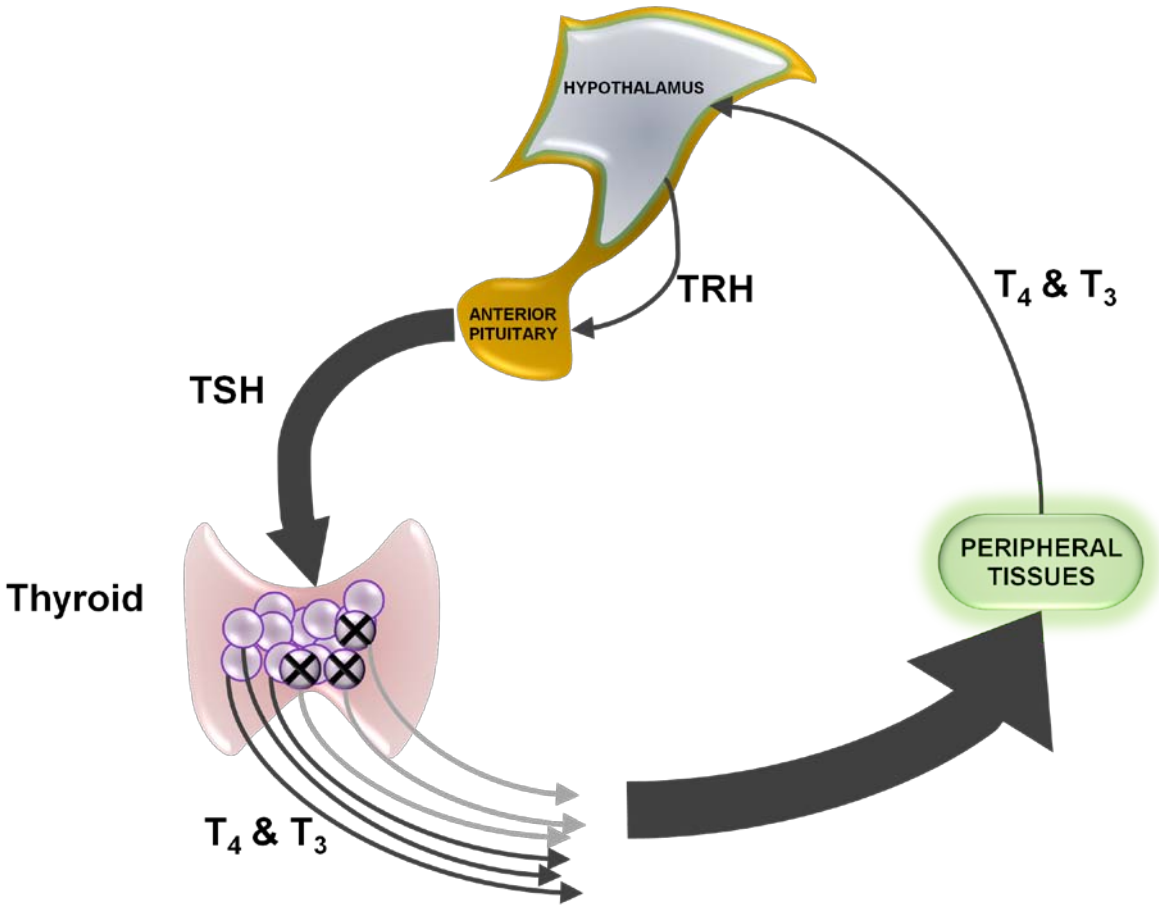
1.4.3 Hashimoto's thyroiditis

The mechanism outlined in the section 1.4.1 describes the cyclical process of the normal control of thyroid hormone production, in HT this cycle is disrupted and hypothyroidism ensues. The death of thyrocytes begins to increase beyond the rate at which they are replaced in a healthy

euthyroid individual the thyroids ability to produce T_3 and T_4 is reduced. This is due simply to a reduction in the number of cells able to produce the hormones. In turn this leads to a decrease in the serum levels of T_3 and T_4 and hence the levels of these hormones that are detected by the hypothalamus. In reaction the hypothalamus increases the output of TRH stimulating the anterior pituitary to increase the production of TSH in an attempt to stimulate the thyroid to produce greater quantities of T_3 and T_4 (see Figure 1.2). Contrary to the eventual outcome of hypothyroidism this can in fact lead to a transient state of hyperthyroidism caused by the excessive production of T_3 and T_4 as a result of this feedback loop. This transient thyrotoxicosis in combination with an initial and incorrect diagnosis of GD may also be the reason for some theories which postulate that GD can transform in to HT.

In 1912 Hakaru Hashimoto first described four patients who had attended his clinic over the course of 6 years (Hashimoto 1912). All four patients he noted were over 40 years of age and female. These patients presented with slightly varying symptoms, the one common symptom to all however was the appearance of a large ‘tumour’ in the neck. Hashimoto performed surgery on all four patients and found that the thyroid gland was enlarged in each. He also ruled out the possibility of this being a normal ‘tumour’ as no metastases were reported and all other organs

Figure 1.2: Mechanism of action of thyroid destruction



TRH: Thyrotropin releasing hormone

T_4: Thyroxine

T_3: Triiodothyronine

seemed healthy at the time (Hashimoto 1912). Following the operations Hashimoto kept tissue samples to further investigate the cause of this enlargement. His most prominent finding was that lymph tissue was abundant in the thyroid of these individuals, a fact which resulted in the alternate and original name for HT, Struma lymphomatosa (Hashimoto 1912).

These early findings provided the first insight into the disease. It was not by coincidence that the 4 patients Hashimoto investigated were all female as a high female preponderance is displayed in HT with women being five to seven times more likely to be affected than men (Vanderpump *et al.* 1995). Again the ages of the four initial patients coincide with the later finding that HT most commonly occurs in the fourth or fifth decade of life (Vanderpump *et al.* 1995).

Diagnosis

Initially the symptoms of HT may not be apparent. As the disease progresses and hypothyroidism becomes more severe, visible symptoms may occur. Common symptoms include intolerance to cold, mild weight gain, fatigue, constipation, dry skin, hair loss, heavy and irregular menses, difficulty concentrating or thinking, joint stiffness and facial swelling (Hashimoto 1912). In some cases mild thyrotoxicosis (a sometimes transient elevation of free T_3 and T_4. hyperthyroidism is a subset of this) and in extreme cases myxedema (dermal and cutaneous edema caused by deposit of large amounts of mucoproteins in the tissues) leading to a coma may occur. An enlarged swelling in the neck or goitre may also be present as a result of either inflammation or hashitoxicosis and late in the disease a small or atrophic thyroid gland. If a goitre is present it may be unilateral or bilateral and will be diffuse and firm with pyramidal lobe enlargement.

If HT is suspected then levels of T_3 and T_4 should be investigated to confirm biochemical hypothyroidism. Furthermore antibody tests are carried out to confirm the diagnosis with positive antibodies to Tg or TPO indicating HT.

Treatment

The effects of the disease cause decreased levels of T_4 to be present hence levothyroxine sodium, a synthetic form of T_4, is used as a replacement treatment. By replacing the T_4 only, the lost T_3 can also be regained via outer ring deiodination of T_4 as would occur in a euthyroid patient (see Figure 1.1). Whilst it is generally accepted that levothyroxine therapy is a suitable course of treatment the actions of the levothyroxine and the dosage required are often brought in to question. The dose required must be carefully adjusted throughout the course of the disease. Some cases of HT result in a euthyroid state and therefore the withdrawal of therapy entirely may be necessary in some instances (Lorini *et al.* 2003).

Other treatments have been postulated and studied, one of the most notable being the use of selenium. The deiodination of T_4 to produce T_3 (see Figure 1.1) occurs via enzymes containing selenocysteine. Whilst three types of deiodination enzyme are present in the body, each acting in different tissues all three are reliant on adequate resources of selenocysteine, a source of selenium. In addition to this glutathione peroxidases are selenium based antioxidant enzymes which catalyse the breakdown of reactive oxygen species such as hydrogen peroxide. A reduction in the normal levels of these selenoproteins can lead to inefficient and incomplete breakdown of

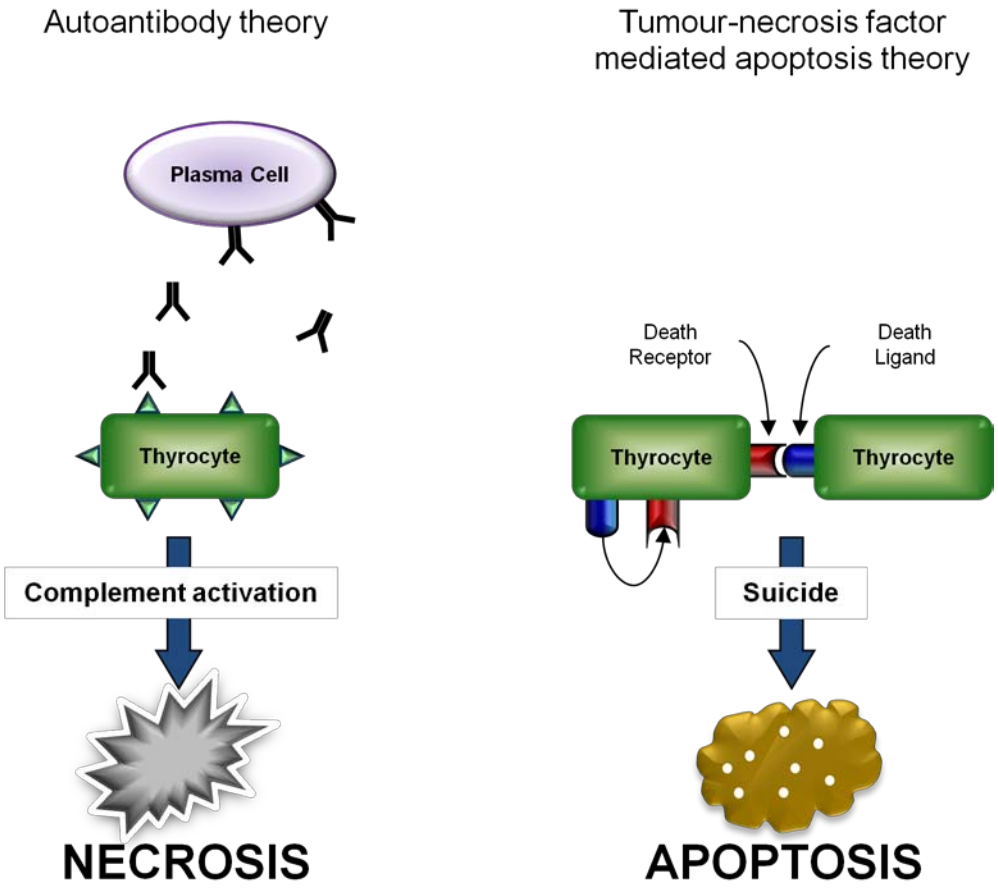
the reactive oxygen species and result in inflammation. This may be a causal factor in not only HT but several AIDs (Duntas 2008). In support of these theories and findings selenium deficiency has been shown to have an adverse effect on the deiodination rate in rats (Stefanou and Wooster 1975) whilst selenium therapy has been shown to have a suppressive effect on TPO antibodies in human studies (Mazokopakis and Chatzipavlidou 2007). This preliminary evidence suggests that selenium may be beneficial in the treatment of HT. There is however no evidence to indicate that selenium improves thyroid function significantly and is therefore not used routinely as a treatment for HT.

Levothyroxine treatment manages HT resulting in lifelong medication, rather than cure it. There has been little investigation in to the possibility of more aggressive treatments involving targeting the autoantibodies themselves or a more permanent cure.

1.4.4 Pathogenesis of Hashimoto's Thyroiditis

There is little available data on the pathogenesis of HT. Whilst some aspects of the etiology are known (see section 1.5) the actual mechanisms and pathways that result in the formation of disease are harder to elucidate. The involvement of the death of thyrocytes and the resulting impact on T_4 and T_3 plasma levels is accepted. However, although thyrocyte death is a known fact of HT the mechanism behind it remains unclear. There are several theories and studies which support various possible mechanisms of either apoptosis or necrosis of the thyrocytes (see figure 1.3).

Figure 1.3: Possible pathogenic pathways of Hashimoto's thyroiditis



Tumour-necrosis factor mediated apoptosis

As thyrocyte death is a feature of HT changes within the pathways causing apoptosis may be linked to HT onset. One of the theories suggested centres around tumour-necrosis factor receptors, in particular the death receptor Fas (also known as TNF receptor superfamily, member 6) and the Fas ligand (FasL) and their roles in apoptosis. One method by which apoptosis occurs is that death receptors such as Fas on the target cell interacts with the ligands present on the T cell such as FasL causing the apoptotic chain to commence. The first step within apoptosis involves the activation of pro-caspases 8 or 10. Active caspases 8 or 10 initiate the mitochondrial membrane of the target cell to become damaged, cellular respiration to cease and finally the activation caspases 3, 6 and 7 which lead to fatal changes in the cell membrane, cytoskeleton and nucleus causing cell death (Lumachi and Basso 2002). The study of Fas and FasL highlights the opposing natures of HT and GD. Several studies have shown that Fas/FasL were upregulated and expressed in thyrocytes of HT patients indicating that this raised level may be responsible for the thyrocyte destruction seen as the disease progresses (Salmaso *et al.* 2002, Xu *et al.* 2007, Stassi and De Maria 2002, Chistiakov 2005).

The Fas mediated thyrocyte death seen in HT is not just due to the presence of T cells, these thyrocytes are able to produce both Fas and FasL on their cellular surface (Giordano *et al.* 2001). This allows fratricidal apoptosis to occur where one thyrocyte induces the death of another independent of T cell interaction (Lumachi and Basso 2002, Chistiakov 2005). Interestingly, Interleukin-1β (IL-1β) is expressed in the thyroid, which stimulates Fas expression in normal thyrocytes pointing towards a potential pathway for the upregulation seen of Fas/FasL. It remains

unclear however whether the upregulation of Fas/FasL seen in HT patients is independent of IL-1β or not.

Autoantibodies

Although it is clear that autoantibodies are present in HT including TPO and Tg, their exact role in the onset and pathogenesis of the disease remains to be validated. At present it is unclear whether the autoantibodies present are the trigger or the result of an autoimmune attack. This is due in part to the variable titres of autoantibodies detected in HT patients and the presence of autoantibodies (albeit in lower levels) in healthy euthyroid patients (Nielsen *et al.* 2008).

The TPO autoantibodies found in HT are able to fix complement. As such, a theory has been proposed suggesting that TPO autoantibodies may be responsible for the thyrocyte death seen in HT (Blanchin *et al.* 2003, Stassi and De Maria 2002). The complement pathway plays an integral role in the innate immune system and may also be utilised by the adaptive immune system. There are three complement pathways, classical, alternative and lectin which, whilst different in the initial stages, all result in similar final stages of the pathway. The classical complement pathway is the only one which is typically due to a specific immune response and is mediated by antibodies (the alternative pathway is stimulated by direct contact with pathogen surface molecules whereas the lectin pathway is stimulated by mannose binding lectin). At the end of the complement pathway the membrane attack pathway is activated and subsequently the membrane attack complex is formed from cytokine fragments C5b, C6, C7 and C8. This complex is a

transmembrane channel which effectively changes the osmotic pressures on the cell causing necrosis.

Environmental cause

One theory suggests that HT is either caused, or the onset of disease induced in an already predisposed individual, by an inflammatory event. An environmental causal factor such as variation in iodine levels or bacterial/viral infection may affect the thyroid causing an inflammatory response and an influx of dendritic cells and macrophages which could be the trigger to activate other immune cells resulting in an autoimmune response. (see section 1.5.1) (Chistiakov 2005). This theory also links to the Selenium therapy mentioned in the discussion of the treatment of HT in section 1.4.3. A deficiency in the levels of selenium would cause a prolonged and intensified inflammatory response (Duntas 2008). This may then cause a heightened sensitivity to pathogens resulting in self antigens being recognised as non-self.

1.5 Genetic and environmental causal factors of AITD

The vast majority of the original work concerning AITD was performed using either solely GD patient cohorts or mixed cohorts where GD and HT were grouped together. As a result the work discussed in the following sections is concentrated on GD and mixed AITD cohorts.

Some of the most striking evidence for a genetic basis to AITD can be seen in the twin studies performed. In two studies of Danish twins GD concordance rates of 0.35-0.36 and 0-0.07 were found for monozygotic (MZ) and dizygotic (DZ) twins respectively (Brix *et al.* 2001, Brix *et al.*

1998). In a similar Californian based study concordance rates of 0.17 and 0.019 were found for MZ and DZ twins respectively (Ringold *et al.* 2002). A further study by Brix et al. attempted to investigate HT in a Danish twin cohort. Only 24 HT patients were used with 3 MZ pairs displaying concordance and 0 DZ pairs showing concordance. The low numbers were taken into consideration and "crude" concordance rates of 0.55 for MZ twins and 0 for DZ twins were reported (Brix *et al.* 2000). Although this does point towards a genetic component to HT the study requires replication in a much larger cohort to verify the findings. This method of using the comparison of MZ and DZ twin pairs is well established for demonstrating the existence of a genetic component in the causal factors of a disease (Osborne *et al.* 1958). Whilst MZ twins share one hundred percent of their genetic material DZ twins do not, as each is formed from a separate fertilised egg. In most cases however, both MZ and DZ twins share environments hence the comparison of concordance rates between MZ and DZ twins can help elucidate whether the causal factors for a disease are genetic and/or environmental factors. The two sets of concordance rates gained from the Danish and Californian GD studies along with a concordance rate of 0.036 between probands and first degree relatives (defined as a relative who is separated by a single meiotic division from the proband and hence shares around 50% of genetic information, i.e. parent, sibling, child) in the Californian (Ringold *et al.* 2002) indicate that there is a definite genetic influence; however they also demonstrate that there are other aspects which need to be investigated concerning the causal factors affecting the development of AITD. With a disease whose only causal factors are genetic, a concordance rate approaching 1.00 would be expected for the MZ twin sets. The concordance rates for MZ twins in the GD studies fall far below the

1.00 mark indicating that the development of GD in an individual is influenced by factors other than a germline variant, be it a somatic variation or environmental factor.

These and other familial clustering and twin studies have clearly demonstrated that AITD does not occur because of a single gene defect and does not follow a simple pattern of Mendelian inheritance (Evans *et al.* 1967, Stenszky *et al.* 1985, Farid 1981). With a single gene defect a clear path of inheritance from parent to child would be expected, although AITD clusters in families it does not necessarily passed from one generation to another and often is found to skip generations (Farid 1992). This irregular pattern of inheritance is characteristic of many of the common medical conditions which are now referred to as complex diseases in which both genetic and environmental factors contribute to disease development (Simmonds and Gough 2004a). This pattern may also imply that AITD is caused by genetic variations conforming to the 'two-hit mutation' theory (Nordling 1953, Knudson 1971). This theory suggests that a germline polymorphism or mutation is indeed inherited but that this alone is not enough to cause disease. For the disease to develop a second somatic or germline polymorphism or mutation must also be present. Although referred to as the two hit mutation hypothesis a disease may require three or more mutations to fully develop (Tomlinson *et al.* 2001). This theory may well account for the erratic pattern of inheritance seen in AITD. If a single mutation is inherited then the progeny will have the potential to develop AITD but will require at least one other mutation to occur for the eventual disease state to develop. This has led to the use of statistical modelling as a possible means of identifying the genetic contribution to AITD. The previously mentioned study by Brix *et al*. which investigated the concordance rates in Danish twins used statistical modelling to

determine the impact of genetic contribution to GD which they proposed was around 79% (Brix *et al.* 2001). This indicates that the inherited component to AITD is likely to arise from numerous common and also rare genetic variants, with contributions from all over the human genome, producing a "DNA fingerprint" that confers an increased risk for the development of disease (Brickman and Shoenfeld 2001). Although the genetic factors are almost certainly the main causal factors in AITD the impact of potential environmental factors should not be ruled out.

1.5.1 Environmental Factors in AITD

To investigate the majority of environmental factors patients are interviewed and the relevant history taken including family history if applicable. This method of data collection may cause inconsistencies in the data for these studies due to the reliance on patient testimony for a number of environmental factors. Embarrassment for example may lead a patient to under-quote the number of cigarettes smoked a day or units of alcohol consumed. For the vast majority of environmental factors however previous notes and documentation may be available to aid with this possible pitfall. A range of statistical tests may be performed on the collated data including assessment of the mean for factors such as age of onset, comparison of environmental factors against physical or biochemical findings such as smoking versus NOSPECS score or variations in physical and biochemical findings between males and females.

Infection

It remains unclear as to whether infection is a significant causal factor for HT (see section 1.4.3 for further information on the pathogenesis of HT). There has been some evidence however for

bacterial infections, in particular *Yersinia enterocolitica* as causal factors for both HT and GD (Corapcioglu *et al.* 2002). *Y.enterocolitica* displays molecular mimicry to TSH whereby the bacteria closely mimics the binding sites displayed on TSH without being identical. As the binding sites are not completely identical the host system recognises the presence of a foreign antigen and launches an immune attack accordingly. The similarity of the *Y.enterocolitica* binding sites to the TSH binding sites is so great however that cross reactive antibodies, those which respond to TSH rather than *Y.enterocolitica* can occur. Whilst some weak correlation between autoantibody titres and *Y.enterocolitica* infection have been shown (Corapcioglu *et al.* 2002) there is little evidence to support this particular model of infection as a causal factor for AITD.

Smoking

Conventionally smoking has been associated with increased risk of AITD and associated symptoms, in particular the severity of thyroid eye disease in GD (Manji *et al.* 2006, Fukata *et al.* 1996, Holm *et al.* 2005, Vestergaard 2002). The effects are presumed to centre on two constituents of cigarette smoke, nicotine and tar. They have been shown to affect the expression HLA, most notably that of HLA-DR (Muller *et al.* 1995). This change in expression causes changes in the presentation of antigens by APCs hence the actions of T cells during positive and negative selection (Vestergaard *et al.* 2002) (see section 1.2). There is some evidence to suggest that smoking can have a positive effect on at least one aspect of HT though. Several studies have shown negative correlations between smoking and TPO antibodies in patients with AITD (Strieder *et al.* 2003, Goh *et al.* 2004, Belin *et al.* 2004). It is thought that one explanation is the

inhibitory effect of thiocynate, a component in cigarette smoke, on iodide organification in the thyroid (Fukayama *et al.* 1992). As organification is catalysed by TPO a reduction in the TPO levels may give rise to a smaller immune response against that particular antigen.

Iodine

Iodine is a central part of the normal mechanisms of the thyroid with both T_3 and T_4 being iodine based hormones (see section 1.4.1). Several animal based studies have shown the ability of increased iodine intake to affect normal thyroid function and induce hypothyroidism and thyroiditis (Rasooly *et al.* 1996, Follis 1964, Allen *et al.* 1986). Furthermore there is evidence that Tg reactive T cells show a greater affinity for highly iodinated Tg than low iodinated Tg (Sundick *et al.* 1987). An increased dietary intake of iodine may therefore lead to a heightened immune response increasing the possibility of an inappropriate response such as one directed at self.

It is thought that the involvement of the TPO autoantibodies play a more prominent role than those of the Tg autoantibodies in HT (see section 1.4.3). Increased dietary iodine has been shown to cause hypothyroidism, in particular when iodine supplements are introduced in geographical areas of poor iodine intake (Harach *et al.* 1985). However this observed hypothyroidism may not be associated with the autoimmune forms seen in AITD.

1.6 Genetics of AITD

It has long been established that there is a genetic component to both HT and GD. The primary concern of early AITD studies was to determine whether there was a genetic component to both GD and HT rather than assessing the exact causal loci. As has been previously mentioned, little work has been done concerning HT alone and the majority of the early genetic studies have been performed using either mixed cohorts or solely GD cohorts. A variety of different methods have been used to investigate the genetics of AITD including case control and genome wide studies.

1.6.1 *Identification of Genetic causal factors*

Candidate Gene studies

Candidate gene studies are based on a prior knowledge or theory of the pathogenesis of the disease or of similar diseases. This may be either from a general hypothesis, such as the investigation of the genes involved in the immune response in autoimmune diseases or from a previous non-genetic study where a protein or molecule was found to be present in abnormal amounts in the sera of affected patients. In AIDs there is a certain amount of crossover of genetic causal factors. The common autoimmune association means that a gene associated with one AID may well be associated with another, in particular if the gene or gene region in question plays a major role in the autoimmune response in a healthy individual.

Once a gene has been chosen variations within that region are investigated in candidate control studies by comparison of unrelated affected subjects with control subjects who are unrelated and unaffected and are matched to cases for sex, age, ethnicity and all other variables with the

exception of disease state . Alternatively in family based studies the affected subjects may be compared with unaffected individuals from the same family.

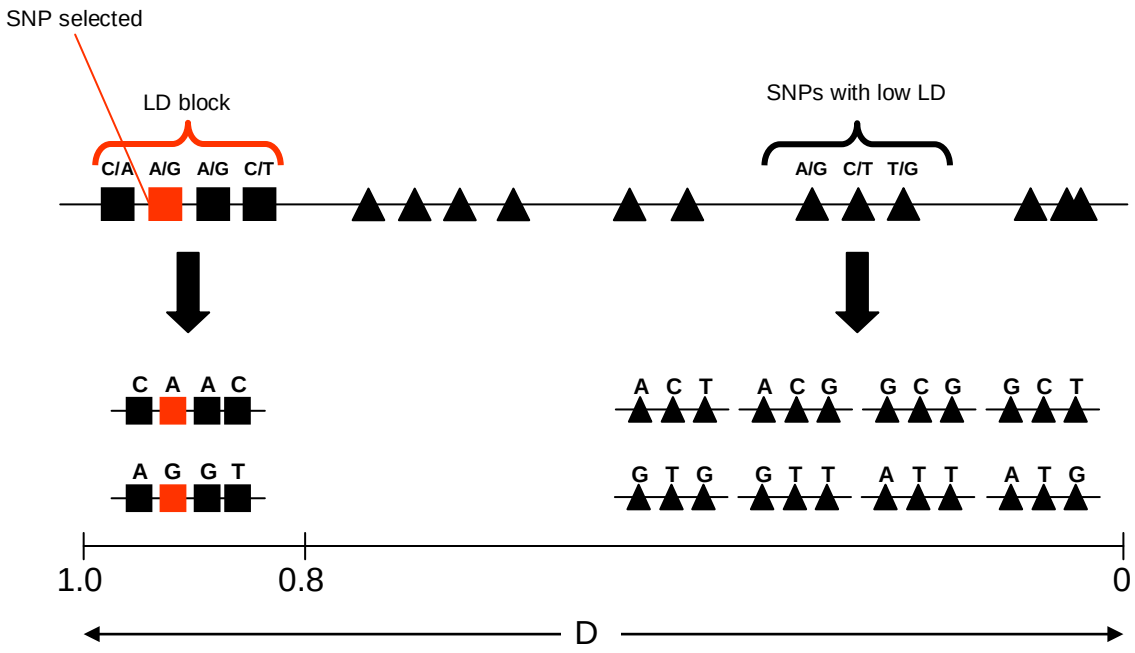
Linkage disequilibrium-mapping and Tag Single nucleotide polymorphisms (SNPs)

Early candidate gene studies examined only one or two polymorphisms due to the constraints of time, expense and knowledge of the gene region. This lead to some genes being falsely excluded as susceptibility loci as the small portion tested was not associated but other areas within the same gene region were later discovered to be associated such as the early work performed on *TSHR* (Cuddihy *et al.* 1995). Linkage disequilibrium (LD) is a measure of the probability that two markers will be inherited on the same haplotype (see chapter 2 section 2.8.4 for further detail). Utilising the strong LD seen in some regions one SNP can be chosen which will give a good representation of the associated LD block and several other SNPs within that block allowing a more comprehensive coverage of the gene region of interest (see Figure 1.4). This allows for an estimation of a large number of genotypes by only typing a few that capture, or tag, a block of LD (Johnson *et al.* 2001).

Genome wide linkage studies

Genome-wide screens can be used to investigate entire genomes for loci which show signs of association with disease. One strength of genome wide screens is that a prior hypothesis is not required. One method used is based on the presence of short tandem repeats (STRs) within the genome. These STRs are normally composed of di-, tri- or tetra-nucleotide repeats and are known as microsatellites. Microsatellites are highly polymorphic; in combination with their

Figure 1.4: Haplotype formation of SNPs in high and low LD



Complete LD allows for only one haplotype to be formed. Low levels of linkage allow for several different SNP combinations.

frequent appearance in the genome they can be used as markers to identify associated loci.

Following the completion of the first family based genome wide linkage study in type 1 diabetes (T1D) (Davies *et al.* 1994), many investigators adopted this approach to identify novel susceptibility loci for complex diseases. Using microsatellite markers, a number of linkage studies and genome wide scans have been conducted in AITD (Tomer *et al.* 1999, Tomer *et al.* 2003, Taylor *et al.* 2006, Sakai *et al.* 2001). One of the first of these was performed by Tomer *et al* in 1999 on 56 multiplex families and identified six regions of linkage to GD, HT and AITD, AITD-1 (6p), GD-1 (14q31), GD-2 (20q11.2), GD-3 (Xq21), HT-1 (13q32) and HT-2 (12q22) (Tomer *et al.* 1999). HT-1 was found to show linkage in all HT families within the cohort whereas HT-2 was linked only in the European HT families and not in the North American families. Upon expansion of the dataset GD-3 and HT-1 were not replicated and three new markers D7S502 (7q), D8S284 (8q) and D10S537 (10q) were proposed as susceptibility loci (Tomer *et al.* 2003). Disparity is also seen when comparing these studies to that of Taylor *et al*, where only 3 areas of possible susceptibility were identified 18p11, 2q36 and 11p15 (Taylor *et al.* 2006).

Whilst putative loci have been reported for AITD, few if any linkages have been convincingly replicated and none of the chromosomal regions of linkage, including HT-2, have led to the identification of a novel locus. The conclusions drawn from many of these studies is that linkage analysis alone is unlikely to deliver a significant number of new loci in complex disease, largely in part, because of inadequate sample size (Wang *et al.* 2005).

A recent initiative has aimed to overcome many of the problems seen in previous genome-wide screens and linkage studies. The Wellcome Trust Case Control Consortium (WTCCC) has conducted several large association studies in which the cohort size is far greater than previous studies. The second major improvement made in these studies is the switch from microsatellite markers to the use of SNPs. Whilst new STRs appear more frequently than new SNPs the overall number of SNPs is normally greater than the overall number of STRs in a given region (Xing *et al.* 2005, Ulgen and Li 2005). For this reason the use of SNPs in this project has allowed a more detailed analysis of the genome by investigating a greater number of markers.

The WTCCC investigated 14,436 non-synonymous SNPs throughout the genome and 897 SNPs from within the HLA region, in 4 diseases; GD, breast cancer, multiple sclerosis and ankylosing spondylitis. For each disease a cohort of 1000 samples was used and a common control cohort of 1500 samples. Several associated SNPs were discovered for GD including numerous associations within the HLA region, 3 SNPs in *FCRL5* and one in each of *FLJ32784, FCRL3, MRPL53, HDLBP, PPP1R3B, ADRA1A, ZNF268, TEKT1, ADCYAP1, AMH* and *UTX*. An associated SNP was also found in *TSHR* in an expanded data set (Burton *et al.* 2007).

Problems surrounding the identification of the genetic causal factors for HT

The primary difficulty in elucidating the genetic components of AITD is that the individual genetic effects at each locus are small. As previously stated AITD is not a monogenic disease, the collective effect of the multiple mutations and polymorphisms may be considerable and indeed

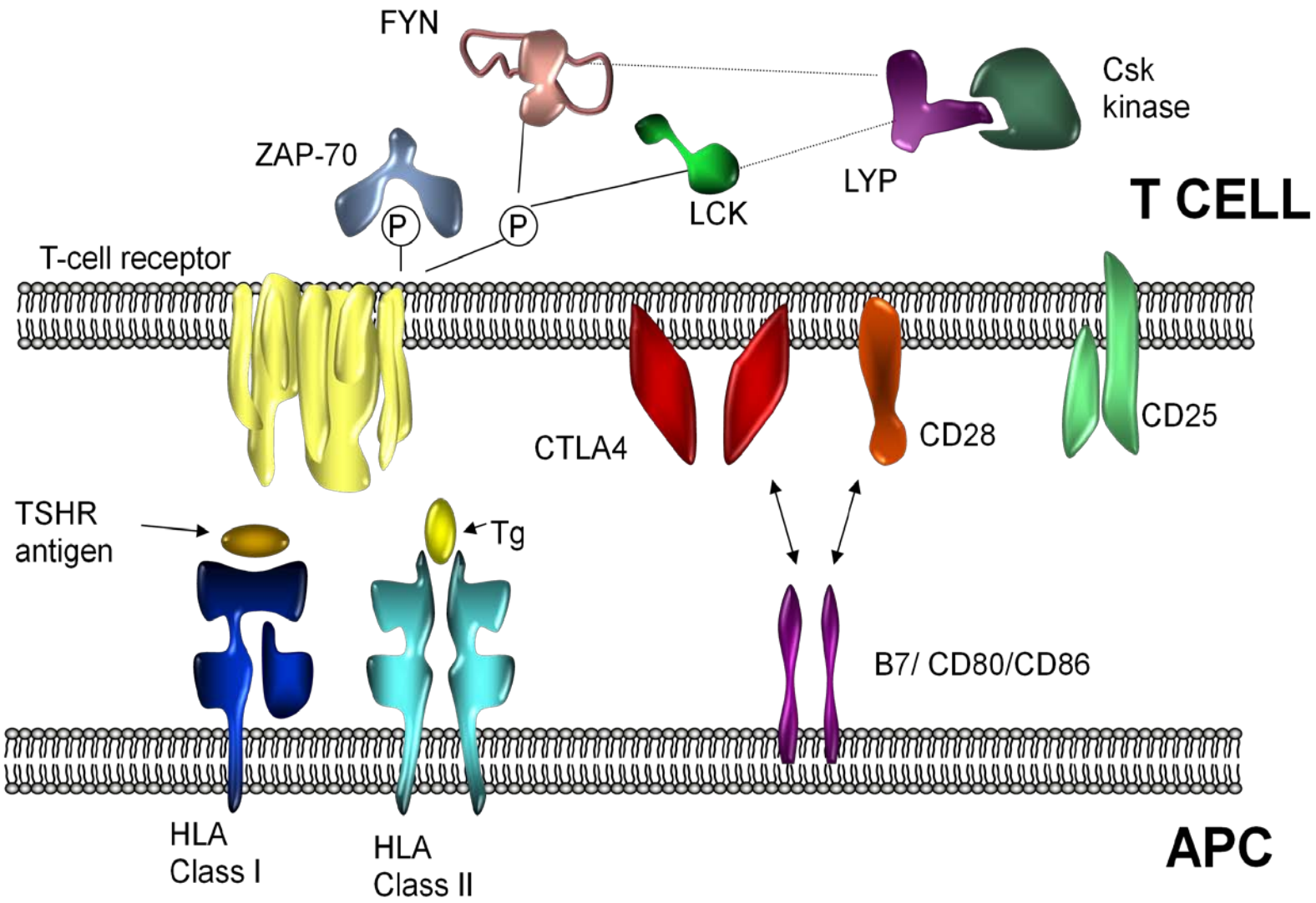
enough to confer disease. However it seems likely that the majority of individual loci confer odds ratios (OR) for the development of disease between 1.2 and 1.5 (Wang *et al.* 2005).

As a consequence, the sample sizes in almost all the early candidate gene population based case control studies, have been too small to detect these effects, whilst at the same time generating non-reproducible false positive results (Ioannidis *et al.* 2001). Sample sizes and power calculations depend greatly on allele frequencies and the size of effect expected. Generally, therefore, whilst some small studies have identified and excluded true specific effects, those with less than 500 cases and 500 controls could be viewed with caution as they may be too small to provide reliable, reproducible data (Dahlman *et al.* 2002).

1.7 Genetic Causal Factors for AITD

There are few indentified genetic causal factors for HT. Of note are the HLA region (discussed in chapter 3) and *CTLA4, Tg and PTPN22.* It is worth however investigating the studies showing associated regions to GD and general AITD cohorts as they may provide some insight into future candidate genes for HT. (see diagram 1.5 for the schematic of the interactions of the molecules encoded by these genes)

Figure 1.5: Some of the suggested and confirmed susceptibility loci for GD involved in antigen presentation to T cells



CTLA4

CTLA4 is a negative regulatory molecule central to the immune system. It inhibits T cell responses by opposing the action of CD28, molecule which promotes T cell actions (Gough *et al.* 2005). CTLA4 was first reported to be associated with GD (Yanagawa *et al.* 1995), since then it has also been shown to be associated with HT (Ueda *et al.* 2003) and many AIDs (Gough *et al.* 2005). Initially only four polymorphisms were known and investigated including an (AT)n repeat sequence in the 3' untranslated region which was associated with potential functional effects (Braun *et al.* 1998, Vaidya *et al.* 1999, Yanagawa *et al.* 1995, Heward *et al.* 1999a).

At this stage the primary disease determinants within this gene region remained unknown. A large resequencing effort and fine mapping of all common variants detected within the gene allowed GD susceptibility to be mapped to four SNPs (CT60, J030, JO31 and JO27_1) within a non-coding 6.1kb region. The common allelic variation (G allele) of the CT60 SNP was associated with lower messenger RNA levels of the soluble alternative splice form of *CTLA*. The CT60 SNP was shown to have an association with HT (OR=1.45, 95%CI=1.17–1.80); *P*=0.0005) in the same study (Ueda *et al.* 2003). Association of the CT60 SNP with GD has since been confirmed by several independent studies including a recent meta-analysis using data collected from 7246 GD patients which showed a significant association (Kavvoura *et al.* 2007). The association has also been confirmed in HT (Donner *et al.* 1997). In contrast to the large numbers available for the GD studies and meta-analysis it should be noted that the two HT studies had patient cohorts of only 228 and 73 respectively and that similar studies showing association of *CTLA4* with HT have also used small patient cohorts (Kotsa *et al.* 1997, Nithiyananthan *et al.*

2002). These findings have yet to be replicated in large numbers. The mechanism by which polymorphism of *CTLA4* acts to inhibit autoimmunity and specifically the role of soluble, as opposed to full length CTLA4, currently remains unknown (Gough *et al.* 2005), but is the subject of ongoing functional analysis.

Tg

Two genome wide linkage studies have reported evidence for linkage between AITD and chromosome 8q24 (Sakai *et al.* 2001, Tomer and Greenberg 2004). This is however inconsistent with the findings of the largest linkage study to date which observed no evidence for linkage between chromosome 8q24 and AITD nor GD or HT (Taylor *et al.* 2006). Tg maps to chromosome 8q24 and a number of candidate gene case control association studies have been conducted examining both microsatellite (Collins *et al.* 2003) and SNP markers (Ban *et al.* 2003, Collins *et al.* 2004a) The original study by Collins *et al.* demonstrated weak association of the intron 27 Tg microsatellite with AITD ($P < 0.001$) (Collins *et al.* 2003). It was however presumed at the time that this may be a false result. This conclusion was strengthened by the second study performed by Collins *et al.* in which no association of Tg with AITD was noted (Collins *et al.* 2004a). Whilst no association was found in these studies Ban et al. described association of a SNP cluster in exons 10 and 12 and also of a separate SNP within exon 33 (Ban *et al.* 2003). Although Tg autoantibodies are seen in both HT and GD, making Tg a viable candidate gene, findings have been inconsistent with the largest association study to date providing no evidence for association between *Tg* markers and AITD (Collins *et al.* 2004a). However, as was the case with the early CTLA4 association studies only a fraction of the gene

has been tested for association with AITD. This may be improved upon in further years following the resequencing of all exons within *Tg* and the genotyping of all exonic SNPs (Ban *et al.* 2003).

PTPN22

PTPN22 located on chromosome 1p13 encodes a protein PTPN22 (also known as LYP). PTPN22 inhibits T cell signalling by dephosphorylating autophosphorylation sites on Src kinases hence preventing activation of T cells. PTPN22 interacts with several accessory molecules including protein tyrosine kinase Csk and Grb2 (Brand *et al.* 2005, Bottini *et al.* 2004). Initially association of *PTPN22* was reported in T1D (Brand *et al.* 2005, Smyth *et al.* 2004) and this was soon followed by positive associations in AITD (Smyth *et al.* 2004, Velaga *et al.* 2004) and also in other autoimmune diseases including systemic lupus erythematosus (Kyogoku *et al.* 2004) and rheumatoid arthritis (RA) (Begovich *et al.* 2004).

The association of *PTPN22* with several AIDs suggests that this may be a general susceptibility locus for AID. This theory is supported by the functional knowledge of both the general role of PTPN22 as mentioned above and also the specific effects of the C and T allelic variants of the associated 1858 SNP (rs2476601). It has been demonstrated that the change from the major allele C to the minor allele T causes a change of the amino acid sequence encoded by this region at position 620 in PTPN22 (Bottini *et al.* 2004). The common allele encodes arginine and the rarer allele tryptophan. This change in amino acid also changes the binding properties of PTPN22 with protein tyrosine kinase Csk with the rarer allele form tryptophan leading to a reduction in the ability of PTPN22 to inhibit T cell activation. This results in a change from arginine in the

common form to tryptophan in the rarer allele, which in turn affects the binding properties of PTPN22 with protein tyrosine kinase Csk. The rarer T allele leads to a reduction in the ability of PTPN22 to inhibit T cell activation, resulting in an exaggerated immune response (Bottini *et al.* 2004).

The rs2476601 SNP appears to be common to a number of AIDs, however other independent associations within the PTPN22 gene region have been detected, with different patterns of association observed in individual diseases (Michou *et al.* 2007, Heward *et al.* 2007, Carlton *et al.* 2005, Onengut-Gumuscu *et al.* 2006). In GD several disease specific haplotypes have been identified, in particular the strongly associated AATTCT $6.77x10^{-8}$ and the strongly protective AACTTT $3.7x10^{-5}$ (both corresponding to alleles from each of the following SNPs (rs2488458 , rs12730735, rs2476601, rs1310182, rs1217413 and rs3811021) (Heward *et al.* 2007). These disease specific haplotypes suggest that the mechanism by which *PTPN22* confers susceptibility to GD may be different, for example, to T1D and RA.

In addition there is one notable report of HT specific association in a cohort of size. In a study by Criswell *et al.* 256 multiplex families which contained at least two different autoimmune diseases within the family were investigated for association with PTPN22. From the families chosen 746 affected individuals, of which 194 were affected with HT, were compared to 2,064 unaffected control subjects. An association of the T allele of rs2476601 SNP with HT was noted with OR=1.77, 95%CI=1.31-2.40 (Criswell *et al.* 2005).

Commonality and distinction in AITD genetic causal factors

Whilst for many genetic studies HT and GD are combined and referred to as AITD, which suggests a single diseases state, more information is becoming available to suggest that these two diseases are distinct conditions. HT and GD represent two extremes of the AITD spectrum. HT is primarily a hypothyroid disorder characterized by the presence of hypothyroidism together with positive antibodies directed against Tg or TPO. In contrast GD is typified by hyperthyroidism and autoantibodies directed against the TSHR in addition to those against Tg and TPO. Many of the genetic studies focusing on AITD have either grouped both HT and GD patients together as a single cohort or have focused solely on GD. This is in part due to the lack of large cohorts available for HT studies; hence to obtain the power necessary for a viable statistical result the cohorts have been predominantly GD where larger cohorts are more readily available. This, in turn, means that whilst many of the causal genetic factors for GD have been established there are few confirmed results for HT. Interestingly although GD and HT share several genetic factors, there are genetic differences already established between these diseases for the CD40 and TSHR genes.

CD40

Genome wide microsatellite screening has linked chromosome 20q11.2 to GD and has been designated GD-2 (Tomer *et al.* 1999, Tomer *et al.* 2003). Further weak support for this result was present in 12 UK Caucasian sib-pairs who appeared to have dominantly inherited GD (Pearce *et al.* 1999), although no evidence for linkage was found in the largest linkage study to date of 1119 Caucasian sib-pairs (Taylor *et al.* 2006). *CD40* has been mapped close to the initial region of

linkage and a single Kozak SNP has been reported to be associated with GD in a study by Tomer *et al.* (Tomer *et al.* 2002a). Whilst this result has been replicated in Koreans (Kim *et al.* 2003) and Japanese (Ban *et al.* 2006, Mukai *et al.* 2005) this has not been the case in two large UK Caucasian cohorts (Houston *et al.* 2004, Heward *et al.* 2004). The Kozak SNP investigated by Tomer *et al.* was shown not to be associated with HT (Tomer *et al.* 2002a) and to date, no association has been reported with HT.

TSHR

Autoantibodies targeted against the TSHR can both stimulate and inhibit the thyroid gland with the stimulating antibodies leading to hyperthyroidism and the classical clinical features of GD (Weetman 2000) (see section 1.4). Using a combination of the TagSNP approach and SNPs previously examined in earlier studies, a total of 40 SNPs were examined in two large independent AITD case control cohorts; one International cohort with 2030 subjects and one exclusively UK with 2427 subjects (Dechairo *et al.* 2005). Convincing, replicated statistical evidence for association of a number of intronic SNPs with GD in the Caucasian population was found. A lack of association with HT identified the *TSHR* as the first GD specific susceptibility locus.

Clustering of Autoimmune Diseases

The clustering of apparently separate AIDs within one individual has been recognised as relatively common, occurring in around 25% of individuals with an existing AID (Mohan and Ramesh 2003). The presentation of three or more separate AIDs in one individual has been

classified as multiple autoimmune syndrome of which there are three groupings. Type I includes myasthenia gravis, thymoma, polymyositis and giant cell myocarditis, Type II includes Sjögren's syndrome, RA, primary biliary cirrhosis, scleroderma and AITD and Type III comprises AITD, myasthenia gravis and/or thymoma, Sjögren's syndrome, pernicious anaemia, idiopathic thrombocytopenic purpura, Addison's disease, insulin-dependent diabetes, vitiligo, autoimmune haemolytic anaemia and systemic lupus erythematosus (Humbert and Dupond 1988). The clustering of more than one type of AID in families and also in individuals indicates that there may be a shared component in the cellular or molecular pathways responsible for the manifestation of some AIDs. This theory of a common cellular or molecular pathway is further supported by the one common theme in all AIDS, the presence of self reactive antibodies.

When investigating the causal factors of a single disease it is unquestionable that a significant result, be it a positive or negative association will further the understanding of that disease. Taking note of the theory of common causal components between AIDs it can be seen though that a significant finding in one AID may also aid in the future research and understanding of other AIDs. Extending this theory further it may also mean that a greater understanding of autoimmune disease in general can be gleaned from findings of common or similar genetic and environmental causal factors. For this study the HLA gene region is being investigated for association with HT, one of the contributing factors in the choice of this region was the previous associations seen with not only GD but also with other AIDs (see Chapter 3).The results from this study and others like it will in turn go on to provide starting points for the investigation of causal genetic factors and pathogenic pathways for other AIDs.

The association of CD40 and TSHR and several other genes (see Table 1.1) with GD but not HT does not necessarily imply that they are not common AITD susceptibility loci. As previously stated most HT genetic studies have been performed on small cohorts, many of which would have been greatly underpowered had they revealed an association. The multifactorial nature of HT means that many, if not all genetic causal factors will have a low odd ratio (OR) making them almost impossible to reliably detect in small cohorts (see Chapter 2, section 2.8 for the definitions and explanations of power and OR). On the other hand these GD only associations may be just as they seem and may be GD specific associations not shared by HT. The extreme difference between both the symptoms of GD and HT and also between the cellular pathways of pathogenesis for these diseases suggests the presence of at least some separate causal factors.

One genetic region where there is much confusion over whether HT and GD are identical is at the HLA class II region. HLA class II molecules play a crucial role in peptide presentation to T cells both in the periphery and in thymic selection. For this reason HLA class II has been investigated as a candidate gene for a wide range of AIDs (Simmonds and Gough 2004a). HLA class II has already been shown to have associations not only in GD (Heward *et al.* 1998) but also in several other autoimmune diseases such as T1D (Todd 1995), systemic lupus erythematosus (SLE) (Graham *et al.* 2002) and RA (Weyand *et al.* 1992). Due however to the aforementioned use of small HT datasets combined with a lack of replication between studies, progress in determining HLA class II associations in HT has been much slower. This leaves a degree of uncertainty pertaining to whether GD and HT share the same susceptibility loci within this region (see Chapter 3).

Table 1.1: A summary of genes investigated for association with Graves' disease

Locus	Reference
CONSISTENTLY REPLICATED	
CTLA-4	(Furugaki *et al.* 2004), (Ueda *et al.* 2003), (Vaidya *et al.* 1999), (Yanagawa *et al.* 1995)
HLA Class II	(Ban *et al.* 2004), (Chen *et al.* 2000), (Simmonds *et al.* 2005c), (Ban *et al.* 2004), (Wongsurawat *et al.* 2006)
PTPN22	(Heward *et al.* 2007), (Smyth *et al.* 2004), (Velaga *et al.* 2004)
TSHR	(Dechairo *et al.* 2005),
AWAITING CONFIRMATION	
CD25	(Brand *et al.* 2007)
IDDM2	(Taylor *et al.* 2006)
IFIH1	(Sutherland *et al.* 2007)
IRF1	(Yang *et al.* 2005)
LMP2	(Heward *et al.* 1999b), (Vives-Pi *et al.* 1997)
LMP7	(Heward *et al.* 1999b), (Vives-Pi *et al.* 1997)
PDCD1	(Newby *et al.* 2007)
TCR-β	(Demaine *et al.* 1989), (Pickerill *et al.* 1993), (Zhang *et al.* 2000)
TNFα	(Nakkuntod *et al.* 2006a), (Simmonds *et al.* 2004)
TPO	(Kotani *et al.* 1986a, Kotani *et al.* 1986b) , (Ludgate and Vassart 1990), (Pirro *et al.* 1995)
UGRP1	(Yang *et al.* 2005)
INCONSISTENT FINDINGS	
CD40	(Heward *et al.* 2004), (Houston *et al.* 2004), (Jacobson *et al.* 2005, Jacobson *et al.* 2007), (Tomer *et al.* 2002a)
FCRL3	(Kochi *et al.* 2005), (Owen *et al.* 2007), (Simmonds *et al.* 2005a)
FOXP3	(Ban *et al.* 2007), (Owen *et al.* 2006)
HLA Class I	(Farid *et al.* 1976), (Grumet *et al.* 1974), (Huang *et al.* 2003), (Mather *et al.* 1980)
IL-13	(Bednarczuk *et al.* 2003), (Bednarczuk *et al.* 2003), (Simmonds *et al.* 2005b)
IL-1RN	(Blakemore *et al.* 1995), (Muhlberg *et al.* 1998)
IL-4	(Heward *et al.* 2001), (Yang *et al.* 2005)
Tg	(Collins *et al.* 2003, Collins *et al.* 2004a), (Tomer and Greenberg 2004, Tomer *et al.* 2002b),
TNFβ	(Nakkuntod *et al.* 2006b)
Vitamin D receptor	(Ban *et al.* 2000a, Ban *et al.* 2000b), (Collins *et al.* 2004b) , (Ramos-Lopez *et al.* 2005), (Stefanic *et al.* 2005)
X Chromosome (various loci)	(Barbesino *et al.* 1998), (Brix *et al.* 2005), (Imrie *et al.* 2001), (Ozcelik *et al.* 2006), (Tomer *et al.* 2003)

Consistently replicated loci: Associations confirmed by replication

Loci awaiting conformation: Preliminary associations found but awaiting replication

Inconsistent findings: Conflicting results from independent studies

For this reason the investigation of HLA class II susceptibility factors in a large HT only cohort is important for elucidating the differences between the associated loci in HT and GD in this area. In addition the HLA class I region has been associated in several AIDs (see Chapter 4). Hence this will also be investigated to determine if components of this region are associated with HT. The aim of this thesis therefore is to investigate HLA for association with HT in a large cohort.

Chapter 2 will look at the methods used in preparation for genetic analysis from blood samples. It will also look at the specific protocols used in analysis of DNA samples via polymerase chain reaction (PCR), allele specific PCR (ASPCR) and TagSNP analysis.

Chapter 3 will look at HLA class II as a causal factor for autoimmune disease and will investigate the following aims:

i. Genotype a large cohort comprising of HT patients and controls at the DRB1, DQA1 and DQB1 loci in the HLA class II region to determine the predisposing and protective genetic factors in the region.
ii. Analyse the combined DRB1, DQA1, DQB1 haplotypes for association with HT
iii. Investigate possible phenotype/genotype interactions.

Chapter 4 will look at the HLA class I MICA and MICB molecules as causal factors for AID and will investigate the following aims:

i. To genotype a large cohort comprising of HT patients and controls using TagSNPs to cover the majority of common polymorphisms with a minor allele frequency of $\geq$ 20% within *MICA* and *MICB*.

To determine the predisposing and protective factors within the MICA and MICB genes via statistical analysis of TagSNP frequencies.

CHAPTER 2: METHODS AND MATERIALS

2.1 Subjects

Unrelated white Caucasian patients of United Kingdom (UK) origin with no history of thyroid surgery, radioiodine therapy or GD were recruited from specialist clinics in Birmingham, Bournemouth, Cambridge, Cardiff, Exeter, Leeds, Sheffield, and Newcastle, (UK), as part of the UK AITD National collection (see Appendix, 6.1). HT was defined by the presence of biochemical hypothyroidism and positive autoantibodies to thyroglobulin (Tg) or Thyroid peroxidase (TPO), the presence of lymphocytic infiltrate in a fine-needle aspirate, or the presence of a diffuse goitre on physical examination by a thyroid specialist (Manji *et al.* 2006). Two sets of ethnically and gender matched control subjects with no family history of AITD were used in this study. Blood samples from control subjects were collected at Blood Transfusion Services (Birmingham, UK and Oxford, UK), Birmingham Heartlands Hospital and the Queen Elizabeth Hospital (Birmingham, UK) as part of the Birmingham control collection. A set of controls were also selected from the 1958 British birth cohort taken from individuals from across the UK who were all born within a single week in 1958 (UKDA 2002, CLS 2005).

2.2 Blood Pelleting and DNA Extraction

The following procedures of blood pelleting, DNA extraction and PicoGreen® were performed by myself and other members of the thyroid research group (see Appendix, section 6.1).

DNA was extracted from 18ml of whole blood collected in two 9ml vacuette tubes (Greiner Bio-One Ltd, UK) which contained sodium ethylenediaminetetraacetic acid (EDTA) to prevent

clotting. The vacuette tubes were stored at 4^{o}C overnight to allow the plasma to separate from the erythrocytes. Following this 1ml of plasma was pipetted from each vacuette tube and placed into a labelled cryovial (Alpha, UK) and stored at -80^{o}C for potential microsomal and Tg antibody testing. The remaining blood samples were each poured into a 50ml falcon tube (TRP, Switzerland) and Reagent A (see Appendix, section 6.2) added to the sample to make a total volume of 40ml. The samples were inverted several times and then shaken at 170rpm for 4 minutes on a shaker (Geneflow, UK), then centrifuged at 2400rpm for a further 4 minutes. Once the supernatant had been removed, without disturbing the white pellet, 15ml of Reagent A was added and the above process repeated to ensure purity of the sample. Once more removing the supernatant, 2ml of Reagent B (see Appendix, section 6.2) was added and the tube briefly vortexed to re-suspend the pellet. The sample was transferred into a 5ml Cryovial (Alpha, UK) and 500µl of Sodium Perchlorate (see Appendix, section 6.2) added to each sample to denature the protein. Chloroform (stored at -20^{o}C) was added to further aid protein denaturation and create a total volume of 5ml. The Cryovial was inverted 10 times to mix and then centrifuged at 3000rpm for 5 minutes creating two phases, a supernatant and a protein layer. The supernatant was gently removed by pipette and transferred into a fresh Cryovial with care taken not to include any of the protein layer. Hundred percent ethanol (-20^{o}C) was then added to precipitate the DNA and make up a final volume of 5ml. The presence of precipitated DNA was characterized by a white mass within the solution (if no DNA was apparent then the tube was incubated at -80^{o}C for 30 minutes to aid precipitation). The sample was then centrifuged at 3000rpm for 10 minutes to pellet the DNA and the ethanol then removed and discarded.

To the pellet, 1ml of 70% ethanol was added; the tube was inverted several times, followed by the sample being centrifuged for 5 minutes at 3000rpm. The ethanol was then removed and the tube left exposed to air for at least 3 hours to allow the samples to dry (indicated by the pellet turning from a milky white to transparent). Once dry, 200µl of T.E. Buffer (see Appendix, section 6.2) was added to each sample and the samples incubated at 37^{o}C for 2 hours to allow the DNA to re-suspend.

2.3 PicoGreen® DNA Quantification

PicoGreen® reagent (Molecular Probes, Eugene, Oregon, USA) was used to quantify the DNA samples collected from both HT individuals and control subjects. PicoGreen® is a fluorochrome which binds to double stranded DNA (dsDNA) and fluoresces highly when bound. The non-bound reagent has little fluorescence hence produces little 'noise' when samples are processed using a standard fluorimeter. PicoGreen® has an excitation maximum at 480nm and an emission peak at 520nm.

Sigma Calf Thymus DNA (Sigma Aldrich, UK) was re-suspended overnight in 1.0ml of T.E. buffer to create a concentration of 1mg/ml. Four µl of re-suspended Calf Thymus DNA and 1246 µl T.E. buffer provided the starting concentration of 1600ng/ml for a serial dilution. Using T.E. buffer as the dilutant, the resulting concentrations of calf thymus DNA were produced to complete the standard curve: 800ng/ml, 400ng/ml, 200ng/ml, 100ng/ml, 50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml.

Two µl of each DNA sample needing to be quantified was diluted in 198 µl T.E. buffer in a sterile 0.5 ml microtube (Alpha, UK) in duplicate and left overnight at 4^{o}C to re-suspend.

Using a sterile 96 flat well plate (Alpha, UK) 50 µl of T.E. buffer was added to the first two wells in order to serve as controls for the procedure. Fifty µl of each of the standard curve dilutions were added to the remaining wells on the first row with duplicates added to the second row. Five µl of each of the duplicated DNA samples were added to the remaining wells on the plate, allowing 36 samples to be analysed in total(see Figure 2.1).

1194 µl T.E. buffer was added to 6 µl of thawed PicoGreen® reagent in an amber microtube (Alpha, UK) to create PicoGreen solution A. To create PicoGreen solution B 7580 µl T.E. Buffer was added to 20 µl PicoGreen® reagent. As the PicoGreen compound displays light sensitivity all solutions containing the compound were prepared and stored in amber/foil covered vessels. Fifty µl of PicoGreen solution A was added to each of the standards and T.E. controls whilst 95 µl of PicoGreen solution B was added to each of the DNA samples. The plate was read immediately on a Wallac Victor 3™ Multilabel counter (Applied Biosystems, UK) using standard fluorescein wavelengths, excitation 480nm and emission 520nm.

Figure 2.1: Layout of the 96 flat well plate for the Picogreen® protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	CONTROL	800ng/ml	400ng/ml	200ng/ml	100ng/ml	50ng/ml	25ng/ml	12.5ng/ml	6.25ng/ml	EMPTY	EMPTY	EMPTY
B	CONTROL	800ng/ml	400ng/ml	200ng/ml	100ng/ml	50ng/ml	25ng/ml	12.5ng/ml	6.25ng/ml	EMPTY	EMPTY	EMPTY
C	Sample 1	Sample1	Sample 2	Sample 2	Sample 3	Sample 3	Sample 4	Sample 4	Sample 5	Sample 5	Sample 6	Sample 6
.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.
H	Sample 31	Sample 31	Sample 32	Sample 32	Sample 33	Sample 33	Sample 34	Sample 34	Sample 35	Sample 35	Sample 36	Sample 36

Wells A1 and B1 show blank controls containing 50 µl of T.E. buffer. Wells A2-A10 and B2-B10 show concentrations of the serial dilution of calf thymus DNA forming the standard curve. Rows C through H show DNA samples in duplicate.

The standard dilutions formed the basis of a standard curve against which the DNA samples can be compared. Repeat tests were performed on any 2 samples for which there was a difference of greater than 20%. Using the concentrations determined each DNA stock sample was then diluted in T.E. Buffer to a concentration of 100ng/ul and then to 20ng/ul to create a uniform DNA dilution for all samples. The 20ng/ul samples prepared were used in the MICA and MICB study (see chapter 4). Neat DNA was used in the HLA study (see chapter 3)

2.4 Polymerase chain reaction (PCR)

The PCR technique for DNA amplification was used for both the HLA and MICA and MICB studies. The following gives an overview of the general principles of PCR whilst the exact methods for each study can be found later in Chapter 3 and Chapter 4.

The PCR technique is designed to amplify a section of DNA. Oligonucleotides are designed around the region of interest which act as primers in the reaction. The two oligonucleotides are different in sequence and are complimentary to the known areas of sequence either side of the desired region for amplification. The PCR reaction also requires Taq polymerase, magnesium chloride and the presence of deoxyribonucleoside triphosphates (dNTPs) which act as free base pairings for the forming strands.

The amplification of the DNA is achieved by first denaturing the template double stranded DNA in the presence of the primers. The cooling step following this allows annealing of the primers to their target sequences on the template DNA. The Taq polymerase then allows the extension of the primers to form complementary DNA strands across the length of the desired region utilising the

free dNTPs available. This cycle of denaturing and annealing is repeated numerous times allowing the strands formed from the last cycle to serve as the templates for the following cycle. With each cycle the quantity of the target DNA is doubled. (see Figure 2.2)

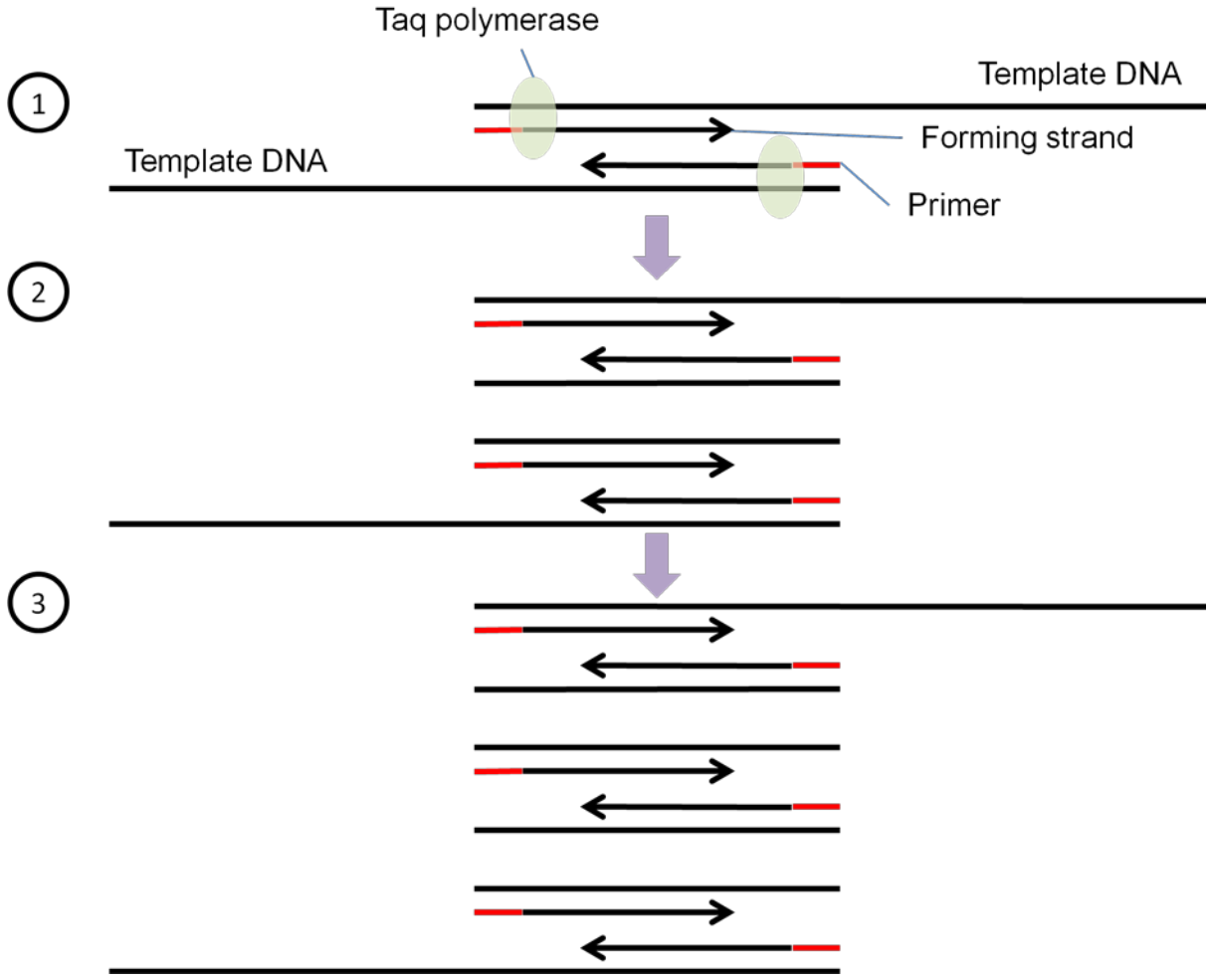
Oligonucleotide primers

To avoid unnecessary amplification of unwanted areas the primers must only anneal to the flanking region and hence a sequence unique to that region must be chosen as the template for the primers. The two sequences should exhibit minimal complementarity to reduce the likelihood of the formation of primer dimers (Fuchs and Podda 2005). Ideally the primers should be between 20-30 bases in length. A high CG content requires a high annealing temperature, so it is preferable to use a sequence with a low CG content (around 50% or less) for the primers. CG base pairs are more stable than AT pairs due to the triple hydrogen bond between the nucleotides (as opposed to the double bond in AT pairs). Therefore high GC content, causes a high melting temperature to be required (Ausubel *et al.* 2003, Gray and Honigberg 2001). Similarly the CG content of the two primers should be similar so that the annealing stage occurs at a similar temperature.

Taq Polymerase

PCR is catalysed by Taq DNA polymerase a form of polymerase originally isolated from the bacterium *Thermus aquaticus. T.aquaticus* reside in hot springs, hence the polymerase derived from these bacteria is able to withstand the unusually high temperatures (of up to 95^{o}C) required

Figure 2.2: cycle of DNA replication via the polymerase chain reaction.



1. Taq Polymerase allows the extension of the primers to from two new complimentary strands

2. The cycle repeats using both the original dsDNA as a template for the new strands in addition to the 2 short sections of DNA formed from the first cycle.

3. The cycle continues, with each repetition the short DNA sections from the previous cycle are used as templates in the new cycle. This allows for exponential growth in the number of strands formed in each cycle.

for PCR protocol. The amount of Taq polymerase used in each reaction must be carefully determined. Increasing the volume used increases the efficiency of the reaction, however this can also increase the frequency of non-specific primer binding. Most common forms of Taq polymerase used in PCR reactions do not have 3'-5' proof reading ability.

dNTP concentration

The dNTPs present in the PCR reaction serve as the raw material from which the new DNA strands are formed. If the concentration is too low then there is not enough raw material to complete the number of cycles needed. Increasing the concentration increases the efficiency of the reaction; however above a certain point increasing the concentration may cause the error rate of the Taq polymerase to increase. An increased concentration of dNTPs will also affect the $MgCl_2$ concentration required as dNTPs chelate magnesium. For 12.5µg of synthesised DNA, 200µM of each dNTP allows half of the dNTPs to become incorporated (Ausubel 2001).

Buffer and Magnesium chloride

PCR reactions require a buffer to be used to maintain the pH at a level in which Taq polymerase can function. Magnesium chloride is used as a source of magnesium in the PCR reaction. Magnesium binds to single nucleotides forming a substrate which allows Taq polymerase to bind. The optimal $MgCl_2$ concentration must be determined for each primer pair used in PCR reactions separately as the concentration required is dependent on the DNA sequence. The optimal concentration is determined via titration and is normally found to be within the range of 0.5mM – 4.0mM. Concentrations below this range do not allow the PCR product to be visualised whilst concentrations above the stated range may result in non-specific banding.

Purity of PCR components

Due to the repetitive nature of the PCR cycling a small anomaly can become greatly magnified. If DNA foreign to the desired sample is present or a mutagenic component is introduced then the final PCR product will show anomalies. To minimise this the reagents used in the PCR reaction must be free from contamination. The reagents mentioned previously must be of high quality and purity and in addition MilliQ water (NANOpure Diamond^{TM}, UK) is used to dilute where required as MilliQ water contains lower levels of DNase, RNase, microorganisms and other contaminants than normal distilled water. To test for DNA contamination at least one well on each plate is left blank, containing only the reagents but none of the sample DNA. Any amplification seen in this well will indicate if a foreign source of DNA in present in any of the reagents used or has been transferred onto the plate from equipment used during the PCR protocol.

Thermal cycling

Two different PCR thermal cycling methods were used in this study. They both however follow the same basic pattern with the main difference being an additional primary step for the MICA and MICB thermal cycling protocol. For this the temperature was first raised to 50^{o}C for a period of 2 minutes to facilitate activation of the Taq polymerase. The following steps of denaturation and annealing were common to both studies.

For the denaturation step the temperature is kept within the range of 93 ^{o}C to 96^{o}C. This range allows for separation of the DNA double strand whilst conferring little or no decrease on the activity of the Taq polymerase. The exact temperature used is again dependent on the CG content

of the template with higher CG content requiring higher temperatures. This is a rapid stage and does not normally exceed 1 minute.

The temperature is lowered for the annealing stage (in this thesis temperatures ranging between 55^{o}C and 70^{o}C were used). The temperature must be such that the DNA template remains in a semi-denatured state whilst allowing the primers to anneal stably to the template and 55^{o}C is the average optimal temperature for this. However this process is once again dependent on CG content. Primers with a CG content which is either higher or lower than 50% may require annealing temperatures of either higher or lower than 55^{o}C respectively. Using non-optimal temperatures in this stage increases the occurrence of non-specific primer annealing.

In these studies extension temperatures of between 60^{o}C and 72^{o}C were used. The optimal working temperature for the Taq polymerase used is 75^{o}C, hence using temperatures around this region will allow for efficient use of Taq polymerase whilst allowing the primers to remain attached to the template DNA. The length of the DNA section which is required to be amplified affects the length of time needed for the extension procedure and in these studies times of 25 seconds to 90 seconds were used.

Conventionally in PCR reactions the annealing and extension stages are separate with the annealing stage being at a lower temperature than that of the extension. However in the MICA and MICB study the primers were designed by Applied Biosystems UK to anneal at a higher temperature, meaning that a separate stage is not required. As well as making the PCR much

quicker the samples are at less risk of contamination because the temperature is kept higher at this stage.

2.5 Allele Specific PCR(ASPCR)

ASPCR uses two or more primers which are able to discriminate between the different alleles of a given locus. The primers are designed to be able to discriminate between the different alleles by insertions, deletions, single base polymorphisms or other small base pair changes found in the DNA region of interest. Each primer will therefore only bind to the single strand of DNA for which it is complementary (see Figure 2.3).

In each reaction control primers which amplify a DNA region outside the region of interest are used. These primers amplify products of different sizes to those produced from within the region of interest. Presence of the control bands in a PCR reaction ensures the PCR reaction was successful if allele bands are absent from the sample.

2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise PCR products in the HLA class II study (See Chapter 3). This method allows the DNA fragments to be separated by size and charge. A solid agarose gel is created, the concentration of which is proportional to the size of the products to be analysed. These gels are placed in gel tanks and covered with buffer. The DNA samples are loaded through the layer of buffer into wells cut into the gel. A general outline of the method is given below with further details of the volumes and measure used given later in Chapter 3.

Figure 2.3: Primers used in PCR reactions

1. In a PCR reaction primers are designed as complimentary to the forward or reverse strand as appropriate

2. In an ASPCR reaction the primers are designed similarly but 2 separate primers are used for each reaction, each containing a different base at the SNP locus.

A fixed volume of electrophoresis buffer (0.5% x TBE; see Appendix, section 6.2) was prepared for both the creation of the gels and to be used as buffer in the gel tanks. Five grams of electrophoresis-grade agarose (Sigma, UK) was added to 500ml 0.5% TBE to create the gels. This was heated using a hotplate and magnetic stirrer (Geneflow, UK). The gel was heated until the agarose powder had fully dissolved, at which point the solution was allowed to cool. Once the solution had cooled to below 55^{o}C an appropriate amount of ethidium bromide (Sigma, UK) was added to facilitate later visualisation. A casting tray with gel combs and casting gates was prepared and the gel solution poured into it. Once the gel had hardened the casting gates and gel combs were removed and the gel still in the casting tray placed into a gel tank. A volume of TBE (see Appendix, section 6.2) was added to the gel tank so as to completely cover the gel and again an appropriate volume of ethidium bromide was added to this. The DNA samples were mixed with loading buffer (see Appendix, section 6.2) and loaded into the wells in the gel. The gel was then run at an appropriate voltage for a set amount of time to allow a current to be created across the length of the gel and the DNA fragments to separate and move down the gel. When finished the gel was removed and visualised under UV light with a photograph taken as a permanent record of the results.

2.7 Tag SNPs

The allelic discrimination in the MICA and MICB study was performed using single nucleotide polymorphisms (SNPs). SNPs are loci where the DNA differs by a single base and the rare allele occurs at a frequency≥ 1% in the general population (Pasternak 2005). Genotyping data for the MICA and MICB gene regions was obtained from the International Haplotype Mapping Project web site [www.hapmap.org]. The data was then incorporated into the Haploview program

[http://www.broad.mit.edu/mpg/haploview/] which allowed the number of SNPs with an allelic frequency greater than 5% to be calculated. The tagger function on the Haploview program was used to calculate the linkage disequilibrium (LD) between the selected SNPs and determine groups of SNPs which were in strong LD with each other. For each group a tag SNP was chosen which was in strong LD (D'>0.80) with those which it tagged (Gabriel *et al.* 2002) (see section 2.8.4 Linkage Disequilibrium for further information)

Assays

Pre-validated assays were purchased from Applied Biosystems 'assay on demand' service [www.appliedbiosystems.com] for 8 SNPs. For the remaining 7 SNPs no assay had previously been designed. For these SNPs sequencing data surrounding the SNP of interest was obtained from the NCBI SNP web site [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Snp] was used to design an appropriate assay and the sequences were then submitted to Applied Biosystems 'assays by design' service [www.appliedbiosystems.com].

Taqman Probes

All assays contained two probes; VIC and FAM to detect alleles 1 and 2 respectively of the SNP being assayed. Initially the fluorescing portion of the probe, either VIC or FAM, is prevented from fluorescing by a non-fluorescent quencher (NFQ). As the forming strand reaches the location of the probe the fluorescent portion is displaced hence it is no longer attached to the NFQ and is seen to fluoresce (see Figure 2.4). The probe will bind to the DNA present in accordance with the allele exhibited; in a heterozygous sample VIC and FAM will both bind, in a homozygous sample only one type of probe will bind. The probes fluoresce with different

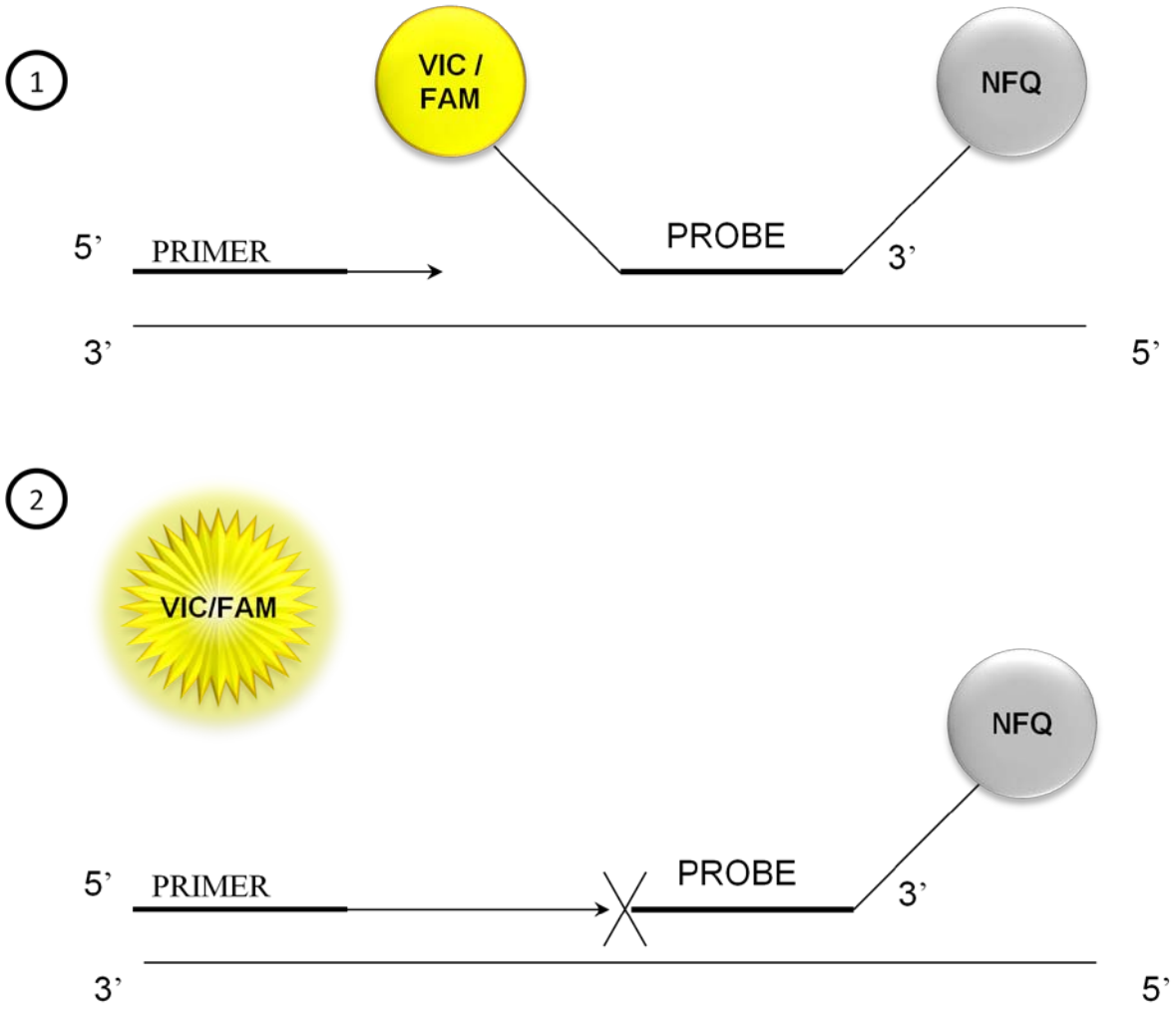
wavelengths enabling the sample to be read using the ABI7900 prism (Applied Biosystems, UK).

2.8 Statistical analysis

2.8.1 Power calculations

Association studies have been commonly seen as a useful method for detecting small genetic contributions to disease. Unfortunately association studies also commonly show a high degree of variation in results between replication studies. As a means of combating this problem power calculations can be used to define the probability that a test can identify a given difference in the population frequency of a marker between cases and control subjects in a given dataset as being significant (P=0.05) and is based on a null hypothesis (Barcikowski 1981, Risch and Merikangas 1996, Lalouel and Rohrwasser 2002). Power calculations can be used to determine the probability that a false null hypothesis will be rejected. It is assumed that any test with a statistical outcome of $P< 0.05$ indicates that the outcome was not by chance or coincidence. Conventionally a power of >80% is accepted (see Chapter 1, section 1.6.1 for further discussion).

Figure 2.4: Probe binding and fluorescence



2.8.2 Case control analysis

All analysis of case control data was performed using the χ^2 test or for small numbers the Fisher's exact probability test.

The χ^2 test was used in conjunction with a 2 x 2 contingency table as shown below.

	Positive for marker	**Negative for marker**
Hashimoto's thyroiditis patients	a	b
Control subjects	c	d

The χ^2 value was calculated and probabilities assigned (see Appendix, section 6.3 for χ^2 calculation). All χ^2 calculations were performed using Excel (Microsoft® Excel, © 1985-2003 Microsoft Corporation).

The Fishers exact probability test was used when any number in the contingency table had a value of 10 or less and all calculations were performed using the MINITAB statistical package (MINITAB Release 14.1 © 1972–2003, Minitab Inc, USA.) (see Appendix, section 6.3 for Fishers exact probability test calculation).

All P values are presented as uncorrected and $P<0.05$ was considered significant. All P values presented have not been corrected for multiple tests. Statistical correlations are not commonly presented for the methods of genetic analysis use in this thesis.

For any result found to be significantly associated with disease the odds ratio (OR) and 95% confidence interval (95% CI) was calculated using the method of Woolf with Haldane's modification for small numbers (Woolf 1955) (see Appendix, section 6.3 for OR and 95% CI calculations). The OR test is a form of the χ^2 test where the probability of an individual with the major allele exhibiting HT is compared to the probability of an individual with the minor allele exhibiting HT. The 95% CI calculation is a measure of variation. It implies that although the OR calculated may be incorrect there is only a 5% chance that the confidence limits generated do not contain the true OR (BMJ 2006). If the OR is greater than 1 and the lower boundary of the 95% CI is greater than 1 then there is a significant risk of exhibiting the disease phenotype if the mutant allele is present (Shephard 2005).

2.8.3 Hardy Weinberg Equilibrium (HWE)

The law of HWE proposes that polymorphisms become stable in the general population after one generation of random mating. HWE calculations were performed for all successfully genotyped Hashimoto's thyroiditis patients and control subjects in both studies using a program based on one designed by Dr. Heather Cordell, University of Cambridge, UK.

2.8.4 Linkage Disequilibrium (LD)

The global D prime (D') calculation (Hedrick 1987) may be used to calculate the LD between two given genetic markers. LD gives a measure of the likelihood that two markers will be inherited as a haplotype. The calculation compares the probability at which the markers would be inherited as a pair if linked compared to the rate at which they would be inherited if combined randomly. A strong LD is often indicative of a physically close relationship between the two markers. A D' of 1 is complete LD, a D' greater than 0.8 is considered to be strong LD, a D' between 0.2 and 0.8 is incomplete LD and less than 0.2 is considered negligible (Ardlie *et al.* 2002). For the Tag SNP study SNPs which displayed LD greater than 0.8 were selected.

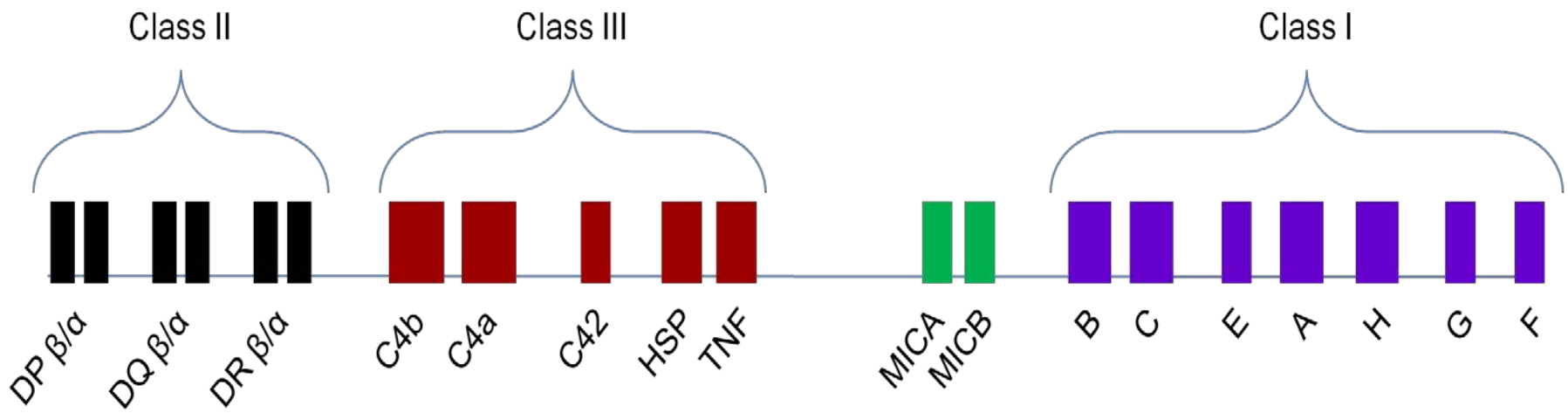
CHAPTER 3: HLA CLASS II

3.1 Introduction

The HLA region is located at chromosome 6p21-23 (NLM 2008). The region consists of a group of genes primarily concerned with humoral and cell mediated immune response. Within this region three major groupings are apparent, HLA class I, HLA class II and HLA class III (see Figure 3.1 for HLA class arrangement). The HLA class I and class II gene regions encode membrane bound glycoproteins which display structural and functional similarity. In contrast the HLA class III region genes encode proteins which differ in both structure and function to the class I and class II loci. In addition the HLA class III genes also encode a variety of other secreted products. The class I region spans approximately 2Mb and contains the classical class I genes HLA-A, HLA-B and HLA-C and non-classical class I genes including HLA-E, HLA- F, HLA-G, HFE, HLA-J, HLA-X, MICA, MICB, MICC, MICD and MICE. (see chapter 4 for further discussion of HLA Class I). The class III region encodes various components of the innate and adaptive immune response (see Chapter 1, section 1.2 for further discussion of the immune response) including the complement components C4, C2 which are involved in the mannose-binding lectin pathway and factor B which is involved in the alternative complement pathway. Several inflammatory cytokines including tumour necrosis factor α (TNFα), lymphotoxin α, heat shock proteins and several other products are also encoded in this region.

Whilst most genetic studies in autoimmune disease has concentrated on the role of HLA class I and class II regions in disease, some work has explored association of the class III genes with an association of TNFα with GD (Simmonds *et al.* 2004) and a tentative association with

Figure 3.1: Schematic of the HLA gene region



C: Complement

HSP: Heat shock protein

TNF: Tumor necrosis factor

MIC: MHC class I chain related

HT (Chen *et al.* 2006) being demonstrated. For this chapter the role of HLA class II as a susceptibility locus for HT is discussed.

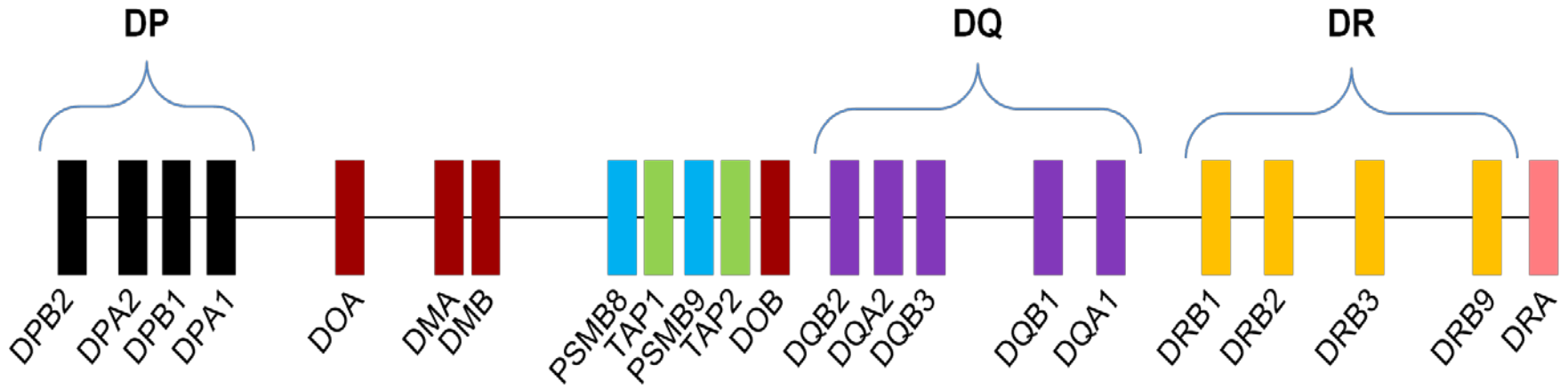
3.1.2 HLA Class II

The HLA Class II gene region spans approximately 1 x 10^6 bp and contains genes encoding polymorphic, heterodimeric, bound glycoproteins mainly expressed on antigen presenting cells such as macrophages, dendritic cells and B cells in addition to many other molecules. The best characterised of these genes, DP, DQ and DR encode proteins that display processed antigenic peptides for recognition by T helper cells (T_H cells) (see Figure 3.2).

The HLA Class II molecules are comprised of an α and β chain encoded by two separate genes. The α and β chain are indentified by having a letter A or B after the molecule being encoded for example the DR alpha chain is called DRA and the DR beta chain is called DRB. Both the α and β genes contain a leader exon, α1 or β1 exon, α2 or β2 exon, a transmembrane exon and one or more cytoplasmic exons. The DR and DQ chains all have similar substructures.In each the first exon encodes the signal sequence, the second the α1 or β1 domain, the third the α2 or β2 domain, the fourth the transmembrane domain and part of the cytoplasmic domain and the fifth again part of the cytoplasmic domain. DRA, DRB and DQA also share the encoding of the cytoplasmic domain over a sixth exon whilst DQB completes the cytoplasmic domain and terminates at exon 5 (Goldsby and Goldsby 2003).

The DQA1, DQB1 and DRB1-9 (specifically DRB1) regions are all highly polymorphic. DQA1 contains 6 loci and 22 alleles, DQB1 5 loci and 46 alleles and DRB1 13 loci and 335 alleles (http://www.ebi.ac.uk/imgt/hla/links.html). This makes these regions ideal for this

Figure 3.2: schematic of HLA class II gene region



PSMB: proteasome subunit beta-type

TAP: transporter associated with antigen processing

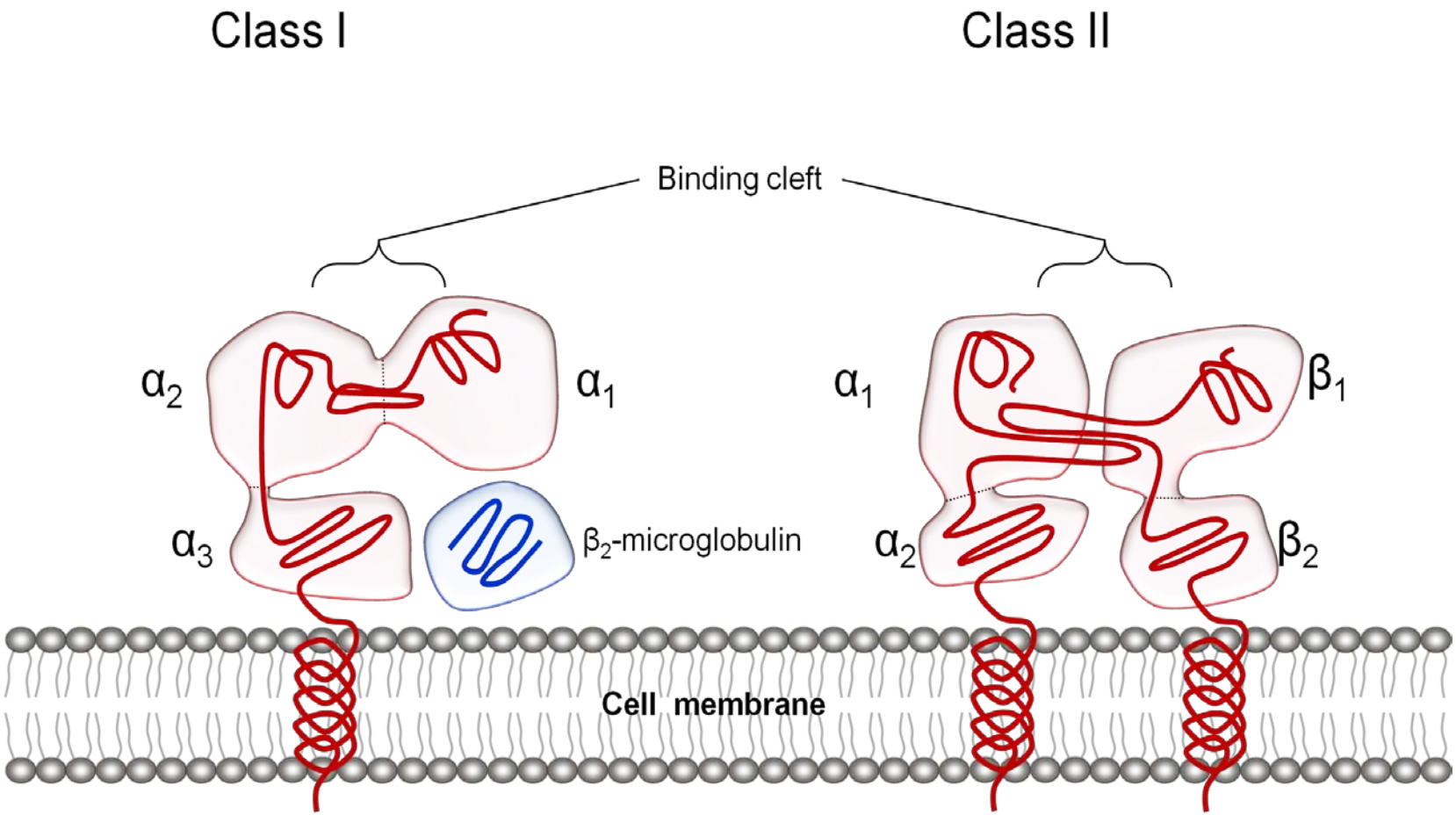
study as the numerous polymorphisms allow for a high density scan of this region. These polymorphisms are reflected in the nomenclature, for example, DRB1*04 denotes that DRB1 is the beta chain in question and that the locus is 04. The DRA region however contains relatively few polymorphisms and for this reason was not investigated (http://www.ebi.ac.uk/imgt/hla/links.html).

Structure and function of the HLA Class II molecules

The HLA Class II molecules are comprised of two polypeptide chains, a 33kDa α chain and a 28kDa β chain. These two chains are linked by non-covalent interactions to form a membrane bound glycoprotein. Each chain consists of two external domains α1 , α2 or β1 , β2 , a transmembrane segment and a cytoplasmic segment. (see Figure 3.3). The outermost portions of the molecule, the α1 and β1 segments, form the antigen binding cleft

The bound peptides originate via the exogenous pathway from antigen presenting cells (APCs) including macrophages, immature dendritic cells, B cells and $CD4^+$ T cells. Membrane bound proteins and other external proteins are internalised by APCs via phagocytosis, endocytosis or a combination of both. Internalised antigen then enters the endolytic processing pathway. The exogenous antigen passes through three compartments, early endosomes, endolysosomes and finally lysosomes. Each compartment contains hydrolytic enzymes and is more acidic than the last at around pH 6.0-6.5, pH 5.0-6.0 and pH 4.5-5.0, respectively and is degraded into peptide. This results in oligopeptides of around 13-18 residues in length which are optimal for binding by HLA class II molecules.

Figure 3.3: HLA class I and class II molecule structures



HLA class II molecules are synthesised in the rough endoplasmic reticulum. Once synthesised they are immediately able to bind peptides. To prevent unwanted peptides or unfolded proteins or endogenous antigens from attaching to the binding cleft the HLA class II invariant chain (Ii) binds in its place. By association with Ii the HLA class II molecules can then move to a low pH endosomal compartment to allow peptide binding to occur. For peptides to bind in the HLA Class II binding cleft the Ii must first be removed. This is done in a series of steps where the Ii is cleaved into shorter fragments. The remaining fragment, the HLA class II associated invariant chain peptide (CLIP), must either be displaced or must dissociate to allow binding of other peptides to occur. Once a peptide has bound the HLA class II molecule then moves to the cell surface for presentation to CD4+ T cells and Tregs as part of peripheral tolerance. HLA class II molecules also present antigens to CD4+8+ thymocytes during positive selection during central tolerance (see Chapter 1, section 1.2). If however no peptide binds then the molecule becomes unstable and degrades in the acidic environment of the endosome.

3.2 Association of HLA with Autoimmune Thyroid disease

As previously stated the majority of studies investigating the genetic causal factors of AITD have focused primarily on GD. This has been in part due to the previous assumption that the causal factors for HT were common to GD also. The early results obtained for HT were pertinent due to the findings in HLA class II association studies in GD. One example of this can be seen in the common AITD predisposing loci found in genome wide scans (see Chapter 1, section 1.6.1). Due to the vast amount of work performed concerning the association of the HLA region with GD it is important to investigate these findings in addition to the comparatively few studies performed on HT cohorts.

Graves' disease

Early studies had shown association of the DR3 haplotype (DRB1*03-DQB1*02-DQA1*05) and its component alleles with GD (Dahlberg *et al.* 1981, Farid *et al.* 1980) The association of DR3 with GD has been validated with consistent reports finding association of the DR3 haplotype with GD in studies using large cohorts (Boehm *et al.* 1992, Heward *et al.* 1998, Simmonds *et al.* 2005, Mangklabruks *et al.* 1991, Badenhoop *et al.* 1992)..

The nature of the association of the DR3 haplotype with GD has been brought into question. It has been proposed that DQA1*0501 was associated with GD independently of the DR3 haplotype as a whole. This was based on increased prevalence in GD patients in a study of USA Caucasian subjects (Yanagawa *et al.* 1993). The question of whether the DR3 haplotype is associated with GD as a result of strong LD between the included alleles or whether the haplotype as a whole is associated has attempted to be answered by several studies (Cuddihy and Bahn 1996, Hunt *et al.* 2001, Yanagawa *et al.* 1994, Maciel *et al.* 2001, Heward *et al.* 1998). The work performed by Heward *et al*. (Heward *et al.* 1998) in one such study was furthered by regression mapping of the HLA region in a study performed by the same group (Simmonds *et al.* 2005). Using stepwise logistic-regression DQB1 did not improve allele based models containing either *DRB1* (P=0.154) or *DQA1* (P=0.04). When an allele based model containing *DQB1* was used however both *DRB1* (P=1.88×10^{-8}) and *DQA1* (P=3.99×10^{-8}) improved the model. This indicated that the main associated locus within the HLA class II region was not contained within the DRB1 gene. It could not be ruled out however whether the main etiological variant within the HLA class II region was contained in DRB1 or DQA1 (Simmonds *et al.* 2005).

Some moves have been made to further narrow the associated locus within the DRB1 region of HLA class II. Two studies have shown association of Arginine at position β74 of the DRB1 chain (Ban *et al.* 2004, Simmonds *et al.* 2006). Using stepwise logistic regression this result was further examined in comparison to other amino acids in the DRB1 chain with the finding that β74 remained the most strongly associated, although a series of other amino acid positions were also found to be associated (β10, β11, β12, β13, β14, β25, β26, β30, β32, β47, β58 and β71) (Simmonds *et al.* 2005). Whilst this association was reported in both the studies by Ban *et al*. (Ban *et al.* 2004) and Simmonds *et al*. (Simmonds *et al.* 2006) the relation of the finding to the previously reported DR3 association is still in question. Although the former study found that the β74 association was independent of the DR3 association the latter study could not confirm this.

Other associations of HLA class II have also been shown with GD, DRB1*07 and the DR7 haplotype (DRB1*07-DQB1*02-DQA1*02) have been shown to have a protective effect against onset of GD (Simmonds *et al.* 2005). DRB1*08 has also been shown to be associated with GD in a North American Caucasian cohort ($P<0.01$, $OR=3.2$) (Chen *et al.* 1999).The study showed however that DRB1*08 was only significantly associated with early onset GD ($P=0.001$, $OR=5.0$) and no significant association with late onset GD was reported. It should be noted that by splitting up the cohort of 92 GD patients only 30 were classified as affected by early onset GD. This is an incredibly small dataset on which to perform age of onset correlations and requires confirmation in a larger cohort.

Hashimoto's Thyroiditis

As with many genetic studies in HT there have been few performed concerning HLA Class II associations. Those which have been performed have often been on small numbers using cohorts of under 500 HT cases and 500 controls. In early association studies using serologic markers DR3 and DR5 haplotypes were shown to be associated, although with differing results. In 1978 a study using a cohort of 262 Canadian Caucasian individuals (127 cases, 135 controls) showed the DRw3 (now known as DR3) haplotype to be associated with HT (RR = 3.5) (Moens *et al.* 1978). In 1980 a study using a cohort of 199 Austrian Caucasian individuals (39 cases, 160 controls) showed association of DR5 (RR = 3.2) (Weissel *et al.* 1980). In 1981 these first two studies were seemingly supported by the findings of a Canadian study where both DR3 and DR5 were found to be associated in a Caucasian cohort of 120 individuals (40 cases and 80 controls) (Farid *et al.* 1981). Again in 1987 an association of DR3 with a cohort of 68 Eastern Hungarian HT patients was observed (Stenszky *et al.* 1987). In 1991 using a UK Caucasian dataset of 186 (86 cases and 100 controls) DR3 (OR = 3.3) and DQw2 were found to be associated (Tandon *et al.* 1991). The association of DQw2 in this study may not be an independent risk factor as it is known to be in strong LD with the DR3 haplotype; hence this may be as a result of the DR3 association.

Following these early results studies began to use larger cohorts in an attempt to provide more accurate results. In 2001 two larger Caucasian studies attempted to provide insight into the HLA Class II association with HT. In a UK Caucasian cohort of 344 individuals an association of HLA-DRB1*03 (P=0.02, OR=1.9) with HT was observed (Hunt *et al.* 2001). In the same year an Italian study performed by Petrone *et al.* using a Caucasian cohort of 427 (126 HT patience and 301 controls) showed association of DRB1*04-DQB1*0301 with HT

(Petrone *et al.* 2001). Although these later studies used much larger cohorts the disparity in the results may indicate that the cohorts were still too small to detect associations reliably.

In addition to the tentative associations proposed for DR3 and DR5 haplotypes, other alleles and haplotypes have been investigated. In 1986 Farid *et al.* found association of the DR4 haplotype (HLA-DRB1*04, HLA-DQA1*03, HLA-DQB1*03) in a small cohort of 192 (52 cases, 140 controls) (OR = 3.1) (Farid and Thompson 1986). This finding was replicated in Caucasians in Petrone's 2001 study as previously mentioned (Petrone *et al.* 2001). A study utilising a Japanese cohort of 388 individuals with 71 HT patients and 317 controls showed associations of the DR4 haplotype (RR = 4.5) (Wan *et al.* 1995). However, due to the differing genetic backgrounds seen in different ancestral geographical regions, these results may not be comparable to those obtained in studies using Caucasian cohorts.

In addition to these findings Badenhoop *et al.* found association of DQA1*0201/*0301 heterozygotes and also DQA1*0301/DQB1*0301 (RR = 4.7) with HT (Badenhoop *et al.* 1990). Also a protective effect has also been proposed by Tamai *et al.* in a Japanese cohort of 105 HT patients and 317 control subjects for DQA1*0102 (RR = 0.3) and DQB1*0602 (RR = 0.1). This, however, is yet to be replicated.

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There have been many other studies which have investigated the associations of HLA Class II with HT (see Table 3.1). However the cohorts used in these were very small, often with the entire dataset being under 100 hence the results of these studies are not considered in detail.

Table 3.1: Past associations of HLA class II with HT

Author	Cases	Controls	Total cohort	Nationality	Allele/haplotype	OR/ RR	
(Badenhoop *et al.* 1990)	64	98	162	-	DQA1*0201/*0301 heterozygotes	-	-
					DQA1*0301/DQB1*0301	-	4.7
(Hunt *et al.* 2001)	77	267	344	UK*	DRB1*03	-	-
(Moens *et al.* 1978)	127	135	262	Canadian*	DR3	-	3.5
(Santamaria *et al.* 1994)	17	71	-	N. European	DQB1*0201	-	-
(Shi *et al.* 1992)	46	50	96	Caucasian	DQA*0301	-	-
	32	50	82		DQB*0201	-	-
(Stenszky *et al.* 1987)	68	-	-	Hungarian	DR3	3.3	-
(Tamai *et al.* 1994)	105	317	-	Japanese	DQA1*0102 (protective)	-	0.3
					DQB1*0602 (protective)	-	0.1
(Tandon *et al.* 1991)	86	100	186	English*	DR3,DQA1*0501/DQB1*0201	-	-
(Wan *et al.* 1995)	71	317	388	Japanese	DRB1*0403	-	3.2
					DR4*0101	-	4.5
(Weissel *et al.* 1980)	39	160	199	Austrian*	DR5	-	3.2

*denotes that the cohort was Caucasian
- denotes that no data was given

This proposed study aims to compare HLA class II associations found in GD patients with class II associations in HT patients. The HT patients used in this study were recruited as part of the UK AITD national collection. These patients were recruited from the same geographic regions as GD patients in the UK national collection. In addition the same control cohort was used for this study as several previous GD association studies. This provides a basis for a direct comparison of the HT and GD associated loci within the HLA class II region.

3.3 Aims of Chapter 3

The primary aim of this study was to:

i) Genotype a large cohort comprising of HT patients and controls at the DRB1, DQA1 and DQB1 loci in the HLA class II region to determine the predisposing and protective genetic factors in the region.

ii) Analyse the combined DRB1, DQA1, DQB1 haplotypes for association with HT

iii) Investigate possible phenotype/genotype interactions.

3.4 HLA methods

3.4.1 Subjects

As described in Chapter 2, section 2.1. In total, DNA was obtained from 640 HT patients and 621 control subjects taken from the control set for the Birmingham control collection. The 621 control subjects used in this study had all been previously genotyped (Simmonds *et al.* 2005)

3.4.2 Genotyping

The *HLA-DRB1, DQB1* and *DQA1* regions were amplified using the phototyping method of allele specific PCR (ASPCR) as previously published (Bunce *et al.* 1995) (see Appendix, section 6.4 for primers). To screen these three regions, 48 ASPCR reactions were performed. For each of the 48 ASPCRs a given allele or set of alleles for the *HLA-DRB1, DQB1* and *DQA1* loci were amplified. A set of control primers (which amplified a 796bp fragment from the third intron of HLA-DRB1) were also included in each reaction to ensure that the PCR had not failed and that non-amplification of allelic bands was due to their absence.

Primers

All primer sequences and working primer concentrations for *HLA-DRB1, -DQA1* and *-DQB1* were obtained from previous work by Bunce et al (Bunce *et al.* 1995) (see Appendix, section 6.4). All primers were obtained from Sigma Genosys, UK. The primers were reconstituted with 1 ml of TE Buffer to stabilise the primers. The primers were then diluted to their working concentration at a final volume of 1ml using the following formulae:

Primer amount (μM) = Primer OD x 100 / (1.5(A) + 0.71(C) + 1.2 (G) + 0.84(T))

Primer amount (μl/ml) = required working primer conc. x 1000 / primer amount

Where A C G T refer to the frequency of each base in the primer sequence.

All primer working concentrations were frozen at -20^{o}C until required.

Five μl of each of the prepared primer solutions were pipetted into 48 wells of a 96 well plate (Greiner, UK) with two sets of ASPCR per 96 well plate. Ten μl of mineral oil (Sigma, UK) was added to each well to cover the primer and reduce evaporation in the later PCR process. The prepared 96 well plates were frozen at -20^{o}C until required.

PCR

For each patient and control sample a solution of 300 µl TDMH (see Appendix, section 6.2) 169 µl Millq water, 9 µl DNA sample and 2.25 µl Taq (Bioline, UK) was produced, then briefly vortexed before 8 µl of the resulting solution was added to each of the 48 wells on the PCR plate containing the ASPCR primers. The plate was then sealed with a plastic sealer (Griener, UK), and centrifuged at 1000rpm for 1 minute. The plates were then run on M J Research PTC-225 Peltier Thermal cycler machine (Genetic Research Instrumentation, UK) using the following program:

60 seconds denaturation – 96^{o}C

5 cycles:	20 seconds – 96 ^{o}C
	45 seconds – 70 ^{o}C
	25 seconds – 72 ^{o}C
21 cycles:	25 seconds – 96 ^{o}C
	50 seconds – 65 ^{o}C
	30 seconds – 72 ^{o}C
4 cycles:	30 seconds – 96 ^{o}C
	60 seconds – 55 ^{o}C
	90 seconds – 72 ^{o}C

300 seconds holding at 20^{o}C.

Three µl of loading buffer (Appendix, section 6.2) was then added to each well of the ASPCR reactions.

Agarose gel electrophoresis of samples

Agarose gels were created using 5g agarose (Sigma, UK) and 500ml 0.5xTBE (Appendix, section 6.2) with 12 µl ethidium bromide (Sigma, UK) added once the gel had cooled but before setting to aid visualization of the samples (see chapter 2, section 2.6). These were placed in a gel tank (Geneflow, UK) with a sufficient volume of 0.5xTBE to allow complete submersion of the gel. Twelve µl of ethidium bromide was also added to the surrounding TBE to further aid visualization of the samples. The initial volume of 12µl of ethidium bromide used in both the creation of the gels and in the surrounding TBE was later changed to 15µl as visualisation was poor when using the lower volume. The gel was electrophoresed for a total of 15 minutes using the following program:

350 Volts

300 amps

150 Watts.

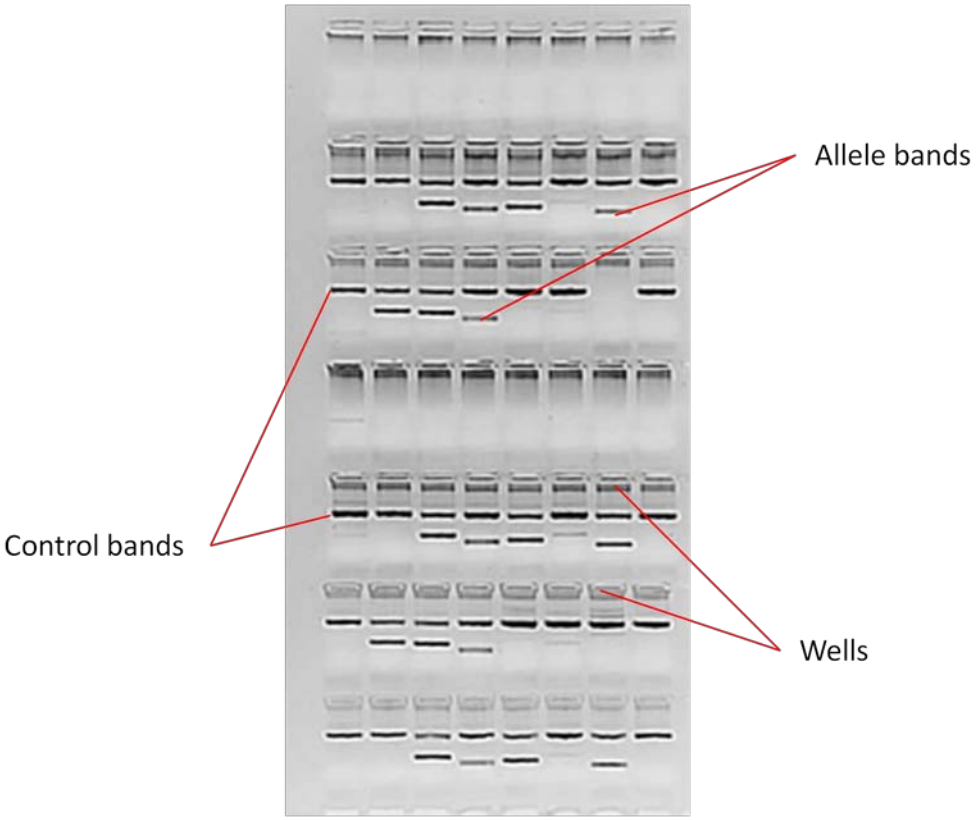
The results were then visualised using an ultraviolet light box (Geneflow, UK) (see figure 3.4)

DYNAL

For some samples ambiguous results were obtained. This may have been due to several reasons including the degradation of the stored DNA sample, inefficient visualisation due to non-optimal volumes of reagents used or cross contamination. In order to resolve these, DYNAL *AllSet*$^{+™}$ SSP kits (Invitrogen, UK) were employed to provide clarification of results via in depth analysis of specific HLA DRB1, DQA1 or DQB1 loci.

For each patient and control sample a solution of 480µl of the DYNAL master mix, 57µl Millq water, 57µl DNA sample and 4.8µl Taq (Bioline, UK) was produced and 10µl of this

Figure 3.4: Visualisation of agarose gel electrophoresis



solution added to each of the 48 wells on the PCR plate containing the ASPCR primers provided by DYNAL. The plate was then sealed with a plastic sealer (Griener, UK), and centrifuged at 1000rpm for 1 minute. The plates were then run on M J Research PTC-225 Peltier Thermal cycler machine (Genetic Research Instrumentation, UK) using the following program:

120 seconds denaturation – 96^{o}C

10 cycles: 15 seconds – 96 ^{o}C

60 seconds – 65 ^{o}C

20 cycles: 10 seconds – 96 ^{o}C

50 seconds – 61 ^{o}C

30 seconds – 72 ^{o}C

Three µl of loading buffer (Appendix, section 6.2) was then added to each well of the ASPCR reactions.

All other conditions were as stated in the previous PCR section with the exception that 25µl ethidium bromide was added to the agarose gel during preparation in place of 15µl ethidium bromide.

Any samples which remained ambiguous or failed completely were not included in the final results.

3.4.3 Statistical analysis

Analysis of case control data was performed using the Chi-squared (χ^2) test with $P < 0.05$ considered significant, with the Fisher's exact test performed for samples with counts lower than 10. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using the method of Woolf with Haldane's modification for small numbers (Woolf 1955). The Fisher's exact test was performed using the MINITAB statistical package (MINITAB Release 14.1 © 1972–2003, Minitab Inc, USA.); all other calculations were performed using Excel (Microsoft® Excel, © 1985-2003 Microsoft Corporation). (See Chapter 2, section 2.8)

3.5 Results

Genotyping results

All cases and controls for each genotype were in Hardy Weinberg equilibrium. In total 625 HT patients and 619 control subjects were successfully genotyped at the *DRB1*, *DQB1* and *DQA1* loci (Table 3.2). One allele from each of the DRB1, DQB1 and DQA1 regions was observed to have a strong association, DRB1*04 (P=0.002, OR=1.37, 95% CI 1.13-1.67), DQB1*0301/4 ($P=3.47x10^{-4}$, OR=1.43, 95% CI=1.17-1.73) and DQA1*03011/12 ($P=1.53x10^{-4}$, OR=1.44, 95% CI=1.19-1.74). Small predisposing effects for HT were detected at DRB1*03, DRB1*08, DQB1*04, and DQA1*0401 (P=0.034-0.017, OR = 1.25-1.93).

Protective effects against the development of HT were also seen at DRB1*07, DRB1*13, DQB1*06, DQA1*01012/3 and DQA1*0201 (P=0.002, OR=0.72-0.79). No other associations were detected.

Strong LD observed between the *DRB1*, *DQB1*, and *DQA1* regions allowed analysis of classical HLA haplotypes to be performed (Table 3.3). A strong predisposing effect was seen for the DR4 haplotype ($P=6.79x10^{-7}$ OR=1.98 [95% CI=1.51-2.59]). A strong association of each of the loci within this haplotype was also seen as mentioned previously; DRB1*04 (P=0.002), DQB1*0301/4 ($P=3.47x10^{-4}$) and DQA1*03011/12 ($P=1.53x10^{-4}$). A weaker predisposing effect for DRB1*08-DQB1*04-DQA1*04 (DR8 haplotype) (P=0.038, OR=1.80, [95% CI=1.01-3.18]) was observed, all loci within this haplotype also demonstrated association. Protective effects were detected for the DR7 haplotype (P=0.013 OR=0.70 [95% CI=0.53-0.93]), DRB1*13-DQB1*06-DQA1*01 (DR13) (P=0.001 OR=0.61 [95% CI=0.45-0.83]) and DRB1*15-DQB1*06-DQA1*01 (DR15) (P=0.034, OR=0.76 [95% CI=0.59-

0.98]). All alleles within these haplotypes were shown to have association when investigated individually barring the DQB1*02 locus (P=0.703) within the DR7 haplotype. Although the DRB1*03 allele was seen to be associated with HT (P=0.034), only a borderline association of the DRB1*03-DQB1*02-DQA1*05 (DR3) haplotype with HT was detected (P=0.050) (Table 3.3). No other associations were detected (Table 3.4).

Phenotype/genotype associations

Data was only available to investigate associations between the presence or absence of diffuse goitre and serum TSH levels at time of diagnosis. Data on the presence or absence of diffuse goitre was only available for a total of 399 HT patients. Data for serum TSH levels was only available for 277 HT patients. No significant differences were seen between HLA class II alleles or haplotypes and individual phenotypes in presence or absence of diffuse goitre (Table 3.5) and HT sub-classified on the basis of TSH levels, 5.5-15 mIU/liter and > 15 mIU/liter (Table 3.6).

Table 3.2: Distribution of DRB1, DQB1 and DQA1 alleles in patients with Hashimoto's thyroiditis and control subjects

HLA class II alleles	Hashimoto's thyroiditis n = 1250† (%)	Control subjects n = 1238† (%)	X^2	P*	OR	95% CI
DRB1						
01	110 (8.80)	130 (10.50)	2.064	0.151		
03	**247 (19.76)**	**204 (16.48)**	**4.514**	**0.034**	**1.25**	**1.02 - 1.53**
04	**285 (22.80)**	**219 (17.69)**	**10.055**	**0.002**	**1.37**	**1.13 - 1.67**
07	**152 (12.16)**	**185 (14.94)**	**4.115**	**0.043**	**0.79**	**0.63 – 0.99**
08	**41 (3.28)**	**24 (1.94)**	**4.399**	**0.036**	**1.70**	**1.02 - 2.83**
09	24 (1.92)	21 (1.70)	0.175	0.675		
10	8 (0.64)	3 (0.24)	FISHER	0.226		
11	77 (6.16)	81 (6.54)	0.153	0.695		
12	21 (1.68)	19 (1.53)	0.083	0.773		
13	**104 (8.32)**	**132 (10.66)**	**3.975**	**0.046**	**0.76**	**0.58 - 1.00**
14	25 (2.00)	35 (2.83)	1.808	0.179		
15	148 (11.84)	177 (14.30)	3.307	0.069		
16	8 (0.64)	8 (0.65)	0.000	0.985		
DQB1						
02	360 (28.80)	348 (28.11)	0.146	0.703		
0301/4	**297 (23.76)**	**222 (17.93)**	**12.796**	**$3.47x10^{-4}$**	**1.43**	**1.17 - 1.73**
0302	103 (8.24)	111 (8.97)	0.417	0.518		
03032	53 (4.24)	60 (4.85)	0.528	0.468		
0305	0 (0.00)	0 (0.00)	-	-		
04	**37 (2.96)**	**20 (1.62)**	**5.023**	**0.025**	**1.84**	**1.06 - 3.18**
05	154 (12.32)	173 (13.97)	1.491	0.222		
06	**246 (19.68)**	**304 (24.56)**	**8.587**	**0.003**	**0.75**	**0.62 - 0.91**
DQA1						
0101/4	140 (11.20)	165 (13.33)	2.618	0.106		
0102/3	**249 (19.92)**	**311 (25.12)**	**9.647**	**0.002**	**0.74**	**0.61 - 0.90**
0201	**137 (10.96)**	**180 (14.54)**	**7.168**	**0.007**	**0.72**	**0.57 - 0.92**
03011/12	**325 (26.00)**	**243 (19.63)**	**14.333**	**$1.53 x10^{-4}$**	**1.44**	**1.19 - 1.74**
0302	1 (0.08)	0 (0.00)	FISHER	1.000		
0401	**37 (2.96)**	**19 (1.53)**	**5.743**	**0.017**	**1.93**	**1.11 - 3.38**
0501/2	361 (28.88)	317 (25.61)	3.363	0.067		
0601	0 (0.00)	3 (0.24)	FISHER	0.123		

P* - uncorrected P values
X^2 = Chi-squared, OR = Odds ratios, 95% CI = 95% confidence interval
† - Number of HT patients = 625, number of control subjects = 619. Each sample produces 2 haplotypes due to the diploid nature of the human genome

Table 3.3: Distribution of associated DRB1, DQB1, DQA1 haplotypes in patients with Hashimoto's thyroiditis and control subjects

HLA class II haplotypes (DRB1, DQB1, DQA1)	Haplotype	Hashimoto's Thyroiditis n=625 (%)	Control subjects n=619 (%)	X^2	P*	OR	95% CI
03, 02, 0501/2	DR3	235 (37.60)	200 (32.31)	3.827	0.050	1.26	1.00 - 1.59
04, 0301/4, 03011/12	DR4	180 (28.80)	105 (16.96)	24.673	6.79×10^{-7}	1.98	1.51 - 2.59
07, 02, 0201	DR7	108 (17.28)	142 (22.94)	6.205	0.013	0.7	0.53 - 0.93
08, 04, 0401	DR8	34 (5.44)	19 (3.07)	4.284	0.038	1.8	1.01 - 3.18
13, 06, 01012/3	DR13	83 (13.28)	124 (20.03)	10.222	0.001	0.61	0.45 - 0.83
15, 06, 0102/3	DR15	143 (22.88)	174 (28.11)	4.48	0.034	0.76	0.59 - 0.98

Table showing individuals displaying at least one copy of specified haplotype
P* - uncorrected P values
X^2 = Chi-squared, OR = Odds ratios, 95% CI = 95% confidence interval

Table 3.4: Distribution of non-associated DRB1, DQB1, DQA1 haplotypes in patients with Hashimoto's thyroiditis and control subjects

HLA class II haplotypes (DRB1, DQB1, DQA1)	Hashimoto's thyroiditis n = 625 (%)		Control subjects n = 619 (%)		X^2	P*
01, 0301/4, 0101/4	0	(0.00)	0	(0.00)	-	-
01, 05, 0101/4	107	(17.12)	122	(19.71)	1.388	0.239
03, 02, 0501/2	235	(37.60)	200	(32.31)	3.827	0.050
03, 06, 0501/2	5	(0.80)	1	(0.16)	FISHER	0.218
04, 0302, 03011/12	93	(14.88)	110	(17.77)	1.903	0.168
07, 02, 03011/12	12	(1.92)	4	(0.65)	FISHER	0.075
07, 03032, 0201	24	(3.84)	38	(6.14)	3.471	0.062
07, 03032, 03011/12	2	(0.32)	0	(0.00)	FISHER	0.500
08, 0301/4, 0401	1	(0.16)	0	(0.00)	FISHER	1.000
09, 03032, 0301	20	(3.20)	21	(3.39)	0.036	0.849
10, 05, 0101/4	6	(0.96)	3	(0.48)	FISHER	0.506
11, 0301/4, 0501/2	68	(10.88)	78	(12.60)	0.889	0.346
11, 05, 0501/2	2	(0.32)	0	(0.00)	FISHER	0.500
11, 06, 0501/2	3	(0.48)	0	(0.00)	FISHER	0.249
12, 0301/4, 0501/2	1	(0.16)	0	(0.00)	FISHER	1.000
12, 05, 0501/2	0	(0.00)	0	(0.00)	-	-
12, 06, 0501/2	1	(0.16)	0	(0.00)	FISHER	1.000
13, 02, 0201	0	(0.00)	0	(0.00)	-	-
13, 02, 0301	0	(0.00)	0	(0.00)	-	-
13, 0301/4, 0201	0	(0.00)	0	(0.00)	-	-
13, 0301/4, 03011/12	0	(0.00)	0	(0.00)	-	-
14, 0301/4, 03011/12	0	(3.52)	0	(0.00)	-	-
14, 05, 001/4	22	(0.00)	35	(5.65)	3.240	0.072
15, 0301/4, 0102/3	2	(0.32)	0	(0.00)	FISHER	0.500
16, 0301/4, 0101/4	0	(0.00)	0	(0.00)	-	-
16, 05, 0101/4	3	(0.48)	5	(0.81)	FISHER	0.504

Table showing individuals displaying at least one copy of specified haplotype
P* - uncorrected P values
X^2 = Chi-squared, OR = Odds ratios, 95% CI = 95% confidence interval

Table 3.5: Distribution of DRB1, DQB1 and DQA1 alleles in patients with Hashimoto's thyroiditis with a diffuse goitre and patients with Hashimotos thyroiditis with no goitre present

HLA class II alleles	Diffuse goitre present n=442† (%)		No goitre present n=356† (%)		X^2	P*	OR	95%CI
DRB1								
0101/2/4	40	(9.03)	36	(9.86)	0.163	0.686		
1501-5	58	(13.09)	38	(10.41)	1.374	0.241		
1601-6	1	(0.23)	5	(1.37)	FISHER	0.096		
0301-5	75	(16.93)	68	(18.63)	0.397	0.529		
0401-22	108	(24.38)	78	(21.37)	1.023	0.312		
0701	57	(12.87)	47	(12.88)	0.000	0.997		
0801-11	14	(3.16)	10	(2.74)	0.123	0.726		
0901	10	(2.26)	7	(1.92)	0.112	0.738		
1001	4	(0.90)	0	(0.00)	FISHER	0.131		
1101-21	22	(4.97)	28	(7.67)	2.523	0.112		
1201-3	11	(2.48)	4	(1.10)	2.114	0.146		
1301-22	35	(7.90)	34	(9.32)	0.513	0.474		
1401-21	7	(1.58)	9	(2.47)	0.809	0.369		
DQB1								
0305	0	(0.00)	0	(0.00)	-	-		
02	120	(27.09)	99	(27.12)	0.000	0.991		
04	11	(2.48)	10	(2.74)	0.052	0.819		
05	54	(12.19)	52	(14.25)	0.743	0.389		
0601-9	94	(21.22)	71	(19.45)	0.384	0.535		
0301/4	97	(21.90)	92	(25.21)	1.223	0.269		
0302	**46**	**(10.38)**	**22**	**(6.03)**	**4.927**	**0.026**	**1.79**	**1.05 - 3.03**
03032	20	(4.51)	18	(4.93)	0.078	0.781		
DQA1								
0101/4	49	(11.06)	46	(12.60)	0.459	0.498		
0102/3	94	(21.22)	72	(19.73)	0.273	0.601		
0201	51	(11.51)	42	(11.51)	0.000	0.998		
03011/12	124	(27.99)	91	(24.93)	0.959	0.327		
0302	1	(0.23)	0	(0.00)	FISHER	1.000		
0401	12	(2.71)	9	(2.47)	0.047	0.829		
0501/2	111	(25.06)	104	(28.49)	1.210	0.271		
0601	0	(0.00)	0	(0.00)	-	-		

P* - uncorrected P values
FISHER = denotes that Fisher's exact test was performed due to small sample numbers
X^2 = Chi-squared, OR = Odds ratios, 95% CI = 95% confidence interval
† - Number of HT patients with goitre present = 221, number of HT patients with no goitre present = 178. Each sample produces 2 haplotypes due to the diploid nature of the human genome

Table 3.6: Distribution of DRB1, DQB1 and DQA1 alleles in patients with Hashimoto's thyroiditis with TSH levels between 5.5-15mIU/liter and patients with Hashimotos thyroiditis with TSH levels greater than 15mIU/liter

HLA class II alleles	TSH = 5.5 - 15mIU/liter n=256† (%)		TSH >15mIU/liter n=298† (%)		X^2	P*
DRB1						
0101/2/4	20	(7.78)	35	(11.71)	2.387	0.122
1501-5	31	(12.06)	29	(9.70)	0.802	0.371
1601-6	0	(0.00)	2	(0.67)	FISHER	0.502
0301-5	42	(16.34)	66	(22.07)	2.900	0.089
0401-22	65	(25.29)	63	(21.07)	1.390	0.238
0701	33	(12.84)	39	(13.04)	0.005	0.943
0801-11	7	(2.72)	5	(1.67)	FISHER	0.560
0901	7	(2.72)	5	(1.67)	FISHER	0.560
1001	1	(0.39)	0	(0.00)	FISHER	0.462
1101-21	13	(5.06)	21	(7.02)	0.930	0.335
1201-3	5	(1.95)	5	(1.67)	FISHER	1.000
1301-22	26	(10.12)	24	(8.03)	0.738	0.390
1401-21	6	(2.33)	4	(1.34)	FISHER	0.525
DQB1						
0305	0	(0.00)	0	(0.00)	-	-
02	66	(25.68)	96	(32.11)	2.764	0.096
04	6	(2.33)	5	(1.67)	FISHER	0.762
05	31	(12.06)	42	(14.05)	0.477	0.490
0601-9	51	(19.84)	54	(18.06)	0.287	0.592
0301/4	65	(25.29)	70	(23.41)	0.266	0.606
0302	22	(8.56)	20	(6.69)	0.693	0.405
03032	15	(5.84)	11	(3.68)	1.443	0.230
DQA1						
0101/4	25	(9.73)	38	(12.71)	1.223	0.269
0102/3	57	(22.18)	53	(17.73)	1.727	0.189
0201	27	(10.51)	36	(12.04)	0.324	0.569
03011/12	78	(30.35)	73	(24.41)	2.461	0.117
0302	1	(0.39)	0	(0.00)	FISHER	0.462
0401	5	(1.95)	5	(1.67)	FISHER	1.000
0501/2	63	(24.51)	93	(31.10)	2.974	0.085
0601	0	(0.00)	0	(0.00)	-	-

P* - uncorrected P values
FISHER = denotes that Fisher's exact test was performed due to small sample numbers. X^2 = Chi-squared, OR = Odds ratios, 95% CI = 95% confidence interval
† - Number of HT patients where TSH=5.5 - 15mIU/liter = 128, number of HT patients where TSH >15mIU/liter = 149. Each sample produces 2 haplotypes due to the diploid nature of the human genome

3.6 Discussion

Although progress has been made in determining the genetic predisposition to GD, less is known about the genetic predisposition to HT, due in part to the use of small datasets and the assumption of genetic homology between both diseases. This study has investigated association of the HLA class II region, long known to be associated with several AIDs, in a large UK Caucasian HT case control dataset.

The strongest association in this study was between the DR4 haplotype and HT. This was closely followed by the associations of the individual alleles included in this haplotype (DRB1*04, DQB1*0301/4 and DQA1*03011/12). As previously mentioned in section 3.2 this is not the first time that association of the DR4 haplotypes and its included loci with HT has been proposed. The DR4 haplotype has been shown to be associated in two Caucasian cohorts (Petrone *et al.* 2001, Farid and Thompson 1986) and DRB1*04 has been associated in a Japanese HT cohort (Wan *et al.* 1995). Whilst the previous associations have been based on small cohort sizes this study does serve to support this association.

Although there is limited data on previous associations with HT, the DR4 haplotype has long been associated with type 1 diabetes (T1D) (Cucca *et al.* 2001, Thomson *et al.* 1988). The shared epitope, a highly conserved amino acid sequence encoded in several DRB variations, including *0401, DRB1*0404, DRB1*0405 and DRB1*0408 has also been associated with rheumatoid arthritis (RA) (Gourraud *et al.* 2006). This may indicate that the DR4 haplotype is a common susceptibility haplotype for autoimmune disease, or more specifically for autoimmune disease centred around cellular destruction such as HT, T1D and RA. It was not possible to assess the impact of T1D and/or RA on the HLA class II associations found in this

study due to lack of available phenotype data concerning the autoimmune history of the patient or family history. This would however be useful to investigate as it may elucidate whether the association of DR4 was due to the coexistence of T1D and/or RA or if it is an independent association

To understand this association and the possible general autoimmune connection, the function of the DR4 haplotype and included alleles must be investigated. Schonland et al demonstrated that individuals with the DRB1*04 allele exhibited shortening of the telomeres in both CD4+ T cells and granulocytes in the first two decades of life (Schonland *et al.* 2003). Telomeric shortening is known to lead to replicative senescence (Harley *et al.* 1990)however this is normally a gradual process which occurs over the course of a lifetime. The early onset telomeric shortening seen in the DRB1*04 positive individuals may lead to an accelerated replicative senescence of CD4+ T cells and granulocytes resulting in a decrease in cell turnover rates. This change in the nature of the T cell population may reduce the efficacy of the immune system in response to antigenic stimuli. It may be that the T cells entering senescence are unable to prevent autoreactive cells from expanding. Alternatively it may be that the senescent T cells, which are less responsive to controlling stimuli, attack the hosts own cells (Sakaguchi 2000, Schonland *et al.* 2003).

The DRB1*03 allele and DR3 haplotype have however been previously associated with GD (Simmonds *et al.* 2005). In particular the strong DR3 association with GD seen in a study by Heward *et al.* and then expanded upon in a study by Simmonds *et al.* can be compared to the results in this thesis with some accuracy (Simmonds *et al.* 2005, Heward *et al.* 1998). The HT

patients recruited for this study are UK Caucasians and are part of the UK national AITD collection (Manji *et al.* 2006). They are taken from the same sample population as that used to recruit GD patients in both Heward's and Simmonds' GD studies and the same control set has been used for all three studies hence allowing comparative analysis. The strong association of DR4 and weak association of DR3 in this HT study are in marked contrast to Heward's GD study where DR3 was the strongest association noted (Heward *et al.* 1998). This study was furthered by Simmonds *et al.* and again showed an increase in the DR3 haplotype and further confirmed the association of the DRB1*03 allele with GD by showing not only an association of a single copy of DRB1*03 (OR 1.94; 95% CI 1.52–2.48) but that individuals homozygous for DRB1*03 showed even stronger association (OR 3.49; 95% CI 1.77–6.87) (Simmonds *et al.* 2005). This provides a strong case for the theory that the genetic causal factors of GD and HT are, at least in part, different.

Although both HT and GD are centred around autoimmune attack of the thyroid gland there are many differences in the etiology and pathogenesis of the two diseases (see chapter 1, sections 1.4.2 and 1.4.3). This may imply that the relationship of genetic causal factors between HT and GD are similar to that between HT and other autoimmune diseases. Whilst some causal factors will be common to both, some will show association only with one. One of the striking differences between HT and GD is the involvement of TSHR autoantibodies. Whilst in GD the formation of TSHR autoantibodies results in increased thyroid hormone production and is a key factor in the pathogenesis of the disease, TSHR autoantibodies are not seen in HT (see Chapter 1, section 1.4). Association of the TSHR gene with GD has also been reported in GD (Brand *et al.* 2009) but not in HT (see chapter 1 section 1.8). It may be

therefore that the DR3 haplotype is involved in the production of TSHR autoantibodies, a feature unique to GD, hence the lack of association seen in this HT study. Similarly the DR4 haplotype, seen to be associated with HT but not GD may be involved in the as yet poorly understood mechanism of action for thyrocyte destruction in HT.

In this study protective roles were detected for DRB1*07 and the DR7 haplotype and a trend towards a predisposing role for the DRB1*08 allele and DR8 haplotype. In contrast to the differences observed with the predisposing associations, the DR7 and DR8 haplotypes have also been identified as having a protective role in GD (Simmonds *et al.* 2005). Whilst there are many differences between the pathogenesis of these two AITDs there are also some common aspects such as the presence of Tg and TPO autoantibodies. It may be that the DR7 and/or DR8 haplotypes protect against one or both of these autoantibody colonies forming.

CHAPTER 4: MICA AND MICB

4.1 Introduction

4.1.1 HLA Class I

The HLA class I region spans approximately 1.8Mb on chromosome 6. There are potentially 118 genes in this region however only 34 have been shown to be expressed, the remainder being pseudogenes, sequence tagged sites, potential coding sequences or expressed sequences (Shiina *et al.* 1999). The HLA Class I loci are subdivided into the classical and non-classical genes. The classical genes are further grouped into HLA-A, HLA-B and HLA-C whilst the non-classical genes comprise of the HLA-D, HLA-E, HLA-F and HLA-G groups, the MIC genes and a variety of other genes. (Bahram *et al.* 1994, Klein and O'HUigin 1994).

Structure and function of the HLA Class I molecules

The HLA Class I genes are all remarkably similar in basic structure. All HLA Class I genes begin with a 5' leader exon which encodes a short signal peptide. This region is followed by 3 exons which encode the extracellular α1, α2, α3 domains of the HLA Class I molecule. The exon encoding the transmembrane region of the molecule follows this and finally a 3' terminal exon encoding the cytoplasmic domains. All HLA Class I genes follow this pattern with the only apparent differences being whether the α chain is encoded by 5 or 6 exons and whether there are 1 or 2 terminal exons (Goldsby and Goldsby 2003).

The HLA Class I molecule is comprised of a 45 kilodalton (kDa) α chain non-covalently associated with a 12 kDa β_2-microglobulin molecule (Goldsby and Goldsby 2003). Whilst the α chain is encoded by the HLA Class I genes on chromosome 6, the β_2-microglobulin molecule is encoded by a highly conserved gene located at 15q21-q22. The hydrophobic

transmembrane region and hydrophilic cytoplasmic tail of the α chain anchors the α chain and β_2-microglobulin dimer in the plasma membrane (see Figure 3.3)

The primary function of the HLA class I genes is to encode glycoproteins involved in the presentation of endogenous peptide antigens to cytotoxic T cells (T_c cells) (see chapter 1, section 1.2).

HLA Class I molecules bind peptides derived from endogenous intracellular proteins. Proteins present within the cell such as those from expressed viral genes, become associated with molecules of ubiquitin. The ubiquitin-protein complex is then degraded by a multifunctional protease complex called a proteasome, including specialised proteases proteasome subunit beta-type 9 (PSMB9), proteasome subunit beta-type 8 (PSMB8) and proteasome subunit beta-type 10 (PSMB10), within the central hollow of the proteasome into short length peptides for association with HLA class I molecules. This process is part of the cytosolic processing pathway present in all cells that allows the proteins, once digested in the cytosol, to be transported to the cisternae of the endoplasmic reticulum which is aided by the transporter associated with antigen processing (TAP) heterodimer. The TAP heterodimer is comprised of TAP1 and TAP2 protein subunits (Goldsby and Goldsby 2003). Each of the subunits is similar in configuration and has a domain projecting into the lumen of the rough endoplasmic reticulum (RER) and a domain that projects into the cytosol. The domain projecting into the cytosol also binds adenosine triphosphates (ATP), which facilitates TAPs function in the ATP-dependent transport of amino acids, sugars, ions and peptides. Although TAP mediates several transport of several molecules it shows the highest affinity for peptides of 8-10 amino

acids and peptides with hydrophobic or basic C-terminal amino acids, which are also the optimal length and peptide anchor residues for binding by HLA class I molecules.

The mature HLA class I molecule consists of an alpha chain and a β_2-microglobulin chain which are synthesised as separate units on polysomes present on the (RER) of the cell. A chaperone molecule, calnexin associates with the α chain aiding in the folding of the protein chain. The β_2-microglobulin chain then binds to the α chain causing the calnexin molecule to be released and a new chaperone molecule, calreticulin, to associate with the now joined α and β_2-microglobulin chains. For the HLA class I molecule to become a stable unit which can then exit the RER a peptide must be present in the binding site of the molecule. The TAP assisted protein (tapasin) associates with the HLA class I around the same time as the association of calrecticulin occurs. Tapasin brings the TAP complex and HLA class I molecule together allowing transport of the peptides through the membrane of the RER. This HLA class I tapasin complex allows capture of the peptide formed from the proteasome before it can be degraded by the RER lumen. The now stable HLA class I complex can leave the RER and move towards the cell surface. Each HLA Class I molecule binds unique sets of peptides and on average a single cell can express around 10^5 copies of each class I molecule, enabling a great variety of different peptides to be expressed simultaneously (Goldsby and Goldsby 2003).

4.2 MICA and MICB

Although the classical HLA class I genes are the most commonly investigated the non-classical HLA class I genes, are also worthy of investigation. The six MHC class I chain-related (MIC) genes, MICA, MICB, MICC, MICD, MICE and MICF are all non-classical

class I HLA genes. Only two of these genes, MICA and MICB are expressed and produce protein products (Janeway 2005, Frigoul and Lefranc 2005). Unlike the majority of the HLA region MICA and MICB were only identified relatively recently in 1994 (Bahram *et al.* 1994) and as a result there still much to be learned about their structure and function.

MICA and MICB gene structure

MICA is located at 6p21.3, 46.4kb centromeric to *HLA-B* and *MICB* 70kb centromeric to *MICA*. The initial study in 1994 concluded that *MICA* consisted of a single long open reading frame of 1149bp and encodes a 383 amino acid long polypeptide with a relative molecular mass of 43kDa (Bahram *et al.* 1994). Since then it has become apparent that a microsatellite polymorphism in exon 5 consisting of between four to ten GCT repeats encoding four to ten alanine amino acids means that MICA is between 360 and 366 amino acids in length (Frigoul and Lefranc 2005).

The structure of the MICA gene and protein is similar to that of classical HLA class I genes and encoded proteins. There are three external domains, α1, α2, α3, two distal G-like domains, a C-like domain, connecting region, transmembrane region and a cytoplasmic tail. The protein is highly glycosylated with 3 glycosylation sites in the G like domains and 5 in the C-like domain. In marked difference to the classical HLA class I molecules the expression of the MICA protein does not require, nor has found to be associated with either peptide or β2 microglobulin (Rodriguez-Rodero *et al.* 2007). Whilst similar to that of classical HLA class I genes, the structure of the MICA gene has some variations. Exon 1, which is 70bp in length and encodes the L-region. Similarly to classical HLA class I molecules this first exon encodes a signal peptide. Exon 2 is 255bp and encodes the first extracellular distal G-like domain of

85 amino acids. This is in contrast to the single exon of classical HLA class I molecules. Exon 3 is 288bp and encodes the second of the distal G-like domains, 96 amino acids in length. Exon 4 is 279bp in length and encodes the 279 amino acid C-like domain. Exon 5 contains the microsatellite polymorphism hence can be between 132 and 150bp. Exon 5 encodes the 12 amino acid connecting region, the transmembrane region which can be between 19 and 25 amino acids in length owing to the polymorphism and finally 13 amino acids of the cytoplasmic region. A very large intron of 2551bp separates exon 5 from exon 6 which is itself 125bp in length and codes the remaining 42 amino acids of the cytoplasmic region and 3' untranslated region. This is in contrast to the separation of the exon encoding the cytoplasmic tail and untranslated regions in classical HLA class I genes.

Whilst there are similarities in the overall genetic and protein structure of MICA and MICB to classical HLA class I genes and molecules there is little similarity in the genetic sequences with the MIC genes showing only 15-30% sequence identity (Goldsby and Goldsby 2003). *MICA* and *MICB* are extremely similar sharing 91% identity in their coding sequences (Bahram *et al.* 1994). Although this primary knowledge is known about *MICA*, there has been little work on the structure and function of *MICB* and the associated protein products.

Function of MICA and MICB genes

It was initially presumed that the expression of MICA and MICB was restricted mainly to intestinal epithelial cells (Groh *et al.* 1996). However a more recent study has shown that MICA and MICB were transcribed in every tissue type examined with the exception of the central nervous system indicating that it has the potential to be transcribed in nearly all tissue types (Schrambach *et al.* 2007). MICA and MICB are expressed in response to cellular stress

or injury, in particular in response to heat shock protein formation due to either heat or oxidative stress from hydrogen peroxide (Frigoul and Lefranc 2005). The MICA and MICB protein chains are expressed on the surface of these cells where they can be recognised by a receptor on natural killer (NK) cells, and a specific subset of T cells, γδT cells and CD8+ αβT cells (Bauer *et al.* 1999). Of particular interest is the γδT cell subset which carry the activating C-type lectin NK receptor killer cell lectin-like receptor, subfamily K, member 1 (KLRK1) which MICA and MICB molecules bind to, with a single MIC molecule binding to each KLRK1 homodimer. This then induces apoptosis of the affected cell.

4.2.1 Previous associations of MICA and MICB with AID

There has not been a great deal of previous work performed concerning *MICA* association with autoimmune disease (AID) and even less concerning *MICB* however a few associations have previously been reported. Five alleles of exon 5 (see MICA and MICB gene structure), each consisting of GCT trinucleotide repetitions have been identified. These are denoted as MICA4, MICA5, MICA6, MICA9 and MICA5.1, each referring to the number of trinucleotide repeats present in that allele. MICA5.1 is so named due to the repetition consisting of 5 GGCT rather than the trinucleotide GCT (Gambelunghe *et al.* 2000).

Only one previous study has shown evidence of association of *MICA* with HT. In a German cohort of 129 GD patients, 56 HT patients and 206 controls weak association of MICA9 was observed (P=0.016). In addition weak association of the MICA5.1/MICA9 haplotype was seen (*P*=0.025, OR=2.5, 95%CI=1.2-5.4) (Ide *et al.* 2007). This study was performed in a mixed dataset using a very small HT cohort; the results therefore should be treated with caution until replicated in a larger dataset.

In a study performed by Gambelunghe *et al.* on a cohort of 195 type 1 diabetes (TID) patients and 158 controls association of the MICA5 allele was observed (OR=5.1; corrected *P(Pc)* < 0.0005) MICA5.1 was also seen to be associated with adult onset T1D in this study (OR= 7.0; corrected *P (Pc)* < 0.001) (Gambelunghe *et al.* 2001). The MICA5 and MICA5.1 alleles were not however found to be associated in a Korean cohort whereas a borderline association of MICA6 was reported. In addition to this the same Korean study also found evidence for MICA4 as a protective factor against T1D (Relative risk (RR)=0.44, *P*c<0.01) (Park *et al.* 2001). Whilst it should be noted the associations found in both studies were weak the lack of replication does not necessarily point to a false positive in either study. It may be that the different genetic backgrounds of the different ethnic groups used in the two studies contributed to this discrepancy. This phenomenon has been seen previously in the TSHR gene in GD when association within UK Caucasians was located to intron 1 and in Japanese GD patients was located in intron 7 (Dechairo *et al.* 2005, Hiratani *et al.* 2005). Additional associations of MICA5.1 and MICA-129met have also been observed in Addison's disease (OR=22, Pc<10^{-3}) (Park *et al.* 2002) and ankylosing spondylitis (*P*c=0.006, OR=2.57; 95%CI=1.29–5.1) (Amroun *et al.* 2005) respectively.

In a study performed by Lo *et al.* on a cohort of 129 GD patients and 396 controls several associations were observed (Lo *et al.* 2003). The MICA5/MICA5 haplotype was associated (RR=2.49, 95% CI=1.52–4.10, 0.00024, *Pc*=0.0035) as was the MICA5/MICA5.1 haplotype (RR=2.13, 95% CI=1.31–3.47, *P*=0.0020, *Pc*=0.030). In addition the MICA5 allele was strongly associated (RR=2.12; 95%CI=1.59–2.82; *P*=1.9 x 10^{-7}; *Pc*=9.5 x 10^{-7}). The MICA5.1/MICA5.1 haplotype was seen to be protective (RR=0.09, 95% CI=0.01–0.66, *P*=0.0030, *Pc*=0.044) (Lo *et al.* 2003). It can be seen that as MICA5.1/MICA5.1 was

protective but MICA5/MICA5.1 was not, two copies of the MICA5.1 alleles are required to confer protection. An unusual factor about this study is that was performed on children with GD and as GD does not commonly present until the fourth decade of life the results may be specific to early onset GD.

It should also be noted that of all the associations mentioned, with the exception of the A5 allele in the study by Lo *et al.*, all were relatively weak associations. Due to lack of consistent replication of these studies it is difficult to say whether this is due to the MICA gene having only a low impact on pathogenesis of AID or whether some of these may be false positives due to underpowered studies (see Chapter 2, section 2.8.1)

Although little work has been done concerning the MICB gene some associations have been recorded. The MICB0106 allele was found to be strongly associated with coeliac disease (P_c< 0.000001, OR=5.6, 95%CI=3.1–10.1) (Gonzalez *et al.* 2004) and the MICB004 allele with rheumatoid arthritis (*Pc*=0.01, OR=2.2, 95%CI=1.3–3.7) (Lopez-Arbesu *et al.* 2007). Both studies did however suggest that the association of these MICB alleles may be due to the strong LD of the area causing other HLA associations to be interpreted as a MICB, independent association.

4.3 Aims

Due to the important role of the HLA region in encoding key immune response genes it is possible that additional susceptibility loci may be present within this gene dense region above and beyond the known HLA class II association I have detected previously (refer to Chapter 3). The role of MICA and MICB in the causation of apoptosis could indicate that these genes play a role in the thyrocyte destruction seen in HT. Although the results for MICA and MICB in autoimmune disease have varied, only a very limited amount of work has been performed in AITD

i. To genotype a large cohort comprising of HT patients and controls using TagSNPs to cover the majority of common polymorphisms with a minor allele frequency of $\geq$ 20% within *MICA* and *MICB*.
ii. To determine the predisposing and protective factors within the MICA and MICB genes via statistical analysis of TagSNP frequencies.

4.4 Methods

4.4.1 Subjects

The sample cohort comprised of 745 individuals with HT and 617 control subjects (comprising of 288 control subjects from the Birmingham control collection and 329 control subjects taken from the 1958 birth cohort control set) as described in Chapter 2, section 2.1.

4.4.2 Genotyping

Genotyping data for the *MICA* and *MICB* regions was obtained from the International Haplotype mapping project web site [www.hapmap.org] (using the July '06 release and a minor allele frequency (MAF) of 0.2) and then processed using the Haploview program (version 3.32) [www.hapmap.org/haploview_help.html]. Power calculations determined that with this smple cohort and a MAF of 0.2 there was power to exclude an effect of OR=1.3 or above (see Chapter 2, section 2.8.1). For *MICA* a region of 11.7Kb was identified containing 10 *TagSNP*s covering a total of 14 variants. For *MICB* a region of 12.9Kb was identified containing 5 *TagSNP*s covering an additional 14 variants. For both gene regions all SNPs with an r^2>0.80 were tagged.

Pre-designed genotyping assays for rs1131896, rs1131904, rs2256175, rs2844519, rs2855812, rs2853977, rs3130615, and rs9267390 were purchased from Applied Biosystems, UK. No previous assays had been designed for rs2523497, rs2844514, rs2844518, rs2853975, rs3131638, rs6934187 and rs7383312 so the sequences surrounding these SNPs (see Appendix, section 6.5) were submitted to Applied Biosystems, UK and genotyping assays designed and validated. All assays were genotyped on an ABI7900 HT using TaqMan® genotyping technologies (Applied Biosystems, UK).

4.4.3 Method

The DNA was prepared as previously described in Chapter 2 sections 2.2 and 2,3.

DNA samples were previously diluted to a concentration of 20ng/ul and stored in 96 well plates. 2.25 µl of each 20ng/ul DNA sample was aliquoted from four different 96 well plates of samples into separate wells on a 384 well plate (Applied Biosystems, UK). On each plate

2.25 µl of Millq water was used in place of DNA in a minimum of four wells to be used as non-template controls (NTC) to check for plate contamination.

A reaction solution comprising of 2.75 µl Applied Biosystems genotyping master mix (Applied Biosystems, UK), 0.18 µl of genotyping assay (Applied Biosystems, UK) and 0.09 µl millq water was aliquoted into each well of the 384 well plate including the NTCs. The plate was then sealed with an optically clear sealer (Griener, UK), and centrifuged at 1000rpm for 1 minute. The plates were then run on M J Research PTC-225 Peltier Thermal cycler machine (Genetic Research Instrumentation, UK) using the following PCR program:

120 seconds Taq activation - 50^{o}C

600 seconds denaturation - 95^{o}C

40 cycles: 15 seconds denaturation - 92^{o}C

60 seconds annealing and extension - 60^{o}C

Conventionally in PCR reactions the annealing and extension stages are separate with the annealing stage being at a lower temperature than that of the extension. However in this process the primers have been designed by Applied Biosystems to anneal at a higher temperature, thus a separate stage is not required (http://www.appliedbiosystems.com). This reduces the time required for the PCR process and also reduces risk of contamination by allowing the temperature to remain high during this stage.

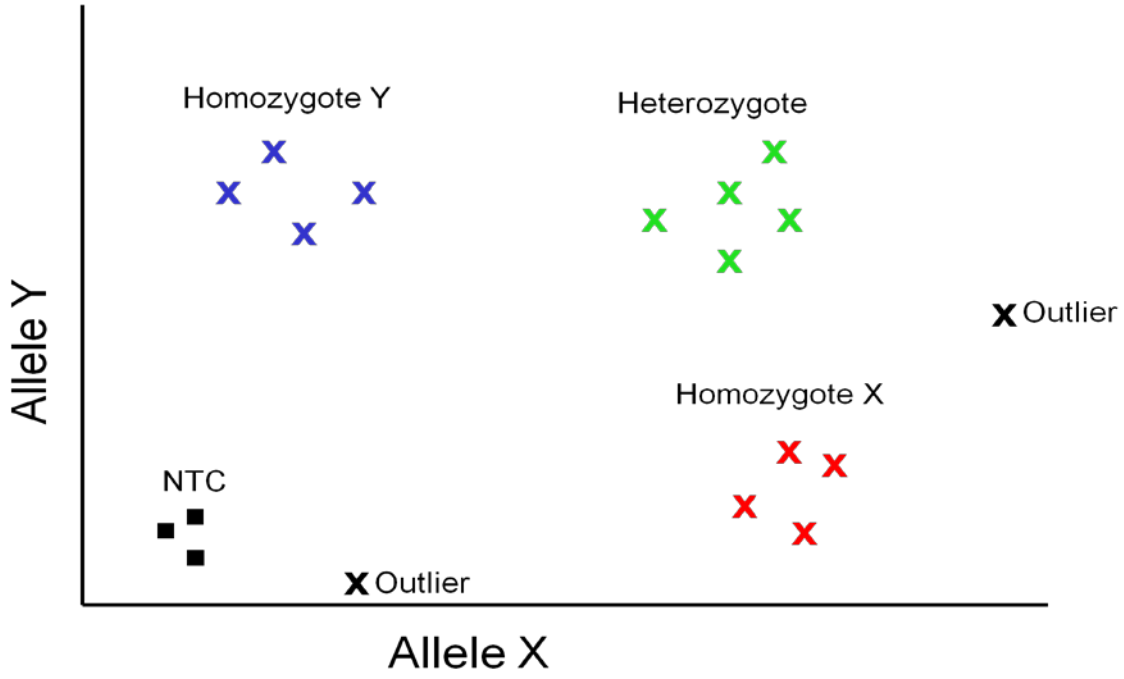
All assays contained two probes; VIC and FAM to detect alleles 1 and 2 respectively (see Figure 2.4 for binding process) of the SNP being assayed. Each assay is provided with

information concerning which allele of a given SNP represents allele 1 and which allele presents allele 2. The probe will bind to the DNA present in accordance with the allele exhibited; in a heterozygous sample VIC and FAM will both bind in a homozygous sample only one type of probe will bind. The probes fluoresce with different wavelengths enabling the sample to be read using the ABI7900 prism (Applied Biosystems, UK).

4.4.4 Analysis

The results from the *ABI7900* prism were incorporated into the SDS software (Version 2.2.2, Applied Biosystems, UK). The preliminary results were displayed in both a graphical and tabular form (see Figure 4.1). From this the initial validity of the results could be assessed. Using the graphical representation any results which did not appear to fall into one of the two homozygote or the heterozygote groupings (and which were not previously defined as NTCs) could be considered as outliers and repeated or eliminated from further analysis. Any of the samples defined as NTCs which did not fall into the NTC grouping demonstrated a possible contamination of the plate by extraneous DNA and would necessitate the repetition of the entire plate. If the results did not appear to group at all or an abnormal grouping was observed then the validity of the results was checked via the tablature representation. By comparing the strength of fluorescence for each of the two probes in each individual well it could be determined whether the grouping was an anomaly due to the failure of the assay or the DNA.

Figure 4.1: Schematic of allelic results



4.4.5 Statistical analysis

Analysis of case control data was performed using the Chi-squared (χ^2) test with $P < 0.05$ considered significant, with the Fisher's exact test performed for samples with counts lower than 10. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using the method of Woolf with Haldane's modification for small numbers (Woolf 1955). The Fisher's exact test was performed using the MINITAB statistical package (MINITAB Release 14.1 © 1972–2003, Minitab Inc, USA.); all other calculations were performed using Excel (Microsoft® Excel, © 1985-2003 Microsoft Corporation).

4.5 Results

Genotyping results

All cases and controls for each assay were in Hardy Weinberg equilibrium. No differences were observed between HT cases and controls for either genotype or allele frequencies for 13 of the SNPs assayed (rs1131904, rs2256175, rs2523497, rs2844514, rs2844518, rs2844519, rs2853977, rs2855812, rs3130615, rs3131638, rs6934187, rs7383312 and rs9267390). A small association (P=0.024) was observed for the TT genotype of MICA SNP rs2853975 SNP with a similar association (P=0.030, OR=1.21, 95% CI= 1.02-1.43) for the T allele. (see Tables 4.1 and 4.2).

Although the assay for the rs1131896 SNP was purchased and initial genotype investigations carried out, information from this assay was unavailable for the final data analysis. Despite several replications no fluorescence was seen in samples processed with this assay indicating that the probe had not bound to the target DNA. As results were gained from all other assays it can be concluded that the DNA was of sufficient quality and rather the design of the

sequence used in the rs1131896 SNP assay was at fault. As no other SNPs were present in this TagSNP group there was no viable alternative. Had more time been available for this project a redesigned sequence could have been submitted to attempt to reassess the association of this SNP.

Phenotype/genotype associations

Insufficient data was available for phenotype correlations. Only partial data had been collected or was available on the various phenotypes of the HT patients used in this study. The numbers of patients with available data for each phenotype was so small as to greatly underpower the analysis.

Table 4.1: Distribution of alleles and genotypes in MICA SNPs in patients with Hashimoto's thyroiditis and control subjects

SNP	Allele/ Genotype	Hashimoto's thyroiditis n = 754 (%)	Control subjects n = 633 (%)	X^2	P	OR	95% CI
rs1131904	**AA**	418 (62.48)	312 (62.28)	0.214	0.643		
	AG	224 (17.09)	166 (33.13)				
	GG	27 (1.98)	23 (4.59)				
	A	1060 (79.22)	790 (78.84)	0.050	0.823	0.98	0.80-1.19
	G	278 (20.78)	212 (21.16)				
rs2256175	**GG**	235 (36.27)	168 (32.56)	3.438	0.064		
	GA	303 (46.76)	273 (52.91)				
	AA	110 (16.98)	75 (14.53)				
	G	773 (59.65)	609 (59.01)	0.096	0.757	0.97	0.83-1.15
	A	523 (40.35)	423 (40.99)				
rs2523497	**AA**	309 (45.64)	247 (46.78)	0.586	0.444		
	AG	295 (23.03)	231 (43.75)				
	GG	73 (5.12)	50 (9.47)				
	A	913 (67.43)	725 (68.66)	0.409	0.522	1.06	0.89-1.26
	G	441 (32.57)	331 (31.34)				
rs2844514	**AA**	106 (15.21)	78 (15.23)	3.211	0.073		
	AG	346 (49.64)	227 (44.34)				
	GG	245 (35.15)	207 (40.43)				
	A	558 (40.03)	383 (37.40)	1.713	0.191	1.12	0.95-1.32
	G	836 (59.97)	641 (62.60)				
rs2844518	**AA**	81 (13.04)	82 (15.05)	1.017	0.313		
	AG	290 (29.23)	242 (44.40)				
	GG	250 (16.76)	221 (40.55)				
	A	452 (36.39)	406 (37.25)	0.182	0.669	0.96	0.81-1.14
	G	790 (63.61)	684 (62.75)				
rs2844519	**GG**	453 (65.18)	364 (62.65)	1.052	0.305		
	GA	219 (31.51)	193 (33.22)				
	AA	23 (3.31)	24 (4.13)				
	G	1125 (80.94)	921 (79.26)	1.118	0.290	0.90	0.74-1.09
	A	265 (19.06)	241 (20.74)				
rs2853975	**TT**	**76 (11.24)**	**46 (7.93)**	**5.078**	**0.024**		
	TA	**294 (43.49)**	**244 (42.07)**				
	AA	**306 (45.27)**	**290 (50.00)**				
	T	**446 (32.99)**	**336 (28.97)**	**4.712**	**0.030**	**1.21**	**1.02-1.43**
	AA	**906 (67.01)**	**824 (71.03)**				

SNP	Allele/ Genotype	Hashimoto's thyroiditis n = 754 (%)	Control subjects n = 633 (%)	X^2	P	OR	95% CI
rs2853977	**TT**	190 (27.90)	127 (24.19)	2.329	0.127		
	TA	336 (49.34)	283 (53.90)				
	AA	155 (22.76)	115 (21.90)				
	T	716 (52.57)	537 (51.14)	0.484	0.487	0.94	0.80-1.11
	A	646 (47.43)	513 (48.86)				
rs6934187	**GG**	340 (54.31)	314 (55.28)	2.959	0.085		
	GC	236 (19.63)	223 (39.26)				
	CC	50 (3.84)	31 (5.46)				
	G	916 (73.16)	851 (74.91)	0.947	0.331	1.10	0.91-1.32
	C	336 (26.84)	285 (25.09)				

Allelic variations on the forward strand are shown.
X^2 = Chi-squared, OR = Odds ratios, 95% CI = 95% confidence interval

Table 4.2: Distribution of alleles and genotypes in MICB SNPs in patients with Hashimoto's thyroiditis and control subjects

SNP	Allele/ Genotype	Hashimoto's thyroiditis n = 754 (%)	Control subjects n = 633 (%)	X^2	P	OR	95% CI
rs2855812	**GG**	339 (49.78)	308 (54.13)	1.909	0.167		
	GT	294 (43.17)	225 (39.54)				
	TT	48 (7.05)	36 (6.33)				
	G	972 (71.37)	841 (73.90)	2.001	0.157	1.14	0.95-1.36
	T	390 (28.63)	297 (26.10)				
rs3130615	**TT**	391 (57.33)	303 (56.95)	0.094	0.760		
	TC	254 (19.14)	198 (37.22)				
	CC	37 (2.64)	31 (5.83)				
	T	1036 (75.95)	804 (75.56)	0.049	0.824	0.98	0.81-1.18
	C	328 (24.05)	260 (24.44)				
rs3131638	**CC**	33 (4.90)	27 (4.66)	0.077	0.781		
	CT	230 (24.57)	195 (33.68)				
	TT	410 (23.35)	357 (61.66)				
	C	296 (21.99)	249 (21.50)	0.087	0.768	1.03	0.85-1.24
	T	1050 (78.01)	909 (78.50)				
rs7383312	**AA**	75 (10.93)	80 (13.89)	2.888	0.089		
	AG	318 (29.47)	267 (46.35)				
	GG	293 (17.60)	229 (39.76)				
	A	468 (34.11)	427 (37.07)	2.390	0.122	0.88	0.75-1.04
	G	904 (65.89)	725 (62.93)				
rs9267390	**AA**	293 (45.08)	245 (46.85)	0.301	0.583		
	AG	288 (23.40)	224 (42.83)				
	GG	69 (5.04)	54 (10.33)				
	A	874 (67.23)	714 (68.26)	0.281	0.596	1.05	0.88-1.25
	G	426 (32.77)	332 (31.74)				

Allelic variations on the forward strand are shown.
X^2 = Chi-squared, OR = Odds ratios, 95% CI = 95% confidence interval

4.6 Discussion

For association of *MICA* with HT 10 TagSNPs covering a total of 14 variants were investigated and for association of *MICB* 5 TagSNPs covering an additional 14 variants were investigated. Of the 15 investigated SNPs one was not able to provide genotype data (see section 4.5). Of the remaining 14 SNPs investigated weak association with HT was seen in one, rs2853975. Only one previous HT study has been performed investigating association of MICA where a small positive correlation was observed for the A9 allele (P=0.016) and A5.1/A9 genotype (P=0.025, OR=2.5, 95%CI=1.2-5.4) (Ide *et al.* 2007). This is a similar level of effect to the small positive correlation observed in this study for the TT genotype (P=0.024) and T allele (P=0.030, OR=1.21, 95% CI= 1.02-1.43) of rs2853975. Whilst this study has added further evidence for the role of *MICA* in HT by performing this investigation in a larger cohort, 745 HT patients were analysed in comparison to 56 in the study performed by Ide *et al.(Ide et al. 2007),* both results were weak positives. Had a smaller P value been observed in this study then it would seek to confirm the association of *MICA* with HT, however as neither study showed association stronger than P=0.024 there may be other factors to consider. Although it is considered a 'significant' association, being below the boundary of P=0.05 it is only just so, the weak association may be due instead to a coincidentally high proportion of MICA polymorphisms in the affected individuals rather than to the associated polymorphisms being causal factors for disease pathogenesis.

The strong LD within this region should also be taken into account when viewing both the results from this study and that by Ide *et al.* As stated previously one of the main problems with determining a reliable result in the HLA region and extended region is the issue of strong LD. The findings of this study may not reflect an independent association but rather an

association of a nearby region within strong LD. To determine the likelihood of LD playing a role in the association observed in this study further analysis would need to be performed however due to the time constraints of this investigation it was not possible to do so for this thesis. The primary goal would be to attempt to identify the common LD blocks within the region surrounding and including *MICA*. This could be performed via use of data available at the International haplotype mapping project web site [www.hapmap.org] and further processed using the Haploview program [www.hapmap.org/haploview_help.html]. Loci which then proved to be in strong LD with MICA polymorphisms, in particular with the associated SNP rs2853975, could be genotyped in the same cohort as used for the initial study.

Although LD only usually extends up to 200kb, LD within the HLA region can extend much further including LD seen between the HLA class II and class I regions (Pritchard and Przeworski 2001, Chapman *et al.* 2003, Gabriel *et al.* 2002). Several associations within the HLA class II gene region with HT have been detected (see Chapter 3), including very strong association of the DR4 haplotype and individual included alleles (See chapter 3). LD analysis between MICA/MICB and components of the DR4 haplotype would therefore be of benefit to determine if this weak association detected in rs2853975 could be due to weak LD with DR4.

The HLA gene region in general is of great interest concerning autoimmune disease due to the involvement of numerous HLA class I, class II and class III genes with the immune response (see chapter 1, chapter 3 and section 4.1 for further details). As HT is a multifactorial autoimmune disease it may be that there are several causal factors within the entire HLA gene region contributing to HT. However, it may be that there are a small number of highly

associated casual factors within this region that are in areas of strong LD. If this is the case then it is not unlikely that any number of falsely associated loci would be detected in studies investigating the association of the HLA gene region with HT.

Due to time constraints further screening was not possible. Ideally to further this work work the remainder of the genetic variation within MICA and MICB and the surrounding areas would be investigated. SNPs with at least a MAF of 0.20 were chosen for this study. For MICA 10 *TagSNP*s capturing a total of 14 variations were genotyped, decreasing the MAF to 0.10 would allow 13 *TagSNP*s to cover a total of 24 variations. For MICB 5 *TagSNP*s covering 14 variants were identified and genotyped, decreasing the MAF to 0.10 would allow 8 *TagSNP*S to cover a total of 27 variations ([www.hapmap.org] using the July '06 release). By increasing the number of genotyped SNPs within the MICA and MICB genes a denser map of the area may be obtained. This may highlight associated SNPs which are not in strong LD with the original set of *TagSNP*s investigated. In order to detect these additional SNPs the size of the cohort used would also need to be increased to ensure sufficient power (see Chapter 2, section 2.8.1). Furthermore the additional SNPs could be screened for LD with HLA class II to determine if new associations were independent of the already demonstrated HLA class II associations (see Chatper 3).

HLA-B, and HLA-C lie close to the MICA and MICB genes and have also shown to be strongly associated with GD (Simmonds *et al.* 2007). Screening of these genes, in addition to other portions of the HLA class I gene region such as HLA-A would therefore be beneficial. Again these areas could be assessed for LD with both the MICA and MICB genes and also the HLA class II region.

CHAPTER 5: DISCUSSION

5.1 Discussion

Whilst much work has been done to investigate autoimmune thyroid diseases (AITDs) Graves' disease (GD) and Hashimoto's Thyroiditis (HT) in combined datasets and to investigate GD, there has been minimal work on the determination of the causal factors of HT alone (see Chapter 1). Although there is a commonality between HT and GD in that they are both exhibit autoimmune attack against the thyroid there are a few differences, a fact which highlights the need for separate investigation of these two diseases.

Several studies have previously been conducted which investigate association of the HLA class II region with HT however they have all been on small data sets and many have been in mixed GD and HT datasets (Moens *et al.* 1978, Weissel *et al.* 1980, Farid *et al.* 1981, Stenszky *et al.* 1987, Tandon *et al.* 1991, Petrone *et al.* 2001, Hunt *et al.* 2001, Tomer *et al.* 2003a). The primary aim of this study therefore was to perform a candidate gene association study to determine the genetic causal variants present in the HLA class II region in a large HT only cohort.

Strong association of both the DR4 haplotype as a whole and the strong association of its component alleles (DRB1*04, DQB1*0301/4 and DQA1*03011/12) were detected within this study and serve to reinforce smaller previous studies that identified the DR4 haplotype as a causal factor in two Caucasian HT cohorts (Petrone *et al.* 2001, Farid and Thompson 1986).

Although previous studies have found association of the DR4 haplotype and alleles in both Type 1 Diabetes (Cucca *et al.* 2001) and Rheumatoid arthritis (Gourraud *et al.* 2006) no association has been observed in GD. The fact that the DR4 haplotype appears to be a common susceptibility locus to autoimmune disease (AID) in general but not for the more specific grouping of autoimmune thyroid disease may be explained by the different nature of the diseases in question. Whilst HT and GD share a commonality, in that they affect the same organ, they have very different effects on the thyroid, with cellular destruction a key feature of HT but notably absent in GD. The differences between HT and GD are further highlighted in this study by the lack of association of DR3, a strongly associated GD locus.

HT and Type 1 Diabetes are centred around the destruction of cells pertaining to a specific organ. Rheumatoid arthritis similarly is centred around cellular destruction of specific tissue types. It may be that the DR4 susceptibility locus plays a role in this specific aspect of AID by affecting the normal patterns of telomeric shortening and as a result replicative senescence of T-cells and granulocytes. A theory also supported by the work performed by Schonland *et al* concerning the effects of DRB1*04 on telomeric shortening (Schonland *et al.* 2003).

As the HLA region contains immune system regulatory genes throughout the entire region it is unsurprising that associations have been found in genes outside of HLA class II. This, in combination with the strong LD exhibited in the HLA gene region meant it was also important to assess whether any associations in the HLA class II region were independent of possible associations in the HLA Class I region. Hence the secondary aim was to perform a preliminary study into the associations present in the HLA Class I region with HT.

A small association was found for the rs2853975 SNP in the MICA gene with HT and no other associations were detected. This finding is similar to that of the only other previous study concerning association of MICA with HT where a small positive correlation was observed for the A9 allele (Ide *et al.* 2007). This may be a true small, independent association however no significant associations have as yet been found for either MICA or MICB with any of the other AIDs. It is therefore prudent to consider the possibility that this result is either a false positive or is a result of the strong LD seen in the HLA region. To fully investigate the LD full linkage analysis needs to be performed between this area and the associated alleles in the HLA class II region. It is also necessary to perform an in-depth association study of the whole HLA Class I region and once again perform a linkage analysis to determine if any associations are independent of the known HLA class II associations.

5.2 Future Work

Phenotype correlations

Whilst a strong association was found for the DR4 haplotype with the cohort in general it may be that a specific feature such as age of onset of HT is influenced by this causal factor. It may also be that the DR4 haplotype becomes a susceptibility factor of note when found in conjunction with an environmental factor such as smoking. The cohort used for this study did not provide sufficient phenotype data to perform an in depth phenotypical analysis of the results with only TSHR serum antibody presence and presence of diffuse goitre correlations analysed. Although my work has been performed on the largest HT dataset collected worldwide, to date, it would be prudent to perform further work using a cohort with complete data for age, sex, TPO and Tg serum antibody levels and smoking. If the size of the cohort

was also increased then it would also be possible to perform phenotype genotype correlations using finer subdivisions of the phenotypes and environmental factors.

Whilst a significant difference in the genetic causal factors between those for GD and HT has been found in this study HT remains part of the general group of AIDs. It is known that there are several common susceptibility factors between the AIDs; hence it would also be beneficial to investigate the incidence of other AIDs within the probands immediate family. With this information the correlation of AID incidence and discovered HT genetic causal factors could be investigated. This would help determine whether the presence of another AID was enriching HT patients for AID susceptibility loci.

HLA Class I loci

Whilst a preliminary study into the association of HLA Class I encoded *MICA* and *MICB* loci with HT was performed as part of this investigation, the full gene region was not explored. The initial results suggest that due to lack of strong association in the MICA and MICB genes the associations found in the HLA Class II region were independent and not reliant on the associated loci being in LD with potential causal factors in the HLA Class I region. The genes investigated, MICA and MICB were however non-classical HLA Class I genes and an extensive investigation of both the classical HLA Class I genes and the remaining non-classical HLA Class I genes is required to fully rule out LD as the cause of the reported HLA class II associations. This is particularly important following the work performed by Simmonds *et al.* which showed the association of HLA-C with GD to be stronger than the effects of associated HLA class II (Simmonds *et al.* 2007).

To investigate classical HLA class I genes the photoyping method of allele specific PCR could be used. This is the same method as described in chapter 3 for the investigation of HLA class II associations. The alleles used would be those detailed in Bunce et al (Bunce *et al.* 1995). For non-classical HLA class I genes the Tag SNP method detailed in chapter 4 for the investigation of the non-classical HLA class I genes MICA and MICB may be used. Alternatively HLA SNP chip panels, such as those designed for the WTCCC non-synonymous SNP screen (WTCCC 2007) or those commercially available from companies such as illumina (illumine, N America), may be used to screen the entire HLA region.

Genome Wide Scans

Further investigation into the HLA region and other candidate genes such may only complete part of the picture. As HT is a multigenic disease where the two, three or multi-hit mutational hypothesis may apply, it is important to fully investigate all possible sources of genetic causal variants. Previously genome-wide screens have been used to investigate entire genomes for loci which show signs of association with disease. One method, based on short tandem repeats which are composed of di-, tri- or tetra-nucleotide repeats known as microsatellites, has been used to investigate GD, HT and common AITD loci (Tomer *et al.* 2003a, Tomer *et al.* 1999, Tomer *et al.* 1998). The first of these studies produced two possible susceptibility loci for HT (HT-1 (13q32) and HT-2 (12q22)), three for GD and one for AITD (Tomer *et al.* 1999). When this genome screen was repeated with an expanded dataset the results changed dramatically. One locus for HT (HT-1) and one for locus for GD were not replicated and three new susceptibility loci were suggested, D7S502 (7q), D8S284 (8q) and D10S537 (10q) (Tomer *et al.* 2003b). These inconsistencies highlight the danger of using genome-wide screens to assess causal factors for complex diseases. In monogenic diseases this method can provide a definite

locus for further fine mapping however in complex diseases where the frequency of each susceptibility loci may be low it is easy to gain false positives and miss legitimate association loci.

Whilst the precise method may be flawed the idea of scanning the entire genome to hunt out causal loci is sound and could produce susceptibility loci which would not have been considered as candidate genes. A new technique, genome wide association studies, whereby association analysis is performed across the entire genome may provide a means to rectify the flaws of the genome-wide screen method (Duerr *et al.* 2006, Hampe *et al.* 2007, Sladek *et al.* 2007, Gaffney *et al.* 1998, Jorgenson and Witte 2006, Steer *et al.* 2007). This method allows for association analysis to be performed on over 500,000 SNPs in large cohorts of around two to three thousand samples. Although the HT cohort used in this thesis is one of the largest used to date it is still small by comparison to those used in genome wide scans. To perform a genome wide association analysis a much larger cohort, one of roughly 2,000 cases and 2,000 controls, similar to those used in other genome wide association analyses (WTCCC 2007), would need to be collected first to ensure sufficient power for the study. In addition independent similarly sized cohorts would need to be available to replicate and association detected

Whilst it is not possible to perform a genome wide scan on an HT cohort at this time the results from genome wide association analyses on other AIDs may provide useful candidate genes which can be investigated as common AID susceptibility loci. In particular the Wellcome Trust Case Control Consortium's (WTCCC) [http://www.wtccc.org.uk] association

studies include one performed on a large GD cohort in which 14,436 non-synonymous coding SNPs were typed in 1,000 cases and 1,500 controls (Burton *et al.* 2007). This may allow identification of novel AITD susceptibility loci.

5.3 Conclusions

This study has shown a strong predisposing association of the DRB1*04, DQB1*0301/4 and DQA1*03011/12 loci with HT. Furthermore, the DR4 haplotype (DRB1*04-DQB1*03-DQA1*03) was shown to have a predisposing effect. Weaker predisposing effects and protective effects were also observed in this area demonstrating the importance of the genetic factors in the HLA class II region for HT. As only a small association was seen in the MICA region and no associations were observed in the MICB region further work is required to clarify the association in the HLA Class I region. Larger datasets are now required, combined with more thorough screening of the entire HLA region in order to determine the exact location and extent of susceptibility loci within this region.

APPENDIX

6.1 Disclaimer

Collection of AITD samples

All UK AITD samples were collected by specialist doctors and nurses from clinics in Birmingham, Bournemouth, Cambridge, Cardiff, Exeter, Leeds, Sheffield, and Newcastle.

DNA Extraction and quantification

All procedures of blood pelleting, DNA extraction and PicoGreen® were performed by Dr Joanne king, Miss Helen Foxall, Ms Sarah Gibson, Dr Matt Simmonds, Mr Paul Newby and myself.

6.2 Solutions

Reagent A: 2L distilled H_2O
275g Sucrose
2.5ml1.0M Tris HCL pH8.0
2.55ml 4.9M MgCl
25ml TritonX

Reagent B: 200ml 1.0M Tris HCL pH .0
60ml 0.5M EDTA pH 8.0
15ml 5.0M NaCl
205ml distilled H2O
20ml 25% SDS.

5M Sodium Percholate: 200ml distilled H2O
140.46g NaCl O4

T.E. Buffer: 1.0 ltr distilled H2O
186.1g EDTA
10g NaOH

Loading Buffer: 0.25g bromophenol blue
40 sucrose
Made up to 100mls with distilled water

TDMH: 169µl 10 x buffer
12µl dNTPs (200 µM)
135µl 25mM $MgCl_2$
334µl distilled water

6.3 Calculations

Chi-squared

$$\chi^2 = (ad-bc)^2 \times (a+b+c+d)/(a+d)(b+c)(a+b)(c+d)$$

Fishers Exact Probability Test

$$P= (a+b)!(c+d)!(a+c)!(b+d)!/a!b!c!d!(a+b+c+d)!$$

Woolf's method of OR calculation

$$OR=(a+0.5)(d+0.5)/(b+0.5)(c+0.5)$$

95% Confidence intervals

$$95\% \quad CI \quad = \quad (\ln OR \pm 1.96\sqrt{(1/a \quad + \quad 1/b \quad + \quad 1/c \quad + \quad 1/d)})$$

6.4 HLA class II primers used to type the DRB1, DQA1 and DRB1 regions

Primers used to type *DRB1* region

DRB1 Allele(s) Amplified	Forward Primer(s)	Reverse Primer(s)
DRB1*0101/2/4	CCGCCTCTGCTCCAGGAG	TTGTGGCGCTTAAGTTTGAAT
DRB1*0103	CCCGCTCGTCTTCCAGGAT	TTGTGGCGCTTAAGTTTGAAT
DRB1*1501-5	CCACCGCGGCCCGCGC	TCCTGTGGCAGCCTAAGAG
DRB1*1601-6	CTCCGTCACCGCCCGGT	TCCTGTGGCAGCCTAAGAG
DRB1*03011/012/2/3/4/5, 1107	GTCCACCCGGCCCCGCT	GTTTCTTGGGTACTCTACGTC
DRB1*0301/4	CTGCACTGTGAAGCTCTCCA	GACGGAGCGGGTGCGGTA
DRB1*0302/5, 1302/5, 1109/20, 1402/3/9/13/19	CTGCACTGTGAAGCTCTCAC	TACTTCCATAACCAGGAGGAGA
DRB1*0401-22, 1410, 1122	CTGCACTGTGAAGCTCTCAC CTGCACTGTGAAGCTCTCCA	GTTTCTTGGAGCAGGTTAAACA
DRB1*0701	CCCGTAGTTGTGTCTGCACAC	CCTGTGGCAGGGTAAGTATA
DRB1*0801-11, 1415	CTGCAGTAGGTGTCCACCAG TGTTCCAGTACTCGGCGCT	AGTACTCTACGGGTGGTGTT
DRB1*0901	CCCGTAGTTGTGTCTGCACAC	GTTTCTTGAGCAGGATAAGTTT
DRB1*1001	CTGCACTGTGAAGCTCTCAC	CGGTTGCTGGAAAGACGCG
DRB1*1101-21, 1411	CTGGCTGTTCCAGTACTCCT	GTTTCTTGGGTACTCTACGTC TTCTTGGAGTACTCTACGGG
DRB1*1201/2/3	CTGTTCCAGGACTCGGCGA	AGTACTCTACGGGTGGTGTT
DRB1*1301/2/4/8/15-17/19/20/22, 1416, 1102/3/11/14/16/20/21	TCCACCGCGGCCCGCTC	GTTTCTTGGGTACTCTACGTC TTCTTGGAGTACTCTACGGG
DRB1*1301/2/8/9/16/20, 1116/20, 1416	TCCACCGCGGCCCGCTC CCACCGCGGCCCGCGC	GTTCCTGGACAGATACTTCC

DRB1 Allele(s) Amplified	Forward Primer(s)	Reverse Primer(s)
DRB1*1301/2/(6)/10/15/16, 1116/20, 1419/21	CCCGCTCGTCTTCCAGGAT TCCACCGCGGCCCGCTT	TACTTCCATAACCAGGAGGAGA
DRB1*1305/6/10, 1109, 1419/21	ACCGCGGCCCGCCTGTC TCCACCGCGGCCCGCTT	TACTTCCATAACCAGGAGGAGA
DRB1*1303/10, 1419/21	TCCACCGCGGCCCGCTT	GTTTCTTGGGTACTCTACGTC
DRB1*1303/4/12/21, 1413	TGTTCCAGTACTCGGCGCT	GTTTCTTGGGTACTCTACGTC
DRB1*1305/7/11/14/18/21, 1101/4/6/9/10/(11)/12/15	CCCGCCTGTCTTCCAGGAA	GTTTCTTGGGTACTCTACGTC
DRB1*1301/2/5-9/10/11/14-16/18/19/20/22, 1402/3/6/9/12/14/17/19-21, 0301-5	CTGTTCCAGTACTCGGCATC	GTTTCTTGGGTACTCTACGTC
DRB1*1401/3-5/7/8/10-12/14/15/18, 1318, 0809, 1117	TCTGCAATAGGTGTCCACCT CTGCAGTAGGTGTCCACCAG	GTTCCTGGACAGATACTTCC GTTCCTGGAGAGATACTTCC
DRB1*1401/7/16	CTGTTCCAGTGCTCCGCAG	GTTTCTTGGGTACTCTACGTC
DRB1*1402-3/6/9/12/13/17, (1418/19/21), (0301/2/3/5), 1318	CTGCAGTAGGTGTCCACCAG CCGCCTCTGCTCCAGGAG	TACTTCCATAACCAGGAGGAGA

Primers to type DRB3, DRB4 and DRB5 regions

DRB Allele(s) Amplified	Forward Primer(s)	Reverse Primer(s)
DRB5*0101/2, 0201/2/3	GCTGTTCCAGTACTCGCG	GTTTCTTGCAGCAGGATAAGTA
DRB3*0101	CTGCAGTAATTGTCCACCCG	TTTCTTGGAGCTGCGTAAGTC
DRB3*0201/2/3	CCCGCGGTACGCCACCTC	GGAGTACCGGGCGGTGAG
DRB3*0201, 0301	CTGCACTGTGAAGCTCTCCA	GTTTCTTGGAGCTGCTTAAGTC
DRB4*0101101, 0102/3	CTGGTACTCCCCCAGGTCA	GATCGTTCGTGTCCCCACAG
DRB4*0101102N	TCCCCCAGGTCACTGTTGT	GATCGTTCGTGTCCCCACAA

Primers to type DQB1 region

DRB1 Allele(s) Amplified	Forward Primer(s)	Reverse Primer(s)
DQB1*0305	TGCACACCGTGTCCAACTC	GCTACTTCACCAACGGGACC
DQB1*02	CGTGCGGAGCTCCAACTG	GTGCGTCTTGTGAGCAGAAG
DQB1*04	TGGTAGTTGTGTCTGCATACG	GCTACTTCACCAACGGGACC
DQB1*05	CCCGCGGTACGCCACCTC	ACGGAGCGCGTGCGGGG
DQB1*0601/2/3	CCGCGGAACGCCACCTC	TTTCGTGCTCCAGTTTGGC GACGTGGGGGTGTACCGC
DQB1*0603-9	TGCACCCCTGTCCACCG	GGAGCGCGTGCGTCTTGTA GGAGCGCGTGCGTCTTGTA
DQB1*0301/4	CGTGCGGAGCTCCAACTG	GACGGGCGCGTGCGTTA
DQB1*0302	CTGTTCCAGTACTCGGCGG	GTGCGTCTTGTGACCAGATA
DQB1*03032	CTGTTCCAGTACTCGGCGT	GACGGGCGCGTCGCTCT

Primers to type the DQA1 region

DRB1 Allele(s) Amplified	Forward Primer(s)	Reverse Primer(s)
DQA1*0101/4	CATGAATTTGATGGAGATGAG	ATGATGTTCAAGTTGTGTTTTGC
DQA1*0102/3	CATGAATTTGATGGAGATGAC	ATGATGTTCAAGTTGTGTTTTGC
DQA1*0201	ACGGTCCCTCTGGCCAGTT	CAGGATGTTCAAGTTATGTTTTAG
DQA1*03011/12	TTCACTCGTCAGCTGACCAT	CAAATTGCGGGTCAAATCTTCT
DQA1*0302	TTCACTCGTCAGCTGACCAC	CAAATTGCGGGTCAAATCTTCT
DQA1*0401	ACCCATGAATTTGATGGGC	CACATACCATTGGTAGCAGCA
DQA1*05011/12/13, 0502	CGGTCCCTCTGGCCAGTA	AGTTGGAGCGTTTATCAGC
DQA1*0601	ACGGTCCCTCTGGCCAGTT	GGTCAATCTAAATTGTCTGAGA

6.5 SNPs used to genotype the MICA and MICB gene regions

SNPS used to gentotype the MICA gene

SNP	Sequence
rs1131896	AGGAACTACGGCGATATCTAGAATCC[A/G]GCGTAGTCCTGAGGAGAACAGGTAC
rs1131904	CTGCAAAATGTTAGTAGATATGAGGC[A/G]TTTGCAGCTGTGCCATATTAATTGG
rs2256175	CTGCCCACTCCTCACCTGCAGCAGGA[A/G]GATGCCACAGCTGGGCGTTTGACCC
rs2523497	AAAAGGTAGAAGTAACCCAAGTGTCC[A/G]TTGTCTGAGGGATGGATAACCAAGA
rs2844514	ATTCCCCAACTTTCATCCCCTGTTAT[A/G]GAAGCCTTGTCACCAACATGCCTAT
rs2844518	GGGGCCACCATGAGGCAGGGTCCAGA[A/G]CAGGCATCTGCACTGGAGGGGAGGG
rs2844519	AAAGGGAAGATGCCAGCCAGAAGCAG[A/G]AAGACCGGGCCCAGCCCCATGGCCC
rs2853975	TTGTGTTTATTCCCTGGGGACTTTTC[A/T]CTCTTCAGTTGCTCCAAAACCAGAT
rs2853977	ACACTGGATGGTCCAGTCCCTGACCC[A/T]CTGTCTTTATCCAGTGGCTCTAACA
rs6934187	GGCCTCCCTGAGTTTCTTGCAGATGA[C/G]ATGGATGAGTAGATAAGCAGATGTC

SNPs used to genotype the MICB gene

SNP	Sequence
rs2855812	CTGAAAAGATGGACTTACTCTTTGA[G/T]ACCCTGTCACCTGCCCACCCCAGTG
rs3130615	AAGACGTAGGTGACAAGGCTGCTGG[A/G]ACAGGGGATGGAAGCTGGGGTATTT
rs3131638	ATAAGGGGTTCCTGCCTCTGTGTTTT[G/T]TTTTTCCTCTATTAGATTATCTGGC
rs7383312	CGTGTATGTGGAGTACTATTCAGCCA[C/T]CATGTCATTTGCAAAGAACTGGAGG
rs9267390	GAGTGCTGGAGCATATGACAGCTCT[A/G]TTATATTTTTAGTTTTTGGAAGAAC

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