Genetic and Immunological Characterisation of Patients with Latent Autoimmune Diabetes in Adults (LADA)

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A thesis submitted to the Nuffield Department of Clinical Medicine at University of Oxford for the degree of Doctor of Philosophy

October 2005

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THESIS

For Amar,

because sometimes,

miracles do happen.

MEMORANDUM

The work in this thesis is the original work of the author. Experiments were carried out at the Oxford Centre for Diabetes, Endocrinology and Metabolism under the supervision of Dr A Clark. HLA typing was performed by the author in collaboration with the Tissue Typing Laboratories, Churchill Hospital. The statistical analyses in Chapter 3 were performed by Dr Carole Cull at the Diabetes Trial Unit, Oxford Centre for Diabetes, Endocrinology and Metabolism.

I hereby state that no part of this thesis has been submitted for any degree at this or any other university.

Minal Desai

October 2005

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GENETIC AND IMMUNOLOGICAL CHARACTERISATION OF PATIENTS WITH LATENT AUTOIMMUNE DIABETES IN ADULTS (LADA)

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A thesis submitted for the degree of Doctor of Philosophy *Michaelmas 2005*

Abstract

Autoimmune diabetes is a disorder in which the β-cells in the pancreatic islets of Langerhans are specifically destroyed resulting in absolute insulin deficiency; typically this is a childhood-onset disease, Type 1 Diabetes (T1D). Type 2 Diabetes (T2D) is a metabolic disorder usually developing in adults resulting from defects in insulin secretion and action. Latent Autoimmune Diabetes in Adults (LADA) is a form of diabetes that shares autoimmune disease pathology with T1D but a clinical presentation similar to T2D; LADA patients develop diabetes as adults (>25 years) and do not immediately require insulin treatment for survival. They are therefore often misdiagnosed with T2D.

The aims of this work were to characterise immunological and genetic aspects of LADA using a large cohort collected from various patient repositories the United Kingdom to determine if it is a separate disease entity or an age-related extension of T1D. Both T1D and LADA are characterised by autoantibodies to the islet cell protein glutamic acid decarboxylase 65 (GADA) at diagnosis. The persistence and titre of GADA post-diagnosis in LADA was examined at 0.5, 3 and 6 years. GADA persisted in 93% of patients for 6 years; GADA titres decreased between 0.5 and 3 years post-diagnosis and either stabilised or increased again between 3 and 6 years. GADA titre was not associated with age at diagnosis, glycaemic control, β-cell function or other clinical features. GADA titre at 0.5 years was associated with a greater likelihood of requiring more intensive anti-hyperglycaemic therapy but did not predict therapy or insulin requirement at 3 and 6 years. Autoantibodies against IA-2 plus GADA compared to GADA alone at diagnosis predicted increased therapy requirement by 3 and 6 years and insulin requirement by 3 years post-diagnosis.

Variants of the Human Leukocyte Antigen (HLA) genes *DRB1* and *DQB1,* are associated with susceptibility for T1D. An analysis of these variants in LADA (n = 378) revealed that the predisposing and protective variants in LADA are similar to those reported in T1D; DR3 (in linkage disequilibrium, LD with DQ2) and DR4 (in LD with DQ8) were the main predisposing variants whereas DR2 (in LD with DQ6) was most the protective against LADA. 85% of LADA patients possessed the DR3 and DR4 specificities, compared with 95% seen in T1D, suggesting a reduced predisposition in LADA compared with T1D. Synergistic effects of the DR3 and DR4 specificities occurred in LADA and the *DRB1*0401* allele within the DR4 specificity was predisposing to disease, as seen in T1D. No other predisposing variants were identified in LADA. As reported for T1D, DR11, DR13, DQ5, DQ7 and DQ9 were protective against LADA; DQ6 was positively correlated with age at diagnosis.

Association analysis of the insulin gene region in LADA (n = 400) showed that the variable number of tandem repeats (VNTR) locus primarily confers susceptibility to disease. Overall, the short Class I alleles predisposed to disease whereas longer Class III alleles conferred dominant protection, as reported in T1D. Fine-structure analysis showed that the Class I haplotypes 'IC+/ID+' and 'ID-' both conferred susceptibility for LADA – unlike in T1D, where the ID- haplotype has been reported to have protective effects. The Class III 'Protective' and 'Very Protective' haplotypes, conferred equal protection in LADA, as reported for T1D.

In conclusion; GADA persist post-diagnosis but are not markers for disease progression of LADA. Patterns of susceptibility at the HLA and insulin gene regions in LADA are similar to that reported for T1D. LADA is likely to represent an age-related extension of T1D rather than a separate disease entity.

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Chapter 1:

General introduction

CHAPTER 1: GENERAL INTRODUCTION

1.1 CLASSIFICATION OF DIABETES

1.1.1 Evolving definitions of diabetes mellitus: classification based on treatment to aetiology

Diabetes mellitus describes a heterogeneous collection of disorders arising from different pathologies but resulting in a common end-point - hyperglycaemia. Historically, two paradigmatic forms of the disease have been described based upon clinical examination: (1) an acute juvenile-onset form, occurring with ketosis (or ketonuria), severe muscle wasting, polydipsia and polyuria that is associated with need for exogenous insulin for survival. This form was described by the WHO in 1985 as 'insulin-requiring diabetes mellitus' (IDDM) (WHO 1985).

(2) a less symptomatic form of the disease, characterised by heavy glycosuria but no ketosis and presentation later in life usually associated with obesity (Himsworth and Kerr 1939; Zimmet *et al.* 1999). The WHO 1985 report described this form as 'non-insulin requiring diabetes mellitus' (NIDDM) (WHO 1985).

However, these definitions and nomenclature have been modified over the years. Since this classification of diabetes was based on clinical definition (age and insulin dependence) it was inadequate, as many patients do not strictly fall into either category. Specifically, diagnosis of IDDM is not confined to children (Molbak *et al.* 1994) nor does NIDDM occur only in adults (Bloomgarden 2004). Furthermore, not all children with IDDM may be clinically insulin dependent at diagnosis (Lohmann *et al.* 2000) nor can all patients with NIDDM be maintained with diet or oral agent therapy (Zimmet *et al.* 1999).

As a result, the pathogenesis of different forms of diabetes has been incorporated into diagnostic criteria, and has led to replacement of the terms IDDM and NIDDM with 'Type 1

diabetes' and 'Type 2 diabetes', respectively (Alberti and Zimmet 1998). With these new definitions, those cases that did not fall into either the IDDM or NIDDM categories defined by the WHO 1985 report criteria and had previously been declared 'unclassifiable' upon clinical examination could be now characterised by their underlying pathology:

***Type 1 diabetes (T1D)*:** This subgroup was further subdivided according to aetiology into autoimmune (T1aD) or idiopathic/ unknown mechanisms (T1bD), both of which culminate in β-cell self-destruction and thus absolute insulin deficiency. Together these forms of T1D comprise 5-10% of all cases of diabetes (American Diabetes Association 2004). T1aD probably occurs as a result of an abnormal genetically programmed immune response that, through interaction with the environment (e.g. infection), precipitates disease (see Section 1.2). Patients with T1aD can be characterised by the presence of circulating autoantibodies to islet cell proteins.

***Type 2 diabetes (T2D)*:** This form of diabetes arises from metabolic dysfunction in patients exhibiting varying degrees of defects in insulin secretion and insulin action. T2D is the most common form of diabetes, accounting for ~90% of the diabetic population (American Diabetes Association 2004). This form of diabetes occurs in genetically predisposed individuals although the environment plays a major role in disease susceptibility. Obesity, caused by a sedentary lifestyle and an over-indulgent diet has been strongly associated with T2D (Zimmet 1995). Therefore, an entirely glucocentric view of the pathogenesis of T2D is no longer considered to be valid, and abnormalities in lipid handling and metabolism in this disease are suspected (Krauss 2004).

In addition to the classical form of T2D, a number of rarer subgroups of T2D exist. Genetic analyses have revealed forms of T2D which are collectively called 'Maturity Onset Diabetes of the Young' or MODY; these are caused by autosomal dominant mutations in genes involved in β-cell differentiation and function. These cases comprise around 2% of the T2D population (Owen and Hattersley 2001). In addition, mitochondrial DNA mutations have been shown to cause the rare syndrome, mitochondrial diabetes associated deafness

(MIDD) (Walker and Turnbull 1997). Also, genetic defects of insulin action have been identified (Alberti and Zimmet 1998).

1.1.2 Problems of diagnosing adult-onset diabetes: a multitude of phenotypes

In clinical practice, differentiation of T1D from T2D is made phenotypically using five main factors; age at onset, apparent abruptness of onset of hyperglycaemia, the presence of ketosis, BMI and whether there is an immediate need for insulin treatment. An adult patient presenting with hyperglycaemia could have any one of the forms of diabetes described above (Zimmet *et al.* 1999). The clinical distinction of T1D from T2D is recognised to be imperfect and has limitations. In children and adolescents, T1D is relatively easy to diagnose as it is commonly associated with a classical acute onset of hyperglycaemia, ketosis, low BMI and an immediate need for insulin therapy. However, autoimmune diabetes occurring in adults can have a more insidious onset such that the patient is often misdiagnosed with T2D even though they have an underlying T1D-like pathology. These adult-onset patients with autoimmune diabetes may retain residual β–cell function that is sufficient to prevent ketoacidosis for a number of years (Zimmet 1995). Since the deterioration in metabolic control is slow, it may take many months/ years after clinical presentation to progress to insulin dependency. However, these patients progress more rapidly to insulin requirement than do patients with classical T2D (Turner *et al.* 1997). This subgroup of patients is usually only easily distinguished from classical T2D by testing for autoantibodies against β-cell proteins and comprises a group that is known today as Latent Autoimmune Diabetes in Adults (LADA) (Zimmet *et al.* 1994). This group of patients has also been described as slowly-progressive IDDM, latent type 1 diabetes, type 1 diabetes masquerading as type 2 diabetes and type 1 ½ diabetes. LADA patients are estimated to represent a variable proportion (2 - 22%) of the T2D population depending on the ascertainment criteria used (Reviewed by Pozzilli and Di Mario 2001; Schernthaner *et al.* 2001). Most large population-based studies in Caucasians agree that the prevalence is

~10% of all T2D diagnosed cases (Landin-Olsson *et al.* 1990; Turner *et al.* 1997; Humphrey *et al.* 1998; Tuomi *et al.* 1999). The prevalence of recognised T2D in the UK population is ~4% (Sicree *et al.* 2003). If up to 10% of these are GADA-positive, the prevalence of LADA is at least half of that of classical T1D (Narendran *et al.* 2005).

Since there is now a diabetes epidemic (Wild *et al.* 2004), it has become increasingly important to identify LADA patients early in the disease to ensure their appropriate management (e.g. monitoring to ensure insulin treatment is given when required). Today the diagnosis of LADA becomes even more of a challenge given that the age of onset of classical T2D appears to have moved several decades to younger age groups, particularly in populations with a high prevalence of this disease (Rosenbloom *et al.* 1999).

The underlying disease pathology of LADA is not well understood. It is likely, given the apparent autoimmune nature of LADA that patients with this form of diabetes possess immunological and genetic characteristics similar to those that have been observed in classical T1D occurring in children and adolescents. However, we do not know the factors that govern the later age of onset and milder phenotype (less severe disease progression) of LADA compared with T1D. Nor do we know whether this subgroup of slowly-progressing T1D represents a unique entity. By comparing markers of autoimmunity found in classical T1D with those found in LADA, we can obtain clues which relate not only to the disease process of LADA but to T1D too. This would also enable one to determine what factors govern the different disease courses of these two forms of autoimmune diabetes. In the next section, our current understanding of disease pathology in T1D is discussed.

1.2 AETIOPATHOGENESIS OF TYPE 1 DIABETES

Our understanding that T1D is caused by an autoimmune destruction of insulin-producing β-cells in the islet of Langerhans within the pancreas came from three key discoveries; (1) the occurrence of an 'insulitis' (lymphocytic infiltration) in this organ in patients, (2) the existence of antibodies in patient sera that reacted with islet proteins, and (3) the association of the disease with major histocompatibility (MHC) genes. The contribution of the first two discoveries to our knowledge of T1D is described below whereas the role of MHC is described in Section 1.2.2: A genetic component.

1.2.1 An immunological component

1.2.1.1 Insulitis in the pancreas

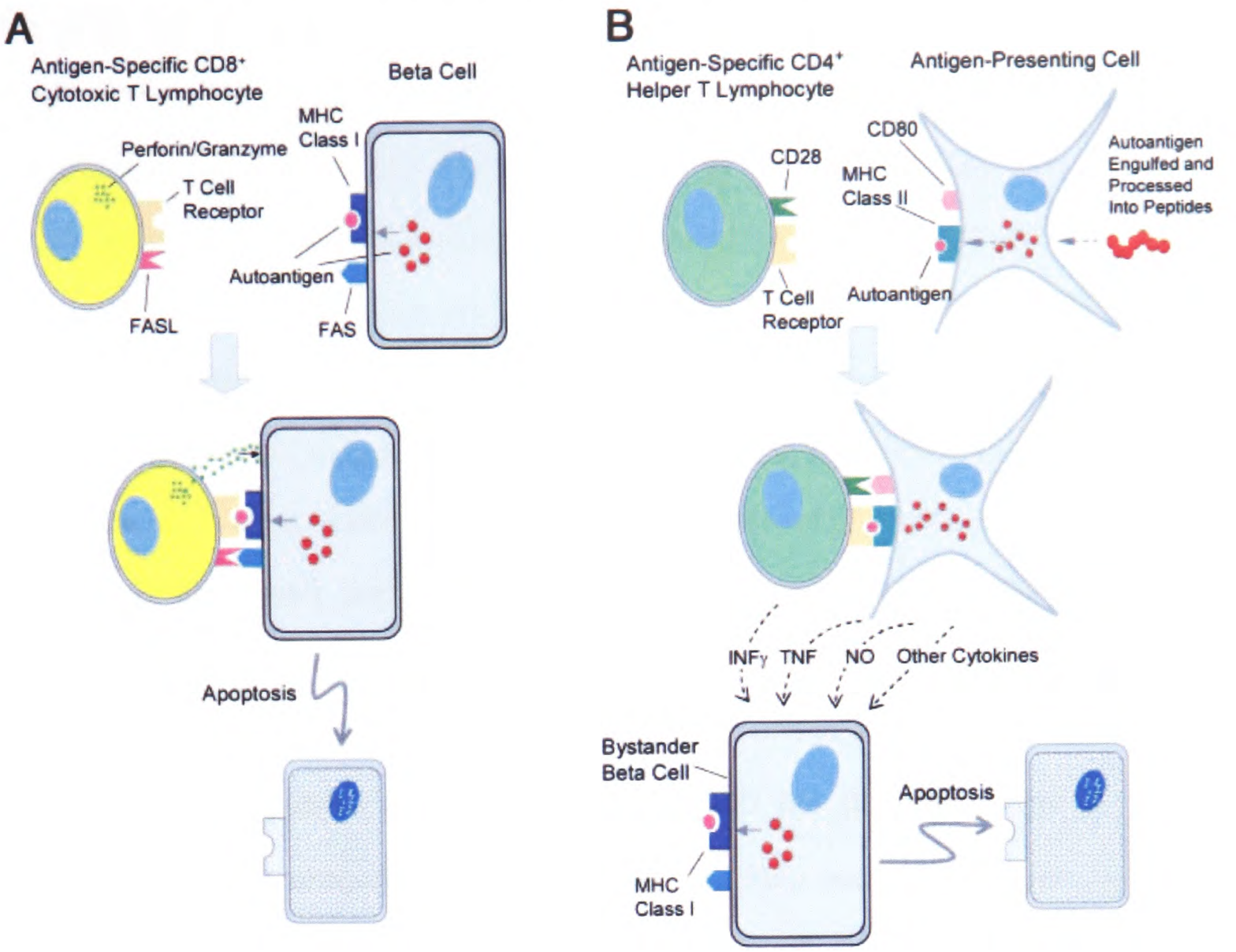
Gepts noted that islet lesions characterised by the presence of inflammatory infiltrates occurred in pancreata of young children who died within 6 months of diagnosis. On this basis he concluded that T1D must have an 'immunological origin'. He speculated that the insulitis heralded the later stages of the acute attack since a substantial loss of β-cells was observed (Gepts 1965). Later, Foulis demonstrated that this insulitis was a specific but progressive autoimmune attack of insulin-secreting β-cells while α-cells, δ-cells and pp-cells were preserved (Foulis and Stewart 1984; Foulis *et al.* 1986). At clinical onset of disease in T1D, 60-80% of the β-cells are believed to have been destroyed (Foulis *et al.* 1991; Notkins and Lernmark 2001).

Further immunohistochemical analysis of human islets showed that the composition of the invading leucocytes included a predominance of $CD8^+$ T cells as well as $CD4^+$ T cells, macrophages and B cells (Itoh *et al.* 1993). It is postulated that T1D is primarily a $CD4^+$ T_H1-mediated disease. Several lines of evidence support the pathogenic role of T cells: a delay

of disease progression is seen in patients given immunosuppressive therapy (Bougneres *et al.* 1988); recurrence of β-cell destruction occurs when a pancreatic graft from a non-diabetic monozygous twin is given to a diabetic twin (Sibley *et al.* 1985); 'adoptive transfer' of diabetes is possible upon transplantation of bone marrow from a diabetic donor to a non-diabetic recipient (Lampeter *et al.* 1993) and finally, circulating autoreactive T cells are found in T1D patients, albeit at low frequency (Roep *et al.* 1990).

Given the difficulties in obtaining pancreatic material during the acute phase of the attack, spontaneous rodent models of diabetes (non-obese diabetic (NOD) mouse and the BioBreeding (BB) rat) have been investigated in order to attempt to elucidate the exact mechanisms of cell death in T1D. To date, the mechanisms of β-cell destruction remain largely a mystery, however, two major hypotheses have been proposed, involving the 'direct' and 'indirect' killing of β-cells (Figure 1.1) (reviewed by Mathis *et al.* 2001; Notkins 2002). Although these models have mostly been derived from rodent studies and many differences in the pathological process exist compared with man (Roep and Atkinson 2004), a few studies of human pancreatic biopsies of prediabetic and newly-diagnosed subjects support these ideas. Hyper-expression of MHC class I molecules was shown to occur on β-cells which could enable selective death of these cells via the Fas-mediated apoptotic pathway (Moriwaki *et al.* 1999; Imagawa *et al.* 2001).

Figure 1.1: Mechanisms of T-cell mediated β-cell death in T1D.



(A) Direct killing. In this model, antigen presentation in the context of a MHC Class I molecule by islet β-cells act as 'Signal 1' for activation of autoreactive T cells. $CD8^+$ cytotoxic T cells directly recognise the MHC Class I–peptide complexes via engagement with their T cell receptor. This leads to apoptosis via regular pathways: Fas-FasL interaction and perforin-granenzyme B mediated cell lysis via activation of caspases. (B) Indirect killing. This is also referred to as 'bystander activation'. Here a professional antigen presenting cell presents peptide in the context of a MHC Class II molecule to a $CD4^+$ helper T cell. The consequent activation of the $CD4^+$ helper T cell results in an inflammatory response that may kill β-cells via secretion of cytotoxic cytokines (e.g. TNF-α, IFN-γ, IL-1) and through induction of cytocidal activity of macrophages in the vicinity leading to a delayed type hypersensitivity response. It is worth noting that these pathways are not mutually exclusive; cytokines can induce Fas expression on β-cells and activated $CD4^+$ T cells can also directly kill via the Fas death receptor pathway. (Adapted from Notkins 2002).

1.2.1.2 The discovery of islet cell autoantibodies

In the 1970s, two studies concomitantly published the findings that antibodies present in the sera of patients with various polyendocrinopathies (including T1D) were able to bind to pancreatic islets (Bottazzo *et al.* 1974; MacCuish *et al.* 1974). The observation that these islet cell antibodies (ICAs) could precede diabetes development (Lendrum *et al.* 1976) sparked a flurry of interest to deduce the specificities of these antibodies and identify the autoimmune target(s) in this disease. Immunoprecipitation experiments revealed that ICA in patient sera precipitated antigens from radiolabelled islet lysates predominantly of 64 kDa in size (Baekkeskov *et al.* 1982). This suggested the existence of one islet autoantigen. However, mild proteolysis (partial trypsinisation) of this complex revealed the existence of three distinct protein species migrating at 50 kDa, 40 kDa and 37 kDa on SDS-PAGE gels (Christie *et al.* 1990b; Christie *et al.* 1993). These were found to correspond to fragments of the β-cell proteins, glutamic acid decarboxylase (GAD 65) (Baekkeskov *et al.* 1990), insulinoma-associated antigen-2 (IA-2) (Payton *et al.* 1995) and IA2β/ phogrin (Lu *et al.* 1996), respectively.

In addition, insulin was later identified to be a major autoantigen (Palmer 1987) since it is difficult to detect on frozen sections of islet tissues to be picked up in the ICA reaction. A number of other putative autoantigens have also been considered as part of the variable ICA cocktail; these include carboxypeptidase H (Castano *et al.* 1991), ICA69 (Pietropaolo *et al.* 1993), Imogen 38 (Arden *et al.* 1996) and the glycolipid GM2-1 (Dotta *et al.* 1995). However, because autoantibodies to these proteins have been reported at considerably lower frequency than GADA, IA-2A and insulin autoantibodies (IAA), they have not been used in routine clinical studies and will not be discussed further.

The major autoantigens in T1D

GAD65

This enzyme which is expressed in α-, β- and δ- cells of the islet, was found to comprise the major component of ICA reactivity (Richter *et al.* 1992; Atkinson *et al.* 1993). GAD 65 is 585 amino acids in length and catalyses the formation of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA) from glutamine (Baekkeskov *et al.* 1990). In the β-cell, it is associated via its N-terminal domain with the cytosolic face of membranes of small synaptic-like vesicles (Christgau *et al.* 1992; Shi *et al.* 1994). The role of the transmitter in the islet is unknown but it has been proposed that GABA acts as a paracrine signalling molecule, switching off glucagon secretion from juxtaposed α-cells in the islet (Wendt *et al.* 2004). Also, GABA may be an important β-cell energy reserve as it can be fuelled into the Krebs cycle, generating NADH and ATP via the GABA shunt (Michalik and Erecinska 1992). In addition, it may play a role in regulating insulin secretion (Sorenson *et al.* 1991; Braun *et al.* 2004).

Two nonallelic isoforms of GAD exist, GAD 65 and GAD 67 and are encoded on chromosomes 2 and 10, respectively (Bu *et al.* 1992). They differ in length by 8 amino acids and share 65% overall homology in primary sequence with most variation occurring in the first 95 residues (Karlsen *et al.* 1991; Bu *et al.* 1992). Both are expressed in the brain but only the GAD 65 isoform is expressed in β-cells of human pancreas (Karlsen *et al.* 1991).

Autoantibodies to GAD 65 (GADA) are not exclusive to autoimmune diabetes. They are also found circulating in patients with Stiff-Man Syndrome (SMS), a rare T_H2-biased autoimmune disorder where the GABA-nergic neurons of the brain are attacked (Solimena *et al.* 1990), in Autoimmune Polyendocrine Syndrome (Powers *et al.* 1999) and a rare autosomal recessive neurodegenerative disorder, Batten disease (Chattopadhyay *et al.* 2002). In SMS, autoantibodies against both isoforms of GAD may be present, however, in T1D antibodies

are primarily targeted to GAD 65 (Hagopian *et al.* 1993b; Velloso *et al.* 1993; Bonifacio *et al.* 2000). Virtually the entire surface of GAD65 is targeted by GADA in T1D (Schwartz *et al.* 1999). GADA have been found in both prediabetic and newly-diagnosed patients (Baekkeskov *et al.* 1987; Christie *et al.* 1988). Later studies have shown that around 70-80% of T1D patients at diagnosis have autoantibodies to GAD 65 compared to 1-2% of healthy individuals (Hagopian *et al.* 1993b; Petersen *et al.* 1994; Verge *et al.* 1994). GADA are generally considered to have a high diagnostic sensitivity in T1D, especially in older onset autoimmune diabetic patients (Vandewalle *et al.* 1995; Sabbah *et al.* 1996).

The atypical protein tyrosine phosphatases: IA-2 and IA-2β

Full-length IA-2 is a 979 amino acid protein weighing 106 kDa. This protein is also known by its truncated fragment as ICA512, which was identified through antibody screening of a human islet expression library using sera from T1D patients (Rabin *et al.* 1992). It is predominantly expressed in both α and β cells of islets as well as neuronal cells and is located in insulin secretory granules (Lan *et al.* 1994; Lu *et al.* 1996). It is described as an atypical member of the transmembrane protein tyrosine phosphatases (PTP) family because it lacks enzyme activity as a result of critical mutations within its catalytic domain (Lan *et al.* 1994; Lu *et al.* 1994). IA-2β or phogrin shares 74% homology with IA-2 (in their intracellular domains) and is also an inactive PTP occurring in neuroendocrine tissues (Lu *et al.* 1996). The function of both these molecules is unknown but their association with insulin-secretory vesicles suggests a role in granule trafficking or maturation (Lieberman and DiLorenzo 2003).

Autoantibodies against IA-2 (IA-2A) and IA-2β are targeted against their C-terminal PTP-like domains and in the juxtamembrane region (Lampasona *et al.* 1996). Blocking studies have shown that autoantibody reactivity is primarily directed to IA-2A and binding to IA-2β occurs through inter-molecular cross-reactivity (Bonifacio *et al.* 1998). Around 55-75% of patients

have autoantibodies to IA-2A at diagnosis; these autoantibodies occur more frequently in children with T1D compared with adult-onset patients (Myers *et al.* 1995; Bingley *et al.* 1997; Gorus *et al.* 1997; Hawa *et al.* 2000; Sabbah *et al.* 2000).

Insulin

Autoantibodies to insulin (IAA) are the only β-cell specific targets of autoimmunity in T1D. The mature form of the 6 kD insulin molecule consists of an A chain and a B chain covalently linked by two disulphide bonds. Insulin epitopes of low-affinity IAA map mainly to the C-terminal end of the B chain, whereas high-affinity IAA are dependent on A chain residues 8-13 (Achenbach *et al.* 2004a). These antibodies are the first to appear in at-risk children (Ziegler *et al.* 1999). Recently, it was demonstrated in double knockout *ins1*$^{-/-}$ and *ins2*$^{-/-}$ NOD mice, that the replacement of insulin with a non-immunogenic modified form could prevent insulitis and development of diabetes (Nakayama *et al.* 2005). Also, Kent *et al* (Kent *et al.* 2005) have shown that of the lymphocyte population taken from the pancreatic lymph nodes of two T1D patients, ~50% of them specifically reacted with the A chain of insulin. Taken together, these findings imply that the initiating autoantigen in T1D may be insulin.

IAA are present at a frequency of 18-69% at diagnosis (Palmer *et al.* 1983; Landin-Olsson *et al.* 1992; Bingley *et al.* 1997). As with IA-2A, an age-dependent prevalence is observed, with a higher proportion of children being IAA-positive compared with adults (Karjalainen *et al.* 1989; Vandewalle *et al.* 1995; Sabbah *et al.* 2000).

Autoantigens and initiation of disease

Since autoimmune diabetes is caused by a specific attack of pancreatic β-cells, the presence of IAA in patients is understandable. It is not known why GAD65 and IA-2 are also major autoantigens. This autoimmunity is unlikely to be explained by genetic variation in the

genes encoding these proteins (Cai *et al.* 2001; Johnson *et al.* 2002). It is interesting that the subcellular localisation of these and other putative autoantigens occur largely within the components of the secretory pathway, especially in insulin secretory granules, the synaptic-like microvesicles, the ER and Golgi network (Lieberman and DiLorenzo 2003). Perhaps exposure of these molecules to the outside of the cell could trigger an autoimmune response against them. However, since both the antigenic portions of GAD and IA-2 are orientated toward the cytoplasm of the β-cell, they are unlikely to be targets of complement-mediated attack. One possible scenario is that lysis of the β-cell via autoreactivity against an initiating autoantigen (possibly insulin, in light of the recent evidence discussed above), will expose molecules such as GAD and IA-2 that are normally sequestered within the cell. This exposure can then lead to their attack by the autoreactive repertoire of immune cells and propagate the self-attack.

Rationale for studying autoantibodies and their specificities in T1D

Although autoimmune diabetes is a T-cell mediated disease, the identification and characterisation of T cell specificities is hampered by the inaccessibility of islet tissue during the autoimmune process and the low prevalence of autoreactive T cells in systemic circulation. Therefore, many workers in the field have turned their attention to towards autoreactive B cell responses. The introduction of reliable autoantibody detection methodology has made humoral responses relatively easy to measure in plasma samples in a non-invasive manner.

The rationale behind studying the specificity of the B cell autoantibody response is that they reflect the overall specificity of autoreactive T cell responses, since a reciprocal nature of T and B cell activation occurs. Therefore, the analysis of humoral targets may provide clues as to the nature of T cell epitopes. For example, autoreactive T cells with specificities for GAD 65 epitopes have been found in T1D (Panina-Bordignon *et al.* 1995; Reijonen *et al.*

2002). However, it is possible that B cells themselves, and/or their antibodies could play a role in disease.

A pathological role of autoantibodies?

Autoantibodies may participate in antigen clearance by several effector mechanisms including antibody-dependent cell cytotoxicity - release of inflammatory mediators via stimulation of F_c receptors on natural killer cells, macrophages or mast cells, opsonisation of antigen which promotes phagocytosis by macrophages and activation of complement factors resulting in formation of membrane attack complexes in target cells (Janeway *et al.* 2001). However, the role of autoantibodies in the pathogenesis of autoimmune diabetes in addition to their direct effector functions is unclear. In studies of the NOD mouse, B cell-deficient animals are protected from spontaneous islet cell destruction (Serreze *et al.* 1996; Falcone *et al.* 1998; Noorchashm *et al.* 1999). Also, the function of B cells as antigen-presenting cells (APCs) for autoreactive T cells has been shown to be important for the initiation of insulitis in these mice (Falcone *et al.* 1998; Serreze *et al.* 1998; Noorchashm *et al.* 1999; Wong *et al.* 2004). However, disease resistance in B-cell deficient mice is not absolute (Chiu *et al.* 2001) and some studies in humans do not support the contention that B cells have an essential role in T1D development. Martin *et al* reported the existence of a patient with X-linked agammaglobulineamia who developed T1D despite having severe B cell lymphopenia (Martin *et al.* 2001). Other studies questioning the pathogenicity of B cells showed that in newly-diagnosed T1D patients, the presence or absence of antibodies had no impact on the occurrence of cyclosporin-induced, remission of insulin dependence or the rate of loss of glucagon-stimulated C-peptide secretion (Mandrup-Poulsen *et al.* 1990; Petersen *et al.* 1994).

However, recent *ex vivo* studies suggest that autoimmune GAD 65-specific B cells and the antibodies they secrete (GADA) can modulate the processing and presentation of GAD 65 to $CD4^+$ T cells. Rejionen *et al* showed that when GADA from the sera of T1D patients is

complexed with antigen and fed to peripheral blood APCs, an enhanced presentation of a GAD 65 T cell determinant (spanning residues 274-286) is seen compared to when they are fed the antigen alone (Reijonen *et al.* 2000). Further support for an immunomodulatory role of GADA was later obtained by Jaume *et al* in their study of antigen presentation by autoreactive EBV-transformed *DRB1*0401*-positive B cell lines derived from a T1D patient to a panel of *DRB1*0401*-restricted murine T cell hybridomas (Jaume *et al.* 2002). The presentation of GAD 65 T cell determinants distant from the membrane IgG antibody epitope or 'footprint' of each B cell line was enhanced whilst the presentation of determinants overlapping this site was inhibited. This effect was also seen by these authors when soluble GAD 65 immune complexes were fed to splenocytes from humanised transgenic MHC class II mice. The ability of antibodies to suppress efficient processing and the presentation of certain epitopes within their footprint has been demonstrated previously and is believed to be due to the high affinity of their binding, preventing dissociation of peptide and antibody in endosomal processing/ MHC class II loading compartments (Watts and Lanzavecchia 1993; Simitsek *et al.* 1995). In a recent study using the same B-cell lines as Jaume *et al* (2002) but with *human* T cell hybridomas, additional factors were shown to be important for presentation of particular peptides aside from their topographical characteristics including the affinity of the antibody in processing compartments and overall processing capacity of the B cell line studied (Banga *et al.* 2004).

Therefore, the evidence presented above suggests that the peptide specificity of antibodies can manipulate T cell responses. One can speculate that this provides a mechanism by which antibodies may influence not only epitope spreading and hence disease progression (Mamula *et al.* 1992; Simitsek *et al.* 1995) but contribute to the actual precipitation of disease in the first place, by revealing otherwise cryptic T cell determinants to which tolerance has not been achieved during development (Lanzavecchia 1995).

Nevertheless, whether or not B cells themselves are of pathogenic importance or if the antibodies they secrete occur merely secondary to β-cell destruction, it is clear from many studies that antibodies present in the pre-diabetic period are invaluable markers of impending disease as discussed in the next section.

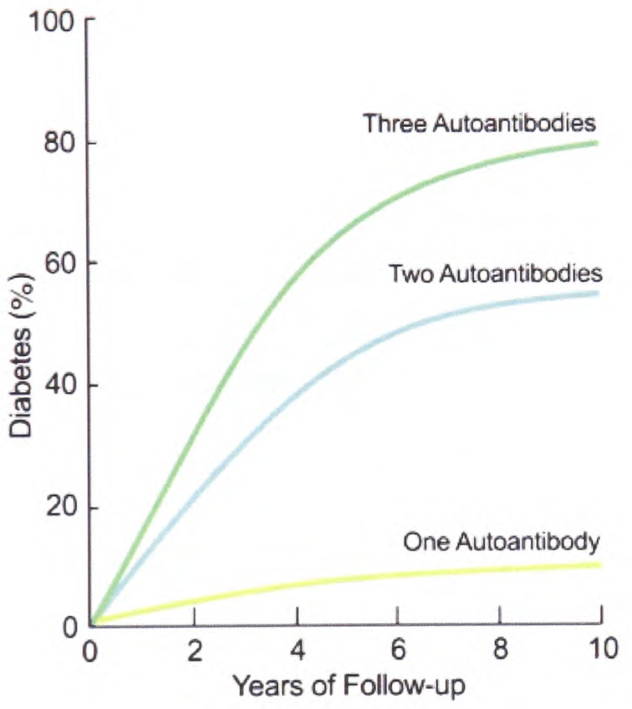
Antibodies as predictors

By virtue of the existence of a long prodromal period in T1D, predictors of clinical onset of disease can be sought to devise potential secondary prevention strategies. Bingley *et al* showed that the prediction of diabetes in at-risk individuals (first degree relatives) is best determined by loss of first-phase insulin response (FPIR) to the intra-venous glucose tolerance test (Bingley 1996). However, this method of metabolic testing is labour-intensive, invasive and difficult to standardise. For these reasons, attention has been diverted to measurement of autoantibody markers as indirect measures of functional residual β-cell mass. The fact that 92% of T1D patients have one or more β-cell autoantibodies at diagnosis suggests that they are highly predictive markers for disease (Gorus *et al.* 1997). Many studies have been conducted to evaluate the risk and rate of progression and the severity of clinical disease based on autoantibody measurements; that is the type, number and titre of disease-associated autoantibodies.

Studies of diabetes risk in first-degree relatives indicate that the number of antibodies rather than elevated levels of any one marker autoantibody, is predictive of T1D (Bingley *et al.* 1994; Verge *et al.* 1996a; Kulmala *et al.* 1998; Maclaren *et al.* 1999; Ziegler *et al.* 1999) (see Figure 1.2). Since ~85% of T1D occurs sporadically (Dahlquist *et al.* 1985; Maclaren *et al.* 1989), it is reassuring that this has also been demonstrated in the general population (Bingley *et al.* 1997; LaGasse *et al.* 2002; Maclaren *et al.* 2003). However, the type of autoantibody present in multiple autoantibody-positive individuals also appears to be important in determining risk. Decochez *et al* recently reported that IA-2A positivity was the

best predictor of diabetes development rather than the presence of multiple antibodies *per se* (Decochez *et al.* 2002). A correlation of IA-2A positivity with a more rapid progression to disease has been reported by others (Bingley *et al.* 1994; Christie *et al.* 1994; Gardner *et al.* 1999; Achenbach *et al.* 2004b). On the other hand, isolated GADA positivity or high-titre GADA have been shown to confer a lower risk and delayed onset (Schmidli *et al.* 1994; Yu *et al.* 1994; Verge *et al.* 1996b; Gardner *et al.* 1999). Earlier investigations of patients with a 'restricted ICA' pattern characterised by high GADA levels was found to associate with a more slowly-progressing form of autoimmune diabetes (Ujihara *et al.* 1994).

Figure 1.2: The risk of developing T1D in individuals with one, two or three antibodies (taken from Notkins and Lernmark 2001).



Percent diabetes represents an approximation from several longitudinal studies on first-degree relatives of type 1 diabetes patients.

The progression to overt diabetes is also predicted by a general high-titre of autoantibodies (Bonifacio *et al.* 1990; Bingley 1996; Borg *et al.* 2001). As shown in a study of IAA, the affinity of autoantibody could also be important for risk determination (Achenbach *et al.* 2004a). Additionally, young age (Bingley 1996; Williams *et al.* 2002) and the HLA type[1] of the individual may affect diabetes risk (Kukko *et al.* 2005).

[1] This is a genetic risk marker for T1D, discussed in Section 1.2.2.

Taken together, these studies suggest that the overall intensity of the humoral response may determine the extent of β-cell destruction. However, the use of autoantibodies and HLA markers for predicting the exact *rate* of progression to disease is limited. Gardner *et al* (Gardner *et al.* 1999) demonstrated that individuals who progressed to disease within 5 yrs of initial sampling, compared to those progressing later had similar autoantibody and HLA type profiles, suggesting that although multiple antibodies and high-risk alleles may predict *eventual* development of T1D, the time to progression can not be predicted solely by these markers alone. Moreover, inter-individual differences have been reported; some at-risk subjects with multiple autoantibodies at initial sampling do not progress to disease whereas others with only one specificity do so (Kulmala *et al.* 1998; Greenbaum *et al.* 1999). Furthermore, those with transient autoantibody positivity are less likely to develop frank diabetes (Barker *et al.* 2004b). These problems limit autoantibody screening purposes to research studies only (Bingley *et al.* 2001).

The use of IgG subclass and epitope specificity of autoantibody markers as predictors have been used to attempt to 'refine' risk levels. A recent study by Achenbach *et al* (Achenbach *et al.* 2004b) showed by multivariate analysis that multiple IgG subclass usage rather than titre and epitope specificity of IAA and IA-2A most significantly improved prediction of diabetes in at-risk individuals.

1.2.2 A genetic component

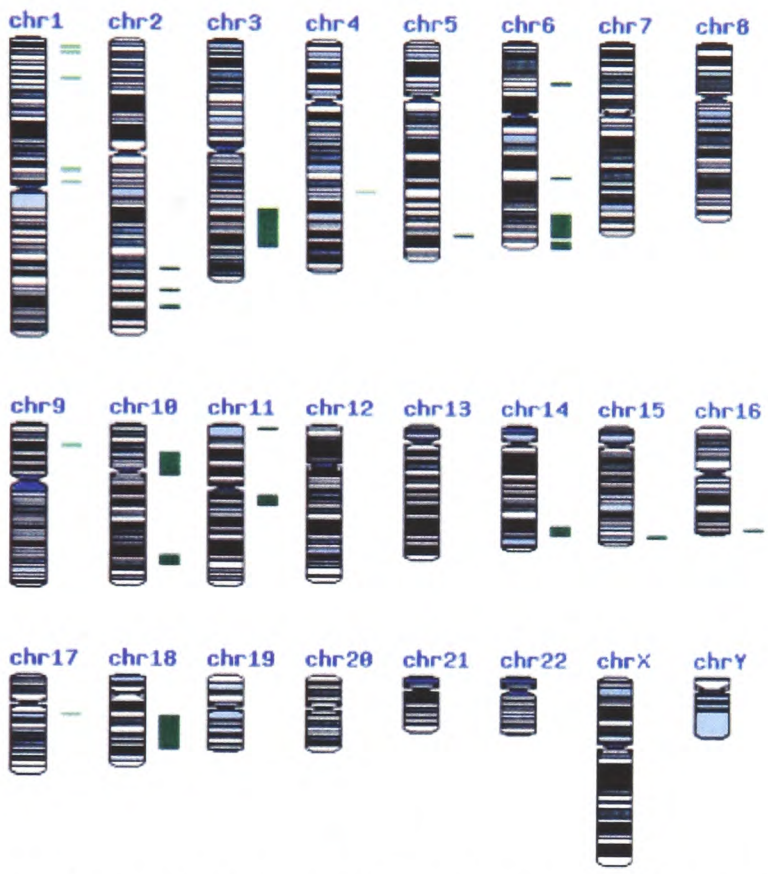
Evidence for a genetic component in the aetiology of T1D comes from family and twin studies. A higher frequency of the disorder occurs in close relatives of T1D patients than in the general population. To illustrate, the sibling risk ratio, λ_{sib} of developing the disease by age 30 is 15 (Risch 1987) and the frequency of disease in offspring of T1D parents is 3-6%, with a higher risk conferred by diabetic fathers than mothers (Reviewed by (Field 2002)). The strongest evidence of genetic involvement in disease susceptibility is the higher concordance of diabetes occurrence in monozygotic twins compared with dizygotic twins (Reviewed by (Field 2002; Hirschhorn 2003)). These twin studies have estimated that as much as 66-72% of the risk of developing T1D may be attributed to genetic factors, although other studies have yielded lower estimates (Hirschhorn 2003).

Identifying genetic factors involved in T1D could provide new clues about the disease aetiology and pathogenesis through investigation of biological pathways on which the proteins encoded by susceptibility genes reside. Genetic variants may also help identify high-risk individuals that would be useful for prediction of disease and their enrolment into prevention or treatment trials.

Since the initial discovery of a Human Leukocyte Antigen (HLA) gene association with T1D, a systematic search for other predisposing genes has been performed by means of genome-wide scans (Reviewed by (Onengut-Gumuscu and Concannon 2002)). Figure 1.3 shows the location of susceptibility loci identified by this approach. It has been definitively shown that the HLA gene complex at 6p21.3 (designated the *IDDM1* susceptibility locus) is the major inherited genetic risk factor predisposing to T1D, accounting for 44% of total genetic risk (Field 2002). The insulin gene region at 11p15.5 (*IDDM2,* Section 1.2.2.2) has also been confirmed in genome scans (Cox *et al.* 2001). It has been estimated to contribute ~10% to the total genetic risk of T1D (Todd 1995). The *CTLA4* gene (*IDDM12)* (Ueda *et al.*

2003) has also recently been confirmed as a minor susceptibility gene for T1D. Other regions of susceptibility have been more difficult to identify in genome scans but on the basis of biological relevance and replication of association studies, they have been considered as strong candidates e.g. the discovered, *PTPN22* gene (Bottini *et al.* 2004).

Figure 1.3: T1D susceptibility loci identified by genome-wide linkage studies.



Locus	Localisation	Markers
IDDM1	6p21.3	*HLA-DRB1, DQB1, DQA1*
IDDM2	11p15	*INS* VNTR
IDDM3	15q26	D15S107
IDDM4	11q13	*FGF3*, D11S1337
IDDM5	6q25	*ESR*
IDDM6	18q21	*JK*, D18S487
IDDM7	2q31	*HOXD8*, D2S152
IDDM8	6q27	D6S264, D6S446
IDDM9	3q21-q25	D3S1576
IDDM10	10p11-q11	D10S193
IDDM11	14q24.3-q31	D14S67
IDDM12	2q33	*CTLA4*
IDDM13	2q34-q35	D2S164
IDDM15	6q21	D6S283
IDDM16	14q32.3	D14S542, *IGH*
IDDM17	10q25	D10S554
IDDM18	5q33-q34	*IL12B*
Unnamed	1q42	D1S1617
Unnamed	16q22-q24	D16S3098
Unnamed	19p13	D19S247
Unnamed	19q13	D19S225
Unnamed	Xp13-11	DXS1068
Unnamed	7p13	*GCK*
Unnamed	12q14-q15	*IFNG*

Taken from (Atkinson 2005). Loci supporting an association with T1D are indicated in green on the chart. Candidate genes in the susceptibility regions are indicated in the table; loci highlighted in red had 'significant' evidence for linkage and those in blue are those with 'suggestive' evidence according to data from (Cox et al. 2001).

Our understanding of the role of *IDDM1* and *IDDM2* in susceptibility for T1D will be discussed in the following section. It is worth noting that susceptibility alleles at these loci, as with any complex disorder, are not 'mutant' alleles since sequences found in patients are also found in controls. Rather, the disease is associated with certain wild-type HLA alleles which may render a subject susceptible to disease as they modify the risk of developing disease but are not causative. Therefore, a particular allele is neither necessary nor sufficient to precipitate disease; although it may act as a risk-modifier (Todd *et al.* 1987).

1.2.2.1 The IDDM1 *susceptibility locus: the HLA genes*

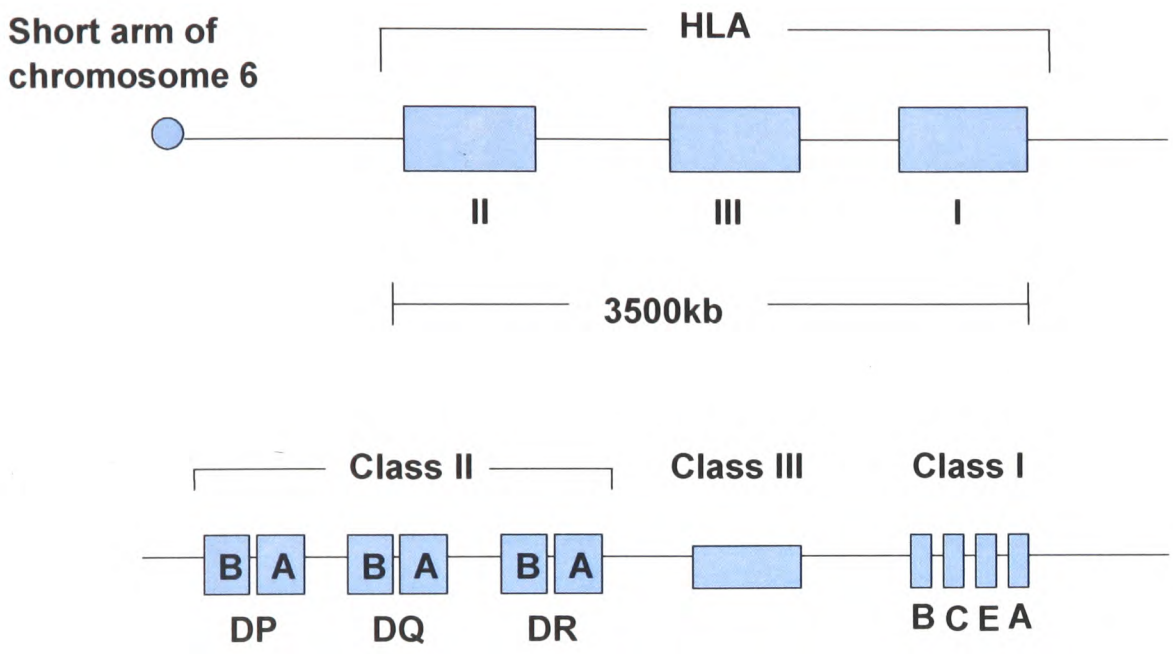
The HLA complex

The ~200 genes in the HLA complex can be divided into three main classes; the Class I region, the Class II region and the Class III region. Some HLA molecules are involved in antigen presentation to T-lymphocytes which in turn drive adaptive immune responses. These are encoded by Class I and II genes that comprise the 'Major Histocompatibility Complex' (MHC) situated on chromosome 6p21.3 (Figure 1.4). These molecules occur in the form of membrane bound heterodimers, originally defined by their ability to provoke vigorous rejection of grafts exchanged between different members of a species (Marsh *et al.* 2000). The MHC genes form a large complex of extremely polymorphic genes. MHC genes in humans are commonly referred to in the literature simply as HLA genes. This nomenclature will be used.

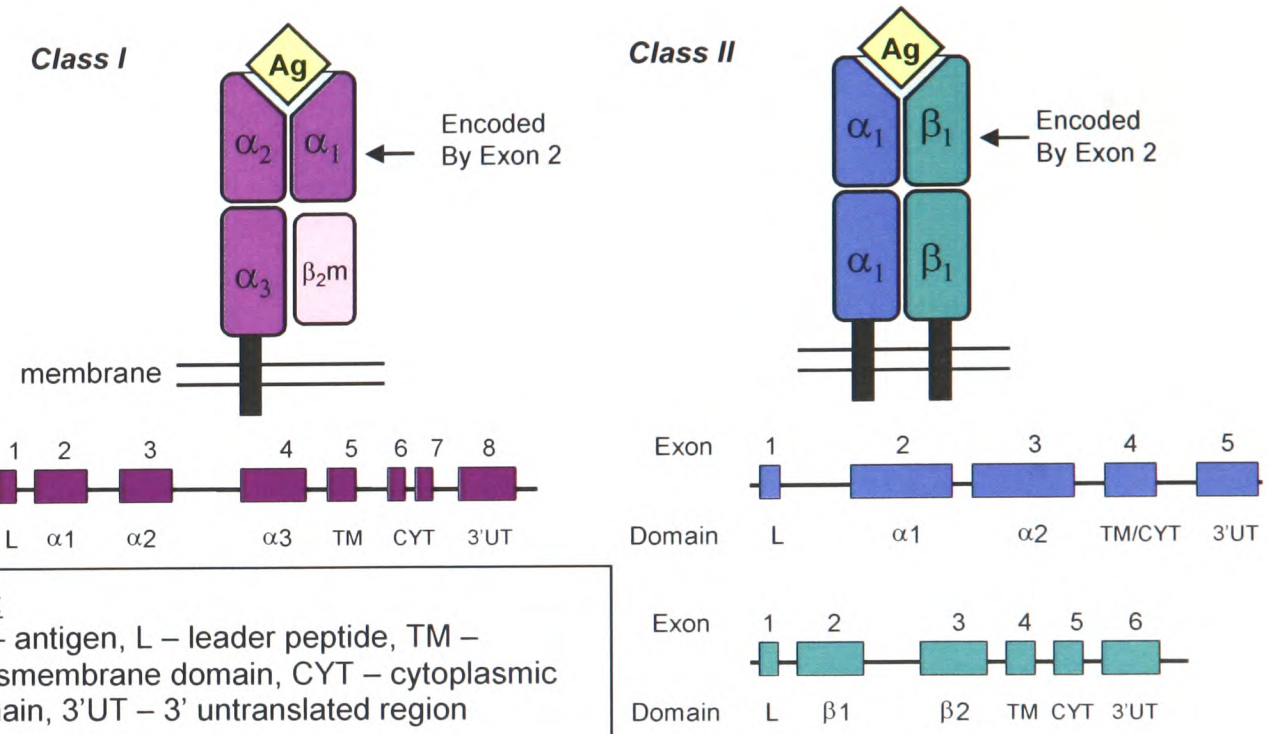
An important feature of HLA genes is that they are highly polymorphic with multiple alleles. This polymorphism is located mostly in the antigen binding site. Such variation has a strong selective advantage and has been maintained throughout evolution. This is because the plethora of peptides derived from pathogens are more likely to be recognised and bound by the highly polymorphic HLA molecules, facilitating their elimination by the adaptive immune system, enabling the body to fight infections and promote survival. However, there is a 'trade-off' between maximising protection against microorganisms by generating diverse specificities and producing MHC molecules capable of recognising self-molecules which would lead to autoimmune destruction. Normally, autoreactive T-cells are destroyed or rendered inactive by central (thymic) and peripheral tolerance mechanisms. Indeed, given the pivotal role of HLA molecules in mediating the immune response, it is not surprising that strong genetic associations at this gene cluster have been found with autoimmune diseases including coeliac disease and rheumatoid arthritis (Rose and Mackay 1999).

Figure 1.4: The HLA complex.

(a) Map of the HLA complex.



(b) Protein and gene structure of HLA molecules.



Key:
Ag – antigen, L – leader peptide, TM – transmembrane domain, CYT – cytoplasmic domain, 3'UT – 3' untranslated region

Class I. *The Class I genes encode (HLA-A, HLA-B and HLA-C) the original 'transplantation antigens'. They consist of a glycosylated heavy peptide chain of 43kDa (called the α-chain) which is non-covalently bound to a smaller peptide encoded on chromosome 15 called β_2-microglobin (a). The Class I molecules bind 8-10 residue peptides by their α1 and α2 domains (b) and are presented on the surface of every nucleated cell in the body. They are involved in the activation of $CD8^+$ T cells, which elicit cytotoxic responses to intracellular organisms that infect cells.* ***Class II (HLA-D).*** *The Class II loci (HLA-DP, HLA-DQ and HLA-DR) also encode transmembrane glycoproteins. Unlike Class I genes, each Class II loci encodes two types of peptide chain, the α (34kDa) and β (28kDa) chains from two different genes called A and B genes, respectively (a). Each of the DP, DQ and DR loci contain at least one A and one B gene. The Class II molecules bind polypeptides less than 30 residues long by their α1 and β1 domains (b). They are only expressed on the surface of specialised immune cells called 'professional antigen presenting cells' (macrophages, dendritic cells and B-lymphocytes). They bind peptide antigens that are derived primarily from extracellular pathogens. The Class II molecules activate $CD4^+$ helper T cells, resulting in a humoral response against the ectopathogen.* ***Class III.*** *These genes encode other proteins involved in shaping the immune response. These may be complement factor proteins, inflammatory molecules and proteins involved in antigen presentation.*

In the case of autoimmune diabetes, it is theorised that autoimmune adaptive responses would require autoantigen presentation by 'aberrant' HLA molecules present on islet β-cells themselves or on professional antigen presenting cells (e.g. dendritic cells) located in the islet. A strong association is seen between certain alleles of the Class I and Class II HLA genes and T1D. As a result of strong linkage disequilibrium between the MHC genes, the true 'etiological' variant(s) are difficult to dissect out and have yet to be confirmed.

Historical perspective: susceptibility at the HLA gene region (*IDDM1*)

The initial association was seen between the Class I antigens; HLA-B8 and HLA-B15 (Singal and Blajchman 1973; Cudworth and Woodrow 1974; Nerup *et al.* 1974). At the time, only serological methods were in place to detect Class I antigens. Upon discovery of the Class II antigens, it became apparent that the Class I association was secondary to linkage disequilibrium with the Class II genes, where the primary susceptibility determinant(s) were believed to exist (Sachs *et al.* 1980; Platz *et al.* 1981). Linkage to the HLA class II region with T1D was demonstrated by Thomson (Thomson 1988) and Spielman *et al* (Spielman *et al.* 1989). Alleles at the *DRB1*, *DQA1* and *DQB1* genes have been found to confer both susceptibility and protection against autoimmune diabetes whilst others are neutral. The Class II alleles discussed in the following sections and their corresponding antigen/ serological specificities are shown in Table 1.1. In particular, the sequence of exon 2 in both the DR (β_1-chain) and DQ (α_1 and β_1-chains) genes was found to be highly polymorphic. This exon encodes the protein domains of the HLA molecule which bind peptide antigen (Figure 1b). The recent complete sequencing of the HLA gene region (MHC Sequencing Consortium, 1999) showed that there are no other genes between the DR and DQ loci to explain the susceptibility arising from this genomic region. Also, recent fine mapping studies in a UK population have confirmed that the *DRB1* and *DQB1* genes are the major determinants of *IDDM1* susceptibility (Herr *et al.* 2000).

Table 1.1: HLA serological specificities and their common Caucasoid alleles.

DR serological specificity	Allele(s)[§]	DQ serological specificity	Allele(s)[§]
DR1	*DRB1*0101*	DQ2	*DQB1*0201-*0202*
DR103	*DRB1*0103*	DQ4	*DQB1*0401-02*
DR2	*DRB1*1501-3, *1601*	DQ5	*DQB1*0501-0503*
DR3	*DRB1*0301*	DQ6	*DQB1*0601-0604*
DR4	*DRB1*0401-*0405*	DQ7	*DQB1*0301*
DR7	*DRB1*0701*	DQ8	*DQB1*0302*
DR8	*DRB1*0801*	DQ9	*DQB1*0303*
DR9	*DRB1*0901*		
DR10	*DRB1*1001*		
DR11	*DRB1*1101-04*		
DR12	*DRB1*1201-02*		
DR13	*DRB1*1301-03*		
DR14	*DRB1*1401/04*		

[§]*These are commonly occurring alleles in UK White Caucasoid within each serological group.*

Serological specificities (antigens) are indicated by a two letter prefix (denoting the locus), followed by a number that represents the relevant antigen group in accordance with conventional nomenclature e.g. DR1 represents antigen specificity 1 at the DRB1 *locus. Alleles are presented in italics, prefixed by the gene name and an asterisk, followed by a 4 digit allele name e.g. DRB1*0101 is allele *0101 at the DRB1 gene.*

Susceptibility at DR and DQ

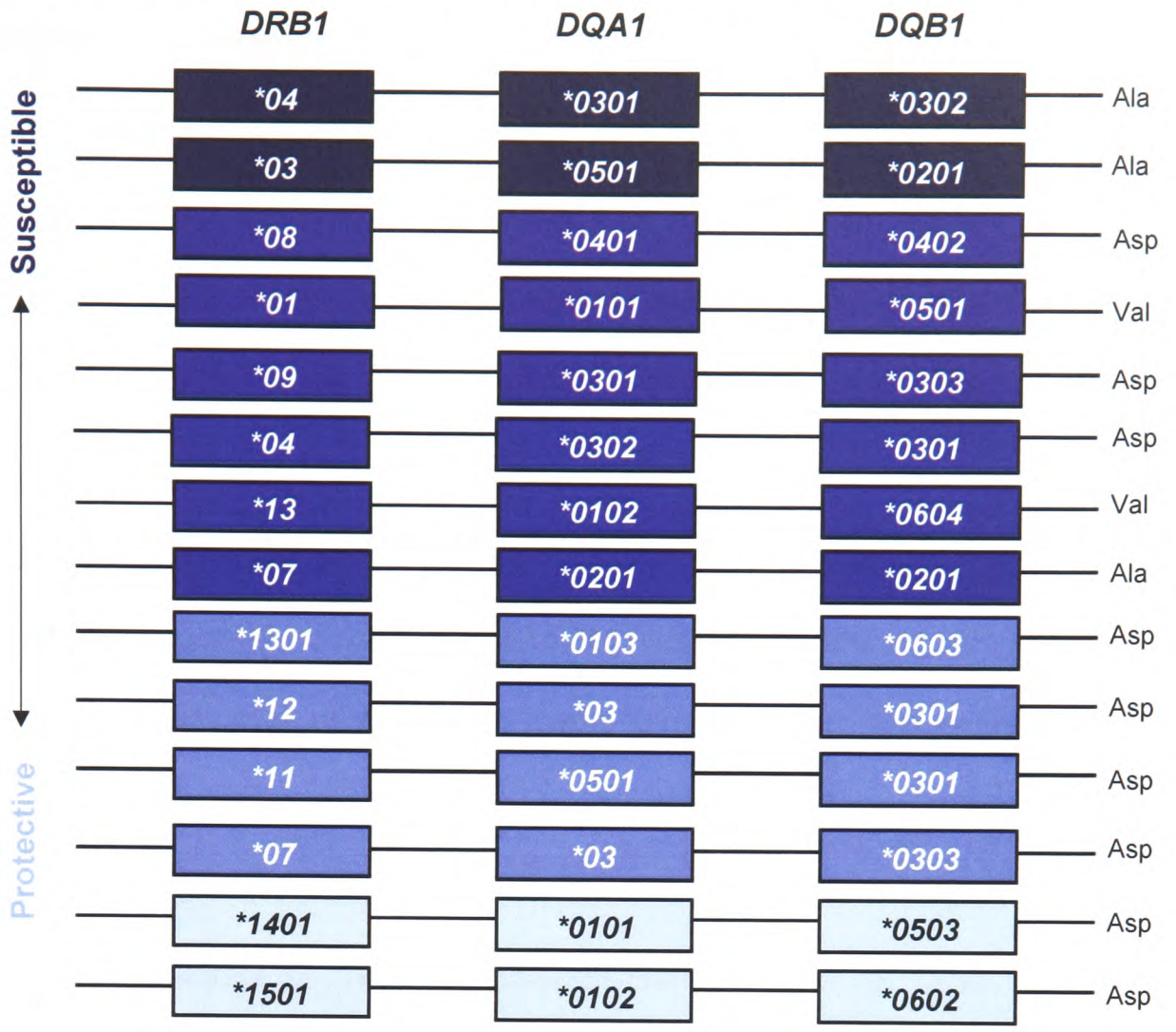
Susceptibility has been defined to the *DRB1* and *DQB1* genes at these loci. Alleles encoding the DR3 and DR4 antigens were found to be present in 95% of classical Type 1 diabetic patients compared with a frequency of 50% in the normal population (Thomson *et al.* 1988; Svejgaard and Ryder 1989). Such a high frequency of these alleles in the general population led workers to believe that they could not be primary susceptibility determinants (Jenkins *et al.* 1991). Attention was thus focused on other susceptibility candidates in the Class II region, in particular, *DQB1*. The first demonstration that *DQB1* modified the disease risk conferred by the DR4 antigen specificity was that *DRB1*04* alleles could be divided into high and low risk categories by their association with a restriction site in the *DQB1* gene (Owerbach *et al.* 1983). The restriction site distinguished between the *DRB1*04* alleles in linkage disequilibrium with DQ7 (*DQB1*0301*) and DQ8 (*DQB1*0302*) (Kim *et al.* 1985). In healthy controls the DQ7 and DQ8 molecules occurred at equal frequencies in DR4 individuals. However in T1D patients, the DQ8 specificity predominated, occurring at 90% (Bohme *et al.* 1986; Nepom *et al.* 1986; Michelsen and Lernmark 1987; Owerbach *et al.* 1989). The DQ7 and DQ8 molecules differ from each other at four residues in the β_1 domain, giving rise to differential binding properties and possibly susceptibility (Sanjeevi *et al.* 1995). Aside from the restriction mapping studies, the finding that low-risk DR alleles occurred on high-risk DQ haplotypes in T1D indicated a stronger and more specific association with the *DQB1* gene rather than the *DRB1* locus (Morel *et al.* 1988). Moreover, transracial mapping studies supported the above findings for a stronger role of DQ genes (Mijovic *et al.* 1991). Lastly, these human studies are supported by murine studies, where the NOD equivalent of the *DQB1* gene, *IA* associates mostly strongly with disease (Hattori *et al.* 1986; Miyazaki *et al.* 1990). Indeed, the structure of DQ8 and I-A^{g7} binding pockets are very similar, suggesting that the same antigen presentation events could initiate disease in mice as in humans (Lee *et al.* 2001). However, recent studies suggest that susceptibility conferred by DR and DQ is complex and a primary susceptibility role of either may vary among haplotypes (Kockum *et al.* 1999).

DRB1-DQB1 *haplotype risk in T1D*

As mentioned earlier, DR3 alleles are predisposing. They occur in LD with the DQ2 antigen, forming the high-risk *DRB1*03-DQB1*0201* haplotype. However, the *DQB1*0201* allele can also occur in LD with *DRB1*0701*, forming a haplotype that is neutral/ protective against disease. Therefore, *DRB1* variants associated with disease may carry differential risks according to the *DQB1* allele that is in linkage disequilibrium with it and vice versa. Thus susceptibility is best described by haplotypic combinations rather than alleles at the individual loci alone (Sheehy *et al.* 1989).

Although the *DRB1*0301-DQB1*0201* and *DRB1*0401-DQB1*0302* haplotypes carry the greatest risk for the development of T1D, around 10% of Caucasian T1D patients and up to 30% of non-Caucasoid populations do not possess either of these susceptibility haplotypes (Ronningen *et al.* 1992). The nature of these 'other' haplotypes that predispose to T1D have been determined in nonDR3/nonDR4 individuals. Dubois-Laforgue and colleagues (Dubois-Laforgue *et al.* 1997) were the first to perform a case-control analysis of 72 nonDR3/nonDR4 French patients with T1D. They showed that in these individuals, the *DRB1*08-DQB1*0402* haplotype was associated with disease. Since this report, a number of association and family studies have identified haplotypes associated with disease in nonDR3/nonDR4 T1D patients (Noble *et al.* 1996; She 1996; Caillat-Zucman *et al.* 1997; Kawasaki *et al.* 1998; Undlien *et al.* 1999; Lie *et al.* 2000; Redondo *et al.* 2000; Cucca *et al.* 2001b; Redondo *et al.* 2001). Figure 1.5 shows the spectrum of susceptibility conferred by the various haplotypes ranging from highly predisposing to strongly protective (Figure 1.5). The risks reported for haplotypes that occur in between the two extremes of the spectrum are not clear-cut from the literature; the intermediate risk haplotypes vary from being considered predisposing or protective to neutral between different studies.

Figure 1.5: Spectrum of risk of T1D-associated haplotypes in Caucasians



*This classification is based on the general consensus obtained from several population and family-based association studies of nonDR*03/nonDR*04 T1D subjects. Boxes represent alleles at each gene. The role of the* DRB1 *and* DQB1 *variants is discussed in text.* DQA1 *variants are included for fuller definition of haplotype structure. The amino acid code refers to the nature of the residue present at position 57 of the DQβ1 chain (see 'Molecular basis of susceptibility at* DQB1*'). Note that this hierarchy has been constructed for Caucasoids and the exact order of risk portrayed is not absolute, and may vary among populations. Not all* DRB1*04-DQB1*0302 *haplotypes are predisposing (see 'DR4 subtypes and differential susceptibility').*

DRB1-DQB1 *haplotype protection in T1D*

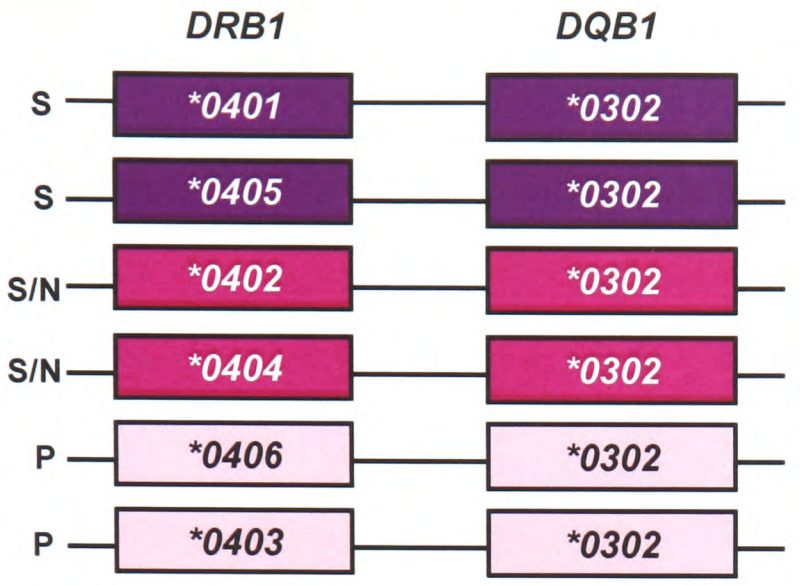
The **1501* allele at *DRB1* and **0602* allele at *DQB1* form a haplotype that strongly protects against T1D (Caillat-Zucman *et al.* 1997). The haplotype occurs in ~1% of T1D individuals compared with ~20% of the general population (Baisch *et al.* 1990). This protection, which appears to be conferred by the *DQB1*0602* allele rather than the *DRB1*1501* allele (Noble *et al.* 1996; Redondo *et al.* 2000), can override the susceptibility conferred by the high-risk *DRB1*03-DQB1*0201* or *DRB1*04-DQB1*0302* haplotypes (Baisch *et al.* 1990). One report

showed that the *DQB1*0602* allele did not associate with progression to overt diabetes in antibody-positive T1D relatives (Pugliese *et al.* 1995), suggesting an immunomodulatory role. However, this protective effect is not absolute since T1D subjects carrying the *DQB1*0602* allele have been reported (Pugliese *et al.* 1999; Rayner *et al.* 2002). The *DRB1*1401-DQB1*0503* haplotype has also been shown to be strongly protective (Redondo *et al.* 2000).

DR4 subtypes and differential susceptibility

A number of studies have shown that not all DR4 haplotypes are equally protective. The risk conferred by the **0302* allele at the *DQB1* gene is depends on the specific subtype (allele) of the DR4 antigen specificity in LD with it on the *DRB1*04-DQB1*0302* haplotype (Figure 1.6) (Sheehy *et al.* 1989; Erlich *et al.* 1993; Cucca *et al.* 1995; Huang *et al.* 1995; Tait *et al.* 1995a; Van der Auwera *et al.* 1995b; Harfouch-Hammoud *et al.* 1996; Noble *et al.* 1996; Sanjeevi *et al.* 1996; Caillat-Zucman *et al.* 1997; Undlien *et al.* 1997; Donner *et al.* 2000; Cucca *et al.* 2001b; Rewers *et al.* 2003; Lambert *et al.* 2004). Since the background frequency of the various DR4 subtypes varies between ethnic populations, different studies have yielded conflicting results even among Caucasians. Therefore, the effect of DR4 subtype in determining overall risk of the *DRB1*04-DQB1*0302* haplotype is unclear. The general consensus is that, in Caucasians, *DRB1*0401*, **0402* and **0405* increase risk independently of *DQB1*0302* whereas **0403* and **0407* appear to confer relative protection. Some reports suggest that the **0403* allele is dominantly protective (Van der Auwera *et al.* 1995b) and that it can override the susceptibility caused by *DQB1*0302*, even in subjects carrying high-risk genotypes such as *DRB1*03-DQB1*0201/DRB1*04-DQB1*0302* (Roep *et al.* 1999).

*Figure 1.6: Differential of risk DRB1*04 haplotypes*



Susceptibility conferred by DQB1*0302 *is modified by the* DRB1*04 *allele subtype in cis with it. Boxes represent alleles at each gene. Key: S, susceptible haplotypes; S/N, susceptible to neutral haplotypes; P, protective haplotypes.*

Genotypic risk in T1D

The complex interaction of all HLA molecules in a given genotype is likely to affect disease risk (Noble *et al.* 1996). The importance in examining an individual's complement of haplotypes, that is, their diplotype on disease risk is evident in those carrying the high-risk *DRB1*03-DQB1*0201-DRB1*04-DQB1*0302* diplotype which is the highest risk susceptibility determinant (She 1996; Caillat-Zucman *et al.* 1997). Around 30-40% of T1D cases possess this determinant compared with only 2% of controls. Many studies have shown that the odds ratio of the diplotype is greater than the sum of the odds ratios for the individual haplotypes, suggesting a synergistic effect of these haplotypes (Kockum *et al.* 1999).

As with haplotype risks, diplotypic risks have been found to vary among studies and therefore no definitive risks have been assigned. However, in most White Caucasian populations, apart from the *DRB1*03-DQB1*0201/DRB1*04-DQB1*0302* heterozygote, homozygosity for *DRB1*04-DQB1*0302 and DRB1*03-DQB1*0201* confer high risk (Kelly *et al.* 2004). Some studies also show the *DRB1*04-DQB1*0302/DRB1*01-DQB1*0501* and *DRB1*04-DQB1*0302/DRB1*08-DQB1*0402* diplotypes to confer risk (Noble *et al.* 1996; Lambert *et al.* 2004). Those genotypes containing *DRB1*04-DQB1*0302* haplotypes with *DRB1*0401*, **0405* and **0404* have been reported to confer high risk (Undlien *et al.* 1997)

whereas those containing **0403* are neutral/ protective (Cucca *et al.* 1995; Roep *et al.* 1999).

Cucca *et al* showed that genotypes carrying protective haplotypes such as those associated with DR2, are negatively associated with disease or neutral, despite the other haplotype being of high-risk (Cucca *et al.* 2001b).

Molecular basis of HLA susceptibility

Molecular basis of susceptibility at DRB1

The molecular mechanism by which DR4 subtypes modify susceptibility is not known. The differential susceptibility conferred by the DR4 subtypes may be accounted for by variation in their encoded peptide sequence. Undlien *et al* reported that protective *DRB1*04* alleles (**0403, *0404* and **0406*) had very similar peptide binding motifs (Undlien *et al.* 1997). Since then, analysis of peptide sequences of the different subtypes have showed consistently that the different degrees of susceptibility of these alleles correlated with differences in key residues in the P1 and P4 binding pockets, which influence peptide selectivity of the molecule (Donner *et al.* 2000; Cucca *et al.* 2001b). Protection conferred by HLA molecules of risk-reducing alleles may occur by their binding of a common protective/ tolerogenic peptide antigen or through prevention of presentation of immunodominant peptides.

Molecular basis of susceptibility at DQB1

Susceptibility at the *DQB1* locus was thought to be governed in part, by the amino acid present at residue 57 in the peptide sequence; this residue is in the peptide-binding motif and may affect antigen selectivity/ binding affinity and possibly molecular stability (Sato *et al.* 1999; Wucherpfennig 2003). The alleles encoding an aspartic acid (asp) residue at this position were proposed to be protective against T1D whereas non-asp (alanine, valine or

serine) alleles were considered predisposing (Todd *et al.* 1987; Horn *et al.* 1988; Morel *et al.* 1988). In line with this, the *DQB1*0201* and **0302* alleles (predisposing) encode an alanine residue whereas the **0602* (protective) encodes an asp residue (Figure 1.5).

However, studies have shown that the asp/non-asp dimorphism cannot fully account for the susceptibility conferred by the *DQB1* gene (Jenkins *et al.* 1990; Sanjeevi *et al.* 1995). In the Japanese, risk is associated with asp57-encoding alleles *DQB1*0401* and *DQB1*0303* (Yamagata *et al.* 1989). Moreover, both DR3 and DR7 haplotypes can contain a *DQB1*0201* allele, although only DR3 haplotypes are predisposing (Figure 1.5) (Erlich 1990). Furthermore, some high-risk *DQB1* genotypes also contain aspartic acid at position 57 (Figure 1.5) (Lambert *et al.* 2004). Therefore, it is unlikely that the residue at position 57 in the DQβ1 chain alone can explain the susceptibility to disease in an 'all-or-nothing' manner.

Khalil *et al* extended the asp57 story and proposed that the presence of an arginine residue at position 52 in the *DQA1* chain formed diabetogenic dimers when in combination with a *DQB1* non-asp chain (Khalil *et al.* 1990). Such susceptibility dimers could form in *cis* or *trans* (Nepom *et al.* 1987) and their effect was found to be dose-dependent (Khalil *et al.* 1992). The phenomenon of transcomplementation was used to explain the synergistic effect of the *DRB1*0401-DQB1*0302/DRB1*0301-DQB1*0201* heterozygote compared to homozygotes since four possible susceptibility dimers vs. two could potentially be formed in such individuals (Khalil *et al.* 1992; Thorsby and Ronningen 1993). However, not all T1D patients in different ethnic groups possess such dimers so this mechanism is not absolute (Penny *et al.* 1993). More recent evaluation of molecular risk of the primary structure of this β-chain has implicated the role of several amino acids (including residue 57) but reports have been conflicting with regards to the exact residues involved in determining susceptibility (Valdes *et al.* 1997; Djoulah *et al.* 1999).

Extended HLA haplotypes and non-DR/DQ encoded susceptibility

The evidence presented above suggests that the *DRB1* and *DQB1* genes in combination act as a 'superlocus' to influence the risk of developing T1D (Cavan *et al.* 1993; Thorsby and Ronningen 1993). However, because the *DRB1* and *DQB1* loci do not wholly account for all risk, it is possible that yet another gene(s) in linkage disequilibrium with these susceptibility loci also contribute. The *DQA1* gene is generally believed to be a component of the superlocus (Cavan *et al.* 1993) but since there are no informative splits of *DRB1-DQB1* haplotypes on the basis of this gene (Cucca *et al.* 2001b), it has been omitted from discussion. Another Class II locus, *DPB1, is* believed to make a small contribution to risk (Noble *et al.* 2000; Cucca *et al.* 2001a; Cruz *et al.* 2004). Among other loci implicated include the Class III genes; *TAP*, *LMP*, *HLA-F* and the *TNF* and *HSP70* gene clusters (Reviewed by (Kelly *et al.* 2004)).

1.2.2.2 The IDDM2 *susceptibility locus: the insulin gene region*

The insulin gene region (*IDDM2* region) is another locus associated with T1D. The region consists of the insulin gene and its surrounding genes on chromosome 11p15.5; with the tyrosine hydroxylase (*TH*) gene positioned telomeric to it and the gene for insulin-like growth factor 2 (*IGF-2*) located centromeric (Figure 1.7).

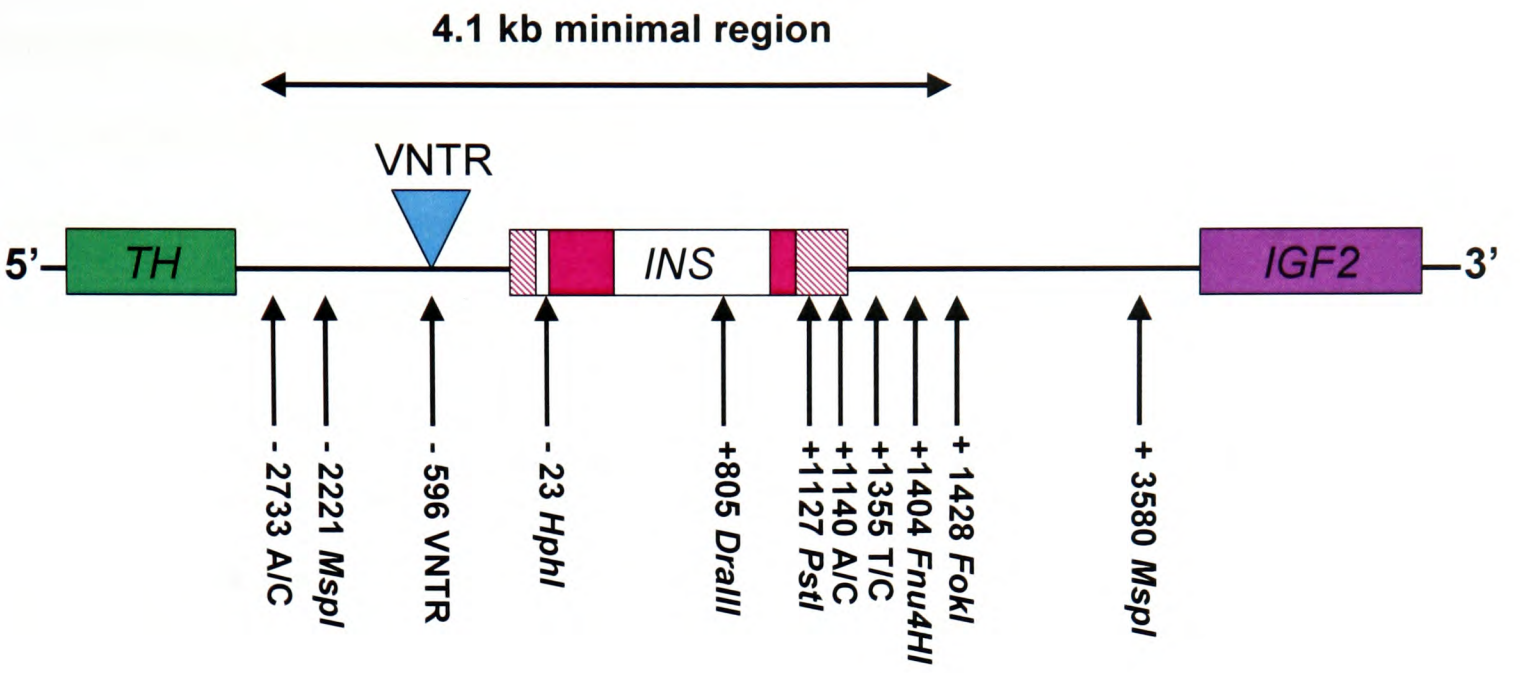
Historical perspective: The insulin gene VNTR is *IDDM2*

Early mapping studies of the *IDDM2* gene region revealed an insertion-deletion polymorphism located upstream of the insulin gene (Bell *et al.* 1981). Further characterisation of this polymorphic region showed it to consist of a minisatellite, comprising tandem repeats of a 14-15 bp sequence with the general consensus sequence 5'-ACAGGGGTGTGGGG-3' (Bell *et al.* 1982). It was named the insulin gene variable number of tandem repeats or *INS VNTR*, and is positioned 363 bp 5' from the transcription start codon (596 bp upstream from the translation start site) (Bell *et al.* 1982). The alleles at the VNTR were found to have a bimodal size distribution in Caucasians, initially identified by

Southern blotting; the shorter Class I alleles contained 26-63 repeats and the longer Class III alleles contained 141-209 repeats (Bell *et al.* 1982; Stead and Jeffreys 2000). The intermediate sized Class II alleles (~80 repeats) are relatively rarely found in Caucasoid populations compared with African populations (Bell *et al.* 1984). A positive association of Class I alleles with T1D was reported in case-control studies (Bell *et al.* 1984; Hitman *et al.* 1985), and was confirmed later in linkage and family-based association studies (Julier *et al.* 1991; Bain *et al.* 1992; Spielman *et al.* 1993). Homozygosity for Class I alleles has been reported to occur in ~75-85% of T1D patients compared with a lower frequency of 50-60% in the general population. Conversely, the longer Class III alleles were found to confer protection against the development of T1D in a dominant fashion (Bennett *et al.* 1996).

To determine whether the VNTR was the functional polymorphism accounting for susceptibility in the *IDDM2* region, or whether it was associated with disease secondary to linkage disequilibrium with the causal variant(s), a search for all candidate aetiologic polymorphisms in the *TH, INS* and *IGF2* gene cluster flanking the VNTR was performed. Fine mapping studies (using RFLP typing) narrowed down the region of susceptibility to a 19 kb interval (Julier *et al.* 1991) and later to a 4.1 kb minimal region believed to harbour the susceptibility variant(s) (Lucassen *et al.* 1993). In total, 9 SNPs in addition to the *INS VNTR* were identified as being most strongly associated with disease and were all located outside of coding sequences; these were identified as the following polymorphisms moving 5' $\rightarrow$ 3' along chromosome 11p15.5: -2733 A/C, -2221*MspI*, -596VNTR, -23*HphI*, +805*DraIII*, +1127*PstI*, +1140A/C, +1355T/C, +1404*Fnu4HI*, +1428*FokI* (Figure 1.7). Polymorphisms are designated by their position with respect to the first base of the translation initiation codon in the *INS* gene. The –23*HphI* site was found to occur in almost 100% LD with the *INS* VNTR; the T allele at this site occurs on almost all haplotypes containing VNTR Class III alleles in Caucasian populations (Bennett *et al.* 1995).

Figure 1.7: Candidate polymorphisms in the insulin gene region.



Key:
VNTR = variable number of tandem repeats
TH = tyrosine hydroxylase
INS = insulin gene
IGF-2 = insulin-like growth factor II

Schematic representation of the TH-INS-IGF2 *region on chromosome 11p15.5 showing the 10 polymorphisms (and their identifying enzymes) contained in the 4.1kb region of strongest susceptibility, plus the +3580*MspI *polymorphism that identifies the ID- lineage of Class I alleles (see section 'VNTR subclasses'). Polymorphisms are designated by their position with respect to the first base of the translation initiation codon in the INS gene. The INS gene is coloured pink; open, closed and hatched boxes depict introns, exons and untranslated regions, respectively. Adapted from (Bennett and Todd 1996), not drawn to scale.*

All of these polymorphisms in the insulin gene region display strong linkage disequilibrium with one another and confer similar relative risks for T1D, which has made it difficult to dissect the causative variants from the 'hitchhiking' ones. 'Cross-match' haplotype analysis and multiple DNA variant association analysis, followed by analysis of VNTR allele subtypes suggested that only variation within the *INS* VNTR itself could account for disease susceptibility (Owerbach and Gabbay 1993; Julier *et al.* 1994; Undlien *et al.* 1995). It was thus considered to be the primary aetiological candidate, with SNPs in the flanking regions

of the VNTR possibly capable of modulating this disease susceptibility (Bennett and Todd 1996). However, a re-examination of the data published by Julier *et al* (Julier *et al.* 1991) and the finding of significant association of a SNP marker 3' of the *TH* gene that appeared to modulate the VNTR susceptibility, challenged the consensus that susceptibility was confined to the 4.1kb minimal region (Doria *et al.* 1996). These data warranted a comprehensive re-sequencing of the *IDDM2* region and association analysis in a large number of individuals. This was done recently by Barratt *et al* who performed a detailed genetic analysis of a 325 kb region containing the *TH-INS-IGF2-H19* genes in nearly 3000 families (Barratt *et al.* 2004). They were able to confirm that susceptibility mapped within the 4.1 kb region, although –23*HphI*, the *INS* VNTR and the +1140A/C polymorphisms were all found to be associated with T1D and it was impossible to determine which conferred primary susceptibility due to the strong LD between them. However, the *INS* VNTR is the most likely functional candidate (see later) since no obvious biological function of the biallelic –23*HphI* and +1140A/C variants is apparent.

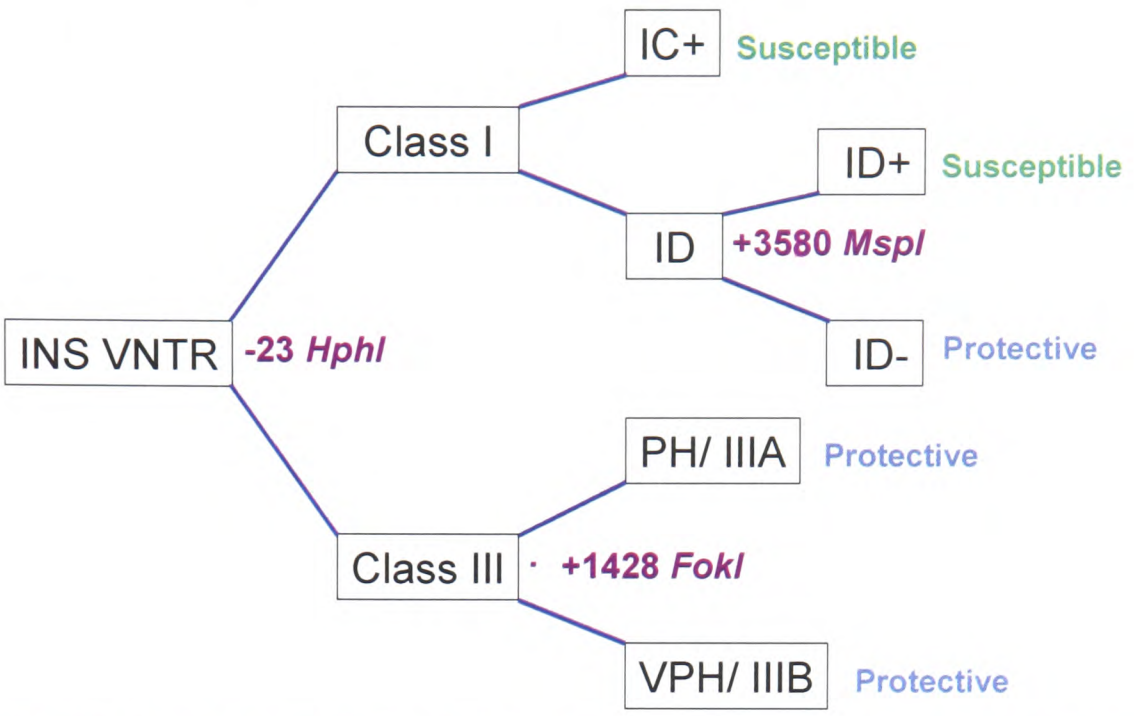
VNTR internal structure – subclasses

Variation at the VNTR can be defined by size into 39 different alleles (Stead *et al.* 2000). However, by structural analysis of alleles, 189 different alleles have been identified (Stead *et al.* 2000). Eleven variant repeats named a-j based on the 14-15 bp consensus sequence were originally identified in Caucasian alleles (Bell *et al.* 1982; Owerbach and Aagaard 1984). The number and distribution of variant repeat sequences within alleles was later determined by minisatellite variant repeat mapping by PCR (MVR-PCR) (Stead and Jeffreys 2000). On the basis of allele structure (via visual inspection of MVR-code) and flanking haplotype, Stead *et al* were able to assign VNTR alleles to 5 main groups (Figure 1.8) (Stead *et al.* 2000; Stead and Jeffreys 2000). By analysing LD between the *INS* VNTR and the *HUMTH01* microsatellite upstream of the *TH* gene, located ~9 kb away, these VNTR groups were found to correspond to true ancestral lineages. Haplotypes of the five groups were dominated by a different allele of *HUMTH01*.

Class I alleles could be divided into two lineages *IC*+ and *ID*, by size, variant composition and repeat distribution (Stead and Jeffreys 2000). All Class I alleles within these lineages shared identical haplotypes between the -2733A/C and +1428*FokI* polymorphisms in the 4.1 kb minimal region of strongest association. However, the *ID* subgroup could be divided further into the *ID*+ and *ID*- lineages by the +3580*MspI* polymorphism located 3' to the *INS* gene (Figure 1.7); the *ID*- lineage can be distinguished from all other Class I alleles by the absence of this restriction site (see Figure 1.8). Otherwise, ID+ and ID- alleles cannot be distinguished specifically by variant repeat composition or distribution, as these do not differ significantly between the two ID subgroups.

Class III alleles of the VNTR can also be divided into the *IIIA* and *IIIB* lineages, defined by size and allele structure (Stead and Jeffreys 2000). These lineages were identified prior to MVR code analysis by flanking haplotype defined by the class of VNTR allele together with the allele present at the +1428*FokI* restriction site (Owerbach and Gabbay 1993; McGinnis and Spielman 1995) or the *TH* gene *HUMTH01* microsatellite (Bennett *et al.* 1995; Stead *et al.* 2000). Thus, the *IIIA* lineage is also known as the 'Protective Haplotype' (PH) and *IIIB* as the 'Very Protective Haplotype' (VPH). As their names suggest, these haplotypes were originally found to associate with differential degrees of protection against the development of T1D (Bennett *et al.* 1995).

Figure 1.8: Subgroups of VNTR Class I and Class III alleles and T1D risk



*The –23*HphI *site is in tight LD with the VNTR and therefore the VNTR classification (Class I/ Class III) can be inferred from genotyping the –23*HphI *site. VNTR alleles can be further subdivided into groups by flanking haplotype that are considered to be 'Susceptible' or 'Protective' in the context of T1D. Class I susceptibility alleles have also been further sub-divided into two lineages by allele structure, IC+ and ID. The absence of the +3580*MspI *site further divided the ID subgroup and distinguishes the ID- lineage from all other Class I alleles. Class III alleles can be divided into the IIIA (or "Protective Haplotype (PH")) and IIIB (or "Very Protective Haplotype (VPH)") lineages. The presence and absence of the +1428*FokI *site can also divide the Class III subgroup into the same lineages.*

VNTR subclasses and differential susceptibility to disease in T1D

Bennett *et al* showed that the high-risk VNTR genotype, Class I/I conferred a relative risk (RR) of 2.68 for development of T1D. The inheritance of one copy of a Class III allele could reduce the risk of developing disease by ~60% since both Class I/III and Class III/III genotypes have an RR of 0.37 (Bennett *et al.* 1996). Although on the whole, Class I alleles are predisposing and Class III alleles dominantly protective, differential degrees of susceptibility and protection to disease, respectively, have been found to occur within these classes of alleles.

Class I subclasses

Differential susceptibility of Class I allele subclasses was first reported by Bennett *et al* (Bennett *et al.* 1995). They reported that the overall transmission of Class I alleles to offspring with T1D was 64%, $p < 0.025$. However, the transmission of one particular allele, defined as *814* (42 repeats in length), did not significantly segregate with disease (%t = 58%, $p > 0.05$). This allele is the most common Class I allele, representing 25% of the total Class I alleles and its transmission was expected to mirror that of Class I alleles collectively. Since this was not the case, it was concluded that not all Class I alleles were equally predisposing for T1D.

In a later study using a larger data set (n = 891 families), the overall transmission of the *814* allele was not found to be significantly different from *non-814* Class I alleles, and thus all Class I alleles appeared to be equally associated with susceptibility to T1D (Bennett *et al.* 1997). However, when the transmissions from *814/III* mothers and fathers were analysed separately, it was evident that the *814* allele was only predisposing when inherited from *814/III* mothers and not from *814/III* fathers (mothers $\%t_{814}$ = 69.1% and fathers $\%t_{814}$ = 51.6%). This suggested that paternally inherited *814* alleles from Class III-positive fathers were protective. Fathers with a *non-814/III* genotype transmitted other Class I alleles significantly more often than Class III alleles, suggesting that this effect was *814*-allele specific. Furthermore, when the frequency of the *814/814* genotypes was compared with *non-814/non-814* Class I genotypes in sporadic T1D cases and controls, the former genotype was not associated with disease (Bennett *et al.* 1997). The protective effects of the *814* allele was replicated in another study (Urrutia *et al.* 1998), warranting further investigation of Class I subtypes.

More recently, the *814* allele was found to represent a subset of alleles that are 42 repeats in length and are contained within the *ID-* lineage (Stead *et al.* 2000). A reduced transmission of *ID-* alleles from *ID-/III* fathers to affected offspring was observed (%t =

41%) compared with *ID-/III* mothers (73%). These findings are in accordance with those reported by Bennett *et al* (Bennett *et al.* 1997) and Urrutia *et al* (Urrutia *et al.* 1998) regarding the anomalous behaviour of the *814* allele. Stead *et al* (Stead *et al.* 2000) also reported that the *ID-* lineage contains other alleles in addition to *814* and the protection associated within the subgroup was associated with all alleles within this lineage, irrespective of size.

Class III subclasses

Class III alleles of the VNTR were originally divided by both size and flanking haplotype into two ancestral lineages which appeared to differ in their degree of protection against T1D, with the so called 'Very Protective Haplotype' (VPH) associating with greater protection than the 'Protective Haplotype' (PH) (Bennett *et al.* 1995; Undlien *et al.* 1995). These protective haplotypes were identified using the class of VNTR allele together with the allele present at the +1428*FokI* restriction site (Owerbach and Gabbay 1993; McGinnis and Spielman 1995) or the *TH* gene *HUMTH01* microsatellite (Bennett *et al.* 1995; Stead *et al.* 2000). The Class III haplotypes have more recently been identified as highly diverged ancestral lineages mentioned above, IIIA (PH) and IIIB (VPH) (Stead *et al.* 2000) (Figure 1.8). The VPH was originally found to be associated with a 4-fold reduction in risk of developing T1D and carried a haplotype relative risk of 0.3 (compared with Class I haplotypes) in a study of 425 families (Bennett *et al.* 1995). However, in the largest study to date, Barratt and colleagues (Barratt *et al.* 2004) could not replicate the nominally significant difference ($p = 0.048$) in protection between the VPH and PH that was stated by Bennett *et al* 1995 (Bennett *et al.* 1995). It now appears that, in classical T1D at least, that both of these Class III haplotypes may confer a similar degree of protection.

Parent of origin effects on susceptibility to T1D

Susceptibility conferred by the *INS* VNTR appears to be subject to complex parent of origin effects and epigenetic phenomena. Intra-familial association studies have demonstrated an excess paternal Class I allele transmission to affected offspring in French (Julier *et al.* 1991), North American (Pugliese *et al.* 1994; Bennett *et al.* 1995) and Canadian population datasets (Polychronakos *et al.* 1995). A mechanism of maternal imprinting was suggested to explain this finding (Bennett *et al.* 1997). However, data from the UK, Sardinia and Denmark contradicted these studies, and Class I alleles were found to be transmitted preferentially from mothers (Bennett *et al.* 1996). This discrepancy suggested that maternal imprinting (although this has been shown to occur in the human yolk sac (Moore *et al.* 2001)) alone could not account for the parent of origin effects at *IDDM2*. Moreover, as described earlier, these parent of origin effects are complicated further since the susceptibility effect of the transmitted Class I allele may also depend on the subclass it belongs to and on the identity of the untransmitted allele (Bennett *et al.* 1997). These findings show that susceptibility at the *INS* gene region is very complicated and further investigation is required to resolve conflicting reports on parent of origin effects and fully understand the genetic associations found.

A functional role of the INS VNTR and mechanism of action in autoimmunity

In the 1980s, the VNTR locus was originally thought to be a marker for a linked causal variant that was responsible for T1D susceptibility since this polymorphism itself does not encode a protein (Bell *et al.* 1984). However, the VNTRs proximity to the *INS* gene promoter suggested that it might play a role in regulation of *INS* gene expression. Indeed subsequent studies have shown that the VNTR can regulate transcriptional expression of the insulin gene.

The effect of allelic variation of the *INS VNTR* on insulin gene expression was initially examined by transfection of reporter constructs into rodent β-cell lines containing the *INS*

promoter and either a Class I or Class III sequence upstream of it. Most studies agree that Class III VNTR alleles are associated with 1.5 to 3-fold reduction in insulin expression (Lucassen *et al.* 1995; Bennett and Todd 1996; Owerbach and Gabbay 1996). Studies involving measurement of steady-state mRNA from each class of allele in pancreatic tissue samples derived from I/III individuals agree with these findings. In normal human foetal and post-natal tissue, a 10-20% reduction in basal *INS* expression was reported to occur with the Class III transcript (Vafiadis *et al.* 1996) and a ~30% reduction in expression was observed in the adult pancreas (Bennett *et al.* 1995; Bennett *et al.* 1996). Moreover, single-nucleotide differences in VNTR alleles affect their binding by the Pur1 transcription factor, which controls basal levels of *INS* expression (Lew *et al.* 2000), suggesting that VNTR variants may differ in their ability to stimulate expression of the gene. It is difficult, however to explain how such a modest decrease of basal pancreatic insulin secretion by Class III alleles could explain the dominant protective effect of these alleles. However, the differential level of *INS* transcription determined by the associated VNTR allele observed in the foetal pancreas gives rise to the possibility that aberrant expression during development could contribute to occurrence of diabetes later on in life.

Attention was focused on the effect of the VNTR on *INS* expression in the thymus since differences in the pancreatic expression was unlikely to explain disease susceptibility. The pattern of Class I- and Class III-linked *INS* gene transcription in this organ was found to be the opposite of what occurs in the pancreas. Several studies have shown a 2 to 3-fold reduction in thymic medullary expression of Class I-linked *INS* gene transcripts compared with Class III-linked ones (Pugliese *et al.* 1997; Vafiadis *et al.* 1997). This has implications for immune self-tolerance for insulin since negative selection of autoreactive T cells is dose-dependent with higher concentrations of peptide promoting clonal deletion (Sebzda *et al.* 1994, reviewed by Pugliese and Miceli 2002). Class I-associated reduction in thymic *INS* transcription during development could therefore result in inefficient purging of autoreactive proinsulin-specific lymphocytes and/or impaired selection of regulatory cells, leading to a

breakdown in central tolerance mechanisms. This would allow the release of autoreactive lymphocytes into the peripheral circulation and pancreatic lymph nodes where they can encounter their target antigen and activate autoimmunity. Evidence for this hypothesis is supported by studies in NOD mice where forced *in vivo* expression of the insulin gene in the thymus prevented diabetes development (French *et al.* 1997). When thymic insulin gene expression is reduced by manipulating gene copy number, peripheral T-cell reactivity to insulin is observed compared to none in mice with normal levels of thymic insulin expression (Chentoufi and Polychronakos 2002). Moreover, complete knockout of *proinsulin2* results in accelerated insulitis and diabetes development (Thebault-Baumont *et al.* 2003). Lastly, knock-out studies of the *aire* transcription factor which mediates thymic expression of several tissue-specific antigens including insulin, results in the Autoimmune Polyendocrine Syndrome -1 (Anderson *et al.* 2002).

It is worth noting that thymic expression studies have shown that not all Class III alleles associated with higher transcription levels of *INS* compared with Class I alleles (Pugliese *et al.* 1997; Vafiadis *et al.* 1997). Some Class III alleles were identified in thymus samples that were called 'thymic-silencing' alleles since mRNA from the *INS* copy linked with these particular Class III alleles was absent. Interestingly, these alleles were found to be transmitted more often to diabetic offspring compared with other Class III alleles, indicating a predisposing effect of these silencing alleles (Vafiadis *et al.* 2001).

1.2.3 An environmental component

Since the concordance rate of T1D in monozygotic twins is not equal to 100%, it is clear that non-genetic factors are involved in the aetiopathogenesis. This notion is also supported by the fact that there has been a dramatic increase in T1D incidence over the past few decades (Onkamo *et al.* 1999; Eurodiab Ace Study 2000); this has been too rapid to be explained simply by evolution of a more diabetogenic genome (Gale 2002b). In fact, the proportion of T1D patients with high-risk HLA haplotypes in the UK appears to have decreased over time (Gillespie *et al.* 2004). In view of this evidence it is generally agreed that the environment has become more permissive for precipitation of autoimmune diabetes either by introduction of risk factors into the childhood environment or loss of protective agents (Gale 2002b). Environmental influences could be prenatal (intrauterine), or related to factors in early childhood such diet, infectious exposure and infant immunisation (Steck and Rewers 2004). Knowledge of these environmental factors and their involvement in the initiation and/or propagation of disease in T1D is limited and a number of conflicting reports have been published. Some popular risk factors are discussed below.

Diet

The consumption of cow's milk soon after birth has been implicated with susceptibility to T1D (Virtanen *et al.* 1993), although this contention is currently controversial and awaits confirmation through large-scale clinical trials (reviewed by (Devendra *et al.* 2004b; Narendran *et al.* 2005)). Also, the timing of introduction of cereals into infant diets has been found to increase the risk for development of T1D-associated autoimmunity (Norris *et al.* 2003; Ziegler *et al.* 2003). Exactly how exposure to milk proteins and gluten may cause/ precipitate islet autoimmunity is unclear. A mechanism involving an aberrant immune response to these dietary constituents in an immature gut immune system in susceptible individuals has been suggested (Ziegler *et al.* 2003; Devendra *et al.* 2004b). Other dietary factors associated with T1D based on circumstantial evidence include nitrates, nitrites or

nitrosamines, artificial sweeteners, caffeine and smoked fish (Devendra *et al.* 2004b; Atkinson 2005).

Viruses

Seasonal variation in the incidence of T1D suggests that a viral trigger for the disease may exist (Steck and Rewers 2004). Congenital rubella, enterovirus (namely Coxsackie B), mumps, rotavirus and chickenpox are among the candidates that have been found to associate with diabetes autoantibodies (reviewed by (Devendra *et al.* 2004b; Narendran *et al.* 2005)). However, only congenital rubella has been convincingly associated with disease; ~40% of children with congenital rubella who are born to mothers infected early in their pregnancy will develop antibodies and around half of these will develop T1D (Menser *et al.* 1978). It is important to note though that since congenital rubella is now rare, this virus cannot be considered a significant contributor to T1D. Although a possible relationship of Coxsackie B virus infection and T1D has been investigated for over 30 years, epidemiological evidence for the involvement of this virus remains highly inconclusive (Green *et al.* 2004). The pathogenic mechanisms by which such viruses may operate to cause autoimmunity are unclear. Among the proposed hypotheses, two current contenders are molecular mimcry[2] and bystander activation (Figure 1.1) (Jaeckel *et al.* 2002). An example of the first hypothesis is the sequence homology between GAD 65 and the Coxsackie B4 viral protein, P2C, which may result in an autoimmune response against GAD 65 following infection, resulting in T1D (Kaufman *et al.* 1992). In support of the bystander activation response, inflammatory responses associated with viral infection, involving α interferon in particular, have been linked to initiation of insulitis in animal models (Moriyama *et al.* 2002), providing a potential mechanism by which autoimmunity may occur.

[2] This is a mechanism whereby self-antigen-specific cells have the ability to cross-react with viral antigens and consequently these cells become activated directly by infected cells presenting viral determinants.

Hygiene hypothesis:

It has been suggested that the increase in incidence of T1D and other autoimmune diseases (T_H1-diseases) as well as atopic diseases (T_H2-diseases) are the result of our 'over-clean' environment. This hypothesis argues that exposure to a range of infective agents in early childhood is necessary for successful maturation of the neonatal immune repertoire. The lack of sufficient immune stimulation early in life may result in a breakdown of immune regulatory events, such that in genetically susceptible individuals, abnormal immune responses may result (Gale 2002a). The indigenous biota of the gut and intestinal parasites have been proposed as key candidates for the hygiene hypothesis (Gale 2002a). However the available epidemiological evidence for this hypothesis in autoimmune diabetes is scanty.

1.3 LATENT AUTOIMMUNE DIABETES IN ADULTS (LADA)

Having discussed our current understanding of the pathophysiology of classical T1D, the last section in this introductory chapter concerns LADA, the focus of this thesis.

1.3.1 Initial discovery of LADA

It became apparent in the late 1970s that some patients diagnosed with T2D actually had a slowly-progressing form of autoimmune diabetes. Irvine *et al* (Irvine *et al.* 1977a) showed that these patients possessed circulating autoantibodies which could bind to pancreatic islets in a similar fashion to that reported previously for sera from patients with classical T1D in the 'islet cell antibody' or ICA test (Bottazzo *et al.* 1974). Also indicative of the existence of an autoimmune aetiology in these 'ICA-positive T2D' patients, there was an increased prevalence of the high-risk HLA markers DR3 and DR4 and a reduced frequency of DR2 in small series of such patients ($n < 60$) (Gleichmann *et al.* 1984; Groop *et al.* 1988). It was recognised that ICA-positive T2D patients had a lower β-cell function, which generally deteriorated faster than that seen in classical T2D patients over a two-year follow-up period

(Groop *et al.* 1986). These patients were found to fail more rapidly on oral hypoglycaemic agent therapy than classical T2D patients (Irvine *et al.* 1977b; Irvine *et al.* 1979; Di Mario *et al.* 1983) and consequently had an increased likelihood for early insulin requirement in the course of the disease (Landin-Olsson *et al.* 1990; Hagopian *et al.* 1993a). However, when compared with classical T1D patients, these autoimmune diabetic patients were found to be able to maintain good blood glucose control on diet or hypoglycaemic agent therapy for several months or even years before insulin therapy became essential (Kobayashi *et al.* 1996). Hence the term 'latent autoimmunity' was adopted when describing these patients (Tuomi *et al.* 1993). Groop *et al* showed that the greatest impairment of β-cell function was seen in T2D patients with both ICA positivity and HLA DR3/DR4, suggesting that both genetic and immunological factors were important for the process of β-cell autoimmunity (Groop *et al.* 1988). Positivity for GADA was later shown to be a more superior measure than ICA in identifying T2D patients with future insulin deficiency (Tuomi *et al.* 1993; Niskanen *et al.* 1995).

Early studies also demonstrated that these patients were younger at diagnosis and leaner compared with T2D patients (Zimmet *et al.* 1994). Zimmet *et al* coined the term 'Latent Autoimmune Diabetes in Adults', and its acronym, LADA, describing its clinical features as follows; age of presentation >30 yrs, no ketonuria and no exogenous insulin requirement within 6 months of diagnosis (Zimmet *et al.* 1994). As a result, LADA patients were later described by the WHO as having a slowly-progressing form of T1D (Alberti and Zimmet 1998). Since the clinical presentation of these autoimmune patients occurs at adult age without the usual pathognomonic features of classical T1D e.g. ketosis, weight loss and requirement of insulin treatment for survival, they are often misdiagnosed as classical T2D patients in clinic. There are currently no guidelines set by the ADA or WHO to clinically define LADA. This is partly due to the very heterogeneous nature of the disease characteristics of this group of patients. Such heterogeneity was best demonstrated by the

two largest population-based studies of LADA to date; antibody-positive patients initially diagnosed with T2D in the UK Prospective Diabetes Study (UKPDS) (Turner *et al.* 1997) and in the Botnia T2D Study in Western Finland (Tuomi *et al.* 1999). These studies have been the most representative and informative assessment of LADA: earlier reports were based on small series of patients (typically n < 60) collected from hospital-based cohorts where selection of more serious cases was likely to have occurred. The clinical, immunological and genetic features of this group of patients with autoimmune diabetes will be described in more detail based mainly on findings from these two studies.

1.3.2 Clinical features of LADA

As shown above LADA patients share clinical features with both classical forms of T1D and T2D. For this reason, LADA has also been described has 'Type 1.5 diabetes' (Juneja and Palmer 1999). Comparisons of clinical features of LADA with T1D and T2D are made.

Comparisons with T1D:

In the largest comparison of LADA patients (n = 104, diagnosed 28-68 years) with classical T1D patients (n = 194, diagnosed <35 years of age), C-peptide levels were found to be higher in LADA (Tuomi *et al.* 1999). This report corroborated the findings of an earlier study of Swedish patients where 11 LADA subjects were found to have a retained β-cell function at diagnosis compared with 17 patients diagnosed with T1D (Gottsater *et al.* 1993). The latter study and others have shown that a faster loss of C-peptide over the post-diagnosis period occurs in T1D patients compared with LADA patients (Gottsater *et al.* 1993; Torn *et al.* 2000; Hosszufalusi *et al.* 2003), in accord with the more slowly-progressive nature of disease progression in LADA compared with T1D.

Upon comparison of BMI, LADA patients are less lean compared with T1D patients (Gottsater *et al.* 1993). In the study of Botnia Finns, patients with the highest GADA titres had BMIs comparable to that observed in T1D patients (Tuomi *et al.* 1999). Tuomi *et al* also

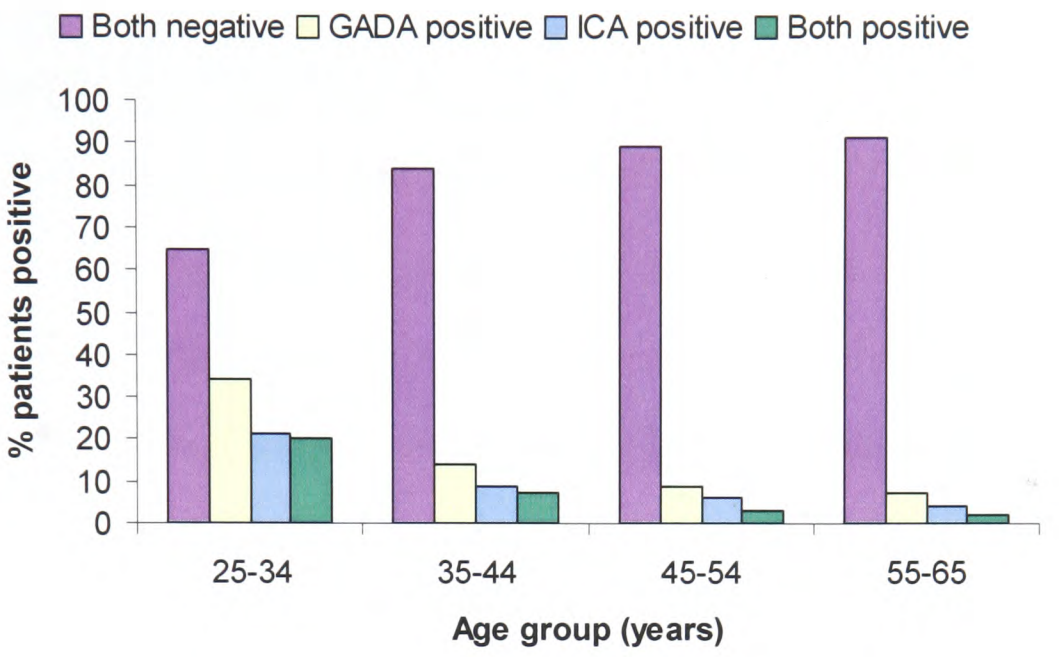
reported that in terms of lipid profile, patients with LADA had higher total cholesterol and triglycerides and lower HDL cholesterol compared with T1D patients (Tuomi *et al.* 1999).

However, more recent studies comparing the clinical profile of adult-onset T1D patients (defined as >35 at diagnosis presenting with ketonuria or ketoacidosis and requiring immediate insulin treatment) and LADA patients (defined as >35 years at diagnosis and not requiring insulin therapy for at least 6 months post-diagnosis) give conflicting results. A small Brazilian study showed that compared with patients with adult-onset classical T1D (n = 45), LADA (n = 54) patients had higher BMI, C-peptide levels, and also had higher degree of insulin resistance, arterial hypertension and dyslipidaemia (hypertriglyceridaemia and reduction in HDL cholesterol) (Rosario *et al.* 2005). However, in a study of Hungarian patients, no differences in BMI, waist-to-hip ratio, HDL cholesterol, total cholesterol, triglycerides, presence of arterial hypertension and β-cell function at diagnosis were observed between their adult-onset T1D group (n = 57) and LADA group (n = 54) (Hosszufalusi *et al.* 2003). Clearly, larger age-matched studies directly comparing clinical features of adult-onset T1D with LADA are required to resolve these inconsistent findings.

Comparisons with T2D:

Both the studies of Turner *et al* (Turner *et al.* 1997) and that of Tuomi *et al* (Tuomi *et al.* 1999) showed that, overall, patients with LADA were diagnosed at an earlier age than those with classical T2D. Both studies also demonstrated an age-related prevalence of LADA, most elegantly shown in the UKPDS study; the percentage of patients diagnosed with LADA on the basis of autoantibody positivity, decreased with decades of age (Figure 1.9). Those patients aged <45 years at diagnosis commonly possessed both ICA and GADA whereas those diagnosed >45 years were more likely to have either ICA or GADA alone (Turner *et al.* 1997). An age-related prevalence of IA-2A also occurred in the UKPDS, with patients diagnosed at younger ages being more likely to be positive for this autoantibody (Bottazzo *et al.* 2005).

Figure 1.9: Age-related autoantibody prevalence in the UKPDS (taken from (Turner et al. 1997))



In the UKPDS, at all ages patients with antibodies (ICA/ GADA/ IA-2A) at diagnosis tended to have a clinical phenotype more similar to T1D in that they were generally non-obese, had high HbA_1c percentage and decreased β-cell function (Turner *et al.* 1997; Bottazzo *et al.* 2005)[3]. This was particularly pronounced in those aged <45 years at diagnosis compared with those >45 years. These findings were agreement with the Botnia study of LADA patients (Tuomi *et al.* 1999).

One of the most important reasons to detect LADA is to identify patients among the T2D-diagnosed population that will require early insulin treatment (Niskanen *et al.* 1995; Littorin *et al.* 1999; Torn *et al.* 2000). Compared with T2D patients, those with autoantibodies were more likely to fail on diet/ oral agent therapy and had an increased likelihood of future insulin requirement in both the UK and Finnish studies (Turner *et al.* 1997; Tuomi *et al.* 1999). In the UKPDS, survival analysis showed that this likelihood was increased in patients when both ICA and GADA were present compared with those with either autoantibody alone and autoantibody positivity *per se,* was the most significant predictor of insulin

[3] With the exception of an age-related association of HbA_1c levels in those positive for IA-2A.

requirement by 6 years post-diagnosis (Turner *et al.* 1997). Knowledge of autoantibody status was particularly important in subjects diagnosed over the age of 45 years, where clinical features alone were not good predictors of insulin requirement since in this age group the BMI and HbA_1c levels of LADA and T2D patients were comparable. A later UKPDS study has shown that knowledge of IA-2A status does not improve prediction of early insulin use relative to predictability when ICA and GADA results are known (Bottazzo *et al.* 2005). In the Finnish study, those LADA patients who required insulin therapy within 1 year of diagnosis resembled the T1D patient group since they were younger, more likely to be IA-2A positive, had lower C-peptide and plasma triglyceride levels compared with T2D patients (Tuomi *et al.* 1999).

Although LADA patients display a more severe defect in maximally stimulated β-cell capacity, they can exhibit insulin resistance (Carlsson *et al.* 2000). In the UKPDS, compared with T2D patients, LADA patients had a lower prevalence of the metabolic syndrome at diagnosis (as assessed by NCEP ATP III criteria (Expert panel on detection 2001): as expected there was also a lower prevalence of the individual components of the syndrome; namely hypertension, obesity and degree of dyslipidaemia (Davis *et al.* 2005). Similar findings were found in the Botnia study (Isomaa *et al.* 1999; Tuomi *et al.* 1999). A follow-up study of Swedish patients has shown that the cardiovascular risk profile in LADA patients at 5 years post-diagnosis is more favourable than that of T2D patients; they had relatively lower BMI, waist-to-hip ratio, total cholesterol, triglycerides, LDL cholesterol and higher HDL cholesterol (Gottsater *et al.* 1996).

The diagnosis of LADA

As mentioned earlier, there are no consensus criteria for the diagnosis of LADA. It is generally agreed that antibodies (particularly GADA) are a better marker in classifying LADA compared to traditional characteristics such as BMI and age at diagnosis (Turner *et al.* 1997; Humphrey *et al.* 1998; Juneja *et al.* 2001). In this thesis, the definition of LADA has been taken as antibody-positive (ICA/ GADA/ IA-2A) patients diagnosed over the age of 25 with T2D on the basis of not requiring immediate insulin treatment at clinical onset (within 3 months of diagnosis). It is recognised that this definition is imperfect (Gale 2005) since this definition is based on largely clinical parameters rather than aetiology. However, this was the definition proposed by the late Professor Turner and has been used for UKPDS patients since.

1.3.3 Immunology of LADA

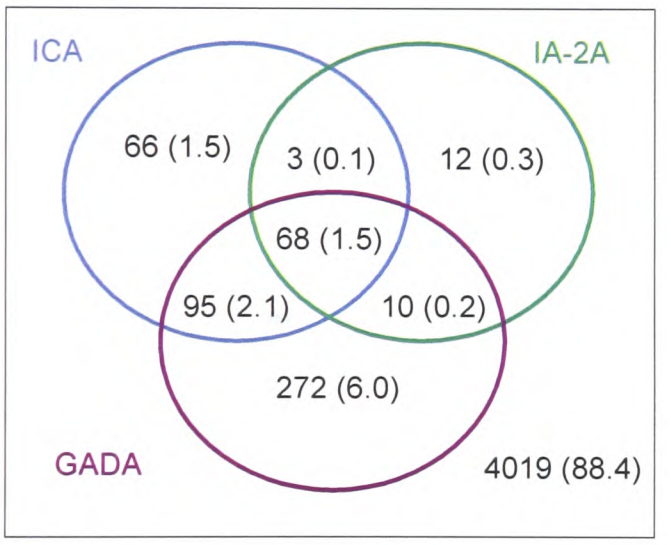
The immunological features of LADA have both similarities and differences compared to classical T1D.

Similarities with T1D

Histological analysis of a pancreatic biopsy from a Japanese patient diagnosed with T2D who had GADA and residual β-cell function, showed that T cell insulitis occurs in LADA (Shimada *et al.* 1999). An infiltrate of predominantly $CD4^+$ T cells was found in islets which remarkably, still contained insulin. The proportion of β-cells compared to non-β-cells in this patient's islets was not different to that observed in an age-matched control subject, indicating that β-cell mass had been preserved. Following this report, Fukui *et al* demonstrated that peripheral blood mononuclear cell (PBMC) responses to GAD 65 occurred in patients with LADA (Fukui *et al.* 1999). Taken together with the findings of Shimida *et al*, these key findings show that insulitis and a cellular immune response to GAD 65 occur not only in classical T1D, but in LADA also.

Autoantibodies commonly associated with classical T1D (GADA, ICA, IA-2A and IAA) have been found to occur in LADA and the relative merits of the measurement of these in identifying LADA cases in the T2D population have been investigated. In Caucasians, GADA are much more prevalent and less transient in LADA compared with ICA, IA-2A and IAA (discussed in Chapter 3). The relative prevalence's of ICA, GADA and IA-2A that occurred in the UKPDS are depicted in Figure 1.10. In this large cohort of patients diagnosed with T2D, at diagnosis, GADA was the most prevalent marker, followed by ICA, whereas IA-2A was relatively uncommon (Davis *et al.* 2005).

Figure 1.10: Prevalence of T1D-associated autoantibodies among T2D patients at diagnosis.



This data is based on 4,545 UKPDS patients diagnosed with T2D measured for autoantibodies (Davis et al. 2005).

The polarity (T_H1/ T_H2 bias) of the autoimmune response in LADA appears similar to that in T1D. IgG subclass analysis of autoantibodies directed against GAD 65 and IA-2, showed that both T1D and LADA patients display IgG_1 restricted antibodies consistent with a T_H1-type immune response (Hawa *et al.* 2000).

Differences with T1D

Brooks-Worrell *et al* compared the PBMC reactivity to islet proteins (by cellular immunoblotting) between T1D and LADA (Brooks-Worrell *et al.* 1999). The overall magnitude of the cellular response was found to be lower in LADA compared with T1D.

Although an overlap in reactivity to certain proteins was observed (defined by molecular weight only), some reactivities clearly differed in magnitude between LADA and T1D. The authors suggested that these differences could reflect those of the pathogenic processes that lead to the different courses of disease in these forms of autoimmune diabetes. Determining whether LADA can be characterised by T cells that can react to specific antigens or epitopes in a distinct manner to T1D awaits improvements in current T cell assay technology.

Seissler *et al* showed that differences in ICA sub-specificities (identified by blocking experiments) exist between T1D and LADA (Seissler *et al.* 1998). They found that pre-incubation of sera with a mixture of GAD 65 and IA-2 completely blocked ICA staining in 60% of T1D patients. However, when analysing sera of LADA patients, only 37.5% inhibited the total ICA reactivity following pre-incubation with these autoantigens. This suggested that in LADA, the majority of ICA could be directed to an uncharacterised target antigen.

Given the potential variation in the humoral response described above, it is not surprising that differences have been noted in the prevalence of autoantibodies between T1D and LADA. A higher frequency of single autoantibody positivity vs. ≥2 autoantibodies is seen in LADA compared with T1D patients (Seissler *et al.* 1998; Schranz *et al.* 2000; Hosszufalusi *et al.* 2003). In view of the fact that multiple autoantibody positivity is more common in T1D, some have hypothesised that these patients lose tolerance to a greater number of islet antigens than do LADA patients, resulting in a more aggressive autoimmune attack (Palmer and Hirsch 2003).

Comparisons of autoantibody titres between T1D and LADA have led to inconsistent findings. No significant difference in GADA titres between adult-onset T1D (n = 45) and LADA (n = 54) was reported by Rosario *et al* (Rosario *et al.* 2005). However, in another

study, titres of GADA and IA-2A were found to be significantly lower at diagnosis in LADA (n = 54) compared with T1D (n = 388) (Schranz *et al.* 2000).

Lastly, a difference in the epitope recognition of GADA has been noted between T1D and LADA. Two studies have shown that reactivity towards the N-terminal portion of GAD 65, which is rare in T1D, is more common in LADA (Hampe *et al.* 2002b; Kobayashi *et al.* 2003). However, these studies have been performed on small numbers of LADA patients (n < 35) and therefore the results need to be substantiated by further investigation.

Use of autoantibody markers to identify more homogenous subgroups of LADA

Lohmann *et al* suggested that LADA could be divided into two clinically distinct types on the basis of autoantibody positivity and titre (Lohmann *et al.* 2001). Those LADA patients with multiple autoantibody positivity (GADA/ ICA/ IA-2A) or high-titre GADA were defined as 'LADA-type 1' since they had clinical features that resembled T1D patients; that is, a reduced β-cell function, BMI and prevalence of hypertension. Those LADA subjects with single autoantibody positivity and low-titre GADA were described as 'LADA - Type 2' as they were phenotypically more similar to T2D patients; that is they had relatively higher β-cell function, BMI and frequency of hypertension. It is important to note that these findings were based on only 51 LADA patients. Although the study supports the notion that autoantibody measurement has clinical utility, larger studies are required before the relevance of these definitions is apparent.

1.3.4 Genetic markers of LADA

The genetic markers of T1D have been investigated in LADA to investigate whether the two forms of autoimmune diabetes share similar genetic aetiologies. In particular, frequencies of variants at the two major T1D susceptibility genes for T1D (*HLA* and *INS* genes) have been determined in LADA and compared with those occurring in T1D and T2D.

Comparisons with T1D

In a genetic analysis of the HLA genes in LADA (n = 95), Tuomi *et al* showed that compared with T1D patients (n = 82), LADA patients show a significant decrease in the frequency of *DQB1*0201/*0302* (37% vs. 13%) (Tuomi *et al.* 1999). However, in a study of Hungarian adult-onset T1D patients and LADA patients, no significant differences in the frequencies of this high-risk heterozygote was found, suggesting that the level of risk conferred by this determinant in these two forms of autoimmune diabetes may be the same (Hosszufalusi *et al.* 2003). However, it is important to note that the sample size of this study was small; only 50 LADA patients were compared with 50 adult-onset T1D patients.

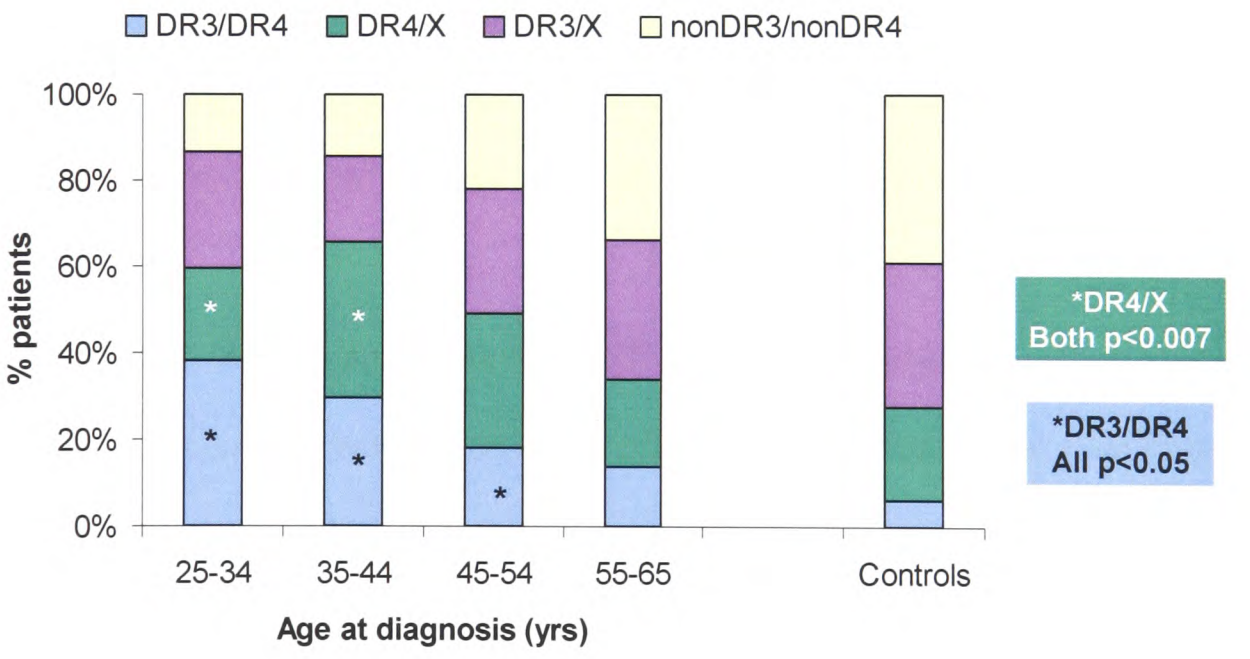
Tuomi and co-workers also reported that the frequency of genotypes comprising the *DQB1*0602* allele was significantly decreased in T1D (9%) relative to controls (30%) but not in LADA (21%) (Tuomi *et al.* 1999). However, in two more recent studies of Swedish LADA patients, a significant decrease of genotypes containing this variant was found to occur in LADA, although it is important to note that only ~50 patients were genotyped in each report (Stenstrom *et al.* 2002; Torn *et al.* 2003). Further studies with a greater numbers of patients are required to clarify these inconsistent findings between T1D and LADA.

The second most important contributor to genetic risk in T1D is the insulin gene region (refer to Section 1.2.2). The only association study performed on this locus when work was started on this thesis was by Tuomi *et al*. These authors reported no significant associations of VNTR class (Class I/ Class III) by genotyping the –23*HphI* site with LADA (Tuomi *et al.* 1999). Subsequently, Cerrone *et al* reported a similar increase in the frequency of Class I alleles in 44 LADA patients (80.6%) as in 88 T1D patients (81.3%) compared to 138 control subjects (69.9%) (Cerrone *et al.* 2004). This *INS* gene region also requires more comprehensive investigation in a larger cohort of patients.

Comparisons with T2D

The largest association study of the HLA gene region in LADA to date (n = 255) was performed on the UKPDS cohort by Horton *et al* (Horton *et al.* 1999). The authors looked at the age-related prevalence's of HLA markers at *DRB1* and *DQB1* in LADA, given the heterogeneity of clinical features of these patients described in this cohort earlier (Turner *et al.* 1997). At *DRB1*, the prevalence of the DR3/DR4 heterozygote was found to decrease as the age at diagnosis increased, whilst the frequency of nonDR3/nonDR4 rose with age at diagnosis (Figure 1.11). In addition, in patients aged <45 years at diagnosis, the prevalence of DR4/X genotypes (where X ≠ DR3) were increased compared with controls.

Figure 1.11: Prevalence of HLA DRB1 *genotypes by age of onset in the LADA group* (*taken from Horton et al. 1999).*



Also in this study, susceptibility at *DQB1* was assessed by the presence or absence of asp and non-asp alleles; patients were divided into asp/asp, asp/nonasp and nonasp/nonasp genotypes. The overall distribution of these genotype frequencies differed between LADA and the control population due to an increased prevalence of the nonasp alleles in LADA. However, no age-dependent effects were noted for these *DQB1* variants. However, it is now recognised that classifying *DQB1* susceptibility using the asp/ nonasp division may no longer be the best way of analysing data (see Section 1.2.2.1).

An association of high-risk genotypes at *DRB1* and *DQB1* with insulin requirement by 6 years post-diagnosis was observed, although multivariate analysis showed that antibody positivity was a greater predictor of insulin requirement by this time-point in the UKPDS than HLA status (Horton *et al.* 1999).

All these studies have demonstrated that HLA *DRB1* and *DQB1* are risk markers for LADA that clearly warrant further investigation. The results have confirmed findings obtained in smaller studies in the 1980s, which suggested that a contribution from T1D susceptibility genes existed in the autoimmune pathology of LADA. However, no studies to date have examined nonDR3 and nonDR4 variants in a LADA population and explored the alleles that comprise the spectrum of susceptibility observed in T1D (Figure 1.5).

Regarding the insulin gene, the frequency of the Class I/Class I genotype was reported to be higher in LADA compared with T2D patients in two small studies (Abe *et al.* 2001; Matejkova-Behanova *et al.* 2004) but the same in another (Tuomi *et al.* 1999).

Aside from the genetic associations found in LADA discussed above, further evidence for a genetic component in LADA was demonstrated by Vauhkonen *et al* (Vauhkonen *et al.* 2000). These authors showed that the early phase insulin secretion of non-diabetic offspring of LADA patients was impaired, although insulin sensitivity was normal (Vauhkonen *et al.* 2000). These subjects also had an increased prevalence of HLA *DQB1*-genotypes conferring a low, but still an increased risk for T1D (Vauhkonen *et al.* 2000). A recent study by this group has shown that the offspring of GADA-positive LADA patients display subtle defects in the processing of insulin precursors (Vauhkonen *et al.* 2005). The authors conceded that the defects in insulin secretory capacity seen in LADA are likely to be familial and genetically determined.

1.4 AIMS OF THESIS

The overall aim of this thesis was to better characterise the immunological and genetic aspects of LADA and relate findings to clinical features. Comparisons with data reported for T1D were made where possible to assess whether LADA represents an age-related extension of T1D or a distinct entity of autoimmune diabetes.

Immunological studies

The GADA response after clinical onset of LADA and its utility as a marker of the continuing underlying β-cell destruction process was investigated. GADA persistence and changes in titre in the post-diagnosis period has not been examined in a large cohort of LADA patients. If this autoantibody reflects changes in β-cell function in the slowly-progressing islet deterioration occurring in LADA, GADA could be used to predict the disease course after clinical onset. In Chapter 3, GADA titres at 0.5, 3 and 6 years post-diagnosis were determined in the UKPDS LADA cohort.

The specific aims were:

- To determine whether GADA titres in LADA subjects from the UKPDS persist in the post-diagnosis period and whether changes in titre occur.
- To examine whether GADA titre relates to clinical features at diagnosis and in the post-diagnosis period.
- To investigate whether the intensity of the humoral response as determined by GADA titre and number of autoantibodies present (GADA + IA-2A vs. GADA alone), acts as a marker for progressive loss of β-cell function (indicated by deterioration of glycaemic control) in the post-diagnosis period.

Genetic studies

Association studies of the major T1D susceptibility loci, the HLA (*IDDM1*) and insulin (*IDDM2*) genes were performed in the largest cohort of LADA individuals to date (n = 453). It was hypothesised that a reduced susceptibility or alternatively an increased protection from T1D-predisposing genes could explain in part the later age of onset and less aggressive disease course of LADA compared with classical T1D. A spectrum of susceptibility variants at the *DRB1* and *DQB1* genes has been identified in classical T1D (Figure 1.5). Likewise, extensive association studies in T1D have identified key variants in the insulin gene region that confer susceptibility (Figure 1.8). Association studies in LADA to date have been relatively small and limited to the main susceptibility/ protective determinants at these loci and fine-structure analyses have not been performed. The aims of the genetic studies in Chapters 4 and 5 were:

- To confirm that the HLA and insulin gene regions confirm susceptibility to LADA.
- To determine if the pattern of susceptibility at these two candidate loci in LADA is similar/ different from that reported in T1D.
- To test whether genetic susceptibility at these loci is associated with age of diagnosis and severity of the clinical course of disease in LADA.
- To determine whether genetic variants at these loci associate with immunological risk markers identified in Chapter 3.

The inter-relationships of the immunological and genetic findings obtained were explored in the final chapter of this thesis and potential underlying mechanisms resulting in the different courses of autoimmune disease in children and adults are discussed.

Chapter 2:

Subjects and Methods

CHAPTER 2: SUBJECTS AND METHODS

2.1 SUBJECTS

2.1.1 Antibody studies

In Chapter 3, the clinical relevance of GAD 65 autoantibodies (GADA) in the post-diagnosis period in LADA will be assessed in patients recruited to the UK Prospective Diabetes Study (UKPDS).

The UKPDS was a randomised controlled trial designed to evaluate the efficacy of different therapies for T2D for the control of hyperglycaemia and prevention of complications. 5102 newly-diagnosed patients were recruited by general practitioner referral from 23 clinical centres based in England, Northern Ireland and Scotland. The patients recruited were aged 25–65 years at onset, and had fasting plasma glucose (FPG) values of above 6.0 mmol/l (confirmed on two independent occasions following diagnosis). Exclusion criteria for the study were: severe vascular disease (myocardial infarction in the past year, current angina or heart failure), accelerated hypertension, proliferative or pre-proliferative retinopathy, renal failure with plasma creatinine greater than 175 μmol/l, other life-threatening disease such as cancer, an illness requiring systemic steroids, an occupation precluding insulin treatment, unfamiliarity with English, and ketonuria greater than 3 mmol/l.

The study was performed in accordance with the Helsinki guidelines. All patients gave informed consent and the protocol received Research Ethics Committee approval. The UKPDS design and protocol has been previously reported (UKPDS Group, 1991). Following a 3-month dietary run-in period, patients were randomised to various therapies according to their level of hyperglycaemia at this point. Patients with FPG values between 6.0 and 14.9 mmol/l and no evidence of hyperglycaemic symptoms were randomised to diet, insulin, sulphonylurea or metformin (if overweight) therapy. Patients with FPG values of ≥15.0 mmol/l after the run-in period, or with hyperglycaemic symptoms were randomised to oral agents or insulin. Finally, patients with FPG

values below 6.0 mmol/l remained on diet alone but were randomised as described above, if their FPG values subsequently rose to 6.0 mmol/l or higher. In the post run-in period, those patients who were not initially randomised to insulin were only given this therapy if their FPG reached ≥15.0 mmol/l or if hyperglycaemic symptoms persisted after therapy with maximum tolerated doses of sulphonylurea and metformin. Further information about the UKPDS can be found at www.dtu.ox.ac.uk

Baseline clinical characteristics of the UKPDS LADA patients studied are given in Table 2.1.

Table 2.1: Clinical characteristics of UKPDS LADA patients (DASP 2003 cohort) included in the GADA study in Chapter 3.

Clinical characteristic at diagnosis	UKPDS LADA (DASP 2003)
N	242
Age (years) [a]	47.0 (10.8)
Sex (% male)	53
FPG (mmol/L) [b]	11.4 (7.9 to 15.5)
HbA_1c (%)[b]	9.0 (7.0 to 10.7)
Weight (kg) [a]	68.1 (13.2)
BMI (kg/m^2) [a]	23.8 (4.4)
Systolic BP (mm Hg) [a]	125 (17)
Diastolic BP (mm Hg [a]	79 (11)
HOMA %B [b]	30 (15 to 58)
HOMA %S [b]	73 (48 to 101)
Plasma triglycerides (mmol/L) [c]	1.19 [0.73, 1.95]
Metabolic syndrome (NCEP ATP III definition)[d], n(%)	106 (44%)

Values are given as: [a] mean (SD), [b] median (IQR) and [c] geometric mean (1 SD interval). [d] NCEP ATPIII definition with BMI >27 kg/m^2 male or >29 kg/m^2 female

2.1.2 Genetic studies

To perform the largest association study of the HLA and insulin gene regions in LADA, DNA samples were collected from GADA/ IA-2A positive probands from the UKPDS, Warren (W2) consortium, Young-onset T2D (YT2D) and Diabetes in Families (DIF) collections. The ascertainment details for each of these studies are given below. In this thesis, patients from these collections were considered to have LADA if they were positive for at least one of the antibodies and were initially diagnosed with T2D. A summary of the clinical characteristics of patients studied in this thesis for genetic analyses are given in Table 2.2.

Ascertainment details

UK Prospective Diabetes Study (UKPDS). Details of the ascertainment criteria of these subjects are detailed above. Only patients who were of White Caucasian ethnicity were studied in the present analysis. Patients with T1D were excluded from the UKPDS on the basis of insulin requirement within 3 months of diagnosis and ketonuria >3 mmol/l at presentation. Patients were included in the genetic analyses if considered to be GADA-positive according to measurements made in this laboratory according to DASP 2003 criteria.

Warren 2 consortium (W2). The aim of this study was to collect DNA from affected sib-pairs diagnosed with T2D in order to perform a genome-wide scan to identify the major loci responsible for T2D. The age at diagnosis of the probands ranged between 25-70 years. Patients were of UK/Irish origin as were all 4 grandparents of each sib-ship collected. Other forms of diabetes, Maturity-onset Diabetes in the Young (MODY), mitochondrial diabetes and T1D were excluded by standard clinical criteria and based on personal and family history, including an absence of first-degree relatives with T1D and an interval of ≥1 year between diagnosis and institution of regular insulin therapy (Wiltshire *et al.* 2001). GADA measurements (made in the laboratory of Professor Franco Bottazzo, Department of Immunology, London Hospital Medical College) of plasma samples taken from the subjects recruited showed that

~5% of the cohort had LADA. Only one LADA patient from each sibship was studied for genetic analyses; selection was made according to DNA availability.

Young-onset Type 2 Diabetes Study (YT2D). The aim of this study was examine the specific aetiologies of patients presenting with apparent T2D (Owen *et al.* 2003). Patients were diagnosed between 18-45 years and were of UK Caucasian origin. T1D was excluded on the basis of requirement for insulin within 3 months of diagnosis. Subjects selected for LADA genetic studies were GADA and/or IA-2A positive (GADA and IA-2A measurements made in the laboratory of Polly Bingley, Bristol).

Diabetes in Families Study (DIF). This sib-ship study was collected as a resource for analysis of quantitative trait loci in T2D. Age at diagnosis of the probands ranged between 35-75 years and they were collected from Oxford and Northamptonshire. White Caucasian subjects were selected for these analyses. Patients with T1D were excluded since anyone requiring insulin within 6 months of diagnosis was not recruited. GADA measurement of plasma samples taken in the post-diagnosis period revealed that 10% of the probands had LADA (Levy *et al.* 2003). (GADA measurements were made in the laboratory of Professor Polly Bingley, Bristol). Subjects selected for LADA genetic studies were either GADA-positive probands or siblings diagnosed with T2D. Only one LADA patient from each sibship was studied; selection was made according to DNA availability.

Control subjects. Genetic data of LADA subjects were compared with that of a non-diabetic population collected as part of the DIF study (Morris *et al.* 1998). These control subjects were considered non-diabetic on the basis of fasting plasma glucose measures <6.0 mmol/l and GADA-negativity (made in the laboratory of Professor Polly Bingley, Bristol). These subjects have been reported in other genetic studies (Gloyn *et al.* 2002; Groves *et al.* 2003).

Whole genome amplification of DNA samples

The quantity of DNA available from our collaborators for analysis of the HLA and insulin gene regions was limited. An inadequate supply of DNA (100 ng) was available in most instances to perform genotyping of the *DRB1* and *DQB1* genes (where typically 1 μg of genomic DNA was required) and typing of insulin gene polymorphisms. Therefore, it was necessary to investigate whole genome amplification (WGA) techniques to enlarge our LADA DNA resource.

On the basis of quality control testing of these techniques (see Appendix 1), WGA products were used for genotyping work in this thesis. For HLA typing, all DNA samples from the various LADA collections and control subjects in this study were amplified by the Genomiphi technique (Amersham Biosciences, Buckinghamshire, UK) except for 47 subjects from the W2 collection who were amplified using the deaza RepliG technique (Qiagen Inc, CA, USA). The Genomiphi method was mostly used because WGA and clean-up could be performed in-house and because of the considerably lower cost of amplification using these kits compared with the deaza RepliG system. For typing of polymorphisms in the insulin gene region, a sufficient quantity of neat unamplified DNA was available for all samples except for the same 47 subjects from the W2 collection (discussed above), that were whole genome amplified by the RepliG technique.

Table 2.2: Clinical characteristics of subjects studied in genetic analyses.

Study (n)	Ascertainment Criteria	Autoantibody positivity*	Age at onset (range) (years)	Age at onset (mean ±SD) (years)	Duration of diabetes (mean ±SD) (years)[§]	BMI (mean±SD) (kgm^{-2})	% male	% insulin requiring by 3 years post-diagnosis
UKPDS LADA (232)	Newly diagnosed T2D: not insulin requiring within 3 months of diagnosis Ketonuria <3 mmol/l	GADA and/or IA-2A	25-65	46.7 ± 10.6	Studied at diagnosis	25.2 ± 4.9 [#]	53	51
W2 LADA (131)	Clinical diagnosis of T2D: not insulin requiring within 12 months of diagnosis	GADA	26-68	47.4 ± 10.0	8.9 ± 6.7 [§]	28.6 ± 5.3 [§]	57	41
YT2D LADA (37)	Clinical diagnosis of T2D: not insulin requiring within 3 months of diagnosis.	GADA and/or IA-2A	29-45	38.4 ± 5.4	11.9 ± 7.7 [§]	27.5 ± 5.8 [§]	43	57
DIF LADA (55)	Clinical diagnosis of T2D: not requiring insulin within 6 months of diagnosis.	GADA and/or IA-2A	35-70	58.1 ± 10.8	10.6 ± 14.2 [§]	27.0 ± 0.9 [§]	53	20.3
DIF Controls (332)	Spouses/ friends of probands collected in the DIF study. Normoglycaemic.	Negative for GADA and IA-2A	20-91 [§]	55.5 ± 19.8 [§]	N/A	25.4 ± 4.0 [§]	45	N/A

n = number of individuals.

UKPDS - UK Prospective Diabetes Study, W2 - Warren 2 consortium, YT2D - Young-Onset T2D study, DIF – Diabetes in Families Study.

[*]Autoantibodies measured at diagnosis in the UKPDS and at ascertainment for the W2 and YT2D cohorts. Antibody measurements were made in the accredited laboratory of Professor Polly Bingley, (University of Bristol) for the UKPDS and YT2D cohorts and in the laboratory of Professor Bottazzo for the W2 collection (Department of Immunology, London Hospital Medical College). [§] At time of sample collection. [#] At diagnosis.

2.2 GAD 65 AUTOANTIBODY MEASUREMENT

The two most common methods of detecting diabetes-associated autoantibodies in current practice are by radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). When this project was begun (2001), the radioimmunoassay (radiobinding) method provided the most specific and sensitive measurement of GAD autoantibodies. However, the accuracy of this method has recently been superseded by a commercially available ELISA kit (RSR Ltd, Cardiff, UK). In addition, newer methods of autoantibody detection are being developed such as the phage-display method of detecting autoantibodies to IA-2 epitopes (Dromey *et al.* 2004) and IAA idiotopes (Devendra *et al.* 2004a).

To measure GAD autoantibodies in our LADA cohort, a 'home-made' radiobinding assay was developed using *in vitro* translated recombinant human radiolabelled GAD 65 (by incorporation of ^{35}S-methionine) as bait. Briefly, plasma from patients was incubated with the biosynthetically labelled ^{35}S-GAD 65 at 4^0C overnight, and immune complexes were precipitated using Protein A Sepharose. Following a series of washing steps, the bound label was counted in a scintillation counter. Results were expressed in WHO units after transforming counts into units from a standard curve. This was derived from a standardisation of the in-house positive control plasma against internationally recognised workshop criteria (Diabetes Autoantibody Standardisation Program 2003, DASP 2003) (See Appendix 2).

2.2.1 Cloning of GAD DNA construct

The GAD 65 DNA construct used in this study was kindly provided by Professor Ezio Bonifacio (Milan, Italy). Full-length human islet GAD 65 cDNA (accession no. M81882) was cloned into a pGEM3 vector.

2.2.2 Amplification of GAD construct

A sufficient amount of plasmid DNA for completion of this project was produced by transformation and culture of bacteria. This method of amplification was used in preference to PCR technology since contamination with incomplete or mutated sequences is less likely to occur.

Transformation of construct and bacterial culture

Day 1: The GAD 65 DNA construct was transformed into JM109 (Promega, WI, USA) or DH5α (Gibco Life Technologies (Invitrogen, Paisley, UK) competent *E.coli* cells by heat-shocking at 42^{0}C for 45 seconds and was grown up on 3.1% of 2x 2YT microbial medium[4] (w/vol)/ 1% agar (w/vol) (Sigma, Poole, UK) media containing 75 μg/ml ampicillin (Sigma). The plate was sealed tightly with Saran wrap and incubated overnight at 37^{0}C. Day 2: A resultant ampicillin resistant colony was picked and seeded into 5 mls of 3.1% 2YT (w/vol) media containing 75 μg/ml ampicillin. This inoculation was incubated overnight at 37^{0}C with shaking (250 rpm). Day 3: The inoculation media from Day 2 was inoculated again into 200 ml of 3.1% 2YT (w/vol) media (75 μg/ml ampicillin). The flask was incubated overnight at 37^{0}C with shaking (200 rpm).

Plasmid DNA extraction and purification from bacterial culture

DNA was extracted from the culture obtained from Day 3 using a large-scale MIDIPREP kit (Qiagen Ltd, Sussex, UK). Briefly, the bacterial cells were spun down and supernatant (media) was removed. The pellets underwent alkaline lysis and the lysate was filtered through an anion-exchange resin. A series of medium-salt washes was performed to remove RNA, proteins, dyes and low-molecular weight impurities. Next, the DNA was eluted using a high-salt buffer, then concentrated and desalted by isopropanol precipitation. The DNA pellet was washed with 70% ethanol, air-dried and suspended in an appropriate amount of nuclease-free sterile water. Tris-EDTA was not used to rehydrate pellets since it chelates Mg^{2+} ions, which are important co-

[4] Contains 16 g/L Tryptone (pancreatic digest of casein), 10 g/L Yeast extract, 5 g/L NaCl.

factors for function of nucleic acid polymerases in the *in vitro* transcription/ translation technique.

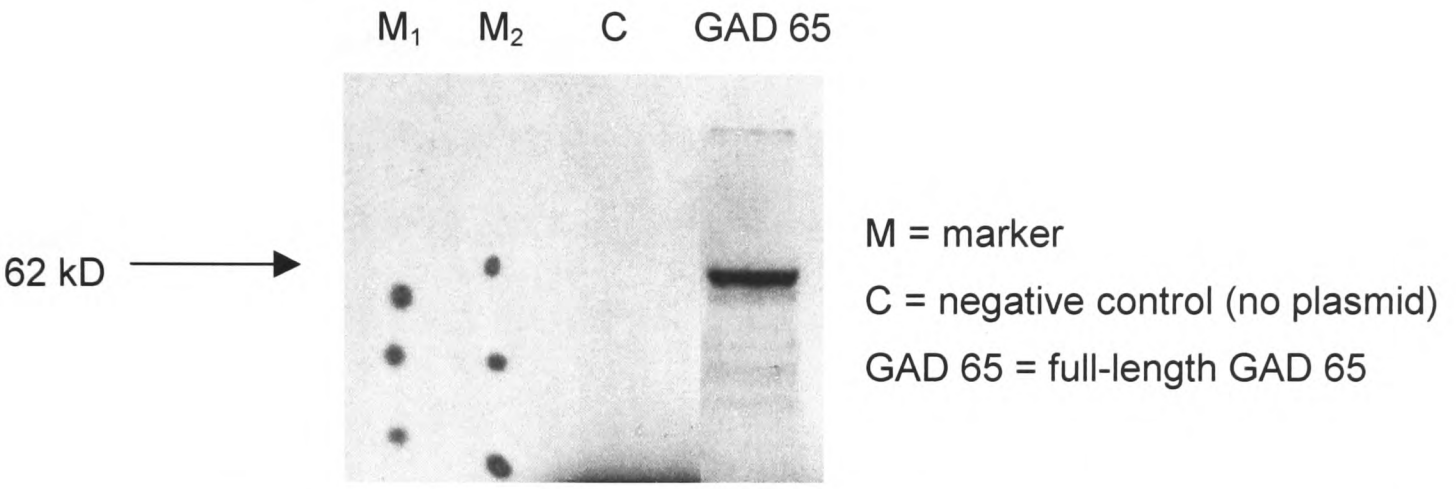
2.2.3 Expression of recombinant GAD 65 protein from DNA construct

^{35}S-labelled GAD 65 was produced by *in vitro* transcription/ translation of GAD cDNA using a rabbit reticulocyte lysate (Promega) with L-^{35}S-methionine (Amersham Biosciences, Amersham, UK) added in the reaction. The TnT Quick Coupled Transcription/ Translation System was used. This reticulocyte lysate master mix contains SP6 RNA polymerase, nucleotides, salts and 'Recombinant RNAisn Ribonuclease Inhibitor' (Promega). The protocol was performed under RNAse free conditions according to the kit manufacturer's instructions. A typical reaction contained 1 μg of GAD plasmid template, 2 μl ^{35}S-methionine (1000Ci/mmol, 10 mCi/ml), 40 μl of lysate master mix and the volume made up to 50 μl with nuclease-free sterile water. A control reaction was run with each experiment without plasmid (GAD negative) to determine background labelling of the lysate. Reactions were incubated in a 30^{0}C water bath for 90 mins.

Verification of cloning products

The GAD 65 plasmid received from Professor Bonifacio's laboratory was cloned and the product obtained from its expression was checked for molecular size as predicted from amino acid sequence. Expression was performed by *in vitro* transcription and translation of the DNA constructs using the TnT SP6-coupled rabbit reticulocyte lysate system (Promega) as described above with ^{35}S-methoinine in the reaction. The radioactive methionine was incorporated into the translated products allowing their identification by SDS PAGE followed by autoradiography (see Figure 2.1). The plasmid was also checked by sequencing in Professor Bonifacio's laboratory.

Figure 2.1 Autoradiograph of the GAD 65 translated product to check for expression and product size.



Translate was labelled with ^{35}S-methionine to enable detection by autoradiography. Samples were boiled in SDS sample buffer and were run out on an 8% SDS polyacrylamide denaturing gel stained and fixed with Coomassie brilliant blue. The gel was dried on a BioRad model 543 gel dryer and exposed to Kodak X-ray film (Sigma) for 3hrs at –20^{0}C. See Appendix 3 for gel constituent details.

The structural integrity of the recombinant product obtained from this construct had been validated previously in Professor Bonifacio's laboratory by testing its ability to be bound by human monoclonal antibodies MICA 4, -6 and -3 (Bonifacio *et al.* 2000).

Expression yield of the recombinant protein

To determine the expression yield of the recombinant ^{35}S-labelled GAD 65, 1 μl of GAD positive and GAD negative translate was spotted in duplicate onto Whatman 3MM filter paper strips and protein precipitation carried out in 10% TCA solution (Sigma) at 65^{0}C. The strips were then dried under a bench lamp for 1 hour, the spots immersed in 4 ml Optiphase HiSafe 4 scintillation fluid (Fisher Scientific UK, Loughborough, UK) contained within 4 ml Pico Provial plastic scintillation tubes (Packard (Perkin-Elmer), Netherlands). Radioactive content was counted for 1 min on a Beckman LS 5801 scintillation counter (Beckman Coulter Ltd, High Wycombe, UK). The background counts obtained from the GAD negative control spots were subtracted from the GAD positive counts to determine radiolabelled GAD content in counts per minute (cpm)/ μl. Typical net counts obtained for the GAD 65 construct was ~150 000 cpm/μl.

Filtering of the translate

Since GAD 65 is a membrane protein, aggregates are easily formed in the translate despite the inclusion of Triton-X100 in the buffer used for the radiobinding assay. Such aggregates stick on the filter plates during the assay, increase background counts and thus cause a reduction in the signal:noise ratio. The translate was therefore diluted in a buffer and filtered through a sterile 0.20 μm Durapore filtration column (Millipore, UK). The resulting filtrate was assumed to contain GAD 65 monomers.

Storage of translate

All translates were stored in aliquots at –20°C until use and were discarded after one week of production.

2.2.4 Autoantibody titre measurement

Sample storage

Archival plasma samples were used for antibody measurement. Whole blood had been collected in EDTA or Heparin tubes and red blood cells separated from plasma by centrifugation for 10 minutes at 3000 rpm. The plasma samples used were stored at –20°C, although they had been defrosted and re-frozen on a number of occasions for other studies.

Radiobinding assay

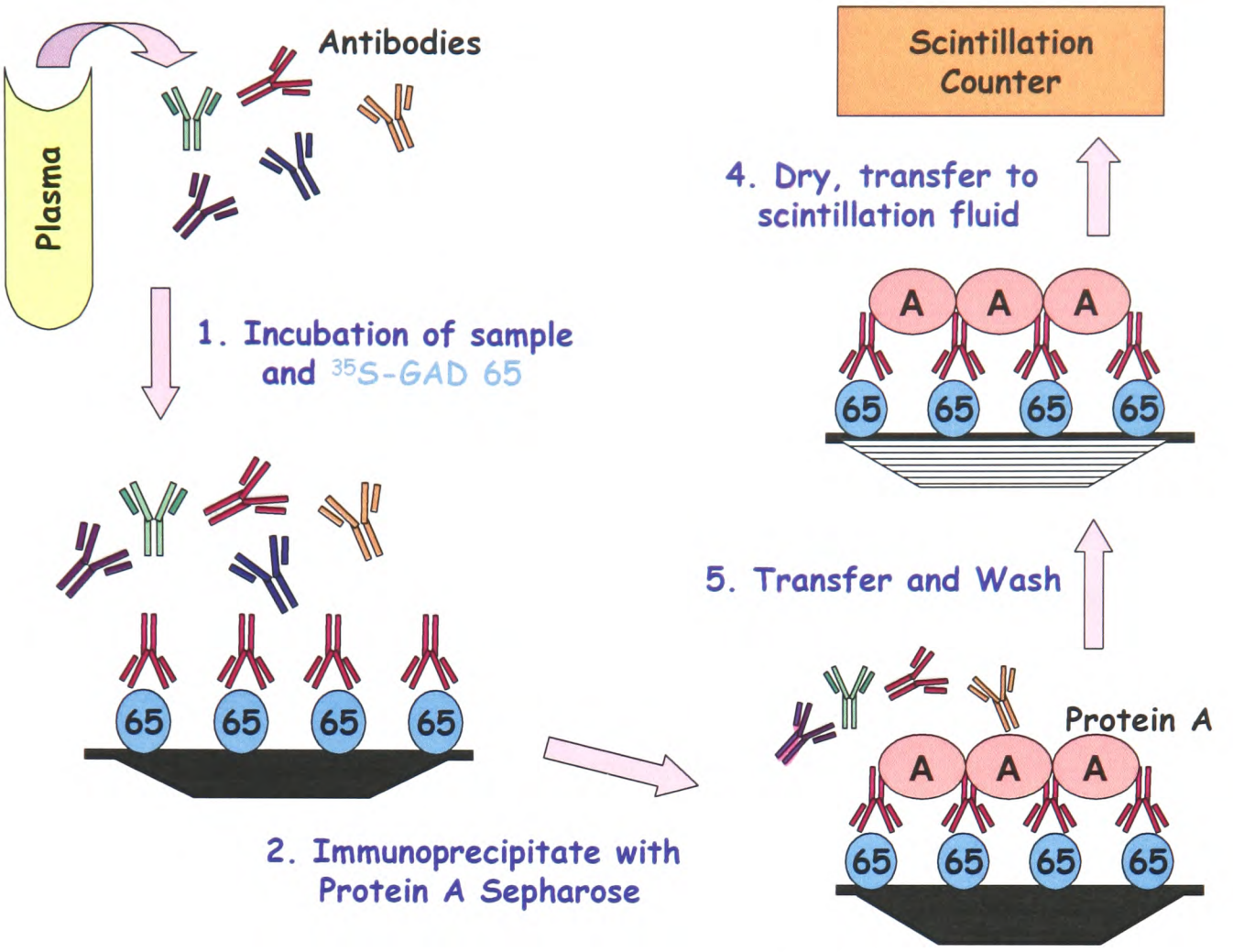
The antibody detection assay is based on the ability of sera from individuals to immunoprecipitate radiolabelled recombinant GAD 65. The procedure takes place over two days (See Figure 2.2 for schematic of assay). Day 1: 5 μl of subject plasma was incubated with 10,000 cpm of buffered ^{35}S-GAD 65[5] in 96-well V-bottom plates (NUNC Fisher Scientific, Loughborough, UK) for 12-15 hours at 4°C during which the autoantibodies bound to

[5] Buffer contained 10mM HEPES (pH 7.4), 150mM NaCl, 0.5 mg/ml BSA, 20mM methionine and 0.5% vol/vol Triton-X-100. It was stored at 4C.

radiolabelled recombinant protein. Day 2: The antibody-antigen complexes formed were immunoprecipitated with a 50% slurry of pre-swollen Protein A Sepharose beads[6] (Sigma) by vigorous shaking on a plate vortex for 45 min at 4^0C. The resultant mixture from each well was transferred to 2 mg/ml BSA-coated 96-well filter plates (Millipore Ltd, Watford, UK). The BSA coat helped to prevent non-specific binding. The antibody-antigen complexes in each well of the filter plate was washed 6 times with 170 µl ice-cold buffer followed by two 170 µl ddH_20 rinses on a Millipore suction gasket device. Lastly, the multi-well filters containing the complexes were dried under a bench lamp for 30 min and transferred using a Millipore punch device into scintillation vials containing 2 ml scintillation fluid (Fisher Scientific). Bound radioactivity was counted for 1 minute on a scintillation counter.

[6] A 50% slurry was a 1:1 mixture of pre-swollen Protein A Sepharose beads and deionised distilled water.

Figure 2.2: Simplified schematic of GAD 65 radiobinding assay protocol.



(1) 5 μl of patient plasma was incubated with 10,000 cpm of buffered ^{35}S-GAD 65 in 96-well plates for 12-15 hours at 4^{0}C during which autoantibodies bound the radiolabelled recombinant protein. (2) The antibody-antigen complexes formed were then immunoprecipitated with pre-swollen Protein A sepharose beads (shaking for 45 min at 4^{0}C). (3) This mixture was transferred to BSA-coated filter plates (to prevent non-specific binding) to undergo extensive washing. (4) The multi-well filters were dried under a bench lamp for 30 min and transferred to scintillation vials where bound radioactivity was counted on a liquid scintillation fluid counter.

Calculation of units

The counts per minute (cpm) obtained for each sample was converted into arbitrary GADA units: a standard curve was generated in each assay by serial dilution of a reliable standard plasma from a Type 1 diabetic patient into plasma of a non-diabetic subject. This positive-control plasma was kindly provided by Dr. M. Christie (King's College and Guy's Medical School, London) and contained suitably high GADA autoantibody titres to use as our 'in-house' standard (referred hereafter as JC). For each experiment the following dilutions were

performed: neat, 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64 and 1 in 128. Neat serum was assigned an arbitrary number of units (see Appendix 2).

For each GAD 65 experiment, the cpm obtained for each subject in the study was converted to nominal GADA units reading from the standard curve of JC which was included in each assay. A negative control was also included. The standard curve was transformed into a linear distribution by plotting log GAD units vs. log cpm for each dilution. Once outliers had been removed, a trendline was applied and it's gradient and y-intercept used to convert the sample cpms obtained for individual samples into GADA units. GADA units were converted into WHO units following standardisation of JC with the reference serum provided in the DASP 2003 workshop. (Please see Appendix 2 for more details).

Establishing cut-offs

The threshold for antibody detection for each of the radiobinding assays was established using 100 control plasma samples from non-diabetic subjects (from the DIF study) and the cut-off for GADA positivity was initially calculated as mean + 3SD binding units. It was anticipated that this threshold would provide the most specific measurement of GADA. Having participated in the DASP 2003 workshop, the optimal cut-off for our assay was 15 WHO units (at which 86% sensitivity and 94% specificity is attained). Please see Appendix 2 for more details.

Assay reproducibility

The on a day-to-day basis, reproducibility of our assay was comparable to that used in other laboratories (M Christie, personal communication). There are no international guidelines on how to assess the reproducibility of GADA assay. In our laboratory, each sample was tested in duplicate and in most instances was re-tested if the two duplicate readings obtained had a coefficient of variation greater than 15%. However, a higher threshold of 20% was set for low titre samples where small differences in cpm were sensitive to greater fluctuations in this measure of variation. The intra- and inter-assay coefficients of variation of the cpm obtained

were higher than 10% and 15%, respectively. Given that this assay is inherently noisy it was very difficult to achieve perfect concordance of readings between assays.

2.3 DNA QUANTIFICATION

2.3.1 UV Spectrometry

A UV plate reader was used to quantify DNA samples using the SPECTRA MAX190 (software: SOFTmax PRO 3.1.1). DNA samples were diluted in 1 x TE and measured in a 96-well plate format with a standard curve on each run generated by dilution of a 1 mg/ml calf thymus DNA stock (Sigma). Samples were measured in duplicate. DNA concentrations were inferred from absorbances obtained for standards at 260nm and DNA:protein ratios calculated from the 260/280 values.

2.3.2 PicoGreen method

Since single-stranded products could be obtained during the whole genome amplification protocols, PicoGreen was a more useful technique to measure DNA content since the assay reagent only binds double stranded DNA. The assay is also 10,000-fold more sensitive than UV absorbance methods. A standard PicoGreen protocol was adopted using a 96-well format with a standard curve on each run. Samples were diluted in 1 x TE solution and PicoGreen assay reagent (Molecular Probes (Invitrogen Ltd), Paisley, UK) added according to the manufacturers instructions. All samples were measured in duplicate. Plates were covered with foil and spun briefly to ensure mixing, then left at room temperature for a 5 min incubation period during which time the assay reagent bound double-stranded DNA in the samples. Lastly, the sample fluorescence was measured (excitation λ_{485nm}; emission λ_{530nm}) using a Cytofluor 4000 TC Series Fluorescence Multi-well Plate Reader with Cytofluor Version 4.2 software (Applied Biosystems, Cheshire, UK).

2.4 POLYMORPHISM DETECTION

2.4.1 Polymerase chain reaction (PCR)

PCR enables enormous amplification (10^8-fold) of specific regions of the genome containing polymorphisms of interest provided that short sequences on either side of the target sequence are known. A typical PCR reaction consists of target double-stranded DNA, two primers (single-stranded oligonucleotides) that hybridise to flanking sequences on opposing (sense and antisense) strands of the target, all four deoxyribonucleoside triphosphates (dNTPs) and a heat-stable DNA polymerase. The reaction is carried out in a buffer with a suitable concentration of $MgCl_2$ that enables the DNA polymerase to work at maximum efficiency. The PCR protocol consists of three steps;

(i) Denaturation – the reaction is heated to 94-95^{0}C for 15-30 seconds to denature the target DNA into single strands that can act as templates for DNA synthesis.

(ii) Primer annealing – the reaction is then cooled to a temperature (annealing temperature or Tm) that is optimised to enable each primer to bind specifically to the two strands of DNA flanking the target DNA. A great excess of primer is added so that the primers bind the flanking sequences making re-annealling of parental DNA less likely to occur.

(iii) Elongation – the temperature is raised to 70-75^{0}C for about 10 mins to enable the DNA polymerase (usually Taq enzyme derived from *Thermus aquaticus*) to elongate each primer to produce a copy of the specific region of interest.

Steps 1-3 are repeated usually for 30-35 cycles during which exponential amplification of the region of interest is achieved – creating 2^n copies of the amplicon (where n = number of cycles).

Primer design

PCR primers are usually designed to clone <400 bp fragments. Their lengths are generally 15-30 bp long with G+C contents of 40-60% with minimal complementary sequences (especially at the 3' end) in order to prevent formation of primer-dimers during PCR. They are also designed

to contain minimal potential to form secondary structure; for example, three C or G nucleotides in succession are avoided. Primers are designed such that annealing temperatures of the forward (antisense strand binding) and reverse (sense strand binding) primers are as similar as possible.

Standard assay reagents and software

All PCR reactions were carried out in thermowell 96 PCR plates (Corning Co-Star, Schiphol-Rijk, The Netherlands) and protected from evaporation using either lids (Corning Co-Star, Schiphol-Rijk, The Netherlands) or sealing mats (Thermo Life Sciences, Hampshire, UK). For PCRs performed, magnesium chloride, buffer and Taq polymerase were purchased from Applied Biosciences unless stated otherwise. Primers were obtained from Sigma Genosys (Haverhill, UK) and dNTPS from Promega. PTC-0200 or PTC-0225 DNA engine gradient thermal cyclers (MJ Research Inc, Essex, UK) were used for thermocycling. Seakem agarose powder for making gels was brought from Flowgen (Staffordshire, UK) and 10X TBE running buffer concentrate from Severn Biotech (Worcstershire, UK). All products were run out on gels using either 5 μl Orange G[7] or 3 μl formamide buffer[8] loading buffers for a typical 10 μl PCR product volume. UV imaging for PCR product visualisation was carried out using a Gel Doc 2000 (Bio-Rad, Hertfordshire, UK) with Quantity One 4.2.1 software ('Gel Doc' application).

Various adaptations of the basic PCR method for amplifying genomic regions of interest were employed to genotype individuals for single nucleotide polymorphisms at the HLA and insulin genes in this thesis.

[7] Orange dye contained 0.25% Orange G, 30% vol/vol glycerol and 0.5x TBE buffer (89mM Tris base, 89mM boric acid, 2mM EDTA, pH 8.0).
[8] Formamide buffer contained 0.1% bromophenol blue (BioRad), 0.1% xylene cyanol (BioRad), 90% formamide (Sigma) and 10mM EDTA.

2.4.2 Amplification refractory mutation system (ARMS)/ allele-specific PCR (ASP) sequence-specific PCR (PCR-SSP)

This method was used to detect alleles at the HLA *DRB1* and *DQB1* genes described in Chapter 4.

Principles

This adaptation of a normal PCR protocol employs a primer that binds the polymorphic base of interest at its 3' end (Newton *et al.* 1989); thereby allowing amplification of only the allele containing the matching base at the 3' end. The primer is synthesised in two forms. The 'WT' form is refractory to PCR on 'mutant' template DNA and the 'mutant' (MUT) form is refractory to PCR on 'WT' DNA. The Taq polymerase used has no 3'-5' exonuclease activity, therefore, depending on whether the WT or MUT primer is added in the PCR, only one allele is detected in one reaction. Sometimes, a single 3'-mismatched base allows amplification to proceed. The specificity of the reaction is improved by introducing an extra destabilising mismatch within the primer or by using a high Tm. The primer sets used in this study had been designed and optimised by staff in the Tissue Typing Laboratories (Churchill Hospital, Oxford) where this genotyping work was performed.

Genotyping:

The PCR-SSP method used to detect for the presence of alleles in the HLA class II *DRB1* and *DQB1* genes was developed by Bunce *et al* (Bunce *et al.* 1995). 48 reactions were required to genotype individuals for the most relevant alleles (T1D-associated) in exon 2 of *DRB1* and *DQB1* (see Table 2.2). Each reaction consisted of sequence specific primers, which amplified groups of alleles or individual alleles comprising a defined serological (antigen) specificity. Also, included in each reaction were control primers that amplified a non-polymorphic 796 bp fragment from the third intron of the *DRB1* gene. The design and concentration of primers in the 48 reactions were optimised to PCR at a Tm of 58-62^{0}C with the specificity dependent

nucleotide on the penultimate or 3'-base. The DNA concentration or Taq polymerase was increased for samples that did not amplify well.

PCR reactions were carried out in 8 μl volumes consisting of; 67mM Tris base (pH 8.8), 16.6 mM ammonium sulphate, 2 mM magnesium chloride, 0.01% (vol/vol) Tween 20, 200 μM of each dNTP, 1-4 μM of allele specific primers, 0.1 μM of *DRB1* control primers and 0.1875 units of Taq polymerase (Advanced Biotechnologies, London). Typically, 400-600 ng of template genomic DNA was required for the 48 reactions. 5 μl of buffer, DNA and enzyme mixture was added to 3 μl primer mix. A 10 μl mineral oil overlay was applied. PCR of two individuals could be performed on one 96 well plate.

Table 2.2: List of alleles detected by the 48 PCR-SSP reactions performed for genotyping of the HLA DRB1 *and* DQB1 *genes.*

Primer set	Fragment size	Alleles detected
1	990	*DQB1*0201*
2	146	*DQB1*0202/03*
3	119	*DQB1*0203*
4	201	*DQB1*0401*
5	201	*DQB1*0402*
6	129	*DQB1*0501*
7	117	*DQB1*0502*
8	87	*DQB1*0502/03*
9	118	*DQB1*0504*
10	112	*DQB1*0602/03/04/07/08/10/11/13/14/15/16/20*
11	176	*DQB1*0603/04/05/06/07/08/09/11/12/14/17/18*
12	249	*DQB1*0601/06*
13	183	*DQB1*0602/15/19*
14	127	*DQB1*0603/07/11/14*
15	167	*DQB1*0302/03/07/11/12, *0604/05/07/09/15/18*
16	218	*DQB1*0302/03/05/07/08, *0602/03/04/07/08/09/10/11/12/13/14/15/16/17/18/19*
17	179	*DQB1*0302/03/07/08/11/12, *0602/03/04/05/07/08/09/10/11/12/13/14/15/16/17/18/19/20*
18	208	*DQB1*0301/04/09/10/13*

Primer set	Fragment size	Alleles detected
19	174	*DQB1*0304*
20	122	*DQB1*0302/07/08/11*
21	131	*DQB1*0203, *0303/06/12*
22	156	*DQB1*0307*
23	183	*DQA1*0301/02/03*
24	234	*DQA1*0501/03*
25	198	*DQA1*0501/05*
26	215	*DRB1*0301/02/03/04/05/06/07/08/09/10/11/12/13/14/15/16/17/18/19/20/21/22/23, *1107*
27	217	*DRB1*0301/04/06/08/10/11/12/13/15/16/18/19/20/22/23, *1327*
28	190	*DRB1*0301/03/06/07/08/10/11/12/13/15/16/18/19/20/22, *1116, *1301/06/09/10/15/18/20/27/28/32/35/40/42/43/51/53, *1406/12/17/18/21/29/33*
29	143	*DRB1*0302/03/06, *0820, *1117/37/41, *1303/07/08/12/13/19/26/32/33/36/37/38/40/47/48/49/53, *1401/02/03/05/06/07/08/09/12/13/14/16/18/19/20/22/23/24/25/26/27/29/32/34/36/37/38/40/41/43/44/45*
30	189	*DRB1*0302/05/14, *1109/20, *1302/05/26/29/31/34/36/39/41, *1402/03/09/19/24/27/30, *1608*
31	178	*DRB1*0401/02/03/04/05/06/07/08/09/10/11/12/13/14/15/16/17/18/19/20/21/22/23/24/25 /26/27/28/29/30/31/32/33/34/35/36/37/38/39/40/41/42/43*
32	174	*DRB1*0401/02/03/04/06/07/08/13/14/18/19/20/21/22/23/25/27/31/32/33/35/36/37/38/39/41/42/43/44*
33	212	*DRB1*0401/02/09/13/14/16/21/26/33/35/37/38*
34	206	*DRB1*0402/14/37*
35	211	*DRB1*0403/06/07/11/17/20/27/39/41*
36	223	*DRB1*0401/02/04/05/08/09/10/13/14/15/16/19/21/23/24/26/28/29/30/32/33/35/36/37/38/40/42/43, *1122*
37	195	*DRB1*0101/02/04/05/06/07/08/09*
38	196	*DRB1*0103*
39	207	*DRB1*1501/02/03/04/05/06/07/08/09/11/12/13*
40	137	*DRB1*1507/11, *1601/02/03/04/05/07/08*
41	133	*DRB1*0701/03/04/05/06/07*
42	214	*DRB1*0801/02/03/04/06/07/08/09/10/11/12/13/15/16/17/19/22/23, *1415*
42	162	*DRB1*0801/03/05/06/10/12/16/17/18/22/23*
43	193	*DRB1*0901/02*
44	204	*DRB1*1001*
45	214	*DRB1*1101/04/06/08/09/10/12/13/15/17/18/19/24/26/27/28/29/31/32/33/34/35/37/38/39/42/43, *1305/06/07/11/12/14/21/25/30/42/44/46/49/50, *1401/02/05/06/07/08/09/13/14/17/18/20/22/23/25/26/29/30/32/33/34/35/36/38/41/42/43/44/45*
46	163	*DRB1*1201/02/03/05/06/07/08*
47	214	*DRB1*1303/10/33/37, *1419/21*
48	212	*DRB1*1113/17, *1401/05/07/08/14/18/23/26/32/34/35/36/38/42/43/44/45*

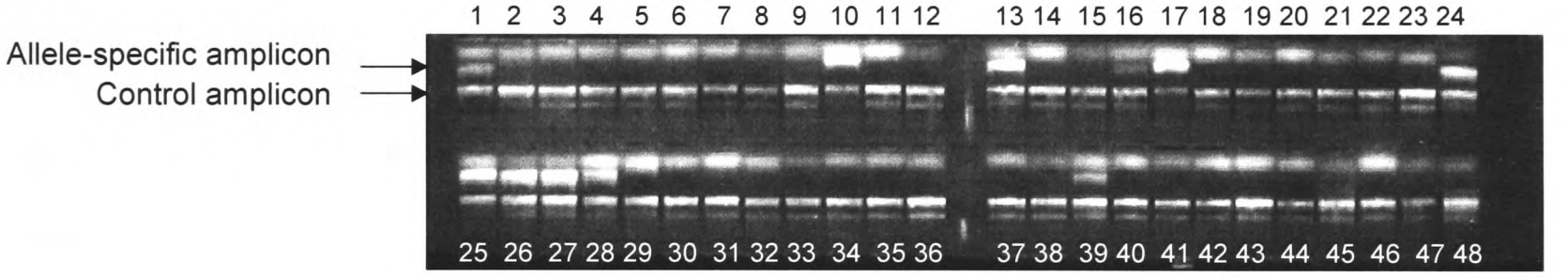
Primer sequences are given in Appendix 4.

Cycling conditions:

No of cycles	Denaturation		Annealling		Extension		Cooling	
1	60 sec	96^{0}C						
5	20 sec	96^{0}C	45 sec	70^{0}C	25 sec	72^{0}C		
21	25 sec	96^{0}C	50 sec	65^{0}C	35 sec	72^{0}C		
4	30 sec	96^{0}C	60 sec	55^{0}C	90 sec	72^{0}C		
1							120 sec	20^{0}C

Gel electrophoresis: PCR products were separated on a 1% agarose gel containing 0.1 μg/ml ethidium bromide at 250V for 23 mins in 0.5 x TBE buffer. Each lane on the gel contained the product of one reaction mix (See Figure 2.3). Products were visualised under UV light.

Figure 2.3: Typical genotyping result of the PCR-SSP technique.



PCR-SSP products tested for 48 groups of alleles (represented by individual lanes on gel) of an individual. An internal control 796 bp (a DRB1 *invariant sequence) was included in every reaction as a positive control. Lanes considered to be positive in this gel are 1 (*DQB1*0201), 10, 13, 16, 17 (*DQB1*0602), 24 and 25 (*DQA1*0501), 26, 27, 28 (*DRB1*0301) and 39 (*DRB1*1501).*

Assigning genotypes: The absence or presence of bands in each lane was used to assign a HLA type to each individual by a process of elimination; a check for a product in several lanes was required to validate whether a particular allele was present since the primers could amplify multiple alleles of a serological specificity. All genotypes were checked using 'AssignIt 2004 version 1.0' software (written by Dr M Barnardo, Tissue Typing Laboratories, Churchill Hospital,

Oxford) and by two independent investigators. Since the *DRB1* and *DQB1* genes are in strong linkage disequilibrium, knowledge of well-known haplotype patterns in Caucasians was also used to validate the results. Where rare haplotypes were obtained, re-genotyping was performed to confirm results. However, where knowledge of common haplotype patterns was not sufficient for accurate/unambiguous allele calling from the 48 primer sets used e.g. if individual was a *DRB1*0301* homozygote, further typing involving two more reactions was performed to distinguish a true *DRB1*0301* allele from a *DRB1*1301* allele.

2.4.3 Amplifluor PCR (K Biosciences)

This technique was used to genotype polymorphisms in the insulin gene region examined in Chapter 5.

This commercially available method of high-throughput genotyping is based on competitive allele-specific PCR employing a single tube format that can simultaneously detect both alleles in the one reaction, without the need for post-PCR processing. The following is a brief description of the Amplifluor method (Chemicon International (Serologicals Corporation), California, USA) that was employed by K Biosciences (Hertfordshire, UK) to whom the work was outsourced to for genotyping of the insulin gene SNPs. The validation and set up for each SNP assay was carried out by K Biosciences.

Principles

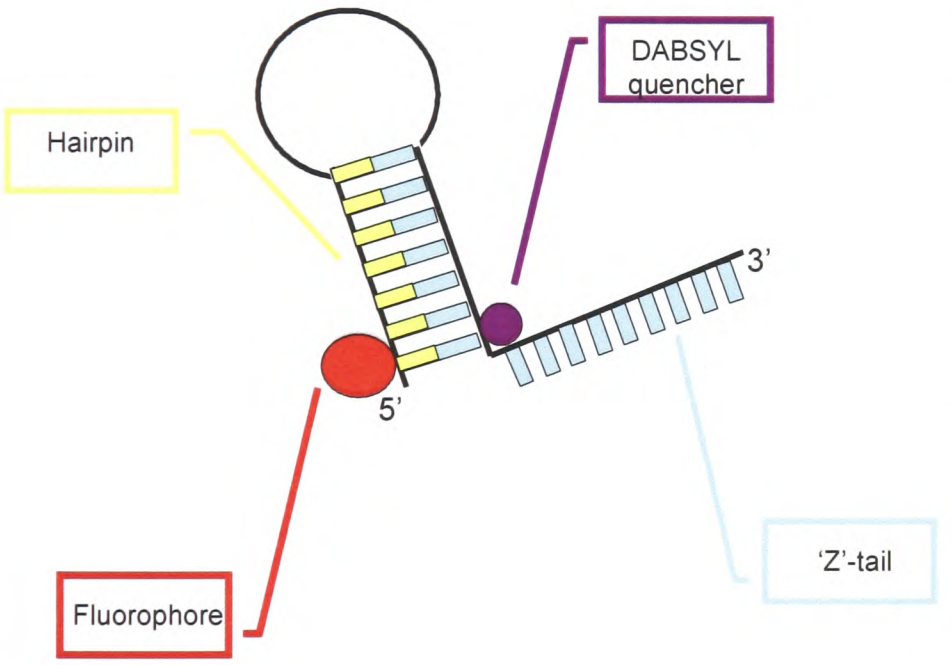
The reaction utilises 2.5 -10 ng of DNA, standard PCR reaction components and 5 primers:

- *Two fluorescently labelled 'Amplifluor SNP Primers®.* These contain a fluorophore and a dimethylaminoazosulphonic acid (DABSYL) quencher that are brought together in close proximity by a hairpin structure, located at the 5' end of the primer; the fluorophores used are fluorescein ('Green Amplifluor SNP Primers®) and either sulphorhodamine or JOE (both 'Red Amplifluor SNP Primers®). Located at the 3' end, is a string of 21 bases called a 'Z-'tail

(See Figure 2.4); this unique tail sequence can extend amplification, beginning with amplicons created from any pair of correspondingly tailed sequence-specific oligonucleotide. For this reason, the Amplifluor Primers® are called 'Universal'. The Z-tails are different for each labelled primer (green or red).

- *Three unlabelled standard desalted oligonucleotides – two forward 'Z'-tailed allele-specific primers (FWT and FMUT) plus a common reverse primer (RCOM).* The 'Z'-tail of the FWT[9] and FMUT[10] primers consist of a 21-nucleotide region identical to the recognition site of one of Universal Amplifluor SNP Primers®, either red or green. The tail is located at the 5' end of the FWT and FMUT primers.

Figure 2.4: The structure of the Amplifluor SNP Primer® molecule.



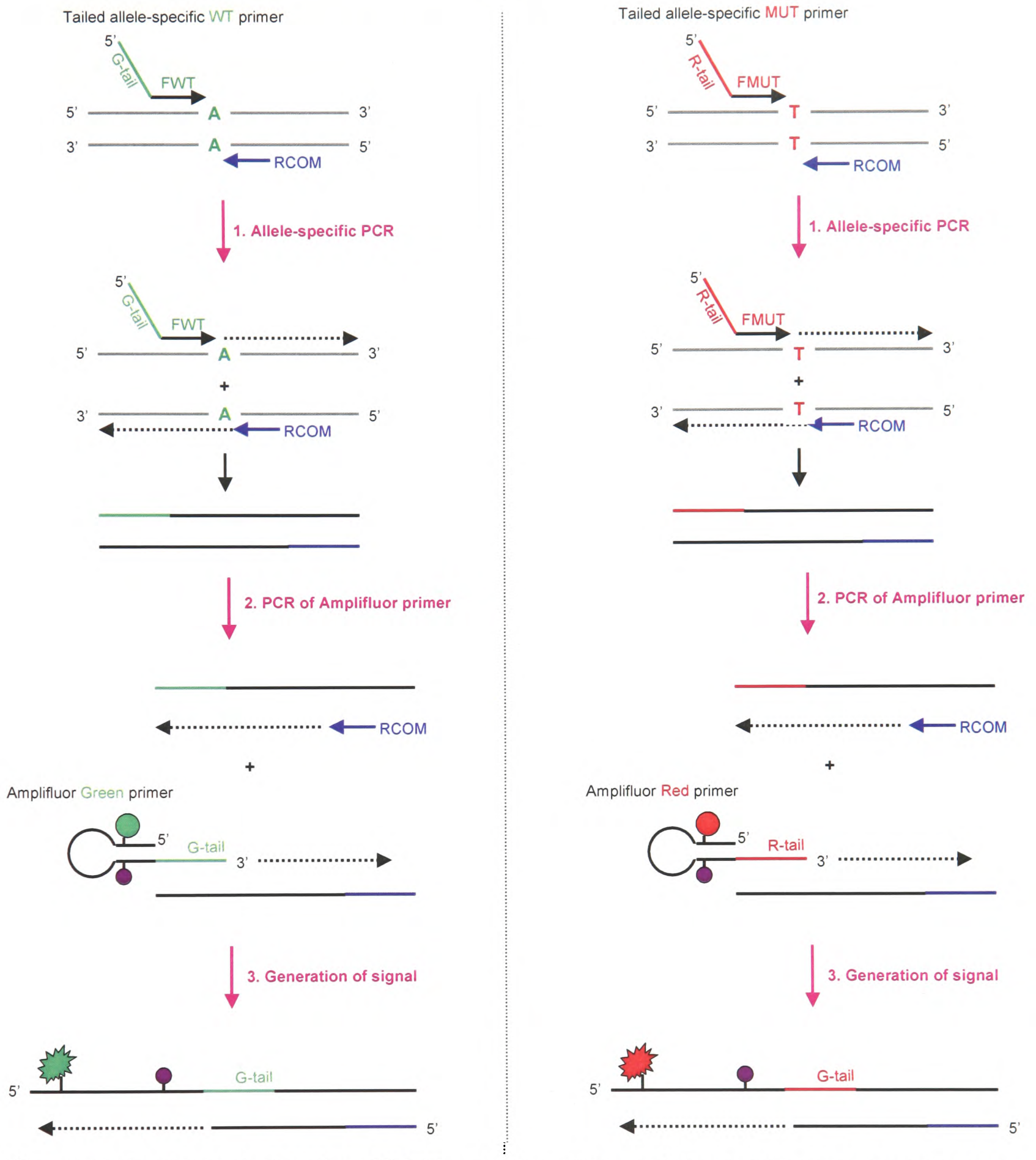
In its native conformation, the intact hairpin stem enables the fluorophore and DABSYL to be in close enough proximity to efficiently quench fluorescence. As a result, unincorporated Amplifluor primers have minimal fluorescence. When the primer is unravelled, no quenching occurs and a fluorescent signal is produced. DABSYL = dimethylaminoazosulphonic acid.

[9] For detection of the wild-type allele.
[10] For detection of the mutant allele.

The mechanism (See Figure 2.5)

The unlabelled primers bind the template DNA and amplify the genomic sequence containing the SNP of interest in an allele-specific manner. The resulting amplicon contains the target sequence for the Amplifluor SNP Primers® since each of the allele-specific primers contains a 'Z'-tail on their 5' end that is incorporated into the amplicon, which is complementary to the 'Z'-tails on the 3' end of each of the Amplifluor SNP Primers®. Therefore, each allele-specific amplicon will target either a Red or Green Amplifluor SNP primer to them. The 'Z'-tail of the Amplifluor SNP Primers® hybridises to the allele-specific amplicons and is extended, incorporating them into the daughter amplicons in the second round of PCR. As a result, the Amplifluor molecule is unravelled, melting its hairpin structure in the process, thus separating the fluorophore from the quencher diminishing the fluorescence resonance energy transfer (FRET) between them, allowing generation of an allele-specific fluorescent signal that can be detected by a fluorescent plate reader. (See Figure 2.5 for schematic of assay).

Figure 2.5: Schematic of SNP detection using Amplifluor technology.



The example of genotyping the -23HphI variant upstream of the insulin gene is given here; where the A allele is wild-type (WT) and T is mutant (MUT). 1. Tailed allelic-specific primers (FWT and FMUT) together with the common reverse primer (RCOM) amplify the region containing the SNP of interest. 2. The unique tails (green, G-Tail; or red, R-Tail) are amplified in the amplicon and their reverse sequences acts as 'recognition sites' for the Amplifluor SNP primers, whose 5' end tails are complementary to them. 3. Consequent amplification of the Amplifluor SNP primers results in unravelling of the 5' hairpin structure in the molecule, thus separating fluorophore and quencher, generating a fluorescent signal. In this case, the presence of an A allele at the -23HphI site on template DNA yields a green fluorescent signal and the T allele, a red fluorescent signal. (Figure adapted from (Bengra et al. 2002)).

Genotyping: The composition of a PCR master mix will vary according to the allele-specific Primers® used. A typical reaction would contain a 1:10 ratio of allele-specific to Amplifluor SNP primers + common reverse primer, as the allele-specific primers are only required for the first few cycles of PCR. Also, the excess ratio of Amplifluor SNP Primers® eliminates non-specificity as these primers will dominate the amplification reaction after the second round of PCR; this helps to minimise the appearance of non-specific amplicons generated by the allele-specific primers. Platinum Taq Polymerase (Life Technologies (Invitrogen)) is often used instead of AmpliTaq polymerase. PCR reactions are set up and performed in optically clear PCR microplates (VWR Scientific Products, Poole, UK) and sealed with plate sealing film (Robbins Scientific Corp, Solihull, UK).

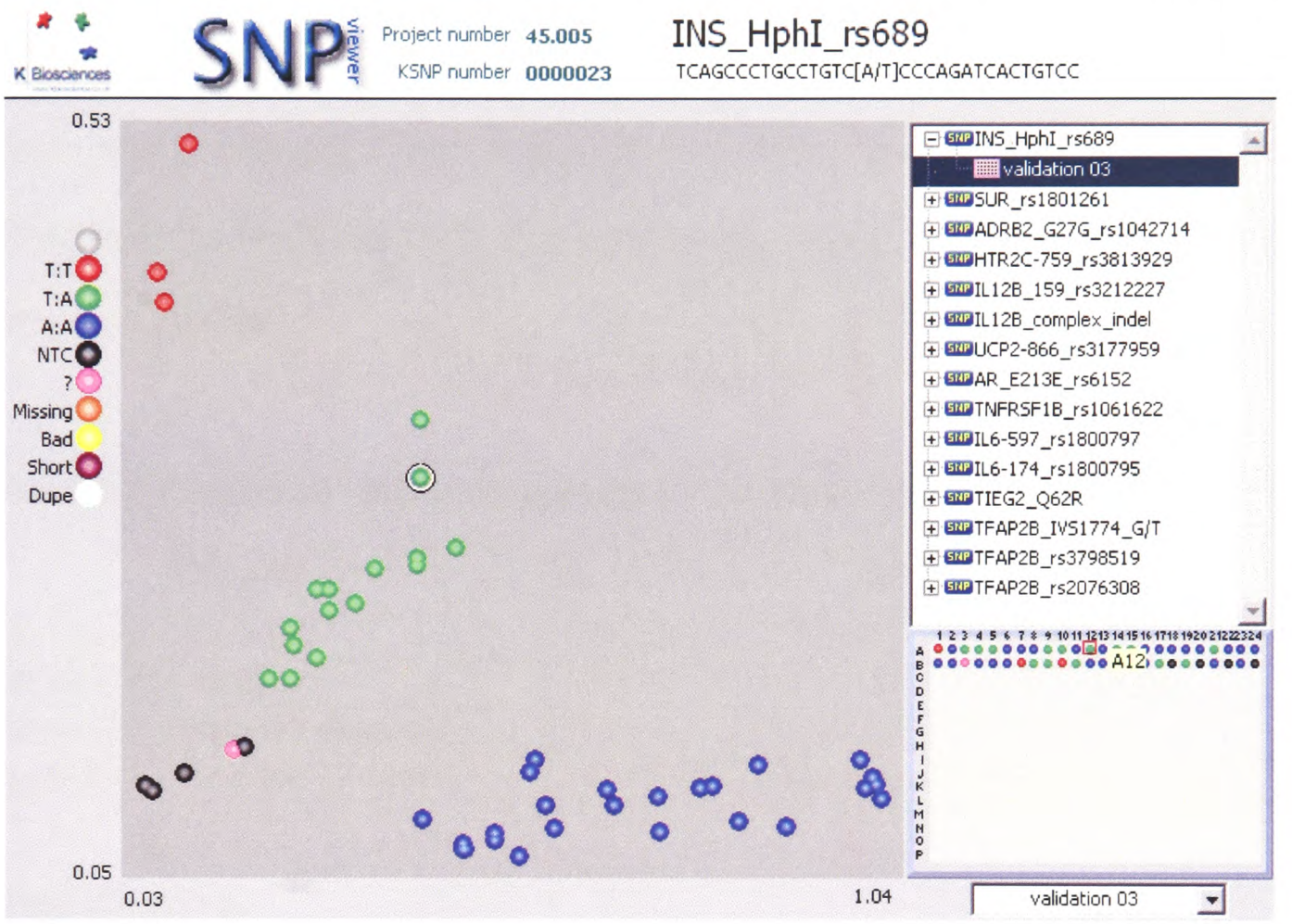
Cycling conditions: Typical hot-start PCR is performed, with the Tm naturally optimised for binding of the allele-specific primers. PCR reactions are held at 20°C until fluorescence measurements are performed.

Fluorescence measurement: Total fluorescence (as relative fluorescence units) of labelled Amplifluor SNP Primer-containing amplicons was quantified through the top of each well of open PCR microplates using an ABI fluorescence plate reader which quantified fluorescein (excitation λ_{485nm}, emission λ_{535nm}) and sulphorhodamine (excitation λ_{485nm}, emission λ_{535nm}).

Assigning genotypes: Fluorescence measurements were plotted with signals from the WT allele (usually form green-labelled primer) plotted on the *x*-axis and signals from the MUT allele on the *y*-axis (usually from red-labelled primer). So, depending on the fluorophore targeted to each allele (Green Amplifluor/ Red Amplifluor primer, a green or red signal is produced in homozygote WT and MUT individuals, respectively, and a yellow/orange signal in heterozygotes. The SNPViewer Program (K Biosciences) plots the fluorescence measurements obtained. The colours used to represent the three different genotypes may vary according to the

SNPViewer Program settings. (See Figure 2.6 for an example). Fluorescence from blank samples (due to unsuccessful PCR) appears on the scatterplot as black spots near the (*x*, *y*) origin.

Figure 2.6: A typical fluorescence scatterplot for SNP detection using Amplifluor technology.



The example of genotyping the -23HphI variant upstream of the insulin gene is presented here. In this plot the AA homozygotes are blue, TT homozygotes in red and heterozygotes in green.

2.4.4 Tetra-primer ARMS PCR

This method was used for checking genotyping of the -23*HphI* SNP in the insulin gene region (Chapter 5). It was also used for quality control testing of whole genome amplified DNA (Appendix 1).

Principles

This is a more sophisticated extension of a single-primer ARMS assay, enabling detection of both alleles at a SNP site in a single reaction mix following gel electrophoresis (Ye *et al.* 2001). The method uses two sets of primers typically ~28 bp in length, called 'inner' and 'outer' primers

at a concentration ratio of 10:1, respectively. The outer primers amplify a region containing the polymorphism of interest - as in a normal PCR. The inner primers are allele-specific, containing an allele-specific mismatch at the 3'-base with additional mismatches at position –2 from the 3'-terminus to increase specificity of the reaction. (See Figure 2.7 overleaf for details of method).

Genotyping: PCR was carried out in a 10μl total volume containing 30 ng template DNA, 5 μmol of each inner primer and 0.5 μmol of each outer primer (see below), 2.5 mM $MgCl_2$, 200 μmol dNTPs, and 1U AmpliTaq Gold *Taq* polymerase (Applied Biosystems). A 10 μl mineral oil overlay was applied.

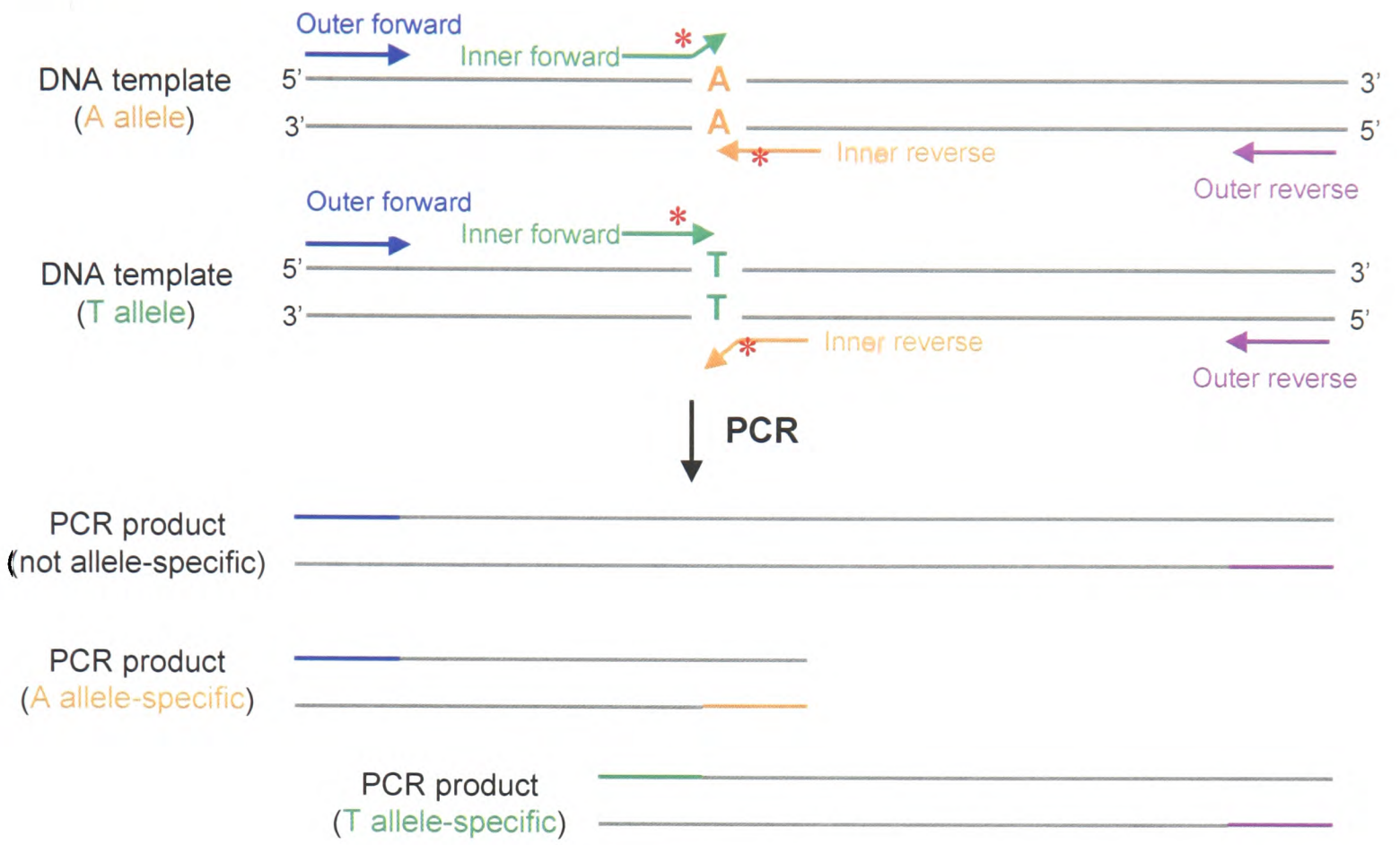
Tetra-primer ARMS PCR primer sequences for genotyping the –23*HphI* polymorphism.

Primers	Sequence
Outer primer - forward	CTCAGCCCTCCAGGACAGGCTGCATCAGA
Outer primer - reverse	AGAGCTTCCACCAGGTGTGAGCCGCACA
Inner primer – forward (A allele)	TCAGCCTGCCTCAGCCCTGCCTGACA
Inner primer – reverse (T allele)	GGCCATGGCAGAAGGACAGTGATCTGCGA

Cycling conditions:

No of cycles	Denaturation		Annealling		Extension	
1	6 min	94^{0}C				
35	30 sec	94^{0}C	30 sec	63^{0}C	45 sec	72^{0}C
1					10 min	72^{0}C

Figure 2.7: Schematic of tetra-primer ARMS PCR method:

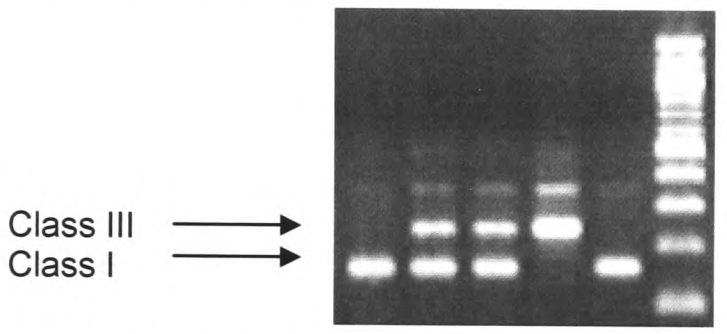


*The example of an A→T substitution is given here as occurs at the -23HphI polymorphic site in the insulin gene. Two allele-specific PCR products are generated using two pairs of primers, one pair producing a product representing the A allele (outer forward, blue and inner reverse, orange) and the other pair representing the T allele (outer reverse, purple and inner forward, green). The inner primers are allele-specific at the 3'-base and contain a second mismatch at –2 from the 3'-base (indicated by *) to enhance specificity. By positioning the two outer primers at different distances from the polymorphic nucleotide, the two allele specific PCR products differ in length, allowing them to be discriminated by gel electrophoresis. Three products can potentially be obtained; the outer forward and outer reverse produce the PCR control band, the outer forward and inner reverse primers amplify the A allele, and lastly, the outer reverse and inner forward primers amplify the T allele.*

Gel electrophoresis: PCR products were separated on a 2% agarose gel containing 0.5 μg/ml ethidium bromide run at 200V for 40 mins in 0.5 x TBE buffer. A 100 bp ladder (New England Biolabs, Hertfordshire, UK) was run alongside test samples. Products were visualised under UV light.

Assigning genotypes: Class I homozygotes were identified by the presence of a single band at 180 bp, Class III homozygotes by the presence of a single band at 260 bp, and Class I/III heterozygotes by the presence of both bands (Figure 2.8).

*Figure 2.8: Gel electrophoresis of –23*HphI *tetra-primer ARMS PCR products.*



The 100 bp ladder has been loaded as marker.

2.4.5 PCR- restriction fragment length polymorphism (RFLP) analysis

This technique was used initially during the course of the thesis when genotyping the +1428*FokI* variant in the insulin gene region (Chapter 5). Since the +1428*FokI* variant is in complete LD with the +1404*Fnu4HI* SNP, the results of +1428*FokI* genotyping by this method was used to check for concordance of genotype calls made for the +1404*Fnu4HI* SNP which was genotyped by the Amplifluor method.

Principles

This technique relies on the SNP of interest being able to create or destroy a restriction enzyme recognition site. A PCR product containing this SNP of interest may (or may not) be cleaved when treated with restriction enzyme, depending on the allele present. For example, a G→A substitution at position +1428 in the insulin gene, downstream of the translation initiation start

site, results in ablation of the *FokI* restriction site at this position. Digested products can be separated on a gel to determine genotype frequencies of this variant.

Genotyping: PCR was performed in a 20 μl volume containing 20-40 ng of template DNA, 200 μmoles of each dNTP, 1.5mM $MgCl_2$, 5 pmol of each primer (see below), and 0.05 U AmpliTaq Gold *Taq* polymerase (Applied Biosystems). A 10 μl mineral oil overlay was applied.

PCR primer sequences for amplifying product containing the +1428*FokI* polymorphism.

Primers	Sequence
Forward	TTAAGCCCTTGAACCAGC
Reverse	CAGCCCAGCCTCCTCCCTCCACA

Cycling conditions:

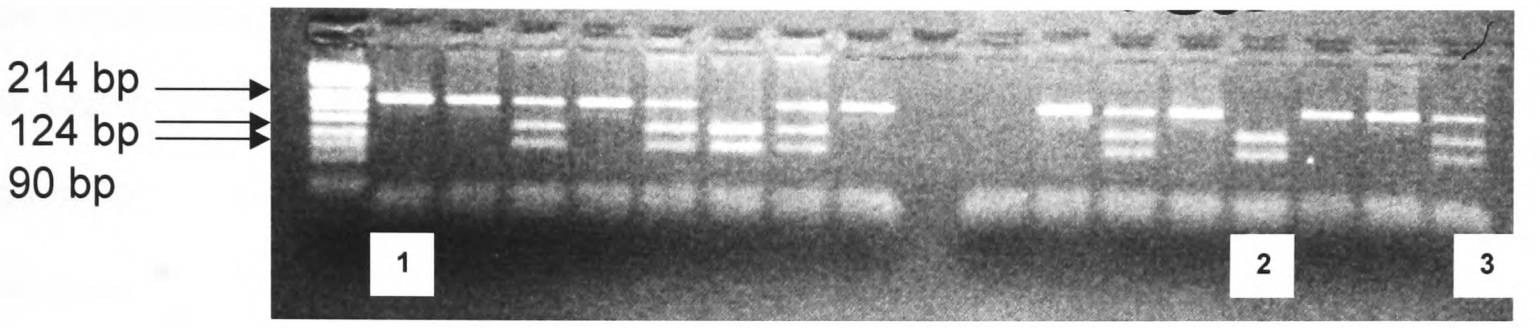
No of cycles	Denaturation		Annealling		Extension	
1	10 min	95^0C				
30	30 sec	95^0C	30 sec	55^0C	30 sec	72^0C
1					10 min	72^0C

Restriction digest: First, 5 μl of PCR product from 5 randomly selected amplified samples per plate was run out on a 2.5% agarose gel to check that the PCR was successful. If successful, the remaining 15 μl of product was digested in 0.25 U of *FokI* restriction enzyme (New England Biolabs) at 37^0C for at least 4 hours.

Gel electrophoresis: PCR products were separated on a 3% agarose gel containing 0.5 μg/ml ethidium bromide at 150V for 35 mins in 0.5 x TBE buffer. Products were visualised under UV light.

Assigning genotypes: PCR of genomic DNA yielded a 214 bp product which when digested with *FokI* restriction enzyme generated 124 bp + 90 bp products if the restriction site was present (wild-type G allele) (See Figure 2.9).

Figure 2.9: PCR-RFLP genotyping of +1428FokI variant.



M = pUC 18 + MspI digest. Pattern 1 indicates an A/A homozygote, Pattern 2 indicates a G/G homozygote and Pattern 3 indicates a G/A heterozygote.

2.4.6 Sequencing

This method was used as an additional measure to check genotyping of individuals considered to be rare homozygotes at the insulin gene +1404*Fnu4HI* SNP by the Amplifluor method. Sequencing was performed in forward and reverse directions.

Sequencing of amplicons containing SNPs of interest was performed using a high-throughput fluorescence-based method (using the ABI 3700 capillary based system).

Principles

An amplicon containing the SNP of interest is amplified by standard PCR using primers that have been designed with M13 tails on their 5' ends. This is followed by a second PCR – the 'sequencing PCR'. In this PCR, extension from only one primer (forward or reverse sequencing primer) per reaction is performed, producing single-stranded products. The sequencing primers are complementary to the M13 tails on primers used in the first PCR to generate the SNP-containing amplicon. In the sequencing PCR reaction, unlabelled dieoxy and fluorescent dye labelled dideoxy nucleotides are incorporated into the sequence during the extension phase of

each cycle. When a dye labelled dideoxy nucleotide is introduced into the sequence during this phase of the reaction, the extension stops. This is referred to as a dye termination reaction. This type of sequencing reaction will produce a number of different size fragments all containing an end labelled dideoxy nucleotide. The number of different size fragments will be equal to the number of bases in the sequence. There are currently four different fluorescent dyes used in cycle sequencing, each one associated with a specific nucleotide. The ubiquitously used 'Big Dye' system (Applied Biosystems) was employed for this work. This method enables each cycle sequencing reaction to utilize all four dyes at the same time in the same reaction. Extension products produced are run out on a gel by a DNA analyser and the sequence is assigned from the electropherogram produced.

PCR 1: Generation of amplicon containing SNP of interest: PCR was optimised in a 20 μl volume containing 20 ng of template DNA, 200 μmoles of each dNTP, 1.5 mM $MgCl_2$, 10 pmol of forward and reverse sequencing primers (see below), and 0.8 U AmpliTaq Gold Taq polymerase (Applied Biosystems). Primers were designed using Primer3 (www.frodo.wi.mit.edu/cgi-bin/primer3) and had M13 tails attached to their 5' ends (see below).

PCR sequencing primer sequences for amplifying product containing the +1404*Fnu4HI* polymorphism.

Primers	Sequence
Forward	TGTAAAACGACGGCCAGTCAGCTCTCTCCACGCTCTCT
Reverse	CAGGAAACAGCTATGACCTCCCTTTCCTGAACAAGGTC

M13 tails are coloured in red

PCR cycling conditions:

No of cycles	Denaturation		Annealling		Extension	
1	10 min	96°C				
34	30 sec	96°C	30 sec	60°C	30 sec	72°C
1					10 min	72°C

PCR purification: First, 5 μl of PCR product from 5 randomly selected amplified samples per plate was run out on a 2.5% agarose gel to check that the PCR was successful. If successful, 1 μl of the remaining of PCR product was purified to remove any residual primers and dNTPs left over from the PCR, which may interfere with the sequencing PCR. A 1 μl of a 1:1 mixture of Shrimp alkaline phosphatase (Promega) and Exonuclease I (20 000 U/ml) (New England Biolabs) was added to 1 μl PCR reaction (called the 'EXOSAP' process).

Sequencing: A 10 μl sequencing reaction containing the 2 μl purified PCR product/ EXOSAP mixture, 1 μl Big Dye terminator mix (contains dideoxynucleotides), 1.5 μl Big Dye Dilution Buffer, 2 μl M13 Sequencing Primer (at 2.5 pmol/ μl) and 3.5 μl of ddH_20. Big Dye version 1.1 reagents obtained from Applied Biosystems were used. One reaction was set up containing the forward M13 primer another containing the reverse M13 primer.

Sequencing cycling conditions:

No of cycles	Denaturation		Annealling		Extension	
25	10 sec	96^0C	5 sec	50^0C	4 min	60^0C

Purification of extension products for Dye-terminator removal: The single-stranded sequencing PCR product was mixed with 10 μl of ddH_20. This mixture was purified by gel filtration using the DyeEx plate method (Qiagen Inc) and samples dried at 70^0C on a thermocycler. 10 μl HiDi Formamide and 10 μl ddH_20 was added to the pellet followed for vortexing to re-suspend the pellet. Samples were then frozen.

Separation of sequencing products: This was performed at the Core Genomics Facility at the Wellcome Trust Centre for Human Genetics (Oxford) using an ABI 3700 DNA analyser. This is a capillary based system which separates on the basis of size using the polymer POP 6.

Assigning genotypes: Sequencing data was analysed using Mutation Surveyor™ (*www.softgenetics.com*). This package compares the sequence obtained to a reference sequence in the GenBank database (accession number L15440 used for insulin gene polymorphism analysis).

Chapter 3:

GADA in the post-diagnosis period in LADA

CHAPTER 3: GADA IN THE POST-DIAGNOSIS PERIOD IN LADA

3.1 INTRODUCTION

3.1.1 Persistence and changes of autoantibody titre during the post-diagnosis period

The handful of studies that have addressed whether autoantibodies (ICA, GADA, IA-2A) persist in the post-diagnosis period in T1D have shown that, remarkably, a significant but varying proportion of patients remain antibody-positive, at least for the first few years after clinical onset despite lack of a preserved β-cell function (Kolb *et al.* 1988; Christie *et al.* 1990a; Kawasaki *et al.* 1992; Muhr *et al.* 1997). Whereas ICA disappears rapidly in the first few years after clinical onset, positivity for GADA and IA-2A is maintained for longer periods (Rowley *et al.* 1992; Yokota *et al.* 1998; Borg *et al.* 2000; Decochez *et al.* 2000b; Chang *et al.* 2004; Scholin *et al.* 2004, Rodacki *et al.* 3004). In addition, GADA titres generally decrease more slowly over time in the post-diagnosis period than do IA-2A concentrations. This occurs in different ethnic populations including White Caucasians (Borg *et al.* 2000), Orientals (Yokota *et al.* 1998) and Arabs (Damanhouri *et al.* 2005). This difference in the degree of persistence has been attributed to the observation that IA-2A are more closely associated with loss of insulin secretion (Savola *et al.* 1998b).

Compared with changes in titre of ICA and IA-2A, the decline in GADA levels over time in the post-diagnosis period of T1D is not uniform (Savola *et al.* 1998b). Seroconversion to GADA positivity or negativity (that is, appearance or disappearance, respectively) is not uncommon and striking fluctuations in GADA concentrations over time have been shown to occur (Batstra *et al.* 1997; Savola *et al.* 1998b). Although overall, most studies have shown that GADA titres decrease over time (Hampe *et al.* 2002a), especially in patients diagnosed before puberty (Savola *et al.* 1998b; Decochez *et al.* 2000b), some patients reach peak titres after clinical onset of T1D, whilst in others, titres remain stable (Kaufman *et al.* 1992; Hagopian *et al.* 1993a; Borg *et al.* 2000; Ronkainen *et al.* 2004).

Small studies (n < 55) of initially non-insulin requiring, antibody-positive, adult onset diabetic patients (probably LADA patients) have demonstrated that GADA also persists after clinical onset in this form of autoimmune diabetes (Hagopian *et al.* 1993a; Gottsater *et al.* 1995; Kobayashi *et al.* 1996; Biesenbach *et al.* 2005). In a similar manner to that observed in T1D, GADA titres can decline over time as the duration of diabetes increases (Davis *et al.* 2000; Borg *et al.* 2002) and persistence associates with high titres at diagnosis (Niskanen *et al.* 1995) and both increases and decreases in titre occurring over time (Hagopian *et al.* 1993a; Borg *et al.* 2002). As in classical T1D, the titres of ICA, GADA and IA-2A decline with time in the LADA patients studied so far and GADA appears to be the most persistent autoantibody of the three (Borg *et al.* 2002; Damanhouri *et al.* 2005).

3.1.2 Clinical relevance of GADA in the post-diagnosis period

Given that loss of β-cell mass may be less complete in T1D patients diagnosed above 7 years of age than in younger children (Pipeleers and Ling 1992), it is important to identify markers that enable one to recognise those patients that are more susceptible to β-cell loss after onset of clinical disease in order to maintain optimal blood glucose control to help prevent diabetes-related complications (DCCT 1993). The discovery that antibody titres are maintained after clinical onset initiated considerable interest over whether a relationship exists between residual β-cell function and humoral autoimmune markers. However, studies investigating the association of antibodies at diagnosis with temporal changes in β-cell function and insulin dose requirement after clinical onset of T1D have revealed conflicting results. For the most persistent autoantibody markers, GADA and IA-2A, some studies report no correlation of autoantibody positivity and/ or titre at or after diagnosis with β-cell function (C-peptide secretion) or insulin dose, whilst others report correlations (Petersen *et al.* 1994; Yokota *et al.* 1996; Hoeldtke *et al.* 1997; Jaeger *et al.* 1997; Ortqvist *et al.* 1997; Bonfanti *et al.* 1998; Decochez *et al.* 2000a; Torn *et al.* 2000; Scholin *et al.* 2004). In many of these reports, the relative importance of GADA or IA-2A as predictors of clinical course after diagnosis is also

debated. In addition, not only the type or titre of any one autoantibody could be important, but also the number of autoantibodies an individual possesses at diagnosis appears to be predictive of a more aggressive clinical course in children as assessed by C-peptide levels, insulin dose requirements and rate of remission (Sabbah *et al.* 1999; Scholin *et al.* 2004).

The clinical utility of autoantibody measurements in the post-diagnosis period of LADA is also unclear. In studies of small numbers of LADA subjects (n < 55), it has been found that higher GADA titres at diagnosis predict a more rapid decline in β-cell function in the post-diagnosis period (Gottsater *et al.* 1995; Kasuga *et al.* 1999; Borg *et al.* 2001; Lohmann *et al.* 2001; Borg *et al.* 2002; Yang *et al.* 2005). At variance with these studies, a recent report suggested that extremely high GADA titres in adult autoimmune diabetes associate with slowly progressing disease; patients with elevated titres had a lower frequency of insulin requirement and longer interval from clinical onset to initiation of insulin therapy compared with those with lower titres (Ishii *et al.* 2005). Also similar to that seen in juvenile-onset T1D, a study of adult-onset T1D that included probable LADA subjects, multiple autoantibody positivity at diagnosis predicted a greater decline in β-cell function post-diagnosis compared with the presence of a single autoantibody (Borg *et al.* 2001).

The clinical relevance of autoantibody persistence in the post-diagnosis period of childhood T1D has not been extensively investigated. This is understandable since in T1D, many children by diagnosis have reached the point of no return of β-cell loss and require life-long exogenous insulin treatment. However, if autoantibody status/ titre relates to underlying islet pathology (i.e. insulitis and residual β-cell reserve), knowledge of this information is particularly important in LADA since patients have a relatively slowly-progressing disease compared to childhood-onset T1D patients, and, by definition, do not immediately require insulin requirement. Therefore, identification of predictors/ indicators of therapy requirements in the post-diagnosis period could help in the clinical management of LADA.

In the UKPDS, patients with newly diagnosed clinical T2D were randomised to different glucose lowering therapies irrespective of autoantibody status. This, together with the population-based nature of patient recruitment provided a unique opportunity to examine objectively the relationship of autoantibody status/ titre to clinical characteristics and therapy decisions over time.

In the first investigation of antibody-positive UKPDS subjects, samples from 3,672 patients diagnosed clinically with T2D were measured for ICA and GADA at diagnosis (Turner *et al.* 1997). The prevalence of GADA was 10%, ICA 6% and 12% of subjects were ICA and/or GADA-positive. These patients with pancreatic autoantibodies were considered to have LADA (n = 440). Of the 3,672 patients studied, the clinical phenotype of 1,870 patients who had not been assigned to insulin therapy at randomisation in relation to autoantibody status was followed-up for 6 years (Turner *et al.* 1997). A key finding of this paper was that, compared with antibody-negative classical T2D patients, the LADA subset of the UKPDS had poor glycaemic control and rapidly progressed to insulin therapy; by 6 years post-diagnosis, 56% of subjects with ICA and 51% of those with GADA required insulin compared with only 4% of antibody negative classical T2D patients.

Davis *et al* extended these findings by examining the prevalence of autoantibodies (ICA/ GADA/ IA-2A) in T2D using a larger cohort of UKPDS subjects (n = 4,545) and evaluated their relationship to glycaemic control in the post-randomisation period of 10 years (Davis *et al.* 2005). LADA was defined by the presence of any one of ICA, GADA or IA-2A. In total, 526 patients with LADA were identified, comprising 11.6% of the subjects studied. Because UKPDS was a randomised clinical trial of therapies for diabetes, analyses of antibody positivity in relation to therapy were performed on an intention-to-treat basis. By UKPDS protocol, patients achieving FPG values between 6.0 and 14.9 mmol/l with no hyperglycaemic symptoms after the 3 month dietary run-in were randomised to conventional (diet) or intensive (oral agents or insulin) therapies whereas those subjects with FPG values ≥15 mmol/l or with

hyperglycaemic symptoms were randomised to intensive policy only (UKPDS Group 1991). The groups defining glycaemic control after the run-in period are referred to as 'randomisation arms'. Davis *et al* examined changes in clinical variables over time in LADA and compared them to those in antibody-negative subjects within each randomisation arm. Over the ten year period studied, a significant gain in weight of the LADA subjects compared with antibody-negative T2D subjects was observed in both groups, which was considered to be a consequence of more of these patients being on insulin treatment. Aside from this, there were no major differences[11] in clinical variables such as glucose measures, blood pressure and lipids in the LADA subjects compared with antibody-negative T2D subjects within either randomisation arm. It was also observed over the 10 year period that the degree of deterioration of glucose control over time (indicated by HbA_1c) in LADA patients and antibody-negative T2D subjects was similar irrespective of their specific allocated therapy within either randomisation arm. These findings suggest that since the glycaemic control of LADA patients over time is comparable to that of antibody-negative subjects in a given randomised therapy group, they can be initially treated with sulphonylurea therapy. However, in this study, it was shown that they were more likely to progress to insulin requirement than antibody-negative classical T2D patients, corroborating the findings of Turner *et al* (Turner *et al.* 1997).

In the study of Davis *et al*, the relationship of autoantibody status and glycaemic response was performed on an intention to treat basis and *actual* therapy changes were not investigated (Davis *et al.* 2005). Therefore, it is not known whether antibody status or titre in the post-diagnosis period affects the rate of progression through the UKPDS protocol; that is, the requirement of more intensive treatment for maintenance of blood glucose control as patients progressed through different treatment modalities of the UKPDS protocol. Since GADA are the most prevalent antibodies in LADA, they are currently considered the marker of choice for diagnostic purposes (Falorni and Brozzetti 2005), and are the most specific marker

[11] The only other significant finding was an increase in HDL cholesterol in LADA vs. antibody-negative subjects in achieving FPG values between 6.0 and 14.9 mmol/l after the dietary run-in period.

for rapid loss of β-cell function (Davis *et al.* 2005). In the present study, the clinical relevance of GADA in the post-diagnosis period was investigated further in relation to progression through the UKPDS protocol. Since progression was dictated by deterioration in blood glucose control, the need for more intensive therapy indirectly acted as marker for diminishing β-cell reserve. This indirect marker had to be used since C-peptide measurements were not made in the UKPDS.

In this study, 441 GADA-positive UKPDS patients were studied, which were a subset of the 526 subjects considered to have LADA reported by Davis *et al* (Davis *et al.* 2005). GADA was measured in plasma samples taken at 0.5 year, 3 years and 6 years post-diagnosis. Our aims were to evaluate in the post-diagnosis period:

- Whether GADA persists and if fluctuations in titre occur over time.
- Whether GADA titre relates to clinical phenotype.
- Whether GADA can be used a marker for deterioration of blood glucose control as determined by progression through the UKPDS protocol and the eventual requirement for insulin therapy.

3.1.3 IA-2A in LADA

In LADA, low prevalences of autoantibodies to insulinoma-associated antigen 2 (IA-2A) (Kasuga *et al.* 1997; Tuomi *et al.* 1999; Gambelunghe *et al.* 2000; Hawa *et al.* 2000; Grasso *et al.* 2001; Lohmann *et al.* 2001; Bottazzo *et al.* 2005; Davis *et al.* 2005) and antibodies to insulin (Kasuga *et al.* 1999; Soriguer-Escofet *et al.* 2002) suggest that these markers at onset have little clinical diagnostic value in LADA, in contrast to that observed in childhood T1D (Hawa *et al.* 1997; Savola *et al.* 1998a; Ziegler *et al.* 1999; Lohmann *et al.* 1997; Decochez *et al.* 2002). In the UKPDS, knowledge of IA-2A status at diagnosis did not significantly improve prediction of early insulin requirement when ICA and GADA results were available (Bottazzo *et al.* 2005). In the present study we also sought to investigate whether IA-2A positivity at

diagnosis in our GADA-positive LADA cohort affected rate of progression through the UKPDS protocol. In our cohort of GADA-positive patients, we sought to determine whether:

- IA-2A positivity at diagnosis is related to GADA titre at 0.5 years.
- IA-2A positivity at diagnosis in GADA-positive individuals is predictive of progression through the UKPDS protocol in the post-diagnosis period.
- IA-2A positivity at diagnosis in GADA-positive individuals is predictive of requirement for insulin therapy post-diagnosis.

3.2 SUBJECTS AND METHODS

3.2.1 Patients

We studied 443 patients from the UKPDS originally described as GADA-positive at diagnosis by JDF 1995 workshop criteria (Rowley *et al.* 1992). This cohort will be referred to as the 'JDF 1995 GADA-positive cohort' as reported in Davis *et al*; this was a subset of the 526 UKPDS patients considered to have LADA on the basis of antibody positivity for GADA, ICA and/or IA-2A (Davis *et al.* 2005). Plasma samples of these patients taken at 0.5 year[12], 3 year and 6 years post-diagnosis were measured for GADA.

3.2.2 Antibody measurement

GADA measurement. As described in detail in Chapter 2, plasma GADA titres were determined by a radiobinding assay that employed recombinant GAD65 labelled with ^{35}S-methoinine as bait, generated by the TnT *in vitro* transcription/ translation system. Antibody concentrations were calculated in each experiment from a standard curve constructed using a serum from a T1D patient: GADA titre of the serum had been standardised against the WHO standard serum provided in the DASP 2003 workshop (Appendix 2). Therefore titres are

[12] Plasma samples at diagnosis were not available for this study so GADA measurements were made at 0.5 years post-diagnosis, since this the closest next available time point for which samples were available.

presented as WHO Units/ ml. The cut-off for positivity was taken as 15 WHO Units/ ml (Appendix 2).

Some samples had titres above that of the top standard (467.5 WHO units/ml) and did not dilute back onto the standard curve in a linear fashion. These samples were therefore considered to have titres above the 'assay limit'. To identify whether those patients with titres above the assay limit were different from those patients with titres within the assay range, clinical characteristics at diagnosis of these two groups were compared. No significant differences were found which would give reason to divide the cohort according to this arbitrary and methodological criterion (Appendix 5).

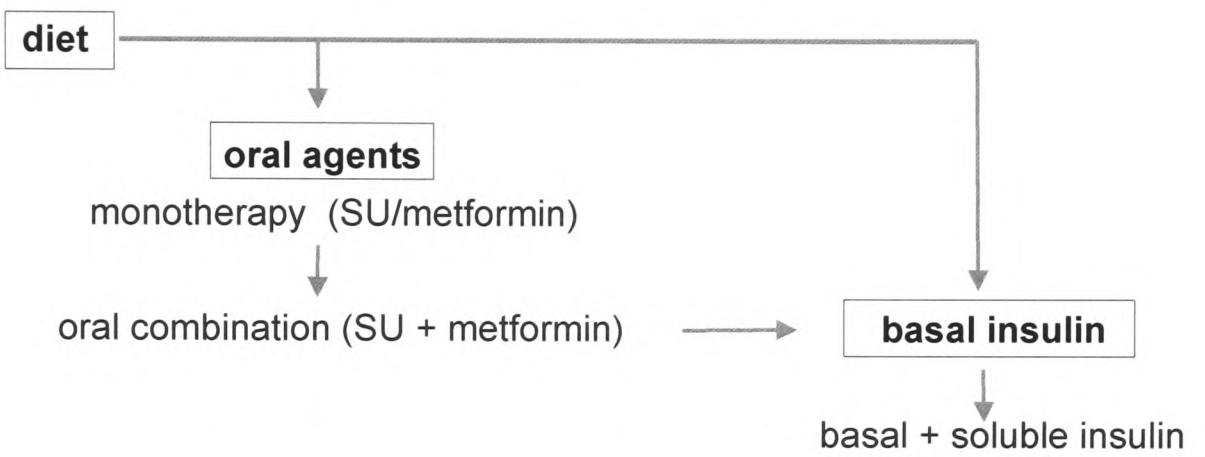
IA-2A measurement. Plasma samples taken at diagnosis had been measured in the UKPDS cohort previously for IA-2A (Bottazzo *et al.* 2005).

3.2.3 Relation of GADA to progression through the UKPDS protocol

The UKPDS patient selection criteria and study design was described in Chapter 2, section 2.1.1. Since C-peptide had not been measured in the UKPDS, it was difficult to evaluate changes in insulin secretory capacity. However, the UKPDS had very well defined criteria for increasing therapy in response to deterioration of glucose control. The progression to a more intensive anti-hyperglycaemic therapy was therefore utilised to estimate decreasing β-cell function/ mass. In the UKPDS study, patients that were initially allocated to diet therapy alone were subsequently randomised either to sulphonylurea, metformin or insulin if their FPG rose > 15.0 mmol/l or if they developed hyperglycaemic symptoms (Figure 3.1). Patients allocated to sulphonylurea either initially or after allocation to diet, were given additional metformin if their FPG rose > 15.0 mmol/l on maximally tolerated doses. Similarly, patients allocated to metformin were given additional sulphonylurea if their FPG rose > 15.0 mmol/l on maximally tolerated doses. Patients on oral combination therapy whose FPG rose > 15.0 mmol/l were given basal insulin therapy. Those on basal insulin therapy whose rose > 15.0 mmol/l were

given soluble insulin therapy. For the purposes of this analysis, patients were considered to have progressed one step through the protocol, if their glycaemic control had deteriorated (FPG >15 mmol/l) such that they required additional anti-hyperglycaemic therapy. Figure 3.1 describes how patients could progress through the protocol. Patients were classified as 'progressors' or 'non-progressors' at each time point studied according to whether or not their actual therapy at the previous time point had needed to be intensified.

Figure 3.1: Progression steps through the UKPDS protocol



A progression step could be: diet → oral agent or insulin, oral monotherapy → oral combination and basal insulin → basal insulin + soluble insulin.

3.2.4 Statistical methods

Since the UKPDS database is only available to designated UKPDS statisticians, Dr Carole Cull (Diabetes Trials Unit, OCDEM) performed the statistical analyses included in this chapter using SAS versions 6.12 and 9.1.3 (SAS Institute, North Carolina, USA). Data are reported as mean (±SD), geometric mean (±1 standard deviation interval), median (interquartile range) or percentages according to the distribution characteristics of the data. Two-sample t-tests or Wilcoxon sign-rank tests were used to compare continuously distributed data according to normality of data distributions. Chi-squared or Fisher's exact tests were used for categorical comparisons, i.e. to compare groups of patients. Spearman's rank coefficients were calculated to measure correlation between two continuous variables. A logistic regression analysis of the probability of progression by 0.5, 3 and 6 years was used to compare progression/ insulin

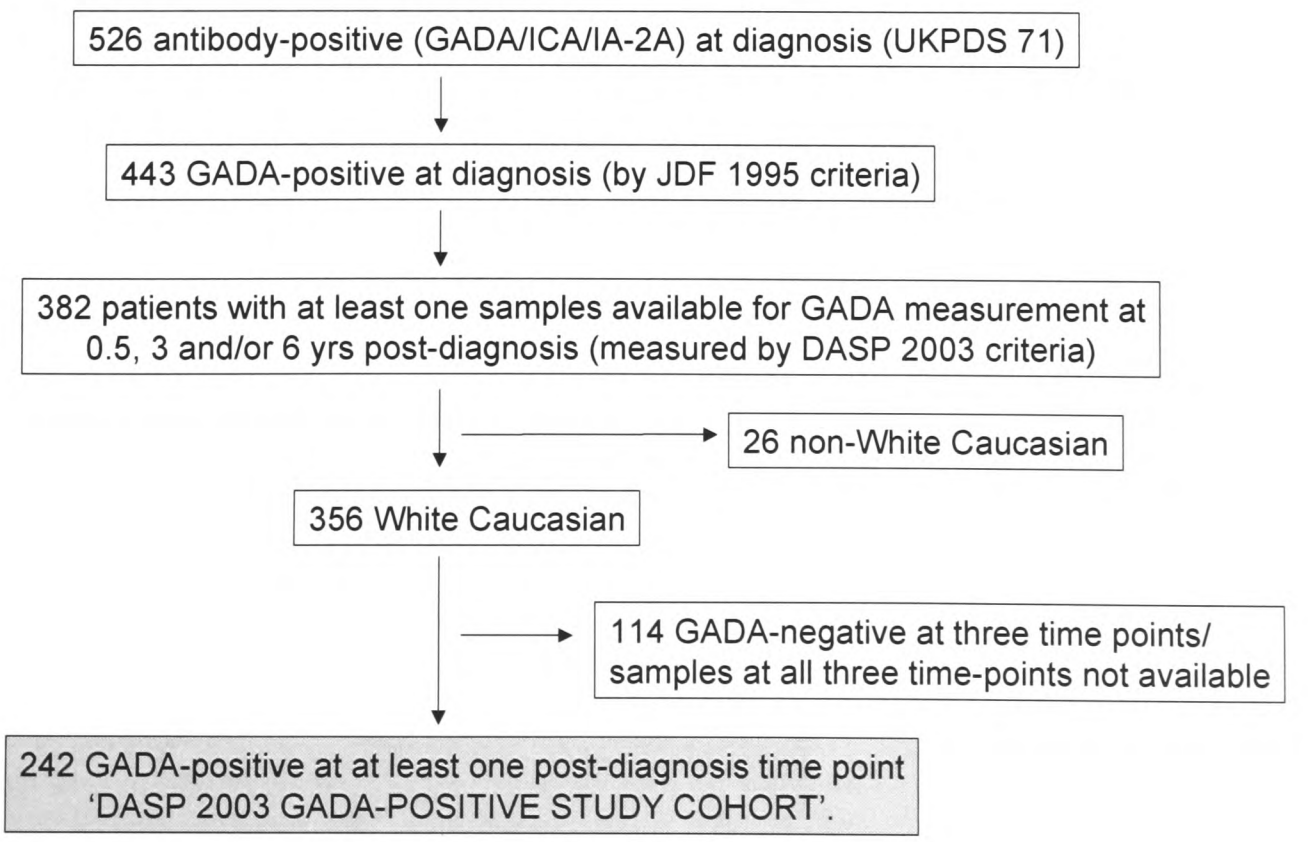
requirement in relation to GADA titre with patients classified according to thirds of autoantibody titre. A conventional significance of $p < 0.05$ was used throughout.

3.3 RESULTS

3.3.1 Establishing the study cohort

Figure 3.2 summarises the process of selection of the GADA-positive cohort used in this study (referred to as the 'DASP 2003' GADA-positive cohort). Plasma samples from the 443 patients identified as GADA-positive at diagnosis by JDF 1995 criteria for positivity were assessed for GADA positivity by DASP 2003 criteria at 0.5 years, 3 years and 6 years post-diagnosis, subject to availability. 382 subjects had samples available for GADA measurement at at least one of these time points. Of these, 356 patients were of White Caucasian origin. Patients of other races (n = 26) were excluded from further analysis. Of the White Caucasian patients, 266 were GADA-positive at the 0.5 year time point, 250 at the 3 year time point and 244 at the 6 year time point. However, not all patients had samples available for GADA measurement at all time points. A total of 242 patients had samples available and were GADA-positive at one or more time points. These patients formed the cohort studied longitudinally in this investigation – called the 'DASP 2003 GADA-positive cohort' (Figure 3.2).

Figure 3.2: Selection of GADA-positive cohort studied.



3.3.2 Clinical characteristics of patients in the DASP 2003 GADA-positive cohort

To determine whether the smaller cohort of 242 GADA-positive patients studied longitudinally (DASP 2003 GADA-positive cohort) were representative of the larger JDF 1995 GADA-positive cohort (n = 443), clinical characteristics at diagnosis of White Caucasian individuals in the two groups were compared with one another and with those of White Caucasian antibody-negative T2D patients in the UKPDS cohort (n = 3619, Table 3.1). As expected, the DASP 2003 GADA-positive cohort contained individuals who were significantly younger, lighter, had higher fasting plasma glucose (FPG), HbA_1c and insulin sensitivity (HOMA %S, (Matthews et al. 1985)) values but lower β-cell function (HOMA %B, (Matthews et al. 1985)) and blood pressure at baseline compared with those in the GADA-negative T2D cohort (Table 3.1). The distribution of age, sex, and HOMA %S values at diagnosis of the two GADA-positive cohorts were similar (Table 3.1). However, patients in the DASP 2003 GADA-positive cohort had poorer glycaemic control and fewer features of the metabolic syndrome (according to the NCEP ATP III definition, (Expert panel on detection 2001) and they were significantly lighter, had lower systolic blood pressure and plasma triglycerides compared with the JDF 1995 GADA-positive cohort. In addition, the β-cell secretory capacity of patients in the DASP 2003 GADA-positive cohort, indicated by the HOMA %B value, was significantly lower than that of patients in the JDF 1995 GADA-positive cohort.

In summary, comparison of clinical characteristics show that the DASP 2003 cohort of 242 GADA-positive subjects represented a more precise identification of LADA patients compared with the JDF 1995 GADA-positive cohort. The DASP 2003 GADA-positive cohort was investigated further in this chapter. Firstly, the persistence of GADA and changes in titre over time in the post-diagnosis period was examined.

Table 3.1: Comparison of baseline clinical characteristics of White Caucasian UKPDS patients included in the DASP 2003 GADA-positive cohort with those GADA-positive by the JDF 1995 assay and those antibody-negative (T2D) for both JDF 1995 GADA and ICA.

Clinical characteristic at diagnosis	JDF 1995 GADA-positive	DASP 2003 GADA-positive	p-value[e]	JDF 1995 GADA and ICA negative (T2D)	p-value[f]
N	443	242		3619	
Age (years)[a]	48.0 (11.0)	47.0 (10.8)	0.18	53.6 (8.3)	<0.00001
Sex (% male)	53	53	0.80	58	0.12
FPG (mmol/L)[b]	10.5 (7.5 to 14.5)	11.4 (7.9 to 15.5)	0.049	8.1 (6.7 to 10.5)	<0.00001
HbA_1c (%) [b]	8.1 (6.5 to 10.6)	9.0 (7.0 to 10.7)	0.017	7.0 (6.0 to 8.4)	<0.00001
Weight (kg) [a]	70.7 (15.1)	68.1 (13.2)	0.000034	79.0 (16.3)	<0.00001
BMI (kg/m^2) [a]	25.0 (5.4)	23.8 (4.4)	<0.00001	28.1 (5.5)	<0.00001
Systolic BP (mm Hg) [a]	129 (19)	125 (17)	0.00021	137 (20)	<0.00001
Diastolic BP (mm Hg [a]	80 (10)	79 (11)	0.18	83 (11)	<0.00001
HOMA %B [b]	37 (20 to 63)	30 (15 to 58)	0.017	57 (36 to 85)	<0.00001
HOMA %S [b]	64 (44 to 93)	73 (48 to 101)	0.13	51 (36 to 74)	<0.00001
Plasma triglycerides (mmol/L) [c]	1.31 [0.78, 2.20]	1.19 [0.73, 1.95]	0.000097	1.63 [0.99, 2.69]	<0.00001
Metabolic syndrome (NCEP ATP III definition) [d], n(%)	147 (33%)	106 (44%)	0.036	2784 (77%)	<0.00001

Values are given as: [a] mean (SD), [b] median (IQR) and [c] geometric mean (1 SD interval). [d] NCEP ATP III definition with BMI >27 kg/m^2 male or >29 kg/m^2 female. [e] JDF 1995 GADA-positive cohort vs. DASP 2003 GADA-positive cohort using distribution tests for equality of means or Chi-squared goodness-of-fit tests. [f] DASP 2003 GADA-positive vs. JDF1995 GADA- and ICA negative (T2D) using two sample t-test or Wilcoxon Sign Rank test.

3.3.3 GADA persistence in the post-diagnosis period

GADA positivity was maintained in 93% of the 242 subjects in the DASP 2003 LADA cohort throughout the 6 year follow-up period studied (Table 3.2). After the 0.5 year time point, seroconversion to GADA positivity or GADA negativity among the cohort of 242 patients was rare and this collectively occurred in 6 patients (Table 3.2). Those patients who were not positive at every time point had very low titres and therefore the failure to detect a titre was probably due to low sensitivity.

Table 3.2: GADA status at each time point for the 242 patients studied longitudinally.

Status at 0.5 years	Status at 3 years	Status at 6 years	n
Positive	positive	positive	225
Positive	positive	negative	2
Positive	negative	negative	3
Positive	negative	positive	7
Negative	positive	positive	3
Negative	negative	positive	1
Negative	positive	negative	1

3.3.4 Changes in GADA titre during the post-diagnosis period

The overall titres of GADA at each time point were examined. The median [IQR] titre was decreased at the 3 year time point (199 [96 to 318]) compared with that at the 0.5 year (331 [134 to 674]) and 6 year (284 [107 to 518]) time points. Figure 3.3 illustrates that, although the overall distribution of GADA titres was lower at 3 years, those patients with high titres at 0.5 years post-diagnosis tended to remain high at 3 and 6 years post-diagnosis and those with low titres remained low. Fewer patients had very high titres at 3 years compared with that at 0.5 years and 6 years (Figure 3.3a and c).

To investigate fluctuations in titre, the change in GADA titre over time was analysed. A significant net decrease in titre by a median of 100 units/ml occurred between the 0.5 year and 3 year time-points ($p < 0.0001$, Table 3.3). Figure 3.4a shows that the decrease in titres between these time points occurred for most patients in the cohort and the extent of this loss in titre appeared to be positively correlated with the titre present at 0.5 years; a greater drop in titre was observed in those with the highest GADA titres at 0.5 years. However, it cannot be excluded that part of this effect might be accounted for by a regression to the mean effect.

The change in titre between the 0.5 year and 6 year time points was not significantly different ($p = 0.89$, Table 3.3). From Figure 3.3b, it can be seen that the majority of titres appear to have remained stable between these two time points. However, fluctuations in some individuals were seen as some titres increased whilst others decreased over time (Figure 3.4b), leading to a non-significant net change in titre between the 0.5 year and 6 year time points.

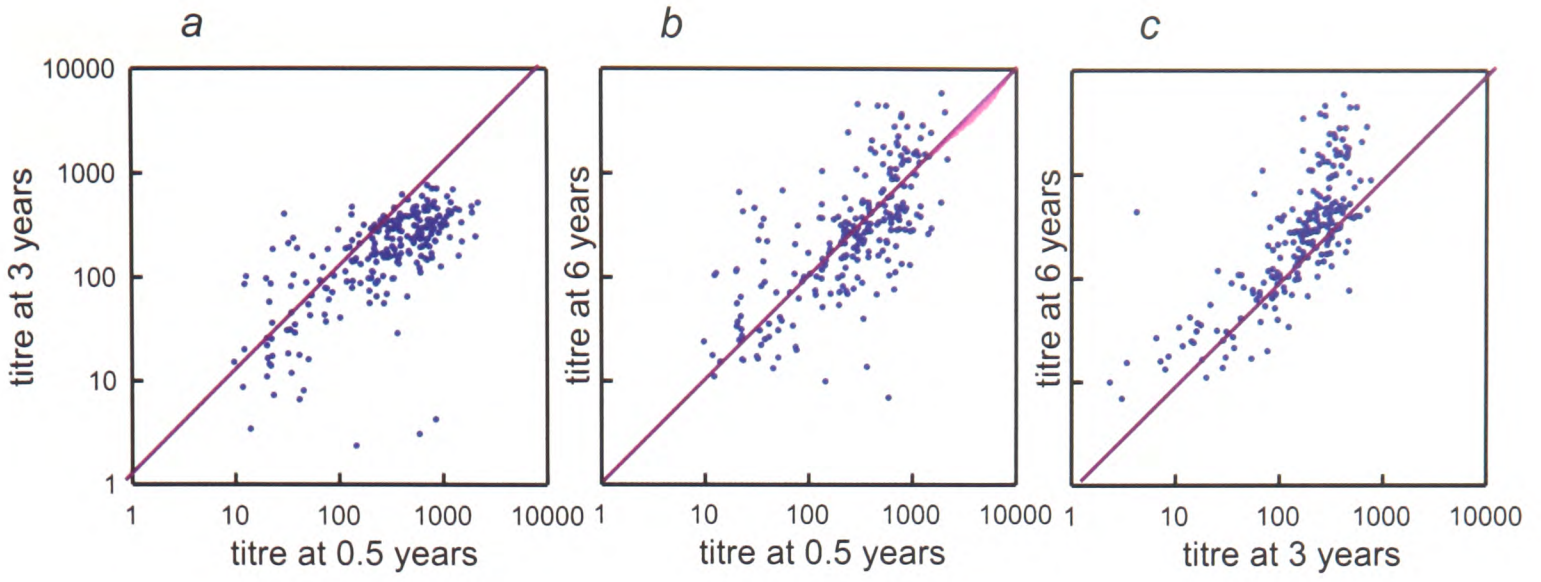
A significant net increase in titres by a median of 53 units/ml occurred between the 3 year and 6 year time points ($p < 0.0001$, Table 3.3). Figure 3.4c shows that this increase in titres of individual patients was less uniform across the group, with the majority of titres of individual patients remaining stable but others increasing or decreasing between these time points. The decrease in titre at 3 years was not related to assay methodology since repeatable data was obtained upon duplicate testing of samples.

Table 3.3: Change in GADA titre over 6 years post-diagnosis in the DASP 2003 GADA-positive cohort.

Time	Titre (WHO Units/ ml)	p-value
Change 0.5 year to 3 year	-100 (-381 to –1)	<0.0001
Change 0.5 year to 6 year	-3 (-138 to 150)	0.89
Change 3 year to 6 year	53 (-9 to 243)	<0.0001

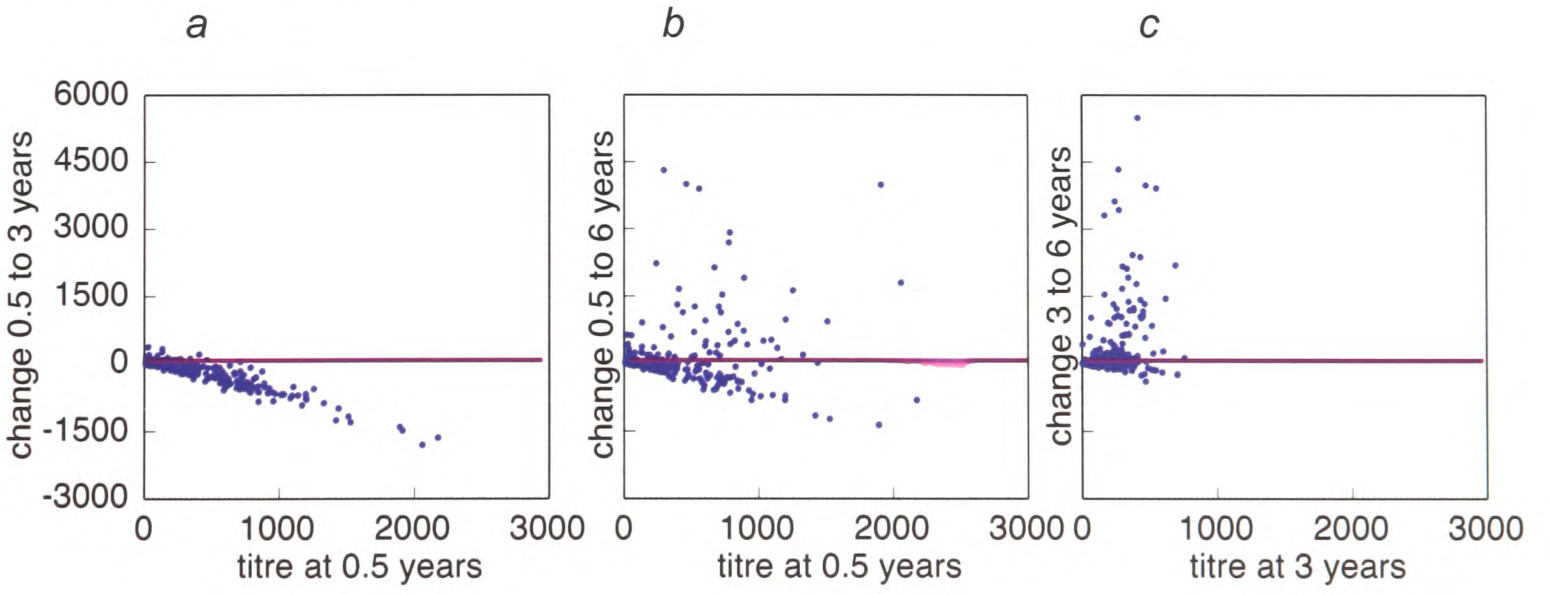
Data are given as median (IQR).

Figure 3.3: GADA titres over the 6 year post-diagnosis period studied in the DASP 2003 GADA-positive cohort



Dots represent individual patients studied longitudinally at 0.5 years, 3 years and 6 years post-diagnosis. (a) GADA titres of patients at 3 years post-diagnosis vs. those at 0.5 years post-diagnosis. (b) GADA titres of patients at 6 years post-diagnosis vs. those at 0.5 years post-diagnosis. (c) GADA titres of patients at 6 years post-diagnosis vs. those at 3 years post-diagnosis. Titres are given as WHO Units/ ml.

Figure 3.4: Changes in GADA titre over the 6 year post-diagnosis period studied DASP 2003 GADA-positive cohort



Dots represent individual patients studied longitudinally. (a) Change in GADA titre between 0.5 years and 3 years post-diagnosis vs. GADA titre at 0.5 years. (b) Change in GADA titre between 0.5 years and 6 years post-diagnosis vs. GADA titre at 0.5 years. (c) Change in GADA titre between 3 years and 6 years post-diagnosis vs. GADA titre at 3 years. Titres are given as WHO Units/ ml.

3.3.5 Relationship of GADA titre to clinical characteristics

It was investigated whether GADA titre was related to clinical features of patients in the DASP 2003 GADA-positive cohort. First, GADA titre at 0.5 years post-diagnosis was analysed in relation to clinical characteristics at diagnosis in the DASP 2003 cohort (Table 3.4). No significant associations were found between GADA titre at 0.5 years and age, FPG, HbA_1c, HOMA %B, HOMA %S, systolic BP or BMI at diagnosis. A significant association was found between 0.5 year titre plasma triglycerides, but the significance of this is unclear.

In addition, the proportion of patients with the metabolic syndrome at diagnosis was compared against GADA titre at 0.5 yrs. 27% of the DASP 2003 GADA-positive patients had metabolic syndrome at diagnosis (Table 3.1), and their GADA titre at 0.5 years was not significantly different from those who did not have the metabolic syndrome (median (IQR): 297 (117 to 613) vs. 356 (158 to 701), p = 0.15). Since the metabolic syndrome is a feature of classical T2D, this result suggests that GADA titre of LADA patients who had more 'T2D-like' features at diagnosis was not different to those who did not.

Table 3.4: Spearman rank correlation coefficients for GADA titre at 0.5 years with clinical characteristics at diagnosis for the 242 patients in the DASP 2003 GADA-positive cohort.

Clinical characteristic at diagnosis	**GADA titre at 0.5 years**	
	r_s	p-value
Age	0.10	0.11
FPG	0.027	0.68
HbA_1c	0.087	0.18
HOMA %B	-0.46	0.057
HOMA %S	0.032	0.67
BMI	-0.065	0.31
Systolic BP*	-0.12	0.069
Plasma triglycerides	-0.16	0.017

** Mean of –1 month and +6 month from randomisation. NB Spearman rank coefficients calculated as GADA titre vs. clinical characteristic studied.*

Secondly, a contemporaneous analysis was also performed where relation of GADA titre at each time point with clinical profile at that time point was investigated (Table 3.6). This was to determine whether changes in GADA titre over the post-diagnosis period associated with changes in clinical profile. A significant association of GADA titre at 0.5 years and plasma triglycerides at 0.5 years and was observed and a nominally significant association found between 6 year titre and plasma triglycerides at 6 years. No other significant associations with clinical characteristics were found.

Table 3.6: Spearman rank correlation coefficients for GADA titre and clinical characteristics at the at 0.5 year, 3 year and 6 year time points for patients in the DASP 2003 GADA-positive cohort.

Clinical characteristic	0.5 years		3 years		6 years	
	r_s	p-value	r_s	p-value	r_s	p-value
FPG	-0.12	0.063	0.12	0.058	0.082	0.22
HbA_1c	0.0044	0.95	0.085	0.19	0.0084	0.90
BMI	-0.044	0.50	-0.030	0.65	0.031	0.63
Systolic BP*	-0.11	0.10	-0.11	0.10	-0.13	0.046
Plasma triglycerides	-0.16	0.014	-0.062	0.35	-0.13	0.042

** 6 months post-randomisation. Spearman rank coefficients calculated as GADA titre vs. clinical characteristic studied.*

3.3.6 Relationship of titre to therapy requirement at each time point

As aforementioned, since C-peptide was not measured in the UKPDS, surrogate markers for loss of islet function were used. We first looked at therapy requirements and then progression through the UKPDS protocol (Section 3.3.7). The proportion of patients [n (%)] in the different allocated therapy groups at randomisation were diet: oral agents: insulin; 27 (11%): 129 (53%): 86 (36%). The proportion of patients in the three therapy groups at the subsequent time points studied are given in Table 3.7. There were significant changes in the proportion on each therapy between allocation (randomisation) and 0.5 years ($p = 0.0071$) because by the 0.5 year time point, UKPDS patients were given the therapy they actually required to maintain glycaemic control ('actual' therapy), rather than continue on randomisation therapy. Between the 0.5 years and 3 years time points, significant changes in the proportions of the different therapy groups occurred ($p < 0.0001$), primarily reflected by an increase in the proportion of patients requiring insulin by 3 years and a decrease of those who could be maintained on diet and oral agents (according to UKPDS protocol criteria). No

significant changes in therapy were observed between 3 years and 6 years post-diagnosis (p = 0.075) but again, a shift away from oral agents to insulin was seen.

GADA titre was not related to the actual therapy patients were taking at each of the three time points. Table 3.7 also shows that no significant differences in the titre of GADA were observed between patients in the three therapy groups (diet, oral agents, insulin) at each time point.

Table 3.7: Proportion and GADA titre of patients in each therapy group at the three different time points.

		Actual therapy at time point			
Time point		**Diet**	**Oral agents**	**Insulin**	**p-value***
0.5 years	N	48 (20%)	100 (41%)	94 (39%)	
	GADA titre[#]	302 (108, 662)	340 (116, 704)	340 (57, 626)	0.24
3 years	N	14 (6%)	71 (29%)	157 (65%)	
	GADA titre[#]	203 (85,300)	198 (91, 334)	205 (112, 305)	0.41
6 years	N	10 (4%)	52 (19%)	180 (74%)	
	GADA titre[#]	279 (104, 383)	250 (78, 788)	285 (118, 494)	0.73

*[#]Titres are given as median (IQR) unadjusted. *P-values have been adjusted for the following factors that may influence protocol defined therapy decisions: BMI, age, sex, smoking status, AHT, systolic BP, diastolic BP, plasma triglycerides, FPG, HOMA2 %B, HOMA2%S.*

3.3.7 Relationship of titre to progression within the UKPDS protocol

The clinical relevance of GADA in the post-diagnosis period was also assessed by determining the relationship of titre to rate of progression through the UKPDS protocol. Progression was defined as the per-protocol stepwise need for increasing anti-hyperglycaemic therapy when fasting blood glucose control deteriorated to > 15 mmol/l (refer to section 3.2.3).

The number of 'progressors' increased significantly between 0.5 years and 3 years (n = 101, p = 0.0037), but not between 3 years and 6 years (n = 32, p = 0.075). This lack of significant change between 3 and 6 years was because the majority of patients (65%) in the cohort had already moved on to insulin therapy by 3 years (Table 3.7). These changes in therapy requirements were those expected because of deteriorating blood glucose control over time in LADA patients as reported previously (Turner *et al.* 1997; Davis *et al.* 2005).

Firstly, the clinical characteristics of progressors and non-progressors were compared at all three time points. As expected, those patients progressing through the protocol by each time point had higher FPG ($p < 0.05$) and HbA_1c values ($p < 0.001$) compared with those who did not progress.

By the 0.5 year time point, those patients who had progressed since randomisation were also younger, had a lower BMI and HOMA %B had a lower prevalence of the metabolic syndrome compared with non-progressors (Table 3.8). In addition, progressors by 0.5 years post-diagnosis had higher overall GADA titres at this time point compared with non-progressors (median [IQR]: 437 [236 - 789] vs. 297 [104 - 626], p = 0.005) (Table 3.8). Moreover, logistic regression analysis showed that when the cohort was divided into thirds of increasing titre, individuals in the upper third were significantly more likely to have progressed through the protocol by 6 months compared with those in the lower third (OR [95% CI]: 3.0 [1.4 – 6.9], p=0.007, Table 3.9).

If GADA titres were related to deteriorating β-cell function/reserve, a difference in titre would be expected between progressors and non-progressors by each time point. However, at the 3 year and 6 year time points, no consistent significant differences in GADA titre were observed with respect to progression (Table 3.9).

Taken together, these results suggest that high GADA titre at 0.5 years was associated with progression through the UKPDS protocol by this time point. However, GADA titre was not found to be a significant predictor of progression through the UKPDS protocol at subsequent time points.

Table 3.8: Clinical characteristics of patients at randomisation and 0.5 years according to progression status

	Progressors	**Non-progressors**	**p-value**
N	56	186	
GADA at 0.5 years[a]	437 (236 to 789)	297 (104 to 626)	0.0054
Sex (% male)	53	54	0.960
Age (years)[b]	44.1 (10.8)	47.8 (10.7)	0.022
FPG at randomisation (mmol/L)[a]	14.3 (12.8 to 18.0)	9.4 (7.4 to 15.0)	<0.0001
FPG at 0.5 years (mmol/L)[a]	8.5 (5.7 to 11.3)	7.5 (6.3 to 9.1)	0.29
HbA_1c at randomisation (%)[a]	10.7 (9.7 to 12.1)	8.0 (6.5 to 10.2)	<0.0001
HbA_1c at 0.5 years (%)[a]	7.6 (6.6 to 8.9)	6.5 (5.8 to 8.0)	0.0046
HOMA2 %B at randomisation[c]	13 (7, 24)	33 (15, 76)	<0.0001
HOMA2 %S at randomisation[c]	72 (36, 142)	73 (39, 134)	0.93
BMI at randomisation (kg/m^2)[b]	22.1 (3.2)	24.3 (4.5)	0.00077
BMI at 0.5 years (kg)[b]	23.5 (3.4)	24.5 (4.5)	0.12
Systolic BP at randomisation (mm Hg)[b]	122 (18)	126 (17)	0.095
Systolic BP at 0.5 years (mm Hg)[b]	122 (18)	127 (18)	0.10
Plasma triglycerides at randomisation (mmol/L)[c]	1.21 (0.70, 2.08)	1.19 (0.74, 1.91)	0.84
Plasma triglycerides at 0.5 years (mmol/L)[c]	0.95 (0.61, 1.50)	1.09 (0.68, 1.73)	0.074
Metabolic syndrome (n(%))	5 (9%)	56 (30%)	0.0014

[a]median (IQR). [b]mean (SD). [c]geometric mean (1SD interval).

Table 3.9: Logistic regression analysis to assess the of probability of progression within the UKPDS protocol at the three time points in relation to GADA titre in the DASP 2003 GADA-positive cohort.

Titre third*	Progression time	N progressing/ N in third	Adjusted for allocated therapy	
			Odds ratio (95% CI)	p-value
0.5 year titre				
15 to 208	0.5 years	10/79	1.0	
208 to 532		20/80	2.3 (1.0 - 5.4)	0.047
≥ 532		36/83	3.0 (1.4 - 6.9)	0.007
15 to 208	3 years	48/79	1.0	
208 to 532		51/80	1.1 (0.6 - 2.2)	0.67
≥ 532		58/83	1.4 (0.7 - 2.8)	0.27
15 to 208	6 years	59/79	1.0	
208 to 532		64/80	1.4 (0.7 - 3.0)	0.39
≥ 532		66/83	1.2 (0.6 - 2.6)	0.61
3 year titre				
15 to 142	3 years	46/80	1.0	
142 to 288		58/79	2.3 (1.1 - 4.3)	0.024
≥ 288		53/83	1.3 (0.7 - 2.5)	0.38
15 to 142	6 years	58/80	1.0	
142 to 288		66/79	2.1 (0.9 - 4.6)	0.068
≥ 288		65/83	1.4 (0.7 - 2.9)	0.37
6 year titre				
15 to 158	6 years	61/80	1.0	
158 to 403		62/80	1.1 (0.5 - 2.4)	0.73
≥ 403		66/82	1.3 (0.6 - 2.8)	0.50

**Data are GADA titre (WHO units/ ml).*

3.3.8 Association of GADA titre with progression to insulin requirement

The final stage of progression through the UKPDS protocol is clinical requirement for insulin therapy, which was the most intensive therapy available. The predictive value of GADA titre for future insulin requirement among the 156 patients not randomised to insulin in the cohort of 242 patients was also ascertained. The proportion of these patients on insulin therapy by 0.5 years, 3 years and 6 years was 16%, 51% and 86%. The proportion of patients requiring insulin therapy at each time point was not related to GADA titre (Table 3.10).

Table 3.10: Logistic regression analysis to assess of probability of requiring insulin in the post-diagnosis period in relation to GADA titre in the DASP 2003 GADA-positive cohort. (Subjects included in this analysis were not randomised to insulin, n = 156).

Titre third*	Insulin requiring by	N requiring insulin/ N in third	Adjusted for allocated therapy	
			Odds ratio (95% CI)	p-value
0.5 year titre				
15 to 214	0.5 years	4/51	1.0	
214 to 581		10/51	3.3 (1.0 - 11.2)	0.057
≥ 581		11/54	2.6 (0.8 - 8.9)	0.13
15 to 214	3 years	23/51	1.0	
214 to 581		25/51	1.2 (0.5 - 2.6)	0.66
≥ 581		31/54	1.6 (0.7 - 3.4)	0.25
15 to 214	6 years	30/54	1.0	
214 to 581		32/54	1.2 (0.5 - 2.6)	0.68
≥ 581		39/54	1.8 (0.8 - 4.1)	0.16
3 year titre				
15 to 142	3 years	24/52	1.0	
142 to 289		30/50	1.8 (0.8 - 4.0)	0.14
≥ 289		25/54	1.0 (0.5 - 2.2)	0.94
15 to 142	6 years	30/52	1.0	
142 to 288		34/50	1.6 (0.7 - 3.6)	0.27
≥ 288		37/54	1.6 (0.7 - 3.6)	0.24
6 year titre				
15 to 158	6 years	31/52	1.0	
158 to 403		36/50	1.8 (0.8 - 4.1)	0.17
≥ 403		34/54	1.2 (0.5- 2.6)	0.66

**Data are GADA titre (WHO Units/ ml).*

3.3.9 Relationship of IA-2A positivity at diagnosis in the DASP 2003 GADA-positive cohort with clinical characteristics

It has been suggested that multiple autoantibody positivity at diagnosis of T1D associates with a faster decline of β-cell function (Sabbah *et al.* 1999). Therefore, the relationship of dual positivity for IA-2A and GADA in the DASP 2003 cohort with clinical progression was examined. Firstly, the relationship of IA-2A status at diagnosis with GADA titre at 0.5 years was investigated. A total of 59 subjects of the 242 patients in the DASP 2003 cohort were IA-2A positive at diagnosis. IA-2A positivity at diagnosis did not correlate with GADA titre at 0.5 years (Table 3.11).

Table 3.11: Relationship between IA2A status at diagnosis and GADA titre at 0.5 yrs in the DASP 2003 GADA-positive cohort.

IA-2A status at diagnosis	**0.5 year GADA titre (thirds)**			***p-value**
	Lower	**Middle**	**Upper**	
positive	13 (22)	27 (46)	19 (32)	0.36
negative	66 (37)	49 (27)	64 (36)	

*Data are n (% IA-2A status). * Cochran-Armitage Trend test.*

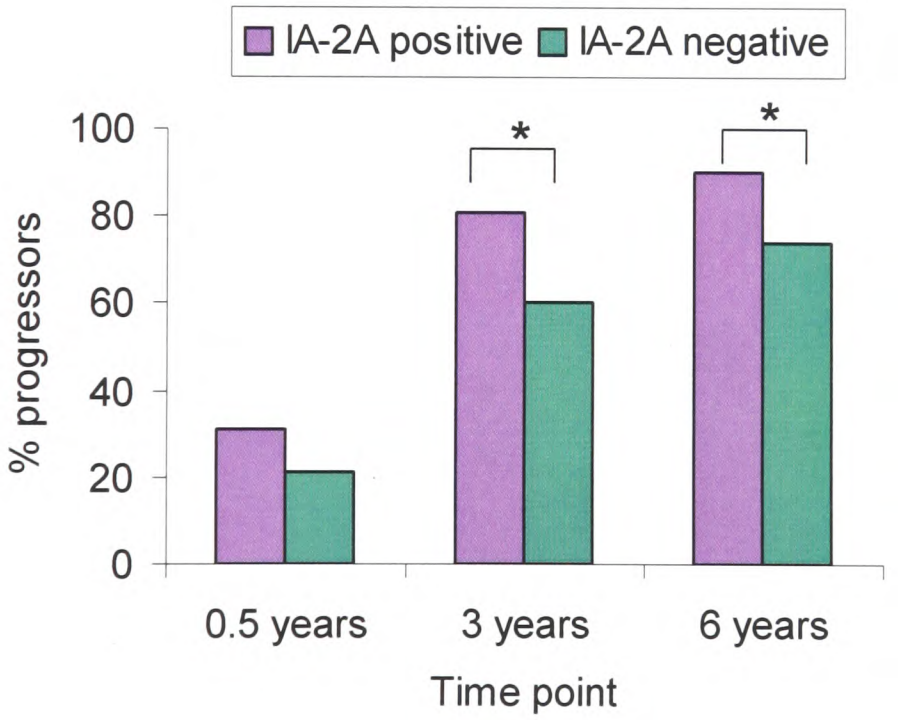
IA-2A positivity at diagnosis in the DASP 2003 GADA-positive cohort was significantly associated with progression through the protocol by 3 years and 6 years ($p = 0.0027$ and $p = 0.011$, respectively, Table 12). These results are depicted in Figure 3.5.

Table 3.12: Relationship of IA-2A positivity at diagnosis to progression through the UKPDS protocol for the DASP 2003 GADA-positive cohort.

IA-2A status at diagnosis	Time point	Progressors	Non-progressors	p-value*
Positive	0.5 year	18 (31)	41 (69)	0.12
Negative		38 (21)	145 (79)	
Positive	3 year	48 (81)	11 (19)	**0.0027**
Negative		109 (60)	74 (40)	
Positive	6 year	53 (90)	6 (10)	**0.011**
Negative		136 (74)	47 (26)	

*Data are n (% IA-2A status). *Chi-square analysis*

Figure 3.5: Proportion of patients progressing through the protocol by the three time points according to IA-2A status at diagnosis in the DASP 2003 GADA-positive cohort:



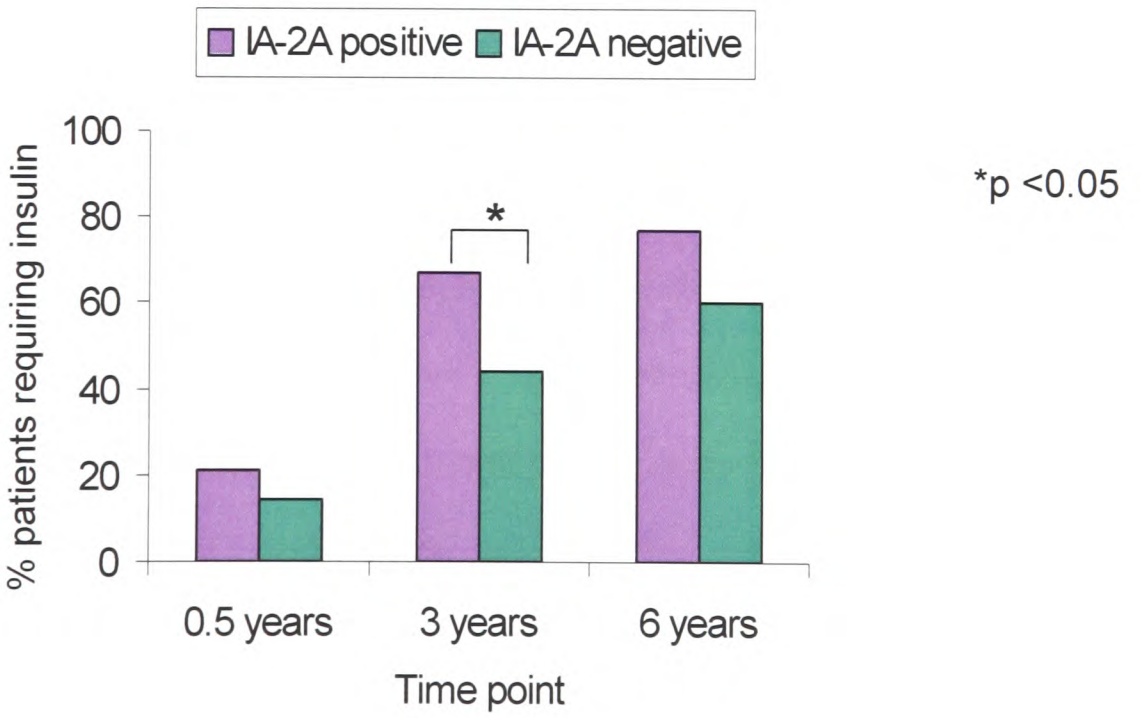
IA-2A positivity at diagnosis in the DASP 2003 GADA-positive cohort was significantly related to requirement for insulin by 3 years (p = 0.0096, Table 3.13) taking into account allocated therapy, but only nominal significance was obtained for insulin requirement by 6 years (p = 0.051, Table 3.13). These results are also depicted in Figure 3.6.

Table 3.13: Relationship of IA-2A status at diagnosis to requirement for insulin for the 156 patients not randomised to insulin among the 242 GADA-positive patients in the DASP 2003 cohort.

IA-2A status	Time point	Insulin requiring	Non-insulin requiring	p-value*
positive	0.5 year	9 (0.21)	34 (0.79)	0.30
negative		16 (0.14)	97 (0.85)	
positive	3 year	29 (0.67)	14 (0.33)	**0.0071**
negative		50 (0.44)	63 (0.56)	
positive	6 year	33 (0.77)	10 (0.23)	0.051
negative		68 (0.60)	45 (0.40)	

*Data are n (% IA-2A status). *Cochran-Mantel-Haenszel test for association adjusting for allocated therapy.*

Figure 3.6: Proportion of patients requiring insulin therapy by the three time points according to IA-2A status at diagnosis in the DASP 2003 GADA-positive cohort:



3.4 DISCUSSION

The DASP 2003 cohort. The clinical characteristics at diagnosis of the current cohort of 242 GADA-positive subjects suggested that this group was a more precise identification of LADA patients compared with those present in the larger GADA-positive (JDF 1995) cohort previously reported (Davis *et al.* 2005). The DASP 2003 cohort contained fewer individuals with features of the metabolic syndrome, which is more characteristic of classical T2D patients. In agreement with this, other studies have shown that LADA subjects possess fewer features of the metabolic syndrome (Tuomi *et al.* 1999; Hosszufalusi *et al.* 2003). A female preponderance of GADA positivity was not seen in our cohort although this has been reported to occur in T1D (Hagopian *et al.* 1995).

Discrepancies were observed concerning GADA status of the JDF 1995 and DASP 2003 GADA-positive cohorts (114 patients were negative by DASP 2003 at 0.5 years but positive by JDF 1995 at diagnosis). These differences were probably due to assay methodology. The GADA measurements made in the JDF 1995 cohort were made using native porcine GAD 65 extracts as antigen (Rowley *et al.* 1992), whereas human recombinant GAD 65 was used in the present study. At the 2nd international GADA workshop (Schmidli *et al.* 1995), the use of animal tissue for preparation of radiolabelled tracer GAD 65 was demonstrated to be inferior to recombinant GAD 65. Therefore, the more accurate identification of patients with LADA included in the DASP 2003 cohort compared with the JDF 1995 cohort was likely due to improvements in assay performance over the last 10 years. It is important to note that the GADA status of the 0.5 year samples in the DASP 2003 cohort agreed with measurements made in these samples in an independent laboratory (Polly Bingley, Bristol University).

Persistence of GADA and fluctuations of titre in the DASP 2003 cohort. This is the first large, longitudinal study to highlight the differences in longevity of GADA positivity in LADA. GADA titres were found to persist for up to 6 years post-diagnosis. As found in T1D, seroconversion

was associated with low level GADA (Hampe *et al.* 2002a). Generally, the titre found at diagnosis, high or low was maintained at 6 years post-diagnosis. In agreement with our results, Borg *et al* 2002 reported that prevalence and titres of GADA were maintained (for up to 12 years) in their study of LADA patients.

Persistence occurred despite the suggestion that there were low existing levels of β-cell antigen as indicated by HOMA %B values at diagnosis. This is similar to that reported in classical juvenile-onset T1D (Christie *et al.* 1990a). The reason why GADA are maintained following clinical onset of disease is unknown. The greater persistence of these autoantibodies in adults compared with young children could be due the larger proportion of β-cell mass that has been found to be retained up to 30 years post-diagnosis in adult-onset patients with autoimmune diabetes (Pipeleers and Ling 1992). Slower pancreatic β-cell destruction in LADA compared to T1D could increase the time over which antigen presentation occurs (Davis *et al.* 2000). Indeed, residual β-cell function has been reported in a LADA case report (Shimada *et al.* 1999). Presumably, the retention of β-cells would prolong release of antigenic peptides and thus the stimulus to sustain the humoral response directed against GAD 65. Another reason for the persistence of GADA could be a greater impairment of antibody clearance in the post-diagnosis period in those developing autoimmune diabetes at older ages. Alternatively, extra-pancreatic sources of GAD 65 or cross-reactive exogenous antigens may maintain the humoral response against this enzyme. Environmental agents could maintain pre-existing immune processes. One such agent that has been implicated in T1D is the Coxsackie B4 virus. The sequence homology between P2-C protein of this virus and GAD 65 (Kaufman *et al.* 1992) suggests that this virus could induce and /or sustain the anti-GAD 65 humoral responses by molecular mimicry. However, the evidence implicating the role of this virus in autoimmune diabetes is currently controversial (reviewed by (Green *et al.* 2004; Filippi and von Herrath 2005)).

Similar to that reported in children with T1D (Batstra *et al.* 1997; Savola *et al.* 1998b), GADA titres in this study, were found to fluctuate over time in individual LADA patients after clinical onset; some titres increased with time, others decreased while others remained stable.

A uniform decrease in GADA titres was observed across the group between 0.5 years and 3 years post-diagnosis. Although there was a net significant increase in titre between 3 and 6 years post-diagnosis, this was due to a small proportion of patients showing quite remarkable increases in titre over this time period, whereas in the vast majority of patients the titres stabilised after the 3 year 'drop'. Our results are similar to those observed in T1D where the majority of studies show that titres generally decline over time after clinical onset in proportion to the titre found at diagnosis (Decochez *et al.* 2000b). However, GADA titre appears to be maintained for longer periods in LADA patients compared with classical T1D patients (Grasso *et al.* 2001).

The reason for dip in 3 year titre seen in this LADA cohort remains unknown. Re-measurement of these samples showed that this reduction in titre compared with 0.5 year and 6 year samples was real and did not appear to be an assay artefact. Also, no obvious differences in the storage of plasma samples collected at the three time points could realistically explain the uniform decrease in titres at the 3 year time point. This fall in titre by 3 years post-diagnosis coincided with the fact that most of the LADA cohort required insulin treatment by this time point (65%). The loss of titre may reflect a waning immune response against GAD 65 as the decline in β-cell function had reached an 'end-stage' by this time point.

Clinical relevance of GADA measurement in the post-diagnosis period. The persistence of GADA positivity over the 6 year post-diagnosis period indicates that this autoantibody may have important diagnostic utility for a long period of time in these autoimmune patients: LADA subjects may otherwise be clinically indistinguishable from those with classical T2D, since LADA patients are also diagnosed at adult age, can be obese and be able to maintain their glucose

levels adequately with diet/ oral agent therapy. Therefore, LADA can be diagnosed years after the onset of hyperglycaemia by means of GADA testing.

However, there is limited clinical relevance of this autoantibody in the post-diagnosis period in terms of association with clinical features or prediction of the rate of deterioration of blood glucose control. GADA titre at each time point did not correlate with clinical variables including blood glucose measures, BMI and blood pressure (Tables 3.4 and 3.5). Our findings do not support the contention proposed by Lohmann *et al* 2001 that LADA patients with high GADA titre have a more 'T1D-like' phenotype (reduced residual β-cell function, lower BMI and lower frequency of hypertension) and those with low titres a more 'T2D-like' phenotype (Lohmann *et al.* 2001; Li *et al.* 2004). Our findings are in agreement with results of a smaller study of Australian LADA subjects, where high GADA levels did not associate with younger age, shorter duration of disease, leaner body mass or dissimilar metabolic control compared to those with lower titres (Davis *et al.* 2000). Also similar to the findings of Davis *et al* (Davis *et al.* 2000), inverse correlations of GADA titre and plasma triglycerides were found in this study. This observation warrants further investigation since the low triglyceride levels in patients with high titre GADA may reflect a subgroup of patients that are relatively insulin sensitive, either as a function of reduced adiposity or as compensation for continuing β-cell destruction and insulin deficiency.

Since a direct measure of β-cell function, such as C-peptide level, was not made in the UKPDS, the requirement of additional anti-hyperglycaemic therapy by each time point (i.e. progression) was used as a surrogate to indirectly examine the relationship of deterioration in β-cell reserve over time with GADA titre. Those LADA patients who had high GADA titres at 0.5 years post-diagnosis were more likely to have undergone a step-wise progression of therapy by this time point. However, progression through the UKPDS protocol between 0.5 year → 3 years → 6 years was not associated with GADA titre at these time points (Table 3.9). This suggests that

GADA titre is associated with an early deterioration in β-cell function post-diagnosis but not in the prediction of the slower long-term progression of disease (i.e. deterioration by 3 years or 6 years post-diagnosis).

No association of clinical requirement for insulin therapy specifically was observed with GADA titre (Table 3.10). This disagrees with results obtained in the Australian LADA study discussed above (Davis *et al.* 2000). Therefore, the results of this study do not provide any clinical indications for monitoring GADA autoantibodies after the diagnosis of diabetes aside from the diagnostic utility of this marker which could help improve therapy management decisions since LADA subjects are likely to rapidly progress to insulin requirement.

It must be remembered that LADA, like T1D, is likely to be a heterogeneous disease in terms of aetiological factors, progression rates, histopathological findings, severity of clinical symptoms and responses to therapy. Therefore, the lack of associations seen may be confounded by our inability to stratify patients according to these factors.

Combination of IA-2A and GADA positivity. In the DASP 2003 cohort, 59/ 242 (24%) of patients were positive for IA-2A at diagnosis. Similar prevalence of IA-2A positivity has been reported for GADA-positive LADA patients in other studies and this is lower to that found in classical T1D (Tuomi *et al.* 1999; Grasso *et al.* 2001). IA-2A positivity was not related to GADA titre in our cohort, in contrast to that seen in smaller studies of Austrian and Finnish LADA patients (Tuomi *et al.* 1999; Schernthaner *et al.* 2001). Generally, the presence of IA-2A at diagnosis, together with GADA positivity, appeared to mark a more aggressive autoimmune process since patients with this dual positivity were significantly more likely to progress through the protocol by 3 and 6 years post-diagnosis. They were also more likely to require insulin treatment by 3 years post-diagnosis. Similar findings of a more aggressive disease process indicated by loss of C-peptide levels in the presence of multiple antibodies have been reported for juvenile-onset T1D (Sabbah

et al. 1999) and in small studies of LADA patients (Lohmann *et al.* 2001; Borg *et al.* 2001; Monge *et al.* 2004).

Future directions. Since LADA patients with GADA represent a target group for interventions at diagnosis may that prevent further loss of β-cells post-diagnosis, it is important to identify factors that indicate/ predict the rate of this decline in these patients. Many LADA patients will not require insulin treatment for a considerable amount of time. The nature of the 'switch' that renders oral agent therapy inadequate in slowly-progressing autoimmune diabetes, is not known but it is possible that this could be a result of increased aggressiveness of the autoimmune response that diminishes the β-cell reserve to below a critical mass. Although associations with overall GADA titre were not observed in this study of LADA patients, a more informative approach could be to observe the temporal dynamics of sequential immune responses to newly arising GADA epitope specificities or subclasses. This would enable one to gain a better understanding of immune disease pathology in LADA and test whether these markers are more specific indicators of deteriorating glucose control and the subsequent need for more intensive therapy. Such investigations have been rare in the post-diagnosis period of T1D. However, one study has shown the potential clinical value of epitope testing in T1D; increasing levels of GADA to the middle region of GAD 65 associated with requirement for lower insulin doses post-diagnosis (Ronkainen *et al.* 2004). Perhaps a gain or loss in a certain epitope specificity would predict the imminent need for insulin therapy in LADA.

Chapter 4:

Association of the HLA gene region in LADA

CHAPTER 4: ASSOCIATION STUDY OF THE HLA GENE REGION WITH LADA

4.1 INTRODUCTION

The HLA gene region (*IDDM1*) is the major susceptibility locus in T1D (Todd 1999), and disease risk has been narrowed down to the *DRB1* and *DQB1* genes (Herr *et al.* 2000). Given the common underlying autoimmune pathology in T1D and LADA, these two genes are therefore also good candidate loci for LADA.

As described in Chapter 1 (Section 1.2.2.1), the main susceptibility effects at the *IDDM1* region in T1D are mediated by HLA haplotypes *DRB1*0401_DQB1*0302* and *DRB1*0301_DQB1*0201*. Over 90% of T1D patients have these haplotypes and they act synergistically, such that the *DRB1*0401_DQB1*0302-DRB1*0301_DQB1*0201* diplotype confers greater risk than either of the two haplotypes alone (Kockum *et al.* 1999).

Heterogeneity in disease risk in T1D has been attributed to different allelic subtypes of the DR4 antigen specificity, such that the risk of the *DRB1*04_DQB1*0302* haplotype is dependent on the *DRB1*04* allele present on it. In White Caucasians, *DRB1*0401*, **0402* and **0405* have been found to increase risk of developing T1D whereas **0403, *0406 and *0407* appear to confer relative protection (She 1996; Rewers *et al.* 2003).

The nature of the other 10% of haplotypes that predispose to T1D or have neutral effects is not clear-cut and associations have been found to differ between studies (as discussed in Chapter 1, Section 1.2.2.1). However, it is widely accepted that the main protective effect at the HLA gene region is mediated by *DRB1*1501_DQB1*0602*.

Susceptibility conferred from loci in the HLA gene region has not been comprehensively assessed in LADA. The UK Prospective Diabetes Study (UKPDS) study showed that the DR3/DR4 genotype was increased relative to nonDR3/nonDR4 in LADA compared to non-

diabetic controls (Horton *et al.* 1999). Also in this study, the *DQB1* alleles were divided crudely according to whether or not an aspartic acid residue was present at position 57. The nonasp/nonasp genotype was over-represented compared to asp/asp in LADA vs. controls (Horton *et al.* 1999). Furthermore, An age-related trend in the prevalence of the DR3/DR4 genotype was observed, with those patients diagnosed between 25-34 years of age more likely to possess this susceptibility determinant compared with those diagnosed between 35-44 years, 45-54 years or 55-65 years of age (Horton *et al.* 1999).

Aims of the current study

The previous investigation of the role of HLA genes in LADA was extended by typing a larger cohort of patients to a higher resolution than that done in the original UKPDS study. Antibody-positive patients misdiagnosed with T2D collected in the Warren 2, Exeter Young-onset T2D study and the Diabetes in Families study were combined with those in the UKPDS cohort and genotyped for HLA *DRB1* and *DQB1* variants to perform the largest association analysis of LADA to date.

The genetic analysis was undertaken in two stages in an exploratory manner to determine the nature of associations with LADA. Firstly, associations with broad serological (antigen) specificities were sought with LADA. Secondly, the resolution of typing analysis was increased to determine association with specific alleles within these serological specificities, according to the degree permitted by the typing method, and where statistically meaningful comparisons could be made. The objective of this analysis was to identify the nature of alleles/ genotypes/ haplotypes and diplotypes that were associated with susceptibility to LADA.

To examine how *IDDM1* susceptibility influences the pathophysiology of LADA, associations of the *DRB1* and *DQB1* loci were investigated in relation to:

- Age of diagnosis
- Requirement for insulin therapy
- Autoantibody status (dual autoantibody positivity [GADA + IA-2A] vs. GADA alone).

The aim of the current study was also to identify more precise associations of HLA genes with LADA and to make better comparisons with data obtained in classical T1D. We wanted to specifically answer the following questions:

- At *DRB1*, are the *DRB1*03* and *DRB1*04* alleles the main susceptibility determinants in LADA as they are in T1D?
- At *DQB1*, are the *DQB1*0201* and *DQB1*0302* alleles the main susceptibility determinants in LADA as they are in T1D?
- Do the *DRB1*1501* and *DQB1*0602* alleles have protective effects in LADA as occurs in T1D?
- Is there a differential risk in allelic subtype of the DR4 antigen specificity as seen in T1D? Specifically, are **0401/*0405* predisposing for and **0403/*0406/*0407* protective against LADA?

4.2 SUBJECTS AND METHODS

4.2.1 Subjects

Genotypic data from LADA subjects from the UK Prospective Diabetes Study (UKPDS, n = 226), Diabetes in Families study (DIF, n = 55), Warren 2 consortium (W2, n = 135) and Exeter Young-Onset T2D study (YT2D, n = 37) were compared with 341 non-diabetic controls from the DIF study. Refer to Table 2.2 in Chapter 2 for patient characteristics. In addition, 13 parent-offspring trios containing a LADA proband from the W2 consortium were genotyped as one of the quality control measures.

4.2.2 Genotyping

The PCR-SSP technique was employed to genotype the cohorts for variants at the *DRB1* and *DQB1* genes (refer to Chapter 2, Section 2.4.2).

4.2.3 Statistical methods

Homogeneity testing. To ensure genetic homogeneity of the LADA groups, serological frequencies were compared between the case groups (UKPDS/ W2/ YT2D/ DIFL) for each gene by contingency table methods in StatXact 6 (Cytel Software, Cambridge, MA, USA). This was done to check that the cases collected from the various repositories came from the same underlying background population and therefore, whether their genotypes could be pooled and analysed as a single group. It was important to check for this since spurious associations may occur as a result of confounding by genetic subdivisions (population stratification). Such subgroups within the population could differ systematically in allele frequencies at many loci, giving rise to spurious associations. Given the presence of rare serological specificities and hence the problem of sparse contingency tables, Monte Carlo simulations were used to generate exact p-values.

Genotypic/ allelic association. Separate case data sets were combined for contingency table analyses only after appropriate homogeneity testing (see above). Genotype-wise and allele-wise p values were considered statistically significant when the p-value was <0.05. Global tests for differences in frequencies of cases and controls at each locus were performed using Monte Carlo simulation. Individual tests for association were only performed when the frequencies of alleles/ genotypes/ haplotypes/ diplotypes exceeded 5% in at least the case or control groups.

Tests for association by allele at each locus (*DRB1*, *DQB1*) (under the multiplicative genetic model) were performed using StatXact 6. Genotypes containing alleles that showed significant evidence of association with disease were tested for association with disease status under the dominant, recessive and additive models of inheritance. If the allele of interest at each locus is denoted 1 and all other alleles at the locus grouped, and denoted as 2, the models were defined as follows. In the dominant model for allele 1, subjects who were carriers of allele 1 were compared with subjects with all other genotypes (i.e. 11 + 12 vs. 22). In the recessive model for allele 1, subjects homozygous for allele 1 were compared against subjects with all other genotypes (i.e. 11 vs. 12 + 22). To test for an additive effect, a test for a linear gradient across the genotypes (11 vs. 12 vs. 22) was performed using the Cochran-Armitage test for trend. Significance for the fit of all these models, ORs and 95% confidence intervals [CI] were calculated in StatXact 6. As appropriate, exact versions of these tests were used.

This analysis was considered exploratory and corrections for multiple testing were not applied.

Haplotype estimation. Haplotype analysis of the *DRB1* and *DQB1* genes was performed. The frequency of the 2-point haplotypes in our cases and controls was estimated by the expectation-maximisation algorithm (EM) using HelixTree®. The EM algorithm enables

calculation of haplotype frequency from genotypes using an iterative method of allele counting to obtain maximum likelihood (ML) estimates of haplotype frequencies. The EM algorithm was re-initialised three times to ensure convergence. Haplotypes with their associated probability distributions were assigned to individuals. Given the presence of extremely strong LD between the *DRB1* and *DQB1* genes and the consequent fact that most diplotypes were associated with a probability of >0.999 (97.9% of cases and 97.8% of controls), the problem of phase uncertainty was minimal. This justified selecting the most probable pair of haplotypes for diplotype analyses.

Haplotype association. Haplotype frequencies were compared between cases and controls by contingency table analysis in StatXact 6. Haplotypes with frequencies of less than 1% were grouped together. Exact p-values were calculated using Monte Carlo simulation.

Quantitative trait association. Association of age with HLA variants was assessed by arbitrary division of the cohort into two groups; those aged <45 years at diagnosis and those >45. Frequency differences between the two groups were determined using contingency table analysis. Association of requirement for insulin therapy by 6 years post-diagnosis and HLA variants was performed using contingency table analysis.

Association with autoantibody status. Association of autoantibody status (dual GADA + IA-2A positivity vs. GADA alone) with HLA variants was performed using contingency table analysis.

Notes on nomenclature used. In this chapter, serological specificities (antigens) are indicated by a two letter prefix (denoting the locus), followed by a number that represents the relevant antigen group in accordance with conventional nomenclature e.g. DR1 represents antigen specificity 1 at the *DRB1* locus. Alleles are presented in *italics*, prefixed by the gene name and an asterisk, followed by a 4 digit allele name e.g. *DRB1*0101* is allele **0101* at the

DRB1 gene. Some alleles within a serological group were impossible to distinguish from one another according to the resolution achieved in our SSP typing method. Such alleles are presented as groups separated by forward slashes or hyphens e.g. an individual with *DRB1*1101/04* may have either *DRB1*1101* or *DRB1*1104*, an individual with *DRB1*1501-06* may possess *DRB1*1501, DRB1*1502, DRB1*1503, DRB1*1504, DRB1*1505* or *DRB1*1506*. Genotypes are presented with a forward slash between alleles e.g. *DRB1*0301/DRB1*0401*. Haplotypes are presented with an underscore separating alleles on the haplotype. The first allele presented always corresponds to that at *DRB1* and the second allele is that at *DQB1* e.g. the **0401_*0301* haplotype consists of the **0401* allele at the *DRB1* gene and the **0301* allele at the *DQB1* gene. Lastly, diplotypes are presented as two haplotypes separated by a hyphen e.g. an individual with diplotype **0401_*0302-*0301_*0201* possesses haplotypes **0401_*0302* and **0301_*0201*.

4.3 RESULTS

4.3.1 Quality control of genotyping data

Quality control checks were carried out to assess the validity of the genotyping data obtained. This was to identify any genotyping errors and population substructure in order to ensure that a robust association analysis could be performed (Lewis 2002; Gordon and Finch 2005).

4.3.1.1 Genotyping success rate

Table 4.1 shows the success rate of the PCR-SSP method for each gene genotyped in the various sample groups.

Table 4.1: Genotype success rates (%) of the HLA genes genotyped in the cohorts studied.

Cohort (n)	*DRB1*	*DQB1*
Controls (341)	96.1	96.1
UKPDS (226)	93.3	93.3
W2P (135)	96.2	95.6
YT2D (37)	100.0	100.0
DIF LADA (55)	74.5	74.5

Data are given as the percentage of samples that were successfully genotyped by the PCR-SSP technique.

On the basis of these data, the DIF LADA cohort was excluded from further analysis due to the poor genotyping performance seen for these samples.

4.3.1.2 Genotyping checks

The validity of the genotyping results was assessed in the following ways:

1. *Duplication.* Due to the expense of this technique and the amount of DNA required (0.5-1.0 μg), only 15 lab control DNA samples could be duplicated. 100% concordance was observed for these samples.

2. *Observation of known linkage patterns.* Genotyping data for all subjects yielded 2 probable haplotypes that conformed to known linkage patterns in White Caucasian UK subjects, as assessed by a tissue typing expert. DNA samples from individuals who had unusual haplotypes were checked by re-genotyping.

3. *Comparison of allele and genotype frequencies with published data.* The allele frequencies obtained at each gene in the control population using the same typing methodology were very similar (exact p-values > 0.05) to those in random UK controls included in the publication of Ahmad *et al* (Ahmad *et al.* 2003) (allele frequency data obtained with permission from authors of this study, see Appendix 6, Tables 1-2). The frequencies were also similar to those in Europid populations published on the dbMHC web-site; *www.ncbi.nlm.nih.gov/mhc/MHC.cgi?cmd=init.*

4. *Check for Mendelian inconsistencies.* 13 parent-offspring trios from the W2 Consortium were also genotyped for the two HLA genes. In informative transmissions, no Mendelian inconsistencies were observed.

4.3.1.3 Combining the LADA cohort

In order to perform the most highly powered association analysis possible, it was desirable to combine data from the UKPDS, W2 and YT2D LADA cohorts. Homogeneity of the different LADA groups was assessed by comparing the distributions of HLA serological specificity frequencies among them using contingency table analysis. An exact χ^2 p value of < 0.05 was considered to indicate a significant difference. Antigen specificity frequencies in the different sample groups for HLA-*DRB1* and HLA-*DQB1* are given in Table 4.2 and Table 4.3,

respectively. The homogeneity tests conducted between the UKPDS, W2 and YT2D groups showed that there were no significant frequency differences at the *DRB1* and *DQB1* genes (exact p = 0.141 and 0.951, respectively). This allowed the data of patients from the different cohorts to be combined and studied as a single group. The UKPDS + W2 + YT2D cohort combination will be referred to simply as 'LADA' or 'All cases' from now on.

Table 4.2: HLA DRB1 *serological specificity frequencies in the control and LADA groups.*

***DRB1* Serological specificity**	**Controls (n = 654)**	**All cases (n = 756)**	**UKPDS (n = 422)**	**W2 (n = 260)**	**YT2D (n = 74)**
DR1	64 (0.098)	53 (0.070)	28 (0.066)	20 (0.077)	5 (0.068)
DR103	3 (0.005)	4 (0.005)	4 (0.009)	0	0
DR2	107 (0.164)	30 (0.040)	10 (0.024)	16 (0.062)	4 (0.054)
DR3	85 (0.130)	236 (0.312)	136 (0.322)	76 (0.292)	24 (0.324)
DR4	139 (0.213)	231 (0.306)	131 (0.310)	77 (0.296)	23 (0.311)
DR7	94 (0.144)	100 (0.132)	54 (0.128)	35 (0.135)	11 (0.149)
DR8	22 (0.034)	16 (0.021)	10 (0.024)	4 (0.015)	2 (0.027)
DR9	9 (0.014)	6 (0.008)	4 (0.009)	1 (0.004)	1 (0.014)
DR10	1 (0.002)	1 (0.001)	0	1 (0.004)	0
DR11	50 (0.076)	28 (0.037)	18 (0.043)	9 (0.035)	1 (0.014)
DR12	10 (0.015)	3 (0.004)	0	1 (0.004)	2 (0.027)
DR13	58 (0.089)	43 (0.057)	25 (0.059)	17 (0.065)	1 (0.014)
DR14	12 (0.018)	5 (0.007)	2 (0.005)	3 (0.012)	

Data are given as n (frequency).

Table 4.3: HLA DQB1 *serological specificity frequencies in the control and LADA groups.*

***DQB1* Serological specificity**	**Controls (n = 654)**	**All cases (n = 754)**	**UKPDS (n = 422)**	**W2 (n = 258)**	**YT2D (n = 74)**
DQ2	149 (0.228)	321 (0.426)	181 (0.429)	105 (0.407)	35 (0.473)
DQ4	21 (0.032)	17 (0.023)	10 (0.024)	5 (0.019)	2 (0.027)
DQ5	90 (0.138)	67 (0.089)	35 (0.083)	26 (0.101)	6 (0.081)
DQ6	155 (0.237)	76 (0.101)	40 (0.095)	32 (0.124)	4 (0.054)
DQ7	127 (0.194)	82 (0.109)	48 (0.114)	27 (0.105)	7 (0.095)
DQ8	75 (0.115)	178 (0.236)	101 (0.239)	58 (0.225)	19 (0.257)
DQ9	37 (0.057)	13 (0.017)	7 (0.017)	5 (0.019)	1 (0.014)

Data are given as n (frequency).

4.3.2 Single-point association analysis: *DRB1*

4.3.2.1 Association of serological specificities with LADA

The overall frequency distribution of serological specificities was significantly different between LADA and controls for the *DRB1* locus (exact $p < 0.0001$).

The frequencies of DR1, DR2, DR3, DR4, DR11 and DR13 appeared by observation to differ between cases and controls (Table 4.2). These specificities were individually tested for association with LADA by 2 x 2 contingency table analysis (Table 4.4). DR3 and DR4 were significantly increased in LADA vs. controls and hence were predisposing to disease whereas the DR2, DR11 and DR13 specificities were significantly decreased in LADA vs. controls and hence were protective (Table 4.4). The next stage was to increase the resolution of these associations by examining specific alleles within these serological groups where our genotyping method and statistical feasibility permitted. In general, tests of association were not performed where frequencies were <5% in both cases and controls.

Table 4.4: Odds ratios and [95% CI] for DRB1 *serological specificities.*

Serological specificity	Alleles[§]	OR [95% CI]	Exact p-value
DR1	**0101*	0.70 [0.47 - 1.03]	0.0742
DR2	**1501-06, *1601*	0.21 [0.13 - 0.33]	**3.12×10^{-15}**
DR3	**0301, *0302*	3.04 [2.29 - 4.05]	**2.01×10^{-16}**
DR4	**0401-*0407*	1.63 [1.27 - 2.10]	**9.01×10^{-5}**
DR11	**1101-*1104*	0.46 [0.28 - 0.76]	**0.0018**
DR13	**1301-*1303*	0.62 [0.40 - 0.95]	**0.0275**

All ORs are presented as LADA vs. controls. All significant p-values are denoted in bold. [§]*These alleles could be identified within each serological group by the typing method employed.*

4.3.2.2 Association of DRB1 *alleles with LADA*

Allele frequencies in the control and LADA groups at *DRB1* are given in Table 4.5. The overall distribution of allele frequencies differed significantly among cases and controls (exact p <0.0001). Looking at the individual allele frequencies, it is possible that this difference was due to an increased proportion of *DRB1*0301* and *DRB1*0401*, and/ or a decreased proportion of *DRB1*1101/04* and *DRB1*1501-06*.

In addition, to investigate the effect of specific DR4 allele subgroups, a separate contingency table analysis was performed for these alleles and significant frequency differences were found between cases and controls (exact p = 0.0194) (Table 4.5). This difference appeared to be due to a susceptibility effect of **0401* and a protective effect of **0403/06/07*.

Table 4.5: HLA DRB1 *allele frequencies in the control and LADA groups.*

***DRB1* allele**	**Serological specificity**	**Controls (n = 654)**	**All cases (n = 756)**
**0101*	DR1	64 (0.098)	53 (0.070)
**0103*	DR103	3 (0.005)	4 (0.005)
**0301*	DR3	84 (0.128)	236 (0.312)
**0302*	DR3	1 (0.002)	0
**0401*	DR4	96 (0.147)	178 (0.235)
**0402*	DR4	2 (0.003)	4 (0.005)
**0403/06/07*	DR4	16 (0.024)	7 (0.009)
**0404*	DR4	22 (0.034)	34 (0.045)
**0405*	DR4	3 (0.005)	8 (0.011)
**0701*	DR7	94 (0.144)	100 (0.132)
**0801*	DR8	22 (0.034)	16 (0.021)
**0901/02*	DR9	9 (0.014)	6 (0.008)
**1001*	DR10	1 (0.002)	1 (0.001)
**1101/04*	DR11	43 (0.066)	13 (0.017)
**1102/03*	DR11	7 (0.011)	15 (0.020)
**1201*	DR12	10 (0.015)	3 (0.004)
**1301*	DR13	27 (0.041)	20 (0.026)
**1302*	DR13	30 (0.046)	22 (0.029)
**1303*	DR13	1 (0.002)	1 (0.001)
**1401*	DR14	12 (0.018)	5 (0.007)
**1501-06*	DR2	97 (0.148)	26 (0.034)
**1601*	DR2	10 (0.015)	4 (0.005)

Data are given as n (frequency).

To clarify which allele frequencies were increased or decreased in LADA, and significantly conferred susceptibility or protection, respectively, further allele-based tests were performed (Table 4.6). From this analysis, *DRB1*0301* and *DRB1*0401* were shown to be significantly predisposing against LADA whereas *DRB1*0403/06/07*, *DRB1*1101/04*, and *DRB1*1501-06* were significantly protective. However, given that the **0403/06/07* allele occurred at a frequency of less than 5% in both cases and controls, these results and subsequent analyses regarding this allele are to be interpreted with caution. This analysis showed that susceptibility or protection at *DRB1* could be attributed to particular alleles within a serological specificity, and demonstrates the value of higher-resolution testing to identify individual alleles associated with disease. However, further resolution of DR13 was not possible in this study due to low frequencies.

Table 4.6: Formal association tests of DRB1 *alleles with LADA*

***DRB1* allele**	**Serological specificity**	**OR [95% CI]**	**Exact p-value**
**0301*	DR3	3.08 [2.32 - 4.11]	**1.01×10^{-16}**
**0401*	DR4	1.79 [1.35 - 2.38]	**3.20×10^{-5}**
**0403/06/07*	DR4	0.37 [0.13 - 0.97]	**0.0410**
**1101/04*	DR11	0.25 [0.12 - 0.48]	**4.48×10^{-6}**
**1501-06*	DR2	0.20 [0.13 - 0.32]	**2.68×10^{-14}**

All ORs are presented as LADA vs. controls.
Significant p-values are denoted in bold.

An exploratory analysis was subsequently performed, calculating effect sizes for each allele under the dominant, recessive and additive models (Table 4.7). The frequencies of all protective alleles (*DRB1*0403/06/07*, **1104/04*, **1501-06*) were reduced in LADA compared with controls and associated with disease under the dominant and additive models. Of the susceptibility alleles, *DRB1*0301* was associated with disease under all models tested, whereas *DRB1*0401* fitted the dominant and additive models only.

Table 4.7: Association of DRB1 *genotypes containing LADA-associated alleles under different genetic models.*

***DRB1* allele-containing genotypes**		**Dominant**	**Recessive**	**Additive[a]**	
				Heterozygote	**Homozygote**
**0301*	OR [95% CI]	3.73 [2.66 - 5.24]	4.52 [1.93 - 12.23]	3.11 [2.32 - 4.21]	9.66 [5.37 - 17.71]
	Exact p-value	**4.65×10^{-16}**	**1.09×10^{-4}**	**2.87×10^{-16}**	
**0401*	OR [95% CI]	2.11 [1.52 - 2.95]	1.35 [0.59 - 3.25]	1.79 [1.35 - 2.39]	3.20 [1.81 - 5.72]
	Exact p-value	**5.14×10^{-6}**	0.5680	**2.94×10^{-5}**	
**0403/06/07*	OR [95% CI]	0.37 [0.13 - 0.96]		0.37 [0.13 - 0.96]	
	Exact p-value	**0.0393**		**0.0319**	
**1101/04*	OR [95% CI]	0.26 [0.13 - 0.52]		0.27 [0.13 - 0.52]	0.07 [0.02 - 0.27]
	Exact p-value	**2.62×10^{-5}**		**6.30×10^{-6}**	
**1501-06*	OR [95% CI]	0.16 [0.10 - 0.27]	0.65 [0.09 - 3.85]	0.20 [0.12 - 0.32]	0.04 [0.02 - 0.10]
	Exact p-value	**8.40×10^{-16}**	0.8419	**1.95×10^{-14}**	

All ORs are presented as LADA vs. controls.

[a] OR [95% CI] for the additive model were calculated using the homozygote for genotypes not containing the DRB1 *allele of interest as comparator. Therefore, the OR [95% CI] for the heterozygote and homozygote of the allele of interest is presented.*

Significant p-values are denoted in bold.

Where cells are left blank, a homozygote of the allele of interest did not exist in the cohorts studied, thereby not permitting this analysis.

4.3.2.3 Association of genotypes at DRB1 with LADA

Genotype frequencies of *DRB1* serological specificities that were greater than 5% in the case or control group are shown in Table 4.8. Where frequencies appeared to differ by observation between LADA and controls, tests for association were performed (Table 4.8). (For a full list of genotype frequencies refer to Appendix 6, Table 5).

Table 4.8: Association of serological specificity genotypes at DRB1.

Serological *DRB1* genotype	**Controls**	**LADA**	**OR [95% CI]**	**Exact p-value**
DR3/DR4	18 (0.055)	82 (0.217)	4.76 [2.74 - 8.62]	**3.60×10^{-10}**
DR3/DR3	7 (0.021)	34 (0.090)	4.52 [1.93 - 12.23]	**1.09×10^{-4}**
DR4/DR4	17 (0.052)	30 (0.080)	1.57 [0.82 - 3.10]	0.1917
DR3/DR7	11 (0.034)	29 (0.077)	2.39 [1.13 - 5.38]	**0.0194**
DR4/DR7	21 (0.064)	26 (0.069)		ND
DR1/DR4	12 (0.037)	20 (0.053)		ND
DR4/DR13	17 (0.052)	13 (0.034)		ND
DR2/DR4	21 (0.064)	9 (0.024)	0.36 [0.14 - 0.83]	**0.01332**
DR2/DR7	21 (0.064)	2 (0.005)	0.08 [0.01 - 0.32]	**1.15×10^{-5}**

Data are given as n (frequency). ORs are represented as LADA vs. controls.
Significant p-values are denoted in bold. ND denotes an association not determined on the basis of a lack of difference in frequencies between LADA and controls upon observation.

The DR3/DR4, DR3/DR3 and DR3/DR7 serological specificity genotypes were significantly increased in LADA vs. controls and hence were predisposing whereas the frequencies of DR2/DR4 and DR2/DR7 were significantly decreased, indicating protective effects against developing LADA.

To perform a higher resolution analysis, genotype frequencies of *DRB1* alleles that were greater than 5% in either the case or control group were also tested for association with LADA (Table 4.9). (For a full list of genotype frequencies refer to Appendix 6, Table 6).

Table 4.9: Association of DRB1 *genotypes with LADA.*

***DRB1* genotype**	**Serological genotype**	**Controls**	**LADA**	**OR [95% CI]**	**Exact p-value**
**0301/*0401*	DR3/DR4	12 (0.040)	62 (0.164)	5.15 [2.68 - 10.69]	**1.91×10^{-8}**
**0301/*0301*	DR3/DR3	7 (0.021)	34 (0.090)	4.52 [1.93 - 12.23]	**1.09×10^{-4}**
**0301/*0701*	DR3/DR7	11 (0.033)	29 (0.077)	2.39 [1.13 - 5.38]	**0.0194**
**0401/*0701*	DR4/DR7	14 (0.043)	22 (0.058)		ND
**0701/*1501-06*	DR7/DR2	17 (0.052)	1 (0.003)	0.05 [0.001 - 0.31]	**3.50×10^{-5}**

Data are given as n (frequency). ORs are represented as LADA vs. controls.
ND denotes an association not determined on the basis of a lack of difference in frequencies between LADA and controls upon observation.

This higher-resolution analysis, demonstrates that the susceptibility effect of the DR3/DR4 genotype can be attributed to the **0301* and **0401* alleles, respectively. Similarly, the susceptibility effect of the DR3/DR3 and DR7/DR3 genotypes can be attributed to the **0301* allele. The protective effect of DR2 in DR7/DR2 was shown to be attributed to the *DRB1*1501* allele.

Summary: Single-point association analysis at DRB1*:*

- DR3 (**0301*) and DR4 (**0401*) are susceptibility determinants.
- DR2 (**1501-06*), DR11 (**1101/04*) and DR13 are dominantly protective.
- Some components of the DR4 specificity (**0403/06/07*) were found to be protective, although these results are to be interpreted with caution since small numbers of patients were examined.

- DR3/DR4 (**0401/*0301*), DR3/DR3 (**0301/0301*) and DR3/DR7 (**0301/*0701*) associate with increased susceptibility to LADA.
- DR2/DR4 and DR2/DR7 (**1501-06/*0701*) associate with protection.

4.3.3 Single-point association analysis: *DQB1*

4.3.3.1 Association of serological specificities with LADA

The overall frequency distribution of serological specificities was significantly different between cases and controls for the *DQB1* locus (exact p <0.0001).

The frequencies of DQ2, DQ5, DQ6, DQ7, DQ8 and DQ9 appeared by observation to differ among cases and controls (Table 4.3). These serological specificities were individually tested for association with LADA by 2 x 2 contingency table analysis (Table 4.10). The frequencies of DQ2 and DQ8 were increased in LADA vs. controls and were found to have a significant predisposing effect, whereas the frequencies of DQ5, DQ6 and DQ7 and DQ9 specificities were decreased in LADA vs. controls and were found to have significant protective effects (Table 4.10). To establish which alleles within the serological specificities are associated with LADA, association analysis of individual alleles was performed where possible given our genotyping method and statistical feasibility.

Table 4.10: Odds ratios and [95% CI] for DQB1*serolgical specificities.*

Serological specificity	Alleles[§]	OR [95% CI]	Exact p-value
DQ2	**0201, *0202*	2.51 [1.98 - 3.20]	**3.58×10^{-15}**
DQ5	**0501-*0504*	0.61 [0.43 - 0.87]	**0.00493**
DQ6	**0601-*0604*	0.36 [0.26 - 0.49]	**7.99×10^{-12}**
DQ7	**0301*	0.51 [0.37 - 0.69]	**9.75×10^{-6}**
DQ8	**0302*	2.39 [1.77 - 3.25]	**3.00×10^{-9}**
DQ9	**0303*	0.29 [0.14 - 0.57]	**1.07×10^{-4}**

All ORs are presented as LADA vs. controls. [§]These alleles could be identified within each serological group by the typing method employed.

4.3.3.2 Association of DQB1 *alleles*

Allele frequencies in the control and LADA groups at *DQB1* are given in Table 4.11. The overall distribution of allele frequencies differed significantly among cases and controls (exact p <0.0001). Looking at the frequencies, it is possible that this difference was due to an increased proportion of *DQB1*0201* and/ or a decreased proportion of *DQB1*0501* and *DQB1*0602*.

NB. **0301* (DQ7), **0302* (DQ8) and **0303* (DQ9) were shown to be associated with LADA in the last section (4.3.3.1).

Table 4.11: HLA DQB1 *allele frequencies in the control and LADA groups.*

***DQB1* allele**	**Serological specificity**	**Controls (n = 654)**	**All cases (n = 754)**
**0201*	DQ2	83 (0.127)	239 (0.317)
**0202*	DQ2	66 (0.101)	82 (0.109)
**0301*	DQ7	127 (0.194)	82 (0.109)
**0302*	DQ8	75 (0.115)	178 (0.236)
**0303*	DQ9	37 (0.057)	13 (0.017)
**0401*	DQ4	0	1 (0.001)
**0402*	DQ4	21 (0.032)	16 (0.021)
**0501*	DQ5	68 (0.104)	59 (0.078)
**0502*	DQ5	10 (0.015)	3 (0.004)
**0503*	DQ5	12 (0.018)	4 (0.005)
**0504*	DQ5	0	1 (0.001)
**0601*	DQ6	4 (0.006)	0
**0602*	DQ6	91 (0.139)	25 (0.033)
**0603*	DQ6	30 (0.046)	15 (0.020)
**0604*	DQ6	30 (0.046)	36 (0.048)

Data are given as n (frequency).

To clarify which allele frequencies were increased or decreased in LADA, and significantly conferred susceptibility or protection, respectively, further allele-based tests were performed (Table 4.12). From this analysis, *DQB1*0201* was found to be significantly predisposing for LADA, whereas *DQB1*0602* was significantly protective. As shown for *DRB1*, susceptibility or protection at *DQB1* could be attributed to particular alleles within a serological specificity. However, further resolution of DQ5 was not possible in this study.

Table 4.12: Formal association tests of DQB1 *alleles with LADA*

***DQB1* allele**	**Serological specificity**	**OR [95% CI]**	**Exact p-value**
**0201*	DQ2	3.19 [2.40 - 4.26]	**1.04×10^{-17}**
**0501*	DQ5	0.73 [0.50 - 1.07]	0.1128
**0602*	DQ6	0.21 [0.13 - 0.34]	**4.09×10^{-13}**

All ORs are presented as LADA vs. controls. Significant p-values are denoted in bold.

As with the *DRB1* single point analysis, the genotypes of alleles found to be associated with disease were analysed under different genetic models. Again, an exploratory analysis was performed, calculating effect sizes for each allele under dominant, recessive and additive models (Table 4.13). As with protective alleles at *DRB1*, protective alleles at *DQB1* (**0301, *0303* and **0602*) were associated with disease under the dominant and additive models of inheritance. The **0201* and **0302* susceptibility alleles were associated with disease under all models.

Table 4.13: Association of DQB1 *genotypes containing LADA-associated alleles under different genetics models.*

***DQB1* allele-containing genotypes**		**Dominant**	**Recessive**	**Additive[a]**	
				Heterozygote	**Homozygote**
**0201*	OR [95% CI]	3.77 [2.69 - 5.31]	5.12 [2.21 - 13.77]	3.14 [2.34 - 4.24]	9.84 [5.50 - 17.99]
	Exact p-value	**2.95×10^{-16}**	**1.46×10^{-5}**	**4.83×10^{-17}**	
**0301*	OR [95% CI]	0.46 [0.32 - 0.65]	0.50 [0.16 - 1.39]	0.52 [0.38 - 0.70]	0.27 [0.14 - 0.49]
	Exact p-value	**7.76×10^{-6}**	0.2124	**1.17×10^{-5}**	
**0302*	OR [95% CI]	2.72 [1.93 - 3.88]	2.84 [1.07 - 8.79]	2.39 [1.76 - 3.28]	5.71 [3.09 - 10.74]
	Exact p-value	**2.82×10^{-9}**	**0.0334**	**3.08×10^{-9}**	
**0303*	OR [95% CI]	0.29 [0.14 - 0.57]		0.29 [0.14 - 0.57]	0.08 [0.02 - 0.32]
	Exact p-value	**1.33×10^{-4}**		**7.25×10^{-5}**	
**0602*	OR [95% CI]	0.18 [0.11 - 0.30]	0.43 [0.04 - 3.03]	0.21 [0.13 - 0.34]	0.04 [0.02 - 0.11]
	Exact p-value	**5.90×10^{-14}**	0.5578	**2.48×10^{-13}**	

All ORs are presented as LADA vs. controls.

[a] OR [95% CI] for the additive model were calculated using the homozygote for genotypes not containing the DQB1 *allele of interest as comparator. Therefore, the OR [95% CI] for the heterozygote and homozygote of the allele of interest is presented.*

Significant p-values are denoted in bold.

Where cells are left blank, a homozygote of the allele of interest did not exist in the cohorts studied, thereby not permitting this analysis.

4.3.3.3 Association of genotypes at DQB1 *with LADA*

Genotype frequencies of *DRB1* serological specificities that were greater than 5% in the case or control group are shown in Table 4.14. Where frequencies appeared to differ between LADA and controls, tests for association were performed (Table 4.14). (For a full list of genotype frequencies refer to Appendix 6, Table 7).

Table 4.14: Association of serological specificity genotypes at DQB1.

Serological *DQB1* genotype	**Controls**	**LADA**	**OR [95% CI]**	**Exact p-value**
DQ2/DQ8	16 (0.049)	86 (0.228)	5.74 [3.24 - 10.73]	**4.12×10^{-12}**
DQ2/DQ2	18 (0.055)	69 (0.183)	3.85 [2.20 - 7.02]	**2.09×10^{-7}**
DQ2/DQ5	15 (0.046)	29 (0.077)	1.73 [0.88 - 3.55]	0.1212
DQ2/DQ6	42 (0.128)	28 (0.074)	0.54 [0.32 - 0.93)	**0.0233**
DQ2/DQ7	27 (0.083)	27 (0.072)		ND
DQ8/DQ8	6 (0.018)	19 (0.050)	2.84 [1.07 - 8.79]	**0.0334**
DQ6/DQ8	20 (0.061)	18 (0.048)		ND
DQ6/DQ7	39 (0.119)	11 (0.029)	0.22 [0.10 - 0.45]	**4.88×10^{-6}**
DQ5/DQ6	24 (0.073)	5 (0.013)	0.17 [0.05 - 0.46]	**9.27×10^{-5}**

Data are given as n (frequency). ORs are represented as LADA vs. controls.
Significant p-values are denoted in bold. ND denotes an association not determined on the basis of a lack of difference in frequencies between LADA and controls upon observation.

The serological specificity genotypes DQ2/DQ8, DQ2/DQ2 and DQ8/DQ8 were significantly increased in LADA and associated with a predisposing effect. On the other hand, DQ2/DQ6, DQ7/DQ6 and DQ5/DQ6 were significantly decreased in LADA, indicating protective effects.

To perform a higher resolution analysis genotype frequencies of *DQB1* alleles that were greater than 5% in at the case or control group were also tested for association with LADA (Table 4.15). (For a full list of genotype frequencies refer to Appendix 6, Table 8).

Table 4.15: Association of DQB1 *genotypes with LADA.*

***DQB1* genotype**	**Serological genotype**	**Controls**	**LADA**	**OR [95% CI]**	**Exact p-value**
**0201/*0302*	DQ2/DQ8	11 (0.033)	73 (0.194)	6.90 [3.54 - 14.68]	**1.25×10^{-11}**
**0201/*0201*	DQ2/DQ2	7 (0.021)	38 (0.101)	5.12 [2.21 - 13.77]	**1.46×10^{-5}**
**0202/*0201*	DQ2/DQ2	8 (0.024)	22 (0.058)	2.47 [1.04 - 6.51]	**0.0390**
**0201/*0501*	DQ2/DQ5	6 (0.018)	19 (0.050)	2.84 [1.07 - 8.79]	**0.0334**
**0302/*0302*	DQ8/DQ8	6 (0.018)	19 (0.050)	2.84 [1.07 - 8.79]	**0.0334**
**0201/*0301*	DQ2/DQ7	17 (0.055)	17 (0.045)		ND
**0602/*0301*	DQ6/DQ7	22 (0.067)	3 (0.008)	0.11 [0.02 - 0.38]	**2.75×10^{-5}**

Data are given as n (frequency). ORs are given as LADA vs. controls.
Significant p-values are denoted in bold. ND denotes an association not determined on the basis of a lack of difference in frequencies between LADA and controls upon observation.

This higher resolution analysis showed that the susceptibility effect of DQ2/DQ8, DQ2/DQ2 and DQ2/DQ5 genotypes was attributed to the *DQB1*0201* allele, not the *DQB1*0202* allele (Table 4.15). However, the **0202/*0201* genotype did reach nominal significance for a predisposing effect, indicating that **0202* is also predisposing. However, the OR of **0201/*0201* was much greater than that of **0202/*0201*, suggesting that **0201* allele is more predisposing to disease than the **0202* allele.

The **0201/*0501* genotype was associated with LADA (Table 4.15), but not DQ2/DQ5 (Table 4.14), indicating the merits of performing higher resolution typing, provided this is a genuine association.

The susceptibility effect of DQ6 in the DQ6/DQ7 genotype was attributed to the *DQB1*0602* allele. Further resolution of DQ2/DQ6 and DQ5/DQ6 could not be performed due to low frequencies.

Summary of single-point association analysis at DQB1*:*

- DQ2 (**0201*) and DQ8 (**0302*) are susceptibility determinants.
- DQ5, DQ6 (**0602*), DQ7 (**0301*) and DQ9 (**0303*) are dominantly protective.
- DQ2/DQ8 (**0201/*0302*), DQ2/DQ2 (**0201/*0201* and **0201/*0202*), DQ8/DQ8 (**0302/*0302*) and **0201/*0501* associate with increased susceptibility to LADA.
- DQ6/DQ7 (**0602/*0301*), DQ2/DQ6 and DQ5/DQ6 associate with protection.

4.3.4 Multi-point association analysis

4.3.4.1 Haplotype association with LADA

To perform a finer-structure analysis of this gene region, multi-point analyses were required given that it is well established in T1D that the combination of *DRB1* and *DQB1* alleles is a more informative assessment of susceptibility (Sheehy *et al.* 1989).

We performed a 2-point haplotype analysis of the LADA group vs. controls. As a result of strong LD between the *DRB1* and *DQB1* genes, haplotype frequencies could be accurately estimated. The global distribution of haplotypes (with a frequency greater than 1% in at least the case or control group) differed significantly between the two groups (exact $p < 0.0001$). The haplotype frequencies (>5%) obtained for cases and controls are given in Table 4.16. (For a full list of haplotype frequencies see Appendix 6, Table 9).

Haplotypes *DRB1*0301_DQB1*0201* and *DRB1*0401_DQB1*0302* appeared to have increased frequencies in LADA vs. controls suggesting a predisposing effect. *DRB1*0101_DQB1*0501*, *DRB1*1101/04_DQB1*0301* and *DRB1*1501-06_DQB1*0602*,

appeared to be decreased in LADA vs. controls, suggesting a protective effect. Haplotypes *DRB1*0701_DQB1*0202* and *DRB1*0401_DQB1*0301* appeared to have neutral effect. Individual contingency table analysis of these haplotypes showed that *DRB1*0301_DQB1*0201* and *DRB1*0401_DQB1*0302* were significantly predisposing whereas *DRB1*1101/04_DQB1*0301* and *DRB1*1501-06_DQB1*0602* were significantly protective against LADA (Table 4.16).

Table 4.16: HLA DRB1_DQB1 *haplotype frequencies in control and LADA subjects.*

Haplotype	Controls	LADA	OR [95% CI]	p-value
**0301_*0201*	83 (0.127)	234 (0.310)	3.08 [2.32 - 4.12]	**1.22×10^{-16}**
**0401_*0302*	48 (0.073)	128 (0.169)	2.57 [1.80 - 3.73]	**4.88×10^{-8}**
**0701_*0202*	64 (0.098)	81 (0.107)		ND
**0101_*0501*	64 (0.098)	52 (0.069)	0.68 [0.46 - 1.02]	0.0598
**0401_*0301*	47 (0.072)	49 (0.065)		ND
**1501-06_*0602*	90 (0.138)	25 (0.033)	0.21 [0.13 - 0.34]	**6.65×10^{-13}**
**1101/04_*0301*	43 (0.066)	13 (0.017)	0.25 [0.12 - 0.48]	**4.48×10^{-6}**

Data are n (frequency). OR are given as LADA vs. controls.
Significant p-values are denoted in bold. ND denotes an association not determined on the basis of a lack of difference in frequencies between LADA and controls upon observation.

Diplotypes containing these haplotypes were assigned to individuals and analysed under different genetic models (Table 4.17). Diplotypes containing *DRB1*0301_DQB1*0201* were significantly associated with LADA under all models. Diplotypes containing *DRB1*0401_DQB1*0302* were associated with disease under the dominant and additive models. The protective haplotypes, *DRB1*1101/04_DQB1*0301* and *DRB1*1501-06_DQB1*0602*, were significantly associated with LADA under the dominant and additive models. These results are in line with those obtained for individual allelic constituents of these haplotypes.

Table 4.17: Association of genotypes containing LADA-associated haplotypes under different genetic models.

Haplotype-containing genotypes		Dominant	Recessive	Additive[a]	
				Heterozygote	Homozygote
*0301_*0201	OR [95% CI]	3.71 [2.65 - 5.22]	4.52 [1.93 - 12.23]	3.09 [2.31 - 4.19]	9.56 [5.31 - 17.55]
	Exact p-value	**6.72×10^{-16}**	**1.09×10^{-4}**	**2.49×10^{-16}**	
*0401_*0302	OR [95% CI]	2.88 [1.94 - 4.30]	3.07 [0.58-30.41]	2.67 [1.85 - 3.93]	7.14 [3.41 - 15.43]
	Exact p-value	**2.31×10^{-8}**	0.2585	**2.65×10^{-8}**	
*1101/04_*0301	OR [95% CI]	0.26 [0.13 - 0.52]		0.27 [0.13 - 0.51]	0.07 [0.02 - 0.26]
	Exact p-value	**2.62×10^{-5}**		**4.82×10^{-6}**	
*1501-06 *0602	OR [95% CI]	0.18 [0.11 - 0.30]	0.43 [0.04 – 3.02]	0.21 [0.13 - 0.34]	0.04 [0.02 - 0.12]
	Exact p-value	**1.04×10^{-13}**	0.5555	**4.26×10^{-13}**	

All ORs are given as LADA vs. controls.

[a] OR [95% CI] for the additive model were calculated using the homozygote for genotypes not containing the DRB1 *allele of interest as comparator. Therefore, the OR [95% CI] for the heterozygote and homozygote of the allele of interest is presented.*

Significant p-values are denoted in bold.

Where cells are left blank, a homozygote of the allele of interest did not exist in the cohorts studied, thereby not permitting this analysis.

4.3.4.2 Diplotype association with LADA

DRB1-DQB1 diplotype frequencies that were greater than 5% in the case or control group were tested for association with LADA (Table 4.18). (For a full list of diplotype frequencies refer to Appendix 6, Table 10).

Table 4.18: Association of DRB1-DQB1 *diplotypes with LADA*

Diplotype	Controls	LADA	OR [95% CI]	Exact p-value
**0301_*0201-*0401_*0302*	6 (0.018)	53 (0.140)	8.73 [3.67 - 25.13]	**1.15×10^{-9}**
**0301_*0201-*0301_*0201*	7 (0.021)	34 (0.090)	4.52 [1.93 - 12.23]	**1.09×10^{-4}**
**0301_*0201-*0701_*0202*	8 (0.024)	22 (0.058)	2.46 [1.04 - 6.49]	**0.0397**

Data are n (frequency). OR are given as LADA vs. controls.
Significant p-values are denoted in bold.

Only 3 diplotypes had frequencies greater than 5%. Diplotypes **0301_*0201-*0401_*0302* and **0301_*0201-*0301_*0201* were significantly increased in LADA compared to controls. A nominally significant predisposing effect of **0301_*0201-*0701_*0202* was also observed. These results are in accordance with sections 4.3.2.3 and 4.3.3.3 where genotype frequencies of *DRB1* and *DQB1* were compared.

Summary of multi-point analyses:

- *DRB1-DQB1* haplotypes **0301_*0201* and **0401_*0302* were significantly associated with disease in LADA.
- *DRB1-DQB1* haplotypes **1501-06_*0602* and **1101/04_*0301* were dominantly protective against LADA.
- *DRB1-DQB1* diplotypes **0301_*0201-*0401_*0302* and **0301_*0201-*0301_*0201* and **0301_*0201-*0701_*0202* were significantly associated with disease in LADA.

4.3.5 Association with age at diagnosis

To determine if HLA-encoded susceptibility was associated with differences in age at diagnosis, patients in the combined LADA cohort were divided into two age groups; <45 years and >45 years at diagnosis. This cut-off was used in order to maximise numbers for statistical comparisons and create an approximately even split of the cohort.

Serological specificity frequencies at *DRB1* differed significantly between patients aged <45 years at diagnosis and those >45 years (exact p = 0.0374, Table 4.19). However after performing further association tests of individual specificities that appeared to drive this global difference between the two age groups, the association observed could not be ascribed to any particular variant. Comparison of serological specificities at *DQB1* of patients aged <45 at diagnosis with those aged >45 (Table 4.20) revealed a significant difference in the overall distribution of frequencies (exact p = 0.0170). Looking at the frequencies, this significant result could be driven by differences in frequencies of DQ2, DQ5, DQ6, DQ7 and DQ8 between the two groups. Further 2 x 2 contingency table analysis of these serological specificities revealed that DQ6 was indeed driving this difference, with those patients aged >45 years about twice more likely to develop LADA compared to those <45 years of age (Table 4.21). It was not statistically feasible to determine which allele of the DQ6 serological specificity was contributing to this lower odds ratio since the individual allele frequencies were too low (<5%) for meaningful comparisons to be made (See Appendix 6, Table 11 for table of allele frequencies in the two age groups).

Comparison of serological genotypes at both *DRB1* and *DQB1* (Tables 4.22 and 4.23) among those under 45 with those older, revealed no significant differences in overall distribution of frequencies (all exact p >0.05). However, it is worth noting that there was a trend towards an increased frequency of the DR3/DR4 genotype in the younger patient group vs. the older one (28 % vs. 17 %, Table 4.22) as was DQ2/DQ8 (29 % vs. 17%, Table 4.23).

Table 4.19: Frequency of DRB1 *serological specificities among patients below and above 45 years at diagnosis in the combined LADA cohort.*

***DRB1* serological specificity**	**<45 years (n = 354)**	**>45 years (n = 392)**
DR1	21 (0.059)	30 (0.077)
DR103	2 (0.006)	2 (0.005)
DR2	10 (0.028)	20 (0.051)
DR3	114 (0.322)	119 (0.304)
DR4	120 (0.339)	110 (0.281)
DR7	46 (0.130)	53 (0.135)
DR8	10 (0.028)	5 (0.013)
DR9	2 (0.006)	4 (0.010)
DR10	1 (0.003)	0
DR11	11 (0.031)	16 (0.041)
DR12	3 (0.008)	0
DR13	14 (0.040)	28 (0.071)
DR14		5 (0.013)

Data are given as n (frequency)

Table 4.20: Frequency of DQB1 *serological specificities among patients below and above 45 years at diagnosis in the combined LADA cohort.*

***DQB1* serological specificity**	**<45 years (n = 354)**	**>45 years (n = 390)**
DQ2	157 (0.444)	160 (0.410)
DQ4	10 (0.028)	6 (0.015)
DQ5	25 (0.071)	40 (0.103)
DQ6	24 (0.068)	51 (0.131)
DQ7	44 (0.124)	36 (0.092)
DQ8	90 (0.254)	88 (0.226)
DQ9	4 (0.011)	9 (0.023)

Data are given as n (frequency).

Table 4.21: Odds ratios and [95% CI] for DQB1 *antigen specificities.*

Serological specificity	***Alleles**	**OR [95% CI]**	**Exact p-value**
DQ2	**0201, *0202*	1.15 [0.85 - 1.55]	0.4000
DQ5	**0501-*0504*	0.66 [0.38 - 1.15]	0.1573
DQ6	**0601-*0604*	0.48 [0.28 - 0.82]	**0.0059**
DQ7	**0301*	1.40 [0.85 - 2.29]	0.1978
DQ8	**0302*	1.17 [0.82 - 1.66]	0.4081

*All ORs are presented as <45 years group vs. >45 years group. All significant p-values are denoted in bold. *These alleles could be identified within each serological group by the typing method employed.*

Table 4.22: Frequency of DRB1 *serological specificity genotypes among patients below (n = 177) and above 45 years (n = 196) at diagnosis in the combined LADA cohort.*

***DRB1* serological genotype**	**<45 years**	**>45 years**	***DRB1* serological genotype cont.../**	**<45 years**	**>45 years**
DR1_DR1	2 (0.011)	0	DR3_DR7	14 (0.079)	15 (0.077)
DR1_DR11	0	2 (0.010)	DR3_DR8	3 (0.017)	1 (0.005)
DR1_DR13	0	3 (0.015)	DR3_DR9	0	2 (0.010)
DR1_DR2	0	1 (0.005)	DR4_DR10	1 (0.006)	0
DR1_DR3	5 (0.028)	11 (0.056)	DR4_DR11	4 (0.023)	4 (0.020)
DR1_DR4	6 (0.034)	11 (0.056)	DR4_DR12	1 (0.006)	0
DR1_DR7	6 (0.034)	2 (0.010)	DR4_DR13	5 (0.028)	8 (0.041)
DR103_DR3	1 (0.006)	1 (0.005)	DR4_DR14	0	3 (0.015)
DR103_DR4	1 (0.006)	1 (0.005)	DR4_DR2	4 (0.023)	5 (0.026)
DR11_DR11	0	1 (0.005)	DR4_DR4	16 (0.090)	14 (0.071)
DR11_DR13	1 (0.006)	3 (0.015)	DR4_DR7	12 (0.068)	14 (0.071)
DR11_DR2	0	1 (0.005)	DR4_DR8	4 (0.023)	2 (0.010)
DR11_DR3	5 (0.028)	2 (0.010)	DR4_DR9	1 (0.006)	1 (0.005)
DR13_DR2	0	2 (0.010)	DR7_DR11	1 (0.006)	1 (0.005)
DR13_DR3	7 (0.040)	8 (0.041)	DR7_DR12	1 (0.006)	0
DR13_DR8	1 (0.006)	0	DR7_DR13	0	4 (0.020)
DR14_DR11	0	1 (0.005)	DR7_DR2	1 (0.006)	1 (0.005)
DR14_DR3	0	1 (0.005)	DR7_DR7	4 (0.023)	8 (0.041)
DR2_DR2	1 (0.006)	2 (0.010)	DR7_DR8	2 (0.011)	0
DR3_DR12	1 (0.006)	0	DR7_DR9	1 (0.006)	0
DR3_DR2	3 (0.017)	5 (0.026)	DR8_DR8	0	1 (0.005)
DR3_DR3	13 (0.073)	20 (0.102)	DR9_DR2	0	1 (0.005)
DR3_DR4	**49 (0.277)**	**33 (0.168)**			

Data are given as n (frequency).

Table 4.23: Frequency of DQB1 *serological specificity genotypes among patients below and above 45 years at diagnosis in the combined LADA cohort.*

***DQB1* serological genotype**	**<45 years (n = 177)**	**>45 years (n = 195)**
DQ2_DQ2	29 (0.164)	39 (0.200)
DQ2_DQ4	5 (0.028)	1 (0.005)
DQ2_DQ5	13 (0.073)	16 (0.082)
DQ2_DQ6	11 (0.062)	16 (0.082)
DQ2_DQ7	15 (0.085)	12 (0.062)
DQ2_DQ8	**52 (0.294)**	**34 (0.174)**
DQ2_DQ9	3 (0.017)	3 (0.015)
DQ4_DQ4	0	1 (0.005)
DQ4_DQ6	1 (0.006)	1 (0.005)
DQ5_DQ5	2 (0.011)	0
DQ5_DQ6	0	5 (0.026)
DQ5_DQ7	5 (0.028)	7 (0.036)
DQ6_DQ6	1 (0.006)	4 (0.021)
DQ6_DQ8	5 (0.028)	13 (0.067)
DQ6_DQ9	0	2 (0.010)
DQ7_DQ4	1 (0.006)	0
DQ7_DQ6	5 (0.028)	6 (0.031)
DQ7_DQ7	5 (0.028)	2 (0.010)
DQ8_DQ4	3 (0.017)	2 (0.010)
DQ8_DQ5	3 (0.017)	11 (0.056)
DQ8_DQ7	8 (0.045)	6 (0.031)
DQ8_DQ8	9 (0.051)	10 (0.051)
DQ9_DQ5	0	1 (0.005)
DQ9_DQ7	0	1 (0.005)
DQ9_DQ8	1 (0.006)	2 (0.010)

Data are given as n (frequency).

4.3.6 Association with insulin requirement

Insulin requirement by 6 years post-diagnosis was used as an indicator of the aggressiveness of the disease course. Within the UKPDS group, the association of serological specificities and genotypes with insulin requirement by 6 years post-diagnosis was tested for (See Appendix 6, Tables 12-15 for frequencies). No association of variants at *DRB1* or *DQB1* was found with insulin requirement by this time point (all exact $p > 0.05$).

4.3.7 Association with autoantibody status

In Chapter 3, it was shown in UKPDS LADA patients that dual positivity for GADA + IA-2A associated with a more severe disease process in the post-diagnosis period than those with GADA alone. Therefore, it was investigated in the UKPDS LADA cohort, whether the frequency of *DRB1* and *DQB1* variants differed among patients with this dual positivity compared to those with GADA alone (See Appendix 6, Tables 16-19 for frequencies). No significant differences in serological specificities and genotypes were found between the two groups (all exact $p > 0.05$).

4.4 DISCUSSION

This study considerably extends the previous association analyses of the HLA gene region in LADA where only the main T1D-associated susceptibility alleles, DR3 (in LD with DQ2), DR4 (in LD with DQ8) and protective alleles, DR2 (in LD with DQ6), have been examined (Tuomi *et al.* 1993; Horton *et al.* 1999).

Single point association analysis.

Serological specificities/ alleles: As observed in T1D, DR3 (*DRB1*0301*) and DR4 (*DRB1*0401*) appear to be the major susceptibility determinants at the *DRB1* gene and DQ2 (*DQB1*0201*) and DQ8 (*DQB1*0302*) at the *DQB1* gene. Together, these specificities/ alleles accounted for ~85% of patients with LADA compared with 95% reported for T1D (Thomson *et al.* 1988). This suggested that other variants at these genes must predispose to LADA. However, analysis of nonDR3/ nonDR4 (nonDQ2/ nonDQ6) variants revealed that no other variants predisposed to disease.

Regarding protective variants, DR2 (*DRB1*1501-06*) and DQ6 (*DQB1*0602*) were the most protective at these loci, as seen in T1D and in smaller studies of LADA (Stenstrom *et al.* 2002). The frequency of these variants was ~3% in our LADA population and is comparable to the frequency of <1% observed in T1D (Caillat-Zucman *et al.* 1997). Other variants were also found to confer dominant protection against the development of LADA (DR11 (*DRB1*1101/04*), DR13, DQ5, DQ7 and DQ9). Similar results have been obtained in T1D (Redondo *et al.* 2001). Although odds ratios found for these variants in LADA cannot be directly compared to those reported in T1D (due to differences in statistical analysis procedures and study design), the overall trends observed appear to mirror those seen in T1D.

DR4 subtypes: In this study, *DRB1*0401* was the only DR4 subtype that conferred risk to LADA. *DRB1*0403* appeared to confer a protective effect, although it is important to note that this allele had a low population prevalence. Larger numbers of patients would need to be studied to substantiate these findings. However, both of these associations are similar to that observed in T1D (She 1996; Rewers *et al.* 2003).

Genotypes: Also similar to that observed in T1D, the DR3/DR4 genotype was associated with the highest risk for disease in LADA, followed by DR3/DR3 (Table 4.8). The association observed with DR3/DR4 (in LD with DQ2/DQ8) confirms previous studies with a smaller number of LADA patients (Horton *et al.* 1999; Tuomi *et al.* 1999; Hosszufalusi *et al.* 2003). Association of the DR3/DR3 homozygote with LADA was also demonstrated by Torn *et al* (Torn *et al.* 2003). The DR3/DR4 heterozygote occurred in ~21% of our LADA cohort compared with 35% in classical T1D (Redondo *et al.* 2001). This suggests that LADA patients could have a less marked predisposition to disease by this high-risk susceptibility determinant for T1D. Similar trends were seen at *DQB1* for DQ2/DQ2 and DQ2/DQ8 (as expected from the extremely strong LD between the *DRB1* and *DQB1* genes. In this study, the T1D-associated DR4/DR4 genotype (Kelly *et al.* 2004) failed to significantly confer susceptibility to LADA (Table 4.8), although a predisposing trend was seen. However, an association with DQ8/DQ8 (*DQB1*0302/DQB1*0302*) was observed in LADA (Table 4.14).

The only other genotypes that were significantly predisposing to disease were DR3/DR7 (*DRB1*0301/DRB1*0701*) and *DQB1*0201/DQB1*0501*.

As seen in studies of T1D (Graham *et al.* 1999), genotypes containing DR2 or DQ6 at *DRB1* and *DQB1*, respectively, were found to be protective in this study (Tables 4.8 and 4.14).

Multi-point association analysis. As in T1D, risk for LADA was refined by studying haplotypes. For example, the risk for disease conferred by *DRB1*0401* was increased when this allele was present on the same haplotype as *DQB1*0302*, but not with *DQB1*0301* (Table 4.16, Figure 1.5 in Chapter 1). The well-known positive synergistic effects of the *DRB1*0301_DQB1*0201* and *DRB1*0401_DRB1*0302* haplotypes reported in T1D (Kelly *et al.* 2004) were also apparent in this study; the sum of the OR [95% CI] of these haplotypes (3.08 [2.32 - 4.12] and 2.57 [1.80 - 3.73], respectively) was lower than that of the *DRB1*0301_DQB1*0201-DRB1*0401_DRB1*0302* diplotype (8.73 [3.67 - 25.13]). This may be explained by transcomplementation; since an increase in concentration of particular combinations of α- and β- chain dimers formed from gene products in *trans* (i.e. *DQA1*0501* and *DQB1*0302*) may be more diabetogenic than others (refer to Chapter 1, Section 1.2.2.1).

Functional effect of HLA susceptibility. The mechanisms by which the *DRB1* and *DQB1* molecules predispose to diabetes are unclear. The polymorphism within these molecules occurs primarily within the antigen binding grooves and exposed surfaces of their β_1 domains. These polymorphisms can therefore influence peptide selectivity and interactions with the T cell receptor complex on $CD4^+$ T cells (Sanjeevi *et al.* 1997). Two main sites of action of HLA molecules have been proposed to be important in determining whether autoimmunity occurs. Firstly, the peptide selectivity and affinity of HLA molecules together with their molecular stability characteristics could influence thymic T cell maturation (Nepom and Kwok 1998). This would dictate whether T cells, including autoreactive ones, would be negatively or positively selected. Inefficient presentation of β-cell peptides to autoreactive T cells could prevent the negative selection of autoreactive T cells and/ or positive selection of regulatory T cells (Nepom and Kwok 1998), rendering individuals prone to autoimmune diabetes. Secondly, given the pivotal role of HLA molecules in T cell activation, it has been speculated that the aberrant binding of self-peptides would enable recognition by, and

subsequent activation of autoreactive T cells in the periphery that have escaped negative selection, triggering an autoimmune immune response (Nepom and Kwok 1998). In the case of diabetes, predisposing HLA molecules are postulated to actively present autoantigens in the pancreas and it's draining lymph nodes, which would represent a key step in the initiation and maintenance of islet autoimmunity. Indeed there is evidence that predisposing HLA molecules can present peptides of key autoantigens in autoimmune diabetes such as GAD 65, IA-2 and insulin and elicit T cell responses (Herman *et al.* 1999; Durinovic-Bello *et al.* 2002; Kelemen *et al.* 2004).

The importance of various amino acids encoded by these HLA genes in disease susceptibility has been debated, particularly the Asp57 residue in the DQβ1 molecule, encoded by alleles such as *DQB1*0602* (refer to Chapter 1, Section 1.2.2.1). It is likely that a collection of amino acid changes occurring in binding pockets, rather than any one single residue influence disease susceptibility (Sanjeevi *et al.* 2002b). Comparison of predicted molecular structures of proteins encoded by susceptibility alleles show that their antigen binding sites contain residues with similar properties and binding preferences in their P1, P4 and P9 pockets[13] (Cucca *et al.* 2001b). The antigen binding sites of predisposing molecules were found to be structurally different from protein products of protective alleles, which in turn were very similar to each other (Cucca *et al.* 2001b). Also, differences in key residues in the P1 and P4 pockets between predisposing and protective DR4 subtypes have been identified (Caillat-Zucman *et al.* 1997; Cucca *et al.* 2001b).

Although the actual mechanism by which different HLA molecules predispose or protect against autoimmune diabetes remains to be understood, it is likely that similar processes operate in classical T1D and LADA. The mystery of exactly how the individual variants lead

[13] The P1 through P9 pockets in a HLA Class II molecule correspond to the anchoring residues of the typically 9-amino-acid core peptide required for binding to the molecule.

to an acute onset and a slowly-progressive is at present unsolved and should be a focus of future work.

Association with age at diagnosis. In this study, HLA profiles in patients <45 years of age and >45 years of age at diagnosis were compared to see if this clinical variable enabled better discrimination of high and low risk variants. The protective variant DQ6, was found to be more prevalent in older patients diagnosed with LADA (Table 4.21). This suggests that the protective effect of DQ6 against the development of LADA is attenuated with age. Unfortunately, we could not resolve which allele within the DQ6 serological specificity was predisposing to disease. This will only be possible with greater numbers of patients. Similar findings of protective effects of this serological specificity have also been reported in a large study (n = 974) of Swedish patients with classical T1D, diagnosed between 15-34 years of age (Graham *et al.* 1999). Due to the low prevalence of this specificity in T1D, only 16 patients positive for DQ6 were available for analysis. However in this group of patients, the frequency of DQ6 was shown to significantly increase with age. Therefore, it appears that DQ6 associates with a decelerated rate of autoimmune-mediated β-cell destruction, although it does not preclude eventual development of autoimmune diabetes. The age-mediated effect of DQ6 remains unexplained.

Age-related associations of the susceptibility variants, DR3 and DR4, have been reported to occur in classical T1D; that is, the frequencies of these variants are negatively correlated with age at diagnosis (Ludvigsson *et al.* 1986; Tait *et al.* 1995b). Such associations were not found to occur in this study. Also, the overall distribution of genotypes at *DRB1* and *DQB1* was not significantly different between the two age groups. To avoid multiple testing issues, association of individual genotypes with age was not performed, although by observation of frequencies, the DR3/DR4 and DQ2/DQ8 heterozygotes appeared to be decreased in the older age group (Table 4.22 and 4.23). This trend with age at diagnosis

has been shown to occur in classical T1D (Karjalainen *et al.* 1989; Caillat-Zucman *et al.* 1992; Caillat-Zucman *et al.* 1997; Gillespie *et al.* 2002).

Association with autoantibody status and insulin requirement. No association of HLA variants with disease severity as assessed by autoantibody status (dual positivity vs. single positivity) and requirement for insulin by 6 years post-diagnosis was observed. These findings, taken together with the fact that the HLA variant, DQ6 associated with age at diagnosis, suggests that while HLA molecules almost certainly predispose to LADA, they do not appear to have a large effect on the aggressiveness of the immune response after the onset of disease. Therefore, it is probable that other genetic factors, and the significant environmental component to T1D susceptibility must act together to influence disease severity post-diagnosis since all HLA at-risk individuals do not display similar degrees of islet autoimmunity or overt diabetes.

Future directions/ improvements to study. Although this is the largest genetic study of the HLA gene region performed in LADA to date, informative assessment of the variants genotyped, especially at the allelic and haplotype level was limited by patient numbers where population prevalence of particular variants was low. A larger cohort size would enable one to substantiate the findings of this study and would provide more power to determine more precisely the nature of HLA associations with LADA.

Since the Class II gene *DPB1* has been found to associate with classical T1D, it would interesting to look at this gene in LADA too (Cucca *et al.* 2001a; Cruz *et al.* 2004). Also, studies in T1D have implicated the role of Class I and Class III variants and extended HLA haplotypes in disease susceptibility (Kelly *et al.* 2004). Examination of these and other variants in the HLA gene region may be important in LADA especially since associations with the major histocompatibility complex Class I chain-related gene A (MICA) (Sanjeevi *et*

al. 2002a; Torn *et al.* 2003) and *TNF*, which encodes TNF-α (Vatay *et al.* 2002) have been reported in smaller cohorts.

Chapter 5:

Association of the insulin gene region in LADA

CHAPTER 5: ASSOCIATION STUDY OF THE INSULIN GENE WITH LADA

5.1 INTRODUCTION

In addition to the HLA gene region, variants at insulin gene (*IDDM2*) have also been implicated in predisposition to classical T1D. This gene region has been confirmed as a minor susceptibility gene through replication of association studies (Bennett and Todd 1996) and genome-wide scans (Cox *et al.* 2001) and thus is another excellent candidate locus for LADA.

As described in Chapter 1 (Section 1.2.2.2), the *INS* VNTR is believed to be the main susceptibility determinant in this gene region. Class I alleles at the VNTR are predisposing, whereas Class III alleles confer protection against development of classical T1D. The +3580*MspI* restriction site allows the subdivision of Class I alleles by flanking haplotype into the predisposing IC+/ID+ ancestral lineage and the relatively protective, ID- lineage (Stead *et al.* 2000). Similarly, the +1428*FokI* restriction site enables identification of the 'Protective' (PH/ IIIA) and 'Very Protective' (VPH/ IIIB) Class III haplotypes, which were initially reported to differ in their degree of protection against T1D, as their names suggest (Bennett *et al.* 1995). However, since a publication by Barratt *et al*, in 2004, it has become apparent that the two Class III haplotypes confer equal susceptibility in classical T1D (Barratt *et al.* 2004). This work was begun prior to this report, thus we continued to assess whether a difference in the susceptibility of these haplotypes occurred in LADA, since it may represent a separate subgroup of autoimmune diabetes - hence its genetic aetiology may vary from that observed in classical T1D.

Therefore, this study investigated whether the insulin gene region is associated with LADA and whether the pattern of predisposition/ protection at this locus is different to that reported

in classical T1D. It was hypothesised that such differences in genetic predisposition may contribute to the delayed onset and milder phenotype of LADA compared with classical T1D.

INS VNTR class (Class I/ Class III alleles) was determined using the –23*HphI* restriction site as a surrogate marker. The modulating effects of the flanking polymorphisms, +1428*FokI* and +3580*MspI* on susceptibility conferred by the VNTR was also assessed.

Aims of the present study:

- To determine whether the *INS* VNTR (using the -23*HphI* site as a marker) is the primary susceptibility determinant in the *IDDM2* region in LADA as occurs in T1D.

- To examine whether the +1428*FokI* and +3580*MspI* polymorphisms modify the risk conferred by the –23*HphI* site. This was achieved by determining whether the Class I and Class III haplotypes identified by these three sites each conferred differential degrees of predisposition to LADA. Specifically, it was addressed whether the ID- haplotype is more protective compared with other Class I haplotypes and whether the Class III VPH is more protective than the Class III PH.

- To evaluate whether variation at the insulin gene region associates with the age of diagnosis of LADA subjects.

- Within the UKPDS LADA cohort, to determine if there is an association of Class I alleles with requirement for insulin therapy by 6 years post-diagnosis. This analysis was performed to evaluate whether susceptibility from the insulin gene affects the rate of progression of disease following the diagnosis of hyperglycaemia.

- Within the UKPDS cohort, to determine if insulin gene polymorphisms associate with dual autoantibody positivity (GADA + IA-2A vs. GADA alone).

5.2 SUBJECTS AND METHODS

5.2.1 Subjects

Genotypic data of LADA subjects from the UK Prospective Diabetes Study (UKPDS, n = 233), Diabetes in Families study (DIF, n = 24), Warren 2 consortium (W2, n = 133) and Young-Onset T2D study (YT2D, n = 37) were compared with 341 non-diabetic controls from the DIF study. See Table 2.2 in Chapter 2 for patient characteristics. In addition, 13 parent-offspring trios containing a LADA proband from the W2 consortium collection were genotyped as one of the quality control measures.

5.2.2 Genotyping

The fluorescence-based Amplifluor technology was employed to genotype all of the cohorts for the -23*HphI* (A→T), +1404*Fnu4HI* (C→A) and +3580*MspI* (C→T) sites (for methods, refer to Chapter 2, Section 2.4.3). The -23*HphI* site was chosen as a surrogate marker for VNTR class. The +1404*Fnu4HI* site was chosen as a surrogate for the +1428*FokI* site, since the latter was not amenable to genotyping using the Amplifluor method. This surrogate was chosen since there is complete concordance between the two sites by virtue of strong LD patterns in this chromosomal region found in Caucasian subjects (Lucassen *et al.* 1993).

5.2.3 Statistical methods

Homogeneity testing. Genotype frequencies were compared between the case groups (UKPDS/ W2/ YT2D/ DIF) for each SNP by standard contingency table methods (Kruskall-Wallis tests) in StatXact 6 (Cytel Software, Cambridge, MA, USA) to test for homogeneity.

Check for Hardy-Weinberg equilibrium (HWE). This check was performed as a quality control measure (Ott 2004). Deviation of observed genotype frequencies at the three SNPs from

expected frequencies was tested in each individual sample group using exact χ^2 tests as implemented in PEDSTATS (Version 6.0, University of Michigan, MI, USA).

Genotypic/ allelic association. Genotypic association with disease status was assessed under dominant, recessive and additive models of inheritance. If the common allele at each SNP is denoted 1 and the rare allele 2, the models were defined as follows. In the dominant model for allele 2, subjects homozygous for allele 1 were compared with carriers of allele 2 (i.e. 11 vs. 12 + 22). In the recessive model for allele 2, carriers of allele 1 were compared with subjects homozygous for allele 2 (i.e. 11 + 12 vs. 22). Significance for the fit of these models, odds ratios (ORs) and 95% confidence intervals [CI] were calculated in StatXact 6. To test for an additive effect, a test for a linear gradient across the genotypes (11 vs. 12 vs. 22) was performed using the Cochran-Armitage test for trend in StatXact 6.

As appropriate, exact versions of these tests were used. Separate case data sets were combined for contingency table analyses only after appropriate homogeneity testing (see above).

Step-wise logistic regression was used to ascertain which of the three SNPs independently contributed to risk (as opposed to association occurring secondary to LD between them) using SPSS (Version 13.0 for Windows; SPSS, Chicago, IL, USA).

Test for linkage disequilibrium (LD). Pairwise measures of LD, D' and r^2, were calculated in HelixTree® for the case groups and control group separately. D' is a measure of LD that takes the value of 1 when there is complete LD between two SNPs, that is, if at most 3 of 4 possible haplotypes are observed. However, intermediate values of D' are difficult to interpret. Also, estimates of D' are strongly inflated in small sample sizes even for SNPs with common alleles and therefore high values of D' can be obtained even if the markers are in linkage equilibrium (Ardlie *et al.* 2002). To guard against false interpretation of D' LD

measures, pairwise correlation coefficient, r^2, values were also measured. r^2 is a measure of LD that takes the value of 1 in the presence of absolute/ perfect LD. This occurs when alleles at the two SNPs have identical frequency, and thus only two out of the four possible haplotypes will be observed. Since r^2 is a measure of statistical correlation between two sites, intermediate vales can be interpreted relatively easily than for D'. In addition, r^2 is considered the most relevant/useful measure of LD in association studies since there is a simple relationship between r^2 and sample size required to detect an association between the susceptibility SNP and marker SNPs (Wall and Pritchard 2003). The power to detect the susceptibility variant indirectly by genotyping a marker SNP in n samples is equivalent to the power to detect it directly using the susceptibility SNP in nr^2 samples.

Haplotype estimation. Haplotype analysis enables capture of a larger part of the genetic variation in the *INS* gene. This is more powerful than single marker association and allows one to detect interactions between the SNPs. The frequency of 3-point haplotypes in our cases and controls was estimated by the expectation maximisation (EM) algorithm using HelixTree®. The EM algorithm was re-initialised three times to ensure convergence. Haplotypes with their associated probability distributions were assigned to individuals.

Haplotype association. To avoid possible errors due to either genotyping or the estimation process, only haplotypes that had a frequency of >1% were considered for further analysis. Haplotype trend regression (HTR) was used for robust comparison of estimated haplotype frequencies and association testing between cases and controls. This analysis was also performed in HelixTree®.

Diplotype association. The most probable haplotype pair that was assigned to each individual was selected for diplotype analyses. An alternative approach would have been to use the weighted diplotype distribution for each individual in order to avoid the problem of phase

uncertainty. However, the completeness of the data (few missing genotypes) and the presence of high LD between the SNPs, meant that the vast majority of haplotype combinations were associated with a probability of greater than 0.99 (98.8% of the combined LADA group, 98.7% of UKPDS LADA, 98.5% of W2 LADA, 100% of YT2D LADA and 97.2% of controls) indicating that phase uncertainty was not a major complicating issue.

Quantitative trait association. Association of age at diagnosis with genotype was assessed by linear regression modelling using SPSS (Version 13). A check for normality was performed on the age distributions of cases (at diagnosis) and controls (at DNA collection) prior to linear regression, and log transformation was not necessary. Association of requirement for insulin and genotype was performed using contingency table analysis.

Association with autoantibody status. Association of autoantibody status (GADA + IA-2A vs. GADA alone) with insulin gene SNPs was performed using contingency table analysis.

Power calculations. Power to detect an association was calculated using Quanto, Version 1.0 (*http://hydra.usc.edu/gxe*).

Threshold for significance. P-values were considered statistically significant when <0.05. Bonferroni correction for multiple testing was considered in order to avoid type 1 error. However, this would be a conservative approach as it assumes independence of the tests performed. Examination of LD between the SNPs in the cohorts studied demonstrated that they were correlated and hence were not independent (See Figure 5.1). Therefore, tests for multiple testing were not applied.

5.3 RESULTS

5.3.1 Quality control of genotyping data

Quality control checks were carried out to assess the validity of the genotyping data obtained. This was to identify any genotyping errors and population substructure to ensure that a robust association analysis was performed (Lewis 2002; Gordon and Finch 2005).

5.3.1.1 Genotype success rate

Table 5.1 shows the success rate of the Amplifluor method for each SNP genotyped in the various sample groups.

Table 5.1: Genotype success rates (%) of the SNPs genotyped in the cohorts studied.

Cohort (n)	-23*HphI*	+1404*Fnu4HI*	+3580*MspI*
Controls (341)	96.0	95.0	96.5
UKPDS LADA (233)	99.1	98.3	99.1
W2 LADA (135)	97.0	96.2	96.2
YT2D LADA (37)	100.0	100.0	100.0
DIF LADA (24)	91.7	83.3	87.5

Data are given as the percentage of samples that were successfully genotyped by the Amplifluor technique.

On the basis of these data, the DIF LADA cohort was excluded from further analysis due to the poor genotyping performance seen for these samples and the small cohort size.

5.3.1.2 Genotyping checks

The validity of the genotyping results obtained by the Amplifluor method that were used in this study was assessed in the following ways:

1. *Negative controls.* Four negative control wells (two water blanks, two empty) per plate of samples were included and showed that no contamination had occurred during genotyping.

2. *Duplication.* 10% of the samples in the DIF control and UKPDS cohorts were assayed in duplicate on the Amplifluor system to assess reproducibility of results and 99.8% concordance was obtained. The YT2D cohort was duplicated entirely and 100% concordance was observed.

3. *Complementary genotyping methods.* The UKPDS cohort of LADA patients and non-diabetic controls from the DIF cohort had previously been genotyped for the -23*HphI* and +1428*FokI* sites using the PCR-RFLP technique (see Chapter 2, Section 2.4.5 for methods). The concordance of results obtained by the PCR-RFLP and Amplifluor techniques was 99%. Any discrepancies were due to incomplete digestion of the RFLP product and hence failure to detect a true homozygote.

4. *Hardy-Weinberg Equilibrium (HWE) of genotype frequencies.* The genotype frequencies at the three sites were checked for deviations from HWE within each cohort separately. (Table 5.2).

Table 5.2: Exact test p-values to assess deviations of genotype frequencies from HWE in the different sample groups.

Marker	Controls	UKPDS LADA	W2 LADA	YT2D LADA	All cases
-23*HphI*	0.692	0.326	0.136	**0.008**	**0.010**
+1404*Fnu4HI*	0.261	0.183	**0.010**	0.066	**0.001**
+3580*MspI*	0.623	**0.024**	0.853	0.233	**0.017**

A modest deviation from HWE was seen for the +3580*MspI* SNP in the UKPDS group (exact p = 0.024). Slight deviations from HWE were also observed for the -23*HphI* SNP YT2D group (exact p = 0.008) and the +1404*Fnu4HI* SNP in the W2 group (exact p = 0.010).

The deviations from HWE observed could have been be due to a number of reasons including poor genotyping, real disease association effects (because one genotype is predisposing to disease, and was therefore be more frequent in subjects ascertained for that disease), non-random mating, admixture and stochastic (sampling) variation due to small sample size.

The deviation at the +3580*MspI* SNP in the UKPDS group was due to an excess of rare (TT) homozygotes. However, since the T allele is predisposing to LADA (see Section 5.3.2.1), this deviation was due to a real disease association effect.

In both the YT2D and W2 cohorts, the departures from HWE observed were due to an excess of rare homozygotes. However, the rare alleles at the –23*HphI* and +1404*Fnu4HI* sites are not predisposing to LADA (Section 5.3.2.1). It is important to note that stringent quality control checks on the genotyping had been performed and have confirmed that these deviations are actually real and not due to genotyping error. Rare homozygotes at –23*HphI* in the YT2D cohort were confirmed by tetra-primer ARMS (See Chapter 2, Section 2.4.4 for methods). Rare homozygotes at +1404*Fnu4HI* were confirmed by sequencing (see Chapter 2 for methods). The possibility that the departures from HWE seen were the effect of non-random mating is difficult to prove and since subjects within each cohort were collected from the same geographical region and were of the same ethnicity, admixture was an unlikely explanation for the deviations observed. Therefore, the most likely explanation for the deviations seen was due to stochastic variation/ chance given the cohort sizes.

5. *Check for Mendelian inconsistencies.* 13 parent-offspring trios from the W2 Consortium were also genotyped for the three SNPs using the Amplifluor technique. In informative transmissions, no Mendelian inconsistencies were observed.

6. *Comparison of allele and genotype frequencies with published data.* The genotype frequencies observed in the control population are very similar to those observed for White Caucasians in other studies ((Lucassen *et al.* 1993), Ensembl genome browser; -23*HphI* (rs689), +1404*Fnu4HI* (rs3842755) and +3580*MspI* (rs2000993)).

7. *Analysis of haplotype frequencies.* An additional measure to check the validity of genotyping data was performed by observation of haplotype frequencies obtained by EM estimation. The occurrence of high frequencies of rare haplotypes in the sample groups studied would imply that genotyping error might have occurred. The appearance of rare haplotypes in the control, UKPDS, W2 and YT2D groups was limited, suggesting that the genotyping data was robust (Table 5.3). Although the DIF LADA group was considered to fail quality control on the basis of genotyping success rate, haplotypes were estimated for the available genotyping data for this cohort (Table 5.3). The occurrence of rare haplotypes in this very small sample group further indicated that the genotyping data for this cohort was not robust and this group definitely should not be included in subsequent analyses.

Table 5.3: Haplotype frequencies estimated by EM among the sample groups studied.

T1D definition	Haplotype*	Controls	UKPDS LADA	W2 LADA	YT2D LADA	DIF LADA
ID-	ACT	0.336	0.440	0.386	0.297	0.279
IC+/ID+	ACC	0.363	0.401	0.378	0.568	0.434
PH	TAC	0.222	0.115	0.190	0.122	0.186
VPH	TCC	0.078	0.045	0.039	0.014	
Rare IC+/ID+	AAC	0.002		0.008		
Rare VPH	TCT					0.101
Rare PH	TAT					6.50×10^{-5}

**3-point haplotypes: -23*HphI_*+1404*Fnu4HI_*+3580*MspI.

5.3.1.3 Defining the LADA cohort

In order to perform the most highly powered association analysis possible, it was desirable to combine data from the UKPDS, W2 and YT2D LADA cohorts to study large numbers of subjects. To pool the three case cohorts together, it was necessary first to check for homogeneity in genotype frequencies to ensure that all groups were sampled from the same underlying background population.

Formal tests for homogeneity among case groups

Homogeneity of genotype frequencies among the different LADA groups was assessed by comparing the genotype frequency distributions among them using contingency table analysis. Genotype and allele frequencies in the different sample groups are given in Table 5.4 and Table 5.5, respectively.

Comparison of genotype frequency distributions among the UKPDS, W2 and YT2D groups revealed borderline significance for heterogeneity (–23*HphI*, p = 0.045; +1404*Fnu4HI*, p = 0.033; +3580*MspI*, p = 0.062). In view of these p-values, results are presented for both the combined group of cases, and for comparisons of the individual case groups with the common control group.

Table 5.4: Genotype frequencies of the insulin gene polymorphisms in the sample groups.

Marker	Genotype	Controls (n*=332)	All cases (n*=400)	UKPDS LADA (n*=232)	W2 LADA (n*=131)	YT2D LADA (n*=37)
-23*HphI*	AA	159 (0.49)	276 (0.69)	165 (0.71)	81 (0.62)	30 (0.81)
	AT	141 (0.43)	102 (0.26)	58 (0.25)	40 (0.31)	4 (0.11)
	TT	27 (0.08)	21 (0.05)	8 (0.04)	10 (0.08)	3 (0.08)
+1404*Fnu4HI*	CC	199 (0.61)	301 (0.76)	182 (0.80)	89 (0.69)	30 (0.81)
	CA	105 (0.32)	78 (0.20)	42 (0.18)	31 (0.24)	5 (0.14)
	AA	20 (0.06)	17 (0.04)	5 (0.02)	10 (0.08)	2 (0.05)
+3580*MspI*	CC	147 (0.45)	151 (0.38)	81 (0.35)	50 (0.39)	20 (0.54)
	CT	143 (0.43)	169 (0.42)	97 (0.42)	60 (0.46)	12 (0.32)
	TT	39 (0.12)	78 (0.20)	53 (0.23)	20 (0.15)	5 (0.14)

Data are given as n (frequency). n = number of individuals in which genotyping was attempted.*The AA genotype at –23HphI indicates the VNTR Class I/ Class I genotype, AT indicates VNTR Class I/ Class III and TT indicates Class III/III.*

Table 5.5: Allele frequencies of the insulin gene polymorphisms in the sample groups.

Marker	Allele	Controls	All cases	UKPDS LADA	W2 LADA	YT2D LADA
-23*HphI**	A	459 (0.70)	654 (0.82)	388 (0.84)	202 (0.77)	64 (0.86)
	T	195 (0.30)	144 (0.18)	74 (0.16)	60 (0.23)	10 (0.14)
+1404*Fnu4HI*	C	503 (0.78)	680 (0.86)	406 (0.89)	209 (0.80)	65 (0.88)
	A	145 (0.22)	112 (0.14)	52 (0.11)	51 (0.20)	9 (0.12)
+3580*MspI*	C	437 (0.66)	471 (0.59)	259 (0.56)	160 (0.62)	52 (0.70)
	T	221 (0.34)	325 (0.41)	203 (0.44)	100 (0.39)	22 (0.30)

Data are given as n (frequencies).

*The A allele at –23*HphI *is a marker for VNTR Class I alleles, the T allele is a marker for VNTR Class III alleles.*

Summary of quality control assessment

Genotyping data for the UKPDS, W2, YT2D and control groups was found to be of good quality on the basis of duplication results and observation of estimated haplotype frequencies. Poor genotyping results were only obtained for the DIFL cohort. As homogeneity testing suggested that the LADA cohorts were sufficiently similar to combine for analysis as a single case group, data were combined to achieve higher power. Individual examination of the UKPDS, W2 and YT2D groups was also conducted to ensure that results obtained in the combined group conformed to those in the separate case groups, and confirm that associations observed in the combined case group were not an artefact of case-group heterogeneity.

Given that deviations from HWE were observed for some of the SNPs in the LADA groups, results obtained using methods that assume HWE, e.g. allele-based frequency comparisons and haplotype-based methods (specifically, estimation using the EM algorithm) need to be interpreted with caution.

5.3.2 Single-point association analysis

5.3.2.1 Genetic modelling

Association tests of each of the 3 SNPs with the three LADA groups compared to controls were performed. The results of single-point association analysis obtained under the dominant model of inheritance for the allele assumed to protective at each SNP are given in Table 5.6, and those under the additive model of inheritance are presented in Table 5.7.

Although the SNPs associate significantly with LADA under both the dominant and additive models of inheritance, the results of the dominant model are discussed since they allow comparisons of our data with that published for classical T1D where there is prior evidence for the fit of this particular model.

*Table 5.6: Single-point association analysis: p-values for significance of association of the –23*HphI, *+1404*Fnu4HI *and +3580*MspI *SNPs with disease in the LADA groups under the dominant model of inheritance.*

Sample group (n*)	LADA vs. controls	-23*HphI*	+1404*Fnu4HI*	+3580*MspI*
UKPDS LADA	OR [95% CI]	0.38 [0.26-0.55]	0.41 [0.27-0.62]	0.45 [0.28-0.73]
(232)	Exact p-value	**9.28×10^{-8}**	**7.18×10^{-6}**	**0.0008**
W2 LADA	OR [95% CI]	0.58 [0.38-0.90]	0.73 [0.46-1.15]	0.74 [0.40-1.40]
(131)	Exact p-value	**0.0138**	0.1923	0.3855
YT2D LADA	OR [95% CI]	0.22 [0.08-0.53]	0.37 [0.13-0.90]	0.86 [0.31-3.00]
(37)	Exact p-value	**0.0002**	**0.0248**	0.9339
All cases	OR [95% CI]	0.42 [0.31-0.58]	0.50 [0.36-0.70]	0.55 [0.35-0.85]
(400)	Exact p-value	**2.81×10^{-8}**	**3.42×10^{-5}**	**0.0059**

n = number of individuals in which genotyping was attempted. The OR [95% CI] were calculated based on the following model of inheritance at each SNP: -23*HphI, *dominant for T allele (VNTR Class III alleles); at +1404*Fnu4HI, *dominant for A allele; at +3580*MspI, *dominant for C allele. Significant p-values are denoted in bold.*

*Table 5.7: Single-point association analysis: p-values for significance of association of the –23*HphI, *+1404*Fnu4HI *and +3580*MspI *SNPs with disease in LADA groups under the additive model of inheritance.*

		-23HphI		***+1404Fnu4HI***		***+3580MspI***	
Sample group (n*)	**LADA vs. controls**	**AT**	**TT**	**CA**	**AA**	**CT**	**CC**
UKPDS LADA (232)	OR [95% CI]	0.45 [0.33 -0.61]	0.20 [0.11-0.38]	0.47 [0.33-0.66]	0.22 [0.11-0.44]	0.67 [0.52-0.85]	0.44 [0.27-0.72]
	Exact p-value	**1.58×10^{-7}**		**5.32×10^{-6}**		**0.0008**	
W2 LADA (131)	OR [95% CI]	0.70 [0.50-0.99]	0.49 [0.24-0.98]	0.86 [0.60-1.22]	0.74 [0.36-1.49]	0.81 [0.60-1.11]	0.66 [0.36-1.22]
	Exact p-value	0.0430		0.4004		0.1736	
YT2D LADA (37)	OR [95% CI]	0.37 [0.16-0.75]	0.14 [0.03-0.56]	0.51 [0.22-1.03]	0.26 [0.05-1.05]	1.19 [0.70-2.08]	1.41 [0.48-4.34]
	Exact p-value	**0.0041**		0.0614		0.5293	
All cases (400)	OR [95% CI]	0.53 [0.41-0.69]	0.29 [0.17-0.47]	0.61 [0.46-0.79]	0.37 [0.21-0.63]	0.75 [0.61-0.93]	0.56 [0.37-0.86]
	Exact p-value	**3.27×10^{-7}**		**0.0002**		**0.0066**	

n = number of individuals in which genotyping was attempted. Exact p-values were calculated based on the additive model of inheritance for each SNP such that the disease risk of the heterozygote lies between that of the two homozygotes. ORs [95% CI] for the heterozygote and protective-allele homozygote were calculated using the susceptibility-allele homozygote as comparator. Significant p-values are denoted in bold.*

*Association at -23*HphI

The frequency distribution of genotypes at this SNP in all LADA groups was significantly different compared with controls, owing to a lower frequency of the T allele in LADA. The observed genotype counts were strongly consistent with a dominant protective effect of the T allele against disease in LADA: OR [95% CI] for the combined LADA group = 0.42 [0.31-0.58], exact p = 2.81×10^{-8} (Table 5.6). Supporting this finding, the ORs obtained for the T allele in the individual case groups vs. controls are also significantly less than 1 (Table 5.6). This association is similar to that reported in classical T1D where the T allele (a marker for VNTR Class III alleles) is considered to be dominantly protective against disease.

*Association at +1404*Fnu4HI

The observed genotype counts for the +1404*Fnu4HI* site differed between cases and controls due to a decreased frequency of the A allele in the LADA groups. The genotype counts were consistent with a dominant protective role of the A allele against disease in the combined LADA group: OR [95% CI] = 0.50 [0.36-0.70], exact p = 3.42×10^{-5}, Table 5.6). Consistent with a protective effect of this allele against LADA, the ORs obtained for the A allele at the +1404*Fnu4HI* site in the UKPDS and YT2D groups separately are also significantly less than 1. Although the OR obtained in the W2 group is less than 1, the association was not found to be statistically significant in this group.

*Association at +3580*MspI

In the combined LADA group, the prevalence of the C allele was significantly decreased relative to controls and a dominant protective effect of this allele was observed: OR [95% CI] = 0.55 [0.35-0.85], exact p = 0.0059, Table 5.6). Upon individual analysis of the case groups, the results obtained in the UKPDS group complemented these data. Although a significant association of the +3580*MspI* site with disease was not seen in the W2 and YT2D groups, the ORs for the C allele in both these groups were less than 1, supporting evidence for a

protective role of this allele against LADA. The lack of significant association observed for these smaller sample groups was possibly due to the lower power to detect association.

5.3.2.2 Logistic regression analysis

Since all three SNPs were found to associate with disease in LADA, step-wise logistic regression was used to ascertain which SNPs were independently associated with LADA. This was performed in the combined case group vs. the control group, which gave the highest power. Individual examination of the variants yielded significant p-values for each; Wald χ^2 $p < 0.001$, in keeping with results from contingency table analysis. However, step-wise logistic regression analysis revealed that only the -23*HphI* site (indicating VNTR class) was independently contributing to the risk of development of LADA (Wald χ^2 $p < 0.001$, Table 5.8). This is similar to that seen in T1D, where the VNTR is considered the primary aetiological variant in the *IDDM2* region. The step-wise entry analysis found the +1404*Fnu4HI* and +3580*MspI* sites to have no additional effect on the risk conferred by the -23*HphI* site for LADA (Score test $p > 0.05$).

Table 5.8: Logistic regression analysis to test for independent association of INS gene SNPs with LADA in the UKPDS group vs. controls.

SNP	Wald χ^2 value	df	OR [95% CI]	p-value
Individual association				
-23*HphI*	31.06	1	0.42 [0.31 - 0.57]	**2.50×10^{-8}**
+1404*Fnu4HI*	17.63	1	0.50 [0.36 - 0.69]	**2.69×10^{-5}**
+3580*MspI*	7.85	1	0.55 [0.36 - 0.84]	**0.0051**
Step-wise entry*				
-23*HphI*	30.83	1	0.42 [0.31 - 0.57]	**2.82×10^{-8}**
+1404*Fnu4HI*		1		0.51
+3580*MspI*		1		0.40

**Forward entry: since the -23*HphI *site had the most significant association with LADA, it was entered into the model first. Additional effects of +1404*Fnu4HI *and +3580*MspI *over the –* 23HphI *site were tested for.*

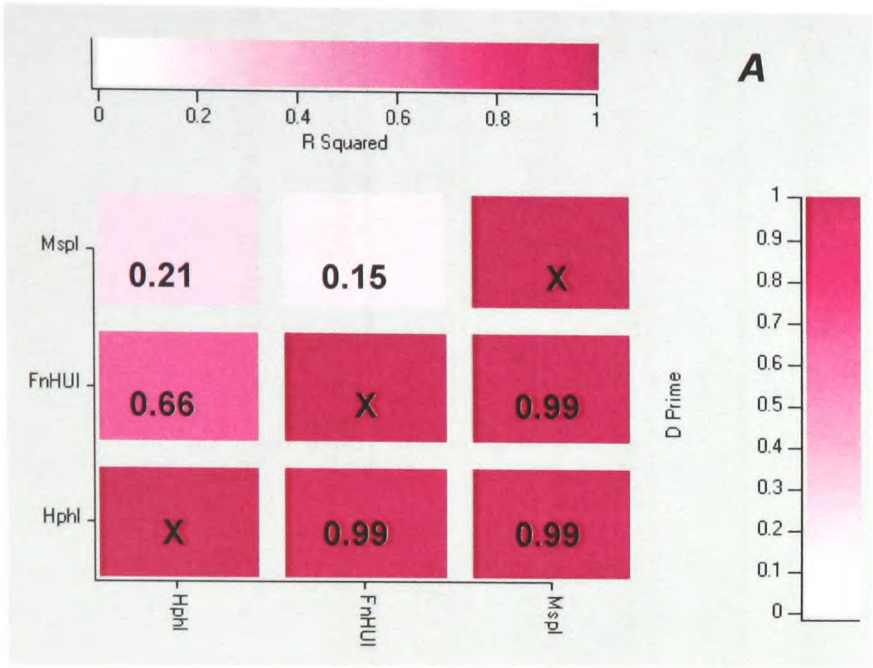
5.3.3 Multi-point analyses

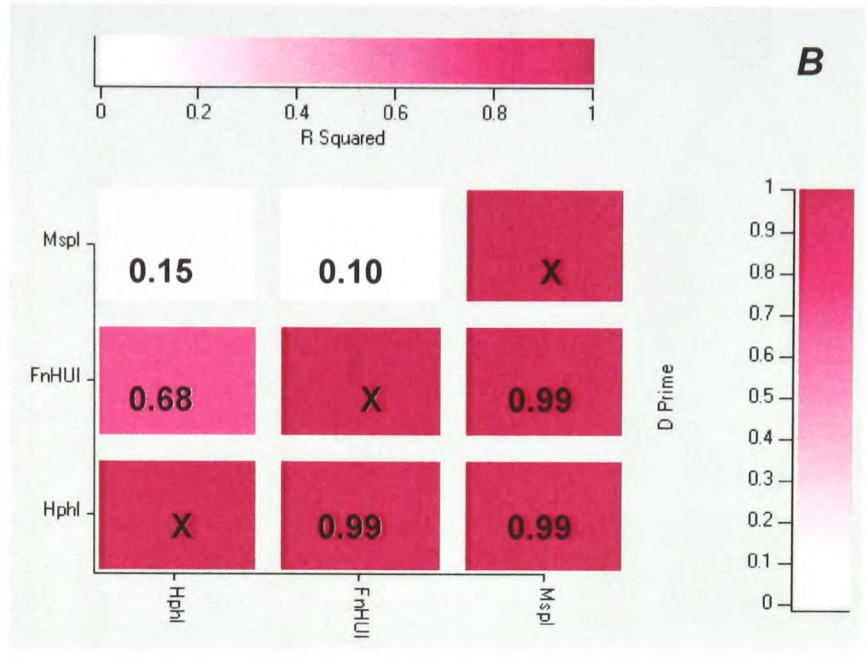
We performed a 3-point haplotype analysis in each of the combined case group vs. controls and in the individual case groups vs. controls. Although the apparent deviations from HWE observed in the LADA groups could potentially invalidate haplotype analyses, the haplotype frequencies obtained from the EM algorithm were consistent when initialised 3 times. This, together with the fact that unexpectedly high frequencies of rare haplotypes were not observed (Table 5.3), suggested that the haplotype estimation process had not been greatly affected by the modest departures from HWE. Therefore, exploratory multi-point analyses were performed, and the results were interpreted with caution – particularly those obtained with the smaller W2 and YT2D groups.

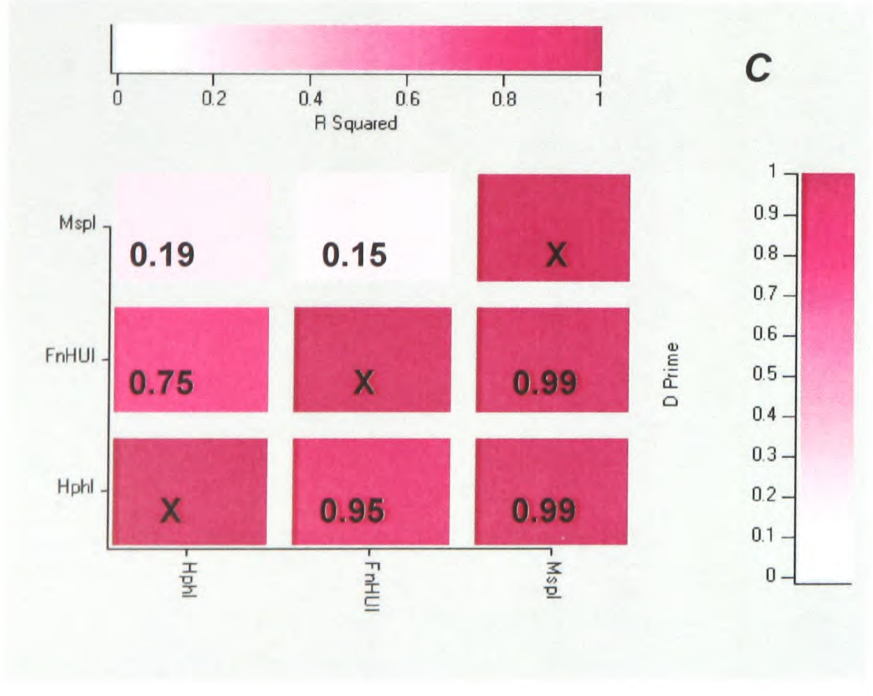
5.3.3.1 Allelic association between SNPs

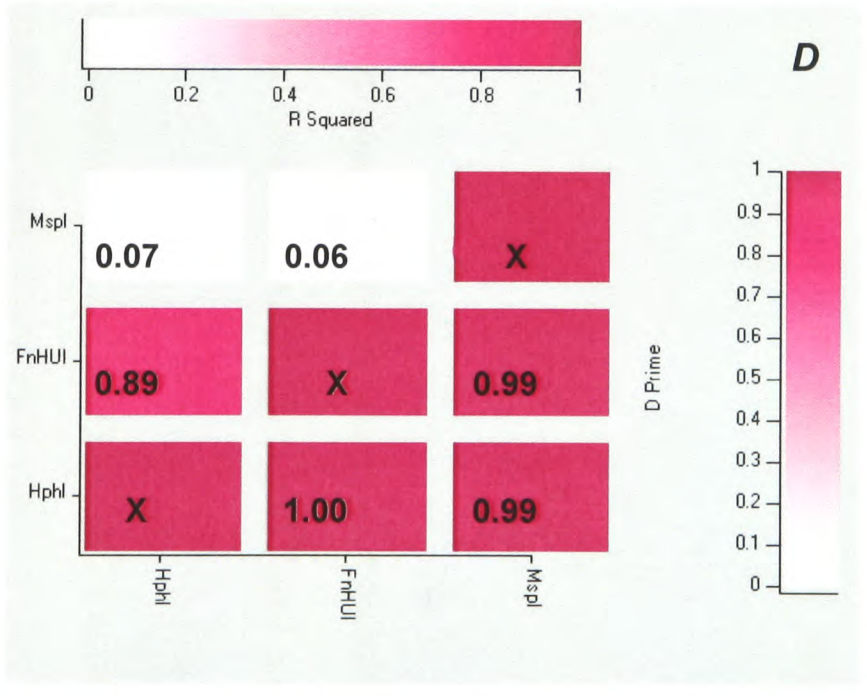
Pairwise LD was determined prior to performing haplotype association analyses (Figure 5.1). As expected, all three SNPs were in LD with one another; D' values were almost 1 and r^2 values were variable, as expected.

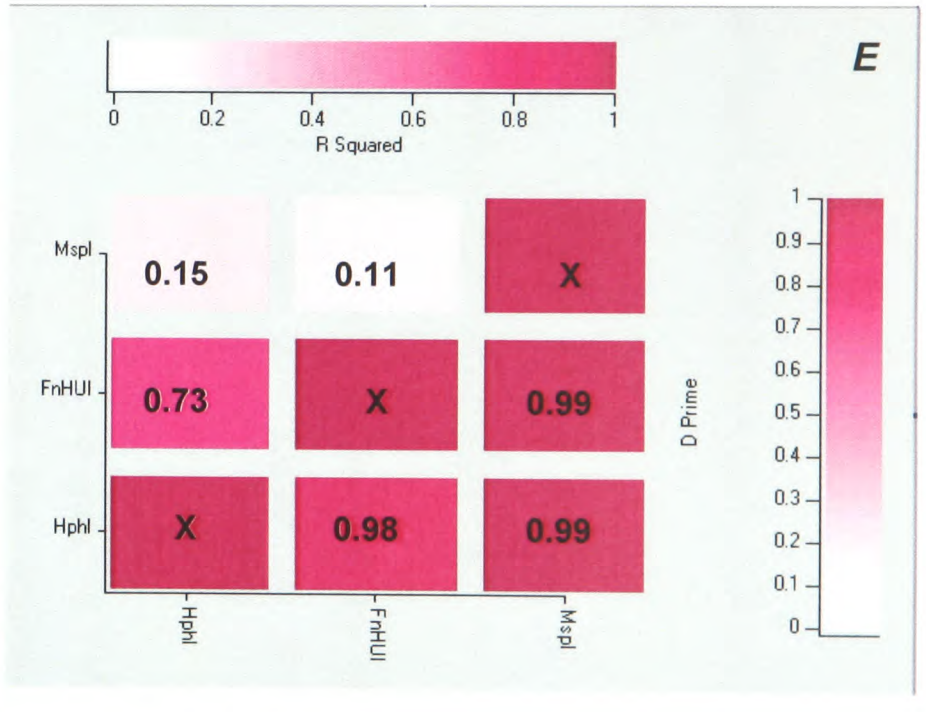
Figure 5.1: Plot of pair-wise r^2 and D' values for (A) control subjects, (B) UKPDS LADA, (C) W2 LADA, (D) YT2D LADA and (E) All cases.









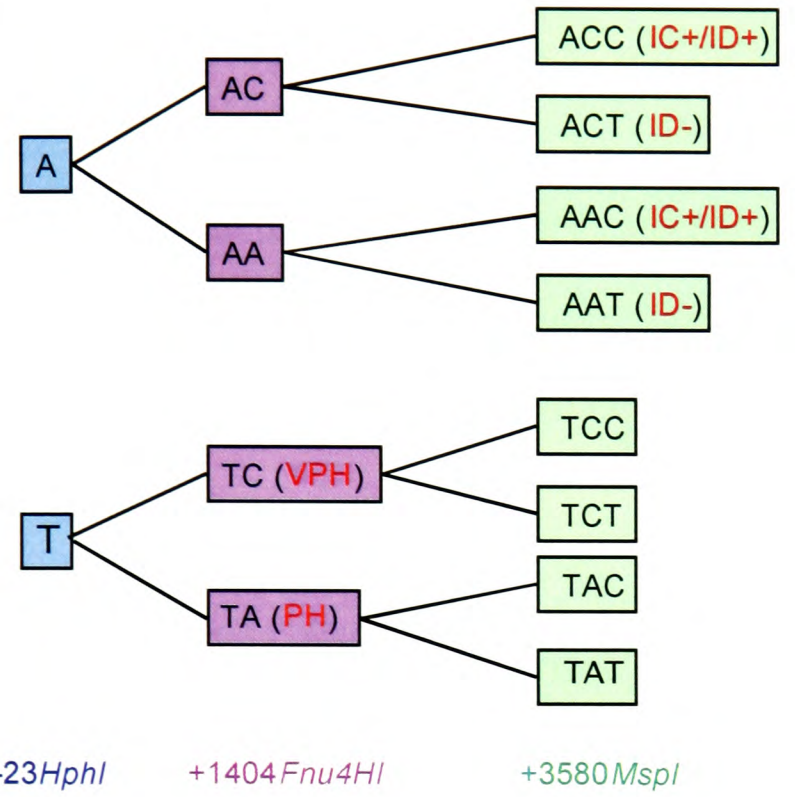


r^2 ('R Squared') values between the SNPs are plotted on the x-axis and D' ('D Prime') values between the SNPs plotted along the y-axis. The colour shading (white → red) indicates the magnitude of LD; where white represents pairwise r^2 and D' values of 0 and red represents values of 1.

5.3.3.2 Haplotypic association

Given that a 3-point analysis was conducted, eight possible haplotypes could potentially be present (see Figure 5.2 below for cladogram).

Figure 5.2: Cladogram to show structure of possible 3-point haplotypes.



Haplotypes formed by the three sites are coded by the alleles present.

Nomenclature used in T1D studies to describe the haplotypes are given in parentheses, coloured in red.

The frequencies of 3-point haplotypes were estimated by the expectation maximisation (EM) algorithm in the control, UKPDS, W2, YT2D and combined LADA groups (Table 5.9).

Table 5.9: Estimated haplotype frequencies in controls and the UKPDS LADA cohort.

Haplotype (T1D-defined)	Haplotype*	Controls (n = 664)	UKPDS LADA (n = 464)	W2 LADA (n = 262)	YT2D LADA (n = 74)	All cases (n = 800)
Class I ID-	ACT	0.336	0.440	0.386	0.297	0.409
Class I IC+/ID+	ACC and AAC	0.364	0.401	0.385	0.568	0.411
Class III PH	TAC	0.222	0.115	0.190	0.122	0.140
Class III VPH	TCC	0.078	0.045	0.039	0.014	0.040

**Allelic combination at –23*HphI*, +1404*Fnu4HI*, +3580*MspI* SNPs.*

n = number of chromosomes.

Four main haplotypes (frequency > 1%) were observed, corresponding to the Class I IC+/ID+[14], Class I ID-, Class III PH and Class III VPH haplotypes. Haplotype trend regression (HTR) showed that the haplotype frequency distribution between the combined LADA cohort and the controls was significantly different ($p = 7.88 \times 10^{-6}$). HTR p-values for the UKPDS, W2 and YT2D groups separately vs. controls were 1.73×10^{-6}, 0.0563 and 9.96×10^{-4}, respectively.

Overall, Class I haplotypes were elevated in the LADA groups compared to controls (suggesting a predisposing effect), whereas Class III haplotypes were decreased (suggesting a protective effect), in keeping with results obtained in classical T1D.

Among the Class I haplotypes, in the combined LADA cohort, the frequencies of both the ID- haplotype and the IC+/ID+ haplotype appeared to be elevated compared to controls (Table 5.9). This result suggested that a protective effect of the ID- haplotype may not occur in LADA, which is in contrast to that suggested by transmission analyses of families in classical T1D (Stead *et al.* 2000). Haplotype frequencies obtained in the individual LADA groups were generally similar to those obtained in the combined group. However, in the YT2D group, the frequency of the IC+/ID+ haplotype was elevated relative to controls but not the ID- haplotype, which was decreased compared to controls.

The frequencies of the Class III haplotypes, PH and VPH, were both decreased in LADA (in both combined and separate groups) compared to controls (Table 5.9), suggesting that they both could have a protective effect against development of LADA.

To substantiate these findings, diplotype analysis was carried out in order to obtain a measure of effect size.

[14] In this study, the Class I IC+ and ID+ lineages were not determined separately as this would require analysis of variant repeat composition and distribution. The ID- lineage was distinguished from all other Class I lineages (i.e. IC+ and ID+) by the absence of the +3580*MspI* restriction site on Class I haplotypes.

5.3.3.3 Diplotype association

The most probable haplotype pair that was assigned to each individual was selected for diplotype analyses (refer to Statistical methods). The diplotype frequencies obtained are given in Table 5.10.

Table 5.10: Diplotype frequencies in controls and the LADA cohorts.

T1D definition	Diplotype	Controls (n = 332)	UKPDS LADA (n = 232)	W2 LADA (n = 131)	YT2D LADA (n = 37)	All cases (n = 400)
IC+/ID+_IC+/ID+	ACC_ACC	42 (0.127)	43 (0.185)	20 (0.153)	15 (0.405)	78 (0.195)
IC+/ID+_ID-	ACC_ACT	80 (0.241)	70 (0.302)	41 (0.313)	10 (0.270)	121 (0.303)
ID-_ID-	ACT_ACT	39 (0.117)	53 (0.228)	20 (0.153)	5 (0.135)	78 (0.195)
IC+/ID+_VPH	ACC_TCC	19 (0.057)	8 (0.034)	4 (0.031)	0	12 (0.030)
ID-_VPH	ACT_ TCC	21 (0.063)	9 (0.039)	5 (0.038)	0	14 (0.035)
IC+/ID+_PH	ACC_TAC	60 (0.181)	22 (0.095)	16 (0.122)	2 (0.054)	40 (0.100)
ID-_PH	ACT_TAC	43 (0.130)	19 (0.082)	15 (0.115)	2 (0.054)	36 (0.090)
PH_PH	TAC_TAC	19 (0.057)	5 (0.022)	9 (0.069)	2 (0.054)	16 (0.040)
PH_VPH	TAC_TCC	8 (0.024)	3 (0.013)	1 (0.008)	1 (0.027)	5 (0.013)
VPH_VPH	TCC_TCC	1 (0.003)	0	0	0	0

Data are n (frequency).

To assess the effect size of each of the four common haplotypes obtained from EM estimation, diplotypes containing the haplotype in question ('positive' diplotypes) were compared against all other diplotypes not containing this haplotype ('negative' diplotypes). Comparisons were made in accordance with the genetic model that best fit in the single-point analysis. This meant that for comparison of Class I diplotypes, the homozygote of the Class I haplotype in question was compared with all other diplotypes (i.e. recessive model). In contrast, for comparison of Class III diplotypes, the homozygotes + heterozygotes of the Class III haplotype in question were compared with all other diplotypes (i.e. dominant model).

The relevant diplotype frequency in the combined LADA group and controls in each category is shown in Table 5.11, together with the OR [95% CI] and p-value for each diplotype comparison obtained by contingency table analysis. This analysis was also performed for the UKPDS, W2 and YT2D groups (Tables 5.12, 5.13 and 5.14, respectively).

Table 5.11: Frequencies of diplotypes and calculated effect sizes in the combined LADA group

Diplotype categorisation	Controls	All cases	OR [95% CI]*	Exact p-value
ID- positive	39 (0.118)	78 (0.195)	1.82 [1.18 - 2.84]	**0.0056**
ID- negative	293 (0.882)	322 (0.805)		
IC+/ID+ positive	42 (0.126)	78 (0.195)	1.67 [1.10 - 2.58]	**0.0161**
IC+/ID+ negative	290 (0.874)	322 (0.805)		
PH positive	130 (0.392)	97 (0.243)	0.50 [0.36 - 0.69]	**2.06×10^{-5}**
PH negative	202 (0.608)	303 (0.758)		
VPH positive	49 (0.148)	31 (0.078)	0.49 [0.29 - 0.80]	**0.0037**
VPH negative	283 (0.852)	369 (0.923)		

*Data are n (frequency). *ORs are given for diplotypes positive for the haplotype in question vs. diplotypes negative for the haplotype in question.*

Table 5.12: Frequencies of diplotypes and calculated effect sizes in the UKPDS LADA group

Diplotype categorisation	Controls	UKPDS LADA	OR [95% CI]*	Exact p-value
ID- positive	39 (0.118)	53 (0.228)	2.22 [1.38 - 3.60]	**7.54×10^{-4}**
ID- negative	293 (0.882)	179 (0.772)		
IC+/ID+ positive	42 (0.126)	43 (0.185)	1.57 [0.96 - 2.56]	0.0728
IC+/ID+ negative	290 (0.874)	189 (0.815)		
PH positive	130 (0.392)	49 (0.211)	0.42 [0.28 - 0.62]	**6.84×10^{-6}**
PH negative	202 (0.608)	183 (0.789)		
VPH positive	49 (0.148)	20 (0.086)	0.55 [0.30 - 0.97]	**0.0371**
VPH negative	283 (0.852)	212 (0.914)		

*Data are n (frequency). *ORs are given for diplotypes positive for the haplotype in question vs. diplotypes negative for the haplotype in question.*

Table 5.13: Frequencies of diplotypes and calculated effect sizes in the W2 LADA group.

Diplotype categorisation	Controls	W2 LADA	OR [95% CI]*	Exact p-value
ID- positive	39 (0.118)	20 (0.153)	1.35 [0.71 - 2.50]	0.3829
ID- negative	293 (0.882)	111 (0.847)		
IC+/ID+ positive	42 (0.126)	20 (0.153)	1.24 [0.66 - 2.28]	0.5463
IC+/ID+ negative	290 (0.874)	111 (0.847)		
PH positive	130 (0.392)	41 (0.313)	0.71 [0.45 - 1.11]	0.1397
PH negative	202 (0.608)	90 (0.687)		
VPH positive	49 (0.148)	10 (0.076)	0.48 [0.21 - 1.00]	**0.0484**
VPH negative	283 (0.852)	121 (0.924)		

*Data are n (frequency). *ORs are given for diplotypes positive for the haplotype in question vs. diplotypes negative for the haplotype in question.*

Table 5.14: Frequencies of diplotypes and calculated effect sizes in the YT2D LADA group.

Diplotype categorisation	Controls	YT2D LADA	OR [95% CI]*	Exact p-value
ID- positive	39 (0.118)	10 (0.270)	2.78 [1.11 - 6.48]	**0.0287**
ID- negative	293 (0.882)	27 (0.730)		
IC+/ID+ positive	42 (0.126)	15 (0.405)	4.71 [2.08 - 10.32]	**1.58×10^{-4}**
IC+/ID+ negative	290 (0.874)	22 (0.595)		
PH positive	130 (0.392)	7 (0.189)	0.36 [0.13 - 0.88]	**0.0207**
PH negative	202 (0.608)	30 (0.811)		
VPH positive	49 (0.148)	1 (0.027)	0.16 [0.004 -1.00]	0.0508
VPH negative	283 (0.852)	36 (0.973)		

*Data are n (frequency). *ORs are given for diplotypes positive for the haplotype in question vs. diplotypes negative for the haplotype in question.*

The diplotype comparisons in the combined LADA group support the notion that both Class I haplotypes, ID- and IC+/ID+, confer similar susceptibility to LADA since overlapping ORs and 95% CIs were obtained for each (Table 5.11). In addition, the Class III haplotypes, PH and VPH appeared to confer similar protection to LADA as comparable ORs and overlapping 95% CIs were obtained for these haplotypes (Table 5.11).

Although not all diplotype comparisons for the individual UKPDS, W2 and YT2D LADA groups were significant when compared against controls (Tables 5.12, 5.13 and 5.14), the OR obtained for each comparison in these smaller data sets complemented the results obtained in the analysis of the combined LADA group vs. controls.

5.3.4 Association with age at diagnosis

Age at diagnosis in the different case groups is shown in Table 5.15. Linear regression analysis showed that none of the genotypes at any of the SNPs tested were significantly associated with age at diagnosis (Table 5.16).

Table 5.15: Age of diagnosis among the LADA groups analysed.

Cohort	Age at diagnosis (median [IQR])	Age at diagnosis (range)
UKPDS LADA	47 [38 - 56]	25 – 65
W2 LADA	47 [40 - 56]	26 – 68
YT2D LADA	40 [32 - 42]	29 – 45
All cases	45 [30 - 61]	25 – 68

Data are given in years. NB Median [IQR] age at DNA collection for control subjects was 56 [38 - 74] years and their range was 20 - 91 years.

Table 5.16: Linear regression analysis of age of diagnosis and genotype among the LADA groups analysed.

Group	Regression output	-23*HphI*	+1404*Fnu4HI*	+3580*MspI*
UKPDS LADA	*β [95% CI]	0.93 [-1.63 to 3.49]	1.50 [-1.46 to 4.46]	-3.75 [-2.20 to 1.45]
	p-value	0.48	0.32	0.69
W2 LADA	*β [95% CI]	-2.48 [-5.21 to 0.26]	-1.97 [-4.75 to 0.81]	1.13 [-1.41 to 3.67]
	p-value	0.08	0.16	0.38
YT2D LADA	*β [95% CI]	0.84 [-2.22 to 3.89]	0.68 [-2.72 to 4.07]	-2.39 [-4.83 to 0.05]
	p-value	0.58	0.69	0.06
All cases	*β [95% CI]	-0.13 [-1.90 to 1.63]	0.07 [-1.84 to 1.98]	0.18 [-1.21 to 1.58]
	p-value	0.88	0.94	0.80

**β is the change in age moving from common homozygote → heterozygote → rare homozygote.*

5.3.5 Association with insulin requirement

To assess whether predisposition from the insulin gene region affected the disease course of LADA after clinical onset, the association of each individual SNP with requirement for insulin therapy by 6 years post-diagnosis was examined. No association of any of the SNPs with this marker of disease progression was found (Table 5.17, $p > 0.05$ for all SNPs).

Table 5.17: Genotype frequencies among UKPDS LADA patients according to requirement for insulin therapy by 6 years post-diagnosis.

Marker	Genotype	Insulin requirement by 6 years	
		No	Yes
-23*HphI*	AA	56 (0.71)	108 (0.72)
	AT	19 (0.24)	38 (0.25)
	TT	4 (0.05)	4 (0.03)
+1404*Fnu4HI*	CC	60 (0.78)	121 (0.81)
	CA	15 (0.19)	26 (0.17)
	AA	2 (0.03)	3 (0.02)
+3580*MspI*	CC	30 (0.38)	50 (0.33)
	CT	32 (0.41)	64 (0.43)
	TT	17 (0.22)	36 (0.24)

Data are n (frequency).

5.3.6 Association with autoantibody status

In Chapter 3, it was shown that in the UKPDS LADA cohort, dual positivity for GADA and IA-2A associated with a more aggressive disease process in the post-diagnosis period compared with those with GADA alone. We therefore investigated whether there was a difference in susceptibility from the insulin gene polymorphisms studied among those with dual positivity compared to those with only GADA. No significant differences were found. (Table 5.18 $p > 0.05$ for all SNPs).

Table 5.18: Genotype frequencies among UKPDS LADA patients according to whether they possessed GADA + IA-2A or GADA alone.

Marker	Genotype	Autoantibody status	
		GADA + IA-2A	GADA only
-23*HphI*	AA	42 (0.76)	159 (0.72)
	AT	12 (0.22)	55 (0.25)
	TT	1 (0.02)	8 (0.04)
+1404*Fnu4HI*	CC	47 (0.85)	175 (0.80)
	CA	7 (0.13)	40 (0.18)
	AA	1 (0.02)	5 (0.02)
+3580*MspI*	CC	17 (0.31)	78 (0.35)
	CT	26 (0.47)	94 (0.42)
	TT	12 (0.22)	50 (0.23)

Data are n (frequency).

5.3 DISCUSSION

This study has established that the *INS* gene is a susceptibility locus for autoimmune diabetes developing in adulthood as well as in children. The –23*HphI* site (indicating the *INS* VNTR) appears to be the main primary disease-associated variant, as observed in classical T1D. It is apparent that a lack of *IDDM2*-encoded genetic susceptibility does not contribute to the different disease courses of LADA and T1D, as had been suggested by the data of Tuomi *et al* (Tuomi *et al.* 1999). The relatively low frequency of the Class III allele in Finns (Undlien *et al.* 1995) and the modest sample size of this study (n=95 LADA) may have compromised the power to detect an association in this particular study. Our findings do however, corroborate another smaller study (n = 44 LADA, n = 138 controls), where a significant association of Class I alleles was found with LADA (Cerrone *et al.* 2004).

From the data available, there is no suggestion that the magnitude of the *INS* VNTR effect size in LADA differs from that seen in T1D. In the combined LADA group, the OR [95% CI] associated with the dominantly protective Class III alleles was 0.42 [0.31-0.58], a value comparable to that found in studies of classical T1D (for example, ORs of 0.36 [0.23-0.55] and 0.22 [0.12-0.39] as calculated from previous reports (Lucassen *et al.* 1993; Bennett *et al.* 1995)).

Fine-structure analysis of the region demonstrated similarities in the association patterns of LADA compared to those reported for T1D. Diplotype analysis of Class I haplotypes showed that the ID- haplotype confers similar susceptibility to LADA as other Class I haplotypes, in keeping with a recent re-mapping and association study of the insulin gene in T1D (Barratt *et al.* 2004). Also in line with the current understanding in T1D (Barratt *et al.* 2004), there was no evidence in this study, that Class III PH and VPH haplotypes differed in protection against LADA. These multi-point association analysis results support those obtained in the step-wise logistic regression analysis.

Although the VPH and PH may not indicate heterogeneity with respect to disease protection within the Class III subgroup, the existence of predisposing 'thymic silencing' alleles suggests that not all Class III may be equally protective (Pugliese *et al.* 1997; Vafiadis *et al.* 1997; Vafiadis *et al.* 2001; Pugliese and Miceli 2002; Anjos and Polychronakos 2004). These alleles are predisposing to T1D since they associate with complete silencing of the *INS* gene in the thymus during development, which could impair the negative selection of autoreactive T cells (see below). Perhaps a lower frequency of these predisposing alleles occurs in LADA compared to T1D. However, given the rarity of these alleles, it is difficult to gauge the global importance these Class III variants may have in determining disease susceptibility in the majority of the LADA population.

Functional effect of IDDM2 *susceptibility in LADA*. It has been suggested that the *INS* VNTR, rather than the –23*HphI* site, is the causative polymorphism in T1D since there appears to be no functional effect of the latter biallelic intronic variant on *INS* expression (Lucassen *et al.* 1995). It is known that the number and distribution of variant repeats differs between the Class I and Class III lineages (Stead and Jeffreys 2000). It was also discovered that differences in the repeat sequence might affect the expression levels of the *INS* gene through their binding by transcription factors. For example, the activity of the Pur 1 transcription factor which binds the VNTR and regulates basal *INS* transcription, may depend on sequence variations within the VNTR since it has a higher affinity for certain repeat variants (Kennedy *et al.* 1995; Lew *et al.* 2000). Class I alleles have been associated with a decreased level of transcription of *INS* compared with Class III alleles in the thymus during development (Pugliese *et al.* 1997). It has been postulated that this difference may affect central tolerance mechanisms such that a low level of insulin transcription associated with Class I *INS* transcripts would impair the negative selection process of autoreactive T cells, and hence increasing the propensity to develop autoimmunity against insulin. Similar susceptibility mechanisms are likely to operate in LADA.

Association with age of diagnosis, requirement for insulin and autoantibody status. In this study, a phenotypic assessment of the insulin gene variants in the UKPDS LADA cohort was performed. A lack of association was seen of *INS* gene polymorphisms with age at diagnosis or requirement of insulin therapy by 6 years post diagnosis. In addition, no association with dual autoantibody positivity (GADA + IA-2A) vs. GADA alone was observed. Although this gene region clearly increases susceptibility to LADA, the complex nature of the disease means that other genes or environmental factors affect the onset and severity of clinical disease in LADA.

Future directions/ improvements to this study. A more comprehensive approach to this study would have been to identify and genotype haplotype-tagging SNPs that would have enabled one to capture all of the variation in this region (Johnson *et al.* 2001). Since this 'pilot' study of the most informative SNPs that are found in T1D has provided a strong indication that the locus predisposes to disease in LADA, such an approach in the future would allow a more thorough evaluation to identify/confirm the main susceptibility determinant(s).

An obvious improvement to the study would be to increase our cohort size to help increase power. The current study had >90% power to detect the Class III dominant protective association with OR $\leq$ 0.5 (for α = 0.05) in the combined case group, and the UKPDS and W2 groups separately. However, considerably larger numbers of subjects would be required to detect subtle differences in predisposition associated with specific Class I or Class III subgroups. For example, the largest study comparing the protective effects associated with PH and VPH in classical T1D featured meta-analysis of data from almost 3000 pedigrees (Barratt *et al.* 2004). Until a suitable cohort size becomes available, this study is the highest powered assessment of the insulin gene haplotypes in LADA.

As with any genetic association study, there is a chance of false-positive findings, given the number of tests performed (Nyholt 2001). Since the present study was testing a specific prior hypothesis (that of dominant protective effects of Class III alleles) based on data from T1D, we have made no corrections for multiple testing and used a relatively liberal threshold for attributing significance. Even so, application of a conservative Bonferroni correction to the lowest p-value obtained ($p = 2.81 \times 10^{-8}$) would still retain significance ($p = 3.01 \times 10^{-6}$), indicating that the observed associations are very likely to be real.

Chapter 6:

Final Discussion

CHAPTER 6: FINAL DISCUSSION

The pathogenic events involved in T1D and the reasons for the heterogeneity in disease course remains an enigma. It is probable that attenuated genetic, immunological and environmental factors in the pathogenesis of LADA lead to the later onset and slower progression to complete β-cell destruction and insulin dependency compared to T1D. In this thesis, both genetic (*HLA* and *INS* genes) and immunological (GADA) factors were explored in LADA to identify markers which could better define the disease progression and where possible, findings were compared to those reported in T1D to seek clues that may explain the different disease courses.

6.1 Immunological analysis

In Chapter 3, GADA persistence in the post-diagnosis period and its role as a marker for deterioration of islet function as indicated by changes in glycaemic control was investigated. GADA is the major autoantibody identified to date in LADA and was examined because this humoral marker could be an indicator of the severity of the underlying autoimmune pathology against the pancreatic β-cell, which is driven by cellular responses. This hypothesis assumes that B and T cell immune responses are coordinated, and are likely to target the same autoantigens. In support of this contention, T cell responses against GAD 65, have been found to occur in LADA patients (Fukui *et al.* 1999).

6.1.1 GADA persistence

A remarkable persistence of GADA titre was observed over the 6 year period with hardly any patients seroconverting to negativity (Chapter 3). Those patients in whom positivity was variable had low titres fluctuating around the cut-off, illustrating the difficulty in deciding what constitutes a GADA positive result (Gale 2005). A similar persistence of GADA post-diagnosis has also been reported to occur in classical T1D (Christie *et al.* 1990a). The persistence of GADA suggests that the GAD 65 autoantigen persists after clinical onset of

disease. Although a critical mass of β-cells must have been lost to induce hyperglycaemia and clinical symptoms, it is probable that some cells still remain to sustain this response. However, there is the possibility that β-cell dysfunction rather than just the loss in cell number causes hyperglycaemic symptoms (Reviewed by (Pipeleers and Ling 1992)). This hypothesis is supported by histological analysis of pancreatic islets of a LADA patient, where it was shown that β-cell mass appeared to be preserved (Shimada *et al.* 1999). However, only 9 islets were examined in this study and further studies are needed to compare the histology of islets from LADA patients with those in T1D. Nevertheless, the large number of insulin-positive islets seen in the biopsy from the LADA patient could also imply that they were simply 'resistant' to immune attack. It has been shown in classical T1D pancreatic specimens that there is heterogeneity in the numbers of β-cells lost from each pancreatic islet (Reviewed by (Pipeleers and Ling 1992)); many islets had reduced insulin positivity whereas some appeared to have been spared from the immune destruction process even after many years of diabetes (Reviewed by (Pipeleers and Ling 1992)). More insulin-positive islets were seen with increasing age of diagnosis of the patient in T1D (Foulis *et al.* 1986). Perhaps a greater number of these 'resistant' islets exist in LADA patients compared with classical T1D patients resulting in the less severe clinical symptoms seen in the former. Also, the GADA response in the post-diagnosis period in both T1D and LADA may be maintained by those islets that are resistant to immune attack. Unfortunately, since pancreata for analysis of LADA patients prior to, during and after the autoimmune attack are even more scarcely available than they are from classical T1D, it not possible to answer these questions at the present time.

Of course, as discussed in Chapter 3, extra-pancreatic sources of GAD 65 and molecular mimicry mechanisms may sustain the response. Lastly, some authors have proposed that β-cell neoformation occurs in the adult pancreas and those β-cells lost through the autoimmune attack may be regenerated in LADA (Pozzilli and Di Mario 2001). However,

whether β-cell regeneration occurs in adults is currently a matter of debate (Meier *et al.* 2005).

6.1.2 Clinical utility of GADA

GADA titre was not found to be a marker for progression through the UKPDS protocol, which was used as a surrogate to gauge β-cell function over the post-diagnosis period (Chapter 3). This suggested that GADA titre *per se* does not reflect the extent of the underlying β-cell destructive process. Rather, it was the number of autoantibody markers a LADA patient had at diagnosis that predicted a faster rate of decline and need for more intensive anti-hyperglycaemic therapy in the post-diagnosis period. This is reminiscent of the situation found in the pre-clinical period of classical T1D where a number of studies have shown that the likelihood of progression to clinical disease in at-risk individuals is largely governed by the number of humoral markers present (see Chapter 1, Section 1.2.1.2). Therefore, the number of autoantibodies, not titre, was an indicator of the severity of the autoimmune process. It is important to note that in other UKPDS studies, GADA titre at diagnosis predicted insulin requirement by 6 years post-diagnosis (Turner *et al.* 1997; Bottazzo *et al.* 2005). So, to answer a question posed by Professor Edwin Gale (University of Bristol) in a recent editorial; 'Do GAD antibodies help us to decide when to start insulin?' (Gale 2005), here is a response; - while it may still be useful to take the quantitative measure of GADA at diagnosis into account when predicting likelihood of early insulin requirement compared with classical T2D subjects, the titre at subsequent time points (0.5, 3 and 6 years) does not appear to have prognostic utility. The exact time at when to begin insulin therapy was not indicated by GADA titre in this study.

In Chapter 3, it was also shown that GADA titre did not associate with the different treatment modalities of the UKPDS (diet, oral agents, insulin). *Davis et al* showed that the initial therapy that a patient was randomised to in the UKPDS did not differentially affect

their glycaemic control over a 10-year period whether they were antibody-positive (of which 85% were GADA positive) or antibody-negative (Davis *et al.* 2005). Taken together, all of the above findings suggest that the use of GADA in predicting therapy changes or in clinical management in LADA is doubtful.

However, the persistence of GADA enables post-diagnostic separation of younger-onset diabetic subjects with autoimmunity from other groups of diabetes, namely classical T2D and those with monogenic disease i.e. MODY subtypes.

6.1.3 Future directions

In this thesis we investigated the role of only GADA and IA-2A in disease progression. However, a study by Seissler *et al* (Seissler *et al.* 1998) suggested that these two autoantigens might not comprise the bulk of ICA reactivity. Perhaps more work into elucidating the role of the vast number of other autoantigens that have been identified in T1D (Lieberman and DiLorenzo 2003) should be performed in LADA. It is important to characterise the constituents of ICA since ICA itself is difficult to measure, given it's strong dependence on the accuracy of observer scoring and quality of the pancreas substrate used.

In addition, identifying the specific epitopes targeted by these antibodies may have clinical utility. In classical T1D, conformational GADA epitopes occur mainly in the middle and C-terminal portions of the GAD 65 molecule, with N-terminal reactivity being relatively rare (Daw and Powers 1995; Falorni *et al.* 1996). The epitope reactivity of GADA can change over time, a phenomenon known as 'epitope spreading' and specificities may be gained or lost. Epitope spreading in the pre-clinical period of T1D generally occurs in the following pattern; middle $\rightarrow$ C-terminal $\rightarrow$ N-terminal and these changes do not predict the clinical onset of disease (Bonifacio *et al.* 2000). However, in the post-diagnosis period of T1D, increasing levels of titres to the middle portion of GAD 65 marked a requirement for lower

doses of exogenous insulin post-diagnosis (Ronkainen *et al.* 2004). As alluded to in Chapter 3, the measurement of epitope reactivities in LADA may be useful in predicting clinical course in the post-diagnosis period. For example, in a study of 17 Japanese LADA patients, Kobayashi *et al* found high levels of reactivity to N-terminal epitopes associated with a faster time to insulin requirement than low levels (Kobayashi *et al.* 2003). However, the widespread significance of these findings in LADA is uncertain since, unlike in the Japanese, N terminal reactivity occurs at low levels in Caucasian LADA patients (M Desai, unpublished observations). Another retrospective study of 61 Italian LADA patients showed that C-terminal reactivity correlated with insulin requirement (Falorni 2000). Large prospective studies of LADA patients are required to evaluate whether spreading or restriction of GADA epitopes occurs in the post-diagnosis period and to determine if this has any clinical utility.

It seems that autoantibodies are not direct indicators of progression of the disease process in autoimmune diabetes. It may be better to investigate T cells themselves, which are the likely pathogenic mediators of autoimmune diabetes, given the T_H1-polarised nature of the disease. As well as characterising the natural history of islet autoreactive T cells, both qualitative and quantitative differences in T cell autoreactivities between T1D and LADA patients need to be characterised. In addition, it will probably also be essential to understand how regulatory T cell networks influence the time course of the disease. A recent report by Arif *et al* showed that T cells in T1D patients presenting at older ages were more likely to raise immune suppressive IL-10 responses to IA-2 and proinsulin peptides (Arif *et al.* 2004). The authors suggested this IL-10 response (speculated to be raised by regulatory T cells) and the immune tolerance it may induce, could explain the latency of clinical onset of T1D occurring in adults. However, the relative levels of cytokine production were not measured (due to limitations of the ELISPOT methodology used) and therefore these differences could not be quantitatively related back to the different rates of disease

onset. However, it is possible that this characteristic of immune tolerance applies to LADA too and warrants further investigation.

6.2 Genetic analyses

Our overall hypothesis regarding our genetic analyses was that the slower progression of autoimmune disease in LADA could be partly explained by a reduced susceptibility or alternatively an increased protection from T1D susceptibility genes.

6.2.1 The HLA genes in LADA

In Chapter 4, the role of variants in the HLA genes *DRB1* and *DQB1* in susceptibility to LADA was investigated. Similarities existed between data from LADA subjects and that reported for T1D regarding general patterns of susceptibility; that is, the nature of predisposing, neutral and protective variants. This implied that the genetic background of the two disease forms may be similar. The main susceptibility variants found in LADA were DR4 (in LD with DQ8) and DR3 (in LD with DQ2), which are the same risk variants found in T1D. The frequency of these variants occurred at 85% in LADA, compared with 95% reported for T1D (Thomson *et al.* 1988). This suggests that the overall risk from T1D susceptibility variants at these loci was reduced in LADA, providing support for our overall hypothesis.

6.2.2 The insulin gene in LADA

In Chapter 5, association analysis of the insulin gene was performed in LADA. The association patterns observed in LADA appeared to mirror those reported for classical T1D. No qualitative or quantitative differences in genetic susceptibility conferred by the insulin gene region could explain the marked variation in disease course and clinical presentation between LADA and T1D.

An obvious question is whether genetic susceptibility at the insulin gene associates with positivity and titre of IAA. Graham *et al* showed that the frequency of IAA increased with the number of Class I alleles present in T1D patients (Graham *et al.* 2002). Also, higher IAA titres have been shown to occur in T1D patients with Class I/Class I genotypes relative to Class III-positive genotypes (Nielsen *et al.* 2005). The low prevalence of IAA known to occur in LADA makes such hypotheses difficult to test.

6.2.3 Future directions

Since both the HLA and insulin gene regions have been shown to be important susceptibility loci for LADA, it would be interesting to perform interaction analyses to see how these genes together influence disease susceptibility. One mechanism by which this may occur is the aberrant presentation of preproinsulin by proteins encoded by HLA susceptibility genes, resulting in diabetes (Bennett and Todd 1996). *HLA-INS* interactions have been reported to occur in T1D; however, conflicting results have been obtained from studies conducted on small numbers of patients (Julier *et al.* 1991; Metcalfe *et al.* 1995; van der Auwera *et al.* 1995a; Laine *et al.* 2004). In the largest study to date (n = 944), Motzo *et al* demonstrated that the effects of Class I alleles are most pronounced in those individuals carrying intermediate or low-risk HLA genotypes (Motzo *et al.* 2004). The numbers of LADA patients available in the present study were not considered sufficient to perform a statistically valid analysis of this type.

It is important to note that a number of other minor susceptibility genes have been associated with T1D. Preliminary candidate-gene association analyses have suggested that many of these genes could also predispose to disease in LADA e.g. *CTLA-4* (Abe *et al.* 2001; Cosentino *et al.* 2002) and MICA (Sanjeevi *et al.* 2002a; Torn *et al.* 2003). In addition, the recently discovered T1D susceptibility gene, *PTPN22* (Bottini *et al.* 2004) is another hot contender for association analysis. Given the strong biological evidence for a role of these

genes in susceptibility to autoimmune diabetes, these represent excellent candidate genes to investigate in LADA.

In addition to susceptibility genes for T1D, perhaps T2D susceptibility genes should be investigated in LADA. It is known that some patients may exhibit insulin resistance (Carlsson *et al.* 2000; Hosszufalusi *et al.* 2003) and as discussed above, β-cell dysfunction may play a role in the polygenic pathogenesis of LADA. Susceptibility genes identified for T2D such as *KCNJ11* (encodes the Kir6.2 K^+-ATP channel) (Gloyn *et al.* 2003), *PPARG* (encodes the peroxisome proliferator-activated receptor-γ) (Altshuler *et al.* 2000) and *CAPN10* (encodes the cysteine protease calpain-10) (Turner *et al.* 2005) could contribute to insulin resistance and β-cell dysfunction in LADA, compounding the problem of β-cell deficiency and causing LADA patients to accelerate faster down the descending slope of the 'Starling Curve of the Pancreas' (DeFronzo *et al.* 2004).

The association studies performed in this thesis should be considered as preliminary as at the present time the veracity of genetic associations (both positive and negative) found with complex diseases is questioned. Larger, independent case-control and family-based studies of the gene regions studied would be required to assess the validity of the results obtained to confirm these associations (Editorial 1999; Dahlman *et al.* 2002; McCarthy *et al.* 2005). Larger cohorts will also enable one to perform stratified analyses of LADA since the heterogeneity of this disease indicated by clinical features may mask disease associations or cause spurious ones.

For genetic comparisons, in an ideal world, one would have a large cohort of classical T1D subjects to directly compare frequencies against to determine the differences in susceptibility to disease from the genes investigated among rapidly and slowly-progressing autoimmune diabetes.

It is important to relate the genetic associations found with disease back to immunological features of the disease. We did not find an association of genetic variants at *IDDM1* and *IDDM2* with the presence of more than one autoantibody at diagnosis. However, this may be too crude a measure. It would be interesting to see if the epitope patterns of B and T cell responses reflect differences in the MHC variants responsible for generation of these responses.

6.3 Difficulties in studying LADA

One of the major difficulties in interpreting results from genetic and immunological analyses of LADA, is taking into account the heterogeneity of this disease. Different researchers adopt various definitions and therefore it is imperative that the classification of LADA, is standardised to enable a fair comparison of data from different studies. However, we currently have a 'catch-22' situation. Definition based on aetiology rather than clinical features is required, especially if one is to make distinctions between juvenile and adult forms of autoimmune diabetes. However, currently we have no specific humoral or T cell markers that distinguish between the two forms, making this impossible. Therefore, it is generally agreed that further knowledge of the natural history and pathogenesis of LADA is required to create objective diagnostic criteria.

It is important to note that environmental factors, which probably account for most of the susceptibility to LADA, will be important to characterise and understand the aetiopathogenesis of both forms of autoimmune diabetes. Their interaction with the immune system and susceptibility genes probably act together to influence disease susceptibility, given the complex nature of the disease. Identification of environmental determinants has become even more important since there is evidence to suggest that the environment has become more permissive for autoimmune diabetes since the prevalence of high-risk HLA genotypes appears to have decreased over time in the T1D population (Gillespie *et al.* 2004). However, few factors have been conclusively associated with susceptibility to

classical T1D but even these risks are not absolute (Chapter 1, Section 1.2.3). Large-scale epidemiological studies are required to assess the role of environmental factors, with great costs involved. At the moment such studies are hindered by the difficulty in recruiting LADA patients with no consensus criteria to diagnose them and results would potentially be confounded by our lack of ability to deal with the disease heterogeneity of LADA.

6.4 Conclusions

The ultimate aim of understanding of the pathology of autoimmune diabetes is to improve clinical management; that is, the quality of life and prognosis of patients with this disease. By preventing this disease we can remove the burden of daily insulin injection and blood testing as well as short and long-term complications including premature death.

The work in this thesis has shown autoantibody number can predict the clinical course of LADA in the post-diagnosis period. Although persistence of GADA occurs in the post-diagnosis period, quantitative measurements of this autoantibody alone after clinical onset are not useful for predicting the need for more intensive therapy. The challenge is now to identify other immune markers of the disease process that could be used to monitor progression of disease to aid prediction of requirement of changes in therapy.

Common genetic susceptibility suggests that at least this component of the underlying disease pathology between T1D and LADA could be the same. Results included in this thesis support the hypothesis that LADA does not represent a unique entity of autoimmune diabetes, but rather an age-related extension of the childhood-onset, garden variety of this disease, a view that appears to be popular in some areas of this field of study (Gale 2005).

Appendices

APPENDICIES

APPENDIX 1: WHOLE GENOME AMPLIFICATION OF LOW QUANTITY DNA SAMPLES

Currently, two reliable whole genome amplification (WGA) methods have been developed; (1) strand-displacement amplification and (2) library based WGA systems. The immediate advantage of these methods over earlier ones (e.g. PEP, DOP) is that they employ the φ29 DNA polymerase whose error rate is 3 in 10^6-10^7 (Esteban *et al.* 1993) compared with native *Taq* DNA polymerase, which has an error rate of 1 in 1000 (Eckert and Kunkel 1991).

The first of these modern methods, involving 'multiple-strand displacement' (MDA), was tested and is discussed in detail below. Briefly, the second library-based method involves the random partial fragmentation of genomic DNA template followed by conversion of these fragments into a library of PCR-amplifiable molecules of defined size. This library is then amplified by PCR using universal primers and a high-fidelity DNA polymerase (Langmore 2002).

Two protocols employing the MDA approach were tested for their ability to perform a viable WGA of DNA samples that were too low in quantity to complete the genotyping required; 1. the Genomiphi system (Amersham Biosciences, Buckinghamshire, UK) and 2. the deaza RepliG system (Qiagen Inc, CA, USA). They both have the advantage of generating amplified DNA in large quantities and of large fragment sizes in a single-tube reaction. A recent comprehensive study of amplification fidelity assessed by the correlation of SNP calls from genomic and amplified DNA showed that the MDA technique achieved almost complete coverage of the genome (99.86%) with minimal amplification bias (Barker *et al.* 2004a). The applicability of these techniques to our work was justified by a recent report that showed the success of the Genomiphi method for accurate HLA typing of the *DRB1* and *DQB1* genes by PCR-SSP of WGA product (Shao *et al.* 2004). The authors concluded that the Genomiphi kit was able to cope with the redundancy of HLA genes, their high G:C content and extensive polymorphisms, with unbiased and high-fidelity amplification of DNA in the 6p21-24 region. However, it has been claimed that the Genomiphi method does not successfully amplify the insulin gene region at 11p15.1 (JA Todd, personal communication). The deaza RepliG technique contains a modified GTP nucleotide (7-deaza-GTP) that disrupts G-quartets found in repetitive DNA elements, breaking down

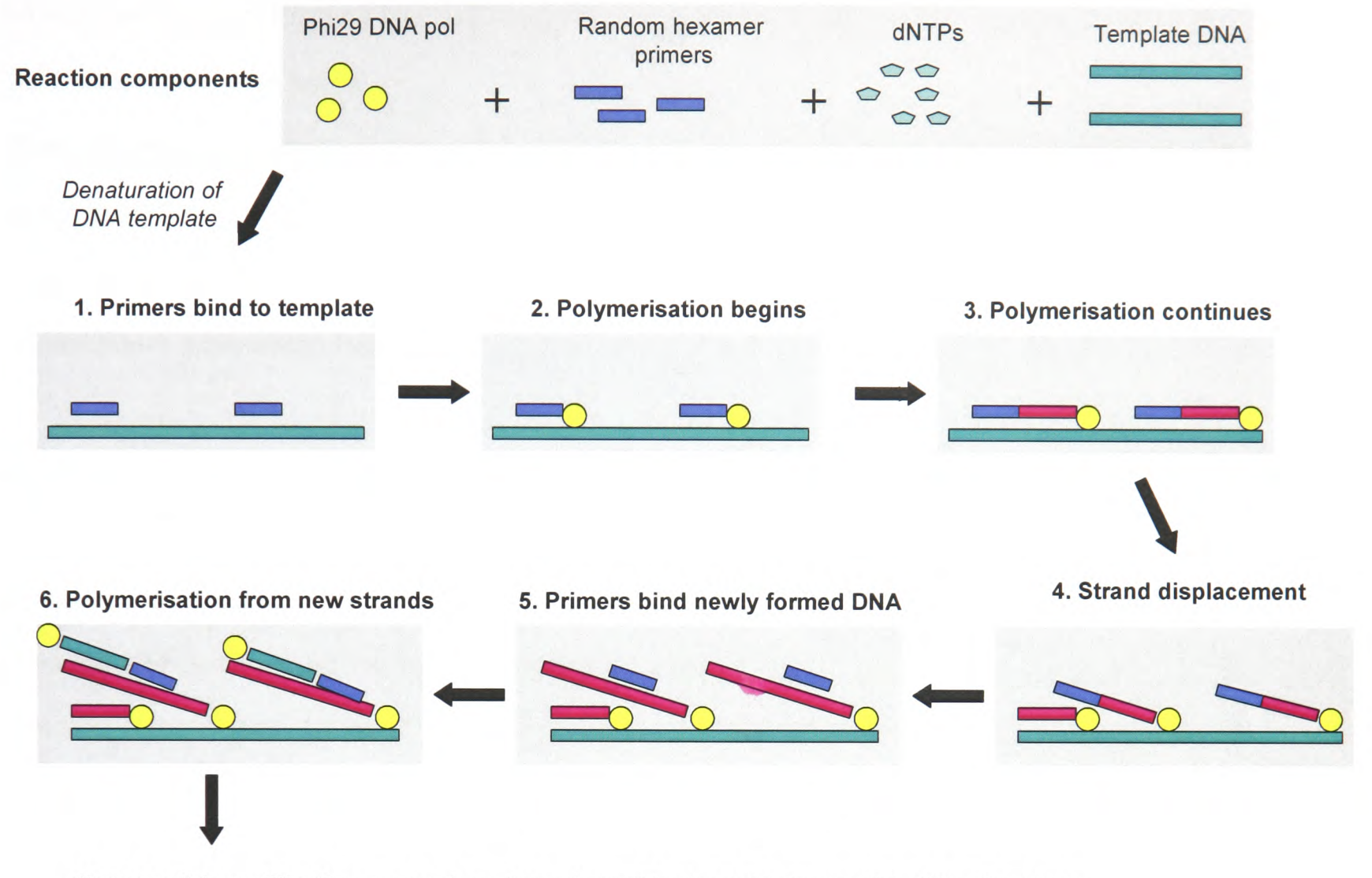
secondary structure which would otherwise prevent the priming reaction and amplification of such DNA regions. This technique was developed to overcome the problem of under-representation these loci in the WGA product. The deaza RepliG protocol was investigated by our laboratory since amplification of the -23*HphI* polymorphism in the insulin gene is affected by the upstream VNTR which contains G-quartet secondary structures. It was therefore determined whether this method provided a better representation of the region compared with that produced by the Genomiphi kit.

Since HLA typing of MDA amplified DNA has been shown to be successful (Shao *et al.* 2004), the primary aim of this work was to determine the capability of the two methods to generate DNA that was amenable to genotyping of the –23*HphI* polymorphism in the insulin gene. Where enough unamplified DNA was present, the ability to genotype polymorphisms in the *DRB1* and *DQB1* genes in the HLA complex was also tested. The success of a method was determined by concordance of genotyping results before and after WGA.

Both Genomiphi and RepliG systems amplify linear genomic DNA using a highly processive DNA polymerase (with 3'-5' exonuclease proofreading ability) by an isothermal (30^0C) strand displacement reaction such that thermal cycling is not required (Dean *et al.* 2002). The enzyme can polymerise 70 kb fragments without dissociating from the genomic DNA template. The amplification of the whole genome is achieved through non-specific extension of random hexamer primers (which are exonuclease-resistant) that bind to template DNA at multiple sites. Microgram quantities of DNA are generated from nanogram amounts of input DNA. Most of the amplified product is of high molecular weight and double-stranded.

Essentially, the two methods are similar in their technology and only differ essentially in the nature of the initial denaturation step. A schematic of how MDA works is shown in Figure 1.

Figure 1: Schematic diagram of the multiple displacement amplification process.



Amplification is achieved at 30^{0}C with random hexamer primers. 1. Primers bind the genomic DNA template. The φ29 polymerase binds the template and extends these primers (2,3). As the newly-synthesised DNA strand grows, it eventually displaces from the template (4). New primers can now bind the single-stranded template of the newly-synthesised strand (5). Polymerisation continues from the newly-bound primers, amplifying the complementary strand of the original template. (Figure adapted from (Mamone 2003)).

Genomiphi system (Amersham Biosciences)

This was tested in-house using the commercially available kit. The amplification protocol was performed in a 96-well format and involved five steps (manufacturers instructions):

1. Denaturation: Genomic template DNA (10 ng) is heat denatured at 95^{0}C for 3 min in 'Sample Buffer', which contains the random primers.

2. Cooling: The reaction is rapidly cooled to 4^{0}C, during which the random primers bind to template DNA.

3. Catalysis of amplification reaction: 'Reaction Buffer' (containing salts, dNTPs, pH optimal for φ29 DNA polymerase) and 'Enzyme Mix' (φ29 polymerase) is added to the reaction followed by incubation at 30^{0}C overnight (16-18 hours).

4. Post-amplification heat inactivation: The φ29 DNA polymerase is heat inactivated by incubating the reaction at 65^{0}C for 10 min.

5. Clean-up of amplified product: This was an optional step to enhance viability of the amplified DNA for downstream applications (see later).

Deaza RepliG system (Qiagen Inc, USA).

Since the deaza RepliG technique was under development, a kit was not available and test samples were sent to Qiagen, who developed the method. The amplification protocol was performed in a 96-well format and also involved five steps (manufacturers instructions):

1. Alkaline Denaturation: 10 ng of genomic template DNA is chemically denatured by application of 'Denaturation Solution' (0.4M KOH, 10mM EDTA (pH 8.0)). Samples were thoroughly mixed by pipetting up and down and were then incubated at room temperature for 3 min.

2. Neutralisation: 'Stop Buffer' (800mM Tris-hydrochloride) is added to each sample to neutralise the alkali. The DNA in each sample is now denatured and ready for primer annealing.

3. Amplification reaction: 'Reaction Master Mix' (containing 37 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM of each dNTP, 50 μM exonuclease-resistant hexamer, 1 unit/ml east pyrophosphatase, 800 units/ml φ29 DNA polymerase) at a pH optimal for the DNA polymerase is added to the reaction followed by thorough mixing and incubation at 30^{0}C overnight (16-18 hours).

4. Post-amplification heat inactivation: The φ29 DNA polymerase is heat inactivated by incubating the reaction at 65^{0}C for 10 min.

5. Clean-up of amplified product: This was an optional step to enhance viability of the amplified DNA for downstream applications (see later).

Purification of WGA products

It was advisable to perform post-amplification clean-up of WGA products for the success of it's use in downstream applications. Two purification protocols were tested; the ethanol precipitation method and the sephadex filtration method. Although both methods remove protein, the sephadex method does not remove excess dNTPs, which can interfere in applications such as sequencing.

Ethanol precipitation:

This was carried out in 96-well plates (AbGene, Surrey, UK). 1/10 of a volume of 125 mM EDTA (pH >8.0) was added to each amplified product together with 1/10 of a volume of 3M sodium acetate solution (pH 5.5), followed by 2.5 volumes of 100% ethanol. The plate was covered by a seal and vortexed briefly. After a 15 min incubation period at room temperature, the plate was spun at 3000 x g at 4^0C for 30 minutes to precipitate the DNA. The seal was carefully removed and the plate spun up-side down at 185 x g for 10-15 sec. Next, 3.5 volumes of 70% ethanol were then added to each well and the plate was spun at 1659 x g at 4^0C for 15 min. This step removed excess salt. The plate was then inverted and spun at 185 x g for 1 min. The wells were then allowed to dry before addition of an appropriate amount of water.

Where DNA quantity was thought to be very low (< 2μg), Pellet Paint NF co-precipitant (Novagen (Merck Biosciences Ltd), Nottingham, UK) was used to aid the precipitation protocol by (i) staining the DNA pellet blue to allow its visualisation and (ii) to weigh the pellet down from the precipitation stage onwards to prevent loss of sample in successive spins. However, unless necessary, it's use was avoided since it interferes with the PicoGreen assay reagent when quantifying DNA concentration. Samples where pellet paint was used were quantified instead by UV spectrometry where a subtraction to absorbance readings was made to account for absorbance of the paint itself.

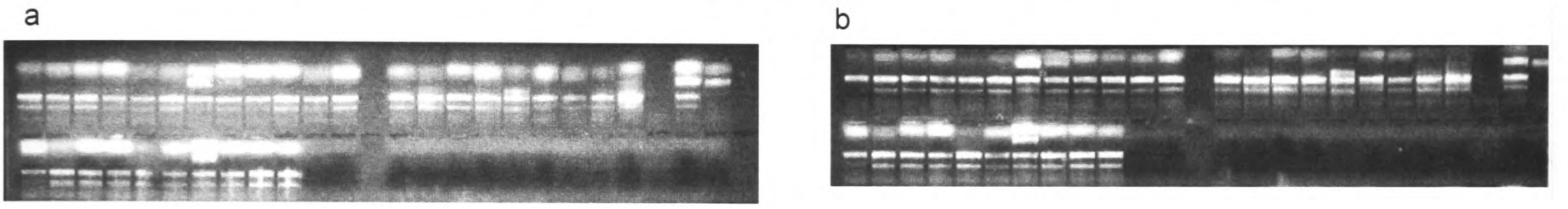
Sephadex filtration method:

This method was also performed in a 96-well format. Sephadex G-50 (Amersham Biosciences (Buckinghamshire, UK) was loaded into Multiscreen-HV assay plates (Millipore, Watford, UK) and swollen with sterile water for at least 3 hours. Excess water was removed by centrifugation at 910 x g for 5 minutes. Amplified DNA product was then applied (>15 μl) to the middle of each well without disturbing the swollen sephadex beads. The Multiscreen-HV assay plate was placed on top of a collection plate (Nalgene NUNC (Fisher Scientific), Leicestershire, UK), carefully aligned so when

spun again at 910 x g for 5 minutes the elutent (containing purified DNA) fell directly into the collection plate.

Purification of Genomiphi amplified product was necessary for downstream applications such as PCR-SSP (Figure 2). However, the RepliG amplified DNA did not require clean-up as good quality/ clean PCR products were observed on gels when performing the PCR-SSP technique and by the Amplifluor method. Both purification techniques yielded DNA of equally good quality but since the EtOH precipitation method was cheaper, this purification method was employed for the Genomiphi amplified DNA.

Figure 2: PCR-SSP of a Genomiphi amplified sample, (a) prior to and (b) after EtOH clean up.



Lanes represent PCR-SSP products obtained from individual PCR reactions for HLA typing of a patient (Chapter 2, Section 2.4.2). The same PCR reactions were performed for uncleaned (a) and cleaned samples (b).

Testing of whole genome amplification (WGA) techniques: Genomiphi and deaza RepliG.

To assess the quality of the products obtained by these WGA methods, genotyping results obtained from DNA samples before and after WGA were compared for a total of 7 samples amplified by the Genomiphi method and 84 samples by the RepliG method. Of the 84 samples sent to Qiagen Inc, USA, 5 failed quality control in their laboratory and were considered 'unusable'[15], leaving 79 samples to quality control test in our laboratory. The 7 Genomiphi-amplified samples and 79 deaza RepliG-amplified samples were tested by genotyping using an in-house quality control PCR, the –23*HphI* polymorphism of the insulin gene and variants in the HLA genes (see Section 2.4 for details of these genotyping methods).

[15]Genotyping of these samples not recommended by Qiagen USA as several loci or alleles may be missing in the amplified DNA.

In-house quality-control PCR

To ensure all samples contained DNA of adequate quality following the WGA procedure, an in-house quality control check PCR was performed. This PCR was also used to check DNA samples for degradation when poor genotyping quality was observed using other methods. This single reaction PCR amplified a region of exon 11 of the PEK gene on Chromosome 2 (coordinates 88714044 – 88714245) in all subjects and a portion of the sex-determining region Y protein, or 'SRY' (coordinates 2698468 – 2698937) of the Y Chromosome in male individuals only.

Genotyping: PCR was carried out in a 10 μl total volume containing 30 ng template DNA, 2.5 pmol of each Chromosome 2 PEK primers (forward and reverse), 3.25 pmol of Y Chromosome SRY primers (forward and reverse) (see below), 2.5 mM $MgCl_2$, 200 μmol dNTPs, and 1U AmpliTaq Gold *Taq* polymerase (Applied Biosystems). A 10 μl mineral oil overlay was applied.

Primer sequences for quality control PCR assay:

Primers	Sequence
Y Chr SRY region – forward	GAATATTCCCGCTCTCCGGA
Y Chr SRY region – reverse	GCTGGTGCTCCATTCTTGAG
Chr 2 PEK Exon 11 – forward	GTATTTCTCCCACTTTTAGC
Chr 2 PEK Exon 11– reverse	GGTGAGAACTGGCTCTC

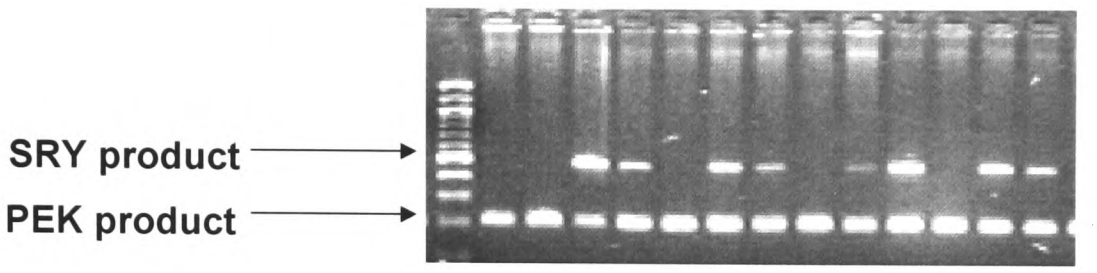
Cycling conditions:

No of cycles	Denaturation		Annealling		Extension	
1	6 min	94^{0}C				
35	30 sec	94^{0}C	30 sec	57^{0}C	45 sec	72^{0}C
1					10 min	72^{0}C

Gel electrophoresis: PCR products were separated on a 2% agarose gel containing 0.5 μg/ml ethidium bromide at 200V for 40 mins in 0.5 x TBE buffer. A 100 bp ladder (New England Biolabs, Hertfordshire, UK) was run alongside test samples. Products were visualised under UV light.

Assigning genotypes: The Chromosome 2 product (PEK) was seen as a band at 200 bp and the Y Chromosome product (SRY) was a band at 510 bp (See Figure 3).

Figure 3: Gel electrophoresis of SRY PCR products.

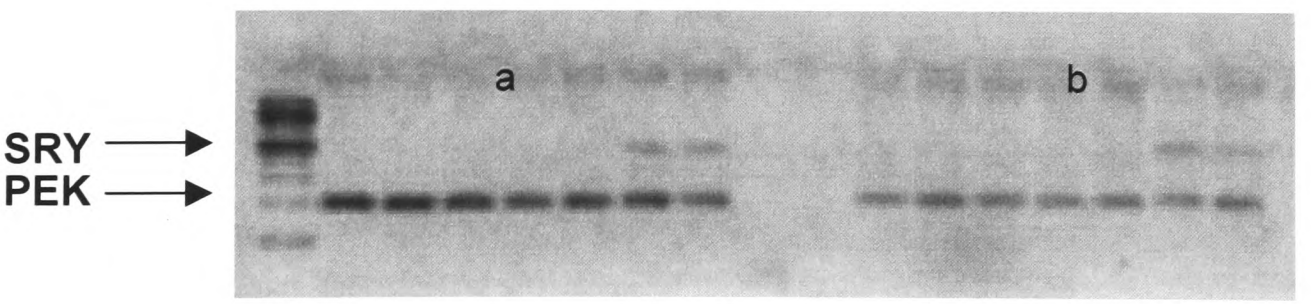


The 100 bp ladder was loaded as a marker. All samples (lanes) contain a Chromsome 2 (PEK) product and samples from males contain a Y Chromosome (SRY) product too.

The Chromosome 2 PEK product was assumed to be a representative region of autosomal sequence in the genome that should be present in all samples, males and females. If this sequence was not successfully amplified, the quality of the whole genome amplification preparation was questioned. Since the Y Chromosome SRY product was only found in males, this was an additional control to check DNA plating of samples.

All (7 out of 7) samples amplified by the Genomiphi method (Figure 4) and 79 samples successfully amplified by the deaza RepliG method (Figure 5) revealed viable Chromosome 2 products. Chromosome Y products were found in all successfully genotyped males. These results suggested that both WGA methods successfully yielded viable products.

Figure 4: SRY PCR of samples prior to (a) and after (b) Genomiphi amplification.



100 bp ladder was loaded in the first lane. Samples were loaded with a two-lane gap separating the 7 unamplified and 7 post-Genomiphi WGA samples (reading left to right). As indicated by arrows, PEK products can observed at 200 bp and SRY products at 510 bp.

Figure 5: SRY PCR of samples that had undergone deaza RepliG amplification.

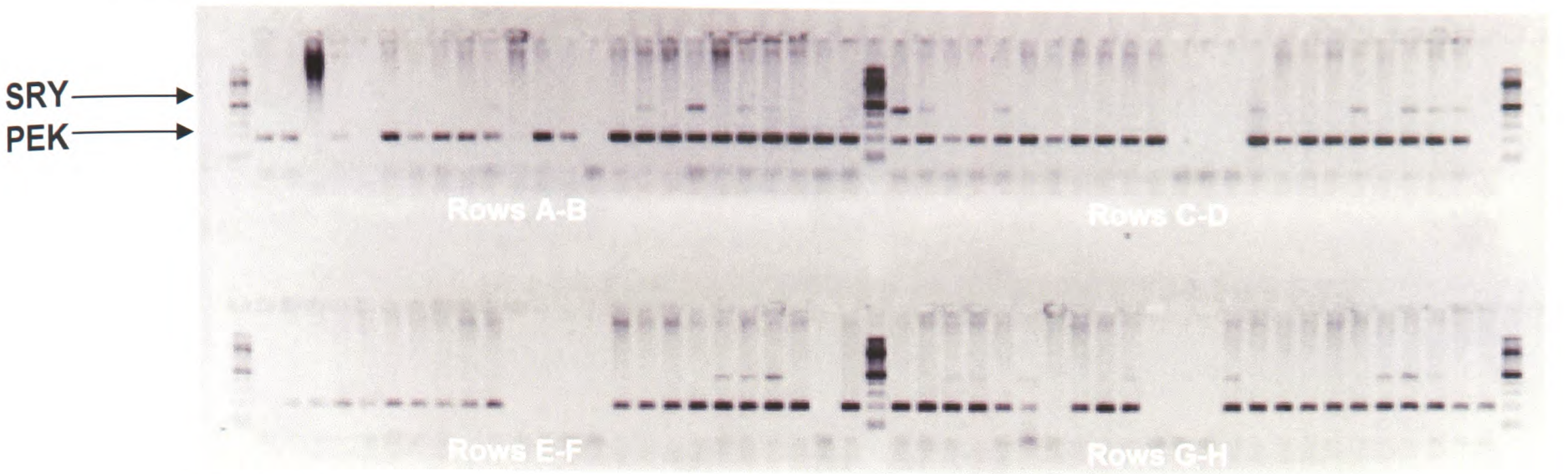


Plate map:

Loading by row →

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1	P8	P15	P23	P30	P38	+VE	T2	+VE	T17	T25	T32
B	P2	P9	P16	P24	P31	P39	Blank	T3	T10	T18	T26	T33
C	P3	P10	P17	P25	P32	P40	Blank	T4	T11	T19	T27	T34
D	P4	P11	P18	+VE	P33	Blank	Blank	T5	T12	T20	T28	T35
E	P5	P12	P19	P26	P34	Blank	Blank	T6	T13	T21	+VE	T36
F	P6	+VE	P20	P21	P35	Blank	Blank	T7	T14	T22	T29	T37
G	P7	P13	P21	P28	P36	Blank	Blank	T8	T15	T23	T30	T38
H	+VE	P14	P22	P29	P37	Blank	T1	T9	T16	T24	T31	T39

100 bp ladder is loaded in the first lane. Samples are loaded with negative controls in blank wells. Samples are labelled P1 to P40 and T1 to T39. P21 has been plated in duplicate as a check for reproducibility. The products of the tetra-primer ARMS PCR are shown on the gel above. Samples have been loaded in groups of 12 (by row). As indicated by arrows, PEK products can observed at 200 bp and SRY products at 510 bp. NB. +VE = positive controls, Blank = negative controls.

Typing of the –23*HphI* polymorphism in the insulin gene region.

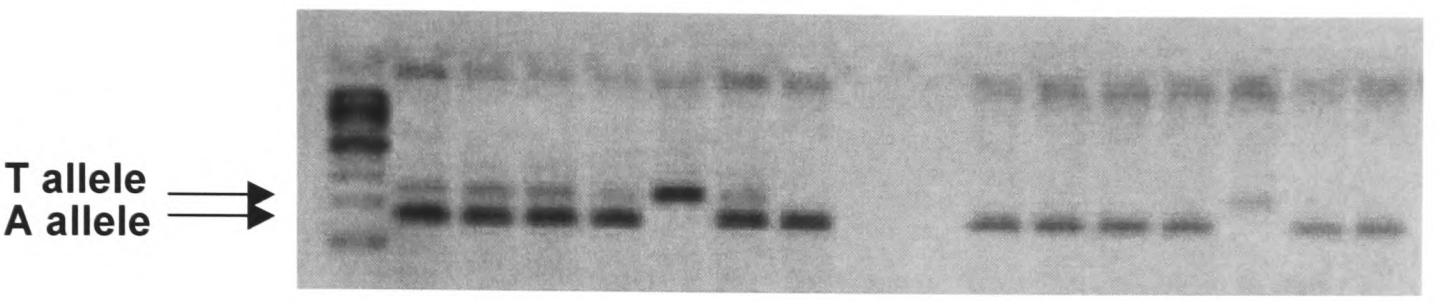
DNA samples prior to amplification were genotyped for this SNP by the tetra-primer ARMS method. (See Section 2.4.4 for details). The post-WGA samples were genotyped for this polymorphism by the tetra-primer ARMS and/ or the Amplifluor method (See Section 2.4.3 for details).

Genomiphi samples:

Genotyping of the 7 test samples by the tetra-primer ARMS methods prior to WGA showed that 6 out of 7 were A/T heterozygotes and 1 was a T/T homozygote (Figure 6). When the Genomiphi products of the same DNA samples were amplified, only the shorter A allele appeared to be successfully amplified in 5 out of 6 A/T heterozygous individuals. The T allele was amplified only in the one T/T homozygous sample. The T allele drop-out seen in heterozygous individuals showed that the ARMS

technique was not successful for genotyping the Genomiphi amplified samples. This could be due to T-allele drop-out during the Genomphi WGA.

Figure 6: Genotyping of –23HphI polymorphism in Genomiphi amplified samples by tetra-primer ARMS.



100 bp ladder is loaded in the first lane. Samples are loaded with a two-lane gap separating the 7 unamplified and 7 post-Genomiphi WGA samples.

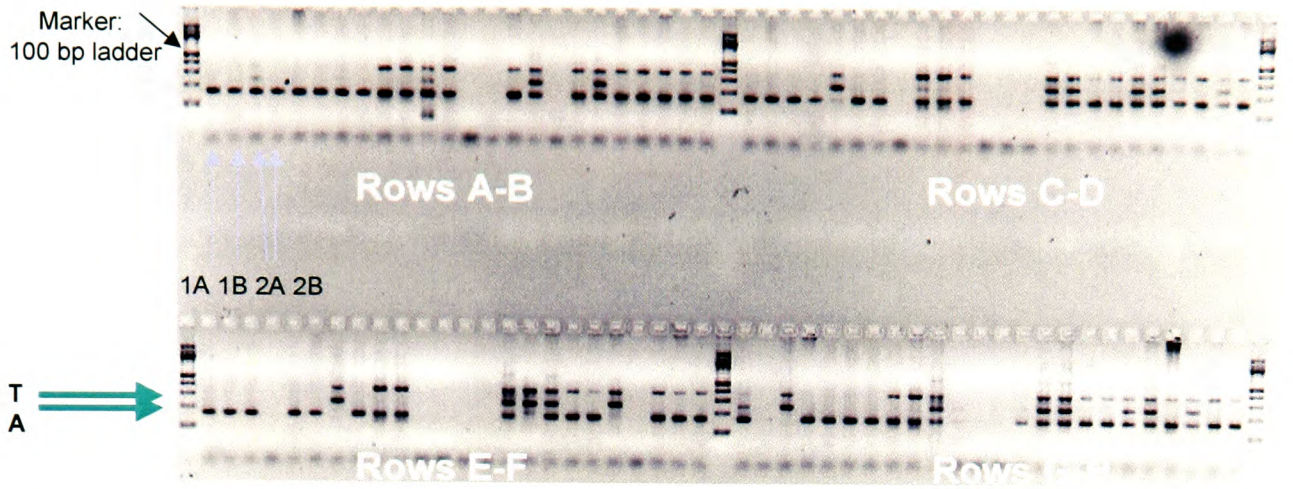
Therefore, the ability to genotype the Genomiphi amplified samples using the Amplifluor technology was tested. It was considered important that the WGA DNA was amenable to such fluorescence-based technologies since they are cost-effective, time-efficient methods for the future. Genotyping of the 7 samples prior to WGA yielded the same genotyping results obtained using the tetra-primer ARMS technique. Genotyping of the same samples post-WGA by the Amplifluor method was not successful at all. It was therefore necessary to test the Deaza RepliG amplified samples for genotyping the insulin gene polymorphisms.

Deaza RepliG samples:

Genotyping of the –23*HphI* site (using tetra-primer ARMS PCR) in the 79 samples before and after deaza RepliG amplification is shown in Figure 7 overleaf. As with the Genomiphi-amplified samples, a drop-out of the T allele occurred in heterozygous individuals.

Figure 7: Genotyping the –23HphI polymorphism on samples whole genome amplified by the deaza RepliG method.

(a) Unamplified samples:

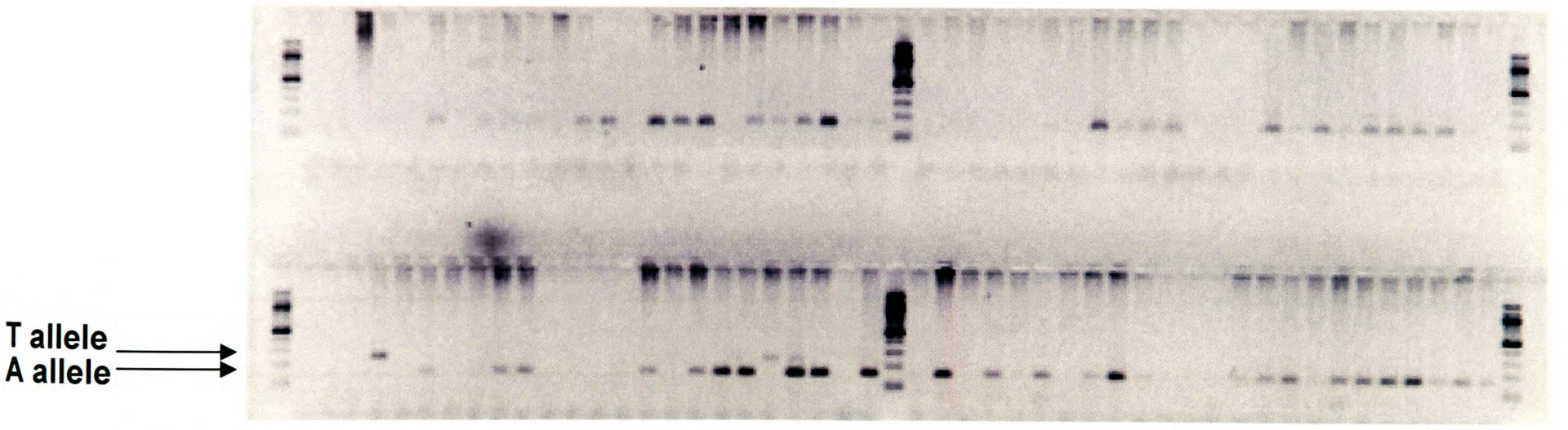


NB: internal duplicate P21 has same genotype

Loading by row →

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1	P8	P15	P23	P30	P38	Blank	T2	Blank	T17	T25	T32
B	P2	P9	P16	P24	P31	P39	Blank	T3	T10	T18	T26	T33
C	P3	P10	P17	P25	P32	P40	Blank	T4	T11	T19	T27	T34
D	P4	P11	P18	Blank	P33	Blank	Blank	T5	T12	T20	T28	T35
E	P5	P12	P19	P26	P34	Blank	Blank	T6	T13	T21	Blank	T36
F	P6	Blank	P20	P21	P35	Blank	Blank	T7	T14	T22	T29	T37
G	P7	P13	P21	P28	P36	Blank	Blank	T8	T15	T23	T30	T38
H	Blank	P14	P22	P29	P37	Blank	T1	T9	T16	T24	T31	T39

(b) Deaza RepliG amplified samples



Samples are labelled P1 to P40 and T1 to T39. P21 has been plated in duplicate as a check for reproducibility. The products of the tetra-primer ARMS PCR are shown on the gel above. Samples have been loaded in groups of 12 (by row). As indicated by green arrows, A/A (Class I) homozygotes were identified by the presence of a single band at 180 bp, T/T (Class III) homozygotes by the presence of a single band at 260 bp, and A/T (Class I/III heterozygotes) by the presence of both bands. N.B. in (b), lanes 7A, 9A, 4D, 2E, 11E and 1H contained positive controls.

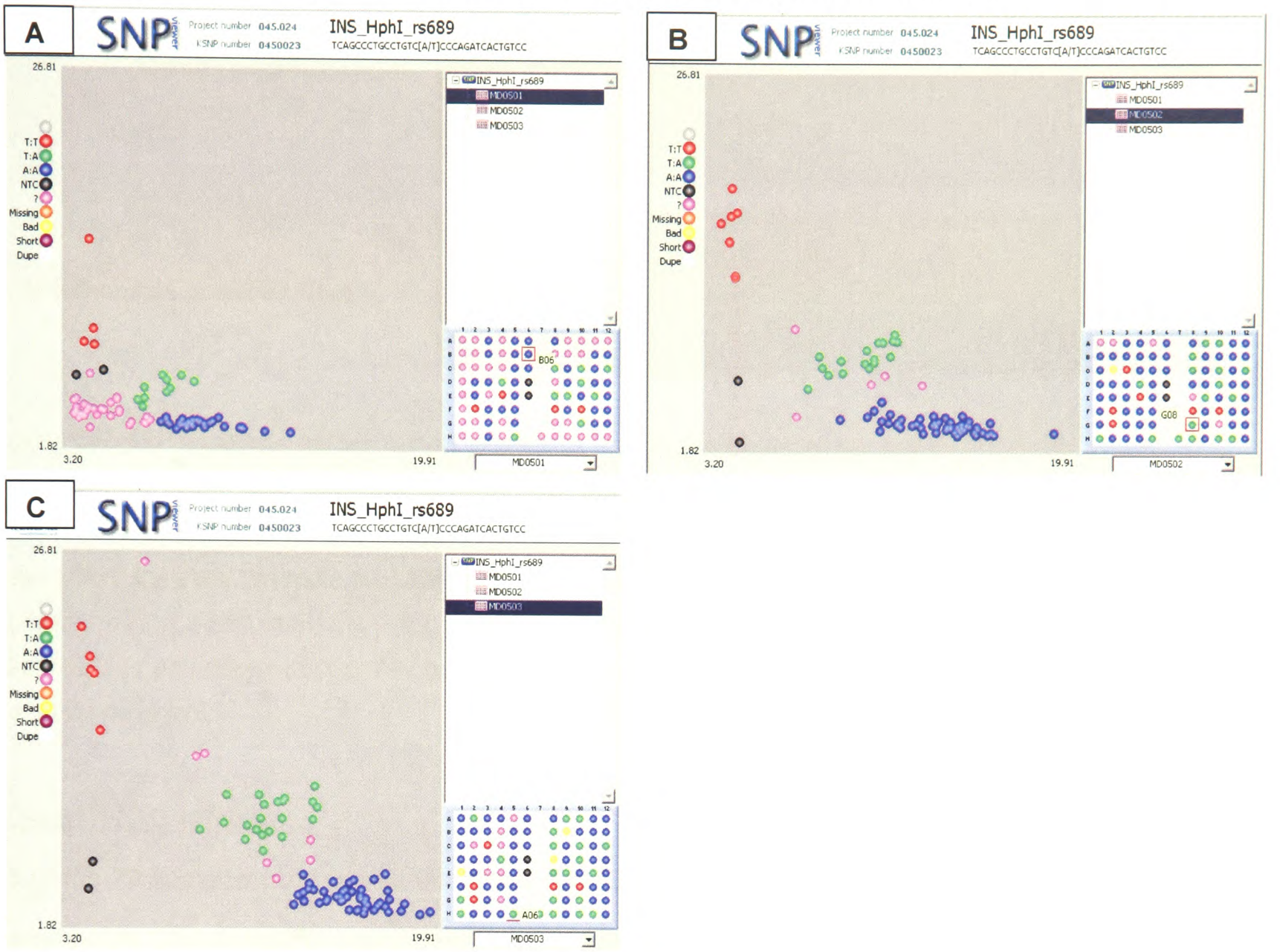
The 79 samples amplified by the deaza RepliG method were successfully genotyped by the Amplifluor method. Samples were sent to K Biosciences at 200 ng/μl, 100 ng/μl, 50 ng/μl, 25 ng/μl and 12.5 ng/μl concentrations. The Amplifluor assay typically performs best at DNA concentrations of 10 ng/μl. The genotyping results of these assays can be seen in Figure 8. From this figure it was evident that decreasing the DNA concentration from 200 ng/μl → 100 ng/μl → 50 ng/μl increased the resolution of the data – the separation between the clusters of homozygotes and heterozygotes was optimal at 100 ng/μl concentration of DNA (Figure 8). Also the number of samples successfully genotyped increased as the concentration of DNA was decreased (fewer samples are coloured pink moving 200 ng/μl → 100 ng/μl → 50 ng/μl). Unfortunately, the samples at 25 ng/μl and 12.5 ng/μl could not be successfully genotyped at all, and therefore these data are not presented. Next, the concordance of actual genotype calls were compared between those obtained for the samples prior to WGA by the tetra-primer ARMS method and those obtained after amplification by deaza RepliG (Table 1). Good concordance was achieved at all three concentrations. A total of three samples did not agree with results obtained by tetra-primer ARMS. However, these three discrepant samples consistently gave the same genotype at all concentrations tested under the Amplifluor technique. Unfortunately, unamplified DNA was not available to re-genotype these individuals to explain the discrepancy. These three samples were excluded from the analysis.

Table 1: Concordance of genotype calls obtained by Amplifluor technique of samples amplified by the deaza RepliG method compared with unamplified DNA.

	200 ng/μl	**100 ng/μl**	**50 ng/μl**
Success genotyping	48 (60.8)	74 (93.7)	73 (92.4)
Concordance of genotype calls with unamplified samples	46/48 (95.8)	71/74 (95.9)	70/73 (95.8)

Data are given as n (%)

Figure 8: Scatter plots of genotyping of the –23HphI SNP of various concentrations of the samples amplified by the deaza RepliG technique. (A) 200 ng/µl, (B) 100 ng/µl and (C) 50 ng/µl:



In these plots, the AA homozygotes are coloured blue, TT homozygotes red, AT heterozygotes green, negatives (blanks) black and those genotypes that cannot be determined by the technique in pink or yellow.

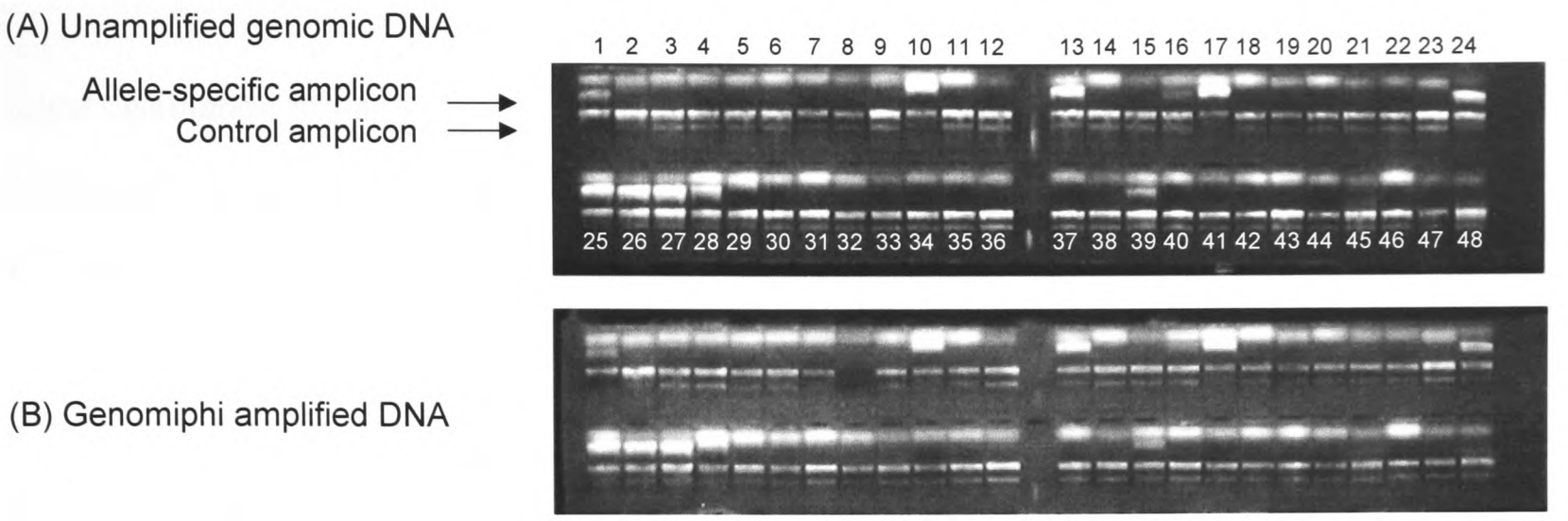
Typing of the *DRB1* and *DQB1* HLA gene variants.

The PCR-SSP technique was used to genotype samples for *DRB1* and *DQB1* gene variants before and after WGA. This could be done in-house (see Chapter 2, Section 2.4.2 for details of method).

<u>Genomiphi samples:</u>

All eight samples tested gave the exact same genotype patterns before and after WGA (see Figure 9 for an example). The quality of the PCR products obtained for HLA typing using the Genomiphi WGA product was comparable to that of the unamplified DNA.

Figure 9: Success of HLA typing by PCR-SSP of Genomiphi WGA product



(A) PCR-SSP products tested for 48 groups of alleles (represented by individual lanes on gel) of individual X using their unamplified DNA. (B) PCR-SSP products tested for 48 groups of alleles (represented by individual lanes on gel) using Genomiphi amplified product of individual X. As can be seen from the gels, the pattern of allele-specific amplicons was identical for unamplified DNA amplified product of the same individual. NB. Equal amounts of unamplified DNA and amplified product was applied and an internal control 796 bp (a DRB1 *invariant sequence) was included in every reaction as a positive control.*

<u>Deaza RepliG samples:</u>

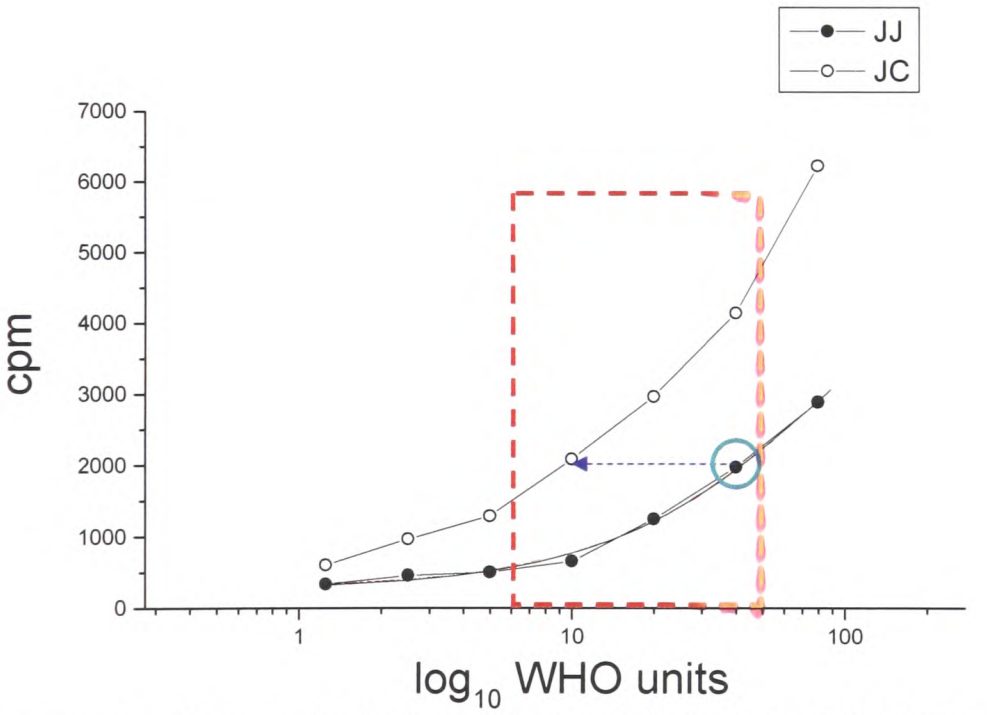
5 of the 79 samples used to test the deaza RepliG WGA samples had sufficient DNA quantities of unamplified DNA to test for concordance of HLA genotyping results. As with the Genomiphi methods, good quality products and 100% concordance were obtained in genotype calls between samples prior to and post WGA by the deaza RepliG method.

APPENDIX 2: CALCULATION OF GADA UNITS

Initial calculation of GADA units

At the start of this project, a serum sample of GADA with WHO units standardised was not available in sufficient quantity from Dr Christie's lab to perform a direct calibration against our in-house standard. Therefore, our standard was indirectly calibrated against another 'reference' plasma from Dr Christie whose neat GADA concentration was estimated at 80 'WHO' GADA units (referred hereafter as JJ) after participation of his laboratory in the DASP 2001 workshop. The WHO GADA content of our in-house standard, JC, was estimated by comparison of the average counts from standard curves generated in three independent calibration runs with that of JJ. The most linear part of the average standard curves was used to crudely compare units (see Figure 1) and estimate our in-house standard undiluted to contain 333 'WHO' units.

Figure 1: Calibration of our in-house standard with a WHO-calibrated serum.



The serial dilutions of positive control plasma tested (circles) for GADA titre moving from left to right are neat, 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64 and 1 in 128. The mean counts per minute (cpm) obtained for each dilution of the reference plasma, JJ (filled circles) is plotted in WHO units/ml with a trendline joining them (neat GADA concentration estimated at 80 'WHO' Units/ml). The mean counts for each dilution of our in-house standard (JC) is also plotted (open circles). The red block encases the most linear part of the curves, where the 1 in 2 dilution of JJ (encircled in green) equates to roughly the 1 in 8 dilution of JC. By comparison of such points along the linear parts of each curve, the computer algorithm estimated JC to have a neat concentration of 333 'WHO' units. This graph is from a representative experiment standardising the positive control. Each point is the mean of a test made in triplicate samples.

During the time-course of this thesis, the method of estimating the GADA units of JC described above was not considered precise enough and it became apparent that our laboratory would need to participate in a workshop to standardise our assay (see below) and assign JC a WHO Unit value more accurately. We participated in the DASP 2003 workshop and our positive standard at neat concentration was estimated at 173.5 WHO U/ml.

Evaluation and international standardisation of the radiobinding assay (DASP workshop)

In order to comply with International regulations regarding autoantibody measurement, our GADA assay was assessed in the Diabetes Autoantibody Standardisation Program (DASP) 2003 workshop to determine the accuracy of our assay. The aim of this workshop was to improve measurement of autoantibodies that are predictive of T1D to enable laboratories to correctly detect disease. A set of 150 reference samples which included 50 true positives (newly diagnosed T1D patients) and 100 true negatives (non-diabetic population controls) are sent to participating laboratories where they are tested blind on their in-house assays. These results are reported back to the DASP organising centre where they calculate true positive and true negative rates, or sensitivity and specificity, respectively. (Please see Table 1 for further definitions of terms used). Whilst a highly sensitive and highly specific test is desirable, there is a trade-off between the two; as one increases, the other naturally decreases. Decreasing the cut-off reduces the number of false-negatives and hence increases the sensitivity of a test. However, decreasing the cut-off will also increase the number of false positives, and thus decrease the specificity of the test. Increasing the cut-off will have an opposite effect – the sensitivity will be lowered whereas the specificity will be increased.

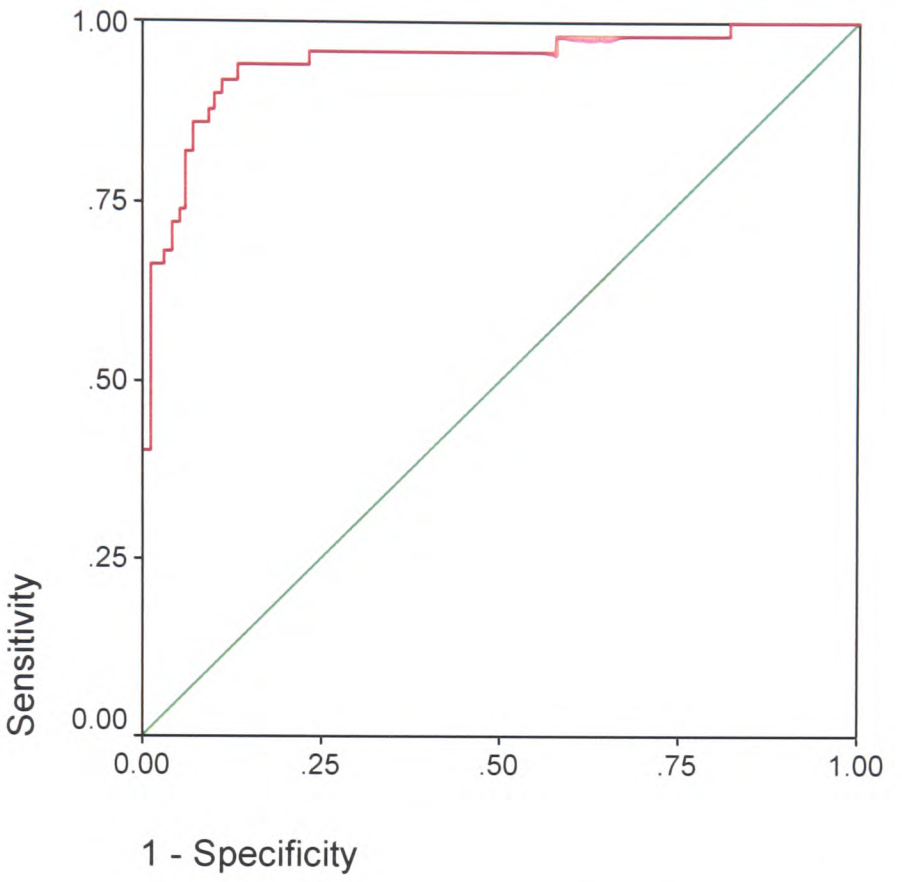
Table 1: Explanation of terms used in DASP workshop evaluation

Term	Definition	Calculated as:
True positive	A test positive with disease present.	
True negative	A test negative with disease absent.	
False positive	A test positive with disease absent.	
False negative	A test negative with disease present.	
Sensitivity	The proportion of patients with the disease who have a positive test. Also called the true positive rate.	True positive/ (True positive + False negative)
Specificity	The proportion of patients without the disease who have a negative test. Also called the false positive rate.	True negative/ (True negative + False positive)
Cut-off level	A test is considered positive if the antibody level of above a certain number of units – the cut-off	Depends on laboratory: can be – • Mean + 3SD of control subject titres. • The unit that defines the 97.5, 99 or 99.5-th percentile of negative control subjects. • Optimum value obtained by ROC curve analysis.
Receiver operating characteristic (ROC) curve	A plot of sensitivity vs. 1- specificity. A more sophisticated assessment of the accuracy of a test over a range of cut-offs.	The points on the curve represent pairs of sensitivity and specificity at the various cut-off levels selected for a positive test result.

Our assay achieved 88% sensitivity and 91% specificity at our working GADA 11.4 WHO unit cut-off, calculated by the mean + 3SD units of 100 in-house laboratory population controls. Since the arbitrary cut-off for positivity that we had established in our assay determined this value, a receiver operating characteristic (ROC) curve (Figure 2) was used to compare the accuracy of our assay over a range of cut-offs. This allowed us to determine the cut-off to use reach the best compromise between specificity and sensitivity. In hospital diagnostic laboratories it is more important to correctly diagnose disease in affected individuals only, i.e. it is considered more important that the assay is specific. By raising the cut-off for our assay to 15 WHO units, we were able to increase specificity to 94%, but this left us with a reduced sensitivity of 86%. However, this is still considered to be in the 'excellent/good' range according to the traditional academic point system used to assess ROC curve results. Moreover, the area under the ROC curve for our assay, a measure of diagnostic performance, was an acceptable

0.945 for our laboratory. (An area of 1 represents a perfect test; an area of 0.5 represents a worthless test).

Figure 2: Receiver operating characteristic curve for our assay.



ROC curves are used to study the sensitivity and specificity in identifying cases with autoimmune diabetes at various cut-off levels for positivity.

Presentation of units:

The DASP workshop also aims to standardise the units used to quantify antibody titres. As part of the evaluation, our in-house positive standard was calibrated against a serum provided by the workshop which had been defined to contain 250 WHO GADA Units/ml. For each experiment, in-house units generated from the standard curve of our serially diluted positive control were converted to WHO units/ml by applying a multiplication factor. The results for GADA titres are presented as WHO units/ml accordingly in this thesis.

APPENDIX 3: AUTORADIOGRAPHY FOR VERIFICATION OF CLONING PRODUCT (SDS-PAGE PROTEIN GEL)

SDS-PAGE

A 8% SDS resolving gel of the following constituents was prepared; 2.3 ml water, 1.3 ml 30% acrylamide + 2.6 % N,N'methylenebisacrylamide (Sigma), 1.3 ml 1.5 M Tris-Cl (pH 8.8), 50 µl 10 % SDS, 50 µl 10 % ammonium persulphate, 2 µl TEMED. A 5% stacking gel was applied on top of the resolving gel (1.4 ml water, 330 µl 30% acrylamide + 2.6 % N,N'methylenebisacrylamide (VWR Scientific Products, Poole, UK), 250 µl 1 M Tris-Cl (pH 6.8), 20 µl 10 % SDS, 20 µl 10 % ammonium persulphate, 2 µl TEMED. Samples were diluted 1:1 in SDS sample buffer and the gel was run in SDS-PAGE running buffer at 200 volts for 40 minutes.

SDS-PAGE running buffer:	25 mM Tris (pH 8.3), 250 mM glycine, 0.1 % SDS.
2 x SDS sample buffer: (pH 6.8)	100 mM Tris-Cl (pH 6.8), 10 % β mercaptoethanol, 4% SDS, 0.2 % bromophenol blue, 20 % glycerol.
2 x SDS sample buffer: (pH 10.4)	25 mM sodium bicarbonate (pH 10.4), 10 % β mercaptoethanol, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol.
Coomassie blue stain:	0.25 % Coomassie blue stain, 45 % methanol, 10 % acetic acid.
Destain solution:	30 % methanol, 10 % acetic acid.
Gel Markers:	M_1 = Benchmark Pre-stained Protein Ladder (Invitrogen). M_2 = SeeBlue Pre-stained Standards (Invitrogen).

APPENDIX 4: PRIMER SEQUENCES FOR HLA TYPING (CHAPTER 2)

Primer set	Sense*	Anti-sense*	Primer set	Sense*	Anti-sense*
1	988	990	25	2737	3578
2	973	974	26	3189	255
3	82	89	27	46	38
4	971	78	28	2489	38
5	972	78	29	3189	491
6	79	967	30	44	37
7	969	853	31	3291	3258
8	969	968	32	47	2510
9	970	853	33	47	498
10	348	350	34	47	499
11	349	350	35	47	500
11	349	351	36	47	501
12	347	111	37	36	39
13	851	622	38	36	40
14	349	622	39	41	252
15	1465	620	40	41	491
16	628	350	41	3596	37
17	88	350	42	50	51
18	181	112	42	50	102
19	347	831	43	46	49
20	353	831	44	53	37
21	1465	1466	45	3189	2516
22	852	987	46	50	256
23	325	327	47	3189	485
24	2737	2969	48	3189	2970

* This is a code for the primer sequences listed in tables below.

Sense primer sequences:

Sense primer code	Primer sequence
988	GTCCGGTGGTTTCGGAATGA
973	TCCGGTGGTTTCGGAATGG
82	GTGCGTCTTGTGAGCAGAAG
971	CACCAACGGGACCGAGCT
972	CACCAACGGGACCGAGCG
79	ACGGAGCGCGTGCGGGG
969	TGCGGGGTGTGACCAGAC
970	GTGCGGGGTGTGACCAGAT
348	GACGTGGGGGTGTACCGC
349	GGAGCGCGTGCGTCTTGTA
347	TTTCGTGCTCCAGTTTAAGGC
851	CCCGCAGAGGATTTCGTGTT
1465	GGACGGAGCGCGTGCGTCT
628	CGTGTACCAGTTTAAGGGCA
88	GACGGAGCGCGTGCGTCT
181	GACGGAGCGCGTGCGTTA
353	GTGCGTCTTGTGACCAGATA
852	CCCGCAGAGGATTTCGTGTA
325	TTCACTCGTCAGCTGACCAT
2737	CACTGGGTCAGCCCAACAT
3189	CACGTTTCTTGGAGTACTCTACGTC
46	GACGGAGCGGGTGCGGTA
2489	ATACTTCCATAACCAGGAGGAGA
44	TACTTCCATAACCAGGAGGAGA
3291	CGGTTCCTGGACAGATACTTCTATC
47	GTTTCTTGGAGCAGGTTAAACA
36	TTGTGGCAGCTTAAGTTTGAAT
41	TCCTGTGGCAGCCTAAGAG
3596	GGCGGTGACGGAGCTA
50	AGTACTCTACGGGTGAGTGTT
53	CGGTTGCTGGAAAGACGCG

Antisense primer sequences:

Antisense primer code	Primer
990	TGCTCTGGGCAGATTCAGAT
974	GTGCTCCACGTGGCAGGT
89	CTGTTCCAGTACTCGGCGT
78	TGGTAGTTGTGTCTGCATACG
967	GCTGTTCCAGTACTCGGCAA
853	TGTTCCAGTACTCGGCGCT
968	GCGGCGTCACCGCCCGA
350	TGCACACCGTGTCCAACTC
351	TGCACACCCTGTCCACCG
111	CCGCGGAACGCCACCTC
622	GCTGTTCCAGTACTCGGCAT
620	CAACTCCGCCCGGGTCCT
112	CGTGCGGAGCTCCAACTG
831	TGGCTGTTCCAGTACTCGGCGG
1466	GCTGTTCCAGTACTCGGCGT
987	CCCCAGCGGCGTCACCA
327	CAAATTGCGGGTCAAATCTTCT
2969	TCAGAAGAGGCTTGTCCAGG
3578	TGCAGTCATAACTCTCCTCAGC
255	GTCCACCCGGCCCCGCT
38	CTGCACTGTGAAGCTCTCCA
491	CTCCGTCACCGCCCGGT
37	CTGCACTGTGAAGCTCTCAC
3258	CGTAGTTGTGTCTGCAGTAG
2510	GGCTGTTCCAGTACTCGGCATC
498	GTCCACCGCGGCCCGCT
499	CGCGGCCCGCTCGTCT
500	TGCAGTAGGTGTCCACCT
501	CTGCAGTAGGTGTCCACCG
39	CCGCCTCTGCTCCAGGAG
40	CCCGCTCGTCTTCCAGGAT
252	CCACCGCGGCCCGCGC
51	CTGCAGTAGGTGTCCACCAG
102	TGTTCCAGTACTCGGCGCT
49	CCCGTAGTTGTGTCTGCACAC
2516	TCCACCGCGGCCCGCC
256	CTGTTCCAGGACTCGGCGA
485	TCCACCGCGGCCCGCTT
2970	CACCTCGGCCCGCCTCC

APPENDIX 5: TABLE FOR CHAPTER 3

Clinical characteristics at baseline of GADA-positive patients according to whether their titre was above or below the assay limit at the 0.5 year time point.

	Within assay limit (<467.5 Units/ ml)	**Above assay limit (>467.5 Units/ ml)**	**p-value[c]**
N	149	110	
GADA titre (WHO Units /ml)[a]	193 (51 to 297)	731 (611 to 938)	
Sex (% male)	54	53	0.87
Age (years)	46.4 (10.3)	47.9 (11.4)	0.29
FPG (mmol/L) [a]	11.1 (7.7 to 15.4)	12.0 (8.1 to 15.7)	0.56
HbA_1c (%)[a]	8.8 (7.0 to 10.5)	9.6 (7.3 to 11.0)	0.14
Weight (kg)	69 (13)	66 (13)	0.13
BMI (kg/m^2)	24.1 (4.3)	23.3 (4.3)	0.091
Systolic blood pressure (mm Hg)	127 (17)	122 (16)	0.066
HOMA2 %B [a]	33 (15 to 58)	29 (15 to 58)	0.76
HOMA2 %S [a]	75 (46 to 103)	69 (50 to 100)	0.99
Plasma triglycerides (mmol/L)[b]	1.27 (0.75, 2.13)	1.08 (0.70, 1.66)	0.018

Mean (SD) except [a] median (IQR) [b] geometric mean (1 SD interval) [c] Chi-squared test

APPENDIX 6: FREQUENCY TABLES FOR CHAPTER 4

Table 1: HLA DRB1 *allele frequencies in published and our control groups.*

***DRB1* allele**	**Ahmad *et al*, 2003**	**Our controls**
**0101*	64 (0.098)	68 (0.096)
**0103*	3 (0.005)	13 (0.018)
**0301*	84 (0.128)	97 (0.137)
**0302*	1 (0.002)	0
**0401*	96 (0.147)	96 (0.136)
**0402*	2 (0.003)	3 (0.004)
**0403*	16 (0.024)	14 (0.02)
**0404*	22 (0.034)	27 (0.038)
**0405*	3 (0.005)	1 (0.001)
**0701*	94 (0.144)	95 (0.134)
**0801*	22 (0.034)	14 (0.02)
**0901/02*	9 (0.014)	10 (0.014)
**1001*	1 (0.002)	0
**1101/04*	43 (0.066)	61 (0.086)
**1102/03*	7 (0.011)	3 (0.004)
**1201*	10 (0.015)	9 (0.013)
**1301*	27 (0.041)	33 (0.047)
**1302*	30 (0.046)	23 (0.032)
**1303*	1 (0.002)	8 (0.011)
**1401*	12 (0.018)	20 (0.028)
**1501-06*	97 (0.148)	105 (0.148)
**1601*	10 (0.015)	3 (0.004)
others	0	5 (0.007)

Data are n (frequency).

Frequencies were found to be similar according to contingency table analysis ($p = 0.09787$).

Table 2: HLA DQB1 *allele frequencies in published and our control groups.*

***DQB1* allele**	**Ahmad *et al*, 2003**	**Our controls**
**0201*	83 (0.127)	97 (0.137)
**0202*	66 (0.101)	71 (0.100)
**0301*	127 (0.194)	169 (0.239)
**0302*	75 (0.115)	60 (0.085)
**0303*	37 (0.057)	33 (0.047)
**0402*	21 (0.032)	13 (0.018)
**0501*	68 (0.104)	80 (0.113)
**0502*	10 (0.015)	3 (0.004)
**0503*	12 (0.018)	20 (0.028)
**0601*	4 (0.006)	1 (0.001)
**0602*	91 (0.139)	104 (0.147)
**0603*	30 (0.046)	30 (0.042)
**0604*	30 (0.046)	27 (0.038)

Data are given as n (frequency).

Frequencies were found to be similar according to contingency table analysis (p =0.09886).

Table 3: HLA DRB1 *allele frequencies in the control and separate LADA groups. (Only frequencies of the combined LADA group have been reported in Chapter 4).*

***DRB1* allele**	**Controls**	**All cases**	**UKPDS**	**W2**	**YT2D**
**0101*	64 (0.098)	53 (0.070)	28 (0.066)	20 (0.077)	5 (0.068)
**0103*	3 (0.005)	4 (0.005)	4 (0.009)	0	0
**0301*	84 (0.128)	236 (0.312)	136 (0.322)	76 (0.292)	24 (0.324)
**0302*	1 (0.002)	0	0	0	0
**0401*	96 (0.147)	178 (0.235)	104 (0.246)	55 (0.212)	19 (0.257)
**0402*	2 (0.003)	4 (0.005)	2 (0.005)	1 (0.004)	1 (0.014)
**0403*	16 (0.024)	7 (0.009)	2 (0.005)	4 (0.015)	1 (0.014)
**0404*	22 (0.034)	34 (0.045)	17 (0.04)	15 (0.058)	2 (0.027)
**0405*	3 (0.005)	8 (0.011)	6 (0.014)	2 (0.008)	0
**0701*	94 (0.144)	100 (0.132)	54 (0.128)	35 (0.135)	11 (0.149)
**0801*	22 (0.034)	16 (0.021)	10 (0.024)	4 (0.015)	2 (0.027)
**0901/02*	9 (0.014)	6 (0.008)	4 (0.009)	1 (0.004)	1 (0.014)
**1001*	1 (0.002)	1 (0.001)	0	1 (0.004)	0
**1101/04*	43 (0.066)	13 (0.017)	5 (0.012)	7 (0.027)	1 (0.014)
**1102/03*	7 (0.011)	15 (0.020)	13 (0.031)	2 (0.008)	0
**1201*	10 (0.015)	3 (0.004)	0	1 (0.004)	2 (0.027)
**1301*	27 (0.041)	20 (0.026)	11 (0.026)	9 (0.035)	0
**1302*	30 (0.046)	22 (0.029)	13 (0.031)	8 (0.031)	1 (0.014)
**1303*	1 (0.002)	1 (0.001)	1 (0.002)	0	0
**1401*	12 (0.018)	5 (0.007)	2 (0.005)	3 (0.012)	0
**1501-06*	97 (0.148)	26 (0.034)	9 (0.021)	14 (0.054)	3 (0.041)
**1601*	10 (0.015)	4 (0.005)	1 (0.002)	2 (0.008)	1 (0.014)

Data are given as n (frequency).

Table 4: HLA DQB1 *allele frequencies in the control and separate LADA groups. (Only frequencies of the combined LADA group have been reported in Chapter 4).*

DQB1 allele	**Controls**	**All cases**	**UKPDS**	**W2**	**YT2D**
**0201*	83 (0.127)	239 (0.317)	136 (0.322)	79 (0.306)	24 (0.324)
**0202*	66 (0.101)	82 (0.109)	45 (0.107)	26 (0.101)	11 (0.149)
**0301*	127 (0.194)	82 (0.109)	48 (0.114)	27 (0.105)	7 (0.095)
**0302*	75 (0.115)	178 (0.236)	101 (0.239)	58 (0.225)	19 (0.257)
**0303*	37 (0.057)	13 (0.017)	7 (0.017)	5 (0.019)	1 (0.014)
**0401*	0	1 (0.001)	0	1 (0.004)	0
**0402*	21 (0.032)	16 (0.021)	10 (0.024)	4 (0.016)	2 (0.027)
**0501*	68 (0.104)	59 (0.078)	32 (0.076)	22 (0.085)	5 (0.068)
**0502*	10 (0.015)	3 (0.004)	1 (0.002)	1 (0.004)	1 (0.014)
**0503*	12 (0.018)	4 (0.005)	1 (0.002)	3 (0.012)	0
**0504*	0	1 (0.001)	1 (0.002)	0	0
**0601*	4 (0.006)	0	0	0	0
**0602*	91 (0.139)	25 (0.033)	9 (0.021)	13 (0.05)	3 (0.041)
**0603*	30 (0.046)	15 (0.020)	8 (0.019)	7 (0.027)	0
**0604*	30 (0.046)	36 (0.048)	23 (0.055)	12 (0.047)	1 (0.014)

Data are given as n (frequency).

Table 5: All DRB1 *serological genotype frequencies in control (n = 327) and LADA group (n = 378).*

Serological genotype	Controls	LADA	Serological genotype cont.../	Controls	LADA
DR3_DR4	18 (0.055)	82 (0.217)	DR4_DR9	1 (0.003)	2 (0.005)
DR3_DR3	7 (0.021)	34 (0.090)	DR1_DR2	15 (0.046)	1 (0.003)
DR4_DR4	17 (0.052)	30 (0.079)	DR2_DR11	9 (0.028)	1 (0.003)
DR7_DR3	11 (0.034)	29 (0.077)	DR11_DR11	4 (0.012)	1 (0.003)
DR4_DR7	21 (0.064)	26 (0.069)	DR4_DR12	3 (0.009)	1 (0.003)
DR1_DR4	12 (0.037)	20 (0.053)	DR7_DR9	2 (0.006)	1 (0.003)
DR3_DR1	6 (0.018)	18 (0.048)	DR8_DR8	1 (0.003)	1 (0.003)
DR3_DR13	11 (0.034)	15 (0.040)	DR14_DR11	1 (0.003)	1 (0.003)
DR4_DR13	17 (0.052)	13 (0.034)	DR3_DR12	0	1 (0.003)
DR7_DR7	7 (0.021)	12 (0.032)	DR3_DR14	0	1 (0.003)
DR2_DR4	21 (0.064)	9 (0.024)	DR4_DR10	0	1 (0.003)
DR3_DR2	12 (0.037)	8 (0.021)	DR7_DR12	0	1 (0.003)
DR7_DR1	10 (0.031)	8 (0.021)	DR9_DR2	0	1 (0.003)
DR4_DR11	5 (0.015)	8 (0.021)	DR13_DR8	0	1 (0.003)
DR3_DR11	11 (0.034)	7 (0.019)	DR1_DR12	2 (0.006)	0
DR4_DR8	2 (0.006)	6 (0.016)	DR1_DR14	1 (0.003)	0
DR7_DR13	6 (0.018)	5 (0.013)	DR2_DR8	6 (0.018)	0
DR3_DR8	1 (0.003)	5 (0.013)	DR1_DR8	3 (0.009)	0
DR11_DR13	9 (0.028)	4 (0.011)	DR1_DR9	3 (0.009)	0
DR1_DR13	5 (0.015)	3 (0.008)	DR2_DR12	3 (0.009)	0
DR2_DR2	5 (0.015)	3 (0.008)	DR14_DR2	3 (0.009)	0
DR4_DR14	5 (0.015)	3 (0.008)	DR7_DR14	1 (0.003)	0
DR1_DR11	2 (0.006)	3 (0.008)	DR8_DR9	1 (0.003)	0
DR7_DR2	21 (0.064)	2 (0.005)	DR11_DR8	1 (0.003)	0
DR2_DR13	7 (0.021)	2 (0.005)	DR11_DR9	1 (0.003)	0
DR7_DR08	6 (0.018)	2 (0.005)	DR11_DR12	1 (0.003)	0
DR1_DR1	4 (0.012)	2 (0.005)	DR13_DR10	1 (0.003)	0
DR7_DR11	2 (0.006)	2 (0.005)	DR13_DR12	1 (0.003)	0
DR3_DR9	1 (0.003)	2 (0.005)	DR14_DR13	1 (0.003)	0

Data are given as n (frequency).

Table 6: All DRB1 *genotype frequencies in control (n = 327) and LADA group (n = 378).*

Genotype	Controls	LADA	Genotype cont.../	Controls	LADA
**0401/*0301*	12 (0.040)	62 (0.164)	**0701/*1302*	4 (0.012)	2 (0.005)
**0301/*0301*	7 (0.021)	34 (0.090)	**0101/*0101*	4 (0.012)	2 (0.005)
**0701/*0301*	11 (0.033)	29 (0.077)	**0701/*0801*	6 (0.018)	2 (0.005)
**0401/*0701*	14 (0.043)	22 (0.058)	**0401/*0103*	0	2 (0.005)
**0401/*0401*	11 (0.033)	17 (0.045)	**0401/*0901*	0	2 (0.005)
**0301/*0101*	6 (0.018)	16 (0.042)	**0301/*0103*	0	2 (0.005)
**0301/*0404*	2 (0.006)	15 (0.040)	**0301/*0403*	0	2 (0.005)
**0401/*0101*	7 (0.021)	14 (0.037)	**0101/*0404*	0	2 (0.005)
**0701/*0701*	7 (0.021)	12 (0.032)	**1102/*0404*	0	2 (0.005)
**0301/*1302*	8 (0.024)	8 (0.021)	**1401/*1101*	1 (0.003)	1 (0.003)
**0701/*0101*	10 (0.030)	8 (0.021)	**1401/*0404*	1 (0.003)	0
**0301/*1301*	3 (0.009)	7 (0.019)	**1501/*1102*	1 (0.003)	1 (0.003)
**0401/*1301*	8 (0.027)	7 (0.019)	**1101/*0403*	1 (0.003)	1 (0.003)
**0301/*1501*	12 (0.036)	7 (0.019)	**0404/*0801*	1 (0.003)	1 (0.003)
**0301/*0801*	1 (0.003)	5 (0.013)	**0404/*0403*	1 (0.003)	1 (0.003)
**0401/*0801*	1 (0.003)	5 (0.013)	**0801/*0801*	1 (0.003)	1 (0.003)
**0401/*0404*	2 (0.006)	5 (0.013)	**0401/*1601*	2 (0.006)	1 (0.003)
**0401/*1302*	4 (0.012)	4 (0.011)	**0401/*1201*	2 (0.006)	1 (0.003)
**0301/*1101*	9 (0.027)	5 (0.013)	**0701/*1101*	2 (0.006)	1 (0.003)
**0401/*1102*	1 (0.003)	4 (0.011)	**0701/*0901*	2 (0.006)	1 (0.003)
**1501/*0404*	3 (0.009)	4 (0.011)	**0301/*0402*	2 (0.006)	1 (0.003)
**0401/*1501*	13 (0.04)	4 (0.011)	**0401/*1101*	3 (0.009)	1 (0.003)
**0701/*1301*	2 (0.006)	3 (0.008)	**0101/*1301*	3 (0.009)	1 (0.003)
**0401/*1401*	3 (0.009)	3 (0.008)	**1302/*1101*	3 (0.009)	1 (0.003)
**1501/*1501*	4 (0.012)	3 (0.008)	**0701/*1601*	4 (0.012)	1 (0.003)
**0401/*0405*	0	3 (0.008)	**0701/*0404*	5 (0.015)	1 (0.003)
**1302/*1102*	0	3 (0.008)	**0101/*0403*	5 (0.015)	1 (0.003)
**0701/*0405*	1 (0.003)	2 (0.005)	**1302/*0404*	5 (0.015)	1 (0.003)
**0301/*0405*	1 (0.003)	2 (0.005)	**0101/*1501*	13 (0.040)	1 (0.003)
**0301/*0901*	1 (0.003)	2 (0.005)	**0701/*1501*	17 (0.052)	1 (0.003)
**0401/*0403*	2 (0.006)	2 (0.005)	**0401/*1303*	0	1 (0.003)
**0301/*1102*	2 (0.006)	2 (0.005)	**0401/*0402*	0	1 (0.003)
**0101/*1302*	2 (0.006)	2 (0.005)	**0701/*1102*	0	1 (0.003)
**0101/*1101*	2 (0.006)	2 (0.005)	**0701/*0402*	0	1 (0.003)
**1501/*1301*	3 (0.009)	2 (0.005)	**0701/*1201*	0	1 (0.003)

Genotype	Controls	LADA	Genotype cont.../	Controls	LADA
**0301/*1401*	0	1 (0.003)	**1601/*1601*	1 (0.003)	0
**0301/*1601*	0	1 (0.003)	**1101/*0801*	1 (0.003)	0
**0301/*1201*	0	1 (0.003)	**1101/*0901*	1 (0.003)	0
**0101/*1102*	0	1 (0.003)	**1101/*1201*	1 (0.003)	0
**0101/*0402*	0	1 (0.003)	**0404/*0405*	1 (0.003)	0
**1302/*0801*	0	1 (0.003)	**0404/*0302*	1 (0.003)	0
**1102/*1101*	0	1 (0.003)	**0801/*0901*	1 (0.003)	0
**0404/*0404*	0	1 (0.003)	**0403/*0901*	1 (0.003)	0
**0405/*1001*	0	1 (0.003)	**0403/*1201*	1 (0.003)	0
**0901/*1601*	0	1 (0.003)	**0101/*1201*	2 (0.006)	0
**0701/*1401*	1 (0.003)	0	**0801/*0103*	2 (0.006)	0
**0701/*0403*	1 (0.003)	0	**0101/*0901*	3 (0.009)	0
**0101/*1401*	1 (0.003)	0	**1302/*1501*	3 (0.009)	0
**0101/*0801*	1 (0.003)	0	**1401/*1501*	3 (0.009)	0
**0101/*1601*	1 (0.003)	0	**1501/*0403*	3 (0.009)	0
**1301/*1201*	1 (0.003)	0	**1501/*1201*	3 (0.009)	0
**1303/*1001*	1 (0.003)	0	**1102/*1301*	3 (0.009)	0
**1302/*1601*	1 (0.003)	0	**1101/*1301*	3 (0.009)	0
**1401/*0403*	1 (0.003)	0	**1101/*1101*	4 (0.012)	0
**1401/*1301*	1 (0.003)	0	**1501/*0801*	6 (0.018)	0
**1501/*0103*	1 (0.003)	0	**1501/*1101*	8 (0.024)	0

Data are given as n (frequency).

Table 7: All DQB1 *serological genotype frequencies in control (n = 327) and LADA group (n = 377).*

Serological genotype	Controls	LADA
DQ2_DQ8	16 (0.049)	86 (0.228)
DQ2_DQ2	18 (0.055)	69 (0.183)
DQ2_DQ5	15 (0.046)	29 (0.077)
DQ2_DQ6	42 (0.128)	28 (0.074)
DQ2_DQ7	27 (0.083)	27 (0.072)
DQ8_DQ8	6 (0.018)	19 (0.050)
DQ8_DQ6	20 (0.061)	18 (0.048)
DQ5_DQ7	16 (0.049)	14 (0.037)
DQ8_DQ7	13 (0.040)	14 (0.037)
DQ8_DQ5	9 (0.028)	14 (0.037)
DQ7_DQ6	39 (0.119)	11 (0.029)
DQ7_DQ7	12 (0.037)	7 (0.019)
DQ2_DQ4	4 (0.012)	7 (0.019)
DQ2_DQ9	9 (0.028)	6 (0.016)
DQ5_DQ6	24 (0.073)	5 (0.013)
DQ6_DQ6	10 (0.031)	5 (0.013)
DQ8_DQ4	1 (0.003)	5 (0.013)
DQ8_DQ9	4 (0.012)	3 (0.008)
DQ5_DQ5	7 (0.021)	2 (0.005)
DQ6_DQ4	5 (0.015)	2 (0.005)
DQ6_DQ9	5 (0.015)	2 (0.005)
DQ5_DQ9	9 (0.028)	1 (0.003)
DQ7_DQ4	4 (0.012)	1 (0.003)
DQ7_DQ9	4 (0.012)	1 (0.003)
DQ4_DQ4	0	1 (0.003)
DQ4_DQ9	4 (0.012)	0
DQ5_DQ4	3 (0.009)	0
DQ9_DQ9	1 (0.003)	0

Data are given as n (frequency).

Table 8: All DQB1 *genotype frequencies in control (n = 327) and LADA group (n = 377).*

Genotype	Controls	LADA	Genotype cont.../	LADA	Controls
**0302/*0201*	11 (0.033)	73 (0.194)	**0603/*0303*	2 (0.005)	0
**0201/*0201*	7 (0.021)	38 (0.101)	**0202/*0602*	1 (0.003)	16 (0.049)
**0202/*0201*	8 (0.024)	22 (0.058)	**0501/*0602*	1 (0.003)	13 (0.040)
**0302/*0302*	6 (0.018)	19 (0.050)	**0301/*0402*	1 (0.003)	4 (0.012)
**0201/*0501*	6 (0.018)	19 (0.050)	**0301/*0303*	1 (0.003)	4 (0.012)
**0201/*0301*	17 (0.055)	17 (0.045)	**0501/*0603*	1 (0.003)	3 (0.009)
**0302/*0301*	13 (0.040)	14 (0.037)	**0604/*0602*	1 (0.003)	3 (0.009)
**0202/*0302*	5 (0.015)	13 (0.034)	**0303/*0502*	1 (0.003)	3 (0.009)
**0201/*0604*	8 (0.024)	12 (0.032)	**0202/*0603*	1 (0.003)	2 (0.006)
**0302/*0501*	6 (0.018)	12 (0.032)	**0302/*0504*	1 (0.003)	0
**0501/*0301*	11 (0.033)	10 (0.027)	**0201/*0502*	1 (0.003)	0
**0202/*0301*	10 (0.030)	10 (0.027)	**0301/*0502*	1 (0.003)	0
**0302/*0604*	8 (0.024)	8 (0.021)	**0604/*0402*	1 (0.003)	0
**0202/*0501*	7 (0.021)	9 (0.024)	**0604/*0401*	1 (0.003)	0
**0202/*0202*	3 (0.009)	9 (0.024)	**0402/*0402*	1 (0.003)	0
**0301/*0301*	12 (0.036)	7 (0.019)	**0501/*0303*	0	6 (0.018)
**0201/*0602*	8 (0.024)	7 (0.019)	**0602/*0402*	0	5 (0.015)
**0302/*0602*	8 (0.024)	6 (0.016)	**0402/*0303*	0	4 (0.012)
**0301/*0604*	4 (0.012)	6 (0.016)	**0501/*0402*	0	3 (0.009)
**0201/*0402*	1 (0.003)	5 (0.013)	**0602/*0303*	0	3 (0.009)
**0302/*0402*	1 (0.003)	5 (0.013)	**0602/*0503*	0	3 (0.009)
**0302/*0603*	3 (0.012)	4 (0.011)	**0302/*0502*	0	2 (0.006)
**0201/*0303*	4 (0.012)	4 (0.011)	**0604/*0303*	0	2 (0.006)
**0202/*0604*	2 (0.006)	4 (0.011)	**0202/*0503*	0	1 (0.003)
**0301/*0602*	22 (0.067)	3 (0.008)	**0202/*0502*	0	1 (0.003)
**0201/*0603*	5 (0.015)	3 (0.008)	**0302/*0601*	0	1 (0.003)
**0301/*0503*	5 (0.015)	3 (0.008)	**0201/*0601*	0	1 (0.003)
**0302/*0303*	4 (0.012)	3 (0.008)	**0501/*0503*	0	1 (0.003)
**0501/*0604*	2 (0.006)	3 (0.008)	**0501/*0502*	0	1 (0.003)
**0301/*0603*	12 (0.036)	2 (0.005)	**0501/*0601*	0	1 (0.003)
**0202/*0303*	5 (0.015)	2 (0.005)	**0301/*0601*	0	1 (0.003)
**0501/*0501*	4 (0.012)	2 (0.005)	**0604/*0502*	0	1 (0.003)
**0602/*0602*	4 (0.012)	2 (0.005)	**0603/*0603*	0	1 (0.003)
**0202/*0402*	3 (0.009)	2 (0.005)	**0603/*0503*	0	1 (0.003)
**0602/*0603*	2 (0.006)	2 (0.005)	**0303/*0303*	0	1 (0.003)
**0302/*0503*	1 (0.003)	1 (0.003)	**0502/*0502*	0	1 (0.003)

Data are given as n (frequency).

Table 9: All haplotype frequencies in control (n = 327) and LADA group (n = 378).

Haplotype	Controls	LADA	Haplotype cont.../	Controls	LADA
**0301_*0201*	83 (0.127)	234 (0.310)	**0404_*0301*	4 (0.006)	1 (0.001)
**0401_*0302*	48 (0.073)	128 (0.169)	**1001_*0501*	1 (0.002)	1 (0.001)
**0701_*0202*	64 (0.098)	81 (0.107)	**1301_*0301*	1 (0.002)	1 (0.001)
**0101_*0501*	64 (0.098)	52 (0.069)	**1303_*0301*	1 (0.002)	1 (0.001)
**0401_*0301*	47 (0.072)	49 (0.065)	**0101_*0504*	0	1 (0.001)
**0404_*0302*	18 (0.028)	33 (0.044)	**0301_*0302*	0	1 (0.001)
**1501_*0602*	90 (0.138)	25 (0.033)	**0301_*0501*	0	1 (0.001)
**1302_*0604*	30 (0.046)	22 (0.029)	**0401_*0201*	0	1 (0.001)
**0801_*0402*	19 (0.029)	16 (0.021)	**0701_*0501*	0	1 (0.001)
**1101_*0301*	43 (0.066)	13 (0.017)	**0701_*0604*	0	1 (0.001)
**1301_*0603*	26 (0.040)	13 (0.017)	**1102_*0201*	0	1 (0.001)
**0405_*0302*	3 (0.005)	8 (0.011)	**1102_*0202*	0	1 (0.001)
**0701_*0303*	30 (0.046)	7 (0.009)	**1301_*0302*	0	1 (0.001)
**1102_*0604*	0	7 (0.009)	**1401_*0301*	0	1 (0.001)
**0901_*0303*	7 (0.011)	6 (0.008)	**1501_*0604*	0	1 (0.001)
**0403_*0301*	13 (0.02)	5 (0.007)	**1601_*0401*	0	1 (0.001)
**1301_*0604*	0	5 (0.007)	**1501_*0601*	4 (0.006)	0
**1401_*0503*	12 (0.018)	4 (0.005)	**1501_*0603*	3 (0.005)	0
**1102_*0301*	5 (0.008)	4 (0.005)	**0801_*0301*	2 (0.003)	0
**0103_*0501*	3 (0.005)	4 (0.005)	**0301_*0301*	1 (0.002)	0
**0402_*0302*	2 (0.003)	4 (0.005)	**0302_*0402*	1 (0.002)	0
**0701_*0301*	0	4 (0.005)	**0401_*0202*	1 (0.002)	0
**1201_*0301*	10 (0.015)	3 (0.004)	**0403_*0402*	1 (0.002)	0
**1601_*0502*	10 (0.015)	3 (0.004)	**0801_*0602*	1 (0.002)	0
**0701_*0201*	0	3 (0.004)	**0901_*0202*	1 (0.002)	0
**0701_*0302*	0	3 (0.004)	**0901_*0302*	1 (0.002)	0
**0403_*0302*	2 (0.003)	2 (0.003)	**1102_*0302*	1 (0.002)	0
**1102_*0603*	1 (0.002)	2 (0.003)			

Data are given as n (frequency).

Table 10: All diplotype frequencies in control (n = 327) and LADA group (n = 378).

Diplotype	Controls	LADA	Diplotype cont .../	Controls	LADA
**0301_*0201-*0401_*0302*	6 (0.018)	53 (0.140)	**1501_*0602-*1301_*0603*	2 (0.006)	2 (0.005)
**0301_*0201-*0301_*0201*	7 (0.021)	34 (0.090)	**0301_*0201-*0405_*0302*	1 (0.003)	2 (0.005)
**0301_*0201-*0701_*0202*	8 (0.024)	22 (0.058)	**0301_*0201-*0901_*0303*	1 (0.003)	2 (0.005)
**0301_*0201-*0101_*0501*	6 (0.018)	16 (0.042)	**0401_*0301-*0404_*0302*	1 (0.003)	2 (0.005)
**0301_*0201-*0404_*0302*	2 (0.006)	14 (0.037)	**0701_*0202-*0405_*0302*	1 (0.003)	2 (0.005)
**0701_*0202-*0401_*0302*	3 (0.009)	9 (0.024)	**0301_*0201-*0103_*0501*	0	2 (0.005)
**0701_*0202-*0701_*0202*	3 (0.009)	9 (0.024)	**0301_*0201-*0403_*0302*	0	2 (0.005)
**0301_*0201-*1302_*0604*	8 (0.024)	8 (0.021)	**0401_*0302-*0901_*0303*	0	2 (0.005)
**0301_*0201-*0401_*0301*	6 (0.018)	8 (0.021)	**0404_*0302-*0101_*0501*	0	2 (0.005)
**0701_*0202-*0101_*0501*	6 (0.018)	8 (0.021)	**0404_*0302-*1102_*0604*	0	2 (0.005)
**0401_*0302-*0101_*0501*	5 (0.015)	8 (0.021)	**0701_*0202-*0701_*0301*	0	2 (0.005)
**0301_*0201-*1501_*0602*	8 (0.024)	7 (0.019)	**0701_*0303-*1301_*0603*	0	2 (0.005)
**0401_*0301-*0401_*0302*	7 (0.021)	7 (0.019)	**1102_*0301-*1302_*0604*	0	2 (0.005)
**0401_*0302-*0401_*0302*	2 (0.006)	7 (0.019)	**0701_*0202-*1501_*0602*	14 (0.043)	1 (0.003)
**0701_*0202-*0401_*0301*	5 (0.015)	6 (0.016)	**0101_*0501-*1501_*0602*	12 (0.037)	1 (0.003)
**0401_*0301-*0101_*0501*	2 (0.006)	6 (0.016)	**0404_*0302-*1302_*0604*	5 (0.015)	1 (0.003)
**0301_*0201-*1101_*0301*	9 (0.028)	5 (0.013)	**0403_*0301-*0101_*0501*	4 (0.012)	1 (0.003)
**0301_*0201-*0801_*0402*	1 (0.003)	5 (0.013)	**0101_*0501-*1301_*0603*	3 (0.009)	1 (0.003)
**0404_*0302-*1501_*0602*	2 (0.006)	4 (0.011)	**0401_*0301-*0701_*0303*	3 (0.009)	1 (0.003)
**0301_*0201-*1301_*0604*	0	4 (0.011)	**0401_*0302-*0701_*0303*	3 (0.009)	1 (0.003)
**0401_*0302-*0801_*0402*	0	4 (0.011)	**0701_*0202-*0701_*0303*	3 (0.009)	1 (0.003)
**0401_*0302-*1301_*0603*	3 (0.009)	3 (0.008)	**1101_*0301-*1302_*0604*	3 (0.009)	1 (0.003)
**0401_*0302-*1302_*0604*	3 (0.009)	3 (0.008)	**0301_*0201-*0402_*0302*	2 (0.006)	1 (0.003)
**0401_*0302-*0404_*0302*	1 (0.003)	3 (0.008)	**0401_*0301-*1101_*0301*	2 (0.006)	1 (0.003)
**0301_*0201-*0701_*0201*	0	3 (0.008)	**0701_*0202-*0901_*0303*	2 (0.006)	1 (0.003)
**0401_*0302-*0405_*0302*	0	3 (0.008)	**0701_*0202-*1101_*0301*	2 (0.006)	1 (0.003)
**0401_*0302-*0701_*0302*	0	3 (0.008)	**0701_*0202-*1301_*0603*	2 (0.006)	1 (0.003)
**0401_*0301-*1501_*0602*	6 (0.018)	2 (0.005)	**1201_*0301-*0401_*0302*	2 (0.006)	1 (0.003)
**0401_*0302-*1501_*0602*	6 (0.018)	2 (0.005)	**0401_*0301-*0403_*0301*	1 (0.003)	1 (0.003)
**0401_*0301-*1301_*0603*	5 (0.015)	2 (0.005)	**0401_*0301-*0801_*0402*	1 (0.003)	1 (0.003)
**0101_*0501-*0101_*0501*	4 (0.012)	2 (0.005)	**0401_*0301-*1302_*0604*	1 (0.003)	1 (0.003)
**1501_*0602-*1501_*0602*	4 (0.012)	2 (0.005)	**0403_*0301-*0401_*0302*	1 (0.003)	1 (0.003)
**0301_*0201-*0701_*0303*	3 (0.009)	2 (0.005)	**0403_*0301-*1101_*0301*	1 (0.003)	1 (0.003)
**0301_*0201-*1301_*0603*	3 (0.009)	2 (0.005)	**0701_*0202-*0404_*0302*	1 (0.003)	1 (0.003)
**0401_*0301-*1401_*0503*	3 (0.009)	2 (0.005)	**1101_*0301-*1401_*0503*	1 (0.003)	1 (0.003)
**0701_*0202-*0801_*0402*	3 (0.009)	2 (0.005)	**1102_*0301-*1501_*0602*	1 (0.003)	1 (0.003)
**0101_*0501-*1302_*0604*	2 (0.006)	2 (0.005)	**0101_*0501-*1102_*0604*	0	1 (0.003)
**0401_*0301-*0401_*0301*	2 (0.006)	2 (0.005)	**0301_*0201-*0404_*0301*	0	1 (0.003)

Diplotype	Controls	LADA	Diplotype cont .../	Controls	LADA
*0701_*0202-*1302_*0604	2 (0.006)	2 (0.005)	*0301_*0201-*0701_*0501	0	1 (0.003)
*1101_*0301-*0101_*0501	2 (0.006)	2 (0.005)	*0301_*0201-*1102_*0201	0	1 (0.003)
*0301_*0201-*1102_*0603	0	1 (0.003)	*0701_*0303-*1501_*0602	3 (0.009)	0
*0301_*0201-*1201_*0301	0	1 (0.003)	*0701_*0303-*1601_*0502	3 (0.009)	0
*0301_*0201-*1301_*0301	0	1 (0.003)	*1101_*0301-*1301_*0603	3 (0.009)	0
*0301_*0201-*1401_*0301	0	1 (0.003)	*1401_*0503-*1501_*0602	3 (0.009)	0
*0301_*0201-*1601_*0502	0	1 (0.003)	*1501_*0602-*1302_*0604	3 (0.009)	0
*0301_*0302-*0401_*0302	0	1 (0.003)	*0301_*0201-*1102_*0301	2 (0.006)	0
*0401_*0201-*0401_*0302	0	1 (0.003)	*0301_*0201-*1501_*0603	2 (0.006)	0
*0401_*0301-*0103_*0501	0	1 (0.003)	*0401_*0302-*1601_*0502	2 (0.006)	0
*0401_*0301-*0701_*0301	0	1 (0.003)	*0403_*0301-*1501_*0602	2 (0.006)	0
*0401_*0301-*1102_*0604	0	1 (0.003)	*0701_*0202-*0404_*0301	2 (0.006)	0
*0401_*0301-*1303_*0301	0	1 (0.003)	*0701_*0303-*0801_*0402	2 (0.006)	0
*0401_*0301-*1601_*0502	0	1 (0.003)	*0701_*0303-*1302_*0604	2 (0.006)	0
*0401_*0302-*0103_*0501	0	1 (0.003)	*0801_*0402-*0103_*0501	2 (0.006)	0
*0401_*0302-*0402_*0302	0	1 (0.003)	*0901_*0303-*0101_*0501	2 (0.006)	0
*0401_*0302-*1102_*0603	0	1 (0.003)	*1102_*0301-*1301_*0603	2 (0.006)	0
*0401_*0302-*1102_*0604	0	1 (0.003)	*1201_*0301-*0101_*0501	2 (0.006)	0
*0401_*0302-*1301_*0302	0	1 (0.003)	*1201_*0301-*1501_*0602	2 (0.006)	0
*0401_*0302-*1301_*0604	0	1 (0.003)	*0101_*0501-*1401_*0503	1 (0.003)	0
*0401_*0302-*1401_*0503	0	1 (0.003)	*0101_*0501-*1501_*0601	1 (0.003)	0
*0402_*0302-*0101_*0504	0	1 (0.003)	*0101_*0501-*1601_*0502	1 (0.003)	0
*0403_*0301-*0404_*0302	0	1 (0.003)	*0103_*0501-*1501_*0602	1 (0.003)	0
*0404_*0302-*0404_*0302	0	1 (0.003)	*0301_*0201-*1501_*0601	1 (0.003)	0
*0404_*0302-*0801_*0402	0	1 (0.003)	*0301_*0301-*1501_*0602	1 (0.003)	0
*0405_*0302-*1001_*0501	0	1 (0.003)	*0401_*0202-*1501_*0602	1 (0.003)	0
*0701_*0202-*0301_*0501	0	1 (0.003)	*0401_*0302-*1102_*0302	1 (0.003)	0
*0701_*0202-*0402_*0302	0	1 (0.003)	*0403_*0301-*1201_*0301	1 (0.003)	0
*0701_*0202-*1102_*0604	0	1 (0.003)	*0403_*0301-*1401_*0503	1 (0.003)	0
*0701_*0202-*1201_*0301	0	1 (0.003)	*0403_*0301-*1501_*0601	1 (0.003)	0
*0701_*0301-*0401_*0302	0	1 (0.003)	*0403_*0302-*0101_*0501	1 (0.003)	0
*0801_*0402-*0801_*0402	0	1 (0.003)	*0403_*0302-*0404_*0302	1 (0.003)	0
*0801_*0402-*1302_*0604	0	1 (0.003)	*0404_*0301-*0701_*0303	1 (0.003)	0
*0901_*0303-*1601_*0502	0	1 (0.003)	*0404_*0301-*0801_*0402	1 (0.003)	0
*1101_*0301-*1102_*0604	0	1 (0.003)	*0404_*0302-*0302_*0402	1 (0.003)	0
*1102_*0202-*1302_*0604	0	1 (0.003)	*0404_*0302-*0405_*0302	1 (0.003)	0
*1102_*0301-*0401_*0302	0	1 (0.003)	*0404_*0302-*0701_*0303	1 (0.003)	0
*1501_*0602-*1501_*0604	0	1 (0.003)	*0404_*0302-*1401_*0503	1 (0.003)	0
*1601_*0401-*0701_*0604	0	1 (0.003)	*0404_*0302-*1501_*0601	1 (0.003)	0

Diplotype	Controls	LADA	Diplotype cont .../	Controls	LADA
**1101_*0301-*1501_*0602*	8 (0.024)	0	**0701_*0202-*0403_*0301*	1 (0.003)	0
**0801_*0402-*1501_*0602*	5 (0.015)	0	**0701_*0202-*0801_*0602*	1 (0.003)	0
**0701_*0303-*0101_*0501*	4 (0.012)	0	**0701_*0202-*1401_*0503*	1 (0.003)	0
**1101_*0301-*1101_*0301*	4 (0.012)	0	**0701_*0202-*1601_*0502*	1 (0.003)	0
**0701_*0303-*0701_*0303*	1 (0.003)	0	**1101_*0301-*1201_*0301*	1 (0.003)	0
**0801_*0301-*0801_*0402*	1 (0.003)	0	**1102_*0603-*1301_*0603*	1 (0.003)	0
**0801_*0301-*1501_*0602*	1 (0.003)	0	**1201_*0301-*1301_*0603*	1 (0.003)	0
**0801_*0402-*0101_*0501*	1 (0.003)	0	**1201_*0301-*1501_*0603*	1 (0.003)	0
**0901_*0202-*0101_*0501*	1 (0.003)	0	**1301_*0301-*1501_*0602*	1 (0.003)	0
**0901_*0303-*0403_*0402*	1 (0.003)	0	**1303_*0301-*1001_*0501*	1 (0.003)	0
**0901_*0303-*0801_*0402*	1 (0.003)	0	**1401_*0503-*1301_*0603*	1 (0.003)	0
**1101_*0301-*0401_*0302*	1 (0.003)	0	**1601_*0502-*1302_*0604*	1 (0.003)	0
**1101_*0301-*0801_*0402*	1 (0.003)	0	**1601_*0502-*1601_*0502*	1 (0.003)	0
**1101_*0301-*0901_*0302*	1 (0.003)	0			

Data are given as n (frequency).

Association with age at diagnosis

Table 11: Frequency of DQB1 *allele frequencies among patients below and above 45 years at diagnosis in the combined LADA cohort.*

***DQB1* allele**	**<45 years (n = 354)**	**>45 years (n = 390)**
**0201*	118 (0.333)	118 (0.303)
**0202*	39 (0.110)	42 (0.108)
**0301*	44 (0.124)	36 (0.092)
**0302*	90 (0.254)	88 (0.226)
**0303*	4 (0.011)	9 (0.023)
**0401*	0	1 (0.003)
**0402*	10 (0.028)	5 (0.013)
**0501*	24 (0.068)	33 (0.085)
**0502*	1 (0.003)	2 (0.005)
**0503*	0	4 (0.010)
**0504*	0	1 (0.003)
**0602*	9 (0.025)	16 (0.041)
**0603*	3 (0.008)	12 (0.031)
**0604*	12 (0.034)	23 (0.059)

Data are given as n (frequency).

Association with insulin requirement

Table 12: Frequency of DRB1 *serological specificities in the UKPDS cohort according to requirement of insulin by 6 years post-diagnosis.*

***DRB1* serological specificity**	**Non-insulin requiring (n = 136)**	**Insulin requiring (n = 282)**
DR1	9 (0.066)	19 (0.067)
DR103	1 (0.007)	3 (0.011)
DR2	3 (0.022)	7 (0.025)
DR3	43 (0.316)	92 (0.326)
DR4	44 (0.324)	85 (0.301)
DR7	19 (0.14)	34 (0.121)
DR8	3 (0.022)	7 (0.025)
DR9	0	4 (0.014)
DR11	5 (0.037)	13 (0.046)
DR13	9 (0.066)	16 (0.057)
DR14	0	2 (0.007)

Data are n (frequency).

Table 13: Frequency of DQB1 *serological specificities in the UKPDS cohort according to requirement of insulin by 6 years post-diagnosis.*

***DQB1* serological specificity**	**Non-insulin requiring (n = 136)**	**Insulin requiring (n = 282)**
DQ2	61 (0.449)	119 (0.422)
DQ4	3 (0.022)	7 (0.025)
DQ5	10 (0.074)	25 (0.089)
DQ6	12 (0.088)	28 (0.099)
DQ7	12 (0.088)	35 (0.124)
DQ8	38 (0.279)	61 (0.216)
DQ9	0	7 (0.025)

Data are n (frequency).

Table 14: Frequency of DRB1 *serological specificity genotypes in the UKPDS cohort according to requirement of insulin by 6 years post-diagnosis.*

***DRB1* serological genotype**	**Non-insulin requiring (n = 68)**	**Insulin requiring (n = 141)**
DR1/DR1	1 (0.015)	0
DR1/DR11	0	1 (0.007)
DR1/DR13	1 (0.015)	2 (0.014)
DR1/DR3	2 (0.029)	8 (0.057)
DR1/DR4	4 (0.059)	5 (0.035)
DR1/DR7	0	3 (0.021)
DR103/DR3	1 (0.015)	1 (0.007)
DR103/DR4	0	2 (0.014)
DR2/DR11	0	1 (0.007)
DR2/DR2	0	1 (0.007)
DR2/DR4	1 (0.015)	2 (0.014)
DR2/DR9	0	1 (0.007)
DR3/DR11	1 (0.015)	3 (0.021)
DR3/DR13	4 (0.059)	6 (0.043)
DR3/DR14	0	1 (0.007)
DR3/DR2	2 (0.029)	1 (0.007)
DR3/DR3	5 (0.074)	15 (0.106)
DR3/DR4	20 (0.294)	28 (0.199)
DR3/DR7	2 (0.029)	10 (0.071)
DR3/DR8	1 (0.015)	2 (0.014)
DR3/DR9	0	2 (0.014)
DR4/DR11	2 (0.029)	4 (0.028)
DR4/DR13	3 (0.044)	6 (0.043)
DR4/DR14	0	1 (0.007)
DR4/DR4	4 (0.059)	12 (0.085)
DR4/DR7	5 (0.074)	10 (0.071)
DR4/DR8	1 (0.015)	3 (0.021)
DR7/DR11	0	2 (0.014)
DR7/DR13	1 (0.015)	0
DR7/DR7	5 (0.074)	4 (0.028)
DR7/DR8	1 (0.015)	0
DR7/DR9	0	1 (0.007)
DR8/DR8	0	1 (0.007)
DR11/DR11	1 (0.015)	0
DR11/DR13	0	2 (0.014)

Data are given as n (frequency).

Table 15: Frequency of DQB1 *serological specificity genotypes in the UKPDS cohort according to requirement of insulin by 6 years post-diagnosis.*

***DQB1* serological genotype**	**Non-insulin requiring (n = 68)**	**Insulin requiring (n = 141)**
DQ2/DQ2	11 (0.162)	24 (0.170)
DQ2/DQ4	2 (0.029)	2 (0.014)
DQ2/DQ5	3 (0.044)	13 (0.092)
DQ2/DQ6	6 (0.088)	9 (0.064)
DQ2/DQ7	8 (0.118)	12 (0.085)
DQ2/DQ8	20 (0.294)	29 (0.206)
DQ2/DQ9	0	6 (0.043)
DQ4/DQ4	0	1 (0.007)
DQ5/DQ5	1 (0.015)	0
DQ5/DQ6	1 (0.015)	3 (0.021)
DQ6/DQ6	0	1 (0.007)
DQ6/DQ8	3 (0.044)	9 (0.064)
DQ7/DQ5	0	4 (0.028)
DQ7/DQ6	2 (0.029)	5 (0.035)
DQ7/DQ7	1 (0.015)	3 (0.021)
DQ8/DQ4	1 (0.015)	3 (0.021)
DQ8/DQ5	4 (0.059)	4 (0.028)
DQ8/DQ7	0	8 (0.057)
DQ8/DQ8	5 (0.074)	4 (0.028)
DQ9/DQ5	0	1 (0.007)

Data are n (frequency).

Association with antibody status (dual positivity for GADA + IA-2A vs. GADA alone)

Table 16: DRB1 *serological frequencies among UKPDS LADA patients according to whether they possessed GADA + IA-2A or GADA alone at diagnosis.*

***DRB1* serological specificity**	**IA-2A + GADA (n = 102)**	**GADA only (n = 304)**
DR1	5 (0.049)	22 (0.072)
DR103	1 (0.010)	3 (0.010)
DR2	2 (0.020)	7 (0.023)
DR3	27 (0.265)	100 (0.329)
DR4	32 (0.314)	96 (0.316)
DR7	15 (0.147)	39 (0.128)
DR8	4 (0.039)	6 (0.020)
DR9	2 (0.020)	2 (0.007)
DR11	5 (0.049)	11 (0.036)
DR13	8 (0.078)	17 (0.056)
DR14	1 (0.010)	1 (0.003)

Data are given as n (frequency).

Table 17: DQB1 *serological frequencies among UKPDS LADA patients according to whether they possessed GADA + IA-2A or GADA alone at diagnosis.*

***DQB1* serological specificity**	**IA-2A + GADA (n = 102)**	**GADA only (n = 304)**
DQ2	39 (0.382)	133 (0.438)
DQ4	4 (0.039)	6 (0.020)
DQ5	7 (0.069)	27 (0.089)
DQ6	9 (0.088)	29 (0.095)
DQ7	16 (0.157)	30 (0.099)
DQ8	25 (0.245)	74 (0.243)
DQ9	2 (0.020)	5 (0.016)

Data are given as n (frequency).

Table 18: DRB1 *serological genotype frequencies among UKPDS LADA patients according to whether they possessed GADA + IA-2A or GADA alone at diagnosis*

***DRB1* Serological genotype**	**IA-2A + GADA (n = 51)**	**GADA only (n = 152)**
DR1/DR1	0	1 (0.007)
DR1/DR11	0	1 (0.007)
DR1/DR13	0	3 (0.020)
DR1/DR3	2 (0.039)	7 (0.046)
DR1/DR4	1 (0.020)	8 (0.053)
DR1/DR7	2 (0.039)	1 (0.007)
DR103/DR3	0	2 (0.013)
DR103/DR4	1 (0.020)	1 (0.007)
DR11/DR11	0	1 (0.007)
DR11/DR13	0	2 (0.013)
DR2/DR11	0	1 (0.007)
DR2/DR2	0	1 (0.007)
DR2/DR3	0	2 (0.013)
DR2/DR4	1 (0.020)	2 (0.013)
DR2/DR9	1 (0.020)	0
DR3/DR11	1 (0.020)	1 (0.007)
DR3/DR13	3 (0.059)	7 (0.046)
DR3/DR14	1 (0.020)	0
DR3/DR3	4 (0.078)	14 (0.092)
DR3/DR4	9 (0.176)	39 (0.257)
DR3/DR7	2 (0.039)	10 (0.066)
DR3/DR8	1 (0.020)	2 (0.013)
DR3/DR9	0	2 (0.013)
DR4/DR11	2 (0.039)	4 (0.026)
DR4/DR13	5 (0.098)	4 (0.026)
DR4/DR14	0	1 (0.007)
DR4/DR4	2 (0.039)	13 (0.086)
DR4/DR7	8 (0.157)	8 (0.053)
DR4/DR8	1 (0.020)	3 (0.020)
DR7/DR11	2 (0.039)	0
DR7/DR13	0	1 (0.007)
DR7/DR7	0	9 (0.059)
DR7/DR8	0	1 (0.007)
DR7/DR9	1 (0.020)	0
DR8/DR8	1 (0.020)	0

Data are given as n (frequency).

Table 19: DQB1 *serological genotype frequencies among UKPDS LADA patients according to whether they possessed GADA + IA-2A or GADA alone at diagnosis*

***DQB1* Serological genotype**	**IA-2A + GADA (n = 51)**	**GADA only (n = 152)**
DQ2/DQ2	6 (0.118)	27 (0.178)
DQ2/DQ4	1 (0.020)	3 (0.020)
DQ2/DQ5	4 (0.078)	11 (0.072)
DQ2/DQ6	4 (0.078)	9 (0.059)
DQ2/DQ7	5 (0.098)	14 (0.092)
DQ2/DQ8	12 (0.235)	37 (0.243)
DQ2/DQ9	1 (0.020)	5 (0.033)
DQ4/DQ4	1 (0.020)	0
DQ4/DQ8	1 (0.020)	3 (0.020)
DQ5/DQ5	0	1 (0.007)
DQ5/DQ6	0	4 (0.026)
DQ5/DQ7	2 (0.039)	2 (0.013)
DQ5/DQ8	0	8 (0.053)
DQ5/DQ9	1 (0.020)	0
DQ6/DQ6	0	1 (0.007)
DQ6/DQ7	1 (0.020)	6 (0.039)
DQ6/DQ8	4 (0.078)	8 (0.053)
DQ7/DQ7	2 (0.039)	2 (0.013)
DQ7/DQ8	4 (0.078)	4 (0.026)
DQ8/DQ8	2 (0.039)	7 (0.046)

Data are given as n (frequency).

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