Immune and metabolic changes leading to diabetes

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Immune and Metabolic Changes Leading to Diabetes

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I dedicate this work to my late parents

Abdullah B Hawa and Fatima Petkar

'Who will always remain close in my Heart'

An inspiration of my father a simple but gracious man, who in times of difficulty said,

"I have six son's each worth a million"

Today I stand proud to say I am your one in a million

My wife Ruksana, who has always believed in me, my children Saiqa, Samena and Yassin and my family who have always stood by me.

"The worldly comforts are not for me. I am like a traveller, who takes a rest under a tree, in the shade and then goes on his way."

Sayings of the Prophet Muhammad Peace and Blessings of Allah be Upon Him.

ABSTRACT

Environmental factors are strong determinants of diabetes-predictive biomarkers. We found these environmentally-determined biomarkers included, advanced glycation end-product serum carboxymethyl lysine (CML), a glycotoxin and diabetes-associated autoantibodies, are largely determined by familial shared and non-familial non-shared effects respectively. Serum CML emerges as an additional diabetes-risk determinant additional to autoimmunity. Low-risk non-diabetic identical twins failed to identify an alteration in insulin secretion or sensitivity predisposing to type 1 diabetes (T1D). Antigen-specific antibodies in autoimmune T1D are also found in adults presenting with non-insulin requiring diabetes known as latent autoimmune diabetes of adult onset (LADA). Antigen specific antibodies in childhood and adult onset diabetes are similar with the same dominant isotype. European LADA patients compared with 'type 2 diabetes' patients are usually non-insulin requiring, younger, leaner and female. LADA is more prevalent than T1D, yet neither encompasses all adult-onset autoimmune diabetes. Prevalence of Metabolic Syndrome is significantly higher, in 'type 2 diabetes' than in adults with LADA or T1D. Excluding glucose as a variable, Metabolic Syndrome is not more prevalent in autoimmune diabetes than in controls. Metabolic Syndrome is not a characteristic of autoimmune diabetes.

Our evidence indicated that autoimmune diabetes is more prevalent in adulthood (9.7% of 6156 patients) than childhood and that presentation with non-insulin requiring diabetes is the norm, not the exception.

Autoantibodies to CD38 antigen reported as a feature of non-insulin requiring diabetes, are not so in childhood onset diabetes and do not add to the panel of diabetes associated auto-antigens.

This thesis suggests that autoimmune T1D of adult onset is prevalent, initially usually non-insulin requiring. Moreover T1D is, in part, non-genetically determined, likely involving more than one non-genetic effect and includes a broad clinical spectrum of severity, not overlapping with ‘type 2 diabetes’. The precise cause or initiating factor of the disease still remains a mystery.

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ABBREVIATIONS

Abs	Antibodies
APC	Antigen presenting cells
GAD_{65}	Glutamic acid decarboxylase
IA2ic	Tyrosine Phosphatase like molecule intracellular fragment
ZnT8	Zinc Transporter
IAA	Insulin Auto Antibodies
ICA	Islet Cell Antibodies
PCA	Parietal Cell Antibodies
TPO	Thyroid Peroxidase
CML	Carboxymethyl Lysine
IDF	International Diabetes Federation
CPM	Counts Per Minute
T1D	Type 1 Diabetes
SMS	Stiff Man Syndrome
WHO	World Health Organizations
ADA	American Diabetes Association
OGTT	Oral Glucose Tolerance Test
IGT	Impaired Glucose Tolerance
MZ	Monozygotic
DZ	Dizygotic
SLE	Systemic Lupus Erethematosus

RA	Rheumatoid Arthritis
MS	Multiple sclerosis
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
NCDs	Non communicable diseases
LADA	Latent Autoimmune Diabetes of Adult Onset
FOXP3	Forkhead Box P3
AIRE	Autoimmune Regulator genes
AGE	Advanced Glycation End Products
HLA	Human Leucocyte Antigen
MHC	Major Histocompatibility Complex
PAS	Protein A Sepharose
TBST	Tris Buffer Saline Tween
DASP	Diabetes Autoantibody Standardization Programme
TP	True Positives
FP	False Positives
FN	False Negatives
TN	True Negatives
WHO	World Health Organization
CPM	Counts Per Minute

UNIT

°C	degrees centigrade
g	Grams
h	Hour
l	Litre
M	Molar (mole/litre)
m	Milli
min	Minute
µ	Micro
%	Percentage
SD	standard deviation

CHAPTER 1

1.10. INTRODUCTION

Diabetes was recognized by the ancient Egyptian, who mention a disease with excessive urinary output. The verbal distinction between diabetes insipidus and diabetes mellitus, where diabetes means passing a fountain of urine, was first made in the 18th century (William Cullen and Johann Peter Frank). When the the physician Aretaeus of Coppadocia (81-138 AD) said that "*Diabetes is a mysterious illness*" his thoughts reflected our current understanding. He described it as 'a melting down of the flesh and limbs into urine', this, reflected the weight loss and excess passing of urine that occurs in acute, undiagnosed diabetes.

Diabetes is a devastating disease, associated with substantial morbidity and mortality. The term diabetes, without qualification, usually refers to diabetes mellitus, which is associated with excessive sweet urine, (known as 'glycosuria') but there are several rarer conditions also named diabetes. The most common of these is diabetes insipidus in which the urine is not sweet (insipidus meaning "without taste" in Latin); it can be caused by either kidney (nephrogenic diabetes) or pituitary gland (central diabetes) damage.

The word "insulin" was coined in 1909 by the Belgian, Jean de Meyer (1878-1934) for the hypothetical pancreatic hormone; later, Frederick Grant Banting (1891-1941) and Charles Herbert Best (1899-1978) adopted this term after having themselves initially proposed the word "isletin". The discovery and introduction of insulin therapy in 1921, one of the 20th century's greatest medical discoveries, heralded the cure of diabetes. Tragically, initial optimism proved unfounded as

insulin treatment did not lower blood glucose levels consistently to within the normal range. As a consequence glucose levels remain elevated with tissues exposed to chronic hyperglycemia and an altered glucose fat and protein metabolism. This in turn lead to devastating complications leading to retinopathy (causing blindness), nephropathy (causing kidney failure) and neuropathy (leading to amputation of limbs), a hallmark of the disease.

T1D is an autoimmune disease in which the target tissue is the endocrine pancreas. Human and murine diseases share a number of similarities. In both the disease is spontaneous, occurs at a young age, have a similar genetic features and progresses in two steps: infiltration of the pancreatic islet cells with lymphocytes (insulitis), followed by the pancreatic islet beta cell (β-cell) destruction, hyperglycaemia ensues together with an altered glucose, fat and protein metabolism. In the non-obese diabetic (NOD) mouse, islet infiltration of T cells, B cells, macrophages and dendritic cells starts at 3-4 weeks of age (Lo et al., 1993), (Miller et al., 1988). The susceptibility to T1D is determined by a combination of both multiple genetic as well as environmental factors (Tisch & McDevitt 1996), (Leslie & Elliot 1994). What initiates the disorder whether a viral infection, an environmental agent or an autoimmune stimulus to a self-antigen is not known, but that the subsequent gradual destruction of the β cells is immune mediated is no longer in any doubt.

1.11. CLASSIFICATION OF DIABETES

For many years clinicians have used the terms for T1D and type 2 diabetes synonymously, with IDDM and NIDDM respectively. The American Diabetes Association, (ADA) 1997 classification abandoned the latter and retained the term T1D, 'type 2 diabetes' impaired glucose tolerance (IGT) and gestational diabetes (Seisler et al., 1998). In this manuscript I will use the ADA classification throughout. More recently the Royal College of General Practioners and NHS Diabetes (Farmer 2011) have suggested changes to the criteria due to the use of the ADA criteria leading to misclassification, misdiagnosis and miscoding in the UK. The latter could however result in incorrect classification or for that matter missed classification of patients with diabetes, as the proposal outlines a simplified categorization based on hyper-glycaemia to separate diabetes from no diabetes with genetic and secondary diabetes placed in separate categories. Diabetes mellitus is a condition characterized by the presence of hyper-glycaemia resulting from autoimmune destruction of the insulin producing β cells.

TID presents mainly in childhood and in early life and accounts for about 20% of cases in Europe and North America, affecting about 35 million people world-wide and may even be double that number. It can be caused at any age and is the second commonest chronic childhood disease after asthma, with a substantially greater morbidity and mortality. It has been established that diabetes mellitus is a genetically and clinically heterogeneous disorder. Given the difficulty in treating the disease, the last 30 years has seen a concerted effort to try and understand the cause of diabetes with a view to better predict and thereby prevent it.

1.12. DIAGNOSTIC CRITERIA

In 1997, an expert Committee of the American Diabetes Association proposed a modification to the diagnostic criteria for diabetes, by lowering fasting plasma glucose at which diabetes can be diagnosed to 7.0 mmol/l (because a fasting value of 7.8 mmol/l defines a greater degree of hyper-glycaemia than a 2 hour value of 11.1 mmol/l) (Expert Committee 1997). This simple fasting value is preferred for diagnosis to the more complex 75g OGTT. Though there have been subsequent modification to this classification, it remains the classification used in this thesis.

Subjects with IGT also have an increased risk of macrovascular complications as well as other cardiovascular disease risk factors including hypertension, dyslipidaemia and obesity. Because of these factors diagnosis of IGT in apparently healthy individuals has important prognostic implications. Subsequent diagnostic criteria have incorporated glycosylated haemoglobin A1c levels but will not be considered here.

1.13. EPIDEMIOLOGY OF TID

T1D is a multifactorial disease, primarily caused by an autoimmune destruction of the pancreatic beta cells leading to an absolute insulin deficiency and a need for insulin therapy for survival. People with TID require exogenous insulin for survival to prevent development of ketoacidosis. The disease is caused by non-genetic, probably environmental, factors operating in a genetically susceptible individual to initiate a destructive immune process. Autoantibodies to the islet cell antigens (ICA), the neuronal enzyme Glutamic Acid Decarboxylase (GAD_{65}),

tyrosine phosphatase like molecule (IA2ic) and the more recently described zinc transporter) a multi-spanning trans-membrane protein that resides in the insulin secretory granules Slc30A8 (ZnT8, can be detected many months or even years before the diagnosis of clinical diabetes and are markers rather than the cause of the disease (Figure 1.1).

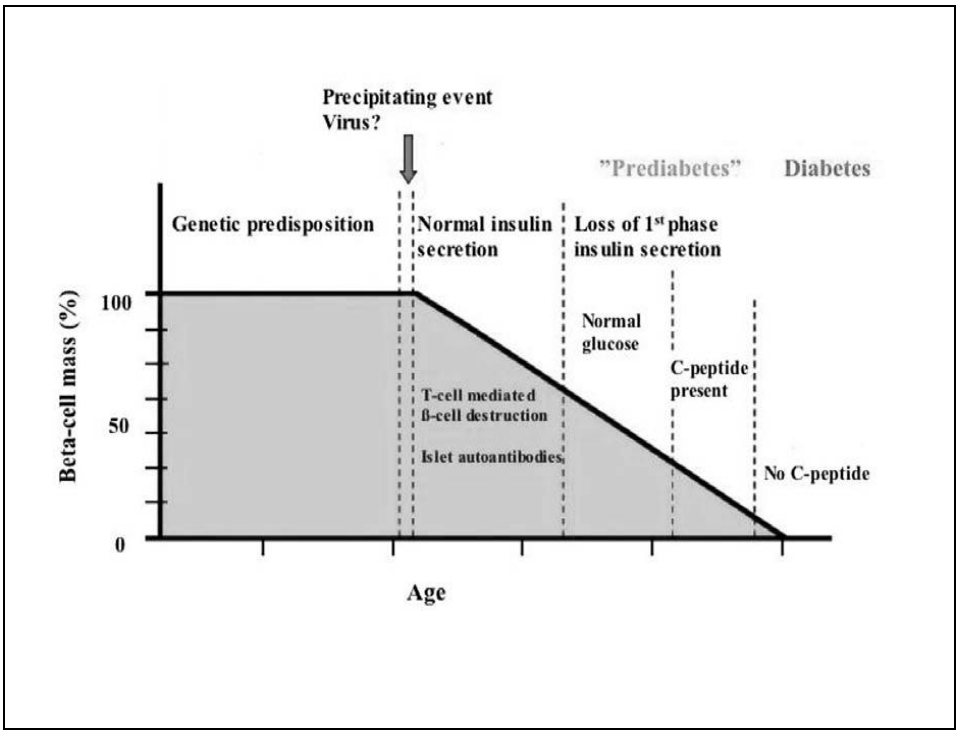


Figure 1.1: A schematic model of development autoimmune T1D.

A genetically predisposed individual exposed to an immunological trigger at a critical time that initiates an autoimmune process, resulting autoimmunity (Islet infiltration and autoantibody formation to Islet cell antigens including GADA, IAA, IA2A and ZnT8) and a gradual decline in beta cell mass, detection of autoantibodies ante-dates the progressive loss of beta cell function leading to impaired glucose tolerance and diabetes. (Modified from Eisenbarth GS. T1D mellitus: a chronic autoimmune disease. N Engl J Med 1986; 314: 360-368)

IAA = Insulin autoantibodies, GADAb = Glutamic acid decarboxylase, IA2Ab = IA2A and ICA 512 Ab and ICA= Islet cell antibodies, ZnT8= Zinc Transporter.

The incidence of TID is rapidly increasing in specific regions and is showing a trend towards earlier onset in different populations with a high variability in the ethnic populations (LaPorte et al., 1985). The overall age adjusted incidence of TID varies and is less frequent in Asia than in Europe. The incidence of TID in Japan and China is about 1:100,000/yr and in South India about 10.5:100,000/yr (Karvonen et al., 1993), (Ramachandran et al.,1996). In contrast, the incidence of TID in Sweden, Finland and Sardinia ranges from 28 to 40:100,000 per year (Green et al., 1992), (Anonymous 2000, EURODIAB ACE Study Group), (Onkamo et al., 2000). There is a large variation in the incidence of TID in the different populations analysed and furthermore the incidence of the disease seems to be increasing in all populations. Furthermore Finland and Sardinia, the two regions with the highest frequency, are 3000 km apart and Estonia Finland's neighbouring country has an incidence rate half that of Finland. It is predicted that the incidence of TID will be about 40-50% higher in 2010 than in 1998 (Struan et al.,2009). This data suggests that there was an equatorial divide for the disease incidence for TID (Karvonen et al., 1993), (Ramachandran et al.,1996), however this is not thought to be as strong as was previously attributed (Muntoni 1999) as high incidence rates similar to the European populations have been noted in Kuwait and Puerto Rico (Atkinson & Eisenbarth 2001). There is a wide difference in the incidence rates within ethnic groups and this is probably due to a combination of both genetic and an environmental background affecting the outcome of the disease incidence in the different geographical locations. The EURODIAB study (Anonymous 2000), indicates an increase in the incidence rate

of TID of 3-4% in the European countries. The incidence of TID varies both with age and sex, with males predominant in postpubertal young adults (Blohme et al., 1992), (Karvonen et al., 2000). Examination of the rates of TID as a function of age at onset shows rates of increase of 6.3%, 3.1% and 2.4% in populations of children aged 0-4 years, 5-9 years and 10-14 years respectively. The increase in incidence has been most obvious in the group under 5 years of age (Tuomilehjto et al., 1999), even though the diagnosis of diabetes is still fairly rare among infants. These findings support the impressions of the health care professionals indicating that they are seeing an increase of cases of TID, especially in the younger children.

Studies have shown that allergic as well as autoimmune diseases are not evenly distributed among continents and countries and suggested a north-south gradient with the incidence of disease decreasing from the north to the south. It is thought that this could be due to environmental as well as genetic factors. In Sardinia the incidence of T1D is high as compared to residents of neighbouring countries as well as Sardinians who migrated to continental Italy (Muntoni et al., 1997). The contribution of the genetic factors seems small in comparison to the environment, though twin studies suggest that the genetic contribution is potent in childhood but decreases thereafter (Salvetti et al., 2000), (Leslie & Castelli 2004). The environmental factors could account for the rapid rise in allergic as well as autoimmune diseases in developed countries.

1.14. AUTOIMMUNE DIABETES IN ADULTS

Autoimmune TID has been classically described as a childhood onset insulin requiring condition but it is now becoming increasingly recognised that this may not be the case as autoimmune diabetes presenting in adults which may or may not require insulin is becoming a major concern to the healthcare system. In fact requirement of insulin and the time to insulin therapy has led to clinicians to postulate the distinction of those who are put on insulin immediately at diagnosis (as TID) from those who remain free of insulin for at least 6 months after diagnosis of diabetes classified as LADA.

LADA is defined clinically by three criteria: 1) Age at diagnosis > 30 years of age; 2) Presence of autoimmune features; 3) Free of insulin therapy for at least 6 months after diagnosis. This condition is sometimes referred to as 'Type 1.5 diabetes', 'Slowly Progressive Diabetes (SPIDDM)', 'Double Diabetes', 'Antibody positive type 2 diabetes' and progressive insulin dependent diabetes (Naik & Palmer 2009), (Palmer et al.,2005). Misdiagnosis of adults as patients with type 2 diabetes is often a feature at presentation of the condition in the general population where such cases may be a little overweight, insulin resistant with signs of metabolic syndrome which clinicians generally associate with type 2 diabetes. LADA is commonly found in the Caucasian populations with 4-12% of patients presenting with type 2 diabetes with autoantibodies at diagnosis (Zinman et al., 2004).

In contrast to type 2 diabetes, defects in insulin secretion through β cell loss and islet cell dysfunction are a prominent features of TID. Numerous studies have

documented the progressive loss of β cell function during the pre-clinical and post diagnosis periods of the disease process. However, the physiological relationship between insulin secretion and insulin resistance still holds and determines the state of glucose tolerance in individuals. It has long been recognised that islet cells when stressed by rising blood sugar levels are up-regulated both metabolically and immunologically (Bjork et al., 1992). Beta cell stress (glucotoxicity) can result from the cells own low functional mass, or due to insulin resistance, both of which reduce the feedback control and blood glucose rises.

Blood glucose concentrations, do not rise linearly with β cell loss or with insulin resistance, but only slowly to the point whereby the capacity of the feedback control loop is exceeded and diabetes ensues. All patients who develop diabetes of whichever type, progress down the same path of deteriorating blood glucose control which can take months, years or decades. Insulin resistance at whatever age it may emerge, increases β cell stress and intensifies an autoimmune response in those individuals who are genetically predisposed to develop diabetes. The phenomenon of insulin resistance with progressive rise in body weight, has been increasingly noted at a younger age at presentation of type 2 diabetes over recent time. This phenomenon might be doing the same for TID by promoting the immunological accelerants of β cell death in a progressively younger age group.

The possibility of preventing ‘type 2 diabetes’ in subjects at high risk became the focus for a number of studies in patients with impaired glucose tolerance (IGT)

(Pan et al.,1997), (Tuomilehto et al., 2001). IGT is defined as hyperglycaemia (with glucose values intermediate between normal and diabetes) following a glucose load (Alberti and Zimmet 1999) (Table 1.1), and affects nearly 200 million people worldwide. IGT represents a key stage in the natural history of 'type 2 diabetes' as these people are at a higher future risk than the general population for developing diabetes (Zimmet et al., 2003). Approximately 40% of subjects with IGT progress to diabetes over 5-10 years, but some revert to normal while others can have IGT without developing diabetes. A proportion of patients presenting with non-insulin requiring diabetes presenting with IGT can have autoimmune changes, fewer features of metabolic syndrome, lower body mass index (BMI) and a lower waist hip ratio as well as lower HDL cholesterol, such that and at diagnosis rapidly go on to insulin requirement (Leslie et al., 2006) such patients are termed as LADA (Pozzilli & DiMario., 2001).

	Glucose concentration in mmol/l (mg/dl)			
	Plasma		**Whole blood**	
	Venous	**Capillary**	**Venous**	**Capillary**
Diabetes mellitus Fasting value	>7.8 (104)	>7.8(140)	>6.7 (120)	>6.7 (120)
Or 2 hour after 75g glucose load	>11.1 (200)	>12.2 (220)	>10.0 (180)	> 11.1
IGT Fasting value	<7.8(104)	<7.8 (140)	<6.7(120)	<6.7 (120)
Or 2 hour after glucose load	7.8-11.0 (140-199)	8.9-12.1 (160-219)	6.7-9.9 (120-179)	7.8-11.0 (140-199)

Table 1.1 WHO diagnostic criteria for diabetes and Impaired glucose tolerance (WHO 1999)

1.15. WHAT ARE THE REASONS FOR THIS DIABETES EPIDEMIC? ENVIRONMENT OR GENES?

The evidence is that T1D is increasing in prevalence because of environmental effects. The reason for this is based on population studies which have demonstrated a dramatic increase in the disease within a relatively short period of time and certainly too short a period to be genetically driven. In support of this proposal, migration studies have shown that children moving from a low risk country to high disease-risk country acquire the disease risk of the new environment. The incidence of T1D in Sardinia is high as compared to residents of neighbouring countries as well as Sardinians who migrated to continental Italy (Muntoni et al., 1997). The contribution of the genetic factors seems small in comparison to the environment. Another trans migratory study of children migrating from the Pakistan and India to the UK reported a rise in the incidence of T1D from 3.1/100000 before migration to 11.8/100000 thereafter, similar to the non Asian children of 12/100000 (Bodansky et al., 1992). Data of migration studies have shown that T1D in children who migrated from Pakistan to the United Kingdom is the same as the non-immigrants in the United Kingdom (11.7 per 100,000) almost 10 times the incidence in Pakistan (1 per 100,000) (Bach 2002). Environmental factors could account for the rapid rise in allergic as well as autoimmune diseases in the developed countries. Both genetic as well as environmental factors (dietary factors and viruses) together are thought to have an impact on the incidence of T1D as well as the national health services.

Finally, the threshold for developing T1D appears to have changed so that children with T1D have less HLA genetic susceptibility today than many years ago, e.g. the Barbara Davis Diabetes Centre have shown over the last 40 years a decrease in the HLA D3/4 in individuals (Steck et al 2011). Thus, the epidemic appears to reflect an interplay between genetic risk and environmental risk in which the environmental risk or effect has increased in Europe and North America while the genetic risk sufficient to lead to the disease has decreased; these principle have been outlined in the 'Threshold Hypothesis' (Wasserfall C et al, 2011).

Environmental and behavioural factors include sedentary lifestyle, nutrition (Low Birth weight) and obesity have been put together to explain the increase in incidence of diabetes. Two schools of thought have been put forward to explain the changing risk of non-communicable diseases (NCD) such as T1D: 'the thrifty genotype' and the 'thrifty phenotype'.

1.16. 'The Thrifty Genotype Hypothesis' (Neel 1962) provides an explanation for the high prevalence of type 2 diabetes in the Pima Indians, Australian Aborigines and Pacific Islanders, as being due to an evolutionary thrifty genotype promoting fat deposition and storage of calories in times of plenty, which would have been advantageous to the hunter gatherer populations. However in the modern world the abundance of food prepares the individuals at risk for a famine that never results leading to obesity. Another pathway to obesity is likely to be the maternal-foetal environment during pregnancy. The effects of hyper-nutrition

during pregnancy as in the offspring of Pima Indians women with type 2 diabetes which accounts for 40% of type 2 diabetes in that population (Dabalea et al., 1998).

1.17. 'The Thrifty Phenotype Hypothesis' on the other hand, is based on the epidemiological observations, linking, low birth weight with the risk of adult disease (obesity, diabetes and hypertension). Hales and Barker, hypothesized (Hales & Barker 1992) that intrauterine malnutrition leads to reduced birth weight and permanent changes in physiological structure and function, which predispose to disease in adult life. Both factors, that is intrauterine environment and low birth weight, are probably relevant given the appropriate environmental and genetic background. Less clear is whether either of these hypotheses are relevant to the origins of T1D.

1.18. HYPOTHESIS FOR AUTOIMMUNE DISEASE

A number of other hypothetical models have been put forward to try and explain the pathogenesis of T1D.

1.181. The 'Accelerator Hypothesis', argues that TID and type 2 diabetes are one and the same, distinguishable only by their rate of beta cell loss and the accelerators responsible (Wilkin 2001). The 'Accelerator Hypothesis' identifies three processes which variably accelerate the loss of beta cells through apoptosis: constitution, insulin resistance and autoimmunity. The first accelerator, a constitutionally (intrinsically) high rate of beta-cell apoptosis, is necessary for

diabetes to develop but in itself is rarely sufficient to cause it. Insulin resistance, the second accelerator, results from weight gain and physical inactivity which further increases beta cell apoptosis and accounts for the rising incidence of TID as well as type 2 diabetes in the industrially developed countries. Finally the third accelerator operates in a genetically defined subset of patients with both an intrinsic lesion of beta cells and insulin resistance developing beta cell autoimmunity.

1.182. The 'Overload Hypothesis' implicates beta cell overload resulting from insulin resistance, high growth rate, physical stress and increased insulin requirement, leading to accelerated beta cell damage (Dahlquist G. 2006). Insulin dependency is the end stage towards which all types of diabetes move to and the notions of TID and non-insulin requiring diabetes as separate diseases becomes irrelevant. Of the three accelerators, one is intrinsic and two are acquired. Insulin resistance, the second accelerator, is associated with visceral fat mass as well as ageing and is widely believed to explain the epidemic rise of type 2 diabetes in the industrially developed world. Evidence that obesity or weight gain are associated with T1D risk is substantial, but evidence that there is another accelerator associated with beta cell apoptosis is less evident (Dahlquist G. 2006).

Other hypothesis try to reconcile aspects of T1D disease process and includes the

1.183. 'Copenhagen model', where beta cell destruction is the result of an interaction of the environment, the immune system and the beta cells themselves releasing cytokine and free radicals in genetically susceptible individuals (Nerup J et al. 1994).

1.184. The 'Hygiene Hypothesis' states that a lack of early childhood exposure to infectious agents, symbiotic microorganisms (e.g., gut flora or pro-biotics), and parasites increases susceptibility to allergic diseases by suppressing natural development of the immune system (Strachan 2000). This hypothesis was originally formulated for asthma (Strachan 2000). Other diseases, such as the rise of autoimmune diseases and acute lymphoblastic leukemia in the young in developed nations, have also been linked to the hygiene hypothesis.

1.185. The 'Fertile Field Hypothesis' implies that microbial infections provides a temporary state conducive to reactivity to self, leading to autoimmunity which has been implicated in TID and multiple sclerosis (Matthias et al., 2003). However, no single micro-organism has been identified in the cause of human auto-immune disease. Multiple hits from the same or different microorganisms could provide an opportunity for the autoimmune disease to manifest in a genetically susceptible individual. Alternatively, multiple hits from a range of non-genetic effects including infections could be relevant.

However while these hypothesis may help in defining the development of T1D in some individuals, none of them quite captures the reason for the general increase in the incidence rate of the disease, a polygenic disease with multiple environmental insults. The importance and contributions of nature (i.e. genetics) over nurture (i.e. environment) in TID has recently been put together by (Wasserfall C et al 2011) in the **'threshold hypothesis'** as a graphical model with genetic and environmental load and the interaction in TID.

1.186. The 'Threshold Hypothesis', is illustrated by arranging the odds ratio for the genetic as well as the environmental components that interplay in this disease. Of the genetic and environmental components, the rare mutation of the Forkhead Box P3 (FOXP3) and the Autoimmune Regulator (AIRE) genes contribute the highest risk for autoimmune diabetes involving beta cell destruction and modulation of the immune system. Of the known environmental factors pancreatectomy and beta cell chemical toxin pose the highest risk component with an odds ration > 7.0. (Figure 1.2).

From the threshold hypothesis we note that the HLA region with an odds ratio close to 7.0, which has the highest genetic association after FOXP3 and AIRE followed by the next closest regions the non MHC loci being INS VNTR, PTPN22, CD25 and CTLA4 before dipping below an odds ratio of 1 exemplifying the HLA-DQB (protective alleles) (Wasserfall C et al 2011). The HLA accounts for the major genetic risk component in children and young adults.

With respect to the environmental factors, the risk posed by toxins or pancreatectomy, outweigh other environmental factors, however dietary factors, infections, perinatal as well as postnatal environment as well as the gut microbiota could well interplay with the genetic susceptibility alleles to provide a combinatorial effect. The threshold hypothesis posits a model as presented in Figure 1.2 to highlight the combined impact of both the genetic and environment factors together to reach a threshold above which disease ensues. The model further confirms that the environmental factors alone or genetic susceptibility alone does not equate to disease but rather the interaction of these factors provide

a combinatorial increased risk factor. With globalisation as well as migration genes find themselves in environments to which they are ill adapted to, leading to increased disease incidence. The threshold model combines the genetic as well as environmental impacts and studies currently underway such as the TEDDY study will allow these questions to be answered.

1.187 The 'Molecular Mimicry hypothesis' is defined as structural similarity between foreign peptides (e.g. virus peptides) and endogenous peptides (e.g. normal component of pancreatic β cells), which is responsible for immunological cross reactivity. An abnormal immune response to that particular virus protein may contribute to the pathogenesis of diabetes through immunity to that particular viral peptide, at the same time, to pancreatic β cell proteins. Molecular mimicry might be one of the mechanisms perpetuating autoimmunity to islet self autoantigens. The cause of autoimmune diseases however is unknown, but it appears that there is an inherited predisposition to develop autoimmune disease in many cases. However in a few types of autoimmune disease (such as rheumatic fever), a bacterial or virus protein triggers an immune response to peptides, such that antibodies or T-cells attack the normal cells because they recognise a part of the peptide structure that resembles a part the infecting microorganism. One such example proposed in TID is the PEVKEK region found on the autoantigen GAD65 and also found on the coxsackie virus.

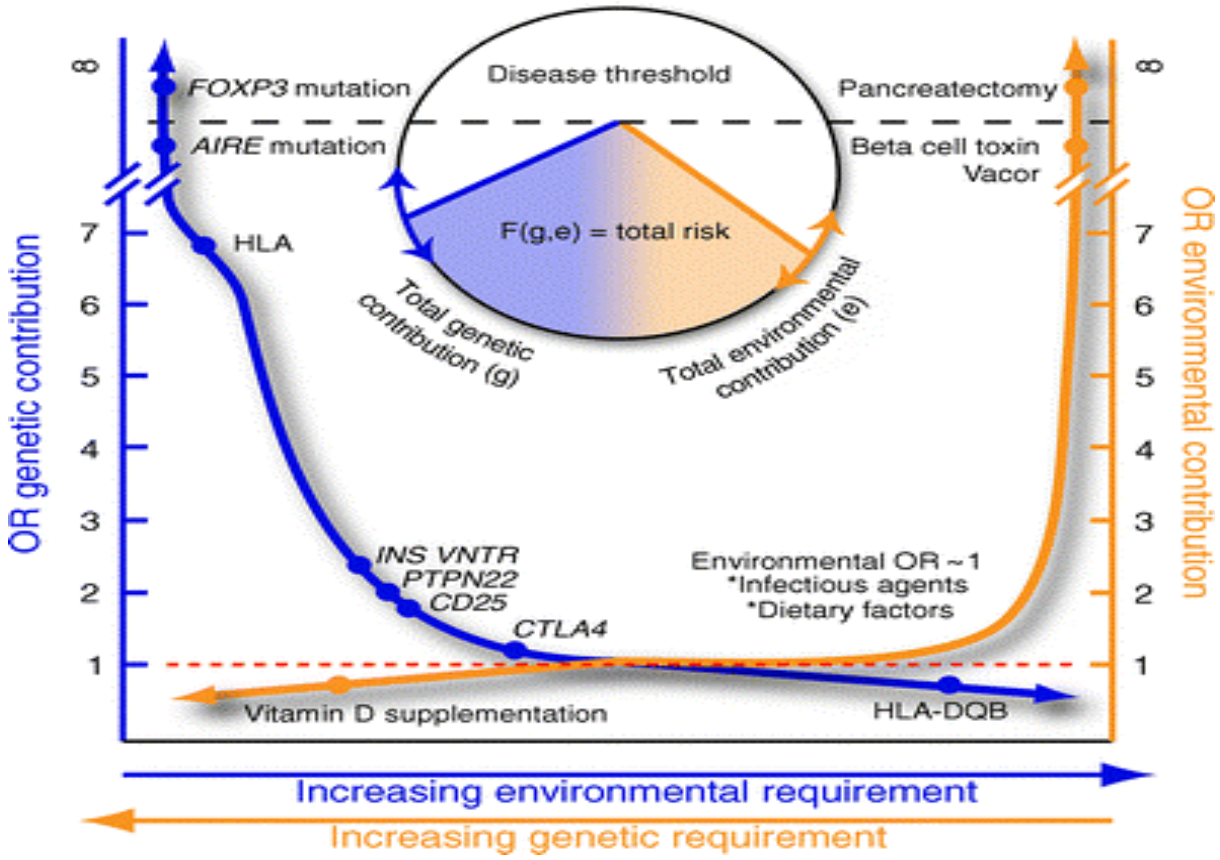


Figure 1.2: A hypothetical model to depict the impact of genetic as well as environmental factors in T1D (Wasserfall C et al 2011).

1.19. PATHOPHYSIOLOGY OF AUTOIMMUNE DIABETES

Diseases of the immune system such as T1D, rheumatoid arthritis, coeliac disease and multiple sclerosis, affect about 5% of Caucasoid populations (Vyse & Todd 1996). These diseases are characterised by features of autoimmunity: the presence of autoantibodies and self-reactive T cells, sensitivity to agents such as cyclosporine that suppresses the immune system and a universal genetic association with the major histocompatibility complex (MHC) on chromosome 6p21. Relatives of patients are at significantly increased risk of developing the disease or, indeed, of other autoimmune diseases; families with TID tend to have increased frequencies of rheumatoid arthritis, coeliac disease, thyroiditis and multiple sclerosis. This familial clustering of disease is consistent with a genetic component of the disease aetiology. It is clear, however that even a sufficient complement of susceptibility polymorphisms at disease associated genes, both inside and outside the MHC, does not always lead to the development of disease at the clinical level, as genetically identical twins are often much less than 100% concordant.

In the spontaneous mouse model of autoimmune T1D, the nonobese diabetic (NOD) strain, the frequency of disease is less than 100%, with about 50-80% of females developing diabetes by 220 days and 10-50% of males (Wicker 1995). By contrast disease frequency decreases if NOD mice are deliberately or accidently infected with bacterial, viral, or parasitic organisms (Todd 1991). These observations challenge the view that TID is initiated by an environmental trigger. Instead, the data from NOD mice are consistent with an intrinsic immune

aetiology, perhaps also a target organ defect, in which the disease penetrance is modified by multiple environmental factors acting in both a positive and negative way during different stages of disease development.

Concordance for T1D in identical twins is consistently less than 100% and approximates to 50% in children and even less as the age of onset becomes older (Leslie & Castelli 2004), (Gardner et al 1997). Incomplete concordance of twins may be a consequence of the typically aggressive microbial environment in which we live. However, other factors could be responsible and include random events and missing heritability one cause of which could be due to gene-gene as well as gene environment interaction together with epigenetic modification (Clarke AJ 2010). It was suggested that one reason TID is increasing in frequency (Strachan 2000) is because standards in sanitation and health care have improved dramatically over the past 30 years. This hygiene hypothesis was originally formulated for asthma and allergic diseases which postulated that infections protected from atopy (Strachan 2000). Consequently, the environment appears to have become more permissive for the development of autoimmunity to the pancreatic insulin producing β cell since our immune system is not as 'educated' as it used to be when antibiotics and sanitation were less evident. A recent study is consistent with this model: multiple infections during the first few years of life were associated with a reduction in risk of T1D (Gibbon et al., 1997).

It may well be that TID can be either accelerated or precipitated by infection. Rubella viral infection is such an example, but even in rare rubella virus syndrome patients also have the established MHC susceptibility markers (Hee

Suk Jun and Ji Won Yoon., 2002). Coupled with the fact that 30% of European cases carry the MHC risk haplotypes, HLA-DR4 and/ or DR3 (Noble et al., 1996) it can be concluded that gene polymorphisms are necessary for the development of disease and could, in many cases, be almost sufficient in an appropriate environment, e.g. FOXP3 or AIRE gene mutations as discussed by Atkinson and colleagues (Wasserfall C et al 2011).

1.20. ENVIRONMENTAL FACTORS

Both genetic and non-genetic factors contribute to the development of T1D. The low concordance rate of TID in identical twins less than 40% (Barnett et al.,1981), though age at diagnosis dependent, suggests that environmental factors play a significant role in the disease penetrance. A number of environmental factors are thought to play important roles in the pathogenesis of childhood onset TID including infections (entero-virus infections), dietary factors (early exposure to cows milk), vitamin D intake, perinatal (uterine environment) as well as psycho social factors (stress early in life, illness, death in family or divorce) have all been implicated. Vitamin D has been reported to have a beneficial protective effect in the regulation of the immune responses and also associated with a lower risk of TID, however to this end there have been conflicting reports. A recent report in the Diabetes Autoimmunity Study in the Young (DAISY) has shown that neither vitamin D nor 25(OH)Vitamin D in childhood were associated with risk to islet immunity or progression to T1D. However despite this, no single factor has been clearly identified as the trigger of autoimmune beta-cell destruction.

Twin studies have helped to define the importance of genetic and environmental factors in the aetiology of disease (Martin et al., 1997), (Smith 1993), (Utz et al., 1993), Brix et al.,1998), (Nanki et al., 1996), Ebers et al., 1986), (Allen et al., 1967), Hassan et al., 1966), Lehtovirta et al., 2000). By measuring concordance rates (both twins affected) in identical (MZ) and non-identical (dizygotic) (DZ) twins, estimates of genetic influence can be obtained.

In primarily genetic disorders, concordance rates should be higher in MZ than dizygotic twins, because MZ twins share the same genes 100% as well as the environment unlike fraternal twins who share 50% of the genes but the same environment. Differences between MZ twins must be due to factors not coded in the germ line, i.e. non-germ-line genetic (e.g. somatic) or environmental factors.

Diabetic twin studies have provided important information about the causes of diabetes including the cause of TID. Twin studies have shown that T1D is due to non-genetically determined factors, probably environmental agents, operating in genetically susceptible individuals. The environmental event operates over a brief period in early childhood to induce an immune process which destroys the islet ß cell. ß cell destruction in some is chronic and progressive, leading to TID, but in others it can remit without diabetes developing. In the months or years before the onset of TID, clinical, immune and metabolic changes can be detected and these changes are predictive of diabetes. The nature, intensity, extent and persistence of these immune changes distinguishes twins who develop TID from those who do not. In an analysis of a combined study of diabetic twins from the United States and United Kingdom of 187 twin pairs many followed for up to 40 years,

indicates that non-germ-line genetic or environmental factors play an important part in both the development of T1D and in the rate of progression to disease (Redondo et al., 2001). In both countries the rates of progression and concordance were similar and it seems likely as a consequence that the genetic and non-genetic factors causing T1D are also similar in the two countries. An age-related heterogeneity, was observed with higher progression to diabetes for twins of patients diagnosed at a younger age (Redondo et al., 2001). Most non-diabetic twins did not develop diabetes at an early age, up to 25% of the twins who progressed did so after more than 14 years of discordance.

A number of environmental factors have been implicated in the cause of diabetes including viruses (Hee Suk Jun and Ji Won Yoon., 2002) and diet in the form of early exposure to cow's milk in infants, each implicated to the increased incidence of diabetes. It is suggested that some components of cow's milk in its pasteurised or homogenised form in particular β-casien may well contribute to the increased triggering of an autoimmune reaction in genetically predisposed individuals. The most abundant protein in Cow's milk is the A1 form of β-casien and its commonest genetic variants are A1, A2 and B β-casein. Cow's milk β-casien consists of 209 amino acids, the A1 and A2 variants differ only at position 67 which has histidine in A1 and proline in A2 milk. Another variant B β-casien has histidine at position 67 and is less frequent than A1 or A2 in cow's milk of European origin. Studies in the NOD mouse model have shown that diabetes development can be delayed by the introduction of dietary modification's including hydrolyzed cow's milk (Elliot et al., 1988), whilst other studies have

shown a differential effect with cow's milk or wheat (Beales et al., 2002) where the highest incidence rate of diabetes was in the control group fed on wheat, corn, soy, alfalfa, Oats, fish meal and cellulose and no milk. In that study (Beales et al., 2002) concluded: "A previous result that A1 β-casein was more diabetogenic than A2 β-casein in NOD mice was not confirmed".

Another hypothesis that A1 and not A2 β-casein may increase diabetes development in genetically susceptible individuals by the release of opoid peptide β Casomorphin 7 (BCM-7) which affects the immune system such that autoantibodies against β-cells are more likely to be formed , however this remains to be confirmed in humans (Monetini 2002). These effects are most prominent after weaning suggesting that early life events in exposure to new food's early in life may have a modifying influence in the progression to diabetes (Daniela et al., 2009). Early exposure to an environmental insult may well initiate an immune response however it is not clear whether cow's milk alone is the causative factor in diabetes development. The TRIGR study, a study that aims to identify environmental factors which cause T1D, should clarify whether weaning to a highly hydrolysed formula may protect children at risk by delaying or preventing the development of diabetes. Preliminary studies by Knip and colleagues (Knip 2010) showed a decreased risk of positivity to diabetes associated autoantibody. There still remains however, the possibility, that an early environmental or nutritional factors, act as co triggers in the development of T1D.

Early exposure to other proteins may well be responsible including heat modified proteins such as Advanced Glycation End (AGE) Products.

1.21. ADVANCED GLYCATION END PRODUCTS (AGE)

Glycosylation is the enzymatic process that attaches glycans to proteins, lipids or other organic molecules. This enzymatic glycosylation is fundamental to the biological function of cellular proteins as well as RNA and DNA and have strucutural as well as functional roles in membrane and secreted proteins in the normal physiology. The general O-glycosylation has also been shown to be present in the Bacteroides Fragilis and is necessary for the bacteria to colonize mammalian intestine (Fletcher et al., 2009). The majority of proteins synthesized in the endoplasmic reticulum (ER) undergo glycosylation which is an enzyme directed site specific process as opposed to the nonenzymatic chemical reaction of glycation of proteins, lipids as well as nucleic acid via the formation of highly reactive intermediates.

This nonenzymatic glycation reaction is also known as the Maillard reaction and the realisation of the importance of this reaction began in the mid 1970's with the study of HbA1c a naturally occurring human hemoglobulin which was elevated in diabetic patients. This nonenzymatic glycation called Advanced Glycation (AGE) is the result of the reaction of glucose with proteins, lipids and nucleic acids to form AGE. AGE accumulates in the body naturally as a result of chronological ageing. Furthermore this process is substantially accelerated under the hyperglycaemic oxidative stress conditions present in a prolonged diabetic cellular milieu.As we age the cumulative exposure of the bodies protein, lipids as well as nucleic acids to reducing sugars in the blood and lymph allows their nonenzymatic glycation to form AGE. These AGE induce the cross linking of

long lived proteins such as collagen and elastin in the extracellular matrix, promoting stiffness and affecting the normal physiological function. AGE formation occurs over months or even years which is why long lived proteins of the extracellular matrix, collagen and elastin are most vulnerable to AGE accumulation. Furthermore AGE bind to AGE specific receptors RAGE, found on all cell types (smooth muscle & endothelial cells) including immune cells (macrophages, monocytes, T-Cells as well as dendritic cells), inducing intracellular signalling leading to increased oxidative stress, production of reactive oxygen species and activation and production of a cycle of pro-inflammatory cytokines leading to micro-vascular as well as macro-vascular damage (Wautier & Schmidt 2004).The level of AGEs increases both during physiological ageing as well as in pathophysiological settings such as diabetes mellitus, RA. Alzheimers disease (AD) and MS (Sternberg et al., 2010).

1.22. GENETIC FACTORS

It is well established that T1D has a strong genetic component. The frequency of the disease is higher in siblings of diabetic patients (in the UK it is 6% by age 30) compared to the general population (0.4% by age 30) (Field 2002). This is unlikely to be due to shared environmental factors as clinic- and population-based studies have demonstrated higher concordance rates for T1D in identical compared with non-identical twins (Salvetti et al.,2000), (Hyttinen et al.,2003).

Genetic influences on T1D are most potent in childhood-onset T1D (Salvetti et al.,2000), (Kumar et al., 1993), (Vyse & Todd 1996). However, even in

childhood-onset T1D the majority of identical MZ twins remain discordant for the disease and the concordance rate falls even further with increasing age at diagnosis of T1D (Gardner et al., 1997), (Leslie and Delli-Castelli 2004). Current explanations for the high rate of T1D discordance between MZ twins include environmental triggers of T1D that operate on one twin and not the other, or differences at the molecular level e.g. mosaicism due to skewed X-chromosome inactivation in females and different repertoires of T-cell receptors and antibodies resulting from somatic rearrangements, or epigenetic effects.

However, although various environmental exposures have been proposed to trigger T1D, including early infant diet, viral infection and toxins, conclusive evidence for an environmental agent is still lacking and there is no proof that the molecular differences mentioned above are the basis of T1D discordance in MZ twins. In light of the facts that only a few genomic susceptibility regions have been found, no environmental agents conclusively identified, and the high rate of disease discordance in MZ twins, it is proposed that penetrance of T1D may, in part, be influenced by epigenetic factors.

1.23. WHAT ARE AUTOIMMUNE DISORDERS

Autoimmune disorders are diseases caused by the body producing an aberrant immune response against its own tissues. The immune system sometimes, will cease to recognize one or more of the body's normal constituents as "self" and will respond to it as foreign and create autoantibodies to it, – antibodies that attack the

cells, tissues, and/or organs. This causes local inflammation and tissue or organ damage which can subsequently lead to autoimmune disorders.

The cause of autoimmune diseases however is unknown, but it appears that there is an inherited predisposition to develop autoimmune disease in many cases. In a few types of autoimmune disease (such as rheumatic fever), a bacteria or virus triggers an immune response, and the antibodies or T-cells attack the normal cells because they recognise a part of the structure that resembles a part of the structure of the infecting microorganism (molecular mimicry).

Autoimmune disorders fall into two general types: those that damage many organs (systemic autoimmune diseases) and those where only a single organ or tissue is directly damaged by the autoimmune process (localized). However, the distinctions become blurred as the effect of localized autoimmune disorders frequently extends beyond the targeted tissues, indirectly affecting other body organs and systems.

A major feature of the immune system is its ability to inflict damage on cells. Therefore, it must confine this activity to cells that are of potential threat, such as cells in the body that have become infected or neoplastic, whilst remaining unresponsive or tolerant to the normal cells of the body. As the regulation of the immune system is very important in the context of human disease, a disordered response may result in a breakdown in tolerance leading to auto-immune disease or immune deficiency.

In general autoimmune diseases can be divided into two categories: organ-specific and systemic autoimmune disease. In organ-specific autoimmune disease, the immune response is directed to a target antigen unique to a single organ or gland, so that the manifestations are largely limited to that organ. In systemic autoimmune disease, the response is directed toward a broad range of target antigens and involves a number of organs and tissues.

1.24. ORGAN-SPECIFIC AUTO-IMMUNE DISEASES

Damage to the target organs in organ-specific auto-immunity can occur as the result of direct cellular damage by humoral or cell-mediated mechanisms or by stimulating or blocking autoantibodies. In this instance, the immune system usually targets discrete antigens present on the tissue. Gradually, the cellular structure of an affected organ is replaced by connective tissue, hence, the function of the organ declines. T1D mellitus and Hashimoto's thyroiditis are good examples of this type of autoimmune diseases (Table 1.2).

Disease	Organ/Tissue
T1D Mellitus	Pancreas, islets
Hashimoto's thyroiditis, Graves' disease	Thyroid Gland
Celiac disease, Crohn's disease, Ulcerative colitis	GI tract
Multiple sclerosis	Myelin sheath
Addison's disease	Adrenal
Primary biliary cirrhosis, Sclerosing cholangitis, Autoimmune hepatitis	Liver
Stiff Man Syndrome (SMS)	Affects axial muscles

Table 1.2: Localized Autoimmune Diseases: Table shows the organs and tissues affected by specific autoimmune disease

1.25. SYSTEMIC AUTO-IMMUNE DISEASES

Autoimmune diseases with systemic manifestations reflect a generalised defect in immune regulation that result in hyperactive T-cells and B-cells. Tissue damage is widespread, both from cell-mediated immune responses and from direct cellular damage caused by autoantibodies or by accumulation of immune complexes. Examples include systemic lupus erythematosus (SLE) and Rheumatoid arthritis (Table 1.3).

Disease	Organ/Tissue
Rheumatoid arthritis (RA) and Juvenile RA (JRA)	(joints; less commonly lung, skin
Lupus [Systemic Lupus Erythematosus]	(skin, joints, kidneys, heart, brain, red blood cells, other)
Scleroderma	(skin, intestine, less commonly lung)
Goodpasture's syndrome	(lungs, kidneys)
Wegener's granulomatosis	(blood vessels, sinuses, lungs, kidneys)
Polymyalgia Rheumatica	(large muscle groups)
Guillain-Barre syndrome	(nervous system)

Table 1.3: Systemic Autoimmune Diseases: Table shows the organs and tissues affected by specific autoimmune disease where there is a generalized defect involving T as well as B cells.

1.26. ELEMENTS OF THE IMMUNE RESPONSE

Functional or structural damage to self-cellular components caused by the immune system is not fully understood. The immune system can suddenly target its own cells tissues and organs for destruction, generally known as autoimmunity. Most of the distinct types of autoimmune diseases are rare, but collectively autoimmune diseases affect millions of individuals throughout the world. Tolerance to self may be broken because normally occurring autoimmunity is not controlled, either centrally due to deficient elimination or lack of functional inactivation or inhibition of auto-reactive lymphocytes in the thymus or in the periphery (Kroemer and Martinez 1992). However, there is no evidence to suggest that the initiating events in the immune response are different whether the antigen is to self or non-self.

1.27. INNATE AND ADAPTIVE IMMUNITY

There are two functionally different types of responses to invading microbes. Innate (natural-genetic) responses we are born with and occur to the same extent however many times the infectious agent is encountered, whereas acquired (adaptive-environmental) responses improve on repeated exposure to a given infection. The innate responses use phagocytic cells (neutrophils, monocytes and macrophages), cells that release inflammatory mediators (basophils, mast cells and eosinophil's) and natural killer cells. The molecular components include complement system, acute phase proteins and cytokines.

On the other hand acquired immune responses involve the proliferation of antigen specific B and T lymphocytes, which occurs upon specific recognition and binding of antigens. Specialized cells, called antigen-presenting cells present the antigen to lymphocytes leading to specific responses. Specific recognition and binding of antigens is dependent on several cells, the following are the most important.

1.28. LYMPHOCYTES

Lymphocytes are wholly for the specific immune recognition of pathogens, so that they initiate the adaptive immune responses. All lymphocytes are derived from bone-marrow stem cells, but T lymphocytes then develop in the thymus, while B lymphocytes develop in the bone marrow. Adaptive immune responses are generated in the lymph nodes, spleen and mucosa-associated lymphoid tissue. These are referred to as secondary lymphoid tissues. In the spleen and lymph nodes, the activation of lymphocytes occurs in distinctive B and T cell compartments of lymphoid tissue. The mucosa associated lymphoid tissues include the tonsils, adenoids and Peyers patches, which defend the mucosal surfaces. Diffuse collections of lymphoid cells are also present throughout the lung and the lamina propria of the intestinal wall (Peter and Roitt 2000).

1.29. B CELLS

Each B cell is genetically programmed to encode a surface receptor specific for a particular antigen. Immunoglobulins (Ig) and T cell receptors (TCR) are two types of proteins produced by B cells. Having recognised its specific antigen, the B cells multiply and differentiate into plasma cells, which produce large amounts of

the receptor molecules in a soluble form which can be secreted, termed as antibody. B cells are essentially little antibody factories, able to switch on high-rate synthesis and secretion of antibody molecules when stimulated by recognition of the "appropriate" antigen.

Recognition and response in B cells are perfectly co-ordinated, because their surface antigen-receptor is the same antibody as they will secrete when stimulated by a specific antigen. Thus only specific antibody molecules are made which can bind to the stimulating antigen and help in its disposal, and thereby production of unwanted antibodies is avoided. It is the formation of plasma antibody that forms the foundation of the so called humoral immune response.

1.30. ANTIBODIES

Structural differences between immunoglobulins are used for their classification. The type of heavy chain an immunoglobulin possesses determines the immunoglobulin "isotype". More specifically, an isotype is determined by the primary sequence of amino acids in the constant region of the heavy chain, which in turn determines the three-dimensional structure of the molecule. Since immunoglobulins are proteins, they can also act as an antigen, thereby eliciting an immune response that generates anti-immunoglobulin antibodies. However, the structural (three-dimensional) features that define isotypes are not immunogenic in an animal of the same species, since they are not seen as "foreign". For example, the five human isotypes, IgA, IgD, IgG, IgE and IgM are found in all humans and as a result, injection of human IgG into another human would not

generate antibodies directed against the structural features (determinants) that define the IgG isotype. However, injection of human IgG into a rabbit would generate antibodies directed against those same structural features.

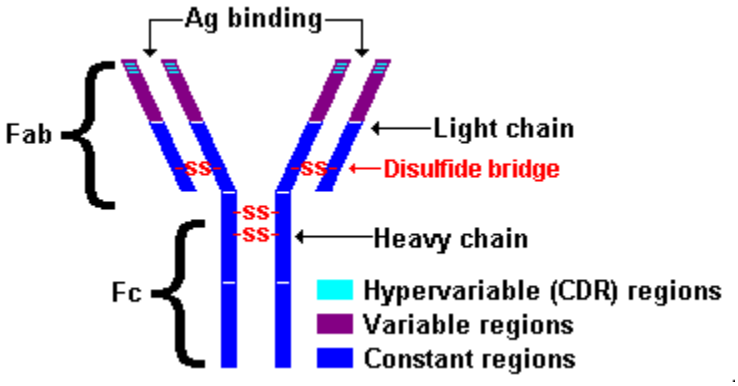


Figure 1.3: Schematic structure of IgG (which exists as a single major subclass) The heavy chain (H) and light (L) chains are composed of variable (V) and constant (C) domains and are linked by inter and intra chain disulphide bonds. IgM is a pentamer and IgA a dimer.

IgG has the general formula of $gamma_2$ $kappa_2$ or $gamma_2$ $lambda_2$ which denotes that one molecule of IgG is composed of two gamma heavy chains and two light chains of either type kappa or type lambda (Figure 1.3). The structure of the IgG molecule has been determined in part by proteolytic digestions and reductive dissociation of the molecule.

The four IgG subclasses (IgG1, IgG2, IgG3 and IgG4) occur in humans in different proportions ranging from 66%, 23%, 7% and 4% respectively and have different properties as shown in table 1.4 (Meulenbroek 2008). The glycoprotein immunoglobulin G (IgG) is a major effector molecule of the humoral immune response in man, accounting for about 75% of the total immunoglobulins in plasma of healthy individuals. The immunoglobulins of the other four classes, IgM, IgA, IgD and IgE each of which has characteristic properties and functions, constituting the other 25% of the immunoglobulins (Spiegelberg 1974). Antibodies of the IgG class express their predominant activity during a secondary antibody response. Thus the appearance of specific IgG antibodies generally corresponds with the maturation of the antibody response, which is switched on upon repeated exposure to an antigen. In comparison to antibodies of the IgM class, IgG antibodies have a relatively high affinity and persist in the circulation for a long time. During an antibody response the isotype and subclass of antibodies can be shifted without changing the specificity. The dominance of IgM in the beginning of an antibody response is switched after re-exposure to the antigen and switched into other classes of antibodies, for example IgG in serum.

	IgG1	IgG2	IgG3	IgG4
Adult serum levels (g/L)	4.9-11.4	1.5-6.4	0.11-0.85	0.03-2.0
1-6 months	1.8-7.0	0.4-2.1	0.2-0.80	<0.1-0.3
6-12 months	2.0-7.7	0.3-2.3	0.2-1.0	<0.1-0.4
Half life (days)	21	21	7	21
Antibody response to proteins	++	+/-	++	+/-
Antibody response to polysaccharides	+	++	_	_
Antibody response to allergens	+	_	_	++
Binding to Protein A sepharose	++	++	_	++

Table 1.4 Modified from Meulenbroek 2000

1.31. IMMUNOGLOBULIN PRODUCTION

The production of immunoglobulins by B-cells or plasma cells occurs in different stages,polypeptide chains of immunoglobulins are encoded by three non-linked clusters of autosomal genes, one cluster coding for the heavy chains of all classes and subclasses, a second one for kappa light chains and a third one for lambda light chains. These three gene clusters are called, the H, κ and λ gene families respectively. In Humans the H gene family is on chromosome 14, the κ gene on chromosome 2 and the λ gene family on chromosome 22.

Each B-cell can make only one heavy chain and one light chain, although the isotype of the heavy chain may change. Initially, a mature B-cell will produce

primarily IgD (and some membrane IgM) that will migrate to the cell surface to act as the antigen receptor. Upon stimulation by antigen, the B-cell will differentiate into a plasma cell expressing large amounts of secreted IgM. Some cells will undergo a "class switch" during which rearrangement of the DNA occurs, encoding the IgG, IgE or IgA. Upon secondary induction (i.e. the secondary response), these B-cells will differentiate into plasma cells expressing the new isotype. Most commonly, this results in a switch from IgM (primary response) to IgG (secondary response). The factors that lead to production of IgE or IgA instead of IgG are not well understood.

1.32. ROLE OF AUTOANTIBODIES IN DIABETES

Autoantibodies to islet cell antigens can be detected many months or even years before the diagnosis of clinical diabetes (Verge et al.,1996), and can appear early in infancy or at any age, Insulin autoantibodies (IAA) appearing early in children but not exclusively (Yu et al. 1996),(Kimpimaki 2001) with a broader antibody pattern in adults. The detection of a low titre single autoantibody positivity may represent a perfectly normal background activity, whereas presence of high titre multiple autoantibodies reflect aggressive beta-cell damage and a rapid progression to overt clinical disease (Kulmala et al.,2001), (LaGasse et al., 2002). Autoantibodies to islet antigens, are not thought to be directly involved in the destructive process as reported previously reported by (Martin et al., 2001) showing that a B cell deficient patient developed diabetes indicating that B cells are themselves not required for disease development (Martin et al., 2001).

Autoantibodies are predictive in high risk patients as well as in the general population, where positivity to multiple islet autoantigens confers the highest risk (Bingley et al. 1994; Bingley et al. 1997; Verge et al. 1998; Kulmala et al. 2001; LaGasse et al. 2002). The Islet Cell Antibody (ICA) assay has largely been replaced with autoantibodies to Glutamic acid decarboxylase (GADA) (Baekkeskov et al. 1990), tyrosine phosphatase like protein antigen (IA2A) (Lan et al. 1996) and more recently ZnT8A (Wenzlau et al. 2007).

1.33. ISLET CELL ANTIGEN (ICA)

Islet cell antibodies (ICA), recognizing islet cytoplasmic antigens, were first described in newly diagnosed T1D patients (Bottazzo et al. 1974) and comprise a number of autoantigens including GADA and IA2A. The presence of organ-specific pancreatic antibodies provided the first evidence for T1D as an autoimmune disease Bottazzo et al. 1974; MacCuish et al. 1974). Insulin autoantibodies (IAA) however are not recognized in the ICA test, because insulin and c-peptide leach out from the unfixed frozen tissue sections during sample preparation. The ICA assay however is difficult to standardize and highly dependent on the operators expertise including, the availability of sufficient antigen in the sections employed. Increasingly, studies use antigen specific autoantibody assays and that is what we have done.

1.34. GLUTAMIC ACID DECARBOXYLASE (GAD_{65})

The enzyme Glutamic acid decarboxylase, catalyzes, the conversion of L-glutamine into γ-amino butyric acid (GABA), the major inhibitory neurotransmitter in the brain. Key experiments carried out by Baekkeskov et al, (Baekkeskov et al.,1990) reported from detergent lysates of biosynthetically labelled human islet cells that the 64 kDa protein was GAD_{65} which is also expressed at lower concentrations in the pancreatic islets of Langerhans (Christie et al. 1992).

There are two isoforms of GAD, GAD_{65} and GAD_{67}, with molecular masses of 65kDa and 67kDa (Bu et al. 1992). Human pancreatic islet cells only express the lower molecular weight GAD65 isoform (Hagopian et al. 1993), an intracellular membrane anchored protein consisting of 585 amino acids and the GAD_{65} gene located on chromosome 10p11 (Bu et al. 1992). Autoantibodies in T1D predominantly recognize the 65 kDa isoform of GAD where as patients with Stiff Person syndrome (SPS) can react with both isoforms, the latter recognizing both linear as well as conformational epitopes and the former recognizing conformational epitopes. Enzymatic methods employed earlier recognized conformational epitopes and patients with SPS recognized both GAD_{65} and GAD_{67} from brain extracts. Most GADA methods measure the ability of sera to immunoprecipitate GAD. They have been applied as enzymatic assays, radiobinding assays (RBA), liquid immunoprecipition assays and enzymes-linked immunosorbent assays (ELISA). Historically RBA have provided stronger

specificity as well as sensitivity in the Diabetes Antibody Standardization Programme (DASP) than ELISA, latest ELISA participation in DASP have performed equally as well as RBA assays.

At diagnosis, approximately 50-80% of newly diagnosed type 1 diabetic patients can have GADA antibodies (Bonifacio et al.) and often persists in sera for many years after the diagnosis (Savola et al. 1998). GADA can also be detected in patients with SPS (Levy et al. 1999), where we found approximately 80% of the patients have GADA, with a broad GADA isotype profile (Lohmann et al)

1.35. TYROSINE PHOSPHATASE LIKE MOLECULES

One of the proteins comprising the islet cell antigens is a transmembrane tyrosine phosphatase like molecule insulinoma-associate antigen- 2 (IA2) comprising of 979 amino acids with a molecular mass of 105847 (Lan et al.,1994). The molecule consists of an intracellular, transmembrane amino acids 605-979 and an extracellular domain with a signal peptide, found in neuroendocrine cells as well as the pancreas. Trypsin treatment of tyrosine phosphatase like molecule IA2 yield a 37 and a 40kD fragment which are immunoreactive with sera from diabetic individuals (Christie et al., 1994), (Lu et al., 1996), (Payton et al.,1995). Autoantibodies to the islet cell antigens comprise of GAD_{65} as well as IA2 and can be detected many months before diagnosis of T1D and we and other have found are highly predictive of the disease (Atkinson and Maclaren., 1994), (Bonifacio et al.,1990), (Christie et al., 1992).

1.36. INSULIN AND INSULIN AUTOANTIBODIES (IAA)

Insulin is a major autoantigen central to glucose control in regulating energy as well as glucose metabolism. Insulin is secreted from the β cells in the pancreas which comprise 70% of the pancreatic islets. Insulin is a polypeptide comprising of 51 amino acids, with a molecular mass of 5808 Da, comprised of the A and B chains of 21 and 30 amino acids held together by disulphide bridges. Insulin is synthesized in the insulin secretory granules as pre-proinsulin and cleaved to release C-peptide and insulin. Insulin autoantibodies (IAA) were first described in untreated diabetic patients (Palmer et al., 1983). Insulin autoantibodies are frequently detected in children and are correlated inversely with age at diagnosis and are less prevalent in subjects diagnosed in adulthood (Zeigler et al., 1999). In another study of twins discordant for diabetes IAA were less frequent in the non-diabetic twins suggesting that IAA do not appear before development of TID and furthermore are not a consequence of the disease; using, an ELISA assay (Wilkin et al., 1985) The original IAA assays using polyethylene glycol (PEG) in conjunction with ^{125}I labeled insulin and ELISA assays have been vastly improved by the incorporation of the use of immunoprecipitation using Protein A Sepharose with ^{125}I labeled insulin in the microassay system (Williams et al., 1997). Despite this difficulties still remain in the standardization of the micro-assay. Furthermore IAA are, most frequently detected in childhood but less frequent in adulthood in whom diabetes is reported to be increasing at an alarming rate worldwide. As an autoantigen IAA are a valuable marker in children at risk of autoimmune diabetes but less so in adults. In this report we elected not to test

for IAA due to difficulty in assay reproducibility as well as the lower frequency of these antibodies in adult cases with autoimmune T1D.

1.37. ZINC TRANSPORTER ANTIGEN (Slc30A8)

ZnT8 is a multispanning transmembrane protein involved in the accumulation of zinc in the intracellular vesicles. It is expressed at high levels in the pancreatic islets (Chimienti et al.,2006). Zinc itself plays an important role in the pancreatic beta cells where its content is the highest incorporating two zinc molecules with six insulin molecules to form hexamers. Approximately 60% of newly diagnosed type 1 diabetic patients are found to be positive to ZnT8 while less than 2% of healthy controls tested positive for ZnT8 (Wenzlau et al., 2007).

Autoantibodies to beta cell antigens GADA, IA2A, IAA and ZnT8 are now extensively used in the prediction as well as a diagnostic tool. The risk of developing T1D is substantially increased in the first degree relative of T1D patients in comparison to the general population (risk general population 0.5%). The risk of developing TID can be stratified from <1% up to >70% by using various combination of risk markers (Zeigler & Nepom. 2010) including younger age, decreased first phase insulin response, presence of multiple autoantibodies, “the greater the combination the greater the risk” as originally described (Christie et al., 1992).

1.38 T CELLS

Resting T cell look very much like resting B cells, but when they respond the difference becomes apparent. T cells have no specificity towards particular antigens. There are several different types of T cells, which have a variety of actions. One group interacts with B cells and help them to divide, differentiate and make antibody. Another group interacts with mononuclear phagocytes and help them destroy intracellular pathogens. These two groups of cells are called T-helper (T_H1, T_H2) cells which carries CD4 marker and mainly helps or induces immune responses (Mosmann et al. 1996). A third group of T cells is responsible for the destruction of host cells which have become infected by viruses or other intracellular pathogens, this kind of action is called cytotoxicity and these T cells carry the CD8 marker and are predominantly cytotoxic cells. $CD4^+$ T cells recognise molecules in association with MHC class II molecules, whereas $CD8^+$ T cells recognise antigens in association with MHC class I. CD8 positive cells differentiate into cytotoxic T cells that kill the infected target cells, while the CD4 positive subset of T cells help to initiate the immune response. The latter can be further subdivided into T helper class 1 and class 2 cells (Th1 and Th2). The Th1 cells stimulate macrophages to stimulate the production of IgG1 antibody isotypes that are effective in opsonising pathogens by phagocytic cells. The Th2 cells on the other hand initiate an immune response by stimulating naïve B cells to produce antibodies and can also subsequently initiate the production of other antibody isotypes, including IgA and IgE including subtypes of IgG (Peter and Roitt., 2000).

Although T cells have similar genetic mechanism as B cells for the generation of T cell receptor (TCR) diversity, they differ in that they react only with antigen bound to class I or class II molecules of the MHC on antigen-presenting cells. The T cell receptor has similarities to the antibody molecule, but with important differences. The T cell receptor can be thought of as a hand reaching out to feel the surface of neighbouring cells, with some fingers devoted to contacting MHC molecules and others probing for peptide bound to them.

1.39 MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (MHC), a cluster of more than a dozen genes, whose products are expressed on a variety of cells, which act as "guidance systems" and enable T cells to recognise antigens on cell surface (Klein & Sato, 1998). The MHC molecules are part of the immunoglobulin "supergene" family and appear to have evolved from the same primordial gene as immunoglobulin and T cell receptor molecules. Human MHC, is also known as the human leukocyte antigen (HLA) system which is located on chromosome 6 and on chromosome 17 in mouse extending over the same region of the DNA molecule. The function of the MHC molecules is to bind to pathogen derived peptide fragments subsequently presented to T-cells leading to the destruction of the pathogen. The nomenclature reflects the way in which these molecules were characterised, i.e. as antigens that allowed alloantibodies to bind and destroy leukocytes. Thus, although they are known as antigens, they are only recognised as such when exposed to non-self immune system. Furthermore because these

antigens are expressed on human leucocytes, the alloantigens became known as human leucocyte antigens (HLA's).

1.391. MHC MOLECULES

Three classes of molecules have been identified as encoded within the human MHC namely Class 1,11 and 111 respectively. Class 1 proteins are the transplantation antigens and are present on every cell of the mammal and are responsible for rejection of foreign tissue. Class 1 proteins are defined serologically by their antigenic properties and in humans the classical transplantation antigens are HLA-A, B and C. Class 11 proteins are found on the surface of both B and T lymphocytes as well as macrophages. These proteins are involved in the cellular interaction necessary to execute an immune response.

The MHC is polygenic and there are several MHC class I and class II genes, encoding proteins with different ranges of peptides-binding specificity. The MHC genes are, in fact, the most polymorphic genes known. These properties of the MHC, polygeny and polymorphism, make it difficult for pathogens to evade immune responses (Piertney & Oliver, 2005).

At least three properties of MHC molecules are affected by MHC polymorphism: the range of peptides bound; the conformation of the bound peptide; and the interaction of the MHC molecule directly with the TCR. Thus the highly polymorphic nature of the MHC has functional consequences, and the evolutionary selection for this polymorphism suggests that it is critical to the role of the MHC molecules in the immune response.

The association of the MHC with T1D has been known for more than 20 years (Nerup J., 1974) (Thorsby & Undlein.,1996), (She., 1996). Experiments with the NOD mouse, in the BB rat model of TID and in humans have led to the current, widely accepted conclusion that it is the MHC HLA class 11 immune response genes themselves, are the major determinants of MHC-associated susceptibility to, and protection from, T1D. Lack of the charged amino acid at position 57 of the class II β chain is the principle genetic susceptibility component, conversely the presence of a charged amino acid is associated with protection from T1D (Wicker et al., 1995), (Morel et al,. 1988). It is also generally accepted that the association of MHC molecules with autoimmune disease is due to the fact that the polymorphic amino acids in their peptide binding sites govern the affinity of peptide binding and therefore the capacity of an individual to mount an immune response to an antigen, whether it be a foreign or self-antigen (Tisch & McDevitt, 1996). Although there are serious gaps in our understanding, we can conclude that a necessary, but not sufficient, step in the development of beta cell destruction is the recognition by T lymphocytes of one or more β cell proteins bound to the MHC class 11 molecules. The HLA region has the highest genetic association as discussed earlier under the threshold hypothesis, after FOXP3 and AIRE. The HLA accounts for the major genetic risk component in children and young adults.

A number of non MHC loci also contribute to the disease risk for TID but have a lesser impact and include PTPN22, (variations in this gene leads to increased autoimmune attack), the polymorphic cytotoxic CTLA4 gene on chromosome 2q33 (Nistico et al., 1996) (a member of the immunoglobulin superfamily

expressed on T helper cells and mutations in this gene have been associated with autoimmune disease including T1D, Graves' disease, Hashimoto's thyroiditis, celiac disease, systemic lupus erythematosus). The HLA class 11 genes on chromosome 6p21 accounts for 30%-50% of TID genetic risk (Noble et al., 1996). Other non HLA TID loci with a relatively smaller effect include the insulin gene (INS) on chromosome 11p15 (Bell et al., 1984), the protein tyrosine phosphatase PTPN22 gene on chromosome Ip13 (Bottini et al.,2004), the interleukin 2 receptor, alpha (IL2RA) and interferon induces helicase C domain 1 (IFIH1) genes as well as other genes discovered with genome-wide association studies (GWAS).

One study has shown that the high risk genotype DQ2/DQ8 was infrequent in LADA compared to juvenile onset T1D (Tuomi et al., 1999), thus the genetic load in LADA is less than that seen in juvenile onset TID but similar to that seen in adult onset diabetes (Tuomi et al., 1999). The genetic impact and load in childhood and adults can show differences for example the INS gene associated with TID is not associated with T2D, whereas other genes (IDE/HHEX, SLC30A8, CDKAL1, CDKN2A/B, IGF2BP2, FTO, and TCF7L2 associated with type 2 diabetes are not associated with T1D. Furthermore TCF7L2 associated with type 2 diabetes is also associated with LADA. So it can be implied that the latter are a distinct group of patients with a decreased genetic load as well as autoimmune features similar to T1D with presence of autoantibodies.

1.40. PATHOGENESIS AND NATURAL HISTORY OF AUTOIMMUNE DIABETES

T1D is believed to be a T-cell mediated autoimmune disease in which insulin producing β cells of the pancreatic islets of the Langerhans are destroyed. The most widely used animal model of autoimmune diabetes, the non-obese diabetic mouse (NOD) (Delowitch & Singh 1997), is one of the rare spontaneous models of the disease that allows us to study the kinetics of the events during prediabetes, particularly in the pancreas. In spite of extensive investigation, the etiology of the autoimmune attack in NOD mice and human patients with TID is still unknown. Many distinction between the two have led to dissatisfaction with the NOD mouse and it will not be discussed further here (Atkinson & Leiter 1999).

1.41. AUTOIMMUNE DIABETES AND GENDER

The susceptibility to autoimmune disease is a characteristic feature and it has been estimated that up to 6.7.million women in the United States are affected with autoimmune disease compared to 1.8 million men. Autoimmune diseases including hyperthyroidism, rheumatoid arthritis, thyroiditis, T1D, pernicious anaemia and multiple sclerosis account for 93% of the total (Jacobson et al., 1997). The overall sex ratio for T1D is reported to be equal in children diagnosed under the age of 15 with an absence of a male sex bias (Dorman et al., 1994), (Beeson 1994)(Table1.4). However, the evidence in patients with T1D diagnosed at a later age is less clear because there are few large studies of these patients (Gale & Gillespie., 2001). Of the few studies of middle-aged patients with LADA the gender ratio is generally not biased , that is the percentage of women in China

LADA (38%)(unpublished data personal communication RD Leslie), CARDS (38% **)** (unpublished data), NIRAD (38%)(Buzzetti et al., 2007) and 4-12% (Zinman et al., 2004). The relevance of these gender biases is that autoimmune diseases are seen to have a female bias and tend to present in middle-age. A glance at the Table 1.5 confirms that female bias and age effect, with the exception of T1D which is predominantly childhood-onset with minimal male excess. An important theme of this thesis is then to what extent T1D is an exception to the general gender and age.

Condition	Peak onset (years)	% Females
T1D	2-15	45%
Multiple Sclerosis	20-35	60%
Rheumatoid Arthritis	20-40	65%
Hashimotos Thyroiditis	30-50	85%
Thyrotoxicosis	30-50	85%
Pernicious anaemia	40-80	60%

Table 1.5 Gender and Autoimmune disease

TID shows a similar prevalence up to the age of 15 with a male preponderance thereafter, unlike other autoimmune diseases where a female preponderance has been reported Data from Beeson. (Beeson 1994)

A number of factors have been implicated in the cause of TID, however, conclusive evidence for an environmental agent in the cause of TID is still lacking. Since only a few genomic susceptibility regions have been found, no environmental agents conclusively identified, and the high rate of disease discordance in MZ twins persists, the penetrance of T1D may, in part, be influenced by epigenetic factors modifying DNA such that genes are either switched on or off such that a particular phenotype is either expressed or repressed respectively.

1.42. EPIGENETICS

Epigenetic modifications, namely DNA methylation and histone modifications, influence gene expression by altering the chromatin state and are indispensable for maintaining cellular homeostasis (Bird 2002). The features of DNA methylation in mammals are that it occurs on cytosine bases that are immediately followed by a guanine (commonly referred to as CpG sites). Examples of epigenetic phenomena in mammals include X-inactivation (the transcriptional silencing of most of the genes on one X-chromosome in female mammals) and parental imprinting (differential expression of an allele depending on whether it was maternally or paternally inherited). Not surprisingly, alterations of the epigenetic profile of the genome are associated with various human diseases, such as cancer.

1.41. EPIGENETICS AND VARIABLE EXPRESSIVITY

Relevant to the hypothesis that epigenetic factors play a role in T1DM aetiology are studies in mice that have shown that the establishment of epigenetic states at some endogenous loci during early development can be stochastic, resulting in incomplete penetrance or variable expressivity of a phenotype among genetically identical mice reared in controlled environments (Morgan et al., 1999), (Rakyan et al., 2003). An example, in mice carrying the *agouti viable yellow* (A^{vy}) allele, methylation upstream of this allele prevents ectopic expression of the gene, resulting in a normal colour coat and normal metabolic phenotype. However when the allele is un-methylated, the resulting phenotype is yellow in colour coat and development of type 2 diabetes. Crucially, this variability of phenotype resulting from an epigenetic change occurs in the absence of genetic variability or any identifiable environmental factors.

Epigenetic variability or epimutations correlating with disease phenotypes, has been observed in humans, e.g. in a recent study of MZ twins discordant for Beckwith-Weidemann Syndrome, only the affected twin had lost epigenetic imprinting within a critical area on chromosome 11 (Weksberg et al., 2002). Considering the high degree of discordance for T1D between MZ twins, it is worth considering the possibility that epimutations influence the susceptibility to T1D. In support of an epigenetic component in the aetiology of T1D, parent-of-origin effects (consistent with parental imprinting) have been associated with T1D. It has been noted that there is an increased risk of T1D in offspring when

the parent with T1D is a father compared to a mother, (Warram et al., 1984), (Bleich et al., 1993), (Bennet et al., 1997) reported that a particular insulin gene allele (that was previously reported to predispose to T1D) does not predispose to disease when paternally inherited, and this effect is influenced by the identity of the father's untransmitted allele.

Interestingly, procainamide and hydralazine, used to treat cardiac arrhythmias and hypertension respectively, are both DNA methylation inhibitors that can induce a lupus-like disease in humans and T-cell autoreactivity leading to a lupus-like autoimmune disease in animal models (Richardson et al., 2004).

There is strong evidence that DNA methylation profiles change with age (Issa 2003). It is therefore possible that accrual of epigenetic variants during ageing in MZ twins, along with environmental insults may precipitate T1D in one twin but not the other, and such differences could account for the relatively greater impact of non-genetically determined factors causing adult-onset T1D as compared with childhood-onset T1D (Salvetti et al.,2000), (Kumar et al., 1993), (Vyse & Todd 1996). Indeed, it has very recently been demonstrated that epigenetic differences can arise during the lifetime of MZ twins (Fraga et al., 2005).

A recent study has shown that T1D–Methylation Variable Position's (MVPs) represent disease associated epigenetic variation that antedates clinical disease. This study noted several MVP–TID associated genes including the HLA class 11 gene, *HLA DQB1* which poses the highest risk for TID, RFXAP, an HLA class II regulating element, NFKB1A, a regulator of apoptosis and inflammatory immune

responses, TNF, a key inflammatory cytokine associated with T1D in animal models, and GAD2 which encodes GAD65, a major T1D autoantigen involved in disease etiology (Vardhman et al., 2011).

These findings including genetic, epigenetic as well as environmental factors alone cannot fully account for the differences in the concordance rates between twins discordant for TID however the interaction of these various factors may have an impact on the disease penetrance.

1.44. Clinical Spectrum of Autoimmune diabetes

The World Health Organization recognizes three main forms of diabetes mellitus: type 1, type 2, and gestational diabetes (WHO 1999) , which have, similar signs, symptoms, and consequences, but different causes and population distributions. The prevalence of affected adults with diabetes according to the WHO has increased from 1994-1995 in 100-135 million to approximately 246 million in 2007, with more than 95% of the cases considered to be type 2 diabetes.

Observations of prevalence (T1D and type 2 diabetes) from Germany has shown, a ten fold increase from 0.6% in 1960 to 6.9% in 2005 (Kolb & Poulsen 2010). This rapid increase cannot be attributed to genetic causes but rather to lifestyle as well as environmental factors including excessive calorie intake, sedentary lifestyle all promoting to the increased inflammatory state as well as obesity. Glucose intolerance of ageing is another additive factor in the increased prevalence

Ultimately, all forms are due to the beta cells of the pancreas being unable to produce sufficient insulin to prevent hyperglycemia. T1D is characterized by genetically determined predisposition, presence of autoimmune markers, aggressive beta-cell destruction, severe insulin deficiency and an immediate need for insulin replacement therapy. The destruction of beta cells leads to insulin deficiency and secondarily to abnormalities in carbohydrate, fat and protein metabolism. Polyuria, polydipsia and weight loss are classical symptoms of insulin deficiency, which if left untreated leads to life-threatening keto-acidosis wasting away and mortality. Patients are dependent on exogenous insulin for the rest of their lives, and even with good care they carry an increased risk of long-term complications such as retinopathy, nephropathy, neuropathy and cardiovascular diseases.

1.45. IMMUNOLOGICAL SPECTRUM OF AUTOIMMUNE DIABETES

T1D can occur at any age and is most often seen in children, adolescents and young adults, characterized by T-cell mediated pancreatic islet cell destruction and loss of beta cell function. On the other hand classic type 2 diabetes is characterized with insufficient insulin secretion and peripheral insulin resistance without showing autoimmune features (Alberti & Zimmet 1998), (Naik & Palmer 1997). Despite these characteristic criteria the distinction between type 1 and type 2 diabetes is not always clear. The clinical evidence identifies a significant overlap between type 1 and type 2 diabetes. In the latter group a further distinction is made in adults presenting with T1D with immediate insulin

requirement soon after diagnosis and Latent Autoimmune Diabetes of Adult onset remaining free of insulin for at least six months post diagnosis.

The discovery of Islet Cell antibodies (ICA) in 1974 in the sera of T1D provided the initial evidence that T1D was autoimmune in nature (Bottazzo et al., 1974). Clinical onset of T1D is usually preceded with the appearance of insulin autoantibodies in particular in the very young. Islet cell antibodies are routinely detected by indirect immunofluorescence technique using group O cryofixed human pancreas although a sensitive assay, it is prone to variability due to the antigenic diversity of the pancreatic substrate as well as the operator experience. The specific antigens that comprise ICA include GAD_{65}, IA2, ZnT8 as well as other antigens, up to 80% of newly diagnosed type 1 diabetics show presence of ICA (Irvine et al., 1977) a highly sensitive assay with one caveat difficulty in standardization of the ICA assay.

Subsequently the second key diabetes auto-antigen Glutamic Acid Decarboxlylase GAD_{65} was identified, followed by IA2 and Zinc Transporter antigen (ZnT8), each adding to the positive predictive value of these auto-antibodies. The ICA assay has now largely been replaced with assays for the auto-antigens GAD_{65}, IA2 and more recently ZnT8 (Baekkeskov et al., 1990), (Christie et al., 1992), (Payton et al., 1995), Wenzlau et al., 2007).

The presence of T cell reactivity as well as autoantibodies provides evidence for underlying autoimmune process in T1D as well as LADA. It has been demonstrated previously that about 11% of subjects with type 2 diabetes were

also positive for ICA's (Irvine et al., 1977). Since ICA are comprised of a number of antigens it has become evident that testing of autoantibodies to GADA and IA2 clearly identifies the majority of ICA positive individuals and therefore by definition the presence of autoantibodies distinguishes patients with classic autoimmune T1D from non-autoimmune diabetes as well as LADA (Fourlanos et al., 2005).

This thesis will focus on immunological features in T1D of adult onset, its prevalence and spectrum of severity which does not overlap with type 2 diabetes as well as provide evidence that TID is non-genetically determined in identical twins.

1.46. SPECIFIC AIMS

1. Whether autoantibodies and Carboxy Methyl lysine (CML) are genetically determined and independently predictive of T1D.

2. Whether autoantibodies, insulin secretion and insulin resistance are predictive and are genetically determined.

3. Whether autoantibody isotype pattern in TID and LADA are similar

4. Whether autoantibodies in adults presenting with type 2 diabetes are prevalent and similar to T1D and whether Metabolic Syndrome is associated with LADA and type 2 diabetes, but not T1D.

5. Whether autoantibodies to CD38 are of any value as biomarkers in T1D, as well as 'type 2 diabetes'.

CHAPTER 2

2.0. WHETHER AUTOANTIBODIES AND CARBOXY METHYL LYSINE (CML) ARE GENETICALLY DETERMINED AND INDEPENDENTLY PREDICTIVE OF T1D.

2.1. Aim: Since both autoantibodies and increased CML levels are associated with T1D, we tested whether both are genetically or environmentally determined and whether they ante date the disease.

2.2. Introduction: Overt clinical diabetes manifests in a genetically susceptible individual only after 90% of the pancreatic beta cells have been destroyed leading to impaired glucose tolerance followed by frank diabetes. Autoantibodies to GADA, IA2A, ZnT8 and IAA all comprise the Islet cell antigens and can be detected many months before the diagnosis of TID, a consequence of which results in hyperglycaemia. Prolonged hyperglycaemia leads on to complications of diabetes associated with the formation of Advanced Glycation End products (AGE's).

Glycosylation is an enzyme controlled reaction that connects a specific sugar to a specific protein or lipid at a specific location which is required for normal physiological function of biomolecules. Glycation on the other hand can occur as a result of intake from the diet or endogenously via non enzymatic glycation and oxidation of lipids and proteins. Advanced Glycation End products are a group of heterogenous compounds formed from the non-enzymatic glycation of proteins lipid as well as nucleic acids and in the process form highly reactive intermediates (Brownlee 1996),(Thomalley 1996). The two most commonly measured AGEs

are, Nε-(carboxymethyl) lysine (CML) (a glycoxidation and lipoxydation product) and carboxyethyllysine (CEL) (derived from methyl glyoxal formed by protein glycoxidation or enzymatic glycolysis with lysine residues), which are glycoxidation products, formed from glucose by sequential glycation and autoxidation reactions. Glycation itself is a random process resulting from the addition of sugar molecules to proteins and lipids without any enzymatic control thereby accumulating end products, affecting normal physiological function and activity. Carboxymethyl Lysine is formed by a process called the Maillard reaction which entails the addition of reduced sugars to amino acids or lipids to form glycated products called Amadori products.

AGEs interact with receptors called RAGE a member of the immunoglobulin superfamily found on endothelial cells, smooth muscle cells, immune cells macrophages, monocytes as well as dendritic cells. Once formed in sufficient quantity the binding of AGEs to RAGE receptors leads to a cascade of reactions with the formation of highly reactive oxygen species as well as further formation of advanced oxidative protein products, oxidative lipid as well as AGE's leading to a vicious cycle of activation, leading to further activation of the RAGE receptors. Binding of AGE's to RAGE receptors initiates signal pathways that amplify inflammation as well as oxidative stress as a result of the formation of reactive oxygen species thereby leading to cellular and tissue damage and death (Kislinger et al., 1999).

It is well known that the levels of AGEs CML and CEL increase as a result of normal physiological ageing as well as pathophysiological changes such as

diabetes, Rheumatoid Arthritis, Alzheimers and Multiple Sclerosis (Sternberg et al.,2010). Altered monocyte/macrophage as well as proinflammatory responses have been documented in T1D including increased formation of AGEs which mediate inflammation through AGE receptors (RAGE) (Beyan et al., 2006),(Mericq et al 2010). The levels of CML in the serum is determined by the endogenously produced CML which has been shown by our group in a classic twin study to be strongly inherited (Leslie et al., 2003), however tissue and serum levels of CML are also affected by the exogenous ingestion and absorption of dietary sources which have undergone AGEs formation via excessive temperatures in cooking process in the dietary intake of resources. Glycotoxins are formed via cooking foods quickly at high temperatures, such as browning of foods and caramelising of sugars. In the food industry such techniques are regularly employed such as the coating of French fries with sugar to enhance the appearance of browning when cooked at high temperatures. The exposure to such AGEs is increasing at an alarming rate in the developed nations and is of concern to the health of the future generations.

2.31. Patient cohorts: We initially tested a population based cohort of samples from Ulm, Germany to test if autoantibodies and CML could predict those at risk. We then went on to test twins discordant for diabetes both MZ as well as DZ twin pairs and healthy twins, to determine the impact of genetic and non-genetic factors on them. School children of European origin (n=7,287) ascertained between 1989-2008 in Germany were also included and of these, 115 (51.3% female) were ICA positive at ascertainment, The latter group together with a

random selection of 2217 subjects were further tested at baseline for GADA, IA2 and ZnT8 antibodies as well as CML. The school children had multiple samples taken and we included the initial and last sample pre-diagnosis of diabetes from these 115 cases in the analysis. Population study samples characteristics are shown in Table 2.1. All subjects participating in the study gave informed consent and the study was approved by Ethical Committee, Ulm.

Variables	**T1D (n=33)**	**No T1D (n=82)**	**p-value**
	Mean (SD)	**Mean (SD)**	
Age at inclusion (SD), in years	13.48 (4.07)	14.24 (4.01)	n.s.
Follow-up [interquartile range], in months	77 [58, 115]	94 [67, 130]	n.s.
Male, n (%)	18 (54.5%)	38 (46.3%)	n.s.

Table 2.1 Characteristics of Population Study samples from 115 ICA positive children tested for CML Means (SD) are given.

2.32. Twin study:

We also tested a cohort of MZ and DZ twins for serum CML and diabetes-associated autoantibodies (GADA, IA-2A and ZnT8A) to determine if they were genetically determined. The Twins cohort sited at St Bartholomew's and The London Hospital at the Blizzard Institute comprises of a unique collection of 367 identical twins pairs which comprise the British Diabetic Twin Study. Of 369 individuals in which the index twin has diabetes, 69 pairs are discordant for T1D and 237 pairs are concordant for diabetes. Of the concordant twins 103 pairs were concordant for type 2 diabetes and 134 pairs were concordant for T1D. Of the remainder 23 pairs have an index twin with Type 2 diabetes and in 41 pairs the type of diabetes is uncertain. We also have 81 pairs of non-identical twins all of whom to date are discordant for Type 1 DM and 8 pairs of non-identical twins discordant for Type 2 diabetes.

2.33. Twin subjects:

Twins from United Kingdom (Caucasian) were ascertained, between 1967 to 2010, with, either T1D or type 2 diabetes were studied, and their type of diabetes was determined by standard guidelines (Expert Committee on Diabetes 1997), cases in which this definition was uncertain were excluded. Monozygosity was established in each twin pair as previously described, (Olmos et al., 1988). Classification of T1D was based on *1*) control of diabetes with insulin from time of diagnosis, *2*) diabetic ketoacidosis or marked ketonuria at time of clinical onset. Twins were followed prospectively until they either developed diabetes or remained free of the disease. The twins included in

the study met the following criteria: 1) European origin, 2) twin pairs initially disease discordant, 3) both twins available for study, 4) neither twin receiving drugs other than human insulin, 5) all had normal plasma creatinine, 6) diabetes initially excluded in the co-twin by oral glucose tolerance test and random whole blood glucose < 7.0 mmol/l. As controls for serum CML, we tested 168 non-diabetic twins (39 MZ, 45 DZ pairs, mean age 51.3 (SD=14.1), range 21-73).

From our collection of twin pairs we identified 32 DZ and 32 MZ pairs discordant for T1D of similar age at diagnosis and disease duration at sampling (Table 2.2). All subjects gave informed consent and East London Health Authority Research Ethics Committee approved the study (Ref 07/Q0604/10).

Twin Cohort (MZ=monozygotic DZ= dizygotic)	**Diabetic Twin**	**Non Diabetic Twin**
MZ Number of Twins	32	32
Male, n (%)	20 (62.5%)	20 (62.5%)
Age at test (SD) yrs	28.09 (16.41)	28.09 (16.41)
Duration of follow up yrs	26.9 (11)	26.9 (11)
Age at diagnosis (SD) yrs	17.1 (12.3)	n.a
Diabetes duration (SD) yrs	11.0 (9.2)	n.a
DZ Twins		
Number of Twins	32	32
Male, n (%)	13 (40.6%)	12 (37.5%)
Age at test (SD) yrs	28.00 (18.16)	28.00 (18.16)
Duration of follow up yrs	19.9 (8.3)	19.8 (8.3)
Age at diagnosis (SD) yrs	17.53 (14.45)	n.a.
Diabetes duration (SD) yrs	10.47 (8.65)	n.a.

Table 2.2: Characteristics of Twins tested for CML

2.4. Assays

2.4. Carboxymethyl-Lysine [CML] assay

AGE-CML was determined in batched assays from population based samples as well as twins samples in a single laboratory (Ulm, Germany) using a competition-based ELISA assay using Carboxymethyl-Lysine specific monoclonal antibody (mouse monoclonal 4G9 (Alteon Inc., New York, NY, USA) as previously described (Wagner et al., 2001). CML levels are expressed in ng/ml serum. Assay sensitivity was 5 ng CML/ml with intra-assay and inter-assay variability less than 4% and 5% respectively.

2.41. Diabetes-associated autoantibody assays: The radioimmunoprecipitation assays for the enzyme Glutamic acid Decarboxylase (GAD_{65}) and IA-2ic (aminoacids 603-979), the putative intracellular fragment and GAD_{65}, all employ in vitro transcription and translation systems (Promega Madison, Wisconsin) Human IA-2ic cDNA in the vector pGEM-4Z a gift from Dr Michael Cristie was in vitro transcribed and translated. Human islet GAD_{65} cDNA in the vector pB 1882 (gift of Dr Thomas Dyrberg, Novo Nordisk, Denmark) was used according to the manufacturers instructions (Promega Madison, WI). For all antibody assays between 0.8-1.0 µg DNA was transcribed and translated with SP6 (IA-2ic) and T7 (for GAD_{65}) RNA polymerase enzymes in a TNT coupled rabbit reticulocyte lysate system (Promega,Madison,WI) in the presence of ^{35}S methionine (0.8 mci/ml)(Amersham UK). Incorporated radioactivity was determined by

precipitation with 10% trichloroacetic acid and scintillation counting. For the immunoprecipitation in each assay 50 µl aliquots of ^{35}S methionine (50,000-75,000 cpm) labelled antigen were incubated overnight with 2µl serum (final dilution 1:25) in tris buffered saline tween. The immunocomplexes were isolated by adding 1 mg protein A-Sepharose, and counted on a multiwell Wallac counter.

The radioimmunoprecipitation assays for the Zinc Transporter antigen ZnT8 assays employ in vitro transcription and translation systems (Promega Madison, WI). ZnT8 cDNA in the vector pCDNA3.1 a gift from Dr John Hutton was in vitro transcribed and translated as described (Wenzlau et al., 2007).

2.42. Transformation of Competent Bacterial cells for GAD_{65}, IA2ic and ZnT8

2.43. Materials:

2.431. Competent cells JM109 Cat No L2001 (Promega) made competent by the method of Hanahan were purchased ready to use for the transformation and insertion of the vector DNA of interest.. Aliquots of the competent cells were stored at -70°C until required.

2.432 Luria Broth Base for the cultivation and growth of bacteria (LB): Dissolve 15.5 g/L in distilled deionised water and autoclave for 15 minutes at 121°C. Allow to cool before use with the addition of the appropriate gene resistant antibiotic namely Streptomycin, Ampicillin, Tetracycline, Kanamycin and or Chloramphenicol as required.

2.433. Luria Base Agar: LB Media is a generic rich media suitable for growing many aero tolerant species of bacteria, including E. coli, Bacillus subtilius, Staphylococcus auerus or Staphylococcus epidermidis and different yeasts including Saccharomyces and Candida species. Many kinds of molds will also grow (Aspergillus or Penicillium, for instance). However, the presence of these organisms on your plate generally represents contamination, and the plate should be discarded. LB agar will not necessarily support the growth of more fastidious organisms such as Streptococci, and pH media and conditions are less than ideal for culturing species of Lactobacilli from yogurt.

2.434. Preparation of Luria Broth Agar (LBA)

a. Suspend 35.6 g of Agar (Sigma Aldrich) in 1 L of distilled water in a sterile autoclave bottle. Components of LBA are shown in the table 2.3.

b. Autoclave for 15 minutes at 121 °C.

c. Allow the sterile agar to cool in a water bath at set at 55°C or until the flask is hand cool to hold. Add 5ml Ampicillin to the cooled agar from the stock of 10mg/ml, to give a final concentration of 50µg/ml.

Tryptone (pancreatic digest of casein)	**10 g/L**
Yeast extract	**5 g/L**
NaCl	**5 g/L**
Agar	**15 g/L**

Table 2.3 Components of LB Agar

2.435. Plasmid DNA for the transformation. A plasmid is a small circular piece of DNA (about 2,000 to 10,000 base pairs) that contains important genetic information for the growth of bacteria. In nature, this information is often a gene that encodes a protein that will make the bacteria resistant to an antibiotic. The GAD_{65} cDNA was in the vector PB 1882 and IA2ic in the vector PGEM4Z. Both GAD_{65} vector PB1882 and PGEM-4Z for IA2ic have the Ampicillin resistant gene in the plasmid construct.

2.436. Plastics: Sterile 15 ml conical bottom Falcon tubes (Marathon Lab Supplies), Eppendorf tubes (Elkay) and sterile Petri dishes (Marathon Lab Supplies**).** Conical bottom 1.5ml screw cap tubes (Sarstedt UK) Sterile pipette tips (Sarstedt UK), 100ml and 50ml Sorval centrifuge tubes (Sorval UK)

2.437. Transformation Protocol

a. Remove one vial of competent cells JM109 (Promega).Catalogue No:L2001, and thaw on ice and pipette 100µl into a sterile Eppendorf tube.

b. Add 10µl of the cDNA either for GAD_{65} or IA2ic as required. Set one tube as a control with no DNA and one tube with control DNA (pGEM-3Z) at a concentration of 0.1ng/µl. Mix gently and leave on ice for 10 minutes mixing gently every 2 minutes.

c. Place the tubes in a water bath for 45-50 seconds at 42°C to heat shock the competent cells to allow the insertion of the test DNA to be inserted into the competent cells.

d. Return the tubes on ice for a further two minutes.

e. Add 900µl of ice cold LB Ampicillin medium to each tube and incubate on a shaking incubator (Grants shaker incubator) at 37°C for 1 hour.

f. From the transformed reaction mixture 100µl of a 1:10, 1:100 and neat undiluted aliquot is inoculated onto LA Ampicillin plates and spread with a spreader after sterilisation in an open flame. The plates are incubated in a 37°C incubator overnight.

g. Colonies growing on the plates indicate the insertion of the gene as only those plates containing the insert grow in the Ampicillin agar plates, control plates should have no colonies growing. Single colonies were picked out and grown in a shaking incubator at 37°C for 4 hours in 10ml of LB Ampicillin medium. The culture was centrifuged and the pellet of cells reconstituted in 1ml of LB Ampicillin media before inoculating the content into an Erlenmeyer Flask containing 500ml LB Ampicillin medium and incubated at 37°C overnight.

h. After the overnight culture the contents were placed into 100ml flasks and centrifuged in a Sorvall RC5B centrifuge at 5000 x g for 10 minutes at 22°C.

Discard the supernatant and reconstitute the pellet in the cell re-suspension solution for the extraction of DNA.

2.438. DNA Extraction Reagents

Plasmid DNA was extracted using the Plasmid Maxiprep System (Promega Catalogue No:A7270) according to the manufacturers instruction.

Materials supplied with the system contains sufficient reagents and columns for 10 isolations from 100–500ml of bacterial culture and includes:

• 150ml Cell Resuspension Solution

• 150ml Cell Lysis Solution

• 300ml Neutralization Solution

• 100ml Wizard® Maxipreps DNA Purification Resin

• 125ml Column Wash Solution

• 10 Maxi/Megacolumns with Reservoirs

10 5ml Syringes

• 10 0.2µm Syringe Filters

2.439. Laboratory Equipment

Sorval RC5B centrifuge capable of 1,300–14,000 × g

New Brunswick shaker incubator

isopropanol (at 22–25°C)

TE buffer

Filter paper (Whatman® #1,GFA or GFC)

2.440. Preparation of column Wash Solution

Before beginning, the Column Wash Solution (provided) was diluted by adding 170ml of 95% ethanol to a final volume of 295ml.

2.441. DNA Extraction Protocol

1. Pellet 100–500ml of cells by centrifugation at 5,000 × g for 10 minutes in a room temperature rotor in a 100ml centrifuge flask. Pour off the supernatant and re-suspend the pellet in 15ml of Cell Re-suspension Solution. (To aid re-suspension, manually disrupt the pellet with a 12-inch applicator stick or by pipetting until no clumps are visible. Complete re-suspension is critical for optimal yields.)

2. Add 15ml of Cell Lysis Solution and mix gently but thoroughly by stirring or inverting. Do not vortex. Cell lysis is complete when the solution becomes clear and viscous (up to 20 minutes). Add 15ml of Neutralization Solution and immediately mix by gently inverting the centrifuge bottle several times. Centrifuge at 14,000 × g for 15 minutes at 22–25°C in a room temperature rotor.

3. Transfer the cleared supernatant by filtering it through Miracloth™ (Calbiochem Corp. Cat.# 475855), filter paper (Whatman® #1, GFA or GFC) into a sterile 50ml falcon tube.

Measure the supernatant volume, then transfer to a centrifuge bottle.

4. Add 0.5 volume of room temperature isopropanol and mix by inversion.

5. Centrifuge at 14,000 × g for 15 minutes at 22–25°C in a room temperature rotor.

6. Discard the supernatant and resuspend the DNA pellet in 2ml of TE buffer. Thoroughly wash the walls of the bottle with TE buffer to recover all of the DNA. At this point, the pellet may not be visible.

2.442. Plasmid Purification

1. Add 10ml of Wizard® Maxipreps DNA Purification Resin to the DNA solution from Step 6 above, Swirl to mix.

Thoroughly mix the Wizard® Maxipreps DNA Purification Resin before removing an aliquot.

2. For each Maxiprep, use one Maxicolumn. Insert the Maxicolumn tip into the vacuum manifold port.

3. Transfer the resin/DNA mix into the Maxicolumn. Apply a vacuum pull the resin/DNA mix into the Maxicolumn.

4. Add 25ml of Column Wash Solution to the Maxicolumn and apply a vacuum to draw the solution through the Maxicolumn.

5. To rinse the resin, add 5ml of 80% ethanol to the Maxicolumn and apply a vacuum to draw the ethanol through the Maxicolumn. Allow the vacuum to draw for an additional 1 minute.

6. Place the Maxicolumn in a 50ml screw cap tube (provided by the user).

Using a centrifuge with a swinging bucket rotor (e.g., Beckman JS-4.3 rotor), centrifuge the Maxicolumn at 2,500rpm (1,300 × g) for 5 minutes.

It is essential that a swinging bucket rotor be used for this step.

Remove the Maxicolumn and discard both the tube and the liquid. Place

the Maxicolumn back on the vacuum manifold.

7. Dry the resin by applying a vacuum for 5 minutes. Remove the Maxicolumn from the vacuum manifold. Place the Maxicolumn in the provided Reservoir (50ml screw cap tube).

8. Add 1.5ml of preheated (65–70°C) nuclease-free water to the Maxicolumn and wait 1 minute. Elute the DNA by centrifuging the Maxicolumn/ Reservoir at 2,500rpm (1,300 × g) for 5 minutes in a centrifuge with a swinging bucket rotor or at 1,300 × g for 5 minutes in a centrifuge with a fixed-angle rotor.

9. A white pellet of resin fines may be present in the final eluate. Whether visible or not, it is important to separate the fines from the DNA. Remove the plunger from one of the 5ml Syringes and set it aside.

10. Attach the syringe barrel to the Luer-Lok® extension of a 0.2µm Syringe Filter and pipet the eluate into the Syringe Barrel.

11. Center the Filter over a 15ml plastic tube. Carefully insert the plunger into the Syringe Barrel and gently push the liquid into the tube.

12. Transfer the eluate to a 1.5ml centrifuge tube. Centrifuge the tube at 14,000 × g for 1 minute. This additional step will remove all resin fines that may be present in the final eluate.

13. Immediately transfer the supernatant to a new micro centrifuge tube. Follow these storage recommendations: DNA is stable in water without addition of buffer if stored at –20°C or below. DNA is stable at 4°C in TE buffer. To store the DNA in TE buffer, add 150µl of 10X TE buffer to the

1.5ml of eluted DNA.

DNA for the autoantigens GAD_{65} and IA2ic as well as ZnT8 were prepared as above and stored at -20°C in 10µl aliquots for use in the in-vitro transcription and translations.

2.443. Determination of the concentration of recovered DNA.

The concentration of the DNA recovered was determined on a Beckman DU 600 analyser and the Optical Densities (OD) determined at 260nM and 280nM. A typical recovery for GAD_{65} and IA2ic are shown below.

2.444. GAD_{65} DNA Concentration

Optical Density 260nM =0.2118

Optical Density 280nM =0.1342

An OD of 1 = 50µg/ml

Therefore 0.2118 x 50 x 1000µL =1059µg/L

This is equal to 1.059µg/µL**.**

2.445. IA2ic DNA Concentration

Optical Density 260nM =0.1934

Optical Density 280nM =0.1263

An OD of 1 = 50µg/ml

Therefore 0.1934 x 50 x 1000µL =0.967µg/L

This is equal to 1.0µg/µL.

2.446. In Vitro Coupled Transcription and Translation of Recombinant Proteins

The TNT® Coupled Reticulocyte Lysate Systems is an in vitro single-tube, coupled transcription/translation system. The TNT® Lysate Systems greatly simplify the process and reduce the time required to obtain in vitro translation results from transcribed RNA synthesized in vitro from SP6, T3 or T7 RNA polymerase promoters. In most cases, the TNT® Lysate reactions produce significantly more protein (two- to six fold) in a 1.5-hour reaction than standard in vitro rabbit reticulocyte lysate translations using RNA templates.

2.447. Components supplied with the TNT® SP6/T7 Coupled Reticulocyte Lysate System.

• 200µl TNT® Rabbit Reticulocyte Lysate

• 20µl TNT® Reaction Buffer

• 20µl TNT® T7 or SP6 RNA Polymerase

• 5µg Luciferase T7 or SP6 Control DNA, 0.5mg/ml(c)

• 25µl Amino Acid Mixture, Minus Methionine, 1mM

2.448. Component not supplied:

Plasmid DNA for GAD_{65},IA2ic or CD38

Screw-cap conical bottom 1.5ml tubes

Water bath at 30°C

Micro-centrifuge (Haeraeus)

35S Methionine (Perkin Elmer) (500µCi stock solution)

RNAsin inhibitor (Promega)

All the components were stored at -70°C and multiple freeze thaw of the reticulocyte was avoided throughout.

TNT® Rabbit Reticulocyte Lysate	25µl
TNT® Reaction Buffer	2µl
TNT® RNA Polymerase (SP6, T3 or T7)	1µl
Amino Acid Mixture, Minus Methionine, 1mM	1µl
*[35S]methionine (>1,000Ci/mmol at 10mCi/ml)	4µl
*Take care when handling Radioactive material	
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
DNA template(s) (1.0µg/µl)	1µl
Nuclease-Free Water to a final volume of 50µl	15µl

Table 2.4 Protocol for the Transcription/Translation of Protein

For the in-vitro transcription/translation of the protein pipette into a 1.5ml screw cap sarstedt tube components as listed in table 2.4. Vortex for 10 seconds and spin in a micro-centrifuge for 10 seconds to allow the reaction mixture to be collected at the bottom of the tube and incubate in a water bath at 30°C for 90 minutes.

2.449. Purification of Translated Protein

The translated proteins were purified by using the NAP 5 column (GE Healthcare Cat No 17-0853-02). The purification of the proteins is by the process of gel filtration. Molecules larger than the largest pores in the matrix are excluded from the matrix and elute first, in our case translated proteins GAD_{65} and IA2ic. Intermediate size molecules penetrate the matrix to varying extents, depending on their size. Penetration of the matrix retards progress through the column; very small molecules elute last such as free amino acids. Remove one column per each translated and place on a suitable column rack holder and allow the contents of the column to pass through the column to remove the preservative out from the column. Equilibrate the column by washing the column with 1 x Tris buffer Saline Tween (5.0ml x 2). The protein's transcribed and translated were purified by passing the reaction mixture after dilution in 200µL of 1 x Tris buffer Saline Tween, through the NAP5 column as depicted in **Figure 2.1**

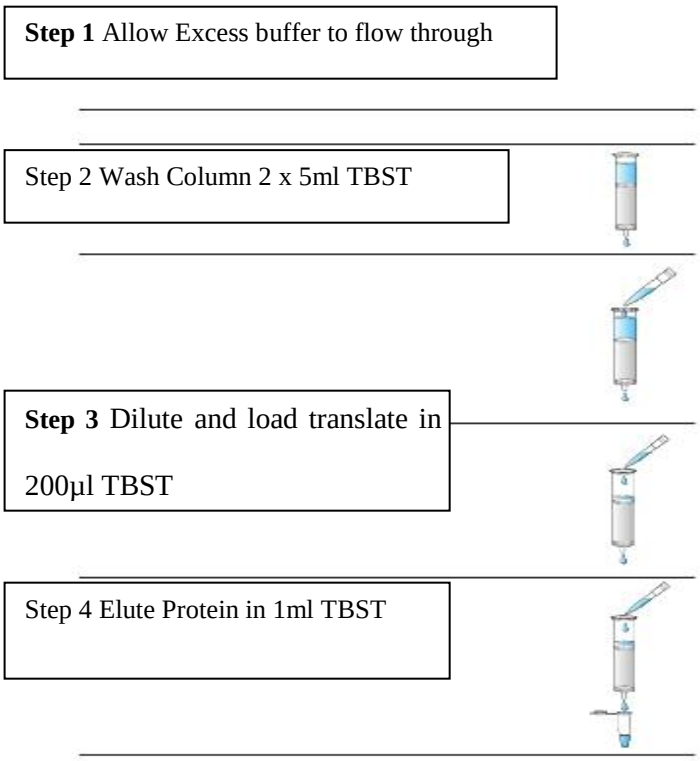


Figure 2.1: Elution of Translated ^{35}S Methionine labelled antigen (GAD_{65}, IA2ic.

All the proteins of interest are eluted in the haemoglobin fraction red in colour, (depicted here in blue in the cartoon) the smaller amino acids including the unincorporated ^{35}S Methionine elutes through the column later and is washed out with excess water to reduce the amount of waste disposed as solid waste.

2.450. Determination of Radioactive Protein Incorporation

The incorporation of protein translated was determined from the 50µl reaction mixture.

1. Remove 2µl of the reaction and add 98µl of 1M NaOH/2%H_2O_2.

Vortex briefly and incubate at 37°C for 10 minutes.

2. Add 900µl of ice cold 25% TCA to precipitate the proteins.
3. Prewash a Whatman GFA glass fiber filter with ice cold 5% TCA. Add 250µl of the TCA reaction mixture. Rinse the filter 3 times with 1.5ml of ice cold 5% TCA.
4. Rinse once with 2ml of acetone, allow the filter to dry at room temperature in the fume cupboard.
5. Put the filter in a scintillation tube add 1ml scintillation liquid and count on the Wallac Micro beta counter.
6. To determine the total counts spot 5µl of the reaction mixture directly onto a dry GFA glass fibre filter, allow to dry for 10 minutes and count after adding 1ml scintillation liquid. This is the CPM of the unwashed filter = Total counts.

The percent incorporation was determined as follows:

(CPM of washed filter (Step 5)/(CPM of unwashed filter (step 6)) x 50) x100

Typical Incorporation of ^{35}S Methionine into GAD_{65},IA2ic and ZnT8

(CPM of washed filter (Step 5)/CPM of unwashed filter (step 6)) x100

GAD_{65} Unwashed CPM Total counts =19428 CPM X50

GAD_{65} washed CPM counts =72578 CPM

Percentage incorporation = 7.5%

(CPM of washed filter (Step 5)/CPM of unwashed filter (step 6)) x100

IA2ic Unwashed CPM Total counts =20147 CPM X50

IA2ic washed CPM counts =90865 CPM

Percentage incorporation = 9.0%

(CPM of washed filter (Step 5)/CPM of unwashed filter (step 6)) x100

ZnT8 4.1 construct Unwashed CPM Total counts =19245 CPM X50

IA2ic washed CPM counts =88865 CPM

Percentage incorporation = 9.2%

On average 5-10% incorporation was achieved from each translation and approximately 20000-25000 CPM per 50µl was used in the radio-immunoprecipitation experiments for all the antigens tested throughout.

2.451. Assay for Glutamic Acid Decarboxylase (GAD_{65})

The radio-immunoprecipitation assay for the enzyme Glutamic acid Decarboxylase (GAD_{65}) employ's in vitro transcription and translation systems (Promega Madison, WI).

2.452. Preparation of 10 x Tris Buffer Saline Tween (TBST) Stock solution

In 1 litre sterile Duran bottle add approximately 600ml of distilled deionised water and place on a magnetic stirrer.

To this add 78.8 grams of Tris Hydrocloride (Sigma).

Add 116.8 grams of Sodium Cloride (Analar grade) (BDH)

Mix well on the stirrer to dissolve Tris Hydrocloride and Sodium Cloride check the PH and adjust to 7.2.

Add 100ml of Tween 20 (Sigma) so that the final concentration of Tween equals 1% after adjusting the volume of the stock solution to 1000ml with pure autoclaved water. The H_2O is autoclaved at 120°C for 15 minutes in order as to extend the shelf life of the stock solution.

The working solution 1x TBST was prepared by diluting 100ml of 10 x TBST with 900ml of pure water (Triple Red water purifier and autoclaved) and stored at 4°C until required for the assay.

Serum From Prediabetic Twin	Negative healthy control serum	Dilution			WHO Units IU/ml
200µl	**0µl**	**Neat**	**0**	**A**	**1209**
100µl of A	**100µl**	**1:2**	**2**	**B**	**702**
100µl of B	**100µl**	**1:4**	**4**	**C**	**417**
100µl of C	**100µl**	**1:8**	**8**	**D**	**250**
100µl of D	**100µl**	**1:16**	**16**	**E**	**126**
100µl of E	**100µl**	**1:32**	**32**	**F**	**89**
100µl of F	**100µl**	**1:64**	**64**	**G**	**51**
100µl of G	**100µl**	**1:128**	**128**	**H**	**25**
100µl of H	**100µl**	**1:256**	**256**	**I**	**-19**
100µl of I	**100µl**	**1:512**	**512**	**J**	**-3**
100µl of J	**100µl**	**1:1024**	**1024**	**K**	**0**

Table 2.5 Prepare a serial dilution of a positive control serum sample standardized to the WHO standard at 250IU/ml.

Serial dilution of the standards was carried out as depicted in Table 2.5.

	1	2	3	4	5	6
A	SB 0 Dil		SB 1:256 Dil		Test 4	4
B	SB 1:2 Dil		SB 1:512 Dil		Test 5	5
C	SB 1:4 Dil		SB 1:1024		Test 6	6
D	SB 1:8 Dil		Negative	Negative	Test 7	7
E	SB 1:16 Dil		Positive	Positive	Test 8	8
F	SB 1:32 Dil		Test 1	1	Test 9	9
G	SB 1:64 Dil		Test 2	2	Test 10	10
H	SB 1:128 Dil		Test 3	3	Test 11	11

Table 2.6 Assay Template for GADA & IA2A in a 96 well Millipore Filtration Plate (Test 1,2 etc= test samples tested in duplicate)

2.453. GAD65 and IA2ic Assay Protocol

1 Pipette 2ul samples in duplicate as shown in the assay template Table 2.6 of the standard curve in a 96 well format plates from Millipore UK (Cat No:MAHVBN4550B).

2 Pipette 2ul in duplicate of the positive control serum from a pre-diabetic twin and negative control serum sample in each well as shown in the template and 2ul in duplicate of the unknown samples.

3 Add 50ul or the translated and diluted GAD_{65}, IA2ic or ZnT8 antigen with approximately 20000 CPM per 50ul per well. Place on a shaker for 5 minutes and incubate overnight at 4°C.

4 Dilute Protein A Sepharose (PAS) (Amersham Pharmacia) so that we have 1mg/well. Dilute PAS 100mg in 25ml of TBST in a universal tube and allowed to swell, (1mg of PAS swells to 4ul of the packed resin volume).

5 Add 50µl of PAS per each well and incubate on a shaker at 4°C for approximately 1.5 hours to allow the immune complexes of Antigen/Antibody to bind to PAS. Protein A Sepharose CL-4B is Protein A covalently bound to Sepharose CL-4B using the CNBr method.

6 Bound immune complexes were washed 12 times with Tris buffer saline and Tween on millipore plates (Catalogue No MHVBN4550B) using a Millipore plate washer system, were allowed to dry at room temperature before the addition of meltilex solid scintillant (Wallac, Turku ,Finland), and were counted on the Wallac 1260 Micro beta scintillation counter.

7 The current assay protocol for all the antigens utilises the Millipore plates MAHAN4B50 plates which eliminates the use of the solid scintillant and instead liquid scintillant Ultima Gold, 50µl per well for aqueous solutions was employed. This reduces the quenching effect on the CPM detected using the solid Meltilex scintillant system previously employed as well as improved the sensitivity of the assay procedure.

2.454. Protein A Sepharose to bind serum immunoglobulins

Note in serum the concentration of IgG is 12.5mg/ml, IgA 2.1mg/ml, IgM 1.25mg/ml IgD 40µg/ml and IgE 0.04µg/ml respectively. This equates to 16mg/ml of total immunoglobulin per ml of serum and 3mg of PAS binds 20mg of immunoglobulin and 1mg PAS will bind 6.66mg of immunoglobulin. PAS added to the assay per well (1mg/well) is added in excess as in 2ul of the serum per well, we have approximately 32µg of immunoglobulin.

The pre-swollen PAS was washed twice in TBST buffer by adding 25ml of buffer mixing by inverting and centrifugation at 4°C for 4 minutes to pellet the PAS. Reconstitute the PAS such that where 100mg was employed for one plate the PAS was reconstituted in 5ml of TBST and for 2 plates 200mg was reconstituted in 10ml of TBST.

Protein A binds to the Fc region of immunoglobulins through interactions with the heavy chain. The binding of Protein A has been well documented for IgG from a

variety of mammalian species as well as for IgM and IgA. Protein A Sepharose CL-4B has been used as a powerful tool to isolate and purify classes, subclasses and fragments of immunoglobulins from biological fluids and from cell culture media. Since only the Fc region is involved in binding, the Fab region is available for binding antigen. Hence, Protein A Sepharose CL-4B is extremely useful for isolating immune complexes.

2.455. Population study autoantibody sample analysis: All subjects were tested for ICA in a single laboratory (Ulm) by indirect immunofluorescence, with detection limit 5 JDF units, and >20 JDF units as positive; assay sensitivity and specificity was 100% in 13th Islet Cell Autoantibody Workshop (1998) (Seissler et al.,1998).

ICA positive subjects were tested for GADA and IA-2A, plus ZnT8A (tested in London, UK) using radio-immunoprecipitation as described in detail chapter 2 and published previously (Hawa et al., 1997). The assay characteristics for Ulm for: GADA 86% sensitivity 95% specificity; IA-2A 73% sensitivity; 99% specificity. Characteristics of ZnT8A assay (London, UK) are described below under twin study.

2.456. Twin study autoantibody sample analysis: All twins were tested for serum autoantibodies to GAD_{65} (GADA), IA-2 (IA-2A) and ZnT8 (ZnT8A) using established radio-immunoprecipitation assays as described above and previously published (Hawa et al., 1997). All twin samples were tested at a single laboratory

(London) in batched assays with values expressed as categorical (positive/negative) and continuous traits. Positive results were duplicated reducing false positives to <0.2%. In the latest Diabetes Antibody Standardization Program (DASP 2008) London assay characteristics: GADA sensitivity 90%, specificity 93%; IA-2A, sensitivity 68%, specificity 95%; and ZnT8A sensitivity 60%, specificity 88% (data unpublished).

2.457. Diabetes Autoantibody Standardisation Programme (DASP)

Over the last 8 years our laboratory has been participating in the diabetes antibody assay standardisation programme (DASP) which entails the analysis of coded samples from healthy volunteers as well as patients with diabetes for the presence of diabetes associated auto-antibodies including ICA, GAD_{65}, IA2ic as well as the more recently described antigen ZnT8. The aims of the first proficiency evaluation of DASP were to assess general implementation of assay methods and to evaluate the new World Health Organization (WHO) reference reagent (250IU) for auto-antibodies to GAD_{65} and IA-2. The reference reagent was used to standardise the in-house assays for GAD_{65} and IA2ic. Forty-six laboratories in 13 countries received coded sera in the first DASP programme from 50 patients with newly diagnosed T1D and 50 blood donor control subjects, together with the WHO reference reagent and diluent serum.

Measurement of diabetes associated auto-antibodies to islet antigens has become invaluable in determining diabetes associated immunity. The measurement of diabetes auto-antibodies is now widespread and is used clinically to identify individuals at risk of developing T1D (Bingley et al.,2001), those requiring

immediate insulin treatment, as an aid to better characterise and classify diabetes, to study the natural history of diabetes and to measure the efficacy of intervention trials in assessing immunity to therapy (Expert Committee 1997),(Zeigler et al., 1999) . In the DASP 2003 workshop our assay for GAD_{65} had a sensitivity of 74% and specificity of 98% and for IA2ic a sensitivity of 62% and specificity of 98% (Bingley & participating Laboratories 2003). In the DASP 2009 workshop our assay for GAD_{65} had a sensitivity of 76% and a specificity of 98%, for IA2ic a sensitivity of 54% and a specificity of 100% and for ZnT8 a sensitivity of 42% and a specificity of 99%. In the data for DASP 2003 workshop the samples were scored positive or negative based on the 99^{th} percentile of 100 healthy controls tested in house.

In the latest DASP 2009 workshop the samples were scored positive or negative based on the end point dilution of the standard curves utilised in the assays. The sensitivity for IA2 in general shows a decrease without a significant decrease in the specificity, this lower sensitivity corresponds with the higher ages of the patients as in general antibodies to IA2 are detected in the younger patients more frequently. The sensitivity is increased significantly by the measurement of combination of antibodies including GADA, IA2icA as well as ZnT8A all comprising the Islet Cell Antigens.

(Publications have resulted from the participation in the DASP (Bingley & participating Laboratories 2003), (Schlosser and participating Laboratories, 2011), (Lampasona V & Participating Laboratories, 2011)

2.5. Results: The results from this study with details of the characteristics of identical and non-identical twins discordant for T1D as well as the population based samples who were ICA positive for CML levels and autoantibody positivity are depicted in Table 2.7, Table 2.8 and Figure 2.2. CML levels were increased in population-based autoantibody positive and pre-diabetic subjects shown in the left panel in Figure 2.2 and the levels were significantly elevated in comparison to the population based healthy controls in both groups. Similarly in the twins, the CML levels were significantly higher in both the diabetic and non-diabetic twins when compared to the normal twins (all $p<0.001$).

Furthermore the diabetic twins irrespective of their zygosity (MZ or DZ) tended to have antibodies more often than their non-diabetic co-twins. Of 64 twin pairs tested MZ and DZ, 37 diabetic twins had autoantibodies compared with 10 non-diabetic twins ($p<0.0001$ with fishers exact chi square analysis) (Table 2.8).

Elevated serum CML in ICA positive subjects in the population study was a persistent and additive predictive marker of progression to diabetes. When analyzed as continuous traits, diabetic twins, compared to their non-diabetic co-twins, had higher values for GADA ($p=0.02$) and IA-2A ($p=0.001$), but not ZnT8A. Neither age nor disease duration affected GADA levels, but older subjects had less presence of IA-2A ($p<0.001$) and ZnT8A ($p=0.002$). Twin correlations (r) were weak for the presence of autoantibodies in this cohort however I have presented data in this thesis of a positive strong correlation for

GADA and IA2A in twins who were concordant for T1D.

Of 64 non-diabetic twins: 62 (96.9%) had a raised serum CML (>1097 ng/ml as 99th centile of normal twin values) as did 57 (89.1%) of the diabetic twins (Figure 2.2); 10 of 64 had diabetes-associated autoantibodies; 2 of these latter 10 subsequently developed diabetes (Table 2.7).

2.6. Predictive Values:

To calculate positive and negative predictive values of a test for example presence of GADA in patients with and without diabetes, a number of parameters need to be recorded.

Patients with diabetes and with GADA are termed True Positives (TP)

Patients without diabetes and with GADA are termed False Positives (FP)

Patients with diabetes and without GADA are termed False Negatives (FN)

Patients without diabetes and without GADA are termed True Negatives (TN)

For example, let us suppose we tested for GADA on the 1000 random people. Among these 100 patients had TID, 95 of them tested GADA positive, and 5 tested GADA negative. Among the 900 people without Diabetes, 90 tested positive, and 810 tested negative.

In this case, TP=95, FN=5, FP=90, and TN=810.

Sensitivity is calculated by dividing TP/(TP+FN) x 100%

95/(95+5) = 95%

Specificity is calculated by dividing TN/(TN+FP) x 100%

810/(810+90) = 90%

Positive Predictive Value (PPV) is calculated by dividing TP/(TP+FP) x 100%

95/(95+90) = 51.4%

Negative Predictive Value (NPV) is calculated by dividing TN/(TN+FN) x 100%

810/(810+5) =99.4%

In the population samples positive and negative predictive values were: raised CML 46.5%, 83.1% (sensitivity 62.5%, specificity 71.9%); GADA 33.7%, 86.2% (sensitivity 87.8%, specificity 30.5%); IA2A 54.8%, 80.0% (sensitivity 50.0%, specificity 82.9%); and ZnT8A 60.0%, 85.0% (sensitivity 63.6%, specificity 82.9%).

In the MZ twin samples positive and negative predictive values were: raised CML 47.9%, 22.2% (sensitivity 89.0%, specificity 3.1%); GADA 85.7%, 60.0% (sensitivity 37.5%, specificity 93.8%); IA2A 77.8%, 54.5% (sensitivity 21.9%, specificity 93.8%); and ZnT8A 50.0%, 50.0% (sensitivity 6.3%, specificity 93.8%).

In the combined MZ/DZ twin samples positive and negative predictive values were: raised CML 47.9%, 22.2% (sensitivity 89.0%, specificity 3.1%); GADA 83.3%, 57,7% (sensitivity 31.3%, specificity 93.8%); IA2A 87.5%, 55.4%

(sensitivity 21.9%, specificity 96.9%); and ZnT8A 42.9%, 50.0% (sensitivity 4.7%, specificity 93.8%).

Twin Cohort (MZ=monozygotic DZ= dizygotic)	**Diabetic Twin**	**Non Diabetic Twin**
MZ Number of Twins	**32**	**32**
GADA	**12**	**2**
IA2A	**7**	**2**
ZnT8	**2**	**2**
Serum CML ng/ml (range)	**2638.5 [1911.5, 3550.5]**	**2248.5 [1708.5, 2991.0]**
DZ Twins		
Number of Twins	**32**	**32**
GADA	**8**	**2**
IA2A	**7**	**0**
ZnT8	**1**	**2**
Serum CML ng/ml (range)	**1776.5 [1185.5, 2077.5]**	**1616.5 [1252.0, 1854.0]**

Table 2.7 Variables in MZ & DZ twins samples (n=64) Means (SD) are given unless indicated differently.

Variables	**T1D (n=33)**	**noT1D (n=82)**	**p-value**
	Mean (SD)	**Mean (SD)**	
CMLinclusion visit, [interquartile range], in ng/ml	669 [524, 761]	512 [424, 619]	<0.001
CMLlast visit, [interquartile range], in ng/ml	773 [611, 831]	424 [381.5, 452]	<0.001
Antibody positivity			
GADA, n (%)	29 (87.9%)	57 (69.5%)	0.04
IA2, n (%)	17 (51.5%)	14 (17.1%)	<0.001
ZnT8, n (%)	21 (80.8%)	14 (28.6%)	<0.001

Table 2.8 Variables in the Population Study samples from 7,287 normal children of which, 115 were ICA positive Means (SD) are given unless indicated differently.

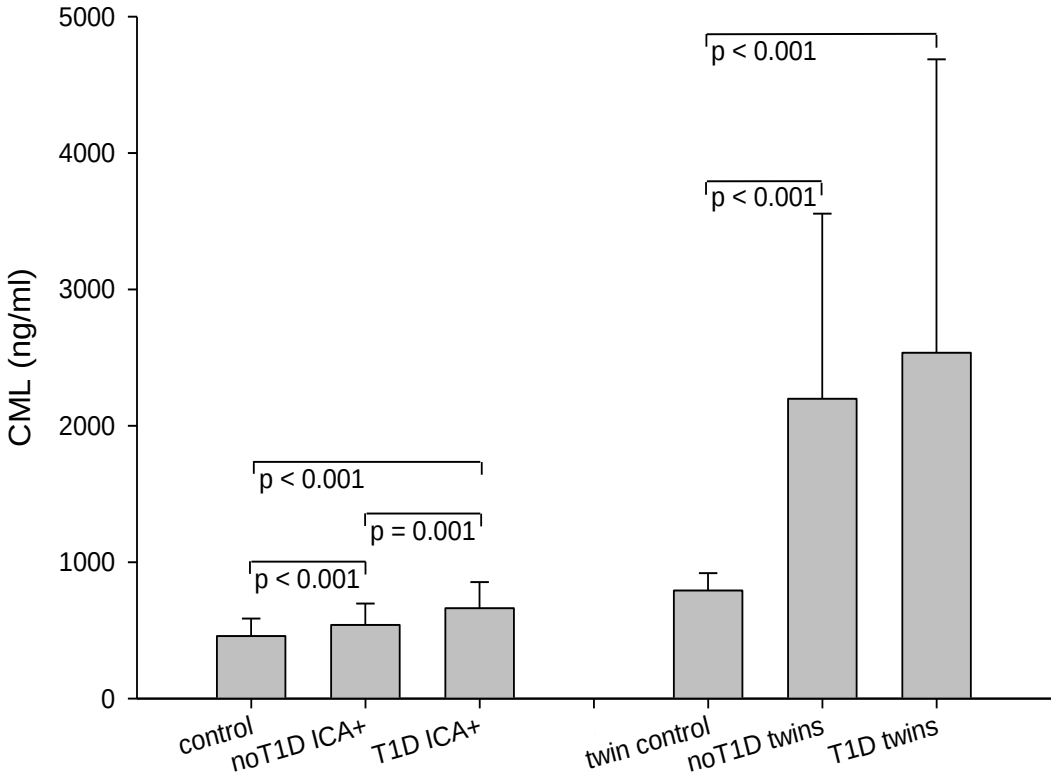


FIGURE 2.2. Mean (SD) baseline serum CML in population (Left panel) and twin study (Right Panel). Control = normal children (n=2102); noT1D ICA+ = non-diabetic child ICA+ (n=82); T1D ICA+ = diabetic child with ICA+ (n=33); twin control = normal twins (n=168); noT1D twins = non diabetic co-twin of T1D twins (n=64); T1D twins = diabetic twin (n=64). There is a graded increase in serum CML from control subjects through to twins with T1D, related to age and diabetes duration. The differences in twins and the population study are potentially related to differential ages and temperature of sample storage.

2.7. Discussion: This study examined CML levels in a population based cohort and confirmed previous findings of elevated levels of AGEs in normal twins, that, the levels of CML are genetically determined, such that, both the diabetic as well as non-diabetic twin had raised but similar CML levels. Interestingly studying CML and auto-antibodies showed that twins tended to be discordant for the latter (which with twin modeling, but not described here showed are determined predominantly by non-shared environmental factors) but not for CML (which with twin modeling, not described here showed are determined predominantly by shared environmental factors) suggesting that non-genetic factors, most probably environmental factors, must contribute to these differences.

If autoantibodies were genetically determined then both the diabetic as well as the non-diabetic twins would show concordance for them, however in this cohort of MZ and DZ twins discordant for diabetes such concordance was not the case. A twin study should ideally be performed prospectively in a population-based cohort from birth to determine the rate of induction of autoantibodies and diabetes, and as our twins were initially disease-discordant, so disease concordance rate is underestimated (Redondo et al., 2008) .

The increased higher level of CML in twins in comparison to the population based children tested could be attributed to the activation of the peripheral immune cells due to chronic ongoing inflammation and oxidative stress however the raised levels were detected in twins discordant for diabetes as well as in the healthy twins, all of whom did not have diabetes associated antibodies. The raised

levels of CML in twins discordant for T1D therefore cannot be attributed to ongoing inflammation or oxidative stress. AGEs CML is known to assert its inflammatory effects via its binding to RAGE leading to the activation of a cascade of signaling molecules which together have an inflammatory response. The cause for the raised CML levels in twins irrespective of their zygosity as well as significantly higher levels in comparison to the ICA positive school children samples is unclear, however this could be attributed to dietary factors, intake of which could be the most likely source. In particular thermally sensitive nutrients, including infant formula cow's milk and heat-treated animal fat, are major sources of AGEs, including CML, which can reach adult serum levels by one year of age which, have been implicated in sustaining an altered inflammatory response (Mericq et al 2010). AGEs levels increase as a result of chronological ageing process most likely due to dietary intake. Increased levels of AGE CML in this study, provides a positive predictive value and furthermore raises the possibility of intervention early in life to modify the course of the disease process. These proposals are supported by the observation that RAGE genetic polymorphisms predicted increased risk of human T1D, and that declining circulating levels of soluble RAGE predict the disease (Forbes et al., 2011). In another autoimmune disease, multiple sclerosis, AGE Carboxy-Ethyllysine (CEL) is elevated and AGE inhibitors have been suggested to have beneficial neuro-protective effect (Sternberg et al., 2010). Moreover, studies in animal models of autoimmune diabetes have shown a reduction of AGE by diet or drug therapy which reduces not only AGE levels, but progression to diabetes (Peppa et al., 2003).

Chapter 3

3.0. WHETHER AUTOANTIBODIES, INSULIN SECRETION AND INSULIN RESISTANCE ARE PREDICTIVE AND ARE GENETICALLY DETERMINED.

3.1. The aim of the study was to investigate the appearance of autoantibodies in twins concordant and discordant for T1D.

3.11. INTRODUCTION:

Twin studies were first described by Sir Francis Galton on the role of genes and environment on human development and behaviour. Twin studies are a powerful tool in scientific research in assessing genetic as well as non-genetic factors in multifactorial diseases, as twins can be monozygotic (MZ) developing from a single fertilized egg and therefore share all of their alleles, or dizygotic (DZ) that is develop from two separate eggs and only share 50% of the polymorphic alleles, which is the same level of shared genetic similarity as found in non-twin siblings. Twin studies are therefore a powerful tool in assessing genetic as well as non-genetic factors in multifactorial diseases. This important difference forms the basis of twin studies to investigate genetic and environmental features in disease and health.

The classic twin study begins by assessing the variance of a behaviour (called a phenotype by geneticists) in a large group, and then attempts to estimate how much of this is due to genetic effects (heritability), and how much appears to be due to shared or unique environmental effects - events that affect each twin in a different way, or events that occur to one twin but not another.

Typically these three components are called A (additive genetics) C (common environment) and E (unique environment); the so-called ACE Model. Given the ACE model, we can determine what proportion of variance in a trait is heritable, versus the proportions which are due to shared environment or unshared environment. Monozygotic (MZ) twins raised in a family share both 100% of their genes, and all of the shared environment. Any differences arising between them in these circumstances are random (unique events not shared). The correlation we observe between MZ twins provides an estimate of A + C . Dizygous (DZ) twins have a common shared environment, and share on average 50% of their genes: so the correlation between DZ twins is a direct estimate of ½A + C .

Monozygotic twins are usually discordant for T1D i.e. only one twin is affected. Studies of twins have shown that the concordance rates for T1D is higher in monozygotic twins than dizygotic twins which is consistent with involvement of genetic factors in the aetiology of T1D (Kaprio et al., 1993). However despite this the concordance rates for immune mediated diseases fall well short of 100% and these rates have been reported to be lower in non-identical twins compared to identical twins. The majority of identical twins with autoimmune disease have an identical twin discordant for the disease as shown in (Table 3.01) (Salvetti et al., 2000). Genetic and Environmental factors have been implicated in the pathogenesis of immune mediated disease, with the most powerful effect coming from the studies of identical twins. It is important to note that while some twins develop the disease soon after the index twin develops the disease, the majority of

twins however remain unaffected even after 40 years of follow up of the unaffected twin (Fava et al., 1998). At diagnosis of T1D, the islets are heavily infiltrated with T Lymphocytes and insulin secretion is markedly reduced or may even be absent (Foulis & Stewart 1984). The disease process is slow with a long prodrome period before diabetes ensues and immune changes (autoantibodies to islet antigens) can be detected many months or even years, before the clinical onset of TID.

Disease	Identical Twin Pairs	Non-Identical Twin Pairs	Reference
MS	26.7	3.5	Ebers & Sadovnick 1994
RA	12.3	3.5	Jarvinen et al 1992
IDDM	13	2.5	Kaprio et al 1993
SLE	33	0	Salvetti et al 2000

Table 3.01 Disease concordance rates in identical and non-identical twin pairs in population-based studies of immune-mediated diseases. (Modified from M Salvetti et al 2000)

Abbreviation: MS Multiple sclerosis, RA Rheumatoid Arthritis, IDDM insulin dependent diabetes, SLE Systemic Lupus Erethematosus

3.12. Methods: To test whether antibodies to Islet auto-antigens GADA and IA2ic are inherited and or can be detected in Twins Discordant and Concordant for Diabetes, available samples from the twin cohort's stored at -20 ºC, were tested for autoantibodies to key auto-antigens using radio-immuno-precipitation assays as previously described.

3.13. Subjects: We tested a cohort of MZ and DZ twins discordant for diabetes and examined the appearance of autoantibodies to GADA, IA2A. Twins from United Kingdom (Caucasian) were ascertained as described earlier. Classification of T1D was based on *1*) control of diabetes with insulin from time of diagnosis, *2*) diabetic ketoacidosis or marked ketonuria at time of clinical onset. Twins were followed prospectively until they either developed diabetes or remained free of the disease. The twins who remained discordant for diabetes more than 5 years from the diagnosis of the index twin, are referred to as low risk twins with an estimated disease risk of less than 2%.

From the Twin Study, consecutive samples were tested from 156 twin pairs; of these 67 subsequently developed T1D (termed Concordant Twin Pairs), and 89 pairs who remained discordant. Stored serum samples were tested both before and at diagnosis of T1D. The mean period of follow-up in the twins who became concordant for T1D was 5.08 years (range 0.33–29.5 yrs), the mean age in concordant twins was 15.97 years (range 0.33–28.4 years), 35 females. From the DZ twins (n=81) sufficient sera was available from 32 pairs and were tested for diabetes associated autoantibodies (Table 3.02).

The mean period of follow-up in the twins who remained discordant for T1D (n=89) was 16.0 ± 10 years (range 1.0–51.9 yrs), the mean age in the discordant twins was 15.97 years (range 1.07–51. years). 50 females. From these 188 twin pairs all available samples were tested for diabetes antibodies in particular GADA and IA2 and in selected cases ZnT8.

3.14. Results: The results from this study are depicted in Table 1.8. Twins when initially referred to the study were discordant for diabetes. Twins tested for autoantibodies were more often concordant for autoantibodies when both twins became concordant for the disease, the concordance for the presence of autoantibodies between twins who became concordant was similar between these pairs (43.4% vs 46.4%) compared to those who were discordant for the disease 46% vs 10% $p < 0.0002$ Chi Square analysis).

There was a positive correlation for age between twin pairs who were concordant for diabetes, (Correlation coefficient (r) = 0.812835 (r^2 = 0.660701)) as shown in **Figure 3.1** showing the correlation for age at diagnosis between the index twin and the co twin. The average follow up period between the twins was 5.3±5.9 years and 28 pairs of the 67 pairs were diagnosed below 18 years of age.

	GADA/IA2 or ZnT8Index Twin	GADA/IA2 or ZnT8 Co Twin	Follow up(Yrs)
Discordant Twins (n=89 pairs)	41 (46%)	9 (10%)	16.0 (10.1) range 1.1-51.9
Concordant Twins (n=67 pairs)	33 (49.2%)	33 (49.3%)	5.1 (5.6) range 0.3-29.5
DZ discordant Twins (n=81) 32 pairs tested	13 (41%)	2 (6%)	9.8 (6.3) Range 2.8-18.8

Table 3.02 Autoantibodies in Concordant and Discordant Twin Pairs

The risk was highest in twins who developed diabetes at a younger age and the mean age at diagnosis was 15.7±9.6 (range 1-47). Of those who became concordant for diabetes under 18 years of age, again there was a positive concordance with a Correlation coefficient (r) = 0.813354 (r^2 = 0.661545) **(Figure**

3.1) and the discordance between twins was less than five years (mean 1.72 ±1.99 years).

Of the twins who became concordant after 18 years of age (n=47) the mean period of discordance was 6.6 ± 5.7 years range 0 -21 years with a mean age at diagnosis of the index twin 31.8 ± 13.3 vs 38.5 ± 13.6 years in the co twin, with a discordance of 6.6 years (Table 3.03). Of the twins who became concordant under 18 years of age the mean period of discordance was 1.7 ± 1.9 years, range 0-9 years with a mean age at diagnosis of the index twin 7.1 ± 3.3 vs 8.8 ± 3.3 years in the co twin, with a discordance of 1.7 ± 1.9 years.

A positive correlation was noted in both groups of twins irrespective of the age at diagnosis. In the twins diagnosed under the age of 18 years the correlation coefficient was (r=0.813354 r^2 =0.6615) (**figure 3.2**) and similarly there was a positive correlation in those twins who became concordant for diabetes after 18 years of age correlation coefficient (r=0.899178 r^2 =0.808521) (**figure 3.3**).

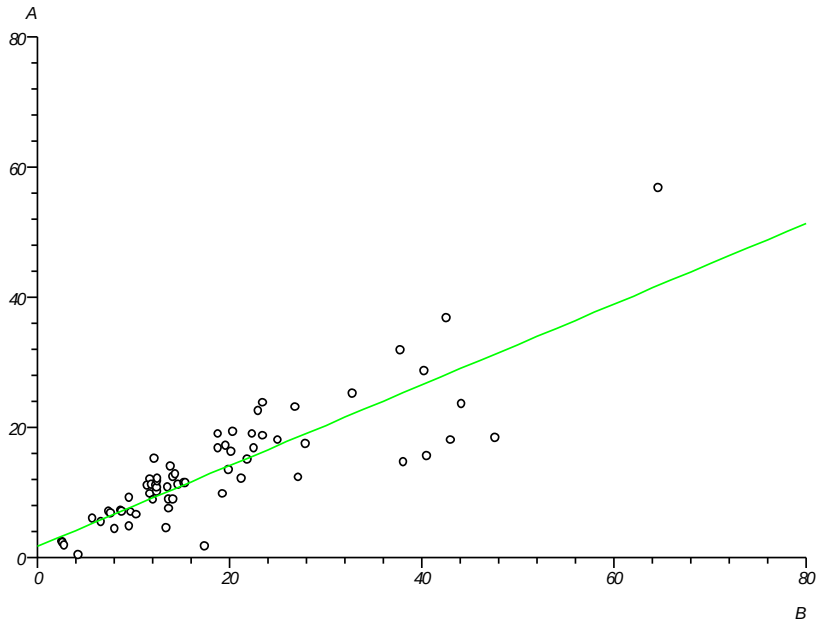


Figure 3.1 Concordance for T1D between MZ twin pairs (n=67) cases plot of correlation using SPSS. 95% CI for population value of slope = 0.52381 to 0.717356. Correlation coefficient (r) = 0.848202 (r^2 = 0.719447) 95% CI for r (Fisher's z transformed) = 0.762758 to 0.90454 t with 64 DF = 12.810961 Two sided P < 0.0001 Power (for 5% significance) > 99.99%

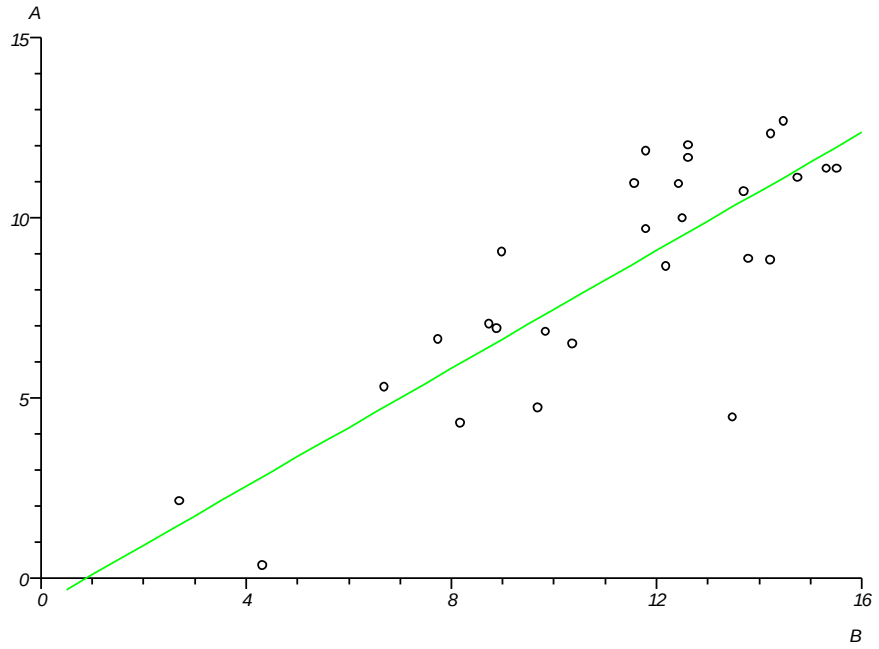


Figure 3.2 Concordance for T1D between MZ twin pairs diagnosed under 18 years of age (r=0.813354 r^2 =0.6615) plot of correlation using SPSS.

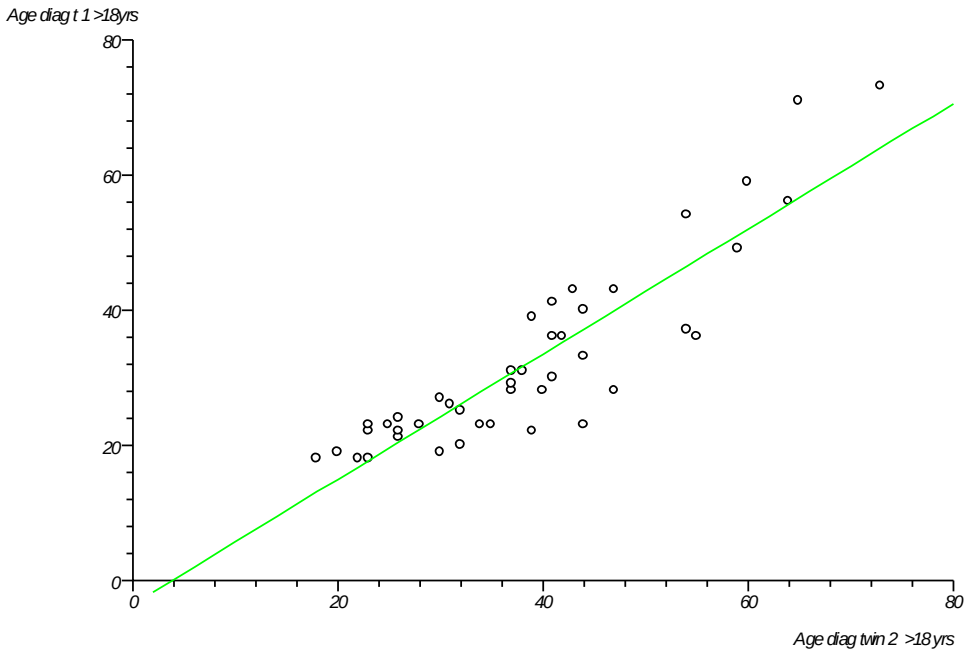


Figure 3.3 Concordance for T1D between MZ twin pairs diagnosed over 18 years of age correlation coefficient (r=0.899178 r^2 =0.808521) plot of correlation using SPSS.

Age at diagnosis Index Twin	Age at diagnosis co twin	Discordance
18.81918	22.47123	3.652055
19.13699	20.47123	1.334247
22.42192	23.08767	0.665753
25.10685	32.86575	7.758904
31.7726	37.90411	6.131507
18.81644	18.90137	0.084932
18.23562	47.7726	29.53699
28.49589	40.33699	11.8411
18.51781	23.52603	5.008219
22.96164	26.96438	4.00274
23.41644	44.26301	20.84658
36.72877	42.64658	5.917808
Mean age 23.7±5.9	Mean age 31.8±10.4	Mean discordance 8.1±8.8

Table 3.03 Concordant Twins diagnosed after 18 years of age on whom data was available for autoantibodies. Correlation coefficient (r) = 0.775205 (r^2 = 0.600943)

3.15. Discussion: In a previous report (Fava et al., 1998) the age of onset in 116 identical twin pairs concordant for T1D was strikingly correlated (correlation coefficient 0.94) and the correlation for age at diagnosis was higher in identical than non-identical twins. The data presented here shows a striking correlation irrespective of the age at diagnosis. A high correlation was noted in twins diagnosed under the age of 18 years age ($r=0.813354$ $r^2=0.6615$), as well as in the twins diagnosed over age of 18 years of age at diagnosis ($r=0.899178$ $r^2=0.808521$).

Of the MZ/DZ Twins tested for autoantibodies, twins were more often concordant for autoantibodies when both twins were concordant for the disease, the concordance for the presence of autoantibodies between twins who became concordant was similar between these pairs (43.4% vs 46.4%) compared to those who were discordant for the disease 46% vs 10% $p < 0.0002$ Chi Square analysis), "No disease No autoimmune changes detected". In the Dizygotic twins discordant for diabetes, diabetes associated autoantibodies were infrequently detected in the non-diabetic twin.

There are a number of limitations of twin studies in assessing genetic impact on disease. First, identical twins may have a more similar environment both in utero and in childhood, leading to an over-estimate of heritability. Second, in contrast to nonidentical twins, identical twins are always the same sex and many autoimmune diseases are more common in females for example, RA, MS and SLE. Third, twin studies are hindered by potential biases in ascertainment. In the traditional 'clinic-

based' approach, identical twin pairs concordant for a disease, or with severe disease, are more likely to be identified. In the 'population-based' approach, individuals are identified first as twins and are then assessed for illness. These population-based studies must ascertain large numbers of twin pairs to detect sufficient numbers of affected twins. Finally, most twin studies have been cross-sectional, but, by following twins for a longer period, it might be possible to detect a higher concordance rate.

Identical twins show a similar genetic regulation of the production of antibodies, even when they are reared apart (Kohler et al., 1985). A study of IgM and IgG rheumatoid factor isotypes in 70 identical and 84 nonidentical twins discordant for RA concluded that genetic factors were important in determining the levels and frequency of these isotypes; for example, IgM and IgG positivity was higher in healthy identical than healthy nonidentical twins (Macgregor et al.,1995). In agreement with such a genetic effect, combinations of IDDM-associated autoantibodies were more often found in healthy identical than healthy nonidentical twins of IDDM patients (Hawa et al., 1997). Furthermore possession of the high risk Hph I insulin genotype in identical twins increases the risk for these twins to be concordant for T1D (Metcalf K et al 2001) , as this gene was found significantly more often in concordant twins in comparison to the discordant twins 87.5% vs 59.5 (p=0.005). Another study suggested that sufficient long term follow up of identical twins discordant for diabetes followed for greater than 40 years eventually develop islet autoimmunity and T1D even if twins were initially discordant for T1D (Redondo et al., 2008). In that study all twins who

were discordant for TID were positive islet cell antigens and therefore were all pre-diabetic and would in theory go on to develop diabetes. In this present thesis data samples were tested from twins followed for more than 40 years who continue to remain free of diabetes associated autoantibodies as well diabetes, the former data remains to be confirmed. The threshold hypothesis indicates the requirement of a combination of both genetic as well as environmental factors reaching a threshold such that disease results, so it is possible in twins who remain discordant for TID, that such a threshold has not been achieved despite being genetically identical, implicating other additive factors are necessary in reaching the threshold. Metabolic changes can also be detected many months or even years before the onset of the disease, whether these changes are inherited can be tested in identical twins discordant for the disease.

CHAPTER 3.2

3.2. IS INSULIN SECRETION AND INSULIN RESISTANCE PREDICTIVE AND GENETICALLY DETERMINED.

3.21. INTRODUCTION: Type 1 diabetes is due to the destruction of the insulin secreting beta cells probably mediated by T lymphocytes recognizing critical beta cell antigens and it is probably caused by environmental factors in a genetically susceptible individual operating at critical time.

There are views that these changes may be due to an early environmental insult or that the altered insulin secretion and sensitivity are genetically determined thereby predisposing an individual to the destructive process (Bennet et al., 1995), (Johnston et al., 1987). If the latter is the case then testing metabolic features could identify such changes in twins discordant for T1D.

3.211. Insulin Sensitivity: Selected twins from the British diabetic twin's cohort were tested to examine if there was any evidence for the alteration of the insulin secretion or sensitivity which predisposed them to TID. We studied prospectively 27 identical twins of patients with TID who were initially non diabetic, as well as 14 healthy control subjects over a period of 18 years. 15 twins remained non diabetic and were estimated as low disease risk twins and 12 developed TID (pre-diabetic twins), **Table 3.20 (Publication Diabetes Care Volume 28 June 2005 pages 1415-1418).** Subjects were tested on at least two separate occasions with an intravenous glucose tolerance test (IVGTT) at least six years apart from the initial test.

Insulin sensitivity: Was estimated as a ratio (HOMA-IR/first-phase insulin response [FPIR]), which is predictive of progression to T1D.

	Control Twins	**Low Risk Twins**	**Prediabetic Twins**
	14	15	12
Age at initial test	21.1±7.5	24.1±9.3	17.4±5.8
Gender (M) n	9	8	5
BMI (Kg/m2)	20.7±2.5	22.2±3.2	20.1±2.5

Table 3.20: The patient group tested for insulin sensitivity Data are represented as mean±SD

3.212. First Phase Insulin Response (FPIR): We estimated FPIR following a glucose load (0.5 g/kg), measured as the area under the 0–10 min curve and above the fasting level.

3.213. Glucose clearance (Kg): calculated as the slope of the regression line of the natural logarithm of glucose between 10 and 30min after glucose infusion and expressed as percent per minute.

3.214. Glucose Estimation: Glucose was determined by the glucose oxidase method on the Yellow Springs Analyser 23A, (Yellow Springs, Ohio, USA). Briefly blood samples are collected in fluoride oxalate tubes, 25ul sample was injected through a membrane into the chamber containing the enzyme glucose oxidase. The reaction between glucose and the enzyme glucose oxidase in the presence of oxygen yields gluconic acid and hydrogen peroxide. The latter is reduced to produce an electrical signal proportional to the amount of glucose in the blood sample. The latter signal records the amount of glucose present in the sample in mMol/L.

3.215. Insulin

Insulin is produced by the β-cells of the pancreatic islets, initially synthesized as a 12 kDa pro-hormone (Pre-proinsulin), which undergoes intracellular processing to a 9kDA, 86 amino acid pro-hormone (pro-insulin) and subsequently packaged in storage granules. Within the granules, disulphide bonds are formed between the A and B chains of the insulin molecule and the C-peptide region is cleaved, resulting in the 51 amino acid, 6kDa mature insulin molecule. Upon stimulation, the islets release equimolar concentrations of insulin and C-peptide, and a small amount of

proinsulin and other intermediates of proinsulin (approximately <5% of the normal insulin secretion.

Insulin is the most important hormone of the fed-state, and is the only physiologic hormone which significantly reduces the circulating blood glucose levels. In response to a number of substrates and stimuli, including glucose and amino acids, insulin is secreted into the hepatic portal circulation. More than fifty percent of the insulin secreted is removed on the first pass through the liver, the remainder, enters the general circulation and is carried to the target tissues to facilitate glucose utilisation. In contrast, C-peptide of insulin does not undergo significant liver or extra renal metabolism and therefore has a longer half-life in the circulation (approximately 30 minutes) and as such is employed to test the insulin secretory capacity of beta cells.

Basal and glucose stimulated insulin concentrations are relatively stable during infancy and childhood, and increase during puberty due to decreased insulin sensitivity (Amiel et al., 1991). Insulin concentrations tend to be higher in obese individuals, particularly those with an increased proportional visceral abdominal fat (Bjorntorp 1991).

The measurement of insulin concentrations can be useful in clinical diagnostics of several conditions. Raised serum insulin concentrations in the presence of low glucose concentrations may be indicative of pathologic hyperinsulinism e.g. nesidioblastosis and islet cell tumour (Haymond 1989). Elevated fasting insulin levels with normal or elevated glucose concentrations are characteristic of insulin resistant forms of glucose intolerance and diabetes mellitus. High circulating

insulin concentrations may be involved in the pathogenesis of hypertension and cardiovascular disease (Reaven 1991). Conversely, low insulin concentrations, in the presence of hyperglycaemia suggests insulin-deficiency, e.g. insulin dependent T1D. The measurement of the first phase insulin secretion after a glucose load is also predictive of T1D development (Robert et al., 1991).

3.216. Insulin Radio-immuno Assay (RIA)

Insulin autoantibodies can be observed in the pre-diabetic phase and can interfere with the insulin radioimmunoassay. Excess proinsulin as well as fragments of pro-insulin can interfere with the estimation of insulin.

In radioimmunoassay for immunoreactive insulin in serum, an unknown serum is compared to a standard curve prepared by the use of increasing amounts of an unlabelled insulin preparation that serves as a standard according to the methods of Morgan and Lazarow (Morgan & Lazarow 1963). The insulin standard is usually dissolved in a buffer solution containing bovine or human (Velasco 1974) serum albumin as the only protein. The absence of human serum from the standard is an uncontrolled variable and can affect the determination of the absolute concentration of insulin.

The reagents for the insulin assay buffer are shown in Table 3.30

3.217. Insulin Assay Reagents:

Table 3.21: Phosphate/albumin buffer pH 7.4 (PO_4) (PBS)

Disodium hydrogen orthophosphate NA2HPO4 (Mwt 141.96)	**4.54g**
Sodium dihydrogen orthophosphate **NaH2P042H20 (Mwt 156.01)**	**1.19g**
Sodium Chloride NaCl (Mwt 58.44)	**9.0g**
Thiomersal	**0.2g**
EthyleneDiaminetriacetic acid EDTA	**3.72g**
Bovine Serum Albumin Fraction V (BDH) **(Radio-immunoasssay grade)**	**5.0g**

Make the contents of Table 3.21 to 1 Litre in distilled H2O.

*Add the Bovine Serum Albumin (BSA) by gently sprinkling over the flask on a magnetic stirrer to allow the BSA to gently dissolve.

Adjust the PH to 7.4 with 1 M NaOH and store at 4°C until required for the assay.

3.218. Radiochemicals:

Monoiodinated TyrosineA^{14} labelled ^{125}I 0.5mg albumin/vial as stock (Amersham Pharmacia). The contents are dissolved in 1 ml of dH2O and 100ul aliquots stored at -20°C until required for use. Add 20 ml of phosphate buffer saline to one 100ul aliquot to give approximately 20000CPM/100ul for the assay.

3.219. 1st Antibody Guinea Pig Anti Insulin antibody (Gift supplied by Nordisk Gentofte). In order to use the Guinea Pig Insulin antibody in the assay, dilution curves were initially prepared by serially diluting from the original 0.5ml stock of 1:1000 dilution to give 1:10000,1:20000, 1:40000, 1:80000, 1:160000, 1:320000 and 1:640000 dilutions respectively.

An antibody dilution curve was set up in duplicate in polypropylene tubes (50 mm X 9.75 mm from Elkay Labs) as follows:

Add 100ul cold unlabelled insulin 1 µIU/ml

Add 100ul first antibody at the different dilutions (Nordisk Gentofte)

Add 100ul Monoiodinated Tyrosine labelled ^{25}I insulin

Add 200ul Phosphate Saline buffer (PBS).

Vortex the tubes and Incubate for 24 hours at 4°C. Add 500ul of the second antibody/PEG complex to the tubes while stirring the solution to allow the mixture to remain homogenous throughout. Incubate for a further 2 hours at 4°C and centrifuge the tubes for 30 minutes at 2500 Revolutions Per Minute (RPM) at 4°C. Decant the supernatant into the designated radioactive waste sink and tap the tubes inverted onto tissues to remove excess liquid. Add 1.5ml of PBS to wash the unbound label by centrifuging for a further 30 minutes at 4°C. Decant the tubes as before and count the tubes on the Wallac Gamma counter for 60 seconds per tube.

From the antibody dilution curves with and without the addition of cold unlabelled insulin an antibody dilution curve was generated from which 50% of maximal binding was determined and used for the subsequent assays for Insulin.

3.220. Second Antibody to 1st Antibody to insulin

Dilute Rabbit antiguinea pig Precipitating serum A-PPT3 (5ml) (iDS Ltd UK):

To 100ml of the phosphate buffer saline, add 0.4g Sodium Azide (preservative) and 15.8g Polyethelene glycol (PEG) (BDH) and mix thoroughly.

To 40ml of the PBS add the contents of the second antibody A-PPT3 mix thoroughly on a magnetic stirrer and add 300ul of the Normal Guinea Pig Serum as a Carrier protein and allow it to mix thoroughly. Combine a & b and make the final volume to 200ml.

3.221. Insulin Standard

The insulin for immunoassay was purchased from the National Institute of Biological Standards Control. The insulin standard was purchased from the World Health Organisation, where one international unit is the activity contained in 1.8233mg of the preparation and when dissolved in 1ml PBS and contained 3.0 I.U (International Units). A 100ul aliquot of this dissolved in 3ml PBS (PH 7.4) to give a solution containing 0.1IU Insulin. This was further diluted taking 200ul into 100ml PBS to give 200 IU/ml and stored at -20°C. Serial dilution of the latter provides the standard curve with 100 IU, 50,25,12.5,6.25,3.125, and 1.56 IU respectively of insulin standard for the assay.

3.222. Preparation of hormone-depleted human serum

Pooled Human serum 100ml from healthy donors was treated with dextran-coated charcoal (400ml). Treatment with Norit NK dextran-coated charcoal: *(a)* prepare a suspension of activated dextran-coated charcoal (Sigma); *(b)* centrifuge at 10,000 X g for 10 minutes at 4°C, *(c)* discard the supernatant; *(d)* resuspend the pellet in 100ml serum to be treated, leave at 4°C mixing overnight for the adsorption of the insulin as well as other hormones; and *(e)* centrifuge as before. On occasion, the last centrifugation may need to be repeated before the charcoal is completely removed. The hormone free plasma is stored in 2 ml aliquots at -20°C.

3.223. Insulin Assay Protocol

Day 1: Prepare tubes and standard as shown in Table 3.22. All standard samples were tested in triplicate and all test samples in duplicate including positive and a negative control sample.

Tube No	Assay Buffer	Test Serum/ Hormone free serum	Insulin Standard	1st Antibody	Standard concentration µIU/ml
1-3	400ul				Non-specific binding tubes
4-6	400ul			100ul	*0*
7-9	200ul	100ul	100ul	100ul	*1.56*
10-12	200ul	100ul	100ul	100ul	*3.125*
13-15	200ul	100ul	100ul	100ul	*6.25*
16-18	200ul	100ul	100ul	100ul	*12.5*
19-21	200ul	100ul	100ul	100ul	*25*
22-24	200ul	100ul	100ul	100ul	*50*
25-27	200ul	100ul	100ul	100ul	*100*
28-29	300ul	100ul		100ul	Test Sample
30-31	300ul	100ul		100ul	Test Sample
32-33	300ul	100ul		100ul	Test Sample

Table 3.22 insulin assay template

Day 2: Add 100ul of ^{125}I insulin label (approximately 20000 cpm/tube) to all the tubes including three extra tubes for the Total Count. Vortex and incubate for 24 hours at 4°C.

Day 3: Add 500ul of the 2nd antibody/PEG complex using the eppendorf repeater pipette to all the tubes ensuring that the complex is kept stirring constantly on ice whilst adding to the assay tubes. Vortex the tubes well and incubate for a minimum of 2 hours at 4°C. Centrifuge the tubes for 30 minutes, at 2500 RPM at 4°C, decant the supernatant. Tap on a tissue to remove excess liquid. Add 1.5ml Phosphate buffered saline to the tubes and centrifuge again for 30 minutes at 2500 RPM at 4°C. Decant as before and count the tubes on a Packard gamma counter for 60 seconds per tube (CPM).

3.224. Results: The results obtained are shown in Table 3.23 at the initial and follow up test in 27 twin pairs discordant for diabetes of whom 12 went on to develop T1D and are termed as pre-diabetic twins of which all were autoantibody positive to GADA and or IA2A. The remaining non diabetic twins were all autoantibody negative and were termed as 15 low risk twins. A group of 14 age and gender matched healthy controls were also tested 6 years apart. Patients and controls were selected to be age as well as gender matched Table 3.20. Twins at low disease risk and healthy controls had similar fasting glucose levels as well as fasting insulin, glucose clearance and First Phase Insulin Response (FPIR) with no evidence of an impaired FPIR. In contrast the pre-diabetic twins had higher fasting insulin levels 10±6.0 compared to the controls 4.6±4.0, decreased glucose

clearance and FPIR as well as a decreased HOMA-IR to FPIR ratio which is predictive of the progression to T1D ($p<0.01$).

	Control subjects initial test	Control subjects Final test	Low risk Non Diabetic Twins Initial test	Low risk Non Diabetic Twins Final test	Prediabetic Twins Initial test	Pre-diabetic Twins Final test
N	14	14	15	15	12	12
Fasting glucose (mmol/l)	4.0 ±0.3	4.4±0.4	4.1±0.2	4.2±0.3	4.0±1.3	4.9±1.2
Fasting insulin (mIU/ml)	4.6±4.0	9.3±10.1	6.2±3.7	4.2±10.3	10.0±6.0	6.1±3.5
6.Glucose clearance (%/min)	2.6±0.8	2.5±0.9	3.0±1.3	2.4±1.9	1.5±0.6	1.2±0.7
FPIR (mIU _ ml_1 _ 10 min_1)	796±622	433±196	465±367	499±293	245±129	138±112
HOMA-IR–to–FPIR ratio	0.00118±0.0009	0.0036±0.002	0.0052±0.0061	0.0028±0.0048	0.0073±0.0057	0.00246±0.0338

Table 3.23 Glucose and Insulin sensitivity: Data are means ± SD. The low-risk twins did not differ from the control subjects, in contrast to the pre-diabetic twins, who showed numerous differences from control subjects. *P _ 0.01 for all differences.

3.225. Discussion: It has previously been reported that monozygotic twins tend to develop T1D within 6 years of diagnosis of the index twin (Fava et al., 1998). Twins discordant for T1D, for more than 6 years have a decreased probability of developing diabetes, estimated at less than 2% and are likely to remain discordant (Tun et al., 1994), (Olmos et al.,1998).

There was no evidence of an impaired beta cell function in the twins who were at low disease risk unlike pre-diabetic twins, in whom we found both reduced insulin secretion and reduced insulin sensitivity relative to levels of insulin secretion and went on to develop diabetes. Failure of the loss of the FPIR, are the hallmarks of loss of beta cell function and are earliest signs leading to hyperglycaemia and glucose intolerance in both T1D as well as type 2 diabetes. There was however, no evidence of any impaired beta cell function in the non-diabetic twins consistent with a non-genetic event which affects the diabetic twin but not their genetically identical non diabetic twin.

Metabolic, as well as immune, changes detected in the pre-diabetic twins however, were not detected in the non-diabetic twins, these features are therefore likely to be determined by non-genetic probably environmental or perhaps even epigenetic factors as a result of stochastic events. It is possible that with better techniques we may be able detect changes in the insulin secretory mechanism as well as immune changes which may pre-dispose the non-diabetic twins to the disease. This is however unlikely as most changes immune as well as metabolic, can be detected months even years prior to the diagnosis of T1D. It should also be noted that patients with metabolic changes can present with diabetes without

showing any immune changes such as the presence of autoantibodies to Islet auto-antigen's namely GADA, IA2, IAA or ZnT8.

Furthermore presentation of impaired glucose tolerance and frank diabetes can occur in the absence of auto-antibodies. Patients can present with impaired glucose tolerance as well as presence of GADA but are non-insulin requiring at diagnosis with a high risk of progression to insulin therapy and are termed as LADA (Pozzilli & DiMario., 2001). T1D can occur at any age and it is known that up to 10% of adults in the developed countries over the age of 30 years present with autoimmune diabetes of adults termed as LADA (Turner et al., 1997). The presentation of T1D in adults appears distinct from diabetes presenting in children in terms of genetic, immune and metabolic as well as clinical features of the disease.

Twin studies have shown that immune changes including presence of multiple autoantibodies to islet antigens as well as metabolic changes can be detected years before diagnosis of T1D. However follow-up date from the British Diabetic Twin study has shown that even genetically susceptible individuals such as identical twins can remain discordant for diabetes for greater that 50 years without showing any signs of immune or metabolic changes confirming that additional non-genetic factors are key in the initiation and induction of the disease which could be due to unique environmental or stochastic epigenetic events.

These results suggest that the disease process is unlikely to be due to shared inherited defects in insulin secretion or sensitivity as they were not noted in the non-diabetic twins however it does not exclude other factors which could

accelerate the disease process or promote metabolic decompensation, such as increased linear growth and childhood obesity (Hypponen et al., 2000), (Bruining., 2000), (Kibirige et al., 2003). In line with this found a disruption in the insulin sensitivity to insulin secretion in autoantibody positive siblings who later developed diabetes (Fourlanos et al., 2004). Notably with the lack of identity of all the diabetes susceptibility genes, twin studies as long as they are not pre-diabetic are the most appropriate to test whether inherited metabolic changes predispose to T1D.

In this dataset we were unable to support the hypothesis that inherited changes in insulin secretion or sensitivity could account for the genetic susceptibility to T1D. In contrast pre-diabetic autoantibody positive individuals can show a broad spectrum of clinical as well as metabolic phenotype, with normal, impaired or diabetic glucose tolerance or in adults as with one of our twin's frank clinical non-insulin requiring diabetes (LADA) with a high risk of progression to insulin requiring diabetes, (Turner et al., 1997) (Greenbaum et al., 2001).

Immune as well as metabolic features are detected in TID as well as a proportion of cases presenting with diabetes later in life termed as LADA. Autoantibodies to Islet antigens have been described previously and are a useful tool in the prediction of TID (Bingley et al., 1994), however changes in antibody isotype pattern could provide a better understanding in the maturation of the antigen specific immune response leading to TID. In this thesis the antigen specific isotypes were predominantly of the IgG1 isotype pattern.

CHAPTER 3.3

3.3. DETERMINE WHETHER THE ISOTYPE OF GADA AND IA-2A IN NON-INSULIN REQUIRING DIABETIC PATIENTS DIFFERED FROM THOSE FOUND IN TYPE 1 DIABETIC PATIENTS.

3.31. INTRODUCTION: The understanding of the pathogenesis of TID comes from random autopsy specimens of patients who died close to diagnosis of the disease. The degree of islet cell destruction varies from islets intact of beta cells to total beta cell destruction. At diagnosis up to 80% of sera from newly diagnosed patients can show reactivity to islet cell antigens (Kulmala et al., 2001), (Hawa et al., 1997), (Leslie et al., 1999). The nature and intensity of the islet cell specific autoantibody responses to IAA, GADA and IA2A identifies individuals at risk of developing TID. Furthermore antigen specific isotype response as well as subclass distribution may reflect the maturation of the immune response in the progression to TID. The IgG subclass response produced by plasma cells can vary depending on the stimulus. The isotype switching is further dependent on the DNA rearrangement in the immunoglobulin gene which further relies on T-helper cells as well as cytokines.

Both ICAs and GAD antibodies (GADA) can be detected in T1D patients before and after the onset of diabetes (Kulmala et al., 1998) in type 2 diabetic patients (Tuomi et al 1999), (Turner et al., 1997), and in patients with stiff - man syndrome (SMS), a rare neurological disorder (Solimena et al 1988). In T1D

patients, ICAs are polyclonal immunoglobulins (Millward et al., 1988), (Dozio et al., 1994).

In general, ICA is restricted to immunoglobulin of the IgG1 isotype belonging to the IgG subclass (Millward et al., 1988), (Dozio et al., 1994). Interferon γ – dependent antibody isotypes in mice are mainly IgG2a and IgG3 (probable human homologs, IgG1 and IgG3) which reflects a Th1 response (Abbas 1996), whereas a Th2 response is predominantly IgE or IgG1 (probable human homologs, IgG4 and IgE) (Abbas 1996). Restriction of ICAs to IgG1 in T1D in humans may therefore represent a Th1 dominant immune response. Antigen-specific antibodies have been found in a fraction of patients who do not need insulin, although many progress to insulin dependency within a few years (Kulmala et al., 1998), (Tuomi et al., 1999). These non–insulin requiring diabetic patients with autoantibodies may actually suffer from a slowly progressive form of T1D or, LADA. Neither has the isotype profiles nor the clonality of antigen-specific antibodies in T1D and non-insulin requiring diabetes been characterized.

3.32. RESEARCH DESIGN AND METHODS

Patients of European origin with either T1D or non-insulin requiring diabetes were studied, and their type of diabetes was determined by standard guidelines (Expert Committee 1997); cases in which this definition was uncertain were excluded. Classification of T1D was based on *1*) control of diabetes with insulin from time of diagnosis, *2*) diabetic ketoacidosis or marked ketonuria at time of clinical onset, *3*) lack of obesity, and *4*) age at diagnosis (40 years). Classification

of type 2 diabetes was based on *1*) adequate control of diabetes on diet alone or oral hypoglycemic agents at time of sampling, *2*) no history of diabetic ketoacidosis or ketonuria, and *3*) age at time of diagnosis (30 years).

Non-diabetic twins were administered oral glucose tolerance tests in doses of 75 g or 1.75 g/kg, whichever dose was less, to confirm that they were neither initially diabetic nor diabetic at intervals there-after. A cross - sectional study of antibodies to GAD_{65} and IA-2 was performed in a consecutive series of selected patients who attended a diabetes center and in a prospective study of a cohort of non-diabetic identical twins of T1D patients.

3.33. Subjects: The patients included 52 newly diagnosed T1D patients (mean age 20 years, range 5–36; 18 females) who were all tested within 1 month of diagnosis; 199 "type 2 diabetic" patients (mean age 57 years, range 30–75; disease duration 4 years, range 0–28; 101 female); and 200 normal healthy control subjects (mean age 29 years, range 14–71; 95 female) who were selected to have a similar distribution for age and sex to the combined groups of 251 diabetic patients. None of the control subjects had a family history of diabetes, were taking drugs, or had clinical signs of illness. The subjects gave informed consent, and the study was approved by the ethical committees at St. Bartholomew's Hospital and the University of Rome, La Sapienza.

Because isotype assays are expensive, the strategy was to test a sample of subjects, to identify, dominant isotypes and then to screen all type 1 and type 2

diabetic patients, for the dominant isotypes. Initially, antibody isotypes IgG1, IgG2, IgG3, IgG4, IgM, and IgE to both GAD and IA-2 were tested in:

1) 10 type 2 diabetic patients (mean age 55 years, range 34–75; five female) who were all GADA positive, and

2) A cohort of 34 non-diabetic identical twins of patients with T1D, 15 subsequently developed T1D, from the British Diabetic Twin study as described earlier and ascertained according to standard criteria, samples were tested both before and at diagnosis (NDDG 1979) (Expert Committee 2003). The mean period of follow-up was 47 months (range 4–156), the mean age at time of the first sample before diagnosis of T1D was 14 years (range 7–31), and the mean age at time of the second sample at diagnosis was 17 years (range 7–44; eight females). The 19 other twins remained nondiabetic (age at entry 17 years, range 4–36; nine females) having been followed for a median of 107 months (range 55–189); the earliest available samples on them were tested, and these twins had an estimated disease risk of 2% (1).

3) To establish a cutoff for positivity for this initial analysis, we also tested 37 control subjects (mean age 26 years, range 9–47; 24 males). Subsequently, selected dominant isotypes were tested in 52 T1D patients and 199 type 2 diabetic patients, and established the cutoff for positivity for dominant isotypes in 200 normal healthy control subjects.

3.34. Diabetes associated Autoantibody analysis: Autoantibodies to GADA and IA2A were measured as previously described in chapter 2.

3.35. GADA & IA2A Antibody Isotype initial experiments.

In vitro transcribed and translated GAD_{65} or IA-2 labeled with [3 5S]methionine were used as the antigens and were prepared as described earlier. A dose of the antigen GAD_{65} or IA2ic containing 50,000 counts/min (CPM)/ 50ul was incubated with 2 µl of test serum samples including a positive and a negative in house control sample, overnight, at 4°C in 96-well Millipore filtration plates (Millipore UK, (Catalogue No MHVBN4550B).

Monoclonal biotinylated Mouse anti-human antibodies was then incubated with the antigen antibody complexes and the immune complexes were then separated by employing streptavidin agarose, which binds biotin. Monoclonal biotinylated Mouse anti-human antibodies were tested from different sources namely Europath Laboratories as well as Southern Biotech (Clone IgG1 Cat No:9050-08, Clone IgG2 Cat No:9060-08, Clone IgG3 Cat No:9070-08, Clone IgG4 Cat No:9190-08, Clone IgG4 Cat No:9200-08 and Clone IgG3 Cat No:9210-08 at the manufacturer's suggested dilution of 1:100 in Tris Buffer Saline Tween as well as Phosphate Buffer Saline Ph 7.4. Positive as well as negative immune-reactivity was confirmed with the standard protein A sepharose protocol described in Chapter 2.

The initial data from these experiments were not encouraging as the immune complexes did not bind sufficiently to the streptavidin agarose and furthermore

gave non-specific binding which may have been due to the source of the biotin anti human antibodies employed as well as the nature of the liquid phase assay performed. The biotinylated antibodies employed here had only been tested in Elisa and western blotting systems and not in a liquid phase radio-immunoassay.

3.36. GADA and IA2ic Antibody isotypes assays

In vitro transcribed and translated GAD_{65} or IA-2 labeled with [^{35}S]methionine were used as the antigens and were prepared as described earlier in chapter 2. A dose of 50 µl of the antigen containing 50,000 counts/min (CPM) was incubated with 2 µl of test serum samples in duplicate overnight at 4°C in 96-well millipore filter plates thereby allowing an antigen antibody complex to be formed. The standard immunoprecipitation assays for GADA and IA2A employ Protein A Sepharose which does not bind all immunoblobulins as shown in Table 1.4, and cannot differentiate between the antibody isotypes. In order as to achieve specificity we employed biotin anti human isotype specific antibodies in conjunction with streptavidin agarose beads, the latter binds strongly to biotin, allowing separation of the antigen specific antibody isotypes in serum samples to overcome this issue.

To the immune complex Monoclonal biotinylated anti-human antibodies (IgG1 clone HP6069, IgG2 clone HP6002, IgG3 clone HP6047, IgG4 clone HP 6046, IgM clone HP6083 [Zymed, Camarillo, CA], and IgE clone G7-26 [Beckton Dickinson U.K., PharMingen, Oxford, U.K.]) at concentrations of 4, 4.8, 2.4, 1.6, 2, and 5 µg, respectively, were added so as to achieve optimal binding to the

immune complexes and incubated at 4°C on a shaker for 1.5 h. Antigen-antibody complexes bound to biotinylated monoclonal anti-human antibodies were separated for 1.5 h at 4°C with 50 µl of immobilized streptavidin agarose immobilized beads (Pierce & Warriner, Chester, U.K.), which binds biotin on the antibody molecules. Bound immune complexes were washed 12 times with Tris buffer saline containing Tween on the Millipore plate washer, were allowed to dry at room temperature before the addition of Meltilex solid scintillant (Wallac, Turku , Finland), and were counted on the Wallac 1260 Micro beta scintillation counter.

The current assay protocol for all the antigens utilises the Millipore plates MAHAN4B50 plates which eliminates the use of the solid scintillant and instead liquid scintillant Ultima Gold, 50µl per well for aqueous solutions was employed. This reduces the quenching effect on the CPM detected using the solid Meltilex scintillant system previously employed as well as improved the sensitivity of the assay procedure.

Each assay for IA-2 and GAD_{65} isotypes included a serum sample from a patient with a rare nerve disease SPS characterized with spasticity of the axial muscles who showed reactivity to all the tested isotypes, a prediabetic individual (a twin who subsequently developed T1D but was not part of this study) who was positive for GAD and IA-2 antibodies, and an antibody negative normal control subject to confirm specificity and reactivity of the antibody isotypes. To establish a cutoff for positivity, the results were calculated in control subjects as the mean

CPM for each antibody isotype minus the blank CPM. Positive results in patients were defined as CPM 3 SD above control mean levels and all samples scoring positive were repeated to avoid false positive signals as well as where duplicate results showed a significant variation

Each assay for IA-2 and GAD_{65} isotypes included a serum sample from a patient with a rare nerve disease SPS characterized with spasticity of the axial muscles who showed reactivity to all the tested isotypes, a prediabetic individual (a twin who subsequently developed T1D but was not part of this study) who was positive for GAD and IA-2 antibodies, and an antibody negative normal control subject to confirm specificity and reactivity of the antibody isotypes. To establish a cutoff for positivity, the results were calculated in control subjects as the mean CPM for each antibody isotype minus the blank CPM. Positive results in patients were defined as CPM + 3 SD above control mean levels and all samples scoring positive were repeated to avoid false positive signals as well as where duplicate results showed a significant variation.

3.37. Clonality

To determine clonality, T1D and non-requiring type 2 diabetes patients positive for the dominant GAD and IA-2 isotype as well as control subjects were tested. Sera were tested after immunoprecipitation as described above, except instead of using biotinylated monoclonal anti-human antibodies, we used biotin conjugated mouse anti-human κ light chain (clone G20-193) and λ light chain (clone JDC-12) monoclonal antibodies at a concentration of 10 μg per well, the reactivity and

the specificity of these subclass specific monoclonal antibodies have previously been established in a large World Health Organization study (Jeffries et al., 1985).

3.38. Results

3.381. GADA & IA2 antigen specific antibody assays: GADA was detected in 29/52 (56%)of the newly diagnosed TID patients, 10/199 (5%) type 2 diabetes and IA2 in 23/52 (44%) of the newly diagnosed TID and 5/199 (3%) of the type 2 diabetes patients. GADA or IA2 were more frequently detected in the newly diagnosed 37 (71%) compared with only 10 (5%) in the type 2 diabetic patients ($p < 0.0001$)**.**

3.382. Screening for dominant isotypes: Selected groups of patients who tested for positive GADA or IA2 were tested for isotypes IgG1, IgG2, IgG3, IgG4, IgM and IgE respectively as these assays were very costly. The groups included 34 identical twins discordant for diabetes followed prospectively and 15 were tested both before and at diagnosis of diabetes, 19 non diabetic twins and 10 type 2 diabetic patients (Table 3.30). The samples tested showed IgG1 dominance for both GAD & IA2 antigens both before occurring in 8 and 8 out of 15 and at diagnosis of diabetes in 7 and 10 out of 15 twins respectively. The isotype profile for isotypes other than IgG1 was noted for GADA in the prediabetic samples for IgG3 (3 of 15), IgG4 (1 of 15) and IgM (1 of 15) and for IA2 IgG2 (3 of 15). The isotype profile for isotypes other than IgG1 for GADA at diagnosis for IgG2 (3 of 15) and for IA2 IgG2 (3 of 15) and IgE (2 of 15) respectively. In these samples both before and at diagnosis of diabetes there was no evidence of a significant

spreading or switching of the isotype response to either of the diabetes associated antigens tested (Table 3.30). Similarly there was no significant difference in the isotype antibody pattern with type 1 and type diabetes and in the latter GADA showed 5 out 10 with IgG1 isotype, no other isotypes were noted for IA2 other than IA2 antibodies.

Of the 19 non-diabetic twins tested GADA antibodies were detected in only 1 patient who also had GADA IgG1 isotype but was negative for both IA2 antibody as well as isotypes and to date remain diabetes free.

Antibodies	Non diabetic twin	TID Twin pre-diagnosis	TID Twin At diagnosis	'Type 2 diabetes ' Patients
N	19	15	15	10
GADA	1	12	10	10
GADA IgG1	1	8	7	5
GADA IgG2	0	0	3	0
GADA IgG3	0	3	0	0
GADA IgG4	0	1	0	0
GADA IgE	0	0	0	0
GADA IgM	0	1	0	0
IA2A	0	13	12	4
IA2A IgG1	0	8	10	0
IA2A IgG2	0	3	3	0
IA2A IgG3	0	0	0	0
IA2A IgG4	0	0	0	0

Table 3.30 Data shows numbers scoring greater than ±3SD of control (n-37) tested for each antibody GADA or IA2 and their antibody isotype. None of the control subjects were positive for any antibodies or antibody isotypes tested.

3.383. Screening for selected isotypes: The initial experiments had shown that the dominant isotypes for both GADA and IA2 was IgG1. A Th1 and Th2 immune response in the mouse is associated with IgG2a and IgG1 antibodies, the human homologues are probably IgG1 and IgE which reflect the Th1 and Th2 immune response. Evidence suggests that the Th2 immunity is associated with IgG4 and IgE responses, because both can be stimulated by the Th2 cytokine IL-4 Lundgren et al., 1989), (King & Nutman 1993). Because IgG1 and IgE could reflect a Th1 and Th2 immune response we elected to screen all 251 patients (52 newly diagnosed T1D and 199 "Type 2 diabetics") for these two isotypes**.**

Of the 52 T1D patients tested GADA IgG1 was detected in 15 patients (29%) and 56% were GADA positive, IA2A IgG1 was detected in 22 patients (42%) and 44% were positive for IA2A (Figure 1.4 & Figure 1.5)

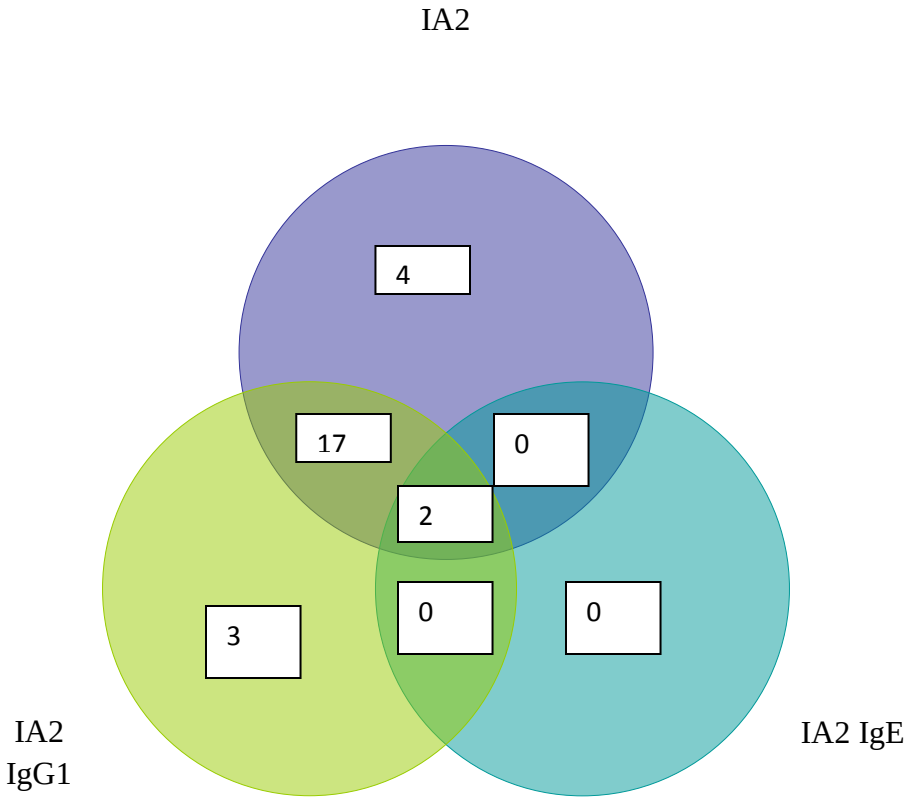


Figure 3.4: IA2A antibodies and IA2 IgG1 and IgE isotypes in newly diagnosed TID (n=52). Intersecting regions indicate number of patients with combinations of antibodies and isotypes.

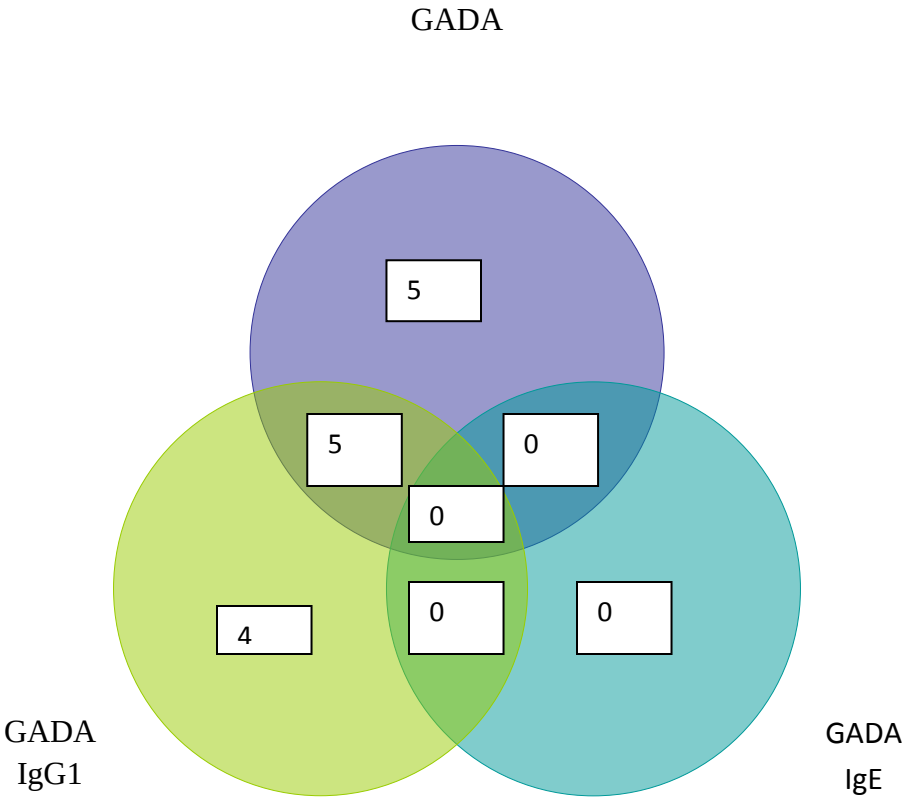


Figure 3.5, GADA antibodies and GADA IgG1 and IgE isotypes in newly diagnosed T1D patients (n=199). ("Of Type 2 diabetics" none had GADA IgE).

3.384. Discussion: The aim of the study was to determine whether the isotype and clonality to key antigens GADA and IA-2A in type 2 diabetic patients differed from those found in T1D patients and to test if there was any evidence of isotype switching before and at diagnosis. By employing biotinylated antihuman isotype specific antibodies in the context of a radiobinding assay we found that the majority of the response to these key antigens was restricted to IgG1 subclass in both T1D as well as 'type 2 diabetes'. Furthermore in the twins there was no

evidence of isotype switch before or at diagnosis with IgG1 as the dominant isotype detected suggesting that a dominant Th1 type immune response may be present from initiation of the disease in these twins.

These findings are consistent with the findings in the BABYDIAB study where diabetes onset and β cell destruction occurred during an early or subsequent IgG1 dominant antibody response (Bonifacio et al., 1999). None of the other isotypes tested to either antigen GADA or IA2 were detected in more than a few of the patients tested. These findings are in agreement with other studies of ICA and GADA in newly diagnosed T1D patients and monoclonal GADA derived from T1D patients, all showing a predominant IgG1 isotype (Millward et al 1988), (Dozio et al., 1994), Madec et al., 1996), (Bonifacio et al., 1999).

A broad isotype response to GADA was shown in our previous study in patients with a rare nerve disease SMS, but an IgG1 restriction in T1D and 'type 2 diabetes' patients (Hawa et al., 1998). There was no shift in the Th1, Th2 phenotype for the antibody isotype response to either GADA or IA2 in this study with IgG1 as the dominant isotype detected. There is evidence for an antibody response being characteristics of a Th immune response phenotype and in humans IgG1 is linked with a Th1 immune response while IgG4 is linked with Th2 immunity. In the BABYDIAB study an IgG4 response was noted to insulin but it was not followed by an IgG4 response to GADA or IA2 indicating that IgG4 response is not antigen specific Bonifacio et al., 1999).

Interestingly none of the healthy controls or T1D patients had GADA IgG1 in the absence of GADA antibodies. Previous reports have shown 'type 2 diabetic' patients who are ICA and or GADA positive tend to have similarities to the classical TID patients, including low or normal body weight, presence of HLA DR3 and DR4 and a tendency to go on to require insulin therapy (Tuomi et al., 1999), (Leslie & Pozzilli 1994), Niskanen et al., 1995). In this present study the frequency of IA2 antibodies in patients with 'type 2 diabetes' was less common than GADA consistent with other reports (Seissler et al.,1998).

These results support the hypothesis that patients with apparent type 2 diabetes because they are initially non-insulin requiring, but with autoimmune features, have similarities in the disease process to autoimmune T1D. If this is true than such cases would potentially be ideal for intervention studies to try and modulate the disease process, thereby avoiding the ethical concerns of intervention studies in antibody positive non diabetic healthy children.

In conclusion these observations confirm that the nature of the antigen specific antibodies in both T1D and adult-onset non-insulin requiring diabetes masquerading as "type 2 diabetes", are similar (with a dominant IgG1 isotype/ sub type) and consistent with a Th1 type immune response.

CHAPTER 4

4.0. WHETHER AUTOANTIBODIES IN ADULTS PRESENTING WITH APPARENT TYPE 2 DIABETES ARE PREVALENT AND SIMILAR TO T1D.

4.01. Aims: 4.01) T**o** characterize adult-onset autoimmune diabetes in European patients, presenting with type 2 diabetes. **4.02)** Are autoantibodies prevalent in patients presenting with type 2 diabetes analysis of the Action Lada and Collaborative Atorvastatin Diabetes Study (CARDS) cohorts **4.03)** Evidence of Bimodality using Quintile Quintle (QQ) Plot for GADA **4.04)** Do autoantibodies differentiate LADA cases? **4.05)** Is there evidence of non-diabetes associated autoimmunity in LADA patients.

4.06. INTRODUCTION: Latent Autoimmune Diabetes of Adults onset (LADA) is defined by three criteria: 1) age of diagnosis >30 years 2) presence of diabetes-associated antibodies and 3) insulin-independence for six months post-diagnosis (Rolandson 2010). The prevalence of LADA in adults presenting with non-insulin requiring diabetes is approximately 10% with 4-12% of Caucasian's presenting with diabetes associated autoantibodies. LADA therefore may be more common or as common as T1D. However there remains extensive contention as to whether LADA can be classified as a separate disease to TID due to their adult age at onset, presence of autoimmunity, that they remain free from insulin therapy for at least 6 months after diagnosis and show less genetic load in comparison to the classic T1D (Desai 2008), (Gale 2005).

Action LADA is a European study which sought to address these questions by ascertaining adult-onset diabetic patients in nine European hospital-based clinics within a defined period and screening them for GADA. We hypothesized that autoimmune diabetes would be prevalent in Europe, that LADA would be more prevalent than T1D and would show phenotypic differences from it.

4.07. Participating countries: Serum samples were obtained from 9 participating centres, Odense University Hospital (coded OUH), Denmark , Royal Victoria Hospital, Belfast UK (coded RGH), University of Rome "La Sapienza" Italy (coded IT, ITA,B,C,D,E,F,G &H), University of Düsseldorf, Germany(coded UKD & DDFI), Rudolfstiftung Hospital, Austria(coded ISF), National Public Health Inst, Finland (coded KTL A,B,C,D & E), Hospital de Sabadell, Barcelona Spain (coded RIH), Hospital Edouard Herriot, Lyon, France (coded UCB) and London (coded L).

Participating centres collected samples from suitable patients fulfilling the inclusion criteria and shipped sample aliquots in duplicate on dry ice to London where they were stored at -20° C prior to analysis. Samples were tested in duplicate in batched assay's to avoid assay variation.

4.08. Research Design: The aim was to screen 10,000 newly-diagnosed non-insulin requiring diabetes cases for GADA. To estimate the prevalence and epidemiology of LADA in Europe a sample size of around 1200 people per centre (nine centres involved in screening) will enable the prevalence to be estimated

with 95% certainty, as being between 8% and 12% (based on the UK study (Turner et al., 1997), by identifying 1,000 LADA cases (about 120 per center).

In the present study a consecutive series of 6809 patients attending European clinics (age range 30-70 years) were examined cross-sectionally and centrally for autoantibodies to diabetes associated antibodies, glutamic acid decarboxylase (GADA), insulinoma-associated antigen-2 (IA-2A) and zinc-transporter8 (ZnT8A). Autoantibody-positive patients were designated T1D (when insulin-treated immediately at diagnosis), and latent autoimmune diabetes (LADA) (when they remained free of insulin treatment for 6 months).

4.09. PATIENTS AND METHODS

4.10. Action LADA Cohort: All patients came from 9 European hospital-based centers involved in the ACTION LADA Project, a European Union-funded multi-centre European study with the aim of identifying immune and clinical risk factors for adult-onset autoimmune diabetes (www.actionlada.org). Patients were designated with diabetes according to standard criteria and LADA was defined as patients aged 30- 70 years of age with autoantibodies to GAD_{65} and who did not require insulin treatment for at least 6 months post-diagnosis (Leslie et al., 2008) .

4.11. Inclusion criteria: Diagnosis of diabetes (with at least 2 fasting blood glucose measurements $\geq$ 7 mmol/L); time from diagnosis < 5 years for all non-insulin requiring diabetes patients; all patients were aged 30 – 70 years at

examination.

4.12. Exclusion criteria: Incomplete data set, pregnant at the time of sampling, renal disease with raised creatinine or proteinuria; acute illness at the time of testing; in addition, patients with non-insulin requiring diabetes > 5 years disease duration were excluded as well as subjects with a family history of autoimmune disease. Data on medication and risk factors were registered by the attending physician based upon the medical files. Serum and plasma samples were collected according to standard procedures and stored at –20°C in a freezer linked to the Tutela Alarm System.

Each subject was tested locally for waist circumference, blood pressure as well as standard demographics where possible. Lipids, and lipoproteins (serum total HDL cholesterol and triglycerides) were determined by standardized assays at each center. All patients were tested for GADA, IA2A and ZnT8 antibodies in the central laboratory (London).

4.13. Collaborative Atorvastatin Diabetes Study Cohort (CARDS): The Collaborative Atorvastatin Diabetes Study (CARDS) was a double-blinded, randomized, placebo-controlled multicentre trial of atorvastatin for the primary prevention of cardiovascular disease in presumed type 2 diabetes patients (defined by WHO criteria, median age 62yrs and duration of diabetes 8yrs) who received atorvastatin or placebo and were evaluated for diabetes outcomes over a median follow up of 4yrs). A total of 2838 patients were included in the study.

4.14. Antibody Measurement: The radio-immuno-precipitation assay for GADA, IA2A and ZnT8 were performed as described in chapter 2. All samples were tested centrally in London in duplicate including positive and negative control standard sera. In the DASP 2010 workshop our assay for GAD_{65} had a sensitivity of 76% and a specificity of 98%, for IA2ic a sensitivity of 54% and a specificity of 100% and for ZnT8 a sensitivity of 52% and a specificity of 98%. Positive samples were repeated to confirm GADA, IA2A and ZnT8 positivity and to reduce false positive rate.

4.15. Thyroid peroxidase (TPOA) and parietal cell (PCA)

TPOA and PCA autoantibodies were measured by ELISA in Germany by Dr Jochem Seissler on all diabetes-associated autoantibody positive patients (n=1130; mean age 52.3 ± 10.1years, range 30-69 years) and compared with a randomly selected cohort of diabetes-associated autoantibody negative patients (n=690; age 54.5 ± 9.0 years, 30-70 years). All serum samples of patients positive for GADA, IA2 or ZNT8 were tested as well as GADA negative cases from five centres Germany, Austria, Belfast, Rome and London where sufficient sera was available were investigated in a blinded fashion for antibodies: thyroid peroxidase (TPO) and parietal gastric cells (PCA).

4.16. Bimodal Distribution: A bimodal distribution in statistics is a continuous distribution of data with different modes and one example would be to look at the heights of men and women which when put together would have two different peaks with two different modes for each of the genders. In the NIRAD study,

(Buzzetti et al., 2007) the distribution of GADA titres showed an apparent bimodal distribution. In that study the patients (n=191 in total) were divided into two subgroups representing the two distributions namely low (<32U) and high (>32U) (Buzzetti et al., 2007) and the data was log transformed. This study based on the GADA titers identified two subgroups of patients with adult onset autoimmune diabetes having distinct clinical, autoimmune as well as genetic features. Patients with high GADA titers had features of insulin deficiency, extended autoimmunity as indicated by the presence of IA2 antibodies as well as TPO antibodies in the NIRAD study. In this present cohort the data set for GADA, IA2A and ZnT8A was similarly analyzed for evidence of bimodality.

4.17. Statistical analyses

The differences between groups were tested with X^2 test or Fisher's exact test when appropriate. Quantitative variables were analyzed with General Linear Model Univariate, a post-hoc analysis was performed with Bonferroni test and data presented as mean (SD). All analyses were performed using SPSS statistical software for Windows. A P value <0.05 was considered statistically significant. Q-Q probability plots were used to analyze the distribution of GADA measurements for normality. Observed antibody values were plotted along the horizontal axis against expected normal values under normality on the vertical axis using Blom's proportion estimation formula. The study protocol is in accordance with the Declaration of Helsinki, and was approved by local ethics

committee in each study center. Informed written consent was obtained from all subjects before blood sampling.

4.18. RESULTS

4.19. QQ Plot Analysis:

A Q-Q plot of GADA positive patients was performed to seek distinct populations, we identified an inflexion point corresponding to 70 and 200 WHO IU consisting with two modes. Further, a plot of log GADA titre according to patient frequency revealed a possible bimodality, and the lowest value between the two modes was at GADA titre 200 WHO IU. Therefore, we arbitrarily analyzed the data according to GADA positivity in those between 70 to 200 WHO units and those greater than 200 WHO units of GADA.

The cut off for positivity was selected arbitrarily based on the end point of the standard curve and further confirmed with Quantile-Quantile (Q-Q) probability plots. QQ plots were used to analyses the distribution of measurements for normality and, for GADA, the vertical line at 70 WHO units indicated the laboratory cut off with values greater than 70 units scored positive **Figure 4.1**, For IA2A at 25 WHO units indicated the laboratory cut off **Figure 4.2, Figure 4.3** and for ZnT8A at 10 Laboratory units indicating the laboratory cut off for positivity **Figure 4.4**. and for the CARDS data set for GADA values greater than 40 units scored positive

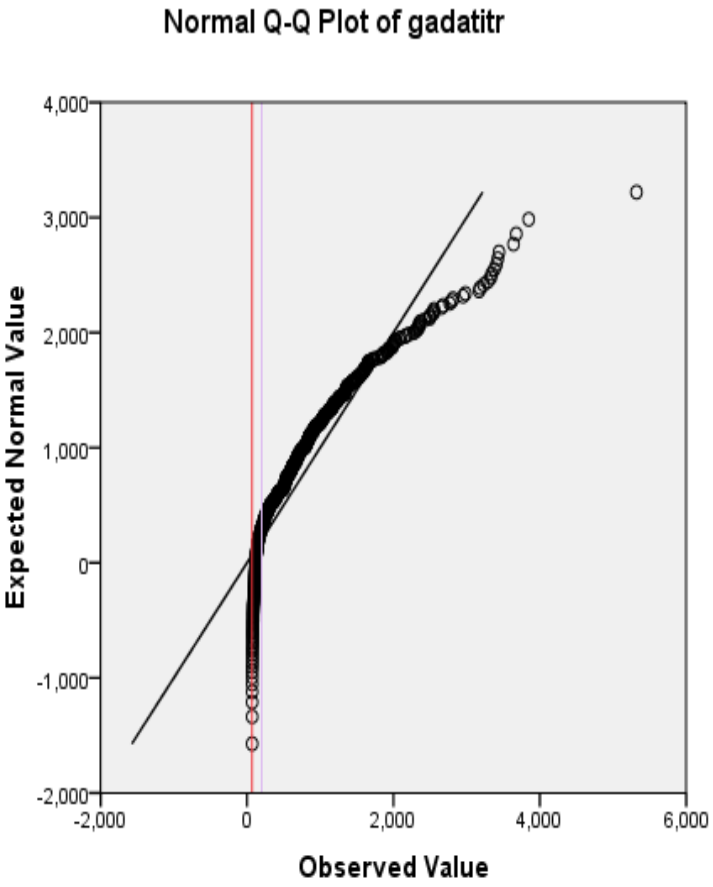


Figure 4.1: QQ plot of GADA positive cases. The red line indicates the cut off at 70 units with a second inflection point at 200 units shown in purple with a further inflection at 400 units.

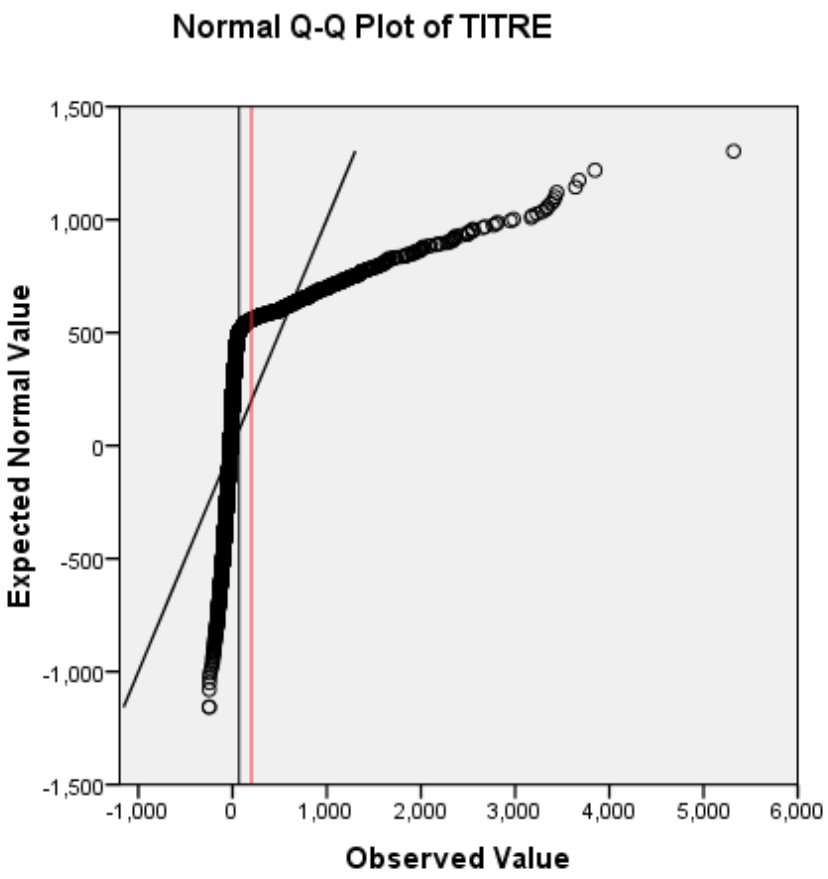


Figure 4.2: GADA QQ plot for all cases, the first vertical line indicates the Laboratory based cut off for positivity for GADA this was set at 70 units based on the end point dilution of the standard curve and as indicated by the vertical line in the QQ plot. The second vertical line indicates another inflection point equivalent to 200 units the limit for high titre GADA. A closer analysis of the dataset shows another inflection point at 400IU.

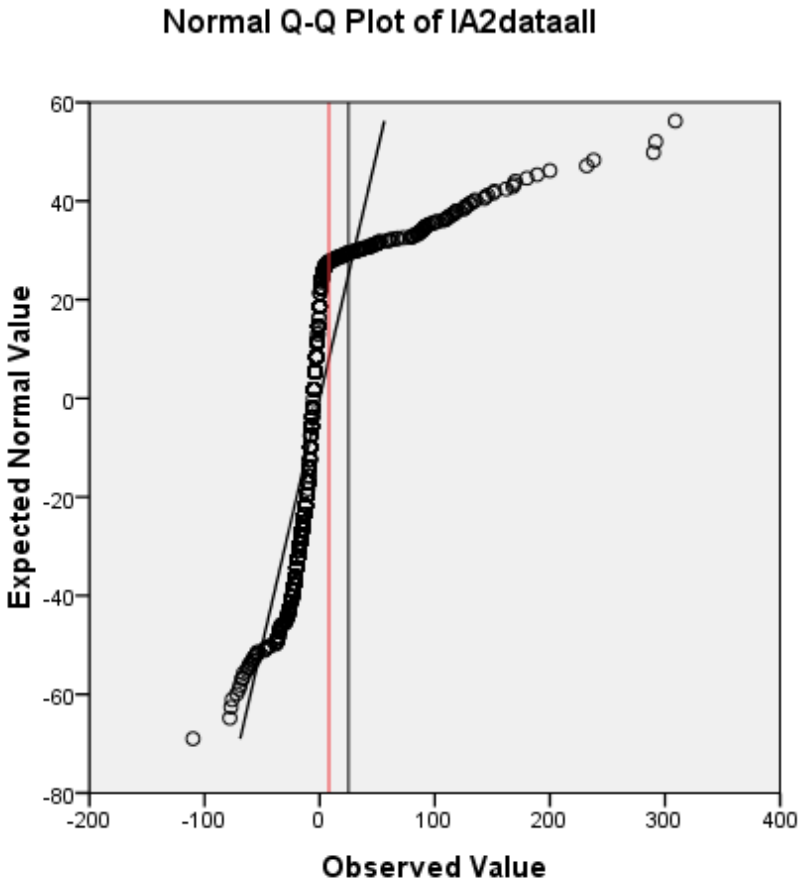


Figure 4.3 IA2A QQ plot for all cases, the vertical line indicates the Laboratory based cut off for positivity for IA2A this was set at 25 units based on the end point dilution of the standard curve and as indicated by the vertical line in the QQ plot. The red line indicates the calculated inflection point at 8 units. Changing to the lower cut off will improve the sensitivity of the assay but this could reduce the specificity of the assay.

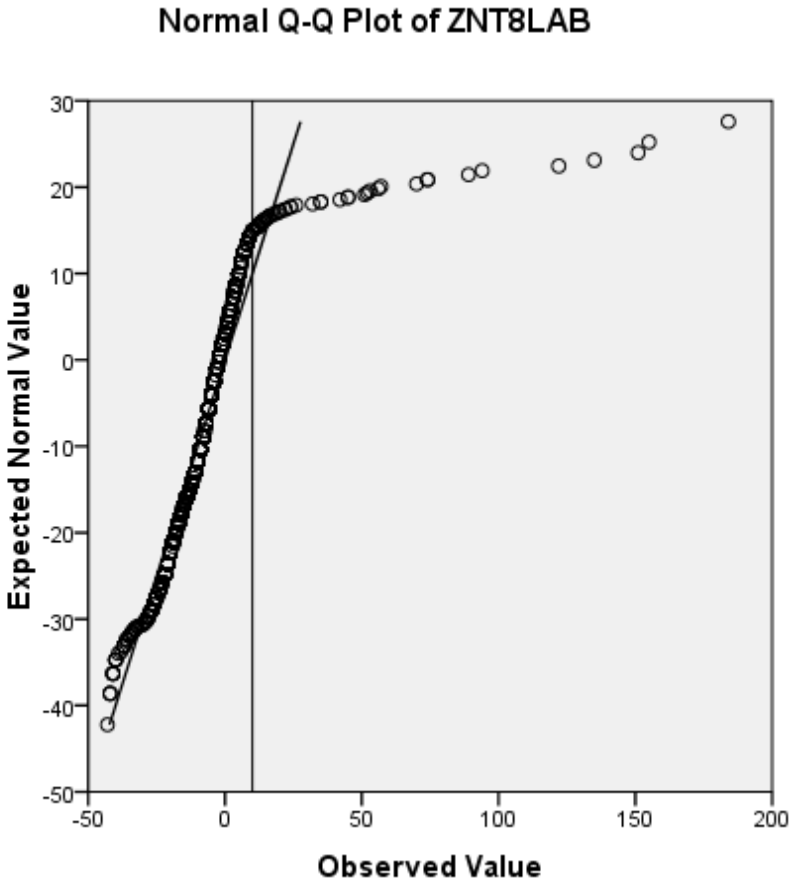


Figure 4.4: ZnT8 QQ plot for all cases, the vertical line indicates the Laboratory based cut off for positivity for ZnT8A this was set at 10 units based on the end point dilution of the standard curve and as indicated by the vertical line in the QQ plot.

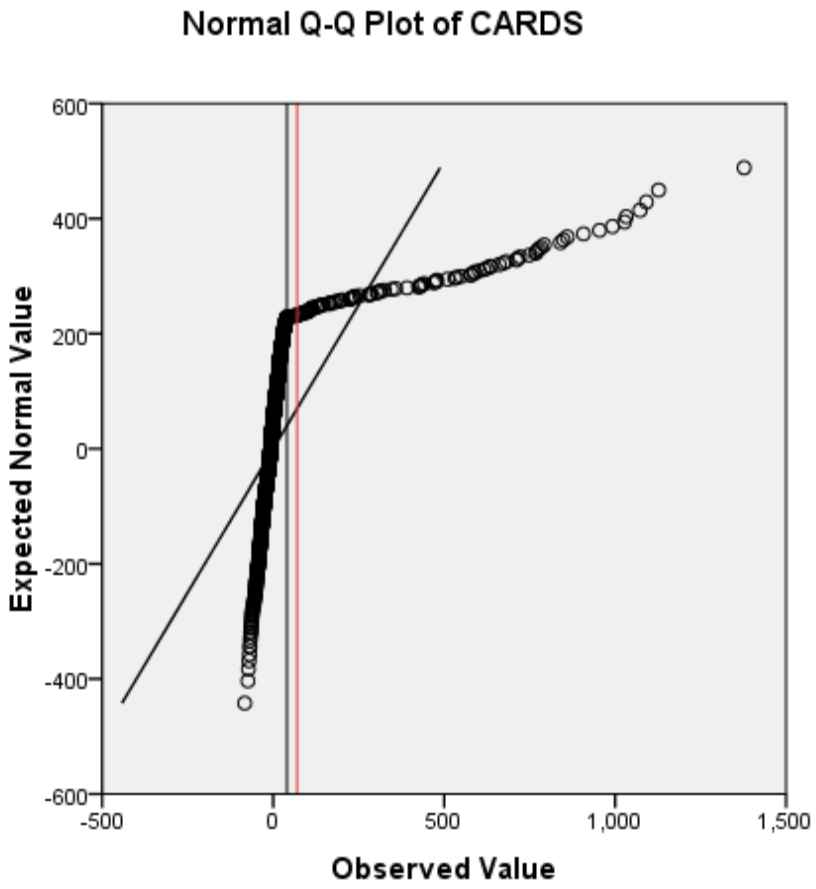


Figure 4.5: CARDS Study GADA QQ plot for all cases, the vertical line indicates the Laboratory based cut off for positivity for GADA this was set at 40 units based on the end point dilution of the standard curve shown in black and as indicated by the vertical line in the QQ plot, the red line indicates the cut off at 70 units.

4.20 Characteristics of adult-onset autoimmune diabetes in Europe in patients presenting with 'type 2 diabetes': A total of 6,809 patients were screened for diabetes-associated autoantibodies from the Action LADA cohort. Of these 654 subjects were misclassified, due to inappropriate age or disease duration, and were excluded. Of 6,155 subjects fulfilling the inclusion criteria: 84.6% were Caucasian, 2.5% Asian, 1.2% African, 4.5% came from Middle East, and 7.2% were other. Their mean (SD) age was 54.4 (9.6) years, mean duration of diabetes was 2.2 (1.6) years and 58.5% were males.

A) Of 6,156 patients aged 30-70 years presenting with diabetes, 598 (9.7%) were positive for at least one autoantibody, with mean age 49.6 (10.8) years, GADA alone was detected in 8.8%. The demographics of the GADA positive compared to GADA negative groups are shown in Table 4.1. The demographics of the any autoantibody positive compared to autoantibody negative groups are shown in Table 4.2. Of autoantibody positive IA2A and ZnT8A(irrespective of GADA) compared with autoantibody negative patients (i.e. patients with 'type 2 diabetes'), mean age and mean age of onset were significantly lower ($p<0.0001$) (Table 4.3).

Analysis of BMI was corrected for age of onset, gender, duration of disease and other analyses were corrected for age of onset, gender, duration of disease, BMI. Of autoantibody positive compared with autoantibody negative patients after this correction: mean age, age of onset, BMI, waist hip ratio (WHR) and waist circumference were significantly lower ($p<0.0001$), while HDL cholesterol levels were higher ($p <0.0001$) (Table 4.2). Similarly and more important to note

patients with auto-antibodies that is having an antibody in the absence of GADA also tended to be younger, leaner and more often on insulin in comparison to the autoantibody negative cases (Table 3).

B) **Of 2838 CARDS patients**, sufficient sera as well as clinical data was available for GADA screening in 2425 patients. (Ethical approval was acquired by the industrial partner Pfizer UK.) The demographics of the GADA positive compared to GADA negative groups are shown in Table 4.4. All CARDS study samples were tested in batched assays to avoid assay variability and all samples scored positive were repeated to avoid false positives. The cut off for positivity was based on the end point dilution of the standard curves in this batched assay was set at 40 WHO IU. The data obtained from the QQ plot analysis (Figure 4.5) again agreed with the cut off value set for positivity, though lower than in the Action LADA Cohort presented earlier where the cut off for positivity was established at 70WHO IU. This lower cut off set at 40 WHO IU however only added an additional 10 cases under the cut off of 70 WHO IU. Therefore caution must be heeded in analyzing such large sets of data and the utilization of tools such as the QQ plot should be considered.

	GADA Positive	GADA Negative	
Cases	**541**	**5615**	
Females*	n=279 (52.0%)	n=2238 (40%)	P<0.0001
Males	n=258 (48%)	n=3335 (59.6%)	
Mean age (years)	49.5 (10.8)	55 (9.6)	P<0.0001
Age of onset (years)	47.4 (11.7)	53. 1 (18.6)	P<0.0001
Duration Disease (years)	2.2 (1.6)	2.2 (1.6)	P=0.75
BMI (kg/m^2)	27 (6.3)	30.9 (6.4)	P<0.0001
Subjects on insulin (%)	259/509 (50.9%)	756/5109 (14.8%)	P<0.0001
Time to Insulin (years)	0.61 (1.03)	0.87 (1.4)	p<0.18
Waist-Hip ratio	0.90 (0.1)	0.953 (0.09)	P<0.0001
Waist Circumference	93.1 (16.1)	103.3 (14.4)	P<0.0001
HDL Cholesterol (mmol/L)	1.47 (0.46)	1.27 (0. 47)	P<0.0001
LDL Cholesterol (mmol/L)	2.3 (1.01)	2.22 (1.01)	P=0.99

Table 4.1. Features of Action LADA Cohort GADA positive versus GADA negative diabetes patients. Mean (SD). *missing information for gender (n=4). More autoantibody positive patients were receiving insulin (50.9% compared with 14.8% autoantibody negative patients; p<0.0001). No differences were seen in LDL cholesterol values.

	Autoantibody Positive	Autoantibody Negative	P value
Cases	**598**	**5558**	
Females*	n=303 (50.7%)	n=2260 (40.7%)	<0.001
Males	n=295 (49.3%)	n=3298 (59.3%)	
Mean age (years)	49.6 (10.7)	54.9 (9.4)	<0.001
Age of onset (years)	47.4 (11.7)	52.5 (10.6)	<0.001
Duration Disease (years)	2.2 (1.6)	2.3 (1.6)	>0.50
BMI (kg/m^2)	27.2 (6.2)	30.9 (6.5)	<0.001
Subjects on insulin (%)	279/564(49.5%)	728/5508 (13.2%)	<0.001
Time to Insulin (years)	0.61 (1.03)	0.87 (1.4)	0.180
Waist-Hip ratio	0.90 (0.1)	0.95 (0.09)	<0.001
Waist Circumference(cm)	93.7 (16.1)	103.8 (14.6)	<0.001
HDL Cholesterol (mmol/L)	1.44 (0.4)	1.29 (0. 39)	<0.001
LDL Cholesterol (mmol/L)	2.05 (0.95)	1.60 (0.82)	>0.50
Systolic BP (mmHg)	116.6 (29.1)	122 (31.0)	<0.001
TGL (mmol/L)	1.5 (1.4)	1.97 (1.5)	<0.001

Table 4.2: Features of any autoantibody positive versus autoantibody negative patients (Mean, SD)

	ZnT8 and/or IA-2A Positive (GADA negative)	Autoantibody Negative	P value
Cases	**57**	**5558**	
Females*	n=21 (36.8%)	n=2260 (40.7%)	>0.5
Males	n=36 (63.2%)	n=3298 (59.3%)	
Mean age (years)	50.4 (10.3)	54.9 (9.4)	<0.001
Age of onset (years)	47.5 (12.4)	52.5 (10.6)	0.005
Duration Disease (years)	1.9 (1.7)	2.3 (1.6)	0.19
BMI (kg/m^2)	28.5 (1.7)	30.9 (6.5)	<0.001
Subjects on insulin (%)	20/57 (35%)	728/5508 (13.2%)	<0.001
Time to Insulin (years)	Insufficient data	0.87 (1.4)	_____
Waist-Hip ratio	0.9 (0.1)	0.95 (0.09)	0.002
Waist Circumference (cm)	96.1 (12.7)	103.8 (14.6)	0.003
HDL Cholesterol (mmol/L)	1.42 (0.33)	1.29 (0. 39)	0.66
LDL Cholesterol (mmol/L)	3.1 (0.6)	1.60 (0.82)	>0.5
Systolic BP (mmHg)	121.9 (22.9)	122 (31.0)	>0.5
TGL (mmol/L)	1.6 (0.4)	1.97 (1.5)	>0.5

Table 4.3: Features of patients positive for autoantibodies other than GADA versus autoantibody negative patients. Mean (SD).

Baseline Characteristics	**GADA Positive (n = 173)**	**GADA Negative (N = 2252)**	**p-value**
Age at diagnosis (Years)	51.51 ± 9.28	54.98 ± 9.43	< 0.0001
Duration of diabetes (Years)	10.16 ± 7.04	7.63 ± 6.24	< 0.0001
BMI (kg/m^2)	27.31 ± 3.65	28.91 ± 3.49	< 0.0001
HbA1c (mmol / L)	8.459 ± 1.509	7.778 ± 1.380	< 0.0001
LDL-C (mmol /L)	3.121 ± 0.687	3.026 ± 0.704	0.0876
HDL-C (mmol /L)	1.559 ± 0.392	1.389 ± 0.319	< 0.0001
TGL (mmol/L)	1.560 ± 0.955	1.978 ± 1.081	< 0.0001

Table 4.4. CARDS Cohort Characteristics GADA Positive versus GADA Negative

4.21. EVIDENCE OF BIMODALITY USING QUINTILE QUINTLE (QQ) PLOT FOR GADA

To further differentiate autoantibody positivity, according to autoantibody titres on the whole cohort we used the QQ plot analysis. The QQ plot confirmed the selection of the laboratory based cutoff for positivity at 70 WHO IU based on the end point dilution of the standard curve employed independently, as well as identifying two distinct cohorts among GADA positive subjects, with an inflection at 70 and another at 200 WHO IU (GADA low) and another inflection at 200 WHO IU (GADA high) (Figures 4.1 & 4.2).

In order to test for bimodality further, GADA titre data from the Action LADA cohort was $\log_{10}$-transformed and a histograms illustrating the distribution of GADA high and low $\log_{10}$-transformed titres were constructed using SPSS shown in Figure 4.6. Normality was analyzed in each of the distributions by the Shapiro-Wilk statistic, which tests the null hypothesis that the sample in question conforms to a normal distribution. However from the statistical analysis using Shapiro-Wilk test for both low titre as well as high titre GADA it was apparent that the data was not normally distributed ($p<0.05$). A similar approach was utilized by Buzzetti et al 2007 to show a bimodal distribution for GADA titres ((Buzzetti et al., 2007) where they log transformed the dataset. Of note log transformation changes the quality of the dataset and cannot be used to justify the presence of bimodality in the original data.

It is important to note the number of subjects in each group, the Action LADA set

of n=701 (high titre (n=511) low titre (n=190)) was substantially larger in comparison to published NIRAD data (Buzetti et al 2007) of which n= 191 (high titre (n=94) low titre (n=97).

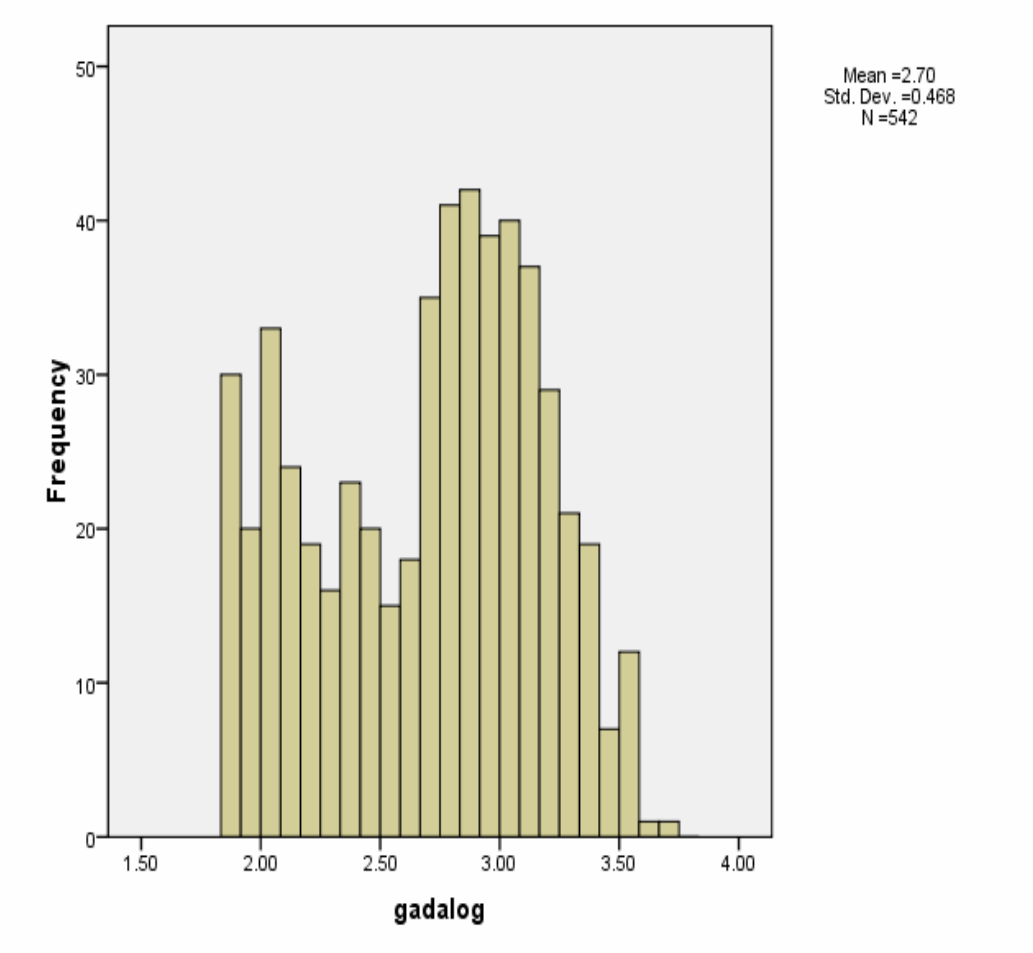


Figure 4.6 Action LADA dataset bimodality distribution analysis. A histogram of the $\log_{10}$ transformed GADA titres for the Action LADA dataset revealed an apparent bimodal distribution. The distributions showed two modes however each mode was not normally distributed.

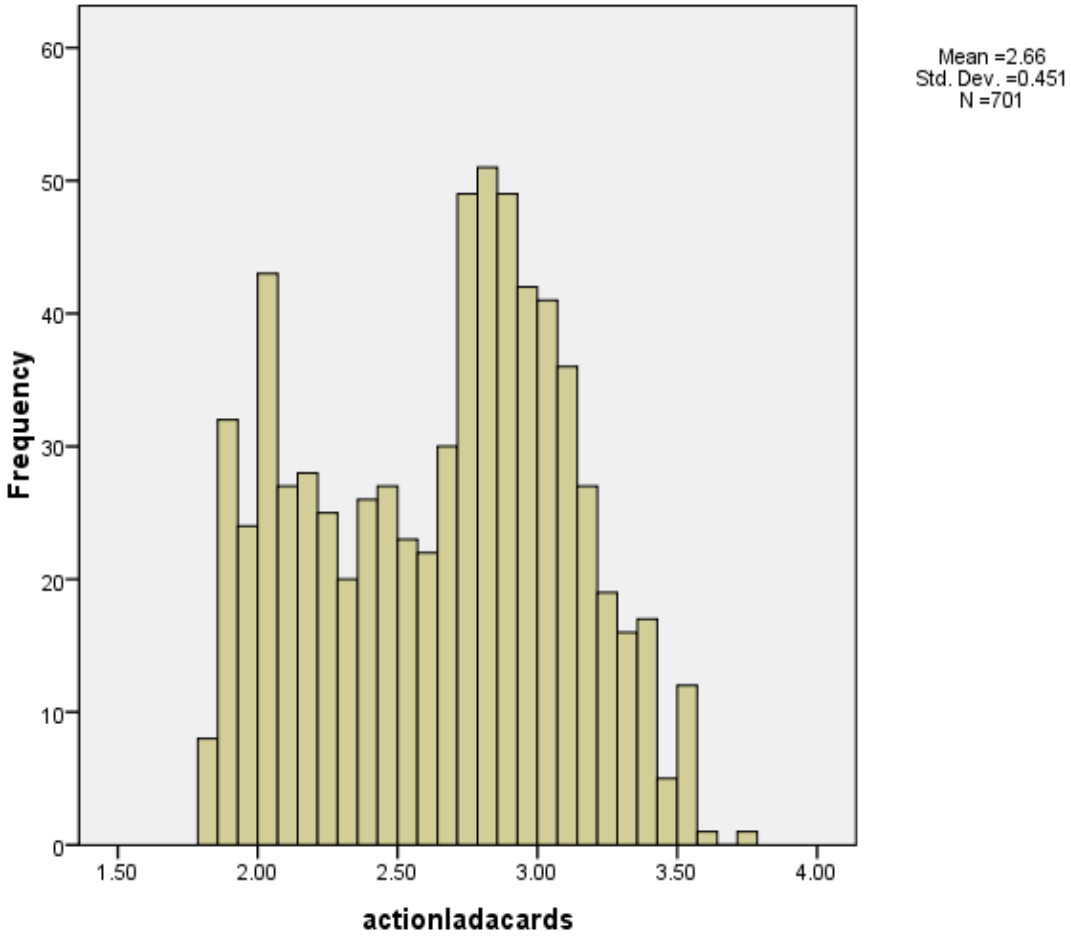


Figure 4.7: Action LADA & CARDS combined dataset bimodality distribution analysis A histogram of the $\log_{10}$ transformed GADA titres for the combined data for Action LADA and CARDS dataset revealed an apparent bimodal distribution. The distributions showed two modes 70-200 and another >200 in a total of 701 cases.

Autoimmune diabetes patients with high titre GADA (n=403), in contrast to low titre GADA (n=138), were more likely to be female, younger, with lower BMI ($p<0.01$ for all comparisons) and lower triglycerides ($p=0.01$) and systolic blood pressure ($p=0.04$) (data not shown) with more on insulin treatment (54.6% versus 39.7%; $p=0.0045$) (Table 4.5). When compared with GADA negative patients, high GADA titre patients had lower waist circumference, WHR as well as higher HDL cholesterol, and more patients were insulin treated ($p<0.0001$ for all comparisons) (Table 4.5). Compared to GADA negative patients, the low GADA titre group also had lower waist circumference, WHR and BMI ($p<0.01$ for all comparisons), as well as higher HDL cholesterol levels ($p=0.02$) and more patients were insulin treated ($p<0.0001$) (Table 4.6). Among all GADA positive patients there was an inverse correlation between GADA titre and BMI ($r= -0.054$, $p=0.243$), WHR ($r=-0.103$, $p=0.047$) and time to insulin therapy ($r=-0.156$, $p=0.032$), but not age at diagnosis ($r=0.129$, $p=0.010$). Time to insulin was positively correlated with age at diagnosis ($r=0.214$, $p=0.007$); the younger the age at diagnosis, the earlier insulin was likely to be introduced.

	GADA High	GADA Low	P value
Cases	**403**	**138**	
Females **Males**	220 (54.4%) 180 (44.6%)	59 (42.8%) 78 (56.5%)	0.02
Mean age (years)	49.3 (10.7)	50.2 (10.9)	0.09
Age of onset (years)	47.0 (14.0)	47.6 (10.8)	0.08
Duration Disease (years)	2.14 (1.6)	2.46 (1.7)	0.07
BMI (kg/m^2)	26.7 (6.1)	28.5 (6.6)	0.008
Subjects on insulin (%)	207/379 (54.6%)	52/131 (39.7%)	0.005
Time to insulin (y)	0.67 (1.0)	0.86 (1.4)	0.81
Waist-Hip ratio	0.90 (0.1)	0.89 (0.1)	0.10
Waist Circumference	92.0 (15.7)	96.7 (16.6)	0.013
HDL Cholesterol (mmol/L)	1.49 (0.5)	1.44 (0.5)	0.6
LDL Cholesterol (mmol/L)	2.36 (0.98)	2.16 (1.08)	0.33
Systolic BP (mmHg)	116.3 (28.8)	116.0 (31.1)	0.96
TGL (mmol/L)	1.23 (0.9)	1.81 (1.7)	0.002

Table 4.5 . Features of GADA High versus GADA Low titre patients. (Mean, SD)

	GADA Low	Autoantibody Negative	P value
Cases	**138**	**5558**	
Females*	59 (42.8%)	n=2260 (40.7%)	>0.5
Males	78 (56.5%)	n=3298 (59.3%)	
Mean age (years)	50.2 (10.9)	54.9 (9.4)	<0.001
Age of onset (years)	47.6 (10.8)	52.5 (10.6)	<0.001
Duration Disease (years)	2.46 (1.7)	2.3 (1.6)	0.076
BMI (kg/m^2)	28.5 (6.6)	30.9 (6.5)	< 0.001
Subjects on insulin (%)	52/131(39.7%)	728/5508 (13.2%)	< 0.001
Time to Insulin (years)	0.86 (1.4)	0.87 (1.4)	>0.5
Waist-Hip ratio	0.89 (0.1)	0.95 (0.09)	<0.001
Waist Circumference (cm)	96.7 (16.6)	103.8 (14.6)	< 0.001
HDL Cholesterol (mmol/L)	1.44 (0.5)	1.29 (0. 39)	>0.5
LDL Cholesterol (mmol/L)	2.16 (1.08)	1.60 (0.82)	< 0.001
Systolic BP (mmHg)	116.0 (31.1)	122 (31.0)	< 0.001
TGL (mmol/L)	1.81 (1.7)	1.97 (1.5)	< 0.001

Table 4.6: Features of GADA low versus antibody negative patients. Mean (SD).

4.22. DO AUTOANTIBODIES DIFFERENTIATE LADA CASES FROM TYPE 2 DIABETES AND TID?

Of the 6156 patient tested 598 were positive for at least one autoantibody GADA IA2A or ZnT8A and 1035 subjects were on insulin therapy. Autoantibodies distinguished LADA from type 2 diabetes cases the former tending to be younger as well as leaner at diagnosis unlike autoantibody negative type 2 diabetes patients (Table 4.2).

Of 598 patients positive for at least one autoantibody (GADA, IA-2A or ZNT8A) 275 (46%) were on insulin. Of 275 insulin-treated patients, information on precise time to insulin therapy was available on 203 cases of whom 114 were designated as T1D, 65 classified as LADA (free of insulin for more than 6 months from diagnosis) and 24 intermediate cases (4%). Of all patients positive for at least one autoantibody, 114 started insulin at diagnosis (19%) (i.e. designated classic T1D) with mean age 44.1 ± 9.9 years and 51.8 % were males. While 323 were on other therapy (oral anti-glycaemic agents or diet) and by inference 388 (65.0.%) did not start on insulin therapy for at least six months from diagnosis (i.e. designated LADA) mean age 51.9 ± 10.5 years and 50.2% were males Table 4.7.

It follows that LADA was more prevalent than classic T1D (Odds Ratio = 3.4).A third group of 24 (4.0%) autoantibody positive patients started on insulin after diagnosis and within 6 months (i.e. designated neither T1D nor LADA); mean age 46.5 (10.2), 45.8% male.

Of autoantibody positive patients, those classified as T1D compared with LADA had a lower age, age of onset, BMI, waist circumference and WHR ($p<0.0001$ for all comparisons), higher LDL cholesterol levels ($p<0.01$) and a higher, but not significant, number with high GADA titre (81% vs 71%) (Table 3.8). It follows that there were limited quantitative, and no categorical, phenotypic differences, between T1D and LADA, the former being younger, leaner, with higher GADA titre and higher LDL cholesterol.

GADA (n=541) autoantibodies identified the majority of cases with autoantibodies (90%), with IA2 (7.2%) and ZnT8 (2.3%) accounting for the remaining 10% of diabetes autoantibodies (Venn diagram Figure 4.8). Measurement of combination of autoantibodies to IA2A, ZnT8A in addition to GADA improved the predictive value of autoantibodies by adding an additional 10%.

Among the autoantibody positive subjects positive for GADA, IA2A or ZnT8A data when separated according to the age groups, 30-40 years, 40-50 years, 50-60 years, for of age at diagnosis similar to the UKPDS analysis, autoantibody positive cases in each age group were significantly leaner with a lower BMI in comparison to autoantibody negative 'type 2 diabetes' cases except. In those in the age group 60-70 years of age at diagnosis no difference was noted between the autoantibody positive and negative group (Table 4.6). It is possible that there is a gradation of the auto-antibody positivity with age as in the patients aged 60-70

years of age at diagnosis there was a similar BMI between antibody positive and negative cases.

	T1D	**LADA**	
Cases	**114**	**388**	
Females*	55 (48.2%)	188(49.8%)	NS
Males	59 (51.8%)	189(50.2%)	
Mean age (years)	44.1 (9.9)	51.9 (10.5)	p<0.001
Age of onset (years)	41.8 (15.2)	49.7 (12.3)	p<0.001
BMI (kg/m^2)	25.6 (5.0)	28.6 (6.6)	p<0.001
Waist-Hip ratio	0.87 (0.08)	0.90 (0.1)	p<0.001
Waist Circumference	88.3 (12.7)	96.8 (16.6)	p<0.001
LDL Cholesterol (mmol/L)	2.8 (1.0)	2.2 (1.0)	P=0.013

Table 4.7. Features of T1D versus LADA patients. Mean (SD)

Age of onset	30-40 years	40-50 years	50-60 years	60-70 years
LADA Cases (with information for age at onset)	549	250	366	192
Autoantibody Positive BMI	130 (23.7 %) 26.4 (6.1)	32 (12.8 %) 27.2 (4.6 %)	31 (8.5 %) 28.1 (4.8 %)	12 (6.3 %) 29.2 (4.9 %)
Autoantibody Negative BMI	419 (76.3 %) 31.9 (8.1)	218 (87.2 %) 31.0 (6.7)	335 (91.5 %) 30.9 (5.8)	180 (93.7 %) 30.5 (6)
P (for BMI)	<0.0001	= 0.0003	= 0.012	= 0.33

Table 4.8. Presence of Auto-antibodies and BMI at different age of diagnosis

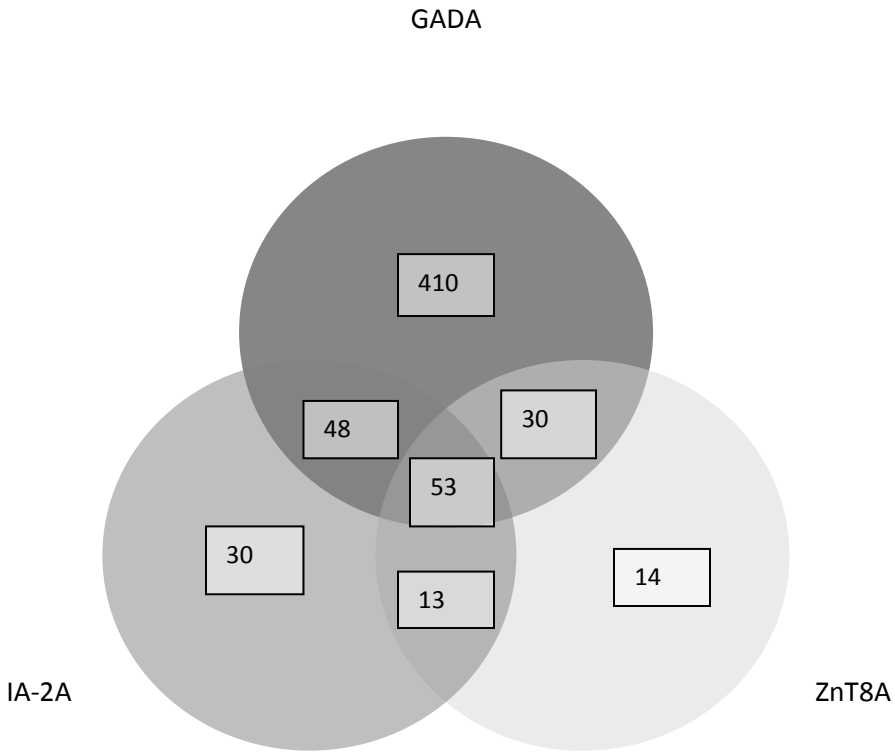


Figure 4.8. Venn Diagram depicts Action Lada patients with GADA/IA-2A or ZnT8A, n=598 of 6156 (9.8%). Of the autoantibody positive samples GADA identified 90% of the samples when an autoantibody was detected, IA-2A and ZnT8A accounting for the remaining 10% of the autoantibodies detected. GADA n=541 (8.8%), IA-2A n=144 (2.3%), ZnT8A n=110 (1.8%)

4.23. EVIDENCE OF NON-DIABETES ASSOCIATED AUTOIMMUNITY IN LADA PATIENTS.

In a subgroup of samples where sufficient sera was available we tested for organ-specific autoantibodies TPO and PCA in 1130 patients (mean age 52.3 ± 10.1years, 30-69 years) of the 6156 sample cohort. In diabetes-associated autoantibody positive and GADA positive patients we found: 28.9% and 29.7% TPO positive respectively ($p<0.0001$ for both) and 15.9% and 16.8% positive PCA. positive respectively ($p=0.069$ and 0.0136 respectively) (Table 4.90 and 4.91).

Importantly, while high titre GADA positive subjects had more TPO autoantibodies than GADA low titre subjects (32.4% versus 21.6% ($p=0.035$), the latter group were more often TPO autoantibody positive than GADA negative patients (9.9%, $p=0.01$) (Table 4.92). Measurement of GADA identified 94% of patients positive for T1D associated autoantibodies in this subgroup. The prevalence of organ-specific autoantibodies was increased in patients with GADA compared to patients negative for GADA.

	Any Ab Positive	Any Ab Negative	P value
PCA	83/521 (15.9%) (28M/55F)	73/609 (11.9%) (37M/36F)	0.069
TPO	151/521 (28.9%) (59M/92F)	55/609 (9%) (22M/33F)	<0.0001

Table 4.9 Non Diabetes associated antibodies PCA & TPO in antibody positive versus antibody negative patients

	GADA Positive	GADA Negative	P value
PCA	80/474 (16.8%) (26M/54F)	76/657 (11.6%) (39M/37F)	0.0136
TPO	141/474 (29.7%) (54M/87F)	65/657 (9.9%) (27M/38F)	<0.0001

Table 4.91 Non Diabetes associated antibodies PCA & TPO in GADA positive versus GADA negative patients

	GADA High >200 units	GADA Low >70 & <200 units	P value
PCA	66/358 (18.4%) (22M/44F)	14/116 (12.1%) (4M/10F)	0.148
TPO	116/358 (32.4%) (41M/75F)	25/116 (21.6%) (13M/12F)	0.035

Table 4.92 Non Diabetes associated antibodies PCA & TPO in GADA High Titre versus GADA Low Titre patients

4.24. Discussion: This dataset of patients collected from a large cohort of adult-onset diabetes patients attending primary and secondary care European centres shows that autoimmune diabetes of slow onset presenting with diabetes associated autoantibodies is prevalent and found in 9.7% of phenotypically type 2 diabetes patients**.** The prevalence of LADA has been reported in adults presenting with non-insulin requiring diabetes at approximately 10% with 4-12% of Caucasian's presenting with diabetes associated autoantibodies (Tuomi et al., 1999), (Zinman et al., 2004), (Leslie 2006), (Turner et al., 1997), (Leslie et al., 2008), (Buzzetti et al., 2007), (Hosszufalusi et al., 2003), (Rosario et al., 2005). The majority of patients presenting with diabetes in adulthood, (approximately 90%) tend to have type 2 diabetes, presenting with abnormal insulin secretion and peripheral insulin resistance (muscle, liver and adipose tissue), the remaining present with autoimmune features.

Of these latter presenting with autoimmune features, at diagnosis, but with similar features as type 2 diabetes, we found that they tend to be younger and leaner. In contrast to type 2 diabetes, T1D usually presents in childhood, with rapid loss of islet cell mass, loss of insulin secretory capacity, aggressive infiltration of the islets with T lymphocytes, detection of multiple islet cell antibodies with a major genetic component. In vitro competitive inhibition studies have shown that GAD and IA2 can block ICA staining in up to 60% of sera from T1D subjects but only 37.5% in sera from LADA cases suggesting that autoantibodies to other antigens other than GAD and IA2 are prevalent in LADA (Seissler et al., 1998), (Falorni et al., 2000), (Hampe et al., 2002). These studies reported GADA from LADA

patients have different recognition patterns as well as conformational epitopes compared with T1D patients using 35S-GAD65/67 fusion proteins with sera of 65% LADA patients binding to the COOH–terminal portion of GAD65 in comparison to 90% of T1D patients. Furthermore 20% of LADA patients recognized the NH2-terminal of GAD65 in comparison to 5% of T1D patients.

T cell responses to multiple islet proteins have been reported in LADA patients with and without autoantibodies (Mayer et al.,2007)(Zaval et al., 1992) in type diabetes patients (Brooks-Worrel et al., 1996) and in subjects at risk of developing T1D before the development of clinical diabetes (Brooks-Worrel et al., 2001).

Whether LADA cases should be collectively classed as a separate group to T1D due to; adult age at onset, presence of autoimmunity, the ability to remain free from insulin therapy for at least 6 months after diagnosis, and, the fact that they show a less genetic load in comparison to the classic T1D, remains to be universally agreed. Nevertheless GADA autoantibodies have been reported in around 10% (range 5-15%) in these non-insulin requiring cases (Hosszufalusi et al., 2003), (Rosario et al., 2005). Our study found GADA autoantibodies in 9.7% of phenotypically type 2 diabetes patients, other similar studies have been much smaller with selection bias selecting patients only with ‘type 2 diabetes’. In this present study all patients attending the clinic were included and benefits from being the largest such study, screening for three diabetes associated autoantibodies. Furthermore unlike other studies we increased the specificity for

all autoantibodies tested by repeating all samples that were scored positive thereby reducing false positive results.

A recent study in subjects aged 0-40 years reported that ZnT8A complements GADA, IA2 and IA2β and may replace IAA in particular in those over the age of 10. This finding is valuable in the use of ZnT8A as an additional marker as reproducibility and standardization of the IAA assays has remained an issue in comparison to the liquid phase radio-binding assays (Ilse et al., 2011). In our study testing for GADA autoantibodies identified the majority of cases with autoantibodies (90%), with autoantibodies to IA2A and ZnT8A accounting for (7.2%) and (2.3%) auto-antibodies respectively. Of 541 GADA positive patients 131 (24%) had IA2icA or ZnT8A, and these latter were younger, leaner as well as a lower waist to hip ratio, however there was no statistical difference in insulin requirement between those who had single (GADA alone) or multiple autoantibodies. Autoantibodies in the absence of GADA were detected to IA2A and ZnT8A in 57 (10.5%) cases. A small Japanese study in 47 GADA positive cases reported that the determination of other antigens namely IAA, IA2icA and ZnT8A improves the prediction of future insulin insufficiency in adult onset autoimmune diabetes as superior to GADA titre (Kawasaki et al., 2010). However, our large study did not show a difference in insulin requirement between those who were GADA alone positive and those with antibodies in addition to GADA. However, the numbers on insulin therapy were higher in those with IA2A or ZnT8A compared to those without autoantibodies.

When analyzing antibodies, it is important to identify thresholds for positivity in antibody measurements which can distinguish the presence or absence of antibody. Most Laboratories participating in the Diabetes Autoantibody Standardization Program (DASP), tend to use the 99^{th} centile of a group of control samples tested historically for the specific auto-antibody tested. Unfortunately this methodology has a flaw in that it does not account the day to day variation in the translated protein employed and variation in the assay. These factors can affect the background noise and as a result the cut off for positivity in particular when historical data is employed to determine the cut off levels. Notably other laboratories have successfully employed the use of the 99^{th} centile in the assays. In our laboratory the use of the end point dilution of the standard curve for the Action LADA Cohort was confirmed by the use of the QQ plot which identifies the change in the shape of the frequency distribution of the whole cohort tested (Figures 4.1 and 4.2).

The use of the normal QQ plot aided the identification of the point at which this change was noted. Similarly in the CARDS cohort we were able to utilize QQ plots to determine the change in the shape of the frequency distribution which for this cohort was less than the Action LADA cohort and again confirmed the cut-off point set from the standard curves employed (Figure 4.5). In this thesis it is evident that where large dataset's not normally distributed are analyzed, then the point of inflection identified from the QQ plots identifies the point where the distribution altered. Interestingly for each of the autoantibody tested this point was consistent with that determined by the methodology of employing the

standard curve for determining the end point. More significantly the QQ plot also identified that the end point for ZnT8A (Figure 5.4) could possibly be reduced further to 8.0 units, however this may affect the specificity of the assay. Therefore in conclusion when analyzing large datasets the use of QQ plots would be a valuable tool to confirm the point above which the data is different from that below the point where the data set follows a normal distribution. This approach further confirms previous report in the BABYDIAB study and adds support to the use of QQ plots in large population studies to define cut-off points (Ziegler et al., 1999).

We identified at least two modes within the GADA positive patients from the QQ plot analysis, with a cut-off of 70 WHO IU (low GADA titre) and another at 200 WHO IU (high GADA titre), remarkably this similar cut off for positivity was found also in the CARDS study cohort. In the latter a lower cut off for positivity was established at 40 WHO IU, but, this point only identified an extra 10 cases, with the majority identified above 70 WHO IU as confirmed by the QQ plots. Those patients with high titre GADA compared with low titre GADA were more likely to be female, younger at onset, leaner, with lower HDL cholesterol, systolic blood pressure and higher triglycerides and risk of insulin treatment. Moreover, patients with low GADA titre, compared to those who were GADA negative, were also younger at diagnosis, leaner with higher HDL cholesterol and proportion treated with insulin. Other studies have shown that high titre GADA positive patients had a tendency to greater HLA genetic disease risk compared to low GADA titre patients (Buzzetti et al., 2007).

A potential bias in this study was the use of hospital-based clinics since patients with autoimmune diabetes are more likely to be ascertained in such clinics given their greater risk of insulin treatment. Such a bias would increase the relative proportion of cases with autoantibodies, and specifically the proportion with T1D compared with LADA, but not the characteristics of autoantibody positive versus negative patients, a bias exacerbated by a tendency to start insulin treatment early if local assays were available to document patients as GADA positive (Sinead et al., 2008),(Davies et al., 2008),. In addition there was an excess of patients presenting with autoimmune diabetes in comparison to T1D (Table 4.1), implying that most cases at presentation of autoimmune diabetes in adults are presented as non-insulin requiring diabetes cases and not as LADA, or T1D.

In a separate study we found that individual components of Metabolic Syndrome were similar in both T1D and LADA, but in each, much less frequent than in type 2 diabetes (Hawa et al., 2009). Our data also shows that metabolic differences between low titre GADA and GADA negative patients extends to another autoimmune biomarker, namely TPOA which was detected more often in GADA positive than GADA negative subjects.

For decades autoimmune diabetes has been considered as a childhood onset or juvenile onset disease however the overwhelming data has shown that the disease affects all ages and in particular those over 30 years of age affecting up-to 9.7% of phenotypically type 2 diabetes patients**.** There is therefore evidence of a

gradation of autoimmune and metabolic features from childhood onset to adult onset autoimmune diabetes and as a result T1D and LADA need to be viewed as a broad spectrum of the same autoimmune disease.

The most prevalent form of diabetes is adult-onset type 2 diabetes which affects about 4-7% of the adult European population. Childhood-onset T1D, on the other hand, affects only some 0.25% of European children and adolescents, though a life-time disease-risk analysis suggested that up to 1% develop classic T1D, with a proportion doing so after age 30 years (Lorenzen et al., 1994). We now show that nearly 10% of adult-onset diabetes patients have autoimmune diabetes, it follows that adult-onset autoimmune diabetes is probably more prevalent than childhood onset T1D. In summary, these results show that the majority of adult-onset autoimmune diabetes patients are non-insulin requiring at diagnosis. In the tendency to be adult-onset, with variable destruction of the target organ, leading to a broad clinical spectrum, autoimmune diabetes can now be seen to resemble other autoimmune diseases.

CHAPTER 5

5.0. WHETHER AUTOANTIBODIES TO CD38 ARE OF ANY VALUE IN T1D AND TYPE 2 DIABETES.

5.01. Aim: To test if antibodies to CD38 were a feature of T1D and Type 2 diabetes patients.

5.02. INTRODUCTION: CD38 is a type II transmembrane glycoprotein, the extracellular domain acting as an ectoenzyme, catalyzing the conversion of nicotinamide adenine dinucleotide (NAD^+) into nicotinamide, adenosine diphosphate–ribose (ADPR), and cyclic ADPR. CD38 is expressed on many types of cells, (white blood cells), including CD4+, CD8+, B and natural killer cells. CD38 mRNA and/or protein have been detected in the mammalian, brain, spleen, heart, liver, kidney, intestine and pancreas (Cinzia et al., 1999), (Ikehata et al., 1998). Human CD38 is a single chain glycoprotein that is commonly used as a marker of differentiation and activation in haematopoietic cells. It is a bi-functional ectoenzyme that catalyzes the conversion of NAD+ to cyclic ADP-ribose (cADPR) and the hydrolysis of cADPR back to ADPR. It is also involved in the glucose induced insulin release from pancreatic islets. CyclicADP-ripose (cADPR) has been shown to be a mediator for intracellular calcium mobilization for insulin secretion by glucose in pancreatic β cells (Cinzia et al., 1999).

CD38 also functions in cell adhesion, signal transduction and calcium signaling. CD38 is a leukocyte differentiation antigen that has been thought to be a phenotypic marker of different subpopulations of T- and B-lymphocytes. The

earliest report of involvement of the cADPR in insulin metabolism came from experiments using a cell-free system of islet microsomes (Takasawa et al., 1993). CD38 was first cloned from rat pancreatic islets and was shown to be homologous to the human CD38 exhibiting 58% overall identity and 76% similarity and is expressed at comparable levels in Wistar rats and GK rats (a genetic model of NIDDM). The presence of CD38 in pancreatic islets suggested that it is involved in insulin secretion via the cADPR pathway (Li et al., 1994). Mice overexpressing CD38 exhibit enhanced glucose-induced insulin release, while CD38 KO mice display severe impairment of β cell function.

A previous report on human diabetes revealed that 13.8% of Japanese type 2 diabetes patients have autoantibodies against CD38 (Ikehata et al., 1998). Their experiments showed that anti-CD38 antibodies inhibited the glucose induced insulin release and recombinant CD38 abolished this effect, suggesting that anti-CD38 autoantibodies may be one of the major causes of impairment of glucose induced insulin release (Ikehata et al., 1998). CD38 is involved in the glucose induced insulin secretion and has been proposed as a candidate auto-antigen in type 2 diabetes patients. Conflicting results to Okamoto's group (Ikehata et al., 1998) have been shown by another study where anti-CD38 antibodies potentiated the release of insulin from isolated human pancreatic islets (Cinzia et al., 1999). Both insulin resistance as well as insulin deficiency are involved in the pathogenesis of type 2 diabetes and autoantibodies to islet antigens ICA, GADA and a protein tyrosine phosphatase like molecule IA2 have been shown in a proportion of these patients. The presence of these antibodies in particular high

titre GADA, have been associated with early insulin requirement (Buzzetti et al., 2007). The presence of autoantibodies to CD38 has previously been evaluated using Western blot analysis in 236 patients with type 2 diabetes and 160 T1D patients with positivity in 9.7% and 13.1% respectively (Cinzia et al., 1999). Okamoto et al showed that of 377 Japanese type 2 diabetes patients tested 13.8% patients had autoantibodies against CD38 (Ikehata et al., 1998), These data suggested that anti CD38 antibodies in 'type 2 diabetes' patients might be a major cause of impaired glucose induced insulin secretion.

These observations as well as the availability of molecular and immunological techniques already employed for other diabetes associated antigens prompted me to employ these analytical procedures using CD38 cDNA, to insert it into a vector allowing in-vitro transcription and translation of CD38 protein, for the subsequent use in a liquid phase immune-precipitation assay to detect autoantibodies in patients with T1D as well as 'type 2 diabetes'.

5.03. Methods: The human CD38 cDNA encoded as the human lymphocyte differentiation antigen CD38 (Jackson & Bell 1990). The gene encoding the human CD38 antigen has been previously cloned from T cells and normal lymphocytes, located on chromosome 4 (Jackson & Bell 1990). The CD38 cDNA a gift from Professor Malavassi at the University of Torino, Italy, was supplied as an agar stab inoculated with E-Coli strain MC1061/PB containing the plasmid pCDM8-HCD38 an expression vector, containing the human CD38 cDNA cloned into the Hind 111 – Not 1 sites with an insert size of 1.4kb. The transfected COS cells, expressed a surface protein of Mr46000 similar to the native CD38

molecule on B cell line recognized by the CD38 specific monoclonal antibody. To isolate and grow the plasmid a stab of CD38cDNA was taken from the glycerol stock and grown in Luria Broth Medium (LB) containing 12.5µg/ml Ampicillin and 7.5µg/ml Tetracyclin.

5.04. Reagents: The reagents required for the procedure included: Ampicillin (Sigma), Luria Broth (Sigma), Tetracyclin (Sigma), CD38 cDNA, Sterile culture plate (Sterilin).

5.05. Preparation of Ampicillin Stock: Dissolve 100mg Ampicillin (Sigma Aldrich UK) in 1ml of sterile H2O and store 100µl aliquots at -20°C and use 62.5µl/500ml LB medium (12.5µg/ml final concentration).

5.06. Preparation of Tetracyclin Stock: Dissolve 100mg Tetracycline in 1ml of sterile H2O and store 100µl aliquots at -20°C and use 37.5µl/500ml LB medium (7.5µg/ml final concentration).

5.07. Prepare Luria Broth Medium (LB)

Components of Luria Broth Millers Modification Sigma Cat No:L3397

Kit Component: Tryptone (pancreatic digest casein) 10g/L

Yeast Extract 5g/L

NaCl 0.5g/L

Dissolve 15.5grams of LB medium (Sigma) in 1 litre of Ultra Pure water, autoclave for 15 minutes at 121°C to dissolve LB media and sterilize.

When the LB medium solution is hand cool to touch add antibiotics as follows:

12.5µg/ml Ampicillin

7.5µg/ml Tetracycline

5.08. Ladder Molecular Weight Marker:

Source Gibco BRL

Cat No:10416-014 (50 base pair DNA Ladder marker)

Low melting point Agarose Gel: 1.0% (1.0g/100ml H_20)

5.09. Prepare 50 x Stock Tris Acetate Buffer (TAE)

242 grams Tris Base

57.1ml Glacial Acetic Acid

100ml of 0.5M Ethylene Di-amine Tetra Acidic acid (EDTA)

Once dissolved adjust the pH to 8.0 and store at 4ºC. From this stock solution prepare a 1 x solution for running the gels to give a final concentration of 0.04M Tris Acetate containing 0.001M EDTA.

5.10. Preparation of CD38 Plasmid:

A. A stab from the original CD38 glycerol stock containing the E Coli strain MC 1061/PB containing the CD38 expression plasmid PCDM8 – HCD38 (Figure 1) was placed in 10ml of LB medium in a 50ml Falcon tube containing Ampicillin and Tetracyclin. The cells were incubated overnight at 37°C in a grants incubator with vigorous shaking at approximately 150 revolutions per minute.

B. After the overnight incubation cells were centrifuged, the cell pellet was reconstituted in 1ml of LB/Ampicillin/Tetracyclin media and the contents added to 500ml of LB/Ampicillin/Tetracycline media in a flat bottom conical flask to further expand the colony as in (**A)** above at 37°C overnight.

C. Following overnight incubation the cells were placed into 100ml centrifuge flasks and centrifuged in a Sorvall RC5B centrifuge at 5000 x g for 10 minutes at 22°C. Discard the supernatant and reconstitute the pellet in cell re-suspension solution for the extraction of DNA using the Promega Maxi Prep DNA extraction kit as described previously in chapter 2.

The pCDM8 4.4kb comprises the CD38 cDNA insert as shown in Figure 5.0, which consists of the T7 promoter site making the vector suitable for in-vitro transcription and translation of the CD38 protein by utilizing the Promega TNT^{TM} transcription and translation kit **as** described previously in chapter 2.

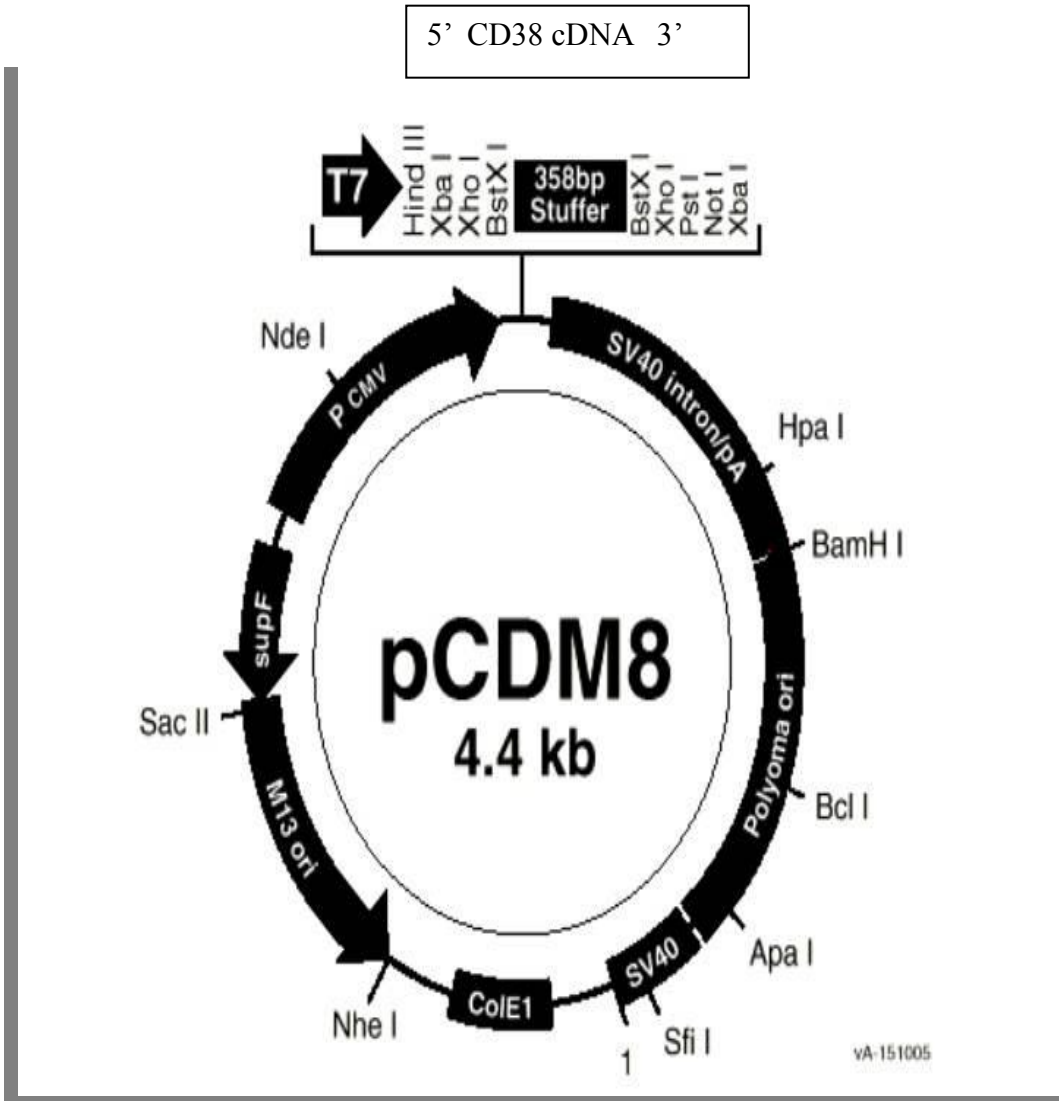


Figure 5.0 Map of pCDM8, the original mammalian CD38 expression vector with T7 promoter site as shown.

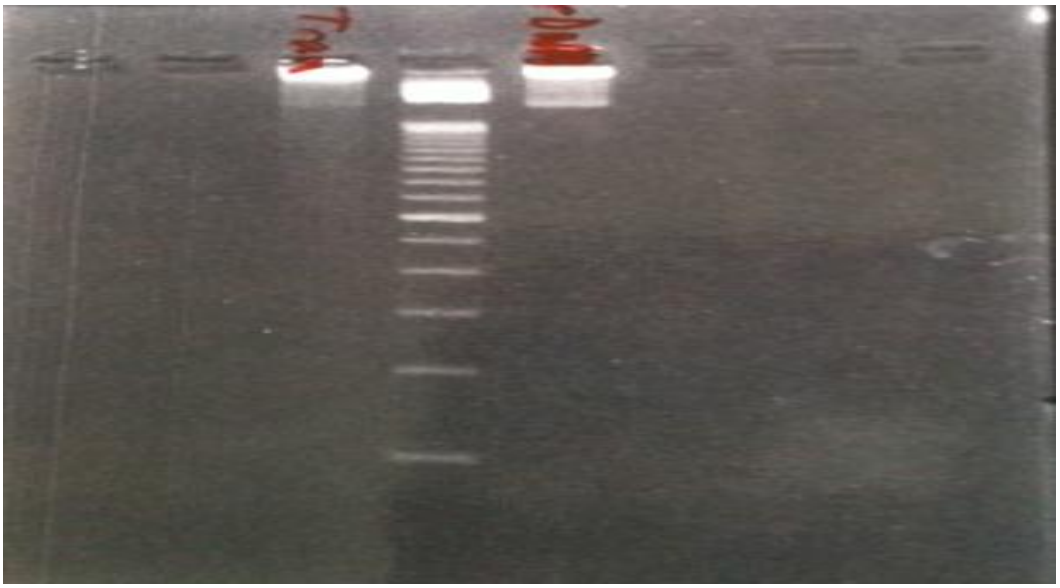
5.11. Initial Results: The extracted CD38 DNA from section C above was purified using the Promega Maxi Prep kit according to the manufacturer's instructions. The extracted DNA concentration was measured by measuring the optical density (OD) at 260nm using the Beckmann spectrophotometer and visualised by low melting point agarose gel electrophoresis in Tris Acetate Buffer as shown in Figure 5.1

OD of extracted DNA = 0.0406

OD of 1.0 = 50ug/ml

Concentration of CD38 Sample = 50 x 0.0406 =2.03ug/ml

5ul = 1ug of DNA



Lanes 1 2 3

Figure 5.1.: Purified CD38 DNA was separated by electrophoresis on a 1% Agarose gel. Lane 1 = CD38 DNA 1 ul , Lane 2 = Ladder molecular weight marker, Lane 3 = 5ul DNA

5.12. CD38 In-vitro Translation: Using the CD38 cDNA prepared as above the protein was subsequently translated using the Promega TNT^{TM} translation kit as previously described in chapter 2 and the translate was electrophoresed on a 10% SDS page gel Figure 5.2.

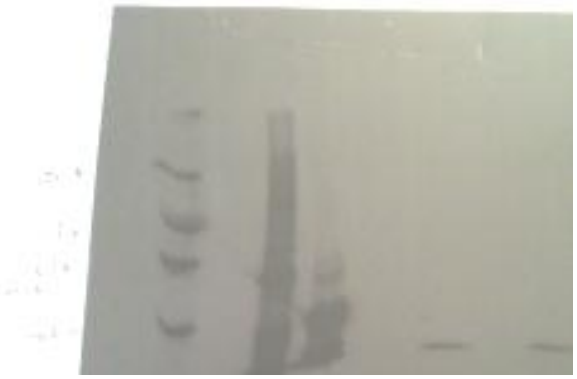


Figure 5.2. Shows autoradiograph of proteins separated on a 10% SDS Page gel Lane 1 = Molecular weight marker (range 200k, 69k, 46k, 30k and 21k respectively), Lane 3 = GAD_{65}, Lane 4 = IA2ic, Lane 6 & 8 = CD38 translated proteins which appear to resolve at the dye front.

Following electrophoresis and autoradiography the proteins noted were unrelated to CD38 Figure 6.3, the only bands visible were that of GAD_{65} and IA2 which were included as control proteins previously translated separately. No band was noted for the CD38 antigen in the 46 kilodalton region, a band was seen in lanes 6

and 8 below the expected region of 46kd and was attributed to non-specific translation.

The question arose whether the PCDM8 CD38 cDNA containing the T7 polymerase promoter enzyme was translating CD38 protein.

5.13. Immunoprecipitation of CD38: To test and confirm if CD38 antigen was present, translated proteins were immuno-precipitated with monoclonal antiCD38 antibody CD38 AT1 Cat No Sc-7325 (Santa Cruz Biotechnology). The mouse CD38 AT1 antibody corresponds to CD38 of human origin reacting with Human CD38 in western blotting, immunohistochemistry and FACS and is not cross reactive with other cell surface markers. The stock solution of CD38 AT1 monoclonal antibody was stored at 4°C.

The immuno-precipitation was performed using the following combinations:

1. In a micro-centrifuge tube add 10µl of the stock CD38 AT1 Monoclonal antibody as a positive control, (Stock solution 200µg/ml equal at 2µg) to 10µl of the CD38 translate. The combination was repeated with 1µg of Monoclonal CD38 AT1 antibody

2. In a micro-centrifuge tube add 10µl of the stock CD38 translate. To this add 2µl of serum sample from a pre-diabetic individual (coded SB) a sample known to be positive for CD38 in another laboratory using western blot technique.

3. In a micro-centrifuge tube add 10µl of the stock CD38 translate. To this add 2µl of serum sample from a healthy individual (coded GM) with no family history of diabetes or any other autoimmune disease.

4. In a micro-centrifuge tube add 10µl of the stock IA2ic translate. To this add 2µl of serum sample from a pre-diabetic individual (coded SB) throughout the text.

5. In a micro-centrifuge tube add 10µl of the stock IA2ic translate. To this add 2µl of serum sample from a healthy individual (coded GM) with no family history of diabetes or any other autoimmune disease.

The above tubes were vortexed and incubated overnight at 4°C. To immuno-precipitate immune complexes Protein A Sepharose was employed. The latter was used at a concentration of 3mg per tube with a binding capacity of 20mg of human IgG per ml. Therefore 1.5mg will bind 20mg of Human IgG and 1mg will bind 6.66mg IgG.

In blood the expected concentration of IgG is 12.5mg/ml, IgA 2.1mg/ml, IgM 1.25mg/ml, IgD 40µg and IgE 0.04µg respectively, equivalent to approximately 16mg/ml of total immunoglobulin per ml of serum.

= at 16mg immunoglobulin per 1000µl of serum.

= at 1.6mg immunoglobulin per 100µl of serum.

= at 0.16mg immunoglobulin per 10µl of serum.

= at 0.016mg immunoglobulin per 1µl of serum or 16µg per ul of serum

5.14. CD38 Monoclonal antibody: CD38 ATI Cat No #SC-3725

Supplier: Santa Cruz Biotechnology Incorporations

Autogen Bioclear, Holly Ditch Farm, Mile Elm, Colne, Wiltshire SN11OPY

Source: CD38 (ATI) is a mouse monoclonal IgG1 antibody, corresponding to the CD38 of human origin.

Product: 200µg IgG1 per ml in PBS containing 0,1% Sodium Azide and 0.2% gelatine.

Specificity: CD38 (ATI) reacts with CD38 of human origin by western blotting, immunochemistry as well as FACS analysis, it is not cross reactive with other cell surface markers. Storage: 4 º C.

For the above the incubation was performed with 1mg of Protein A Sepharose per tube. Now 100mg of Protein A Sepharose swells to 400µl of packed resin volume, therefore the diluted PAS per tube or well swells to 4µl sufficient to bind 6.6mg of IgG. For the immunoprecipitation 2ul of serum was used containing approximately 32µg of IgG to which 1mg of PAS was added which is in excess to attain optimal binding of the antigen antibody complexes.

5.15. . Results:

1. Positive control monoclonal anti-CD38 antibody failed to immuno-precipitate CD38 protein, no band was noted.
2. CD38 was not immuno-precipitated using the control sera SB.

3. As expected negative control sera GM did not immuno-precipitate CD38 molecule.
4. IA2 antigen was immuno-precipitated with the positive control sera SB as expected with IA2 as the antigen source (data not shown)

5.16. Interpretation:

The transformed colony from the original glycerol stock was successfully grown on ampicillin/tetracycline resistant agar plates. The protein translated however failed to immuno-precipitate CD38, the control antigen immuno-precipitated as expected the antigen IA2. These initial experiments raised the question as to whether any CD38 protein was being translated or perhaps the incorporation of methionine into the CD38 was insufficient, such that insufficient protein was translated.

Taking the latter into account the primary sequence of CD38, its nucleotide sequence as well as the predicted amino acid sequence was examined, which indicated that the number of methionine residues (n=5) in comparison to leucine (n=31) was substantially lower (Jackson & Bell., 1990). This could be a reason for the insufficient incorporation of methionine in the translation of CD38 protein. As a result of these initial experiments radioactive Leucine (Amersham Code No: SJ126) instead of Methionine in the translation cocktail was employed. However this also failed to show any significant improvement in the amount of CD38 protein translated.

This raised another question as to whether any transcription from the synthesized CD38 cDNA was occurring. As a result experiments were carried out to test mRNA synthesis.

5.17. CD38 mRNA synthesis:

To test whether transcription using the CD38 cDNA was active we carried out in-vitro transcription assays to generate RNA according to the according to manufacturer's protocol, Promega UK.

1.	5 x Transcription Buffer	4µl
2.	100mM dithiothrietol (DTT)	2 µl
3.	Rnasin Inhibitor	1 µl
4.	ATP	1 µl
5.	GTP	1 µl
6.	UTP	1 µl
7.	CTP	1 µl
8.	Dnase Free H2O	5 µl
9.	CD38 DNA	2 µl
10.	T7 Polymerase	2 µl
	Total volume	**20 µl**

Vortex gently and incubate the mixture at 37°C for 1 hour, the synthesized RNA was electrophoresed on 1% Agarose gel.

The result from this experiment failed to show any RNA band using the original CD38 cDNA.

The vector pCDM8 was an expression vector and it was decided to utilise selected enzymes to excise the CD38 insert of 1.4 Kb from the 4.4Kb pCDM8 vector and re-insert into a suitable translation vector pGEM-11Z to enable translation of the protein of interest namely CD38.

5.18. Protocol to cut the 1.4 Kb CD38 Insert from pCDM8 Plasmid:

The CD38 insert was digested enzymatically using selected restriction enzymes Hind III and Not I to cut out the CD38 insert from the pCDM8 Plasmid. These restriction enzyme sites are shown on the original pCDM8 plasmid as depicted in figure 6.1, each restriction enzyme with specific requirements of buffer, incubation time and concentrations for optimal enzymatic activity.

5.19. Restriction Enzyme Reagents

Hind III Reagents:

1. Hind III (Cat No R604A Product ID:9170302; Promega, UK) 5000U (10U/µl).
2. Multicore ™ 10 x buffer Cat No R999A Product ID:9240804; Promega, UK) (250ul).
3. Buffer E 10 x Cat No R005A Product ID:7544308; Promega, UK (1ml).
4. Bovine Serum Albumin Acetylated Cat No R396E Product ID:8560821; Promega, UK (100 x stock used in dilution)

Not 1 Reagents:

1. Not 1 CQ Cat No R643A Product ID:8827008; Promega, UK (10u/µl)
2. Buffer D Cat No R004A Product ID:7090225; Promega, UK (10 x Concentrate)
3. Multicore Buffer Tm Cat No R999A Product ID:9240804; Promega, UK (10 x Concentrate 0.25ml)

To linearize and digest the 4.4Kb pCDM8/CD38 vector using restriction enzymes HIND 111 and Not 1, the following protocol was employed.

5.20. Protocol to digest CD38 insert from pCDM8/CD38 expression vector using HIND 111**:**

1.	Sterile H_2O	32.6µl
2.	Restriction Enzyme (x10) Buffer E	4.0 µl
3.	Acetylated BSA	0.4 µl
4.	PCDM CD38 DNA 1 µg/ µl	2.0 µl
	(Mix by pipetting then add)	
5.	Restriction Enzyme HIND 111	1.0 µl
Total volume		**40 µl**

The reaction mixture was incubated at 37°C for 4 hours for optimal activity according to the manufacturers protocol for the procedure

5.21. A) Purification of the HIND111 Linearized PCDM CD38 Plasmid

The plasmid from A) above was purified using Promega's Mini Prep DNA purification reagents.

1. Add 1ml of Promega's Wizard miniprep DNA purification resin, mix gently by inversion several times
2. Pass resin bound DNA through the purification column provided with the miniprep kit
3. Wash the column with column wash solution
4. Elute DNA with 1ml TE buffer/ or Dnase free H_2O.

B) After purification the linearized PCDM CD38 DNA template was further digested with the restriction enzyme Not 1, to release the 1.4KB CD38 insert.

5.22. Protocol to digest CD38 insert from pCDM8/CD38 and release insert:

1. From step B above utilise linear CD38 DNA	34.6 µl
2. Add Buffer D	4.0 µl
3. Acetylated BSA	0.4 µl
4. Mix gently by pipetting then add Not1 Restriction Enzyme	1.0 µl

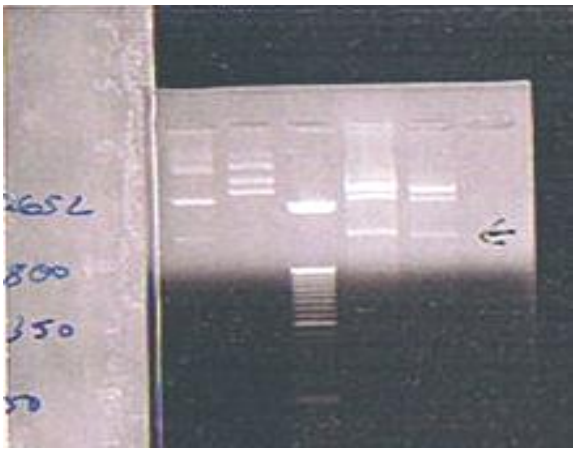
Mix gently, spin in a microcentrifuge for 5 seconds and incubate at 37ºC for 4 hours.

Purify the DNA insert as above using the Promega wizard mini prep DNA purification.

Following the digestion with Hind111 and Not1 enzymes the digestion products were separated on low melting point agarose gel (0.8%).

5.23. Results:

From these experiments we expected a 1.4 kb insert of CD38 and this was visualised after electrophoresis. Figure 5.3



Lane 1 2 3 4 5

Figure 5.3: The pCDM8 vector after digestion with the Hind 111 and Notch 1 enzymes electrophoresed on a 0.8% agarose gel, released a 1.4kb insert indicated by the arrow, from left to right Lane 1 = Uncut pcCDM8 CD38, Lane 2 = Not 1 cut pcCDM8 CD38, Lane 3 =Ladder molecular weight marker, Lanes 4 & 5 Not1 & Hind111 digestion (4µl, 1µl respectively)

5.231. Elution of the 1.4 kb insert

The insert of 1.4kb from above was cut out of the 0.8% agarose gel placed in a sterile eppendorf centrifuge tube, heated to 65º C to elute the DNA fragment. To this add 1ml Wizard DNA binding Gel (Promega UK). This bind's the DNA ready for elution using the DNA wizard miniprep system as described earlier and eluted with 30µl of ultra-pure water heated to 70 º C. The product from this was used for the ligation into PGEM11Z vector.

5.232. Ligation Protocol for PGEM11Z and CD38 insertion:

The insert digested from pcCDM8 CD38 vector using Hind111 and Not1 enzymes was ligated according to the manufacturer's recommended protocol (Promega UK) using T4 DNA ligase which catalyzes the joining of two strands of DNA between the 5' phosphate and the 3' hydroxyl groups of the adjacent nucleotides.

Promega recommend using a ratio of 1:1, 1:2 or 1:3 molar ratio of vector to DNA to be inserted when cloning a fragment into a plasmid vector (Table 5.0).

5.233. Ligation of CD38 insert into PGEM 11Z

For the ligation we used the following ratios of vector and insert **Table 6.0:**

NB: DNA Ligase = 3Units/µl Therefore 1.5U/0.5ul After gently mixing Incubate the tubes containing the ligation cocktail at 4 º C overnight. The ligation products are now ready for the Transformation of competent *E* Coli cells

Table 5.0 Ligation of CD38 insert into PGEM 11Z

	Ligation ratio 1:1	**Ligation ratio 2:1**	**Ligation ratio 3:1**
Vector DNA	2.0µl	2.0µl	3.0µl
Insert CD38	2.0µl	1.0µl	1.0µl
DNA Ligase buffer (x10 conc)	1.0µl	1.0µl	1.0µl
T4 DNA Ligase	0.5µl	0.5µl	0.5µl
Nuclease free H_2O	4.5µl	5.5µl	4.5µl
Total incubation volume	**10. 0µl**	**10. 0µl**	**10. 0µl**

5.234. <u>Transformation protocol of cloned CD38 into competent EColi</u>

Competent Cells Source: Promega Catalogue No: L2001

Product: JM109 Competent cells in FSB (100mM KCL,45mM, MnCl2, 10mM CaCl2, 3mM HACoCl3, 19mMKAcetate, 5% sucrose & 10% glycerol) JM109 cells were made competent by the method of Hanahan)

For the transformation use the Ligation products from the ligation experiment above A,B & C at ratios 1:1, 2:1 and 3:1 and D control DNA.

1. Use 100µl of competent cells add 10µl of the ligation mixture in an Eppendorf tube, mix gently and leave on ice for 10 minutes.

2. Heat shock the contents by placing the tubes in a water bath at 42ºC for approximately 45-50 seconds.

3. Return to ice for a further 2 minutes

4. Add 900µl of ice cold LB Ampicillin medium and incubate at 37ºC for 1 hour in a shaking water bath.

5.235. Preparation of Luria Broth Agar containing IPTG and xGAL:

Prepare fresh Luria Broth Agar (LBA)

Use 30.5g/litre autoclave in order as to dissolve the agar. When hand cool add 50µg/ml Ampicillin (Sigma Aldrich) Stock solution 1000mg/ml used 500µl/1000ml to give a final concentration of 50µg/ml Ampicillin.

Prepare IPTG (Isopropyl β-D-1-thio-galacto-pyranoside) (Molecular Weight 238.31) Stock solution 1 gram dissolve in 50ml distilled H_2O = 0.08Molar

solution. Sterile filter and store at 4º C. Use 6ml/ litre of LBA . xGAL Stock 50mg/ml use 800µl/L of LBA (Final concentration 40µg/ml).

Pour LBA containing Ampicillin, IPTG and xGAL into sterile petri dishes in a hood and allow agar to set. Seal the petri dishes with Para film and store upside down at 4 º C, until ready to use.

5.236. Growth of Transformed cells

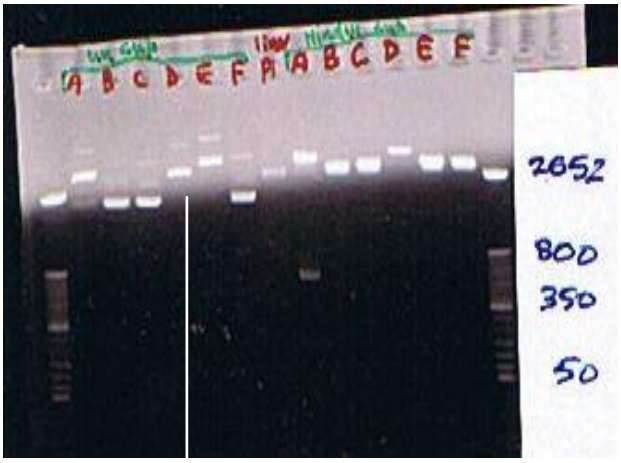
The transformed plasmid remains gal^- and cannot metabolize Xgal to a blue pigment. Resulting insert bearing colonies, grown on LB ampicillin plate supplemented with IPTG and xGAL will remain white. In contrast, a non-insert bearing plasmid complementing the *E coli* host, become gal^+ and form blue colonies on LB/Ampicillin plates with IPTG and Xgal.

Following ligation and transformation single white colonies were picked from the LBAgar/IPTG/Xgal/Amp plates and grown overnight in an incubator at 37ºC containing the insert in the LacZ position after inoculating a 50ml Falcon Tube in 10ml luria broth ampicillin medium (Note each transformation reaction was prepared neat (undiluted), diluted 1:10 and 1:100). The colonies with the insert grow white and those without the insert are gal+ and can therefore metabolise Xgal and produce blue colonies however no Blue colonies were noted.

After overnight growth of the transformed E Coli cells on LBAgar/IPTG/Xgal/Amp plates, five colonies were picked labeled A, B, C, D, E & F respectively and grown in LBA media. DNA was extracted using Promega's SV miniprep DNA extraction kit as previously described.

5.237. Results & Interpretation

Following digestion of the original PCDM CD38 Plasmid with the restriction enzymes and ligation into PGEM11Z vector the DNA was again linearized with Hind 111 restriction enzyme and the DNA was electrophoresed on agarose gel both before and after digestion with the restriction enzyme. Figure 5.4 shows the bands for the products of PGEM11Z vector digested as well as undigested.



8

Figure 5.4: Linearized Vector PGEM11Z with CD38 insert. PGEM11Z uncut in the left panel A-F uncut, right panel A-F digested with Hind111 enzyme. A: refers to the transformation of ligation products (1:1) colony with JM109 competent cells undiluted and CD38 DNA, A, B, C & D: refers to the transformation of ligation products (2:1) colony with JM109 competent cells diluted 1:10 and CD38 DNA. Molecular weight markers are in the first and last lanes. Lane 8 equates to the linearized pGEM11Z vector (labeled 8)

The PGEM 11Z vector employed was 3223bp and the insert of CD38 1.4kb so we would expect a 4623bp linearized product after digestion with restriction enzymes Hind111. However the bands noted in figure 6.5, were running a little higher than 2652bp region (bands A & D). The bands for B, C, E and F were noted to be running at the same level as the vector indicating no insert was present in the construct from which DNA was extracted. In order as to test these products, DNA from A, D and E as a control was tested by translating the proteins of interest using Promega's TNT translation system as previously described with both T7 and SP6 promoter enzymes in chapter 2. The translate proteins were then purified on a NAP5 column as previously described and translated proteins then separated by electrophoresis on 10% SDS page gel as depicted in figure 5.5 following autoradiography.

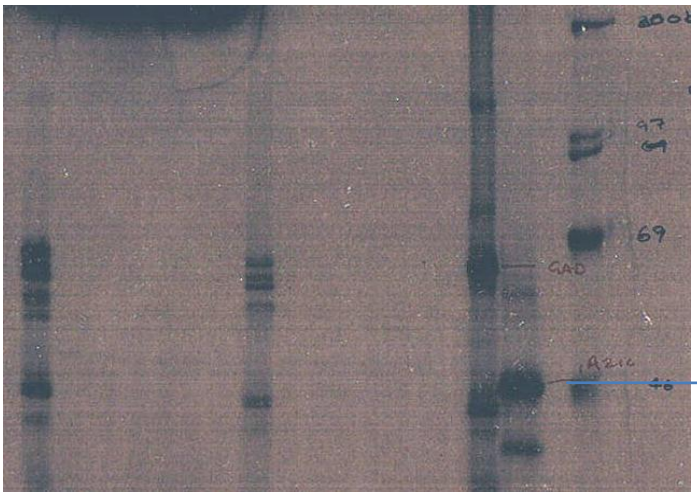


Figure 5.5: The figure shows translated proteins following SDS page gel electrophoresis, autoradiography and purification on a NAP 5 column. The only proteins detected were as indicated in lanes 1 and 8 which separated at the same point as GAD_{65} in lane 15, Lane 16= IA2ic is indicated, Lane 18 Molecular weight Marker. No CD38 translate was detected.

The translated proteins upon electrophoresis and autoradiography for 24 hours showed bands similar to GAD_{65} which was used as a control together with IA2ic intra-cytoplasmic domain (amino acids 605-979). In western blot analysis serum sample from an individual with SPS (CB) reacted with the GAD_{65} and CD38 construct but not IA2ic (data not shown). This further raised a question regarding the identity of the CD38 construct, despite bands detected around the 40kd region as shown in lanes 1 and 8 Interestingly the translations that were note was only with the T7 polymerase enzyme which notably is also present in the GAD_{65} cDNA.

5.238. To test for reactivity of translated protein in a Liquid Phase Immuno-precipitation Assay

A liquid phase immune-precipitation assay was performed using the CD38 protein translated from the CD38 construct prepared earlier, to confirm previous findings on coded samples tested in another laboratory for CD38 using western blot analysis (Eli Ferrannini group) and antibody and for GADA in our laboratory Table 5.1).

Ferrannini Code	**Ferranni Blot result**	**GADA assay result in house**	**IA2ic assay result in-house**	**CD38 translate in house**
263	Negative	Negative	Negative	Negative
145	Negative	Negative	Negative	Negative
241	Negative	Positive	Negative	Positive
214	Negative	Negative	Negative	Negative
281	Negative	Negative	Negative	Negative
177	Positive	Negative	Negative	Negative
48	Positive	Negative	Negative	Negative
194	Positive	Negative	Negative	Negative
227	Positive	Positive	Negative	Negative
S013	Positive	Negative	Negative	Negative
Negative GM	Negative	Negative	Negative	Negative
Control SB	Negative	Positive	Positive	Positive
Control CB	Negative	Positive	Negative	Positive

Table 5.1: Comparison of data tested by western blot and immuno-precipitation (Control Sample CB = SMS patient sera, SB = Pre-diabetic sera, GM = Negative control sera

5.239. Interpretation: These results show a discrepancy between the data for CD38 using Western blotting shown in Table 6.1 (Ele Ferrannini group) and the liquid phase radio-immuno-precipitation assay, as the recognition of the antigen in the two formats was inconsistent. In the former western blot assay linear epitopes are recognized whereas the latter recognizes conformational epitopes. Furthermore immuno-precipitation data gave a similar result with a lack of reactivity of the sera as might have been expected based on the data by Ferrannini et al, with the CD38 antigen. However the reactivity detected following immune-precipitation indicated that the bands following immuno-precipitation, electrophoresis and autoradiography were in the region of 64Kd similar to GAD_{65} Figure 6.7.

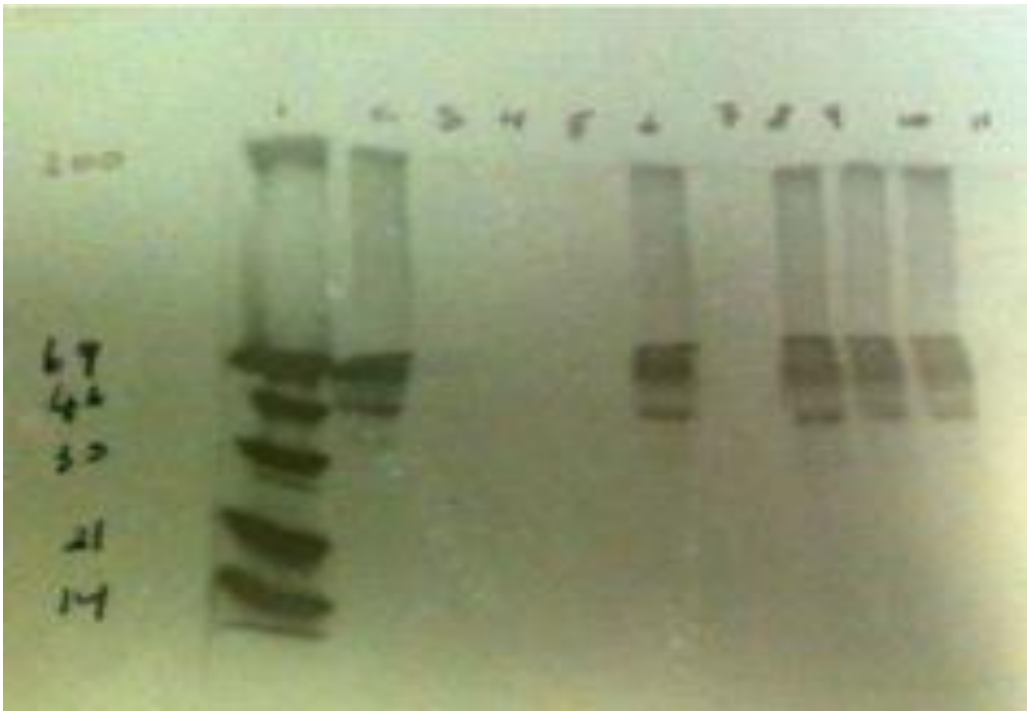


Figure 5.6: Shows an Autoradiograph following Immuno-precipitation experiment to detect CD38 reactivity with serum samples. Lane 1 Molecular weight marker (range 200kd – 14kd)rainbow marker labeled with ^{14}C, Lane 2 Sample 151, Lane 3 Sample 227, Lane 4 Sample 48, Lane 5 Sample 120, Lane 6 Sample 6, Lane 7 Sample 20, Lane 8 Sample 241, Lane 9 CB Positive for GADA, Lane 10 SB Positive for GADA(Table 5.2 Details)

Ferrannini or Lab Code	**Ferrannini Blot result**	**Previous GADA data**
151	Positive	Positive
227	Positive	Positive
48	Positive	Negative
120	Positive	Negative
6	Negative	Positive
20	Negative	Negative
241	Negative	Positive
CB	Negative	Positive
SB	Negative	Positive

Table 5.2 shows findings in routine GADA assay in comparison to western blot data of samples previously tested for Ferrannini in London Laboratory (Tested by M Hawa).

These results indicated that the cDNA prepared could not have been the expected CD38, rather and the data obtained indicated that, it was more closely associated with GAD_{65}.

5.240. Sequencing Data: The CD38 cDNA from above was subsequently sequenced to confirm its precise identity. Sequencing was performed in the Laboratory of Professor Adrian Clarke by Dr Lou Mattheral. Sequencing data produced gave a significant alignments with the enzyme and known auto-antigen Glutamic Acid Decarboxylase ($GAD_{65)}$ and confirmed that the cDNA was GAD_{65} not CD38 Figure 5.7.

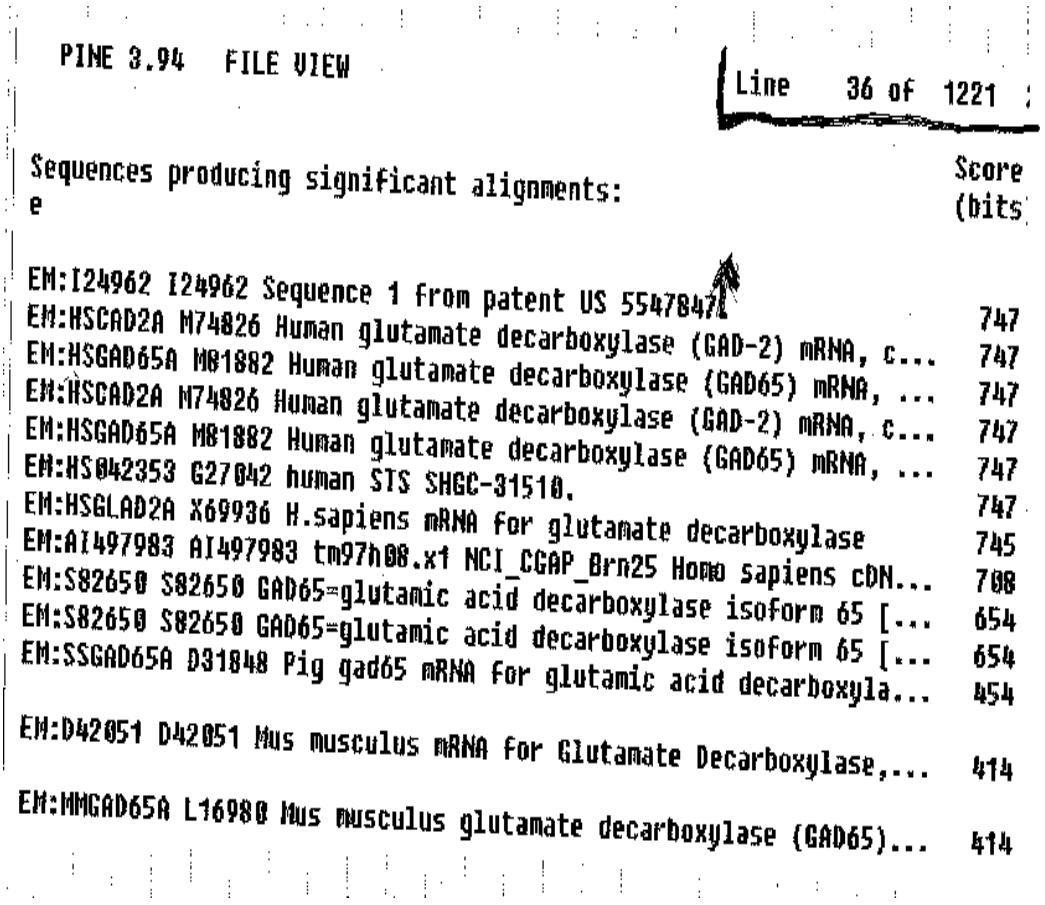


Figure 5.7: Sequencing of the isolated DNA matched with the sequence of GAD_{65}.

5.241. Conclusion: A key message from these experiments is that even with extreme care contamination can occur, in particular when working in a laboratory where work with other plasmids is routinely carried out. The data presented in Table 6.2 from the immune-precipitation experiments was further confirmed by the sequencing data to be GAD_{65} cDNA and not CD38 cDNA.

As a result of these experiments a new batch of PCDM8 CD38 cDNA stock was obtained from the original source (Malavasi et al), to digest out the 1.4kb CD38 insert.

5.25. Digestion of new batch of PCDM8/CD38 Plasmid with restriction Enzymes

Stock PCDM8/CD38 DNA = 150µg was diluted in 150µl to give a concentration of 1µg/µl, 25µl of this was employed in the digestion (25µg). This CD38 DNA was cut with the restriction enzyme Xba1 thereby releasing the 1.4kb insert of CD38.(Xba1 Catalogue No:R6181; lot number:106306; concentration 10u/µl). The enzyme activity is defined as the amount of enzyme required to digest 1µg of DNA in 1 hour at 37ºC in a reaction mixture of 50µl.

5.26. Protocol to digest PCDMCD38 and PGEM11Zf with Xba1 restriction enzyme: The following reaction mixture was set up by pipetting into a sterile Eppendorf tube: as depicted in Table 5.3.

	Tube A	**Tube B**	**Tube C**
H_2O	5.6µl	5.6µl	13.8µl
Restriction Enzyme Buffer D	4.0µl	4.0µl	2.0µl
Acetyl BSA	0.4µl	0.4µl	0.2µl
DNA	25µl CD38	25µl CD38	3.0µl PGEM11Zf
Restriction Enzyme Xba1	5.0µl	5.0µl	1.0µl
Total	40µl	40µl	20µl

Table 5.3: Digestion of PCDMCD38 and PGEM11Zf with Xba1 restriction enzyme

The contents were vortexed and spun in a microcentrifuge for 10 seconds to allow the reaction mixture to be homogenous at the bottom of the tube and incubated at 37ºC for 5 hours.

5.27. Visualization of products following digestion with Xba1 enzyme: Following digestion with Xba1 restriction enzyme of the PCDM-CD38 DNA and PGEM11Zf, products were visualized on a low melting point 0.8% agarose gel resulting in the production of a 1.4kb band for CD38 and a linearized band for PGEM11Zf at 3.223kb Figure 5.8.

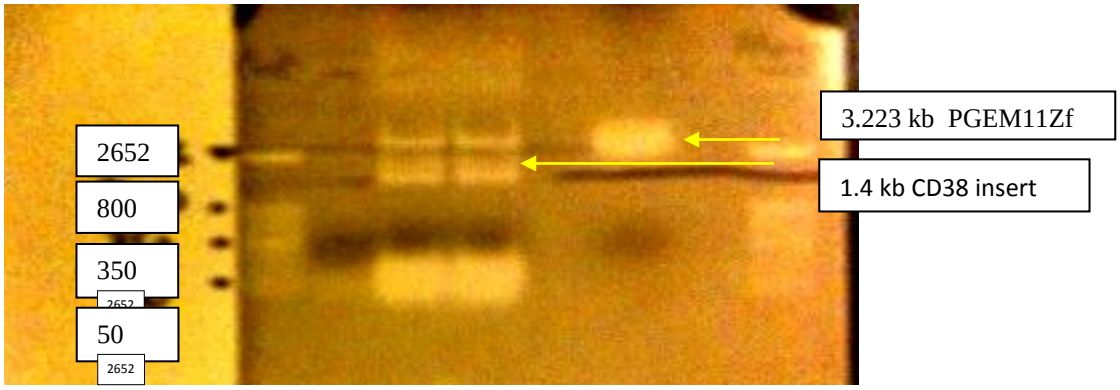


Figure 5.8: Linearized PGEM11Zf vector and CD38 insert following digestion with restriction enzyme Xba1 to release 1.4kb CD38 insert.

5.28. Purification of CD38 insert and linearized PGEM11Zf vector

The 1.4kb CD38 insert was cut out of the low melting point agarose gel and eluted using PCR prep purification system (Promega UK) as described earlier,

and subsequently used for the ligation into linearized PGEM11Zf vector. Starting concentration per tube for CD38 = 25µg and in our sample we recovered approximately 100ng per 1µl. Starting concentration for PGEM11Zf = 3µg and in our sample we recovered approximately 60ng per 1µl.

5.29. CD38 Ligation and Transformation of Competent Cells: Following recovery of the CD38 insert and the linearized PGEM11Zf vector the products were ligated as described earlier and in the details are shown in table 5.4, the ligation products were then used to transform competent cells JM109 (Promega Catalogue No L2001) as described earlier.

	A	B	C	D	E	F
Vector DNA PGEM11Z	2µL	3µL	2µL	2µL	2µL	2µL
Insert CD38	0µL	1µL	6µL	4µL	2µL	2µL control DNA
Buffer x 10	1µL	1µL	1µL	1µL	1µL	1µL
T4 DNA ligase	0.5µL	0.5µL	0.5µL	0.5µL	0.5µL	0.5µL
H_2O	6.5µL	4.5µL	0.5µL	2.5µL	4.5µL	4.5µL

Table 5.4 Ligation of CD38 into PGEM11Zf (A)

Vortex and spin down the reaction mixture and incubate the ligation mixture overnight at 4ºC. Following ligation competent cells (JM109) were transformed as discussed earlier under transformation protocol section 2.42. The presence of the CD38 insert in the transformed cells was again confirmed after digestion of the PGEM11Z plasmid with restriction enzyme XBaI as discussed earlier..

5.30. Sequencing Data to confirm CD38 identity Following agarose gel electrophoresis the insert was cut out, eluted as described earlier and the identity of the insert confirmed by sequencing (performed by Dr Lou Matterhall, Bart's and The London Hospital) (Figure 5.9), giving sequence alignment with CD38 DNA.

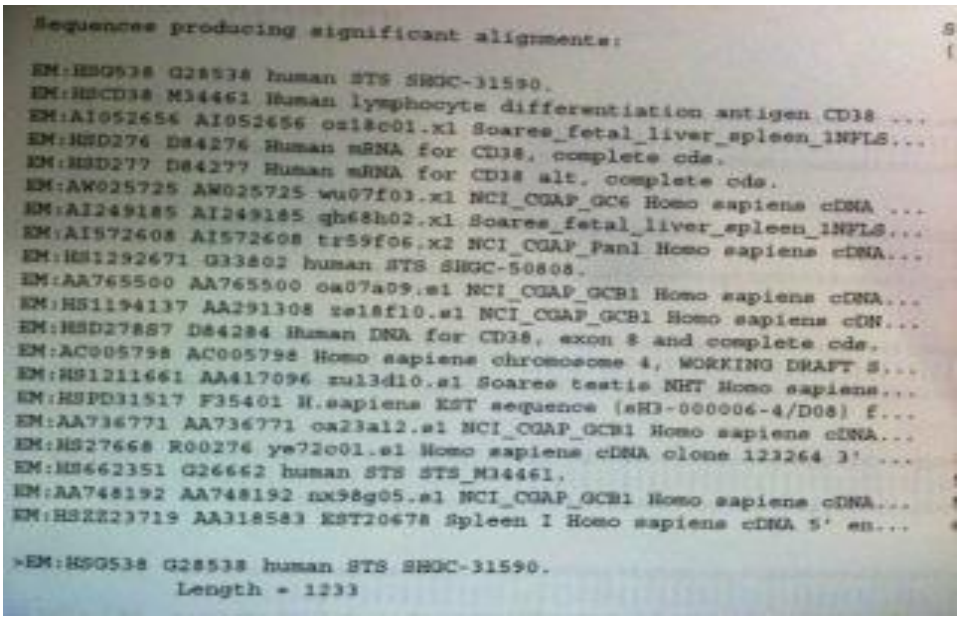


Figure 5.9: DNA sequencing of the insert matched the sequence of CD38 DNA.

5.31. In-vitro transcription and translations using PGEM11Z CD38 DNA:

Translation of CD38 a 42kd protein was carried out using in vitro transcription and translation system (Promega) as previously described. The concentrations of reagents were also optimized to improve the translation of the CD38 protein using both the SP6 as well as T7 polymerase enzymes. To this end optimal concentrations of Mg Acetate (5mM) and KCL (0.5mM) were also employed. Two colonies were tested for the translations respectively using T7 and SP6 polymerase enzymes (Table 5.5) and the translated proteins separated on a 10% SDS page gel.

	Colony 1	Colony 2
Rabbit Reticulocyte	25µl	25µl
Amino acid mix less methionine	1µl	1µl
Buffer	2µl	2µl
Polymerase T7 & or SP6	2µl	2µl
Rnasin Inhibitor	1µl	1µl
CD38 DNA template	2µl	2µl
KCl 90.5mM0	2µl	2µl
Mg Acetate (5mM)	2µl	2µl
35S Methionine	4µl	4µl
H2O Dnase free	10µl	10µl
Total	**50µl**	**50µl**

Table 5.5. In Vitro Transcription & Translation protocol for CD38 synthesis.

5.32. Interpretation: The Translates from both clones gave protein band in the region of 30kd and 38-40 kd region, with no bands in the region of 42kd (data not shown). Furthermore when tested in a liquid phase immuno-precipitation assay as described earlier in chapter 2, CD38 Monoclonal antibody MCA109, T1D sera as well as type 2 diabetic sera previously shown to be positive in western blot analysis in Ferrannini et al laboratory, failed to react or show differences with the translated CD38 protein.

5.33. <u>Detection of CD38 protein by western blotting</u>

The presence of autoantibodies to CD38 has previously been evaluated using western blot analysis in 236 patients with type 2 diabetes and 160 T1Dpatients with positivity in 9.7% and 13.1% respectively (Cinzia et al., 1999). Another report using western blot technique reported that of 377 NIDDM Japanese patients, 13.8% had autoantibodies against CD38. (Ikehata et al.,1998).

CD38 protein was therefore tested in a western blotting system to try and confirm reactivity of the protein with the assumption that, reactivity may be to linear epitopes of the CD38 protein not recognized in the liquid phase immuno-precipitation assay.

5.34. Western Blot Procedure for CD38 antigen: To test the translated CD38 protein 40µl translate from clones 1 and 4 using SP6 and T7 polymerase enzymes respectively were added to 40µl of SDS sample buffer, boiled in a dry block

heater set at 100ºC for 3 minutes and electrophoresed on 10% SDS page gel at 50mVolts for 1 hour.

Following electrophoresis the gel was placed in transfer buffer and the negatively charged proteins transferred onto Nitro-cellulose membrane Hybond NC (Amersham) after preparing a sandwich of Filter paper/ Gel/ Nitro-cellulose/ Filter Paper and transferred at 190 volts for 1 hour in Transfer buffer.

1. The proteins transferred onto nitro cellulose paper, was blocked overnight with Blocking solution containing 0.05% tween 20 in a petri dish.
2. The nitro cellulose membranes were then exposed to the primary antibody anti-CD38 monoclonal antibody (Serotech MCA 109 clone ATB/S) (Primary antibody Mouse Anti-Human CD38 antibody) at 1:1000 dilution on a gentle shaker for 4 hours at room temperature (10µl/10ml solution).
3. The nitro-cellulose membrane was then washed with 10ml wash solution 0.2% Tween Tris Buffer Saline followed by the addition of Anti-mouse Alkaline Phosphatase conjugate (AKP conjugate) at 1:10000 dilution.
4. The membrane was washed with 10ml wash solution 0.2% Tween Tris Buffer Saline followed by the addition of NBT solution which develops the colour to visualize the bands.

5.35. Western Blot Results: The results are shown in figures 6.1 & 6.2 showing a band in the region of 30kd with a weak band at approximately 40kd from both clones 1 and 4 irrespective of the polymerase (T7 or SP6) enzymes employed.

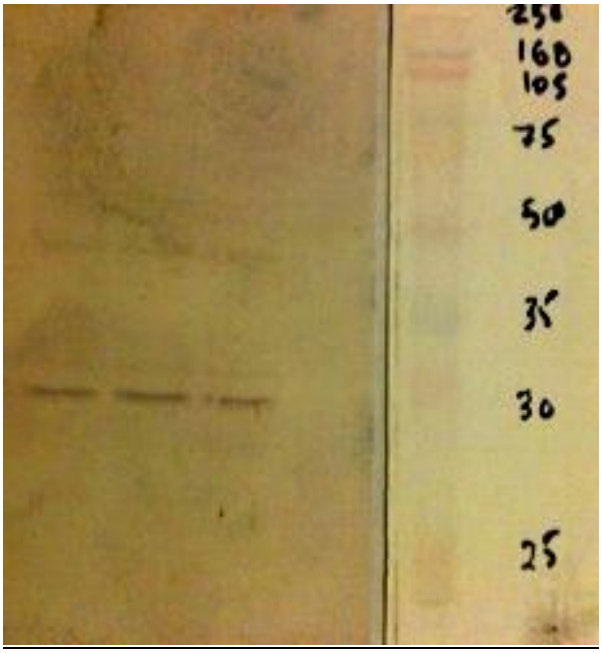


Figure 5.91: Immunoblot showing a band in the region of 30kd and a weak band about 40kd with CD38 protein translated with SP6 polymerase enzyme.

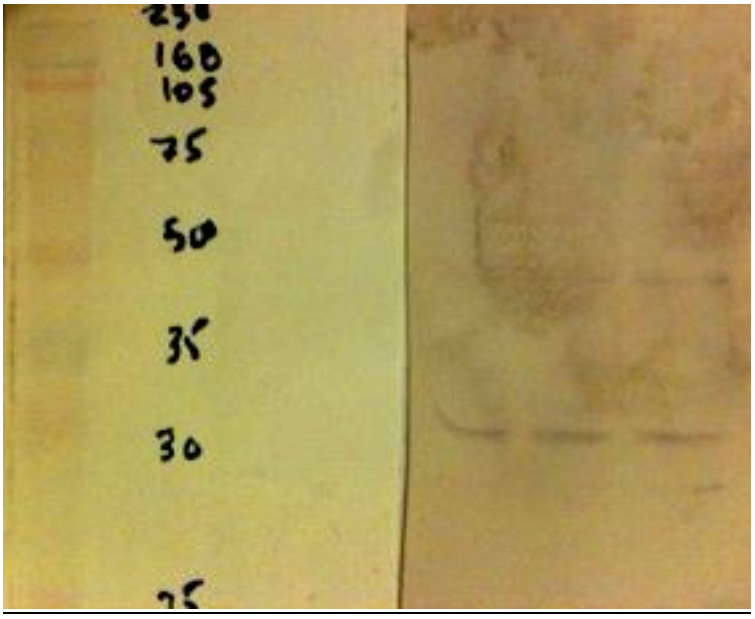


Figure 5.92: Immunoblot showing a band in the region of 30kd and a weak band about 40kd with CD38 protein translated with T7 polymerase enzyme.

5.36. Test serum samples for reactivity in Western Blot: CD38 protein transferred as above in another experiment was tested with serum samples previously tested in the western blot system in Ferrannini laboratory (negative and positive samples) (Tables 5.91 and 5.92) as well as monoclonal anti-CD38 antibody as shown in Figure 5.93.

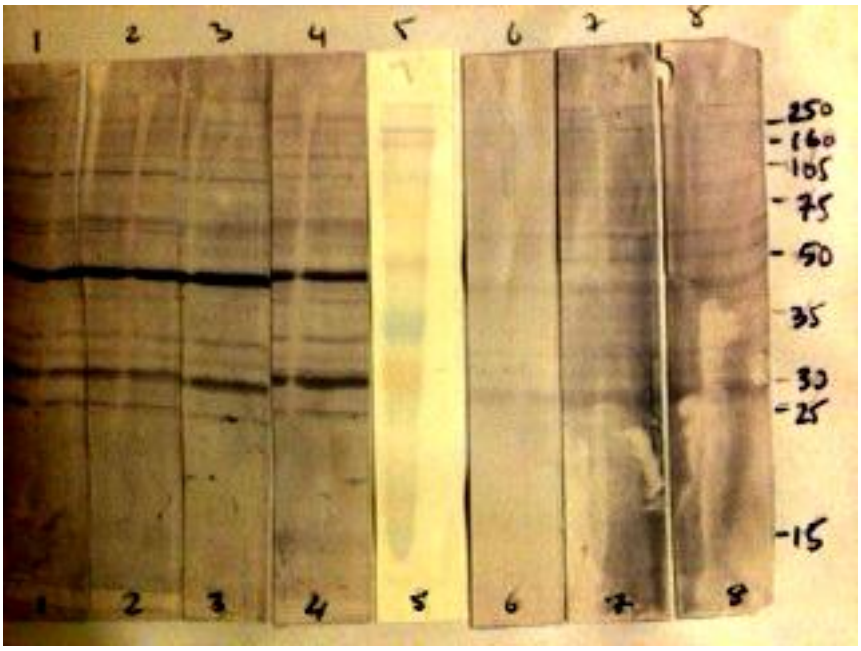


Figure 5.93: Lane 1&2 –Ferrannini sample 151 (positive for CD38), Lane 3 GM (Negative control), Lane 4 Ferrannini 6 (negative for CD38), Lane 5 Rainbow molecular weight marker, Lane 6 Mouse anti Human CD38 (Santa Cruz) antibody 1:1000 dilution, Lanes 7 & 8 Mouse anti Human CD38 (Serotech) antibody 1:1000 dilution.

5.37. Interpretation: Following western blot and probing for immune reactivity using both monoclonal anti-CD38 antibodies from Serotech and Santa Cruz as well as positive and negative serum samples to CD38, bands at approximately 30kd and 40kd were noted. These bands were considered to be irrelevant as when luciferase control DNA (supplied as part of the translation kit) was employed to translate luciferase as a control protein (unrelated to CD38) as well as rabbit reticulocyte lysate alone (with no added DNA), were tested in the immune-blot analysis, both gave similar band of approximately 30kd and 40kd (data not shown). Reticulocytes are juvenile red cells and contain remnants of ribosomes and nucleic acids which are present in larger amounts in the cytoplasm of the nucleated precursors from which they are derived. Luciferase DNA synthesizes a protein of 61kd and with reticulocytes no specific protein should have been translated.

5.37. Conclusion: Our results failed to identify any distinct bands for CD38, that is we were unable to detect CD38A, in patients with type 1 diabetes or type 2 diabetes. Bands were noted in the 30kd and 40kd region, using immuno-precipitation experiments as well as following immune-blot analysis as shown in Figure 5.93 implying proteins were present, as immunoglobulins in patients sera, showed reactivity, however reactivity to these proteins was non-specific, as they were also noted with luciferase and reticulocyte lysate both unrelated to CD38. By implication we found no evidence for the existence of CD38A in diabetes, irrespective of its type.

Glucose induced insulin release has been reported to be attenuated in mice that over express CD38 in the islets (Kato et al., 1995), and attenuated in CD38 knockouts (Kato et al., 1999). In a Japanese study (Fumiko et al 1999) sera containing auto-antibodies to CD38 inhibited insulin secretion from pancreatic islets. While another study (Cinzia et al., 1999) reported that CD38 antibodies potentiate insulin secretion in isolated pancreatic islets and this latter study proposed CD38 as a candidate auto-antigen in type 2 diabetes patients.

The apparent involvement of CD38 in insulin secretion also led to the finding of a mutation of the CD38 gene (Arg ^{140}Trp) in 13% of Japanese type 2 diabetic patients with a family history of the disease (Yagui et al., 1998). Functional and gene studies of CD38 have also been previously reported (Malavassi et al., 2008). The original identification of CD38 autoantibody in diabetes patients (Cinzia et al., 1999) employed, a solid phase immunoblot system. However it was unclear whether conformation of the CD38 molecule was a prerequisite for reactivity to the CD38 protein, as in these experiments only the solid phase assay system was employed to test for reactivity to the CD38 protein. The liquid phase assay systems, unlike the solid phase assay systems, have been shown to be more sensitive as well as specific (Wilkin et al., 1989). This feature has been reported in previous DASP work shop data using different autoantigens (Bingley et al., 2003) and the evidence is that solid-phase systems are less sensitive and less specific than liquid-phase assays, the former recognizing linear epitopes of the antigen as opposed to the liquid phase assays which recognize conformational epitopes The difference between solid or liquid phase assay systems could also

reflect differences in the detection of different subpopulations of antibodies to different auto-antigens. For example with insulin, small amounts of high affinity antibody have been reported to be readily detected in radioactively-labelled binding assays, while large amounts of low affinity antibodies are more readily detected by solid phase ELISA assays (Wilkin et al., 1989).

The low reactivity of the CD38 antigen was a major drawback in this project, this could have been due to the low incorporation of ^{35}S methionine into the CD38 protein (low incorporation even when Leucine was used in place of methionine as discussed earlier). We failed to show clear reactivity to CD38, similar low reactivity and a failure to detect relevant binding of antibodies to recombinant CD38 has also been reported previously using a similar liquid phase assay system (Sordi et al 2005). The data presented confirms previous reports of low reactivity to the CD38 antigen and question its relevance as a marker in differentiating T1D from type 2 diabetes.

6.0. CHAPTER 6

GENERAL DISCUSSION

Diabetes affects about 6% of the population of the United States about half of whom are undiagnosed, with 'type 2 diabetes', accounting for 90% of the cases and in the United Kingdom approximately 2.8 million people suffer from the disease. A large global variation in the incidence of T1D has been reported and the north south gradient was not confirmed (Karvonen et al., 2000). Autoimmune T1D has been described classically as being predominantly of childhood-onset, insulin-requiring and presenting with ketoacidosis and weight-loss. Over the last decade there has been an explosion of cases of diabetes reported in children and young adults presenting as overweight or even obese leading to an excessive stress on the beta cell function and accelerated islet cell destruction. Increasing obesity, including childhood obesity, favouring insulin resistance, autoimmunity and double diabetes, has become a major concern in the youth accounting for the Islet cell immunity through different mechanisms (Naik & Palmer 2009). This increase cannot be explained by an increase in the genetic load in the short space of time but rather points towards a changing environmental impact as well as lifestyle in the youth. The threshold hypothesis describes the putative interaction of the genetic as well as environmental loads and the impact of these in TID (Wasserfall C et al., 2011). The thresholds for developing TID have also changed with increased migration, decreased genetic load and the wide impact of environmental factors on susceptible individuals (Bodansky et al 1992).

WHETHER AUTOANTIBODIES AND CARBOXY METHYL LYSINE (CML) ARE GENETICALLY PREDICTIVE OF T1D.

Advanced Glycation End Products are formed both enzymatically as well as non-enzymatically and the products of AGES activation can have profound effects physiologically, with the production of reactive oxygen species as well as a plethora of reactivation cycles of AGES. Following a prolonged hyperglycaemic state reactive oxygen species, chemokines, as well as gene expression result, leading to the formation of AGES which bind to receptors on endothelial cells, smooth muscle cells as well as immune cells (monocytes, macrophages and dendritic cells), activating formation of reactive oxygen species, as well as further activating the immune system. This activation in turn induces intracellular signaling leading to increased oxidative stress to the cells and tissues, with further production of reactive oxygen species and pro-inflammatory cytokines, that can lead to microvascular as well as macrovascular damage.

The data presented here show ICA positive children at risk of TID had significantly elevated levels of CML both before and at diagnosis of TID in comparison to their controls. Serum CML levels tended to increase in those progressing to T1D. Similarly elevated CML levels were noted in twins discordant for T1D, irrespective of whether they had T1D or not, consistent with a non-genetic environmental effect. CML levels in twins were also higher than in children with TID and their controls. This difference can be accounted as an age effect as the twins were older such that they would be exposed to a significantly

longer period of AGES in comparison to the children but also potentially to storage differences as samples in Germany were stored at -80C and in -20C in the UK. However such differences would not explain the results as control samples were under similar storage conditions in each population.

Raised CML levels in the twins and the school children may be the result of dietary factors, given that they are likely due to shared environmental factors and CML is known to increase due to ingestion of heat-treated food. Raised CML levels could have a harmful effect in the long term, through promoting a pro-inflammatory response, but our data cannot determine this categorically, however raised serum CML is predictive of the disease. Moreover, serum CML was predictive additively with serum autoantibodies: raised CML and presence of autoantibodies gave a PPV in population and twins 46.5% and 47.9%, and 33.7% and 85.7% respectively.

On the other hand autoantibodies were discordant in the twin pairs, in that the non-diabetic twins tended not to have autoantibodies and the diabetic twin did, consistent with a non-genetic determinant event for presence of auto-antibodies unlike CML levels which were elevated in both the index twin and the co-twin. So raised CML in twins discordant for diabetes cannot simply be attributed to an increased auto-inflammation as it was detected in both index as well as their non-diabetic twins, of whom none of the latter had auto-antibodies to GADA, IA2 or ZnT8. But the predictive values imply that these two processes, one

proinflammatory and the other adaptive immunity (as reflected by CML and autoantibodies) act additively to predispose to T1D.

WHETHER AUTOANTIBODIES, INSULIN SECRETION AND INSULIN RESISTANCE ARE PREDICTIVE AND ARE GENETICALLY DETERMINED?

Twin studies have provided a powerful tool to investigate genetic and environmental factors by comparing identical and non-identical twins. It is clear that both immune as well as metabolic changes can be detected many months or even years before diagnosis of T1D, changes that are distinct between twins who develop diabetes and those who do not. These immune as well as metabolic changes can predict the onset of development of T1D.

Autoimmune diseases affect up to 5% of the population and are a major cause of morbidity and mortality. Autoantibodies to key islet cell antigens can be detected in 80% of newly diagnosed T1D patients and are a hallmark for it. Twin studies have provided evidence that both genetic as well as environmental factors are involved in the pathogenesis of autoimmune diseases as the concordance rate for TID ranges from 40-60%, well short of 100%, indicating that other non-genetic environmental factors are involved in the triggering of the disease. The study of twins discordant for TID in the United States of America suggested that with sufficient follow up of twins the concordance rate would be much higher (Redondo M et al., 2008). Studies have shown that the majority of twins with an autoimmune disease have a co-twin who is unaffected and continues to be

unaffected, confirming our data of discordance for T1D even after >40 years of follow-up from the diagnosis of the index twin.

Loss of the FPIR, is a hallmark of beta cell failure and is amongst the earliest signs together with autoimmune changes leading to hyperglycaemia, glucose intolerance and frank diabetes. There was however, no evidence in the data presented of any impaired beta cell function in the non-diabetic twins who remain discordant for diabetes, results which are consistent, with a non-genetic event which affects one twin but not their genetically identical co-twin. In the pre-diabetic twins higher fasting insulin levels as well as lower FPIR and glucose clearance were noted and these twins went on to develop diabetes. The hypothesis that altered islet beta cell function might be genetically determined and predispose to T1D, was rejected. Pre-diabetic twins furthermore showed evidence of autoimmune features with the presence of diabetes associated auto-antibodies to GADA, IA2ic and ZnT8. Auto-antibodies to islet antigens GADA, IA2A and ZnT8 has provided a valuable tool in the prediction of T1D in at risk patients as well as in the general population.

DETERMINE WHETHER THE ISOTYPE OF GADA AND IA-2A IN TYPE 2 DIABETIC PATIENTS DIFFERED FROM THOSE FOUND IN TYPE 1 DIABETIC PATIENTS.

The historical methods employed for the detection of antibodies to islet antigens employed immunoflourescence of human group O cryofixed pancreatic sections. This assay is prone to variability due to: 1) nonspecific binding to islet cell components, 2) variation of the amount of antigen containing tissue, 3) variation

of the results due to operator interpretation which is highly subjective and 4) inability to confirm the identity of the islet antigens involved. In general, ICA is restricted to immunoglobulin of the IgG1 isotype belonging to the IgG subclass (Millward et al., 1988), (Dozio et al., 1994). In the ICA technique it is however impossible to determine the exact nature of the lslet antigen without performing pre-absorption assays using purified antigens, this in itself is an added step with no guarantee of the heterogeneity of the islet cell antigens present in the section employed. Enzyme Linked Immunosorbent Assays have been described using glutamic acid decarboxylase from brain extracts which is rich in the two isoforms GAD_{65} and GAD_{67} for the detection of autoantibodies, however these assays lacked specificity and sensitivity.

In this study the earliest available samples from pre-diabetic Twins, non-diabetic twins and noninsulin requiring diabetes but antibody positive cases were screened for antigen specific isotypes. Antigen specific isotype profile to GAD_{65} and IA2ic did not show any evidence of maturation in the immune response, with IgG1 the dominant isotype detected in the samples before as well as at diagnosis of T1D. Similarly in the noninsulin requiring diabetes GADA positive patients again there was no evidence of antibody isotype's other than IgG1. However SPS patients, tended to have isotypes other than IgG1, in particular, IgG4 as well as subtypes IgE isotype, which T1D patients lacked (Lohman et al 2000). Our findings were consistent with the findings in the BABYDIAB study where diabetes onset and β cell destruction occurred during an early or subsequent IgG1 dominant antibody response (Bonifacio et al 1999).

In conclusion, isotype response to both GAD as well as IA2 were both restricted to the IgG1 isotype in both T1D as well as type 2 diabetes patients confirming that the nature of the antigen specific antibodies in type and type 2 diabetes are similar and consistent with a Th1 type immune response.

WHETHER AUTOANTIBODIES IN ADULTS PRESENTING WITH TYPE 2 DIABETES ARE PREVALENT AND SIMILAR TO T1D.

The American Diabetes Association Expert Committee proposed an etiologic classification of diabetes with type 1A diabetes representing immune-mediated diabetes and type 1B a non-autoimmune idiopathic form of type 1 diabetes (Expert Committee 1997). In this classification autoimmunity was defined as the presence of one or more islet cell autoantibodies to insulin, GADA, IA2A and ICA in 80-90% of the patients being present when hyper-glycaemia is detected. The definition of LADA set by the Immunology of Diabetes Society and Action Lada (www.actionlada.org), age 30-70 at diagnosis, free of insulin for at least 6 months and presence of autoantibodies.

Auto-antibodies to islet cell antigens ICA, Insulin (IAA), GAD_{65}, IA2ic, and ZnT8 are a feature of T1D particularly in children and can also be detected in a small proportion of adults presenting with non-insulin requiring diabetes, termed as LADA (Leslie RD et al 2006). The prevalence of LADA in Caucasian adults presenting with non-insulin requiring diabetes has been reported to be between 4-12%, but usually based on measurement of GADA alone (Leslie RD et al., 2006).

Such LADA patients exist, however, are they distinct from T1D and 'type 2 diabetes' patients. Studies by (Falorni et al., 2000), (Hampe et al., 2002) using fusion proteins indicated that there was a distinct reactivity in GADA positive type 2 diabetes patients termed LADA. However one caveat of employing recombinant fusion proteins, not discussed is that fusion proteins do not maintain the secondary structure of the synthesized proteins. Fusion protein folding as well as epitope recognition by antibodies can be affected when fusion proteins are employed as has been confirmed in studies with fusion proteins using circular dichroism spectroscopy (Wang et al., 2011). Such protein modifications could affect protein conformation which could explain previously reported differences in T1D and LADA, (Falorni et al., 2000), (Hampe et al., 2002).

The majority of typical T1D patients, on the other hand, have auto-antibodies in the serum and these auto-antibodies are predictive of the disease. It has further been reported that a proportion of auto-antibody negative 'type 2 diabetes' patients can also have T cell reactivity to islet extracts transferred onto nitrocellulose membrane, if this is confirmed in larger studies the reported prevalence of autoimmune diabetes in adults would be higher than currently proposed (Brooks-Worrell et al., 2011). However, whilst T-cells appear to have a central role in the pathogenesis of autoimmune diabetes, the study of T-cells is technically laborious and requires extensive expertise in performing T cell activation/proliferation assays. Furthermore the reported studies employed islet extracts from nitrocellulose exposing linear epitopes which can modify both T-cells as well as B-cell reactivity.

The focus of diabetes-prediction studies, have been predominantly to test auto-antibodies as markers of autoimmunity in the course of the disease. In this report of 6156 clinic based patients tested for several diabetes-associated antibodies, the prevalence of autoimmunity was noted to be 9.7%. Other similar studies have been much smaller with a marked selection bias, based on selecting only cases with apparent 'type 2 diabetes' or cases likely to have LADA. In this present study cohort all cases presenting with diabetes and within the selection criteria were included. At diagnosis in the Action LADA study, antibody positive patients were leaner and younger and more often put on insulin therapy in comparison to the antibody negative patients classified as 'type 2 diabetes' patients. GADA antibodies alone were identified in 8.8% accounting for 90% of all cases presenting with diabetes associated antibodies. Retesting of samples that were scored positive for any antibody reduced the false positive errors to <2%. Furthermore in the analysis of the dataset, in house controls were employed including a positive control sample tested by titrating to end point to determine the cut off for positivity. This methodology was further confirmed by employing QQ plot analysis which further confirmed the selected cut off for positivity for each antigen as well as the strategy followed in the BabyDIAB study (Zeigler et al., 1999). The interpretation of the data from this thesis recommends the use of QQ plot where large data sets are analyzed, instead of using percentiles of healthy controls alone. The data also adds to the validity of the use of end point titration curves employed here to evaluate the cut off levels for auto-antibody assays.

Importantly, the consistently high prevalence of patients with adult-onset diabetes who can be ascribed to have autoimmune diabetes i.e. about 10% (5-15%) implies a high prevalence of autoimmune diabetes in general (Akinson & McLaren 1994), (Redondo et al., 2008), (Peppa et al., 2003), Rosario et al., 2005). Indeed, these observations suggest that the most prevalent form of autoimmune diabetes is in adulthood and not in childhood as previously thought. This present study is the largest to date, and benefits from an analysis of all cases attending these clinics, irrespective of clinical features, and screening with three major diabetes-associated auto-antibodies. A small fraction of the patients had diabetes-associated auto-antibodies other than GADA, so the projected shortfall of ascertainment using GADA alone would be small. Interestingly these patients with auto antibodies other than GADA had similar characteristics to the GADA positive cases in that they were leaner as well as younger. Within the GADA positive cases at least two modes were identified using the QQ plot analysis, with a cut-off of 70 WHO IU (low GADA titre) and another inflection at 200 WHO IU (high GADA titre). However there was no clear evidence of a bimodal distribution of the dataset in this analysis unlike the log transformed data presented by the NIRAD study (Buzzetti et al 2007). A bimodal distribution would have to show two normal distributions one for the low GADA titre and another one for the high GADA titre. The NIRAD study was substantially smaller in comparison to the dataset presented here (n=191 versus 701). In this present study, we did not confirm that a bimodal distribution was evident, though

the log transformed data did show two apparent modes, however each mode was not normally distributed.

In this cohort of 6156 clinic based patients presenting with diabetes, autoantibodies to GADA, IA2A and ZnT8 identified 90% of adults with autoimmune features. The information presented here makes the term LADA an increasingly inappropriate term to describe adults presenting with a spectrum of autoimmune features which are similar to T1D and distinct from 'type 2 diabetes' patients.

ARE AUTOANTIBODIES TO THE ADP RIBOSYL CYCLASE/CYCLIC ADP-RIBOSE HYDROLASE (CD38) ASSOCIATED WITH AUTOIMMUNITY IN TYPE 1 AND TYPE 2 DIABETES.

Autoantibodies to CD38 have been reported previously in 13.8% of Japanese Type 2 diabetes patients (Cinzia et al., 1999). Anti CD38 antibodies, both inhibitory as well as stimulatory to the release of insulin have been previously reported (Ikehata et al., 1998), (Cinzia et al., 1999), suggesting that anti CD38 auto antibodies, may be an additional cause of impaired glucose induced insulin release. They, as well as others, employed western blotting, assay procedures, which recognize linear rather than conformational epitopes of the CD38 molecule with a lower specificity and sensitivity.

The aim in this project was to insert the CD38 cDNA into a suitable vector to allow transcription and translation of the CD38 protein, for subsequent use, in a

liquid phase immuno-precipitation assay. As auto-antibodies have been noted to be predictive for the development of TID and ICA comprises auto antibodies to several antigens including GADA, IA2A, ZnT8A as well as other antigens yet to be identified, it was conceivable that the antigen CD38 (antibodies to which were reported in type 2 diabetes patients), could be an additional marker in TID prediction. Since genetic susceptibility through HLA is part of normal genetic variation and since monocyte changes, found by us in T1D and by others in 'type 2 diabetes', are also associated with T1D, it is possible that the spectrum of changes leading to T1D extends across the whole spectrum of diabetes, so that selected auto antibodies could occur in all forms of diabetes.

A number of hurdles were encountered in the process of preparing the CD38 insert into the PGEMIIZ vector which in itself is an important point to make in particular when working in a laboratory where other vectors are routinely employed. It was noted that the initial CD38 insert into the vector PGEM11Z and growth of subsequent colony on agar plates had actually resulted in the insertion of the GAD_{65} cDNA instead of CD38 cDNA.

Despite adherence to good laboratory practice this problem only became evident after performing extensive purification as well as immune-precipitation experiments, confirming an experimental issue with the CD38 cDNA prepared. This was later confirmed to be GAD_{65} following sequencing data analysis of the presumed CD38 cDNA. So an important message from these observations was that experiments must be performed under the strictest care. Furthermore before

embarking on detailed experiments it is vital to confirm the identity of the cDNA being prepared.

In subsequent experiments anti CD38 antibodies using CD38 translated protein did not differentiate between healthy control subjects, TID or cases with type 2 diabetes. This finding has also been confirmed by another researcher (Sordi V et al 2005) and in that study they too failed to show any reactivity to the CD38 antigen in the liquid phase assay system consistent with the data presented here. Auto-antibodies to islet antigens IAA, ICA, GADA, IA2A and ZnT8 identify the majority of autoimmune diabetes patients. The appearance of auto-antibodies does not follow any specific pattern and can vary substantially. However, the presence of multiple auto-antibodies provides the highest positive predictive value for T1D.

T-cells are the earliest hallmark of beta cell infiltration of the islet cells in the pancreas. A recent report by (Brooks-Worrell et al., 2010) identified a group of phenol-typically type 2 diabetic patients, all of whom were autoantibody negative but showed T cell reactivity using islet cell extracts transferred to nitrocellulose, such patients could not have been detected by testing for auto-antibodies alone. However given the lack of a reliable, reproducible and easy to perform T-cell test, measurement of auto-antibodies to GADA, IA2A, ZnT8, IAA and ICA will continue to be the best line of identifying at risk individuals with active autoimmune features leading to T1D.

A number of environmental factors have been implicated in genetically susceptible individuals including, chemicals, early infant diet, virus infection

(Mumps, rubella, cytomegalovirus, entero-viruses such as coxsackie) and exposure to cold environmental conditions have been implicated. The gut microbiome may also play a part in the cause of T1D following infection affecting the gut permeability leading to the activation of the immune system. Exposure to cow's milk early in life has been implicated in both the BB rat model as well as humans. A number of population based studies have been initiated and these include the DIPP, DAISY, BabyDiab, TRIGR and PANDA studies, results from these studies will provide more information on the factors involved in the cause of T1D.

6.1. FUTURE WORK

The concordance rates, for T1D ranges from 10-60%, depending on the ascertainment of the cohorts studied indicating a dominant environmental effect. A preliminary paper by us identified DNA methylation variable positions in monocytes (CD14+) associated with T1D, implying one possible cause of this missing non-genetic effect could be epigenetic. These changes in monocytes could also be found in children at risk of the disease (Vardhman et al., 2011). The large EU programme BLUEPRINT has funded us to examine these questions in more detail. With respect to environmental agents we also intend to study the gut microbiome in twins concordant and discordant for T1D, to determine the impact of the gut microbiota on the disease. Other environmental factors include the food we ingest and we are interested in the character of AGE which might offer a clue relating to the reason high levels of CML in the serum are predictive of T1D.

7.0. CHAPTER 7

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