

TOWARDS PREVENTION OF TYPE 1 DIABETES

Population-based risk assessment
in the Oxford Region

Thesis presented for the degree of Doctor of Medicine
in the University of London

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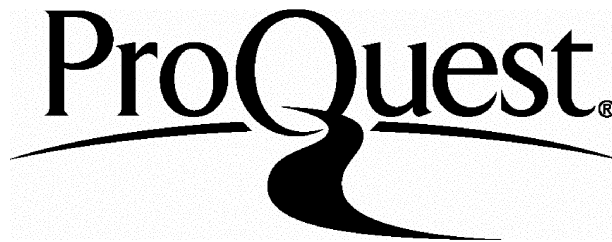
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Abstract: Intervention to prevent or delay the clinical onset of Type 1 (insulin-dependent) diabetes now appears a realistic possibility. In this thesis I review a number of preliminary steps that must be completed before a clinical trial of this type can be undertaken. I describe the population-based, prospective Bart's-Oxford study of childhood diabetes and its use to determine the incidence of Type 1 diabetes in the Oxford Regional Health Authority (RHA). Implications of epidemiological studies for understanding the pathogenesis of childhood diabetes are discussed. I review the advantages and difficulties of the family study approach, and show how these were taken into account in the design of the Bart's-Oxford family study. I review the genetic approach to prediction before describing studies I have undertaken to improve risk assessment in family members using (i) immune markers (islet cell antibodies (ICA) alone and in combination with other humoral markers) and (ii) the intravenous glucose tolerance test. I report a comparison of patterns of basal insulin secretion in ICA positive family members and controls to assess their value as potential early metabolic markers. I describe how these findings can be used in the design of a multicentre intervention study in ICA positive relatives. Only 10% of children with newly diagnosed diabetes have an affected first degree relative and identification of high risk subjects with no family history of diabetes is essential if intervention is to have any impact on the frequency of Type 1 diabetes in the population as a whole. I therefore examined the prevalence and prognostic significance of ICA in the general childhood population, comparing 2,925 schoolchildren in the Oxford RHA with 274 age-matched siblings from the family study. This demonstrated that ICA are considerably less predictive of Type 1 diabetes in the general population than in relatives and, on the basis of my findings, I have proposed a possible two-stage strategy for screening in the general population to overcome these limitations.

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Acknowledgements

Whilst undertaking the work described I was funded by the Medical Research Council. The study has been supported by the British Diabetic Association, the Juvenile Diabetes foundation and the Joint Research Board of St Bartholomew's Hospital. The work described forms part of a large and long running project that has involved the cooperation of a huge number of people. Thousands of family members have supported the study over the years; their generosity and loyalty to the projects have made the work possible. Almost 3,000 schoolchildren agreed to give blood samples, for many it was the first needle they had encountered, but their bravery allowed us to move into a whole new area of disease prediction. The Family Studies have been run by the project administrator, Pam Sawtell, and the field-workers, Cathy Chadburn, Penny Franklin, Sarah Jones, Margaret Keggin, Denise Morgans and Sallie Wall; they have provided most of the samples and data that have allowed the work to be undertaken. Assays have been performed by many people working in the Department of Diabetes and Metabolism, St Bartholomew's Hospital, and the Department of Immunology of the London Hospital Medical College, but in particular I would like to thank Hilary Gillmor, Alistair Williams and Marion Shattock. The studies could not have been contemplated without the support of all the members of the Bart's-Oxford study group whose patients have formed the core of all the family studies. I have worked with many colleagues throughout the world on the ICARUS and EURODIAB projects described and am grateful to them all. In particular I would like to thank Ezio Bonifacio and Franco Bottazzo and, above all, my mentor Edwin Gale for his endless stimulus and support.

CHAPTER 1 - INTRODUCTION AND AIMS

Introduction

Type 1 or insulin-dependent diabetes is the most common endocrine disorder of childhood and young adult life. Its incidence has doubled over the past 20-30 years in many European countries. Late complications of this disease include nephropathy, retinopathy, neuropathy, ischaemic heart disease, and in consequence life expectancy is reduced by about one third.

Over the past twenty years enormous advances have been made in our understanding of the genetic basis of susceptibility to Type 1 diabetes. Further, it has become clear that autoimmune processes are involved in the gestation of the disease, that these develop over many years, and that progression to diabetes can to some extent be predicted. This in turn has raised the intriguing possibility that it might prove possible to intervene in the disease process at a time when glucose tolerance remains normal and the beta cell mass is relatively intact. Indeed, three large intervention trials will shortly be under way.

Many of the developments in basic science which underpin our current understanding of the disease process have yet to be evaluated in clinical practice. Clinical evaluation of this new and unfamiliar technology will however revolve around the familiar issues of risk assessment and risk reduction, based on large carefully planned clinical studies, and employing principles common to many types of screening and intervention. Certain questions are basic to this endeavour. Can low and high risk groups be identified? How do we define risk? How reliable are the markers? How and when might it be possible to intervene? How should such an intervention be planned?

Over the past five years I have been responsible for a large family study programme. I have estimated the background incidence of Type 1 diabetes in our study area, and have studied the natural history of the events leading up to overt diabetes in siblings and parents of children with diabetes. I have helped to evaluate

the prognostic significance of a variety of autoantibodies directed against epitopes on islet cells, and of changes in patterns of insulin secretion which can be identified at a time when glucose tolerance is normal or only mildly impaired. I have been responsible for a screening programme in healthy schoolchildren in our study area, and have compared the prognosis of children with and without a first degree family history of the disease. I have developed an analytic pathway, using a decision tree analysis, which can be used in risk assessment, and have applied this information in the design of a large prospective controlled trial. This work forms the basis of my thesis.

Objective:

The overall aim of the work described in this thesis has been to improve understanding of the aetiology and pathogenesis of Type 1 (insulin-dependent) diabetes mellitus, to improve prediction of the disease, and to prepare the way for intervention trials.

Specific aims:

1) To determine the incidence of Type 1 diabetes in the Oxford region and its relation to disease incidence in other areas and to earlier studies.

2) To improve the prediction of diabetes in family members of children with Type 1 diabetes: This has involved continuing surveillance of the existing clinic-based cohort from the Bart's-Windsor Family Study and the establishment of the population-based Bart's-Oxford Family Study.

The prognostic significance of islet cell antibodies has been re-examined using quantitative measurement of the antibodies, and the value of other autoantibodies used in combination with ICA has been investigated.

3) To investigate metabolic abnormalities preceding the clinical onset of Type 1 diabetes:

This has included

(i) cross-sectional and longitudinal study of the acute insulin response to intravenous glucose in high risk subjects identified from the family studies.

(ii) assessment of the comparability of intravenous glucose tolerance tests performed in different centres around the world and development up of a consensus protocol suitable for general use.

(iii) study of the oscillatory patterns of basal insulin secretion in islet cell antibody positive family members and controls.

4) To measure the prevalence and estimate the prognostic significance of immunological markers of high risk in a population of children with no family history of Type 1 diabetes: This was based on a study in 2,925 schoolchildren in the region who were compared with 274 siblings of children with Type 1 diabetes participating in the Bart's-Oxford family study.

To maintain a logical flow and place my own work within the context of that of other investigators, I have described the main projects I have undertaken in the appropriate section within the subject chapters rather than in separate chapters. The relevant sections are indicated in the introduction to each chapter.

CHAPTER 2 - DEFINING THE DISEASE

2.1 INTRODUCTION:

An initial step towards prevention of insulin dependent diabetes in the general population is to obtain an estimate of the frequency of the disease in a defined population. To do this it is essential that the condition whose frequency is being measured is clearly defined, as is the population in whom it is being studied.

2.2 DEFINITION OF INSULIN DEPENDENT DIABETES MELLITUS:

2.2.1 Diagnosis of diabetes:

The World Health Organisation (WHO) describes untreated diabetes mellitus as being 'characterized by a chronic elevation of the concentration of glucose... resulting from deficient production or action of insulin' (World Health Organization, 1985). From ancient times diabetes mellitus has been defined by description of a characteristic constellation of symptoms with glycosuria (Papaspyros, 1952). The realization that glycosuria reflected hyperglycaemia, together with advances in methods of blood glucose measurement allowed definition of disease to progress from the stage of clinical description to a more precise, quantitative description in terms of blood glucose concentration. After a prolonged period of confusion surrounding the precise definition of the disease (West, 1978), there are now sets of diagnostic criteria for both symptomatic and asymptomatic diabetes, from the National Diabetes Data Group (National Diabetes Data Group, 1979) and from the WHO. Both have defined diabetes in terms of either elevation of fasting or random glucose in the presence of symptoms, sustained elevation of fasting or random glucose in the absence of symptoms or abnormalities of the oral glucose tolerance test. The WHO criteria are most commonly used and have been used throughout this thesis.

In clinical practice, childhood diabetes is generally diagnosed by a combination of typical symptoms, as described by the 6th century A.D. Greek physician Aretaeus of Cappadokia, in association with marked random or fasting hyperglycaemia. In a survey of all newly diagnosed cases of diabetes in residents of Rochester, Minnesota between 1945 and 1969, Melton and colleagues found 12/38 cases

diagnosed before the age of 20 had fasting blood glucose above 16.5 mmol/l (300 mg/dl) and 27/38 (71%) had fasting blood glucose above 11 mmol/l (200 mg/dl). A significant minority, however, presented with mild fasting hyperglycaemia (6 - 11 mmol/l) and 3/38 (8%) were diagnosed only on an abnormal glucose tolerance test (Melton *et al.* 1983). In epidemiological studies, even in childhood diabetes, it is therefore necessary to have the rigorous, standard definition of disease provided by the 75g oral glucose tolerance test (OGTT) as specified by the WHO.

2.2.2 Classification of diabetes:

Table 2.1 The classification of diabetes (National Diabetes Data Group 1979)

Insulin dependent or Type 1	Non insulin dependent or Type 2
HLA associations	Familial aggregation
Abnormal immune reactions	Not insulin dependent or ketosis prone
Insulinopenia	Serum insulin levels normal, elevated or depressed
Ketosis prone	Insulin resistance
Dependent on injected insulin to prevent ketosis and preserve life	

The existence of different subtypes of diabetes mellitus has been described from earliest times. Ancient Hindu writings distinguished between a wasting form in the young and another ascribed to injudicious diet. This difference was more formally encapsulated in the descriptive terms 'diabète maigre' and 'diabète gras' coined by Lancereaux in 1879, or the later 'juvenile onset type' and 'maturity onset type'. In the 1930s Himsforth recognized that there were insulin-sensitive and insulin-insensitive forms of diabetes (Himsforth, 1936) and in 1951 Lister and colleagues coined the terms Type 1 and Type 2 diabetes to describe them (Lister *et al.* 1951). These two subtypes of primary diabetes differ in clinical characteristics, genetics and immunological features (Table 2.1). The nomenclature used can create confusion and Keen has suggested that insulin dependent and non insulin dependent should be used to describe clinical subgroups whilst Type 1 and Type 2 should be applied to aetiologically different classes which may be subdivisions of the two main groups (Keen, 1983). Determining the frequency of Type 1 (insulin dependent) diabetes (IDDM) requires a practical method of distinguishing between this condition and the Type 2 (non insulin dependent) form (NIDDM). The NDDG

definition rests on the association of a constellation of clinical, genetic and immunological features with the fundamental characteristic of 'Dependence on injected insulin to preserve life'(National Diabetes Data Group, 1979). This is fortunately not commonly tested! A variety of studies have, however, attempted to correlate various potential discriminants with dependence on insulin.

Classification by age of onset: The peak age of onset of Type 1 diabetes is seen in puberty as confirmed in many studies. A significant proportion of cases occur, however, after this age and the NDDG specified that 'the insulin-dependent, ketosis-prone type of diabetes....has been inappropriately termed juvenile diabetes. Since it can occur at any age, it is recommended that diagnosis based on age of onset be eliminated.' There is also a subgroup of young people who have non insulin dependent diabetes. (Tattersall *et al.* 1975), otherwise known as maturity onset diabetes of the young (M.O.D.Y.). The age relationships of Type 1 diabetes and Type 2 diabetes were examined in a cross-sectional study of the medical records of all persons diagnosed diabetic by WHO criteria attending municipal health centres in the Kuopio University Central Hospital district in East Finland. Criteria for Type 1 diabetes were (1) treatment initially and thereafter with insulin, and (2) ketonuria or ketoacidosis at the time of diagnosis. Criteria for Type 2 diabetes were (1) initial treatment with diet and/or oral drugs and not insulin treated within one year and (2) no ketonuria or ketoacidosis at the time of diagnosis or later. Criteria were applied irrespective of age at diagnosis. 3389 cases were identified, with 95% case ascertainment in rural areas and 64% in the town of Kuopio (the remainder being under the care of private physicians). 2994 cases were classifiable as Type 1 diabetes or Type 2 diabetes (information was incomplete on 328; 19 had secondary diabetes and 48 were unclassifiable, having been initially treated with diet/oral drugs but started on insulin within 1 year). 281 patients were classified as Type 1 diabetes, 2713 as Type 2 diabetes. The percentage of diabetic persons classified as insulin- dependent and non-insulin dependent by age of diagnosis is shown in the figure. The age of onset of Type 1 diabetes varied greatly but in 63% was less than age 19. Below this age 100% of cases were insulin-dependent whereas between age 20-24 and 25-29 years the

proportions of Type 2 diabetes were 3 and 5% respectively. At age 50 the relative proportion of Type 2 diabetes exceeded that of Type 1 diabetes (57% vs 43%) (Figure 2.1) (Laakso *et al.* 1985).

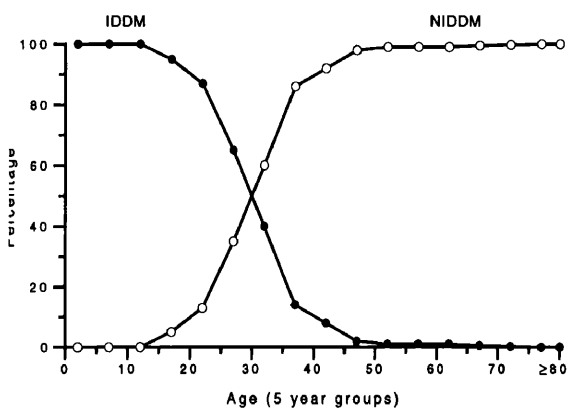


Figure 2.1 Percentage of diabetic persons in Kuopio, Finland classified into the insulin-dependent and non-insulin-dependent types of diabetes by age at diagnosis (Laakso *et al.* 1985)

incidence of diabetes by clinical type in different age groups in the study in Rochester, Minnesota. Only 28/38 (74%) of cases diagnosed before age 20 and 36/62 (58%) of cases diagnosed before age 30 were classified as IDDM (using an extra criterion of relative weight 1.2 and a slightly lower diagnostic blood glucose; 110 mg/dl vs 127 mg/dl). In the younger age group, 4 cases had relative weight >1.2 and 32/38 (84%) were insulin treated (Melton *et al.*

1983). Wilson and colleagues have evaluated the clinical and immunogenetic features of a group of 100 consecutive patients presenting to a diabetic clinic who were judged clinically to require insulin. DR type, islet cell antibodies, C-peptide secretion and ketonuria were determined. 88% of subjects below age 20 were ICA positive, 94% possessed DR3 and/or DR4 antigens, 71% had ketonuria ++ or greater, features designated by NDDG criteria to be characteristic of Type 1 diabetes. In subjects aged 21-30 years, only 47% were ICA positive, 90% had DR3 and/or DR4 and 57% had ketonuria. In this group of insulin treated patients, the clinical decision to start insulin treatment did not appear to be confined to patients with Type 1 diabetes defined by NDDG criteria. In the younger age group however a good correlation was observed. (Wilson *et al.* 1985)

Classification by presentation and symptoms: Children presenting with an abrupt onset of severe symptoms or in established diabetic ketoacidosis are easily classified, but this presentation is becoming less common. It is hoped that, with well-organized diabetes services, early diagnosis and referral will become the

norm, especially since the 'typical' clinical onset may be preceded by months or years of non-specific symptoms that were previously considered to be more a feature of Type 2 diabetes (Tarn *et al.* 1987). Reliance on ketosis alone is unreliable, and in Wilson's series, 29% of patients under 20 years of age did not have ketonuria. A more recent study in Finland found ketonuria in only 50% of children (mean age 9.6 years) at the time of diagnosis (Karjalainen *et al.* 1989). A variety of clinical and biochemical features were examined by Hother-Nielsen and colleagues as predictors of long-term insulin usage in newly-diagnosed patients. Forty-one consecutively referred patients were investigated at diagnosis and followed for more than a year. Ketonuria was found in 10/12 (83%) of the insulin-requiring group but was also found in 10/29 (34%) of those not requiring insulin after one year. Acidosis (total CO₂ concentration < 20 mmol/L) was found in 4/41 patients, 2 of whom were insulin-requiring and 2 non-insulin-requiring after 1 year. The combination of age and weight proved one of the best discriminants; all those under thirty with body weight <100% of desirable body weight were insulin-requiring (Hother-Nielsen *et al.* 1988). Weight loss is however a late feature in the development of diabetes and, with earlier diagnosis, as with ketonuria, it is likely to be seen less frequently. The mean relative body weight of children in the Finnish study mentioned above was 94.5%. In Wilson's study in Nottingham the body mass index at diagnosis ranged between 16 and 26 kg/m² (Wilson *et al.* 1985).

Classification by initial treatment: Initial treatment does not provide a satisfactory basis for classification. In Rochester, 89% of the patients below age 30 who were initially treated with insulin were still on that treatment 10 years later (Melton *et al.* 1983). Patients may be inappropriately classified as having Type 1 diabetes if insulin therapy is started at the time of diagnosis because of marked hyperglycaemia or intercurrent illness. Fashion and the availability of alternative treatments may also be relevant. In Rochester, for example, in 1945-59 all those below age 20 were insulin treated, compared with 17/23 (74%) in 1960-9. The identification of the M.O.D.Y. subtype may have increased the proportion of young people with diabetes who are not immediately started on insulin at presentation.

On the other hand, delay in starting insulin therapy could theoretically result in misclassification of true Type 1 diabetes, though in practice this does not appear to be a significant problem. In Rochester, 96% of patients below 30 years went on to insulin within the first year after diagnosis did so within 1 week, and in a prevalence study in Dundee 99% of insulin-treated patients below age 20 had started insulin within 1 month of diagnosis (Waugh *et al.* 1989).

Classification by residual beta cell function: Insulinopenia is included in the characteristics of NDDG criteria for the classification of diabetes. Residual beta cell function can be assessed even in the presence of exogenous insulin by measurement of fasting and stimulated levels of C-peptide. Hother-Nielsen *et al.* included a glucagon stimulation test in their assessment of newly presenting patients and found that a fasting C-peptide level below 0.3 nM discriminated between insulin-requiring and non-insulin-requiring groups, though there was some overlap. All patients in the insulin-requiring group had stimulated C-peptide below 0.6 nM and all patients with a stimulated value above this were non-insulin-requiring. There were, however, some patients who did not require insulin although their stimulated C-peptide was below 0.6 nM. Therefore the test was able to predict non-insulin-requirement (positive predictive value 100%) but was not specific for insulin-requirement (Hother-Nielsen *et al.* 1988).

Classification by immunological features: Islet cell antibodies (ICA) are characteristic of Type 1 diabetes and are included in the NDDG classification. If present, they support the diagnosis of Type 1 diabetes but many studies have found them in only 70-80% of cases at diagnosis, meaning that failure to detect ICA does not exclude Type 1 diabetes (Karjalainen *et al.* 1989; Irvine *et al.* 1977; Landin-Olsson *et al.* 1989).

Classification by genetic features: Genes coding for class II antigens, especially DR3 and DR4, in the major histocompatibility complex on the short arm of chromosome 6 provide the strongest genetic associations of Type 1 diabetes. These are discussed in more detail later in this thesis. 90% of subjects with Type

1 diabetes possess DR3 and/or DR4 antigens but these are also found in 45% of the general population (Bertrams *et al.* 1984) which limits their value in discriminating between Type 1 diabetes and Type 2 diabetes. Recently described genetic markers, such as the absence of an aspartate residue at position 57 on both DQB chains (Todd *et al.* 1987), may provide higher specificity in classification.

A workable definition of Type 1 diabetes: In conclusion, the ideal definition of insulin-dependent diabetes for use in large scale epidemiological study would be 100% sensitive and 100% specific, including all cases of Type 1 diabetes in the study population and excluding all non-insulin dependent and secondary diabetes. No single criterion can achieve such discrimination. The overwhelming majority of patients diagnosed under 30 are insulin dependent. The proportion of cases that have non-insulin dependent diabetes increases with age; setting the age threshold at 10 years would exclude virtually all cases of Type 2 diabetes but would lead to only a minority of cases of Type 1 diabetes being ascertained. On the other hand most cases of Type 1 diabetes would be ascertained if a threshold of 30 years was used but a small proportion of cases with Type 2 diabetes could be included even if insulin treatment from the time of diagnosis was added to the inclusion criteria. The addition of ketonuria appears to add little in terms of improved specificity and reduces sensitivity considerably. Stimulated C-peptide levels could be useful to confirm insulin dependence but could not be used in large studies of disease frequency. All available genetic markers such as DR type or even non-Asp 57 homozygosity can improve the sensitivity of the classification but are present in such a large proportion of the general population, and therefore also in patients with Type 2 diabetes, that they are not specific. Islet cell antibodies are specific, but not sensitive. Any definition of Type 1 diabetes is necessarily a compromise but the use of a combination of age at diagnosis below 20 with the initiation of insulin treatment within a month of diagnosis seems a practical means to identify a group of patients with true Type 1 (insulin dependent) diabetes. It is only with such careful classification that it will be possible to draw conclusions about the aetiology and pathogenesis of the condition.

CHAPTER 3: HOW COMMON IS IT?

3.1 INTRODUCTION

This chapter consists of (i) a review of the literature on the frequency of Type 1 diabetes in the United Kingdom, available in 1985, in which I have emphasized the lessons that can be learnt from previous studies (ii) the report of my own work in a study of the incidence of Type 1 diabetes in the Oxford Region 1985-6 (*Section 3.3*).

3.2 THE EPIDEMIOLOGY OF TYPE 1 DIABETES IN THE UNITED KINGDOM

Before the inception of the National Health Service, the fragmented nature of health care provision made accurate assessment of the prevalence or incidence of rare diseases almost impossible. In 1948 an annotation in the British Medical Journal commented 'the incidence of diabetes is unknown, although some idea of its prevalence can be obtained from the mortality returns'. Since that time various attempts have been made to quantify the problem of childhood Type 1 diabetes in the United Kingdom. These have used different methods of case ascertainment and of validation and have a number of flaws. Further description of these studies can illustrate many of the problems that may be encountered by the investigator.

Terminology: A confusion that is apparent in many early studies in this field is the apparently interchangeable use of the terms prevalence and incidence of disease. The true meaning of the measurements made requires careful study of the methodology (for example the studies of Henderson (1949) or Beardmore *et al.* (1966)). Prevalence can be defined as 'the proportion of a group possessing a clinical condition at a given time [and] is measured by surveying a defined population containing people with and without the condition of interest, at a single point in time'. Incidence is 'the proportion of a group initially free of the condition that develops it over an interval of time'. (Fletcher *et al.* 1988). Prevalence measurements are most valuable in assessing the scale of a problem and, as they require only cross sectional study, they are usually much easier to collect than incidence figures. Since they are affected by the proportion of subjects recovering or dying, their value is limited in acute disease but this is of less relevance in a

chronic disease such as diabetes. Incidence figures require repeated or continuous surveillance of a population throughout the study period. They can, however, take account of recovery and mortality and are of greater use in identifying aetiological factors.

Case ascertainment levels: The earliest studies relied on detection of cases through schools, sometimes combined with approaches to hospitals and paediatricians. In 1949 Henderson found an prevalence of diabetes of 1/4300 in children aged 5-15 (Henderson, 1949). Robertson, in a report to the British Diabetic Association in 1960, gave a minimum incidence of 1/3,300 schoolchildren aged 7 to 15 years in London and Middlesex (quoted in Beardmore *et al.* 1966). This is said to have provoked the Association to initiate further investigation (Beardmore *et al.* 1966). Beardmore and Reid noticed that, while 14 diabetic schoolchildren were known to the Northamptonshire School Health Service in July 1960 (giving a prevalence of 1 in 3,250), as soon as it became known that the County School Health Service was interested in the condition, the number of reported cases rose sharply. By the end of 1964, 39 children of school age were known to have diabetes, giving an approximate prevalence of 1 in 1,200. Such problems with variations in degree of case ascertainment prove to be a major, recurring difficulty with epidemiological studies in Type 1 diabetes.

Sample size: Three birth cohorts have taken part in longitudinal studies in the National Study of Health and Development (1946), the National Child Development Study (1958) and the Child Health and Education Study (1970). These studies included all the children born in one week throughout the United Kingdom. The cohorts followed comprised 5,362, 15,500 and 13,823 children respectively. The earliest study followed a sample of approximately one third of the children born in the week. The sample was unrepresentative, since all children born to non-manual and agricultural workers but only one in four of children born to manual workers were included. This necessitated statistical weighting of all results to take account of the social class bias. The prevalence of diabetes was measured at age 10 or

11 in each group with 80-90% follow-up achieved in the later groups. The numbers of cases of diabetes in the three groups were 1, 10 and 18 and the small population size resulted in wide 95% confidence intervals (Wadsworth *et al.* 1974; Calnan *et al.* 1977; Stewart-Brown *et al.* 1983).

Table 3.1 : The prevalence of diabetes in 3 British national birth cohorts

Birth cohort	Age of children	Proportion of children with IDDM	Prevalence (per 1000)	95% C.I.
1946	11	1/5,362	0.1	-0.3 - 0.5
1958	11	10/15,500	0.6	0.2 - 1.0
1970	10	18/13,823	1.3	0.7 - 1.9

This method of study, if it can be carried out rigorously, is theoretically ideal. Risk factors can be assessed prospectively in a well-defined population who are repeatedly surveyed. Changes in incidence with age can be dissociated from cohort effects. It is, however, very labour intensive and, in practice, it is often not possible to study sufficient numbers to provide much useful information. Large denominator populations are necessary in studies of relatively rare conditions such as Type 1 diabetes and the information provided will be more useful if random sampling procedures are used.

Small, clinic based studies have been carried out to address specific problems. Often incidence and prevalence figures are almost incidental to the main aim of the study. Jefferson *et al.* compared the clinical features of children presenting age < 5 to those presenting between 5 and 10 years of age. As a minor component of the study they calculated the incidence in each of the groups. This was done retrospectively in what was essentially a prevalence survey. They concluded that the incidence had risen but, as will be described, such methods may well produce an artefactual rise. The number of cases was small, and the 95% confidence intervals, although not given, must have been wide. Statistical analysis of differences in incidence at different ages and of temporal changes is not given

(Jefferson *et al.* 1985). It is tempting to extend the interpretation of such results beyond their limitations and, particularly in small studies of a relatively rare disease, this temptation is often not resisted.

Validation of ascertainment levels: The first nationwide attempt to measure the incidence of Type 1 diabetes in Great Britain and Ireland was the British Diabetic Association sponsored register started in 1972. This relied on notification of new cases of diabetes in children under the age of 16 years by physicians and paediatricians in return for a £1 fee. Results from the first two complete years of the register were published. The total number of cases reported in 1973 and 1974 was 1,216 and 1,058 respectively (Bloom *et al.* 1975). The number of cases reported from each region varied considerably, giving apparent marked geographical differences in incidence. For example 185 cases were reported from Greater London giving an incidence of 5.56 cases/100,000/year compared with 10.61 and 10.15 cases/100,000/year in the adjoining South and South East regions. It seems likely that such findings were due to variation in reporting levels. Unfortunately, since the study was not initially designed to measure the incidence of diabetes, it did not include any attempt to assess the level of case ascertainment. The study demonstrates the need for quantitative validation of the methodology of ascertainment in incidence studies.

Case ascertainment sources and limitations of data: The incidence of childhood diabetes in Scotland since 1968 has been studied using a centralized record of all hospital discharges (Patterson *et al.* 1983; Patterson *et al.* 1988) (Table 3.2). This relied on the assumption that all children who developed diabetes were admitted at the time of diagnosis. During the period covered by the first publication from the group, 1968-76, they reported that this did not constitute a serious source of bias. Scottish diabetologists and paediatricians had confirmed that during this time it had been their policy to admit newly diagnosed cases. A second publication covered a later period, 1977-83. During this time the practice of some centres was beginning to change in that some patients were being started on insulin as outpatients. This problem was not addressed by the authors but demonstrates that

continued vigilance for changing sources of potential bias is necessary. The study also demonstrates that, particularly in large studies such as this, serious limitations in the data are likely to have to be overcome. The computer file used did not distinguish between first and subsequent admissions with a particular diagnosis and data on admissions prior to the start of the study period were not available. This necessitated the use of a correction

Table 3.2 Standardized annual incidence of IDDM per 100,000 < 19 years in Scotland 1968-83; corrected for readmissions (Patterson *et al.* 1983,1988)

Year	Male	Female
1968	10.2	9.8
1969	12.0	10.3
1970	11.3	13.3
1971	13.8	11.4
1972	14.2	13.4
1973	16.0	14.3
1974	15.0	13.9
1975	17.5	14.4
1976	19.4	17.1
1977	18.4	18.3
1978	23.5	23.1
1979	19.2	18.0
1980	25.0	21.2
1981	22.6	19.5
1982	23.4	20.4
1983	20.4	21.2

factor applied to the number of cases identified. This in turn relied on a set of assumptions, most notably that the ratio of first admissions to readmissions remained constant over an eight year period. This assumes that changes in the management of diabetes fail to achieve their goal of reducing readmission with metabolic and other complications; perhaps true, but depressing. Waugh has pointed out that the method also assumes that the recording of admissions is complete and coding correct. He carried out a validation exercise in the Tayside region and found that recording was not quite complete. The first admissions of 3/91 true incident cases were not recorded under the relevant ICD code. One 'false incident' case was also picked up who had an earlier unrecorded admission. One case was incorrectly coded as diabetic. Details within the available data may also be insufficient to identify whether cases actually belong to the denominator population in such a study; for example due to errors occurring in recorded age or area of residence (Waugh, 1985). Once potential problems have been identified it is usually possible to quantify their contribution and, at least for the earlier period, this has largely been done for the Scottish study. They therefore constitute the best previously available incidence figures for a large population within the United Kingdom and Ireland.

Smaller scale studies may provide less information but it is generally more feasible to ensure that the methodology is valid. Again this has proved easier in Scotland. In Tayside, a list of all admissions to hospitals (5 general hospitals in the region have a paediatrician on the staff) was supplemented by examination of case notes. Registers of diabetic school children, records of changeover to U100 insulin, a survey of insulin prescriptions and adult and paediatric diabetic clinic registers were also used (Waugh, 1986). The total number of cases identified was 91. None of the data sources can have been very large and this allowed the use of rigorous methods. The disadvantages of a study of this size is that the denominator population is relatively small, about 105,000 children and the standard errors of the incidence estimates, particularly in subgroups, are large. In designing future incidence studies, careful consideration must be given to the balance between errors caused by the problems inherent in larger studies and the less precise results coming from studies in smaller populations.

Local organization of diabetic care services is a major determinant of the most appropriate method of conducting an incidence study. A prospective register of Leicestershire children with diabetes has been running since 1940. The organisation of services was such that all children were seen in clinics run by Dr Joan Walker and later Dr John Hearnshaw. A Specialist Health Visitor service was started in 1952 (Walker, 1953). In a recent report on the incidence of childhood diabetes in the region, Burden *et al.* validated these sources against in-patient admission books and the U100 register and found no discrepancies between 1940-80 (Burden *et al.* 1989). Such perfect ascertainment methods are unfortunately very rarely available when planning an incidence study.

Conclusion: The available data on the frequency (prevalence or incidence) of Type 1 diabetes in the United Kingdom and Ireland are, for the most part, flawed. Scottish studies provide the best available information and the problems encountered have been identified and quantified since the original publication of the results from the nationwide study. Unfortunately this study is likely to have become less valid in recent years due to alterations in management practice.

Previous attempts to determine the incidence of Type 1 diabetes in England have been unsuccessful and none can be relied on to provide an accurate reflection of the problem.

The studies have shown many potential problems. They have demonstrated the importance of validation of the methods used and that, if this is rigorously undertaken, many of the difficulties that will inevitably be encountered can be overcome. The disease and the measure of its frequency must be clearly defined. The size of the denominator population should be as large as possible whilst maintaining the quality of the study. The population must be clearly defined with good information available both on its total size and on the age, sex and geographical distribution. The most applicable methods of case ascertainment and validation will depend on local circumstances, but it is important to endeavour to use methods that will remain suitable in the future.

3.3 INCIDENCE OF TYPE 1 DIABETES IN THE OXFORD REGION 1985-6

Aim

Despite the fact that insulin dependent diabetes is one of the commonest chronic childhood illnesses and still carries considerable morbidity and mortality, the information available on its epidemiology in England is patchy and unreliable. The aim of this study was to derive accurate incidence rates for Type 1 diabetes in a geographically defined population in England, employing independent validation methods to assess the degree of case ascertainment.

Recent reports have suggested a rapid increase in the incidence of Type 1 diabetes over the last 20 to 30 years. This survey was therefore planned as a baseline for assessment of temporal variation in England. Such a study requires case ascertainment methods that will remain accurate and appropriate for the foreseeable future. We therefore investigated the current management of new cases of Type 1 diabetes, in particular the recent trend toward outpatient care, in

assessing possible methods of ascertainment.

Methods

Denominator population: The study forms part of the Bart's-Oxford (BOX) study of childhood diabetes, which covers the Oxford Regional Health Authority (Figure 3.1) This is divided into 8 Health Districts each containing one or more general hospitals. The area is 3130 square miles (811,700 hectares). The average yearly temperature in Aylesbury, in the centre of the region, is 9.4 degrees celsius, and the average annual rainfall is 6.27 cm. The population during the years covered by the study was 2.4 million, with 750,000 under the age of 21 years (Registrar-General's mid-year



Figure 3.1 The Oxford Region

estimates, Office of Population Censuses and Surveys) (Table 3.3). Estimates of the proportion of non-europid children were made on the basis of the birth place of the head of household of children up to age 15, as recorded in the 1981 census. If the head of the household was born in the New Commonwealth or Pakistan, children were assumed to be non-europid. Such estimates can only be approximate, but precise racial data were not collected in the census.

Inclusion Criteria: Patients with insulin-treated diabetes diagnosed and treated before their 21st birthday between January 1st 1985 and December 31st 1986 and resident in the region at the time of diagnosis were eligible for inclusion, and we sought to ascertain all such individuals. Case records were later examined to

Table 3.3 Office of Population Censuses and Surveys Mid-year estimates for Oxford Region 1985 (unpublished)

	Persons	Male	Female
All ages	2,437,300	1,212,200	1,225,100
<1	32,5000	16,900	15,600
1-4	130,200	67,000	63,200
5-9	155,400	80,100	75,300
10-14	172,100	88,600	83,500
15-19	212,600	112,700	99,900

exclude secondary diabetes and, so far as was possible, maturity-onset diabetes of the young

Table 3.4 The Bart's-Oxford study group

Case ascertainment: The Bart's-Oxford study is being undertaken in collaboration with paediatricians and physicians with a special interest in diabetes throughout the Region

Aylesbury:	RS Brown, AH Knight
Banbury:	RAF Bell
East Berkshire:	JM Cowen, JL Pearce, RDM Scott
Kettering:	AR Davidson, B O'Malley
Milton Keynes:	PJ Latham, RC Paton
Northampton:	CJ Fox, NK Griffin
Oxford:	DB Dunger, HAW Neil
Reading:	JB Burke, NP Mann, KAK North, H Simpson
Wycombe:	CH Cheetham, L Sandler, WB Thomson, T Westcott

(Table 3.4). Primary case ascertainment was from two sources: prospective registration of new cases by collaborating paediatricians and physicians and examination of regional Hospital Activities Analysis records (HAA). Physicians and paediatricians were asked to report all new cases, providing name, address, date of birth, date of diagnosis, hospital registration number and consultant.

HAA data were used to ascertain cases that did not come under the care of participating physicians or paediatricians. HAA data provided a record of all hospital admissions but did not distinguish between the first and subsequent admissions with the diagnosis of diabetes. Centralized records of admissions dating back to 1964 were therefore examined to exclude individuals admitted with this diagnosis prior to the study period. Newly diagnosed cases amongst the remainder were identified by examination of the hospital records of the relevant admission either by the admitting consultant or by me. The Oxford Record Linkage

system was used to identify individuals who died at the time of diagnosis (Goldacre *et al.* 1988).

It is increasingly common for patients to start insulin as outpatients (Wilson *et al.* 1986), which means that they cannot be ascertained from hospital admission records. So far as we know, in our region, this approach has only been applied by teams including a diabetes nurse specialist, and such patients should therefore have been identified by the registration procedure.

Independent validation: To validate these methods, questionnaires were sent to 1276 general practitioners in the region. They were asked to report any cases of Type 1 diabetes on their lists fulfilling the inclusion criteria. They were also asked whether they would normally refer newly diagnosed cases of Type 1 diabetes in this age group to hospital for at least initial assessment as either an in-patient or out-patient and, if so, to which hospital.

Details of clinical and family history: Families recruited into the Bart's-Oxford prospective family study (see below) were visited by nurse-fieldworkers and details of family history of diabetes, symptoms prior to diagnosis and admission at time of diagnosis were collected by interview. Families were asked whether the patient had an intravenous infusion during the admission to obtain some estimate of the severity of illness at the time of presentation. Case notes of patients not recruited into the study were examined for this information.

Statistical Methods: 95% confidence intervals were calculated according to Armitage (Armitage *et al.* 1987). Seasonal variation was assessed by chi-squared and sine curve fitting.

Results

Cases identified and sources: A total of 236 cases of insulin treated diabetes were identified (Table 3.5). One child, aged 4, died at the time of diagnosis, having presented in ketoacidotic coma.

Table 3.5 Ascertainment of cases of insulin-treated diabetes

Prospective registration	207
Hospital activities analysis	144 (23 from this source alone)
General practitioners	115 (5 from this source alone)
Death Certificate	1
TOTAL	237

Secondary diabetes and maturity onset diabetes of the young: Two cases of secondary diabetes (1 cystic fibrosis, 1 steroid therapy) and 1 case of maturity-onset diabetes of the young (positive family history, islet cell antibody negative and 17 month delay between diagnosis and insulin treatment) were identified and excluded from further analysis.

Validation: There was a 78% (990/1276) response from general practitioners to the questionnaire. They reported 117 cases, 112 of whom were already known to us from prospective registration or HAA records, giving a degree of case ascertainment from primary sources in excess of 95%.

Six general practitioners reported that they would not necessarily refer all young patients with newly diagnosed Type 1 diabetes to hospital although they would refer any presenting in ketoacidosis. None had treated new cases during the study period. Doctors from 4 group practices, whose patients were all resident within the region, would refer some patients to hospitals outside the region. Two further practices, whose catchment areas traversed the regional boundary and with a minority of their patients resident in the region, would refer all such patients to hospitals out of the region. No eligible patients had been referred out of the region during the study period.

Overall incidence: The overall incidence rate for the 2 year period was 15.6 cases per 100,000 population per year (95% confidence interval 13.6-17.6). Rates were 16.8 (95% C.I. 14.0-19.7) for males and 14.3 (95% C.I. 11.6-17.1) for females.

There were no significant differences between the rates or between the sexes for the two years. (Table 3.6 and Figure 3.2).

Table 3.6 Overall yearly incidence of Type 1 diabetes (cases/100,000)

YEAR	MALE	95%C.I.	FEMALE	95%C.I.	TOTAL	95% C.I.
1985	15.2	11.3-19.1	14.8	10.8-18.8	14.9	12.1-17.6
1986	18.5	14.2-22.7	13.9	10.0-17.7	16.3	13.4-19.2

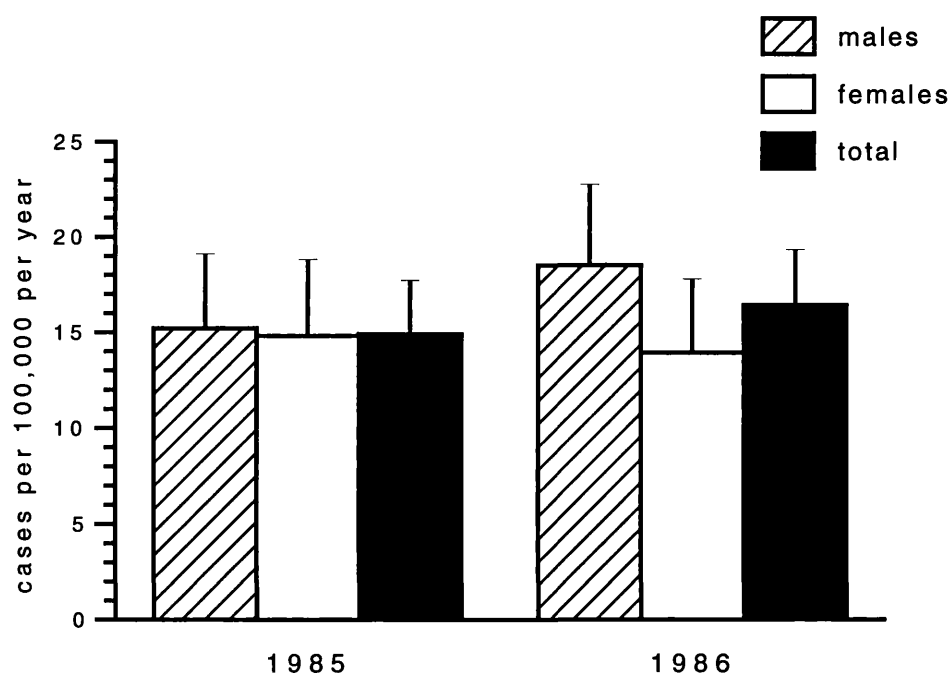


Figure 3.2 Overall yearly incidence of Type 1 dependent diabetes in the Oxford Region

Age specific incidence: The age specific incidence rates are shown in Table 3.7 and Figure 3.3

Seasonal variation: Seasonal variation in incidence was confirmed with more cases diagnosed in the autumn and winter than in summer (Table 3.8).

Management practice: Only 182/230 (79%) patients were admitted to hospital at

the time of diagnosis, the remainder being started on insulin as out-patients. Clinical details of the admission at diagnosis were available from questionnaires on 161 patients. 65/161 (40%) reported having had an intravenous infusion at the time of diagnosis.

Family history of diabetes: Twenty-seven of the 178 patients (15%) on whom the information was available had a first degree relative with Type 1 diabetes.

Table 3.7 Yearly incidence of Type 1 diabetes during 1985-6 stratified by age and sex (cases/100,000)

AGE GROUP (yr)	MALES			FEMALES			TOTAL		
	n	incidence	95%C.I.	n	incidence	95%C.I.	n	incidence	95%CI
0- 4	11	6.5	2.7-10.3	9	5.6	2.0- 9.3	20	6.1	3.4-8.7
5- 9	31	19.0	12.3-25.7	15	9.8	4.8-14.8	46	14.6	10.3-18.8
10-14	46	26.5	18.8-34.1	43	26.3	18.4-34.1	89	26.4	20.9-31.8
15-19	35	15.5	10.4-20.7	33	16.5	10.9-22.2	69	16.2	12.4-20.1

Table 3.8 Seasonal variation in diagnosis of Type 1 diabetes

	Month of diagnosis												Total
	Jan	Feb	Mar	April	May	June	July	Aug	Sept	Oct	Nov	Dec	
No. of cases	28	12	22	13	16	17	12	17	22	29	22	24	234*

* Table excludes two cases of secondary diabetes and one case of maturity onset diabetes of the young

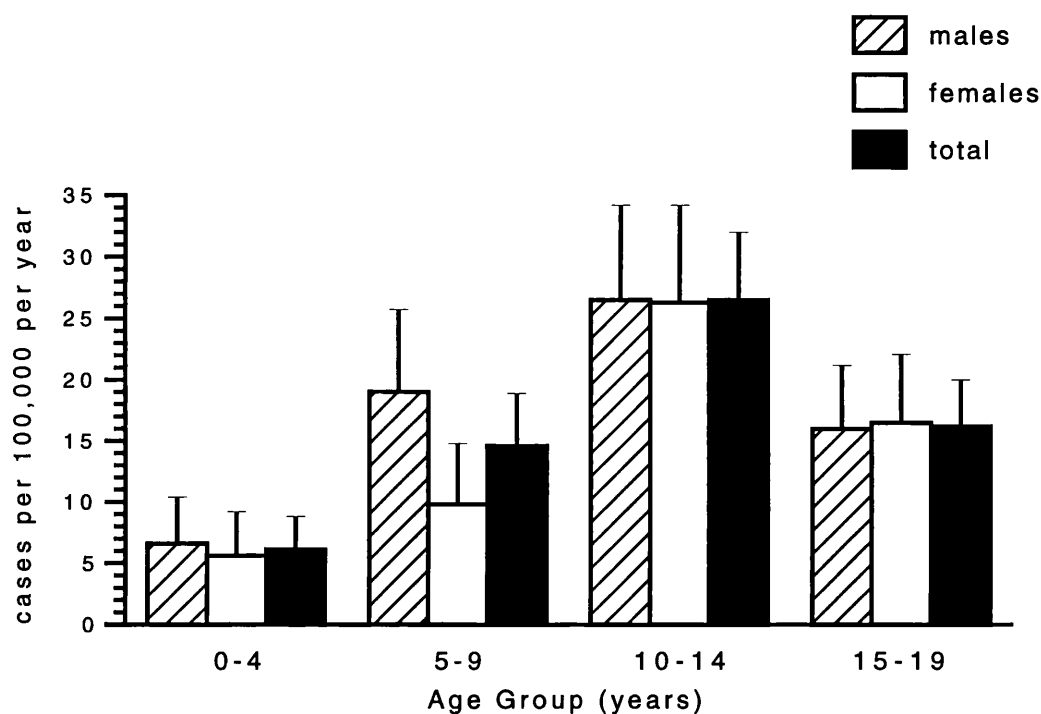


Figure 3.3 Yearly incidence of Type 1 diabetes in the Oxford Region during 1985-6 stratified by age and sex

Discussion

The overall incidence that we report is considerably higher than that previously reported. This apparent difference may be due to inadequate ascertainment in earlier studies, as discussed earlier. Our own ascertainment, in excess of 95%, was validated by a questionnaire to General Practitioners. This also allowed us to establish that cases managed independently by GPs or referred outside the region are not likely to be a significant source of error. Case referral was by paediatricians and physicians with an interest in diabetes throughout the region, and HAA data were used to identify patients admitted under doctors whose primary interest was not in diabetes. The study has been carried out in a geographically defined area for which denominator statistics are available. These may be subject to minor errors, due for example to the time lapse since the 1981 census, but this is likely to result in only minimal error.

This study established a system for prospective surveillance of the incidence of

childhood diabetes in the Oxford region. We have validated the levels of case ascertainment that we are able to achieve, have ensured that it is near complete and have been able to use a system that should not be affected by future changes in practice in the management of Type 1 diabetes. The rigorous design of the study allows several projects to grow from it: first, the system can be used to assess changes in the incidence of Type 1 diabetes within a stable population which, as will be discussed below, is particularly valuable in differentiating the roles of genetic and environmental factors in the aetiology of Type 1 diabetes. Second, since the ascertainment levels have been quantified, the data can be compared with other studies as in the collaborative EURODIAB project covered in the next chapter and, third, the study has formed the basis for a population-based, prospective family study which has been used to study the natural history of Type 1 diabetes without selection bias, to improve prediction of disease, and to form the basis of an intervention trial to prevent or delay clinical onset of the disease.

CHAPTER 4 - LESSONS FROM THE EPIDEMIOLOGY OF TYPE 1 DIABETES

4.1 INTRODUCTION

In this chapter I have reviewed the current literature on the epidemiology of Type 1 diabetes, including the results from my own study, and discussed the relevance to understanding the aetiology of the disease.

4.2 AGE AND SEX DISTRIBUTION

The distribution of age at diagnosis of Type 1 diabetes in the Oxford region is similar to that in most studies throughout the world. It is rare in the first few months of life, rises gradually throughout early childhood to a major peak in the first half of the second decade and then falls fairly sharply.

Although the incidence in young adults is much lower than in children, it has been estimated that some 30% of all cases of Type 1 diabetes are diagnosed after the age of 20 (Laakso *et al.* 1985). The overall distribution of age at diagnosis of Type 1 diabetes throughout life has been drawn by Laakso and Pyorala (Figure 4.1) and gives a curve that is 'skewed to the right', representing a log-normal distribution similar to the distribution of incubation periods in infectious disease (Lilienfeld *et al.* 1980; Sartwell, 1950). This pattern might then be considered consistent with a single or short-lived 'hit', perhaps by an infectious agent, occurring at

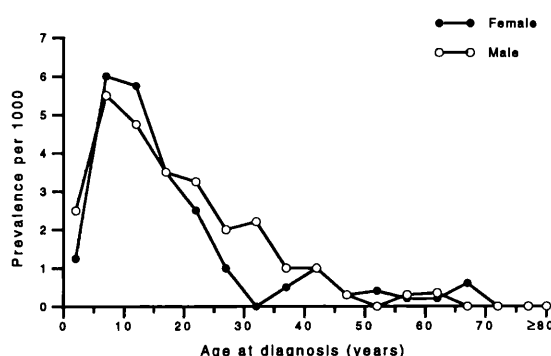


Figure 4.1 The prevalence of the insulin-dependent type of diabetes in Kuopio, Finland by age at diagnosis (5 yr. age groups) and by sex (Laakso *et al.* 1985)

about the same stage of early life in all individuals, followed by a prolonged incubation period. The peak incidence, however, coincides with the marked physiological changes of puberty. In males it generally occurs slightly later than in females, corresponding to their later onset of puberty. Reduced insulin sensitivity has been demonstrated in pubertal non-diabetic children (Amiel *et al.* 1986; Smith *et al.* 1988) and physiological changes occurring around this time are likely to precipitate the clinical onset of diabetes in individuals who already have significant islet cell damage (see below). Minor peaks in incidence have also been observed among children aged about 5 in some studies but this is not a consistent finding. We have found a non-significant male excess in the 5-9 age group.

4.3 SEASONAL VARIATION IN DIAGNOSIS

In the past, considerable weight has been attached to the seasonal variation in diagnosis of Type 1 diabetes in children. A higher incidence in autumn, winter and spring than in the summer months has been found in studies both north and south of the equator (Durruty *et al.* 1979; Mason *et al.* 1987). It is generally less marked in cases diagnosed before the age of five. This variation correlates well with the incidence of various viral infections in the community and prompted a detailed search for a viral trigger. With the identification of the long prodrome that precedes the clinical onset of Type 1 diabetes, often by many years (Gorsuch *et al.* 1981), it now seems unlikely that infection at this late stage initiates the process leading to diabetes, but rather that viral infections at this stage constitute a more non-specific stress factor that precipitates overt diabetes in children whose beta cell reserve is already severely depleted. Other changes in life-style, such as diet and level of physical activity also vary with the season and may be of

some relevance.

4.4 GEOGRAPHICAL VARIATION IN THE INCIDENCE OF TYPE 1 DIABETES

4.4.1 Type 1 diabetes worldwide: The Diabetes Epidemiology Research International (D.E.R.I.) group has compared the results of all studies of the incidence of Type 1 diabetes for the years 1979-80, using comparable definitions of the disease, and with some form of independent validation of the levels of ascertainment achieved (Diabetes Epidemiology Research International Group, 1988). They were able to include results from both the southern and northern hemispheres and found a significant positive correlation between latitude and the incidence of Type 1 diabetes on both sides of the equator; thus diabetes is more common in Northern Europe than the Mediterranean and in the South Island of New Zealand than in the North Island. They went on to establish that there was a significant negative correlation between the incidence of the disease and average temperature, i.e. that the disease is more common in colder countries. The distribution of participating centres was however very uneven, with most studies in western, developed nations and few in the lower incidence countries of the Mediterranean and Third World. Although all centres undertook some form of validation of their methodology, it remains possible that differences in the quality of the component studies may have been a confounding factor. These initial differences do however suggest the need for more detailed study of the variation between populations.

4.4.2 Type 1 diabetes in Europe: There is enormous variation in the frequency of insulin dependent diabetes in different countries in Europe. The results of incidence studies between 1970-90 are shown in Table 4.1. These suggested that a child in Finland appeared about 6 times more likely to develop Type 1 diabetes than one in France. A north-south gradient in disease frequency was suggested (Vaandrager

et al. 1984). Many of the studies performed in Europe were subject to the problems outlined above for British studies and comparable incidence studies performed in well defined populations using independently validated methods of ascertainment were needed. This has recently been possible in the EURODIAB project, in which 26 centres (including the Oxford Region) carried out prospective incidence studies in Europe and Israel. This confirmed the existence of major differences in the incidence of Type 1 diabetes but refuted the idea of a simple north-south gradient (Green *et al.* 1992b) (Table 4.2). The incidence is generally higher in the Scandinavian countries than in the Mediterranean countries but the incidence in Sardinia is second only to that in Finland. A second, east-west gradient has also become apparent; the annual incidence in all the participating eastern and central European countries is below 10 cases/100,000, even in relatively northern countries such as Poland. In another comparative study the incidence in Estonia between 1980-1988 was 10.7 cases/100,000 per year (95% CI 10.0-11.7), compared with a contemporary incidence of 32.8 (31.6-34.0) in Finland, a country with a common ethnic and linguistic background (Tuomilehto *et al.* 1991b). Identification of differences in incidence between countries provokes a series of questions: is the disease the same in all countries, or is there significant heterogeneity? Are there variations in the clinical features, mode of inheritance and evidence of autoimmunity? If no differences are found, then to what extent can differences in the genetic susceptibility of the background population account of differences in incidence and what is the role of variation in environmental exposure? Efforts are currently under way to establish a network of population-based studies throughout Europe which will allow detailed comparisons between low and high incidence populations with respect to genetic markers and environmental conditions. This will allow hypotheses to be generated and tested before more complex and expensive case-

control or prospective cohort studies are undertaken.

Table 4.1 Variation in incidence of Type 1 diabetes in Europe

Country	Study period	Age range	Annual incidence /100,000			Reference
			male	female	total	
Finland	1970-86	0-14	32.5	29.1	30.8	(Akerblom <i>et al.</i> 1985)
Sweden	1977-83	0-14			23.6	(Dahlquist <i>et al.</i> 1982)
Norway	1973-77	0-14	18.8	16.4	17.6	(Joner <i>et al.</i> 1981)
Netherlands	1978-80	0-19	11.6	10.4		(Vaandrager <i>et al.</i> 1984)
Midwestern						
Poland	1982-84	0-16	6.7	6.6	6.6	(Rewers <i>et al.</i> 1987)
Scotland	1977-83	0-19	21.7	20.2		(Patterson <i>et al.</i> 1983)
France	1970-79	0-15	5.1	4.8	5.0	(Hours <i>et al.</i> 1984)
France	1988	0-14			7.13	(Levy-Marchal <i>et al.</i> 1990)
Italy	1981-82	0-19			11.6	(Pagnano <i>et al.</i> 1987)
Hungary	1978-87	0-14			6.1	(Soltesz <i>et al.</i> 1990)
Catalonia	1987-90	0-14	11.4	11.6	11.5	(Goday <i>et al.</i> 1992)
Sardinia	1989-90	0-14	34.1	27.2	30.7	(Muntoni <i>et al.</i> 1992)

Table 4.2 Standardized incidence rates age 0-14 years (EURODIAB ACE 1989-90)

COUNTRY	Males	Females	All
Austria (whole nation)	7.9	7.5	7.7
Belgium (Antwerp region)	9.2	10.4	9.8
Denmark (3 counties)	21.5	21.4	21.5
Finland (2 regions)	47.0	38.8	42.9
France (4 regions)	7.8	7.8	7.8
Greece (Athens region)	10.9	7.7	9.3
Greece (5 northern regions)	5.3	3.8	4.6
Hungary (18 counties)	7.7	7.5	7.6
Israel (whole nation)	4.4	6.7	5.5
Italy (Lombardia region)	7.6	5.9	6.8
Italy (Lazio region)	7.2	5.8	6.5
Italy (Sardinia)	33.5	26.9	30.2
Italy (eastern Sicily)	11.2	9.0	10.1
Luxembourg (whole Nation)	12.1	12.6	12.4
Netherlands (5 regions)	11.2	10.8	11.0
Norway (8 counties)	22.3	19.3	20.8
Poland (9 western provinces)	5.3	5.8	5.5
Poland (3 cities)	5.7	6.0	5.8
Portugal (3 regions combined)	10.1	4.9	7.5
Romania (Bucharest region)	4.6	5.7	5.1
Slovenia	5.2	7.7	6.5
Spain (Catalonia region)	10.5	10.6	10.6
UK (Northern Ireland)	17.8	15.4	16.6
UK (Oxford region)	17.8	14.9	16.4

4.4.3 Correlation with genetic differences: Attempts have been made to relate the incidence of diabetes to the frequency of genes known to be associated with diabetes. The prevalence of the HLA class II antigens, DR3 and DR4, does not differ significantly amongst populations with very different rates of Type 1 diabetes (LaPorte *et al.* 1985). These antigens are however relatively crude markers of genetic risk, since they are present in a large proportion of the non-diabetic population. With the identification of more precise markers such as DR4 subtypes, DQ loci and the presence or absence of an aspartate in position 57 in the DQ beta chain (Todd *et al.* 1987), further studies of this type can be undertaken. Dorman and colleagues estimated the relative risk associated with Non-Asp 57 alleles from case-control studies in a number of populations. They also calculated the genotype specific risk by linking the relative risk and overall incidence of disease in their own population in Allegheny County, Pennsylvania. Finally they applied the genotype specific risk and the relative risk in the different populations. They reported good correlation between the resulting 'expected' incidence with that actually observed

in four other low, medium and high risk populations (Dorman *et al.* 1990). In some high risk populations 'novel' diabetes-associated haplotypes have been described (Tuomilehto-Wolf *et al.* 1989; Carcassi *et al.* 1990).

4.4.4 Variation in incidence within countries: The incidence of Type 1 diabetes may also vary considerably within a given country. Studies in Scotland have found a marked variation in different counties with the lowest rates in the central lowlands which include the cities of Glasgow and Edinburgh. An inverse association was found between the incidence and population density. Even within the city of Glasgow the incidence in each city ward was inversely related to the average number of people per room (Patterson *et al.* 1988). In Norway, however, the incidence is highest in the more highly populated southern part of the country (Joner *et al.* 1989) and in Tasmania a higher prevalence of the disease was found in the urban, as opposed to the rural sector of the community (King *et al.* 1988). These differences remain unexplained, but are certainly partially explained by differences in the level of social deprivation. Deprivation appears to confer protection from diabetes (Patterson *et al.* 1992).

4.5 TEMPORAL CHANGES IN THE INCIDENCE OF TYPE 1 DIABETES

4.5.1 Existing studies: Several recent studies have reported a rapid rise in incidence of insulin-dependent diabetes, equivalent to a doubling period of 20-30 years in some countries. There are problems drawing conclusions from data collected under varying conditions, in different countries, at different times and over different age ranges, but the limited information available do suggest that there has been a genuine increase, at least in Europe; the situation in North America is less clear-cut. The data are summarized in Table 4.3.

Table 4.3 Longitudinal studies of the incidence of Type 1 diabetes

SIGNIFICANTLY RISING INCIDENCE:				
	Dates of study		Method	Reference
FINLAND	1970-83		National Drug register	Tuomilehto <i>et al.</i> 1992
NORWAY	1973-82		Hospital records	Joner 1989 <i>et al.</i> 1989
SWEDEN	1977-83		Prospective register	Dahlquist <i>et al.</i> 1985
DENMARK	1957-68?		Cohorts at conscription	Green <i>et al.</i> 1992a
NETHERLANDS	1960-86		Cohorts at conscription (prevalence)	Drykoningen <i>et al.</i> 1992
UNITED KINGDOM	1957-80		National Birth cohorts (prevalence)	Stewart-Brown <i>et al.</i> 1983
	Scotland	1968-83	Central hospital admission records	Patterson <i>et al.</i> 1983,1988
	Leicester	1951-80	Consultant/specialist nurse records	Burden <i>et al.</i> 1989
POLAND	1970-84		Hospital records and registration	Rewers <i>et al.</i> 1987
HUNGARY	1978-87		Hospital records	Soltesz <i>et al.</i> 1990
SARDINIA	1955-92		Cohorts at conscription	Songini <i>et al.</i> 1993
SIGNIFICANT INCREASE BUT POSSIBLY ARTEFACTUAL:				
USA:				
	Erie county, New York	1947-72	Retrospective health record survey	Sultz <i>et al.</i> 1972
	Michigan	1959-72	Retrospective estimates of incidence from school survey	North <i>et al.</i> 1977
JAPAN		1974-80	Retrospective questionnaire to hospitals	Tajima <i>et al.</i> 1983
NO SIGNIFICANT RISE FOUND:				
USA:				
	Rochester, Minn	1945-70	Case records	Palumbo <i>et al.</i> 1976
	Allegheny County, Pa.	1965-76	Diabetes Mellitus Registry	LaPorte <i>et al.</i> 1981
CANADA:				
	Montreal	1971-77	Hospital records	West <i>et al.</i> 1979
ISRAEL		1975-80	Hospital records	Laron <i>et al.</i> 1985
FALLING INCIDENCE:				
NIL				

The most complete data come from the Scandinavian countries which offer many advantages for epidemiological studies. They have ethnically homogeneous, relatively stable populations together with centralized, well-organized health care systems. They also have the highest documented incidence of Type 1 diabetes in the world. They are therefore especially suitable for longitudinal studies of Type 1 diabetes. The national drug register in Finland provides very accurate data for the incidence of diabetes in those under 20 years of age; with an estimated ascertainment well over 95%. Over a 12 year study period during which the method of ascertainment was unchanged the incidence rose from 27.3 cases per 100,000 per year in 1970-76 to 38 per 100,000 in 1983. Overall, incidence appears to have risen three-fold since the 1950's, with a continued annual increase averaging around 2-3% (Tuomilehto *et al.* 1991a). Prospective registration of all newly diagnosed cases in Sweden started in 1977. With annual checks in hospital records, ascertainment is estimated to be 100%. The results for two three year study periods, 1977-80 and 1980-3, show an overall increase from 22.7 to 25.1 per 100,000 per year (Dahlquist *et al.* 1985), and in Norway the annual incidence rose from 18.5 to 22.7 between the 1973-1977 and 1978-82 (Joner *et al.* 1989). In Denmark, Green and Andersen studied a series of male birth cohorts and identified diabetes from the records of those rejected for conscription on health grounds at the age of 20. They analyzed these data using a regression model and showed a significant increase in incidence from the early 1950s to the mid 1980s (Green *et al.* 1992a). In the Netherlands compulsory registration for conscription has also been used as a powerful source of epidemiological information since it provides almost total ascertainment for the entire male population at a given age. The results demonstrate a steady increase in the cumulative incidence Type 1 diabetes by age 20 from 1.55/1000 in those born in 1960-63 to 2.06/1000 in the 1967-70 birth cohort (Drykoningen *et al.* 1992). Even with the proviso that small alterations in incidence will be magnified when prevalence figures are considered, there does seem to have been a major increase. A conscript study in Sardinia demonstrated a rise in prevalence of Type 1 diabetes at age 20 from close to zero in those born 1936-45 to 3.08 per 1,000 for the 1966 birth cohort (Songini *et al.* 1993). The incidence of Type 1 diabetes in Hungary doubled in only 10 years between 1978

and 1987 (Soltesz *et al.* 1990).

Table 4.4 Cases of Type 1 diabetes per 1,000 medically examined male conscripts (aged 18) for the Royal Dutch Army (Vaandrager *et al.* 1988)

Year	Number
1960-4	0.99
1965-9	1.26
1970-4	1.55
1975-9	1.72
1980-4	1.85
1985-6	1.98

The 3 British national birth cohorts studies referred to above have used essentially comparable methods and have shown that the prevalence of Type 1 diabetes (estimated at age 10-11 years) rose from 0.1/1000 in 1957 to 1.3/1000 in 1980 (Stewart-Brown *et al.* 1983). The incidence of Type 1 diabetes in Scotland has been monitored since 1968 by examination of central records of hospital admissions. The methods used initially required the use of a correction factor to allow for readmissions. A rise in incidence from 10.0 cases /100,000 /year in 1968 to 21.0 in 1977-83 has been reported (Table 3.2) (Patterson *et al.* 1983; Patterson *et al.* 1988). The incidence of childhood diabetes in Leicester has been documented since 1950 and has increased from 5.2/100,000/year in 1951-60 to 14/100,000/year in 1971-80 (Burden *et al.* 1989).

The rise in incidence in Midwestern Poland has been described as an 'apparent epidemic' (Rewers *et al.* 1987). A very gradual rise in incidence over the 10 years 1970-79 was followed by an abrupt increase to almost double the previous rate in 1982-4. Some groups have laid considerable emphasis on the question of 'epidemics' of Type 1 diabetes, since these could provide compelling evidence of intervention of an environmental agent. This type of interpretation is complicated by consideration of the long latency of the disease process, meaning that we need to back-track 5 to 10 years from the change in incidence to the potential causative agent. Further, most studies suggest a steady rise (with superimposed

fluctuations), and no study has as yet documented an actual fall in incidence after a presumed 'epidemic'. Other investigators are more impressed by the evidence of a pandemic of Type 1 diabetes than by the relatively few instances of sharp localized epidemics.

Studies on the incidence of diabetes in the United States have produced diverse results. The study in Rochester, Minnesota achieved complete ascertainment but involved only a small population. Only few cases of Type 1 diabetes occurred during the study period and statistical analysis was not possible (Palumbo *et al.* 1976). A registry of insulin-dependent diabetes in Allegheny county was established in 1965 with almost complete ascertainment. They have found no significant change in the incidence of Type 1 diabetes in either white or non-white children between 1965 and 1976 (LaPorte *et al.* 1981). Two studies have reported an increase in incidence; in Erie County, New York and Michigan. Both, however, used methods that measured the incidence retrospectively and may well have ascertained a higher proportion of cases diagnosed immediately before the analysis was made than of those diagnosed at the beginning of the study periods, 10 and 25 years earlier (Sultz *et al.* 1972; North *et al.* 1977).

Japan is the only Asian country in which longitudinal studies of the incidence of Type 1 diabetes have been carried out. A central registry was established in 1974 and a steady increase in incidence has been reported, but the results published to date cover the earliest years of the registry when its existence was gradually becoming known and ascertainment levels were probably changing. Validation against a smaller study in Tokyo estimated 60% ascertainment levels for the registry (Tajima *et al.* 1985). Unfortunately no results are yet available from longitudinal studies that are in progress in other Asian countries as well as Australasia and parts of Africa.

4.5.2 Potential problems in evaluation of temporal changes in incidence: It is difficult to get a clear idea about changes in incidence over time for any disease.

Apparent rises can easily result from minor flaws in methodology. The definition of the disease may change. The WHO diagnostic criteria for diabetes were only introduced in 1980. This is of major importance in studies of Type 2 diabetes where a large proportion of cases are asymptomatic with borderline OGTT results; it is probably not relevant to Type 1 diabetes in children who usually present with symptoms and unequivocal hyperglycaemia. For this reason it is also unlikely that recent increases in screening or the availability of blood testing strips have caused an artefactual increase in Type 1 diabetes in children. It is very important to ensure that the degree of ascertainment of cases remains constant throughout a longitudinal study which requires validation by some means that is independent of the main ascertainment methods used. This may be particularly difficult if cases are ascertained retrospectively, since it is easier to obtain information about recently diagnosed cases than those diagnosed many years before. Changes in methodology during a period of study are easily introduced; it is tempting to try to increase the time-span covered by adding retrospectively ascertained cases to a prospective study. If fewer children die due to undiagnosed Type 1 diabetes, there will be an apparent increase in incidence. This will be particularly relevant in developing countries but is not likely to be of major importance in the last thirty years in the Western countries where an increase has been demonstrated.

Taking these potential problems into consideration, it does seem that there has been a genuine increase in the incidence of childhood diabetes, at least in parts of Northern Europe. The studies that have been undertaken have generally looked at the incidence up to the age of twenty or below and it has been suggested that the change that has been observed may be due to an alteration in the age distribution of diagnosis, with susceptible cases being culled at a younger age, rather than an overall increase in incidence (Kurtz *et al.* 1988). Longitudinal follow-up of consecutive birth cohorts will be able to answer this question.

4.5.3 Possible role of genetic factors: It is over sixty years since the introduction of insulin therapy and many women with Type 1 diabetes have survived to have children of their own. This means that the population pool of diabetes susceptibility

genes is gradually increasing. However, only a very small minority of children developing Type 1 diabetes are born to mothers who have diabetes and this mechanism can only account for a very minor part of the increase. It has been suggested that diabetes susceptibility genes might be closely linked with genes for some other characteristic that is beneficial to the survival of the species; perhaps something that affects the viability of the gamete or the fertilized ovum. Family studies have shown that genes associated with diabetes seem to be transmitted from parent to child more frequently than would be expected by chance alone (Vadheim *et al.* 1986; Cudworth *et al.* 1979). The major problem with genetic explanations for the observed increases in incidence is that it is rising far too rapidly, since genetic changes manifest themselves very slowly.

4.5.4 Possible role of environmental factors in increasing incidence of Type

1 diabetes: Environmental factors seem to offer a more satisfactory explanation, but clear delineation of the contribution of genetic and environmental factors may be impossible. There have been many changes in the environment over the period during which the incidence has been increasing. Greater affluence and alterations in life-style have resulted in a more highly refined diet and greater consumption of food additives, a reduction in breast feeding and increased stress, all of which have been implicated in the development of Type 1 diabetes (Helgasson *et al.* 1981; Borch-Johnsen *et al.* 1984; Robinson *et al.* 1989). Further investigation of their role by case-control and cohort studies is now underway. Infection rates often vary between and within countries and may change rapidly over time. Changes in life-style can affect infection rates; in recent years there has been an enormous increase in contact between communities which may enhance the spread of infection. At the same time less crowded living conditions may alter patterns of infection to common viruses. It has been suggested that a virus causing Type 1 diabetes might behave like the polio virus where infection in infancy is generally subclinical but confers immunity, whereas infection in adult life is more likely to cause overt disease. With improving living standards, infection in infancy becomes less common and the incidence of clinically apparent disease in older children and

young adults increases (Hillis, 1980). Specific infective agents are discussed below.

4.5.5 Migrant studies:

Migrant studies would provide the best means of separating out genetic and environmental factors, but available studies are inadequate. There is, however, some evidence that migrants from low risk countries take on the risk of their adopted countries. For example French children in Canada have an annual incidence of 8.2/100,000 compared with the 3.7-4.4/100,000 found in contemporary studies of children in France (Siemiatycki *et al.* 1988). The incidence of diabetes in Asian children in the UK has been reported to be considerably lower than that in the indigenous population (Samanta *et al.* 1987) but the rate in Asian children in Bradford rose progressively from 4.26/100,000/year in 1979 to 14.28/100,000/year in 1989, equivalent to that in non-asian children (Bodansky *et al.* 1992).

4.6 ENVIRONMENTAL FACTORS IN TYPE 1 DIABETES.

4.6.1 Evidence from animal models:

In animal models it is possible to manipulate the environment to assess the relevance of various factors in the development of a disease. In genetically susceptible rats and mice, exposure to certain viruses and toxins can alter the proportion developing Type 1 diabetes (Mordes *et al.* 1987). In the BB rat, diet influences the later expression of diabetes (Scott *et al.* 1988). Minor differences in temperature have also been shown to influence the rate of diabetes in the NOD mouse model, with higher incidence in those kept in a cooler environment (Williams *et al.* 1990).

4.6.2 Toxins and dietary factors In humans such experiments are obviously impossible, though 'natural experiments' do occasionally occur. Ingestion of the rat poison PNU (N-3 pyridylmethyl N-p-nitrophenyl) -'Vacor'- results in an insulin-dependent form of diabetes (Prosser *et al.* 1978); maternal consumption of nitrosamine-rich foods such as smoked mutton around the time of conception is

associated with an increased incidence of Type 1 diabetes in the offspring (Helgasson *et al.* 1981). Indirect evidence of the role of dietary factors has come from the finding of significant correlations of the consumption of unfermented milk products and of coffee against the incidence of Type 1 diabetes in different countries (Scott, 1990; Tuomilehto *et al.* 1990). A recent, nationwide case-control study in Sweden has identified differences in protein and nitrosamine consumption in early life in children who develop Type 1 diabetes (Dahlquist *et al.* 1990).

Cow's milk has been under suspicion for a number of years. Alteration of the protein components of laboratory chow can alter the incidence of diabetes in the NOD mouse and BB rat models. Animals reared on a diet free of cow's milk for the first 3 months of life do not develop diabetes (Elliott *et al.* 1984). There is epidemiological evidence to support a role for these proteins in the aetiology of Type 1 diabetes. Several ecological studies have shown a correlation between national average cow's milk consumption and the incidence of diabetes (Dahl-Jorgansen *et al.* 1991), a nationwide case-control study in Sweden found an increased level of protein consumption in children with diabetes (Dahlquist *et al.* 1990) and, in Finland, the risk of Type 1 diabetes was significantly lower in children who were exclusively breast fed for 3 months or in whom supplementary milk feeding was not introduced until they were more than 4 months of age (Virtanen *et al.* 1991). Antibodies to bovine serum albumin were detected in all children in a recent study, but the levels of IgG antibodies were significantly higher in children with newly diagnosed diabetes than in 79 normal children (Karjalainen *et al.* 1992). It has been suggested that a 17 amino acid peptide sequence in bovine serum albumin (ABBOS) may be the reactive epitope. This differs markedly from the equivalent sequence in man and other species, including the rat. Antibodies to this peptide cross-react with a beta cell specific surface antigen (p69) and an elegant hypothesis has been put forward whereby early exposure to bovine serum albumin, at a stage when it is able to cross the immature gut wall, triggers an immune response against the ABBOS peptide in genetically susceptible individuals. The p69 antigen is only expressed on the cell surface when it is induced by gamma-

interferon during unrelated infectious events, but when these occur, the immune system is primed to attack the epitope that this antigen shares with the ABBOS protein. Repeated childhood infections would result in frequent exposure of this self antigen, setting the scene for a smouldering immune attack directed against the beta cell, with both relapses and remissions (Karjalainen *et al.* 1992). Delaying exposure to cow's milk until the gut is mature and therefore impermeable to large peptides such as might this mean that anti-ABBOS immunity (and therefore anti-p69 immunity) could be prevented. Proponents of the hypothesis would add that the rapid rise in incidence may be due to changes in the processing of milk products, for example in the formulation of infant feeds. The milk hypothesis remains speculative but it seems promising, and can be tested.

4.6.3 Infective agents Infective agents, particularly viruses, remain the most popular and plausible environmental "trigger" setting off the autoimmune process that leads to Type 1 diabetes in genetically susceptible individuals. They are compatible with many of the epidemiological features of the disease. Despite much searching there remains no definitely identified infective agent causing Type 1 diabetes. The long 'incubation period' of the disease makes it necessary to look for infection many years before the development of overt diabetes, perhaps in utero. The congenital rubella syndrome provides the strongest link between a virus and the development of Type 1 diabetes. Ginsberg-Fellner *et al* found that 30 of a cohort of 242 (12%) had developed apparently typical insulin-dependent diabetes, while a further 17 had impaired glucose tolerance (Ginsberg-Fellner *et al.* 1985). It is not yet clear as to whether these children have true Type 1 diabetes. Cytomegalovirus (CMV) a relatively common, subclinical intra-uterine infection, has recently been associated with autoimmune type 1 diabetes. A child with congenital CMV infection developed diabetes at the age of 13 months (Ward *et al.* 1979) and CMV inclusion bodies have been found in the beta cells of children dying of disseminated CMV infection (Jenson *et al.* 1980). Pak and colleagues have reported that, using nucleic acid hybridisation techniques, CMV specific viral genome was found in the lymphocyte DNA of 22% of newly diagnosed diabetic

patients compared with 2.6% of controls (Pak *et al.* 1988). This could be the result of transmission of infection in the gametes if viral DNA has been incorporated into the parents genome. It could also be the result of infection transmitted through the placenta or acquired postnatally. Infection in infancy, when the immune system is immature, is particularly likely to result in persistent viral infection (Haywood, 1986). There have also been links with coxsackie B4 and B5, with reports of high titres of antibodies against these viruses in up to 60% of new cases of Type 1 diabetes (Barrett-Connor, 1985). Since these are taken to represent recent infection, and the disease is known to have a long latency, their role is probably to precipitate clinical diabetes in those with established beta cell damage.

4.6.4 Conclusion:

In conclusion, environmental factors probably play an important role in the development of childhood diabetes in genetically susceptible individuals although (and despite much speculation) the nature of the environmental agent or agents responsible is unknown. Since almost 50% of all cases occur by the age of fifteen, and the disease is known to have a long incubation period, exposure to an environmental agent would need to occur early in life or perhaps even in utero. Contact with the agent might trigger a series of autoimmune processes which eventually result in beta cell destruction. An alternative hypothesis would be that most or all genetically susceptible individuals undergo some form of subclinical damage to their beta cells, and that the role of environmental agents is to sustain or enhance this to the point of clinical diabetes. Current research into the pathogenesis of Type I diabetes is directed towards reliable detection before the onset of glucose intolerance, in the hope that the disease can be aborted at this early stage. True prevention of diabetes is a much longer term objective, and despite present uncertainty, is most likely to be achieved by the epidemiological approach.

CHAPTER 5 - METHODS OF INVESTIGATION: FAMILY STUDIES

5.1 INTRODUCTION

The W.H.O. defines diabetes mellitus as 'a state of chronic hyperglycaemia which may result from many genetic and environmental factors, often acting jointly'. Understanding these factors may, in time, lead to prevention of Type 1 diabetes. The disease diabetes has a lengthy prodrome characterized by immune and metabolic changes (Gorsuch *et al.* 1981; Eisenbarth, 1986) and some 90% of beta cells are destroyed by the time of diagnosis (Gepts, 1965). These observations suggest that it may be possible to intervene before the critical stage at which the beta cells fail to produce enough insulin to maintain normoglycaemia. Better understanding of this prodrome should lead to accurate prediction as well as suggesting which modes of intervention are likely to be effective.

In this chapter I have discussed the approaches that can be used to investigate the prodrome of Type 1 (insulin-dependent) diabetes. Study of the aetiology and pathogenesis of Type 1 diabetes was initially retrospective, using a case-control approach to investigate genetic factors and environmental exposure prior to diagnosis. During the last 15 years a variety of prospective studies in high risk groups have been undertaken which have allowed hypotheses concerning predictive markers to be generated and tested. The approach that we have used, the family study, is covered most fully and I have described the subjects and methods used in our own studies (*Section 5.3*).

5.2 FAMILY STUDIES

Family studies have been extensively used as a tool in the investigation of the aetiology and pathogenesis of Type 1 diabetes. They are essential for genetic analysis, and form a readily accessible high risk population. Other advantages may be less apparent. Type 1 diabetes is a disease of childhood and the processes leading up to clinical onset must be studied in children. Parents who have one child with diabetes are highly motivated to help research towards its prevention in their other children. Parental support means that young children can be recruited and that longitudinal studies can be undertaken successfully. Family studies therefore

allow identification and follow-up of children at high risk, and almost all that is known of the natural history of the prodrome of Type 1 diabetes has been derived from this group. Follow-up of large groups of individuals in family studies has allowed the prognostic significance of genetic and immune markers to be evaluated, and predictive models to be developed and tested.

5.2.1 Overall risk: The overall risk that first degree relatives will develop Type 1 diabetes has been derived from both clinic and population based studies. Tillil and Kobberling studied the families of 554 subjects with Type 1 diabetes and calculated the lifetime risk for first degree relatives in three consecutive generations. The overall risk (± 1 SE) for siblings was $6.6 \pm 1.1\%$ and for children was $4.8 \pm 1.7\%$. (Tillil *et al.* 1987). Other studies have also found a risk for siblings between 4-8% (Simpson, 1962; Gottlieb, 1979; Gamble, 1980; Tarn *et al.* 1988). The Pittsburgh group found a higher overall risk of 11% in 442 siblings in 132 families but this was probably due to selection bias resulting in a disproportionate number of multiplex families (Cavender *et al.* 1984).

5.2.2 Types of family study: Family studies fall into two main categories:

- 1) Collections of families of special interest such as monozygotic twins, families with two or more affected members (multiplex families) and large pedigrees with numerous affected individuals.
- 2) Large scale studies for prediction and intervention studies that may be based on a defined population or clinic, or simply on advertising.

Twin studies demonstrated differences in concordance rates for Type 1 and Type 2 diabetes in monozygotic twins, a key observation establishing heterogeneity between the two forms of diabetes (Tattersall *et al.* 1972). The observation that only one in three twins discordant for Type 1 diabetes goes on to develop the disease suggested the potential importance of non-genetic factors. A recent study from Finland, using a population-based twin register, recently found an even lower rate of concordance of around 15% (Kaprio *et al.* 1992). Multiplex families and

large pedigrees with numerous affected individuals are needed for linkage studies. Relatively small numbers may be adequate for this type of study, but full evaluation of the prognostic significance of predictive markers requires the use of population based groups to avoid selection bias. Between these two extremes come the increasing number of studies aiming simply to recruit enough individuals at high risk for intervention studies. Selection bias is then a secondary consideration and methods of recruitment such as advertising may be satisfactory.

5.2.3 Existing studies: Table 5.1 shows the largest and longest running studies. Many of the newer studies do not as yet have results to publish and have not been included. What can be seen, is that very few secondary cases occur even in the largest studies. The commitment needed on the part of families and investigators is enormous and only large and well organized studies are likely to produce useful results.

Table 5.1 Prospective family studies in Type 1 diabetes

STUDY	Established	Families	Unaffected subjects	Secondary cases	Reference
Clinic based					
Barts-Windsor	1978	208	719	17	Tarn et al (1988)
New York	1978-82	64	224	2	Ginsberg-Fellner et al (1982)
Gainesville	1981	-	5229	21	Riley et al (1990)
Seattle	1983	452	724	2	McCulloch et al (1990)
Boston-Sacramento	1982	-	4342	30	Ziegler et al (1990)
Denver	-	448	1169	7	Chase et al (1987)
Population based					
Pittsburgh	1979	-	3270	26	Lipton et al (1992)
Bart's-Oxford	1985	500	1364	10*	
DIME	1986-89	801	720	17	Tuomilehto (1992), Knip (1992)

* cases from whom a blood sample was diagnosed prior to diagnosis (see Table 5.7)

5.3 THE BART'S-WINDSOR AND BART'S-OXFORD FAMILY STUDIES

5.3.1 Introduction: The Bart's-Windsor Family Study has been running since 1978 as a prospective study of the first degree relatives of children with Type 1 diabetes. The Bart's-Oxford Family Study was established in 1985 to overcome the limitations of the Bart's-Windsor Family Study, as outlined below. Three research fellows had worked on the projects prior to 1986. I have outlined their work as it relates to the studies I have undertaken. The overall aims of my own work in these family studies have been (i) to establish a population-based family study for prospective study to develop and test new predictive markers. (ii) to develop a protocol for an intervention study in first degree relatives (iii) to design a strategy for screening first degree relatives. In this chapter I have described the methods and study populations. Specific projects and results are covered in later sections.

5.3.2 Bart's-Windsor Family Study:

Ascertainment and inclusion criteria: Subjects taking part in the study were members of families ascertained through Type 1 diabetic children attending diabetic clinics in East Berkshire and surrounding districts. Inclusion criteria were the diagnosis of Type 1 diabetes before age 20, the availability of at least 1 sibling less than age 20 at the time of entry to the study and informed consent. Half siblings were excluded. No limit was set on the time from diagnosis to entry to the study, nor on place of residence at diagnosis. Recruitment continued until 1984, but most families entered the study between September 1978 and April 1979.

Follow-up: The families have been visited at home by throughout the study. The visits being carried out by the 3 Research Fellows previously involved in the project and, most recently, by myself. Details of family history of diabetes and other autoimmune disease have been recorded. Blood samples have been taken for HLA-typing from all subjects and for repeated ICA determination. Other organ specific antibodies have also been determined. Samples have also been taken in some families for DNA and cell-mediated immunity studies and for random blood glucose estimation (Tarn *et al.* 1988).

At the time I took on the study in November 1986, individual families had received 1-44 visits (median 13 visits) with median follow-up to last blood sample 5.1 years.

The total follow-up of unaffected relatives was 3384 subject-years (Tarn 1988).

Since 1986 follow-up has been concentrated on those identified at being at high-risk for the development of Type 1 diabetes. Visits have been continued if there is an unaffected family member with detectable ICA otherwise contact has been maintained by postal questionnaire.

Subjects: 209 families were recruited to the study of which 198 were suitable for prospective study. After exclusion of family members who were already diabetic, those who were not available for testing and those siblings in whom ex-paternity

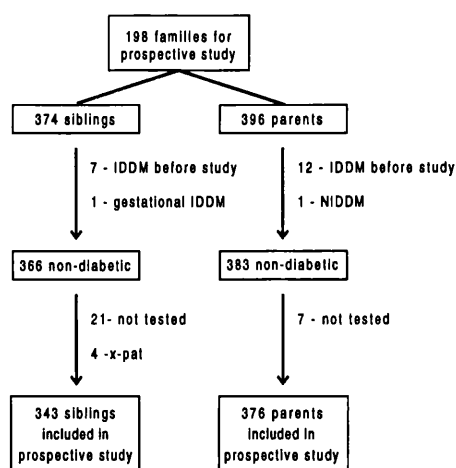


Figure 5.1 Families included in the Bart's-Windsor Family Study

with the proband was found, 719 unaffected family members (376 parents and 343 siblings) were followed prospectively (Figure 5.1).

Secondary cases of Type 1 diabetes: Eighteen family members have developed insulin-dependent diabetes since the study started. Details are shown in Table 5.2.

Table 5.2 Family members developing Type 1 diabetes in the Bart's-Windsor Family Study

ID	Sex	Age at entry	Age at diagnosis	Years from diagnosis to insulin	Years from entry to first ICA+	ICA at entry (JDFu)	Peak ICA (JDFu)	IAA by ELISA	DR	Haplotype sharing [*]
8.6	M	14.6	21.1	0	0	8	8	-	4/3	H1
19.1	M	39.5	40.75	6.4	2.7	4	30	*	4/6	
40.6	M	2.75	5.1	0	0	>80	>80	+	4/*	H1
55.5	F	17.2	17.4	0	0	>80	>80	+	4/8	H2
69.5	M	16.8	23.6	.34	0.08	16	30	-	3/3	*
88.4	F	18.75	21.17	1.75	0	50	50	+	3/3	H2
98.4	F	1.7	9.5	0	0	>80	>80	-	*	H1
100.5	M	5.6	11.7	1.2	0	30	30	-	1/4	H1
101.4	F	1.7	13.1	0	-	<5	<5	*	2/4	H1
103.5	M	6.4	9.5	0	0	80	>80	-	1/4	H2
112.4	M	15.6	23.3	0.67	0	30	50	+	4/*	*
117.1	M	55.7	57.8	2.8	-	<5	<5	-	4/4	
117.3	M	18.25	20.2	0	0	30	30	+	3/4	H1
140.2	F	44.2	46.1	0.1	0	80	80	+	3/4	
153.1	M	36.1	44.2	0	2.5	8	15	-	3/4	
158.1	M	46.9	47.2	Died	0	4	30	-	3/3	
166.2	F	40.4	44.8	.33	0	16	16	-	3/4	
166.4	F	10.2	12.8	2.8	-	<5	<5	-	8/3	H1

^{*} H2 = HLA-identical, H1 = haploidentical, H0= HLA-non-identical

Limitations of the Bart's-Windsor Study

The Bart's-Windsor Family study has certain limitations that needed to be taken into consideration when drawing conclusion from it:

(i) Size: the relatively small size of the population and the small numbers of individuals that have progressed to diabetes. This has meant that it has only been possible to estimate risk within wide confidence intervals.

(ii) Potential sampling bias: As a clinic-based study, there was also the possibility of sampling bias and the population may have not have been truly representative. For example, many of the families entered the study many years after the diagnosis of diabetes in the proband and in several families a second sibling had already developed diabetes before the family joined the study. Many of the susceptible individuals had therefore already been 'culled' before the study started and those that remained may have represented a less susceptible group who may, in theory, behave differently.

(iii) The age of unaffected siblings: The time lapse between diagnosis and recruitment also meant that many of the siblings were adolescent at the time of entry, consequently reducing the overall risk of developing Type 1 diabetes.

(iv) Practical difficulties of continuing follow-up: By 1985 many siblings were adult, and large-scale follow-up was becoming difficult. As the number of high risk individuals decreased the yield of information from prospective study also fell.

5.3.3 The Bart's Oxford Family Study:

Introduction: The Bart's-Oxford study represents an attempt to overcome many of the difficulties outlined above. It is a large population-based study, recruiting families prospectively from the time of diagnosis of the proband.

Aims:

(1) The overall aim is to identify individuals at increased risk of developing diabetes and to study them before and during the prodromal period defined by the appearance of antibodies directed against a variety of islet cell constituents.

The specific aim is to identify markers of incipient diabetes.

- (a) Immunological, in terms of analysis of ICA, insulin autoantibodies and other humoral and cellular elements of the immune response.
- (b) Genetic, in terms of HLA determinants and underlying DNA sequences
- (c) Metabolic, with particular reference to insulin secretory responses to glucose and other secretagogues.

(2) Basing this survey on the Oxford region has enabled the incidence of childhood diabetes to be assessed (*Section 3.3*).

(3) The probands with diabetes themselves constitute a valuable cohort and a study into the rate and pattern of diabetic complications is also being set up within the region to run in parallel with the family study.

Principles of the study:

- (1) A large, well-documented background population.
- (2) Ascertainment of newly-presenting patients under the age of 21.
- (3) Rapid screening of family members.
- (4) Selective follow-up on the basis of estimated risk.

Methods

Inclusion criteria and ascertainment: Family members of all patients presenting since January 1st 1985 with newly diagnosed insulin-dependent diabetes under the age of 21 and resident in the Oxford Region at the time of diagnosis are eligible for inclusion in the study. The methods of ascertainment of probands and the validation of these methods have been described above (*see Section 3.3.3*).

Recruitment: Most are introduced to the study and referred by a paediatrician or physician with a special interest in diabetes, although there are local exceptions. All such families are considered for inclusion in the study. The initial approach to the family is made by the local consultant and, unless the family do not wish to

participate in the study or the consultant feels that there are other contraindications, the family are then contacted by the local field worker, a home visit is arranged, the study is more fully explained and full informed consent obtained. Doctors participating in the study are contacted regularly by the local field worker.

Data collection: At the first visit the field worker obtains demographic details and takes a detailed family history. A manual is provided to each field worker to ensure that uniform and complete data are collected.

Table 5.3 Details collected from families at entry to the study

Proband:

Name and address of proband

Name and address of general practitioner and consultant

Clinical history of onset, admission, and intravenous treatment

Place of birth, birth weight and breast feeding history

Family:

Name and date of birth of all immediate family members

Family history of diabetes in 1st, 2nd and 3rd degree relatives including age at diagnosis, initial and later treatment.

Family history of other autoimmune disease

Sample collection: Blood is taken from all available family members (except infants) for ICA, random glucose and HLA analysis where appropriate. Additional sera and cells are frozen and stored.

Follow-up:

Category A: (Family member with high titre ICA, ≥ 20 JDF units)

Four-monthly visits, venesection of all family members (to avoid invidious concentration on any particular individual) and screening for ICA/activated T

lymphocytes and random glucose. Recruitment for metabolic studies.

Category B: (Family member with low titre ICA, 4-19 JDF units)

Six-monthly visits, screening for ICA and random glucose. Recruitment for metabolic studies.

Category C: (No ICA positive family members)

Annual visits, screening for ICA and random glucose.

Category D: (Families leaving the area or unwilling to undergo regular venesection)

Annual postal contact or telephone follow-up.

Metabolic studies: Family members with high titre ICA (category A) are asked to have an IVGTT once ICA has been confirmed on the second sample and annually thereafter. 75g oral glucose tolerance test is performed in these subjects annually if the first phase insulin response is low or falling. If the IVGTT is normal and stable, OGTT is performed every 2 years. Family members with low titre ICA are invited to have an IVGTT every 2 years. Subjects are also recruited for other metabolic studies as they are developed.

Study organization: Since 1990 a part-time study administrator has been responsible for central organization of the study and coordination of data and sample collection by the field-workers. She is also responsible for communication of results to the referring consultants. A Steering Committee composed mainly of referring consultants meets twice yearly.

Data handling: Identity coded data are stored in a custom-designed database, Genobase, developed by William Howard for use in the Bart's-Windsor Family study. The database design includes integral checking for illogical data and a query facility which was used for data extraction.

Subjects

Recruitment and follow-up: By 31.12.90, 623 families had been notified to the study. Of these, 553 had recruited for prospective study, 17 had refused and 53 were considered unsuitable for prospective study by the referring consultant usually because of social or psychological problems or because the child was not resident with the natural family. Independent validation of our methods of case ascertainment suggested that more than 95% of all eligible cases were notified to the family study so that the families of at least 80% of all patients in the Oxford region with Type 1 diabetes diagnosed before age 21 are recruited into the study.

Table 5.4 Characteristics of the study population recruited by 31.12.90

	Total	Male:Female	Age at entry (mean \pm sd)	Age range
Probands	553	324:229		
Parents	1046	507:539	39.5 \pm 6.9	18.75-68
Siblings*	684	333:351	12.7 \pm 6.5	0-33.8

* Unaffected full siblings only

Blood samples were obtained from 1513 (87%) family members, 952 (91%) parents and 561 (82%) siblings. Table 5.5 give the characteristics of those from whom blood samples were obtained. The total follow-up of these unaffected family members to 31.12.92 was 6834 years, 4268 subject years for parents and 2566 subject years for unaffected siblings.

Secondary cases of Type 1 diabetes: Eighteen family members have developed insulin dependent diabetes after diagnosis of the proband. One sibling was diagnosed on the same day as the proband, and one parent and one sibling had become diabetic between that time and study entry. Diabetes was an incidental finding at the first study visit in one sibling and one parent. Both started insulin

Table 5.5 Characteristics of family members from whom blood samples obtained

	Total	Mean age at entry	Years from diagnosis of proband to first sample	Follow-up to last sample (years)
Parents	952	39.5 ± 6.9	0.52 ± 0.6	2.0 ± 1.5 (range 0 - 5.6)
Siblings	561	13.45 ± 5.8	0.63 ± 0.7	1.84 ± 1.6 (range 0 - 5.6)

soon after. Thirteen have developed Type 1 diabetes since study entry; one parent and 11 siblings. Three of siblings (aged 3, 5 and 12 years) had not given blood samples prior to diagnosis. Details of these cases are given in Table 5.6.

5.4 CONCLUSION

The Bart's-Oxford Family Study has been running since 1985. As a large, population based prospective family study with high levels of ascertainment minimizing selection bias it has provided the basis for the advances in prediction of Type 1 diabetes in family members outlined in the next chapter. It complements the Bart's-Windsor Family Study with its long duration of follow-up. The studies are almost unique in following up both high and low risk family members which allows complete evaluation of the prognostic significance of individual markers. The Bart's-Oxford Study is now amongst the largest and intensively followed in the world and the size of the populations and the number of family members who have developed diabetes during the study are now sufficient to permit thorough analysis of many aspects of the prodrome of Type 1 diabetes. Its main value has been, however, in improving the prediction of Type 1 diabetes, drawing conclusions whose accuracy can be quantified and which can therefore be applied in the design of intervention studies and screening strategies.

Table 5.6 Family members developing Type 1 diabetes in the Bart's-Oxford Family study

ID	Sex	Age at entry	Age at diagnosis	ICA at entry	Peak ICA	Notes
258.6	F	10.3	15.5	13	85	
261.4	F	1.6	8.7	0	85	
325.4	M	11.8	16.6	85	85	
341.5	M		12.4			Diagnosed between diagnosis of the proband and study entry
361.1	M		42.1			Diagnosed between diagnosis of the proband and study entry
367.1	M	30.5	32.7	52	85	
387.3	M	22.7	23.4	0	0	
410.5	M	10.0	12.4			No sample obtained prior to diagnosis
425.3	M	1.3	3.2	0	37	
457.5	M	12.4	16.0	0	9	
460.4	M		3.4			Diagnosed on same day as the proband
592.3	M		9.7	85		Diagnosed on sample taken at entry to the study
608.6	F	6.2	6.9	85	85	
621.3	M	9.6	12.8	49	85	
665.5	M	0.8	2.4			No sample obtained prior to diagnosis
668.5	M	2.1	3.3	85	85	
700.3	F	4.4	5.8			No sample obtained prior to diagnosis
818.1	M		35.3	85		Diagnosed on sample taken at entry to the study

CHAPTER 6 - PRE-TYPE 1 DIABETES: LESSONS FROM FAMILY STUDIES

6.1 INTRODUCTION

For the reasons given in the previous chapter, most of the current knowledge about the prodrome of Type 1 diabetes has been derived from prospective family studies. These provide information about the pathogenesis of the disease and, in turn, allow markers to be developed that can identify family members at high risk of progression to Type 1 diabetes.

In this chapter I have reviewed the current literature on the natural history of the disease process and the use of immune markers in the prediction of Type 1 diabetes in family members as derived from family studies. I have included the work of previous research fellows on the Bart's-Windsor family study within that section. I also report my own work in the Bart's-Windsor and Bart's-Oxford family studies on the prognostic significance of (i) quantitative measurement of islet cell antibodies (ICA) (*Section 6.3.3.2 (b)*) and (ii) combined humoral immune markers (*Section 6.3.3.6*).

The latter part of the chapter discusses the metabolic changes that occur prior to the clinical onset of Type 1 diabetes and their value as predictive markers and limitations of available tests. I report my own work (i) in the development of a standardized protocol for the intravenous glucose tolerance test (*Section 6.3.4.3 (b)*), (ii) the results of intravenous glucose testing in the Bart's-Windsor and Bart's-Oxford family studies (*Section 6.3.4.3 (c)*) and (iii) investigations of patterns of basal insulin secretion in ICA positive family members and controls (*Section 6.3.4.4 (c)*).

6.2 THE NATURAL HISTORY OF THE PRODROME OF TYPE 1 DIABETES.

6.2.1 The nature of the disease process: The pancreas is an inaccessible organ and biopsy has, at least until recently, been considered difficult and dangerous. Evidence about the disease process has therefore been indirect. It appears to be patchy, not affecting all islets at the same time or rate. Examination of the

histopathology of the pancreas in 60 patients who died soon after onset of disease revealed insulinitis in 47/60 (78%) but there was a remarkable heterogeneity in the appearances of the islets within individual pancreata. A single lobe may contain insulin containing islets unaffected by any destructive process or inflammation, insulin containing islets with an inflammatory cell infiltrate and insulin deficient islets (Foulis *et al.* 1984). A similar appearance has been seen in the pancreas of a patient with autoimmune Addison's disease who may have been 'pre-diabetic' (Foulis *et al.* 1988).

The attack on the beta cells appears to be a slow process; the associated humoral immune changes have been detected up to 15 years before the clinical onset of disease. It remains unknown whether it is a slow continuous process or whether it is a stuttering, intermittent process and whether, once initiated, it ever remits entirely. Remission of immune changes has been suggested by twin studies in which ICA and T-cell activation were higher in a group of non-diabetic twins tested near to the time of diagnosis of their cotwin than in a group still discordant after more than 11 years (Millward *et al.* 1986).

Non progression of both immune and metabolic changes in some subjects with persistently detectable ICA is observed over the time scale of most prospective studies. The Seattle Family study has reported stable beta cell function in four of five such subjects followed for up to 42 months (McCulloch *et al.* 1990). At present it is not clear whether all individuals with high titres of ICA will progress, or whether reversible, low titre changes in ICA represent subclinical episodes of insulinitis.

The time of onset of beta cell damage in children is uncertain. Few studies have followed subjects from confirmed ICA negativity to positivity. A small prospective study in children of women with diabetes demonstrated the appearance of anti-islet autoimmunity (ICA and insulin autoantibodies (IAA)) between 9 and 24 months of age (Ziegler *et al.* 1993). Another study has reported that, using a very sensitive assay, ICA always appeared for the first time in family members before age 6 though the levels often continued to rise thereafter (Pilcher *et al.* 1991). If this

finding is confirmed, it suggests that the initial 'trigger' precipitating the autoimmune attack is likely to occur very early in life. Even if this point is identified, the relation between the appearance of circulating autoantibodies and beta cell damage is unclear. Laparoscopic pancreatic biopsy has recently been claimed to be safe (Hanafusa *et al.* 1990), but even this is unlikely to answer this question because of the problem of sampling error due to differences in the degree to which individual islets are affected by the destructive process.

6.2.2 Immune changes:

Changes in humoral immunity: Islet cell antibodies were (ICA) first detected in the serum of patients with polyendocrine autoimmunity and coexistent Type 1 diabetes (Bottazzo *et al.* 1974). They were subsequently found in a high proportion of patients with newly diagnosed Type 1 diabetes (Lendrum *et al.* 1975). They are detected by indirect immunofluorescence on human pancreas but the exact nature of the autoantigen to which they are directed remains unknown (see below). It is unlikely that these antibodies have an active role in beta cell destruction, since it has not been possible to transfer diabetes to experimental animals by injecting ICA. Moreover, they stain all islet cells indiscriminately, while the destructive process is specific for beta cells. Although ICA, for example, have been shown to be cytotoxic to cultured islet cells *in vitro*, it is generally agreed that, *in vivo*, these autoantibodies probably represent an epiphenomenon and that beta cell destruction is the result of cell mediated immune processes. The presence of these autoantibodies many years prior to diagnosis has, however, provided the primary evidence for the long prodrome of Type 1 diabetes and, in prospective studies, they have proved useful markers of increased risk of developing the disease.

Islet cell autoantigens: The antigen against which ICA are directed is still undefined although it has been suggested that they react with carbohydrate determinants of islet cell glycoconjugates (Nayak *et al.* 1985; Colman *et al.* 1988) or Heat Shock Protein 65 (Jones *et al.* 1990). Autoantibodies to an islet cell protein of molecular weight 64,000 identified in 35-S methionine-labelled human pancreatic islet cells have also been described (Baekkeskov *et al.* 1982). The antigen has recently been

identified as the enzyme glutamate dehydrogenase (GAD) (Baekkeskov *et al.* 1990). These antibodies are present in the sera of patients with newly diagnosed Type 1 diabetes and it has been suggested that they may precede ICA and IAA in the diabetic prodrome in both animal models and humans (Baekkeskov *et al.* 1984; Atkinson *et al.* 1988; Baekkeskov *et al.* 1987; Dean *et al.* 1989).

Changes in cell mediated immunity: Changes in cell mediated immunity appear to play a more critical role in the pathogenetic process. *In vitro* peripheral blood leucocytes from newly diagnosed patients can be shown to be sensitized to pancreatic antigens in the leucocyte migration inhibition test (Nerup *et al.* 1974). The predominant cells in the inflammatory islet infiltrate seen at the time of diagnosis of Type 1 diabetes, have been shown to be T lymphocytes. Examination of the pancreas of a child who died soon after diagnosis of Type 1 diabetes showed the majority of these to be CD8-positive cytotoxic/suppressor T cells positive for markers of activation such as interleukin-2 receptors, although other lymphocyte subpopulations were also present (Bottazzo *et al.* 1985b). Increased circulating numbers of activated T cells and raised K cell numbers have been described in newly diagnosed diabetes (Bottazzo *et al.* 1985a; Pozzilli *et al.* 1979). Recently T-lymphocyte clones have been expanded from the peripheral blood of newly diagnosed patients and have shown cytotoxic effects when exposed to HLA-matched islet cells *in vitro* (de Bernadinis *et al.* 1988).

Circulating T cell subsets have been used to study this aspect of the immune response in family studies. An increase in activated lymphocytes and a decrease in the CD4/CD8 (helper:suppressor) ratio have been found prior to the onset of Type 1 diabetes (Al-Sakkaf *et al.* 1989; Riley *et al.* 1989; Faustman *et al.* 1989). A major limitation of this approach is that the cellular changes of interest are localized in the pancreas and may not be reflected in the peripheral blood.

Summary of pathogenesis: The precise sequence of events initiating autoimmune attack on the beta cells remains unclear. Aberrant expression of HLA class II molecules by beta cells, but not other islet cells, has been suggested to be the

earliest abnormality. This is perhaps triggered by some environmental agent and, in turn, allows the target cell to behave as an antigen-presenting cell 'suicidally' presenting its own surface antigens (Bottazzo, 1986a). This would then result in activation of T-helper cells which then stimulate effector B and cytotoxic T lymphocytes. The production of cytokines, potent modulators of class II expression, by invading lymphocytes and macrophages would perpetuate a cycle of HLA gene activation.

Alternatively, cytokines may play a more direct role in beta cell destruction. Interleukin 1 (IL-1), produced by macrophages, is cytotoxic or cytostatic for beta cells. Interleukin 6 and interferon- α have also been demonstrated in beta cells in diabetes and may allow these cells to interact directly with invading immunocytes (Nerup *et al.* 1989).

Cytotoxic T cells appear to play the pivotal role in the final selective destruction of beta cells. This was demonstrated in a fortuitous 'experiment' in which patients with long-standing Type 1 diabetes received pancreatic transplants from their non-diabetic monozygotic cotwins. Unexpectedly, diabetes recurred rapidly in the recipients and histological examination of the pancreas revealed selective beta cell destruction with insulinitis in which cytotoxic T cells were the predominant cell type (Sibley *et al.* 1987).

From the point of view of prevention, one of the most important features of the process is its very slowness. Assuming that circulating autoantibodies reflect autoimmune activation, the process seems to be initiated early in life and may take many years to complete. This offers a large 'therapeutic window' in which it should be possible to intervene.

6.3 PREDICTION OF TYPE 1 DIABETES

6.3.1 Introduction

Intervention during the pre-clinical prodrome of Type 1 diabetes to delay or prevent

the clinical onset of disease requires identification of those in whom the disease process is occurring. While much of the literature talks of 'prediction' this implies a degree of certainty that cannot, as yet, be justified. The exercise that is undertaken is probably best described as 'risk assessment' by which the probability of a non-diabetic developing the disease is estimated. The risk associated with genetic, immunological and metabolic markers has been assessed in case-control and prospective studies. In this chapter I have reviewed the literature covering each group of markers and included the findings from our own studies within each section.

6.3.2 Genetic markers:

Introduction: Delineation of genetic susceptibility to Type 1 diabetes only became possible when the heterogeneity of primary diabetes was clarified so that it was classified into Type 1 (insulin-dependent) and Type 2 (non-insulin dependent) forms. During the 1970's and 1980's much work was done to focus down on the genetic susceptibility and to try to identify, if possible, a single diabetogenic gene. This culminated in the identification of a single amino acid residue on the DQB chain as a potential candidate. More recently, the process has been reversed and it has become apparent that the risk is modified by genes throughout the MHC and by non-MHC genes. The literature on genetic susceptibility is extensive and beyond the scope of this thesis. I have therefore confined my review to those particular aspects relevant to the prediction of Type 1 diabetes in family members and in the general population.

MHC genes

It has been estimated that some 60% of the genetic susceptibility to insulin-dependent diabetes is associated with genes in the Major Histocompatibility Complex (MHC) on the short arm of chromosome 6 (Rotter *et al.* 1984) and it is this component that has been most extensively studied both in population and family studies. In family studies it is possible to define genotypes rather than phenotypes. Investigation can therefore be taken beyond single marker studies and the role of combinations of markers in determining genetic susceptibility for Type

1 diabetes can be clarified.

HLA haplotype sharing: HLA genotyping in 120 diabetic sibling pairs recruited to the British Diabetic Association register established that the risk of developing diabetes was concentrated among siblings who were HLA identical with the proband. Fifty-seven percent of the affected siblings were HLA identical and 6% non-identical, compared with the 25% expected by Mendelian laws (Walker *et al.* 1980). This observation has been confirmed in smaller clinic based studies in Pittsburgh and Windsor where the risk of development of Type 1 diabetes by age 25 has been calculated by life table analysis. The risks were respectively 10.3% and 15.7% for HLA identical siblings, 2.2% and 9% for HLA-haploidentical siblings and 1.0% and 0% for HLA-nonidentical siblings in these studies (Cavender *et al.* 1984; Tarn *et al.* 1988).

MHC class 2 antigens:

DR and DQ: The strongest associations with Type 1 diabetes are found in the genes encoding MHC class 2 antigens, with DR3/DR4 heterozygosity being associated with a relative risk for the development of Type 1 diabetes of 20.

Table 6.1 Frequency of HLA-DR allelic combinations in Caucasian populations (DRX = all DR except DR3 or DR4) (Bertrams *et al.* 1984)

	IDDM	Controls	Relative risk
DR3/X	15%	19.1%	0.75
DR3/3	7.2%	1.3%	5.84
DR3/4	33.6%	2.4%	20.18
DR4/4	7.9%	2.0%	4.13
DR4/X	25.8%	20.8%	1.32
DRX/X	10.2%	54.3%	0.10

90% of subjects with Type 1 diabetes therefore possess DR3 and/or DR4 antigens. These antigens are however found in 45% of the general population and provide very non-specific markers of risk (Bertrams *et al.* 1984). Each of these DR types is found in combination with a limited number of alleles at the DRB1 and DQB1 loci (see Table 6.2).

Table 6.2 Combinations of HLA-DR and HLA-DQ alleles in Caucasian populations (Bodmer *et al.* 1990)

	DRB1 locus	DQB1
DR4	DRB1*0401 (Dw4) * DRB1*0402 (Dw10) * DRB1*0403 (Dw13) DRB1*0404 (Dw14) DRB1*0405 (Dw15)	DQB1*0301 (DQw7, 3.1) DQB1*0302 (DQw8, 3.2) *
DR3		DQB1*0201 (DQw2) * DQB1*0401 (DQw4)

* = alleles associated with IDDM

Only certain of these alleles are associated with Type 1 diabetes and it has recently been demonstrated that the specificity of prediction can be considerably improved if these loci are considered. There is also evidence that the alleles at these locus exert a synergistic effect. Sheehy *et al.* (1989) have demonstrated that the excess risk in DR4 positive individuals is concentrated in those who have *both* DRB1*0401 (Dw4) or DRB1*0402 (Dw10) *and* DQB1*0302 (DQw8). The relative risk in subjects with a high-risk allele at only 1 of these loci is less than 1.0 (Sheehy *et al.* 1989).

Single amino acid residues on DQ α and DQ β chains: In Caucasian populations, the possession of amino-acid residues other than aspartate at position 57 on both DQ- β chains (i.e. non-Asp 57 homozygosity) appears to be associated with increased risk of developing diabetes (Todd *et al.* 1987). In one population (Allegheny County, Philadelphia), phenotype frequency was compared in 27

probands with Type 1 diabetes and 123 healthy unrelated controls. 96% (26/27) probands and 19.5% (24/123) controls were homozygous for non-Asp at position 57. This indicates that the association between the non-Asp 57/non-Asp 57 homozygous phenotype and Type 1 diabetes was significant with an estimated relative risk of 107 (Morel *et al.* 1988). Study of the families of the probands with Type 1 diabetes allowed the association to be confirmed in 'affected' and 'unaffected' haplotypes. Since the sample came from a population-based registry, it was also possible to assess the absolute risk of developing Type 1 diabetes. The overall incidence of Type 1 diabetes in the county was 15/100,000 per year and this was calculated to break down into an incidence of 0.64/100,000 in individuals with at least one Asp gene, and 69/100,000 in non-Asp 57 homozygotes (Dorman *et al.* 1990). This association has appeared less strong in other populations (Yagamata *et al.* 1989; Bao *et al.* 1989), but demonstrates the potential value of a genetic marker in identifying a population at high risk of developing diabetes.

The modifying effect of DQA chain genes: Certain DQA chains are also found more frequently in patients than controls. Khalil and colleagues have shown that the possession of an arginine residue at position 52 on the DQ α chain is also associated with increased risk of Type 1 diabetes (Table 6.3) and have demonstrated that the DQ α and DQ β chain may act synergistically to determine genetic susceptibility.

In any individual, the maternal and paternal genes determining the amino acids at position 52 on the α -chain and position 57 on the β -chain appear to be expressed independently so that 4 possible $\alpha\beta$ -heterodimers may be formed:

- | | |
|--|--|
| (1) maternal α maternal β | (2) paternal α paternal β |
| (3) maternal α paternal β | (4) paternal α maternal β |

If either or both parents possesses susceptible alleles at position 52 of the α -chain and/or at position 57 of the β -chain, a mixture of susceptible and non-susceptible

Table 6.3 Susceptible and protective DQA and DQB chains, based on frequency in 115 patients with IDDM and 108 controls (Khalil *et al.* 1992)

Susceptible	Protective
DQA1*0501	DQA1*0201
DQA1*0301	DQA1*0101
DQA1*0401	DQA1*0102
DQA1*0601	DQA1*0103
DQB1*0201	DQB1*0503
DQB1*0501	DQB1*0601
DQB1*0502	DQB1*0602
DQB1*0604	DQB1*0603
DQB1*0302	DQB1*0401
	DQB1*0402
	DQB1*0301
	DQB1*0303

heterodimers will be produced. The risk of Type 1 diabetes appears to be proportional to the number of these heterodimers that are susceptible; so that the risk is highest if all 4 possible heterodimers are susceptible, intermediate if there are 3, 2, or 1 susceptible heterodimers and zero if there are none (Khalil *et al.* 1990) (Table 6.4). These findings may allow identification of genetically susceptible individuals and give some quantification of the risk.

Table 6.4 Frequencies of different HLA-DQ genotypes according to number of susceptibility (s) or protective (P) DQA1 and DQB1 alleles. (Khalil *et al.* 1992)

Genotype frequencies:							
DQA1*-DQB1*	S-S	S-S	S-S	S-P	S-S	Others	
DQA1*-DQB1*	S-S	P-S	S-P	P-S	P-P		
Possible expressed heterodimers	SS	SS	SS	SS	SP	SP	PP
	SS	SS	SS	PP	PS	PS	PP
	SS	SP	SP	SP	PS	PP	PP
	SS	PS	PS	PS	PS	PP	PP
IDDM/Con(%)	54/3	29/6	8/7 37/13	6/14	0/16 6/30	3/54	
RR	41	5.8	1.2	0.4	0.04	0.03	

A more recent observation is that susceptible heterodimers are not all associated with the equal risk, and the same DQA1-DQB1 combinations confer susceptibility of varying strength depending whether they are formed in cis or trans (Khalil *et al.* 1992; Ronningen *et al.* 1991).

Haplotype analysis: A major advantage of family studies is that genotypes can be defined. This, in turn, permits investigation of the role played by particular haplotypes in determining susceptibility to Type 1 diabetes. This can be undertaken by comparing affected and healthy control families but recruitment of the latter is generally difficult. Various manoeuvres have therefore been used to distinguish 'affected' and 'unaffected' haplotypes within families. The relative risk associated with a particular haplotype can be calculated by comparing the haplotypes of the diabetic child with the remaining two parental haplotypes not present in the affected child. An alternative method is to identify the four parental haplotypes and then designate them 'affected' or 'non-affected' according to the disease status of each family member. Parental haplotypes present only in unaffected family members are defined as 'unaffected' while those present in family members with diabetes are 'affected'.

Four principal 'high-risk haplotypes' for Type 1 diabetes were described in the 9th international histocompatibility workshop:

- a) A1, Cw7, B8, w6 (C4-AQ0, C4-B1, C2-1, Bf-S), DR3, DQw2;
- b) A2, Cw3, Bw62, w6 (C4-A3, C4-B3, C2-2, Bf-S) DR4, DQw8;
- c) A2, Cw3, Bw60, w6 (C4-A3, C4-B3, C2-1, Bf-S) DR4, DQw8;
- d) A30, Cw5, B18, w6 (C4-A3, C4-BQ0, C2-1, Bf-F1) DR3, DQw2;

The first three are found in European and American Caucasoid families and the fourth in Type 1 diabetes patients in Southern Europe (Bertrams *et al.* 1984). A recently described haplotype (A2, Cw1, Bw56, w6, DR4) may explain the very high incidence of Type 1 diabetes in Finland (Tuomilehto-Wolf *et al.* 1989).

Further examination of the data from the Finnish DiMe study has shown that DR4 haplotypes are identical at the DQB1 loci, with high risk DQ β codon 57, were associated with absolute risks for developing Type 1 diabetes varying between 35 and 218 per 100,000 per year. This finding implies that MHC genes outside the DQ region are also important in determining genetic susceptibility to Type 1 diabetes (Tienari *et al.* 1992) (Table 6.5).

Non-MHC genes: The concordance for Type 1 diabetes in monozygotic twins is around 30% (Barnett *et al.* 1981) and in HLA identical siblings is 15-20% (Tarn *et al.* 1988). This difference implies that genes outside the MHC complex must also

Table 6.5 HLA haplotypes and absolute risk of developing Type 1 diabetes in the DiMe study (Tienari *et al.* 1992)

HAPLOTYPE	DQB allele	DQA allele	DQB codon 57	DQ α codon 52	Haplotype specific absolute risk (per 100,000 per year)
A2,Cw4,Bw35,DR4	DQB1*0302	DQA1*0301	NonAsp	Arg	35
A3,Cw3,Bw62,DR4	DQB1*0302	DQA1*0301	NonAsp	Arg	130
A24,Cw7,Bw39,DR4	DQB1*0302	DQA1*0301	NonAsp	Arg	166
A2,Cw1,Bw56,DR4	DQB1*0302	DQA1*0301	NonAsp	Arg	196
A2,Cw1,Bw56,DR4	DQB1*0302	DQA1*0301	NonAsp	Arg	210
A1,Cw7,B8,DR3	DQB1*0201	DQA1*0401	NonAsp	Arg	68
A2,Cw7,B8,DR3	DQB1*0201	DQA1*0401	NonAsp	Arg	103

be relevant in determining genetic susceptibility. Studies in the NOD mouse have identified non-MHC genes on several chromosomes that modulate the major effect of MHC genes; for example a gene on chromosome 3 (analogous to human chromosome 1 or 4) determines frequency and severity of insulinitis and another, on chromosome 11 (analogous to chromosome 17 in humans) may influence the progression from insulinitis to overt diabetes in this animal model (Todd *et al.* 1991). It seems probable that the situation in humans will prove at least as complex. The insulin gene on chromosome 11 and immunoglobulin heavy chain haplotypes do appear to influence susceptibility to Type 1 diabetes (Bell *et al.* 1984; Rich *et al.* 1986). Further progress in this field will be possible by linkage analysis of DNA markers in large numbers of families with two or more affected siblings.

The role of genetic markers in the prediction of Type 1 diabetes: Concordance for Type 1 diabetes in monozygotic twins is less than one in three, and, even allowing for some degree of somatic mutation, this suggests that only a minority of those

genetically susceptible will develop Type 1 diabetes (Barnett *et al.* 1981). Markers of genetic susceptibility are consequently so prevalent in the general population that they cannot be sufficiently specific for use in isolation. Their use for the purposes of prediction and prevention of Type 1 diabetes is limited and it is likely that their main role will be to define high risk groups for surveillance with more specific tests.

6.3.3 Immune markers:

Introduction: Changes in both humoral and cell-mediated immunity have been detected prior to the clinical onset of Type 1 diabetes, but research into the prognostic significance of these changes has been largely confined to the humoral changes. I shall therefore limit my discussion accordingly. In this section I have focused particularly on ICA as the best validated markers but have also described work in our own and other studies into the role of other autoantibodies.

6.3.3.1 Islet cell antibodies:

The appearance of circulating cytoplasmic islet cell antibodies prior to the clinical onset of Type 1 diabetes in patients with other organ-specific autoimmune disease was the first 'pre-diabetic' immune change identified (Bottazzo *et al.* 1974). The observation that these antibodies can be found in individuals who, many months or years later, develop the typical clinical features of insulin dependent diabetes forms the basis of the concept of the long prodrome of Type 1 diabetes (Gorsuch *et al.* 1981). These antibodies to an unidentified islet antigen are present in the serum of newly diagnosed diabetics and are detectable in about 80% at diagnosis, falling to about 30% after 5 years (Lendrum *et al.* 1975). They are usually assayed by indirect immunofluorescence on unfixed human pancreas, though some centres use rat or monkey tissue and other staining methods. Initial measurements of the antibodies were essentially qualitative; the complement-fixing properties of the antibodies provided an indirect measure of quantitative differences (Bruining *et al.* 1984; Bottazzo *et al.* 1980). The first international workshop on standardization demonstrated very wide variation, both within and between laboratories (Bottazzo

et al. 1986b). Three subsequent workshops have been held to improve precision, specificity and comparability, and standardized units (Juvenile Diabetes Foundation -JDF- units) defined. It is therefore now possible to quantify the levels of circulating ICA and to compare results from different centres. The detection threshold still varies considerably between laboratories and it is now apparent that the old designations 'ICA positive' and 'ICA negative' are unsatisfactory and, when used, must be interpreted with caution.

6.3.3.2 ICA in the Bart's-Windsor and Bart's-Oxford Family Studies:

(a) Prediction using qualitative measurement of ICA

The first report of measurement of ICA prior to the diagnosis of Type 1 diabetes in the Bart's Windsor Family Study was published in 1981 after a mean follow-up period of 2.0 years (Gorsuch *et al.* 1981). In this paper conventional and complement-fixing were described qualitatively as being present or absent. At that time 20/313 (6%) parents and 34/288 (12%) siblings were positive for conventional ICA and 6/313 (2%) parents and 12/288 (4%) siblings positive for CF-ICA. 4 siblings and 2 parents had developed Type 1 diabetes and ICA had been present prior to diagnosis in all cases so that 11% of the 54 conventional ICA-positive nondiabetic subjects became diabetic, compared with 0% of those who remained ICA-negative ($2P = 10^{-6}$, Fisher's exact test). The equivalent figures for CF-ICA positivity were 28% and 0.2% respectively ($2P < 10^{-6}$). This gave a relative risk of 73 (95% CI 11-483) for the development of Type 1 diabetes in CF-ICA positive unaffected family members as compared with ICA-negative family members (Gorsuch *et al.* 1982).

The use of ICA in the prediction of Type 1 diabetes within the family study was reconsidered in the paper published by Tarn *et al.* in 1988 (Tarn *et al.* 1988). This covered the first 8 years of follow-up of the 209 families described above, up to October 1986. Since entry to the study 5 parents and 11 siblings had become insulin dependent. The risks for ICA-positive subjects as a whole, and subdivided according to the presence or absence CF-ICA positivity, were compared with that for ICA-negative subjects. Risks were calculated according to cumulative rate, and

to allow to the variable length of follow-up between subjects, the risks were also analyzed according to the total time at risk and by life table analysis as shown in Table 6.6. The study included an evaluation of the balance of sensitivity and specificity of CF-ICA positivity is 1, 3 or 5 tests were considered for each subject. As a result of this, the definition of ICA positivity (positive on 3 occasions at least 3 months apart) was more rigorous than that used in previous analyses.

Table 6.6 Risks of Type 1 diabetes in the Bart's-Windsor family study according to ICA status

	All ICA positive	CF-ICA positive	non-CF-ICA positive	ICA negative negative
n	54	24	30	665
Developed IDDM	14 (26%) ¹	13 (54%) ²	1 (3.3%) ³	2 (0.3%)
DM uncertain type	1	1	-	-
Non-diabetic	40 (74%)	10 (42%)	29 (96.7%)	663 (99.7%)
Attack rate per 100 subject-years (95% CI)	4.89 ⁴ (2.39-7.39)	12.25 ⁵ (6.01-18.49)	0.56 ⁶ (-0.53-1.65)	0.065 (-0.025-0.15)
Cumulative risk of IDDM after 8 years from life table analysis (95% CI)	42.3% ⁷	76.0% ⁸ (53.2-98.2)	3.4% ⁹ (0-9.9)	0.6% (0-1.4)

¹ Cumulative rate all ICA positive vs ICA negative : p < 0.001.

² Cumulative rate CF-ICA positive vs ICA negative: p < 0.001. Relative risk 341 (95% CI 78-1499).

³ Cumulative rate non-CF-ICA positive vs ICA negative: p = NS. Relative risk 14 (95% CI 1.7-106).

⁴ Relative risk all ICA positive vs ICA negative: 75.2.

⁵ Relative risk CF-ICA positive vs ICA negative: 188.5. Relative risk vs all ICA: 25.1.

⁶ Relative risk non-CF-ICA positive vs ICA negative: 8.6. Relative risk vs all ICA: 0.11.

⁷ Cumulative risk all ICA positive vs ICA negative (log-rank test): p < 0.001. Relative risk from life tables: 42.3.

⁸ Cumulative risk CF-ICA positive vs ICA negative (log-rank test): p < 0.001. Relative risk from life tables: 75.3. Cumulative risk CF-ICA positive vs all ICA positive: p < 0.025.

⁹ Cumulative risk non-CF-ICA positive vs ICA negative: p = NS. Relative risk from life tables: 3.5.

(b) Prediction using quantitative measurement of ICA:

Introduction

The first international workshop on the standardization of cytoplasmic islet cell antibodies was held in October 1985 and showed very wide variation in ICA assays both within and between laboratories. Reference sera were then made available (Bottazzo *et al.* 1986b). The second workshop took place in January 1987 evaluating intra-laboratory reproducibility over time and assay precision. This resulted in the definition of international standard units (JDF units), arbitrary units relative to the degree of dilution of the reference (80 JDF unit) serum. Conversion of ICA into JDF units improved precision within the laboratories as well improving concordance between laboratories (Boitard *et al.* 1988). After this workshop the sera from the Bart's Windsor Family Study were re-examined and ICA levels retrospectively converted into JDF units by Dr Ezio Bonifacio and Marion Shattock using the conversion cited above. I worked with Ezio Bonifacio on the following re-analysis of the risks of Type 1 diabetes in the Barts-Windsor Family Study according to quantitative measures of ICA in JDF units.

The cohort of subjects included was the same as that considered by Anne Tarn (see *Section 6.3.3.2 (a)*). Ten years of follow-up were included. No further subjects had developed diabetes, so that since the beginning of the study, Type 1 diabetes had developed in 5 parents and 11 siblings.

Results

At entry to the study, ICA were above the threshold of detection (4 JDFu) in 10/376 (3.6%) of parents and 16/343 (4.7%) of siblings. The distribution of ICA levels at entry is shown in Figure 6.1. There was a highly significant difference ($\chi^2=306$, $p < 10^{-5}$) in the proportion of family members with detectable ICA at entry between those who developed Type 1 diabetes (14/16 (87.5%) with ICA: 10/11 siblings and 4/5 parents) and those who remained non-diabetic (12/703 (1.6%) with ICA: 6/332 siblings and 6/371 parents).

Life table analysis was used to determine the projected insulin-dependent diabetes-

free survival according to peak ICA level (Figure 6.2)

The risk was significantly lower ($p < 0.0001$) in those with undetectable ICA than those with ICA 4-19 JDF units or ICA greater than 20 JDF units. There was also a significant difference between the risk in the latter 2 groups. The estimates of risk of development

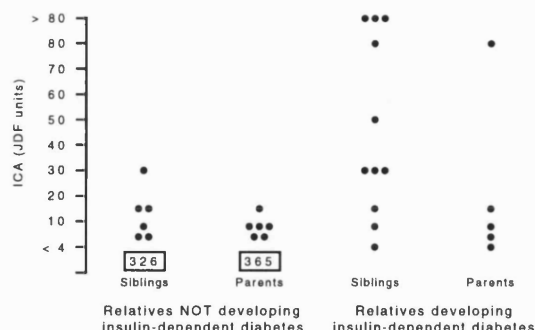
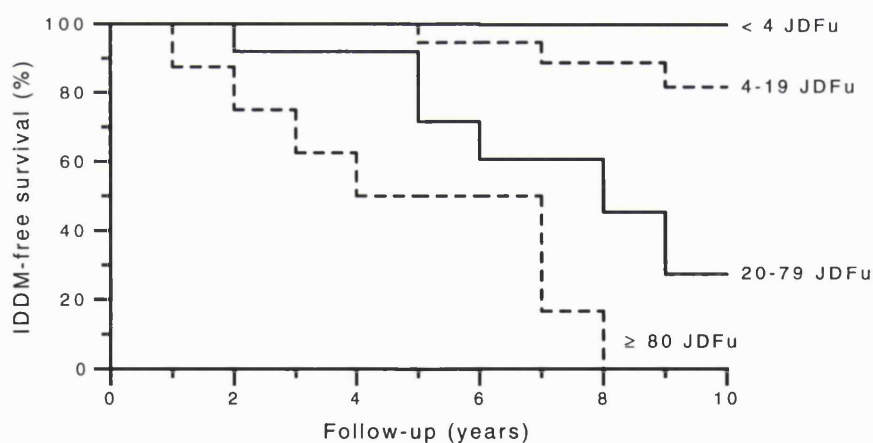


Figure 6.1 The distribution of ICA levels at study entry

of Type 1 diabetes after various periods of follow-up with various ICA thresholds are presented in tabular form in Table 6.7.



< 4 JDFu	692	689	686	684	683	682	678	637	596	534	497
4-19 JDFu	28	26	25	23	19	17	16	15	13	11	5
20-79 JDFu	18	13	11	10	9	7	5	4	3	1	1
≥ 80 JDFu	8	7	6	5	4	3	2	0	0		

Figure 6.2 Cumulative risk of Type 1 diabetes after 10 years by peak ICA level

Table 6.7 Percentages of first-degree relatives in whom Type 1 diabetes will develop by ICA threshold

Years after first positive ICA	Percentage (95% CI) at ICA threshold of		
	4 JDF units n=40	20 JDF units n=19	80 JDF units n=8
1	2 (0-7)	5 (0-15)	12 (0-35)
3	10 (1-19)	20 (1-39)	37 (4-70)
5	18 (6-30)	37 (15-59)	50 (16-84)
7	24 (10-38)	62 (42-82)	83 (44-100)
9	40 (23-57)	73 (45-100)	100 (52-100)

The sensitivity, specificity and positive predictive value of different thresholds of ICA are shown in Table 6.8.

Table 6.8 Peak ICA and development of Type 1 diabetes within 10 years

Threshold (JDF units)	Positive predictive value (%)	Sensitivity (%)	Specificity (%)
4	40	88	97.0
10	48	82	98.0
20	73	69	99.6
40	85	50	99.8
80	100	31	100.0

Discussion

Studies of ICA measured semi-quantitatively in first degree relatives had demonstrated that risk was highest in those with complement-fixing ICA (Tarn *et al.* 1988) which generally corresponded to high titres. Full evaluation of the

predictive value of ICA however required the use of reproducible, quantifiable measurements of the antibodies. The International ICA Workshops showed that early ICA assays were highly variable between laboratories and that even intra-laboratory reproducibility was poor though it could be improved with the introduction of an international standard and the use of standard curves in each assay. The development of standardized JDF units therefore offered the opportunity of comparing data between studies and of evaluating the prognostic significance of different thresholds of ICA.

Life table analysis demonstrated that the risk of development of Type 1 diabetes in first degree relatives of a child with Type 1 diabetes varied with the titre of ICA. The risk was highest in those with ICA ≥ 80 JDF units, all the relatives with ICA of this level had developed Type 1 diabetes within 7 years of follow-up. In contrast to some other studies, we also demonstrated an excess risk in those with low titre ICA (4-19 JDF units) over those with undetectable ICA. The number of subjects studied is small and the confidence intervals around the risk estimates wide. This problem is common to all prospective studies and will only be overcome by pooling standardized data.

With these data it is now possible, for the first time, to consider the practical application of risk assessment in family members. Randomized controlled trials will be necessary to establish the value of any putative treatment. It is essential that such trials have adequate power to provide both negative and positive answers about the efficacy of the treatments tested within the time of the trial. The Bart's-Windsor study provides one of the longest periods of follow-up of a cohort of subjects with ICA. This analysis provides the data from which to estimate the sample size necessary for such studies. These calculations are shown in the next chapter (*Section 7.5.3*).

The threshold of positivity of a test depends on what is being asked of it. Figure 6.3 shows the balance of sensitivity and positive predictive value of various thresholds of ICA. There is an inevitable loss of sensitivity as the positive predictive

value rises as a higher threshold of positivity is considered. It is clear that if ICA are being used to identify subjects for inclusion in an intervention study to test a potentially toxic agent, when only subjects with a very high probability of developing diabetes are wanted, the threshold should be high. Use of a threshold of 80 JDF units might be appropriate in these circumstances but would however only identify 30% of the relatives in whom the disease would develop. On the other hand, identification of all relatives who might benefit from a preventive agent would demand a high sensitivity which could be achieved by using a lower threshold. Specificity might then be improved by combining ICA with another immune or metabolic marker. Use of an assay with a low detection threshold together with quantification of ICA maximizes flexibility. A threshold of 20 JDF units gives approximately equal false negatives and false positives over the 10 year period of follow-up.

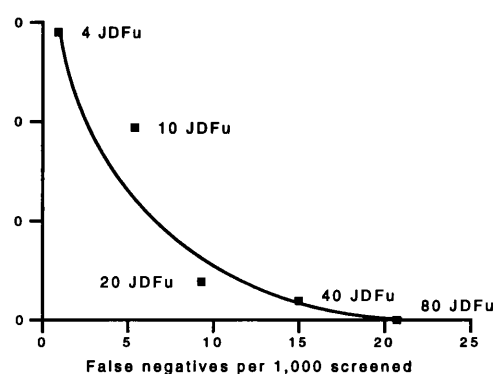


Figure 6.3 The number of false positive and false negative tests per 1,000 individuals screened using different thresholds of ICA

6.3.3.3 ICA staining patterns

The predictive value of ICA is modulated by the staining pattern of the antibodies. It has recently been demonstrated that some ICA stain predominantly beta cells within islets (*beta cell selective* or *restricted* pattern) and are associated with a markedly lower risk of progression to diabetes than ICA that stain all types of islet cells (*whole islet* or *nonrestricted* pattern)(Genovese *et al.* 1992; Gianini *et al.* 1992; Timsit *et al.* 1992). In the Boston-Sacramento family study, using an ICA assay with a high detection threshold, these beta cell selective antibodies accounted for the immunofluorescent staining in approximately 25% of all ICA positive first degree relatives (Gianini *et al.* 1992). ICA of the beta cell selective pattern are completely blocked by pre-incubation with rat brain homogenate and

this inhibition is prevented by pre-clearing the rat brain with sheep anti-GAD antibodies or a serum with selective ICA, but not if a serum with whole islet ICA is used (Genovese *et al.* 1992). These findings suggest that the specificity of the antibody which gives this pattern of staining is directed against GAD.

6.3.3.4 Insulin autoantibodies: Autoantibodies to insulin (IAA) were first described in newly diagnosed Type 1 diabetes before insulin treatment was started (Palmer *et al.* 1983). They have been assayed by both enzyme-linked immunoassay (ELISA) and radio-immunoassay (RIA) which has contributed to the confusion amongst the initial studies of their role in Type 1 diabetes. Four International Workshops on the standardization of insulin autoantibodies have been held. Major differences in detection frequency between RIA and ELISA were found for some sera, possibly due to the particular orientation of the insulin molecule on the solid phase which results in masking of particular binding epitopes, or to iodination-induced amplification of binding affinity occurring in the liquid phase (Palmer *et al.* 1990). Whatever the reason, it now seems that the two assays are not comparable and since RIA are more widely used, only the results from these assays were considered in the 4th Workshop.

IAA have been detected by both ELISA and RIA prior to diagnosis in a prospective studies of twins, family members, subjects with other autoimmune disease and in population studies of normal children (Dean *et al.* 1989; Atkinson *et al.* 1986; Ziegler *et al.* 1989; Wilkin *et al.* 1985; Maclaren *et al.* 1985; Bingley *et al.* 1993b). The tendency to produce IAA is genetically determined and, in general, matches markers of genetic susceptibility to Type 1 diabetes. In newly diagnosed patients and in siblings followed prospectively prior to the diagnosis of Type 1 diabetes, IAA have been found to be less common in those with DR3 than in other genetically susceptible subjects (McEvoy *et al.* 1986). More recently, IAA were found in 28 of 123 relatives of patients with Type 1 diabetes (23%) with at least one DR4 allele, compared with 6 out of 141 (4%) of DR4 negative relatives. The levels were also higher in the DR4 positive group who accounted for 21 of the 22 highest IAA values (Ziegler *et al.* 1991). This finding was not confirmed when IAA were

measured by ELISA in discordant monozygotic twins (Wilkin *et al.* 1985). In a control population of schoolchildren, IAA were only found in children who possessed DR3 or DR4 (Atkinson *et al.* 1986). IAA at diagnosis are more prevalent in younger subjects and a significant negative correlation between insulin binding and age at diagnosis has been found in several studies.(McEvoy *et al.* 1986; Arslanian *et al.* 1985; Vardi *et al.* 1988).

The role of IAA as markers of future Type 1 diabetes has since been investigated along similar lines to those used for ICA. As described above, standardization of measurement of these autoantibodies is not as far advanced and the picture is complicated by the existence of two assay methods, ELISA and RIA, that do not always produce concordant results (Palmer *et al.* 1990). Different studies using either RIA or ELISA have shown that IAA are predictive of Type 1 diabetes in family and twin studies (Dean *et al.* 1989; Atkinson *et al.* 1986; Ziegler *et al.* 1989; Wilkin *et al.* 1985). The main outstanding question is whether IAA have an independent predictive role over and above that of ICA (Atkinson *et al.* 1986; Ziegler *et al.* 1989). All studies have shown that ICA and IAA tend to occur in the same subjects and that they are most prevalent in those with high titres or complement-fixing ICA. Classification of subjects as 'IAA+/ICA+' or 'IAA+/ICA-' is complicated by the use of ICA assays with high detection thresholds. In the Bart's-Windsor Family study, the sensitivity and specificity of ICA >4 JDF units together with IAA is similar to that of ICA \geq 20 JDF units (Dean *et al.* 1989).

There is a negative correlation between IAA and age at diagnosis in newly diagnosed Type 1 diabetes which may be another confounding variable in assessing the role of IAA (Vardi *et al.* 1988). High levels of IAA at diagnosis were found in 100% of children below age 5 and in unaffected family members with ICA > 40 JDF units. It has been suggested that these antibodies might appear early in life in individuals susceptible to Type 1 diabetes and disappear later in life.

6.3.3.5 Autoantibodies to the 64K islet antigen and GAD:

Autoantibodies to the 64K human islet antigen have been detected in 1/14 first

degree relatives up to 91 months prior to the development of Type 1 diabetes and in 5/5 relatives with ICA > 40 JDF units reported to have evidence of impaired beta cell function (Baekkeskov *et al.* 1987). They were also detected in 4/4 ICA+ but 0/3 ICA- school children who progressed to diabetes in a study in The Netherlands (Baekkeskov *et al.* 1989). These antibodies may prove to be useful predictive markers but the difficulties of performing the assay have precluded full assessment of their prognostic significance in large scale studies.

Recently this antigen has been identified as the GABA synthesizing enzyme, glutamic acid decarboxylase (Baekkeskov *et al.* 1990). This which is found in high concentrations in GABA secreting neurons and in pancreatic beta cells, and sera from diabetic patients binds enzyme activity from these tissues (Christie *et al.* 1992). Antibodies to GAD are found in around 80% of relatives studied before diagnosis (Harrison, 1992; Atkinson *et al.* 1990; Thivolet *et al.* 1992). High levels of GAD antibodies are, however, also found in the absence of diabetes. They were first described in 'Stiff man syndrome', a rare neurological disorder (Solimena *et al.* 1990), and GAD may be the autoantigen of beta cell selective ICA, which as we have seen, carry a lower risk of progression to Type 1 diabetes. Their full prognostic significance remains to be clarified. Distinct specificities of the 64K antibody have been demonstrated (Christie *et al.* 1990). Antibodies to a 50,000-M_r tryptic fragment are found in most patients with Type 1 diabetes but are also present in 15% of monozygotic twins who remain discordant for Type 1 diabetes after prolonged follow-up and in all ICA positive polyendocrine patients who did not develop Type 1 diabetes. In contrast, antibodies to 37,000/40,000-M_r tryptic fragments, distinct from GAD, are found in the majority of patients with Type 1 diabetes and correlate well with whole islet ICA. They are found in only 2% of discordant twins and are absent in ICA positive polyendocrine patients who have not developed diabetes (Christie *et al.* 1991; Genovese *et al.* 1991).

6.3.3.6 Use of combined humoral markers in the prediction of Type 1 diabetes in the Bart's-Windsor and Bart's-Oxford Studies

Introduction

Islet cell antibodies (ICA) are the best validated markers of risk of Type 1 diabetes in family members. If an assay with a low detection threshold is used, they provide a sensitive indicator; in the Barts-Windsor family study, 83% of those who have who developed Type 1 diabetes within 10 years had detectable ICA (≥ 4 JDF units) at study entry. At that level, however, they are relatively unspecific markers of future Type 1 diabetes; only 40% of individuals with ICA at or above this level would be expected to develop Type 1 diabetes within 10 years. Their specificity can be increased by raising the threshold, to 40 JDF units for example; above this level the positive predictive value for Type 1 diabetes within 10 years is 85%. This will, however, be associated with a considerable fall in sensitivity, so that instead of detecting 88% of cases, only 50% will be picked up. As described in the previous sections, several other antibodies have been proposed as predictive markers, either in isolation or used in conjunction with ICA. The aim of this study was to investigate whether prediction of Type 1 diabetes within this subpopulation could be improved by additional use of autoantibodies to insulin (IAA), glutamate decarboxylase (anti-GAD) and tryptic fragments of 64kD islet antigens (anti-37k and anti-40k), both singly and in combination. The predictive value of these combined markers was compared with quantitative measurement of ICA.

The study was undertaken in collaboration with Dr Ezio Bonifacio and Dr Michael Christie. The autoantibody assays were performed by a number of people, as listed in the appendix.

Subjects and Methods:

Subjects: One hundred and one non-diabetic first degree relatives (48 parents and 53 siblings) of patients with Type 1 diabetes diagnosed before age 21 were studied. These were all participants in the Bart's-Windsor and Bart's-Oxford family studies who had been found to have ICA greater than or equal to 10 JDF units with detectable ICA (greater than 4 JDF units) on at least one other occasion. The

median age at entry to the study was 19.8 years, range 2 - 56 years. Subjects were followed for up to 14.3 years with repeated sampling for ICA and other autoantibodies. The median duration of follow-up was 3.8 years. Eighteen subjects had developed Type 1 diabetes during the study, 4 parents and 14 siblings (Table 6.9).

Table 6.9 Subject characteristics

n	48 parents:53 siblings
Age	median 19.8 year (range 2-56)
Sex	61 male: 40 female
Follow-up	Median 3.8 year
Progressed to IDDM	4 parents: 14 siblings

Table 6.10 Follow up and the development of Type 1 diabetes in the study cohort

Follow up years	Number at start of year	Withdrawn during year	IDDM diagnosed during year
0-1	101	1	4
1-2	96	19	3
2-3	74	12	1
3-4	61	10	1
4-5	50	15	3
5-6	32	10	2
6-7	20	5	1
7-8	14	0	1
8-9	13	0	2
9-10	11	1	0
>10	10		

The cumulative risk for the development of Type 1 diabetes for the whole group and the duration of follow-up are summarized in Figure 6.4 and Table 6.10.

Autoantibody assays: Autoantibodies were determined on the earliest available sample in which ICA \geq 10 JDF units were detected. If the sample size was insufficient for all tests, a second sample taken within 6 months was used to supplement it. Sequential samples taken at approximately annual intervals between

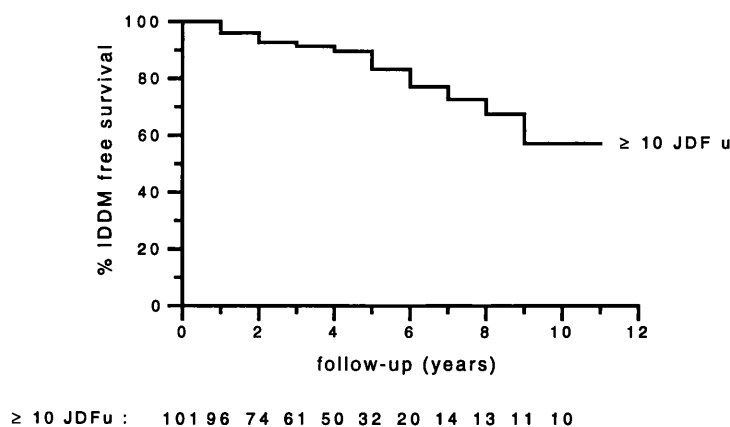


Figure 6.4 The cumulative risk of Type 1 diabetes for the whole study population. The number of subjects included in each year of follow up is shown below the figure.

study entry and diagnosis of Type 1 diabetes were also tested where available. All assays were performed on coded samples.

Details of assay methods are given in the appendix

Individuals were classified as insulin autoantibody positive if the corrected binding was more than 3 standard deviations above the mean of 172 adult blood donors (mean \pm SD; -0.04 ± 0.26). Sera were regarded as positive for anti-GAD antibodies if the relative antibody activity exceeded 2 standard deviations of the activity in sera from a group of 30 healthy control individuals (mean \pm SD; $6.2 \pm 3.4\%$ of positive control). Serum samples were regarded as positive for antibodies to tryptic fragments of islet 64k antigens if a band corresponding to the appropriate polypeptide could be detected on the autoradiogram. Samples with ICA ≥ 20 JDF units were tested for beta cell selective ICA by testing for blocking of immunofluorescence by GAD (Genovese *et al.*1992). ICA were considered beta cell selective if the ICA endpoint titre fell by at least 2 doubling dilutions with the addition of rat brain GAD.

Data Analysis: Life tables were used to estimate the time to development of Type

1 diabetes. Follow-up time for each subject was calculated from the date when ICA ≥ 10 JDFu were first detected. The start of insulin treatment was used as the date of diagnosis of Type 1 diabetes. Survival experience was compared using the Lee-Desu statistic in SPSS-PC.

Results

Prevalence of autoantibodies: Table 6.11 shows the prevalence of autoantibodies detected.

Table 6.11 The prevalence of autoantibodies in the study cohort

	ICA (JDFu)			IAA	GAD	37k	40k
	10-19	20-79	≥ 80				
n	49	38	14	37	40	13	17

Thirty-six individuals had only ICA, 38 had ICA and one other antibody. Three of the 5 antibodies were detected in 16 individuals, 4 antibodies in 7 and all 5 antibodies in 4.

Family members who developed Type 1 diabetes during follow-up: The characteristics of the 18 family members who developed Type 1 diabetes and the autoantibodies detected are shown in Table 6.12.

The effect of age: Twenty individuals were less than 12 years of age at entry to the study. Nine of these developed Type 1 diabetes, compared with 5 out of 33 family members aged between 12 and 25 years and 4 of the 48 more than 25 years of age. The cumulative risk of diabetes within 10 years was 63% in the youngest age group, 38% in those aged 12-25 yr and 33% in those over 25 years ($p = 0.003$). Figure 6.5 shows the survival curves for family members above and below 12 years of age.

Table 6.12 Characteristics of subjects who developed Type 1 diabetes during follow-up

ID	Years before diagnosis	ICA (JDFu)	IAA	GAD	37k	40k
40.6	1.5	>80	-	-	+	+
55.5	0.7	80	+	+	+	+
69.5	6.7	16	-	+	-	-
88.4	4.4	50	-	-	-	-
100.5	7.3	30	+	+	-	-
103.5	1.9	30	+	-	+	+
112.4	8.5	30	+	+	-	-
140.2	0.4	80	-	+	+	+
153.1	8.1	10	+	+	-	-
166.2	4.7	15	-	+	-	-
258.6	5.2	13	-	+	-	-
261.4	6.0	13	+	+	-	-
325.4	4.3	80	+	-	+	+
367.1	2.2	52	-	-	+	+
425.3	0.2	37	+	+	-	-
608.6	0.7	>80	+	+	+	+
621.3	3.2	49	+	+	+	+
668.5	1.1	>80	+	-	-	+



Figure 6.5 left: the cumulative risk of Type 1 diabetes within 10 years by age at entry to the study. right: the age distribution in the cases of diabetes.

Quantitative measurement of ICA: Forty-nine individuals had ICA between 10 and 19 JDF units and 5 of these developed Type 1 diabetes; the cumulative risk of Type 1 diabetes within 10 years in this group was 34%. Thirty-eight had ICA 20-79 JDF units, of whom 7 became diabetic giving a 10 year cumulative risk of 52%. Fourteen had ICA of 80 JDF units or above and 6 developed Type 1 diabetes. The cumulative risk of Type 1 diabetes within 5 years was 63%. Only one individual in this group remained non-diabetic after this time. The survival curves were significantly different in the 3 groups ($p < 0.0001$) (Figure 6.6).

ICA staining pattern: ICA staining pattern was tested in 44 individuals. GAD blocked ICA immunofluorescence in 7; one of these developed Type 1 diabetes. ICA were not blocked in 29, and in 8 samples the results were equivocal.

Insulin autoantibodies: Insulin autoantibodies were positive in 37 family members of whom 11 developed Type 1 diabetes after median follow-up 3.2 years. After 5 years of follow-up there was no significant difference in survival curves of IAA positive and IAA negative groups but 5 family members developed diabetes between 4 and 8.5 years after entry and the survival was significantly different after 10 years ($p = 0.03$). (Figure 6.7)

GAD antibodies: Antibodies to GAD were detected in 60 family members overall and in 12 of those who developed diabetes. There was no significant difference in the survival curves for groups with and without GAD antibodies ($p=0.18$). (Figure 6.8). The median time to diagnosis of Type 1 diabetes in the cases with GAD antibodies was 5.0 years.

Antibodies to 37k and 40 k tryptic fragments: Antibodies to the 37k fragment were found in 13 individuals and to the 40k fragment in 17. Thirteen had antibodies to both fragments, 4 had antibodies to the 40k fragment only, and none had antibodies to 37k only. Nine of the patients who developed Type 1 diabetes had antibodies to one or both fragments. All of these developed diabetes within 5 years of study entry. The median time to diabetes was 1.5 years. The cumulative risk of Type 1 diabetes within 5 years was 76% in those with 37k and/or 40k antibodies compared with 14% in those without. After 10 years, the risk in 37k/40k negative family members was 38%, and 9 cases had been diagnosed within this group.

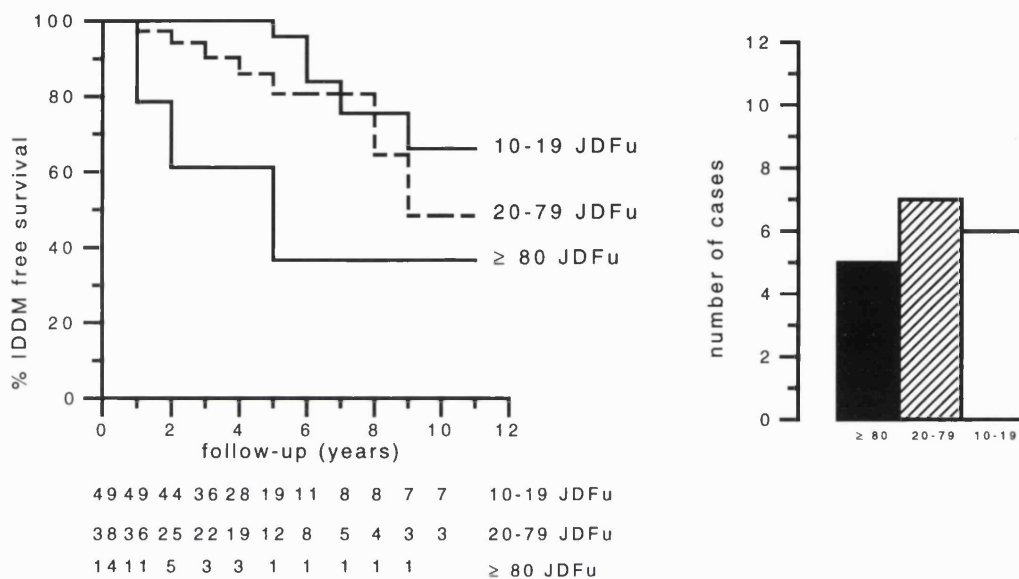


Figure 6.6 left: the cumulative risk of Type 1 diabetes after 10 years by ICA level at entry. right: the distribution of ICA in the cases of diabetes.

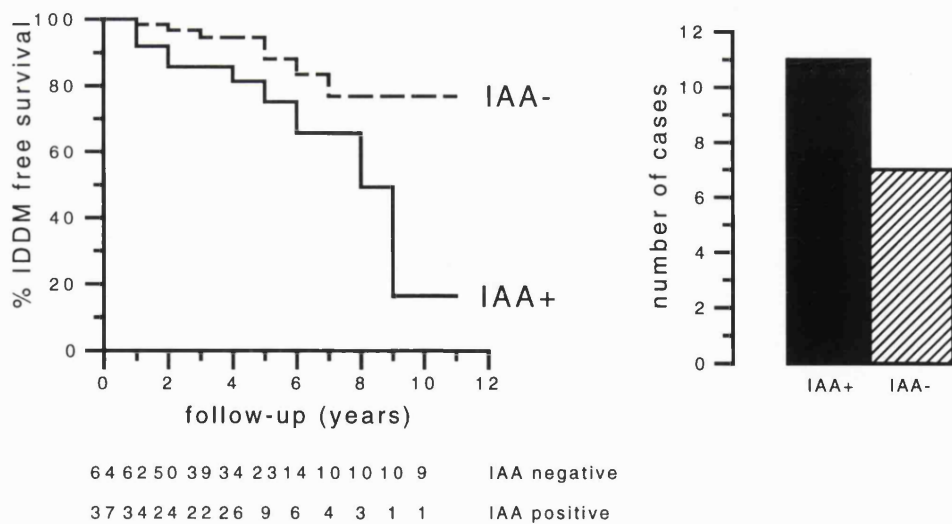


Figure 6.7 left: the cumulative risk of Type 1 diabetes after 10 years by IAA at entry. right: the proportion of IAA positive and IAA negative cases.

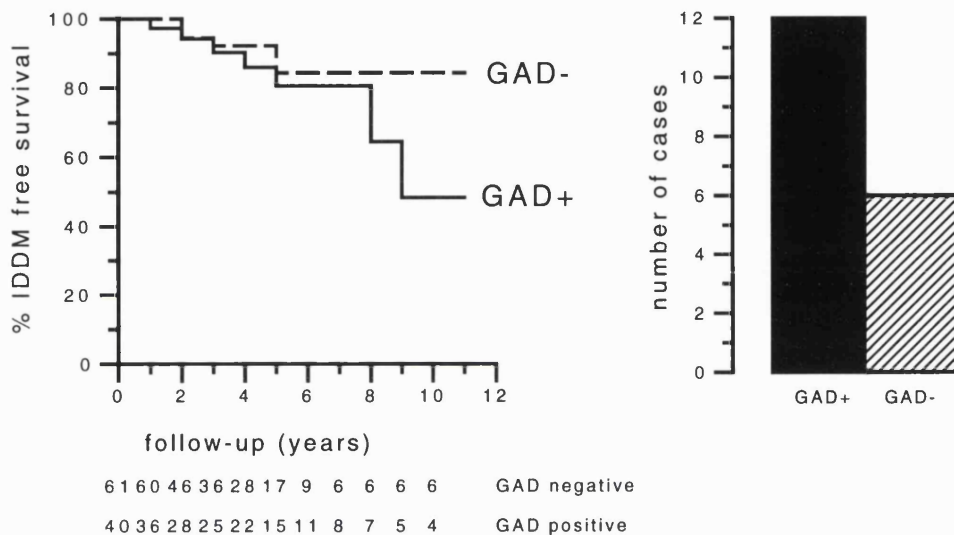


Figure 6.8 left: the cumulative risk of Type 1 diabetes after 10 years by antibodies to GAD at entry. right: the distribution of GAD autoantibodies in the cases of diabetes.

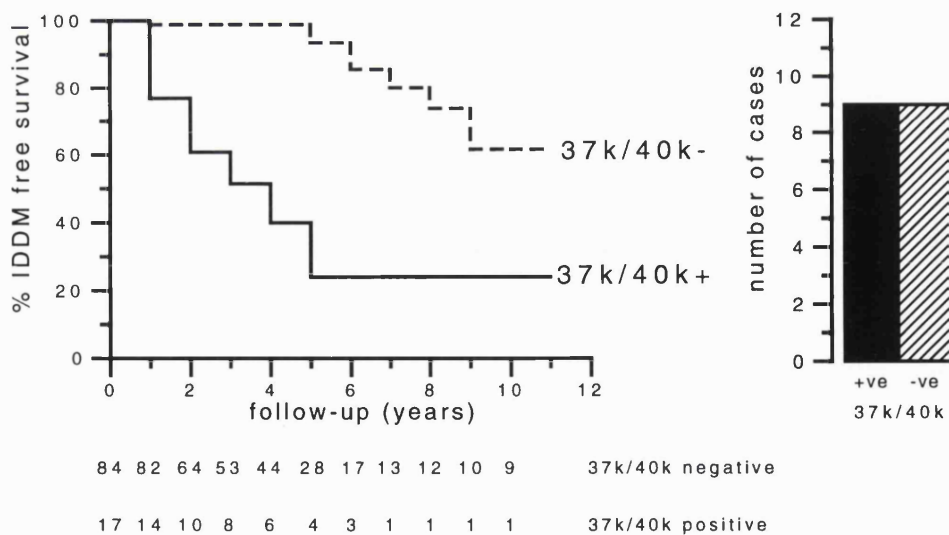


Figure 6.9 left: The cumulative risk of Type 1 diabetes at 10 years by antibodies to 37k and/or 40k tryptic fragments of islet antigen. right: 37k and/or 40k antibodies in the cases of diabetes.

Table 6.13 Autoantibodies detected in individuals who developed Type 1 diabetes

ICA alone	1
ICA + GAD	3
ICA + IAA + GAD	5
ICA + IAA + 40k	1
ICA + 37k + 40k	2
ICA + IAA + 37k + 40k	2
ICA + GAD + 37k + 40k	1
ICA + IAA + GAD + 37k + 40k	3

Combining autoantibodies: The combinations of antibodies detected in individuals who developed diabetes are summarized in Table 6.13. The risk of diabetes increased with number of antibodies detected (Table 6.14).

Table 6.14 The risk of Type 1 diabetes by number of autoantibodies detected

Number of autoantibodies detected	Cumulative risk of IDDM
1 (ICA alone)	8% within 10 yr
2	27% within 10 yr
3	100% within 8 yr
4	65% within 10 yr
5	75% within 4 years

Thirty-six individuals (36% of the cohort) had ICA alone and only 1 of them developed Type 1 diabetes. The cumulative risk of Type 1 diabetes within 10 years in this group was 6%. Thirty-eight individuals had ICA and one other autoantibody; 3 developed Type 1 diabetes. Twenty-seven family members had at least three of the five autoantibodies tested. This group contributed 14 of the 18 cases in the study (median time to diabetes 2.0 years, range 0.2 to 8.5 years) and the cumulative risk of Type 1 diabetes within 10 years was 88% (Figure 6.10).

Family members with 3 or more autoantibodies who were 37k and/or 40k positive has a median time to diagnosis of 1.5 years compared with 7.2 years in those with ICA, IAA and GAD antibodies in the absence of 37k or 40k antibodies ($p = 0.006$).

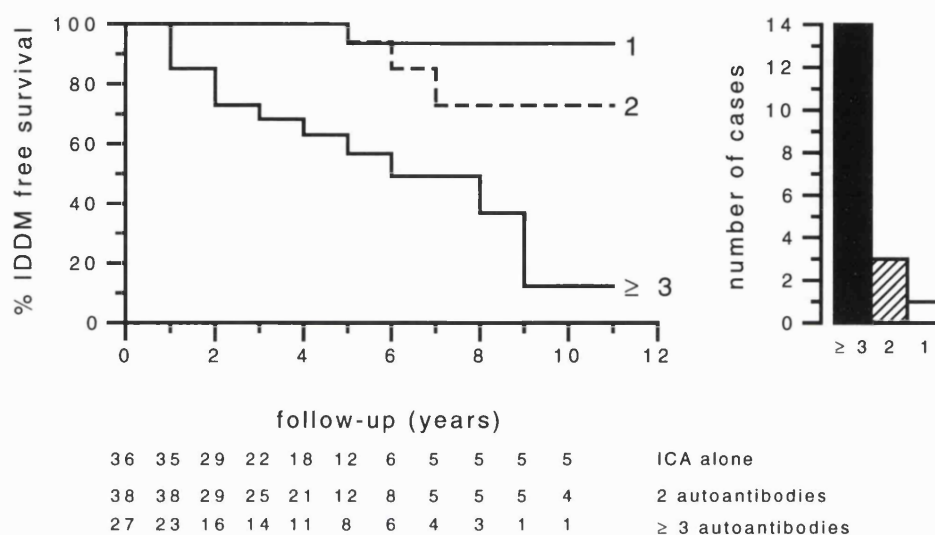


Figure 6.10 left: the cumulative risk of Type 1 diabetes after 10 years by the number of autoantibodies detected. right: the number of the cases of diabetes with 1, 2, or ≥ 3 autoantibodies

Changes in autoantibodies prior to diagnosis: Serial samples were examined from 14 of the family members who developed diabetes. Up to 4 samples per case were tested. One child (40.6) was IAA negative at study entry but had high levels of the autoantibodies 4 months before diagnosis. One parent (153.1) had GAD binding 85% at study entry 8.1 years before diagnosis and this was within the normal range in later samples. No other changes in IAA or GAD antibodies were found. Sequential samples were tested from 8 of 9 individuals who were initially 37k and 40k antibody negative and who later developed diabetes. These latest samples were taken between 0.3 and 2.7 years before insulin was started. Only 1 individual (258.6) appeared to develop 37k or 40k antibodies between entry to the study and onset of diabetes. The antibodies were weakly positive in the last sample taken 2.7 years before diagnosis, but were not detected in samples taken 30 and 18 months earlier.

Discussion

Prediction of Type 1 diabetes in families is carried out with the implicit aim of identifying individuals in whom it may be possible to intervene to delay or prevent the clinical onset of diabetes, ultimately reducing the frequency of the diabetes in the population. This means that there is a need to maximize the *sensitivity* of prediction in order to avoid missing future cases, at the same time having the almost contradictory aim of high *specificity* to avoid false positives and treatment of individuals who may never develop disease even without intervention. In general, the relation of specificity to sensitivity is reciprocal. The more certain we become that individuals will develop diabetes, the greater the proportion of those at risk we thereby exclude from the possible benefits of intervention. A very high degree of specificity of prediction in family members can, for example, be achieved by combining high levels of ICA with loss of first phase insulin response. We have, however, suggested elsewhere that, using a single screening point, such individuals probably account for under 20% of family members who will develop Type 1 diabetes within 5 years, and an even smaller proportion of cases that will occur in the longer term. (Bingley *et al.* 1993a). Decisions on intervention would ideally be founded on an estimate of both likely overall risk of diabetes, both in the short and long term, and of the time to diagnosis. The populations to be screened are likely to be large so that simplicity for both patient and investigator need to be taken into consideration; the yield of information from the first encounter should be as high as possible without need for multiple recalls for further testing. The value of islet cell antibodies as sensitive markers of future Type 1 diabetes has now been confirmed in many family studies (Riley *et al.* 1990; Bonifacio *et al.* 1990) with the proviso that the assay used has a low detection threshold. In the whole Bart's-Windsor and Bart's-Oxford family studies only 7/28 cases were ICA negative at entry to the study and 3 of these had detectable ICA during follow-up. In the Gainesville study, 13/40 cases had ICA < 10 JDF units at entry to the study (Riley *et al.* 1990). The purpose of this study was to work towards a simple screening strategy that could build on the basis of low threshold ICA testing towards maximum specificity with minimum loss of sensitivity.

We were not able to identify any single additional marker that markedly improved prediction with ICA alone. Each marker achieved a modest increase in cumulative risk over the 10 year period, but all were associated with loss of sensitivity. Merely raising the ICA threshold to 20 JDF units increased the cumulative risk from 43% to 53% after 10 years but this meant that 5 of the 18 cases would have been missed. ICA staining patterns were of limited value in this population. They could only be determined in 44% of the cohort, and beta cell selective ICA were only found in 7 individuals. Elimination of these individuals from the analysis of those with ICA greater than or equal to 20 JDF units would increase the cumulative risk in this group to around 61%. In contrast to other studies (Ziegler *et al.* 1989), the addition of insulin autoantibodies was only useful in detecting cases in the later stages of follow-up; the survival curves of IAA positive and IAA negative groups did not differ significantly in the first five years. Antibodies to GAD were more slightly more sensitive markers, found in 66% of the cases of Type 1 diabetes. They added little to the predictive value and there was no difference in the survival curves of groups with and without these antibodies. Antibodies to 37k and 40k tryptic fragments were highly specific markers of risk, associated with a 76% risk of Type 1 diabetes within 5 years. They were, however, relatively insensitive and were found in only 50% of the cases of Type 1 diabetes. This has been observed in other high risk populations such as monozygotic twins discordant for Type 1 diabetes (Christie *et al.* 1991) and patients with polyendocrine autoimmunity (Christie *et al.*, *submitted*).

The most interesting findings in this study came from analysis of the risk associated with combinations of markers. It seems that both the intensity and breadth of the humoral autoimmune activity are important in determining overall risk and time to diabetes. The number of antibodies present reflects the breadth of the immune response. We have measured ICA and antibodies to insulin, GAD, and tryptic fragments of the 64kD islet antigen but it seems likely that similar conclusions would be drawn if other anti-islet antibodies were examined; the list of potential candidates is ever growing (Harrison, 1992). Indeed, other groups have presented preliminary findings that high risk subjects can be identified on the basis

of having 2 out of IAA, antibodies to GAD and to the islet antigen p69 (Pietropaola *et al.*, presented at the 12th International Immunology and Diabetes Workshop, Orlando, 1993). Use of multiple tests in parallel increases sensitivity and generally lowers specificity and positive predictive value. This can be balanced by effectively raising the threshold of a 'positive screen' so that, in this study for example, high risk is associated with three antibodies being detected, ie *three* positive tests not just one.

The type of antibodies appears important in indicating the time to diabetes; antibodies to the 37k and 40k fragments were detected when clinical onset of diabetes was imminent, while the combination of ICA, IAA and antibodies to GAD were associated with similar risk of diabetes over the 10 years but were found in those in whom clinical onset of diabetes was delayed. Our findings in sequential samples taken from family members prior to clinical onset of diabetes indicate that levels of antibodies to 37k and 40k fragments change little during the disease prodrome. They do not seem to appear for the first time towards the time of diagnosis. This suggests that they reflect true differences in the underlying disease process rather than a different stage of the disease process, and are associated with a more rapid destructive attack on the beta cells. Although they were found most often in children, they were also present in 2 parents and one adult sibling. If they are true markers of a rapid disease process, this either questions the dogma of a single event that triggers the autoimmune attack early in childhood or it suggests that the attack may have variable levels of intensity over time. The appearance of these antibodies may perhaps be associated an acceleration in the process resulting from an additional environmental insult.

In conclusion, our findings do not suggest that ICA have yet been replaced as the most sensitive marker of future Type 1 diabetes. Using low titre ICA alone we can identify a group of family members with a 43% risk of Type 1 diabetes within 10 years. Using these four additional autoantibodies we can however identify the third of ICA positive family from which at least 75% of the cases of Type 1 diabetes diagnosed over the next 10 years will be drawn, and from a single screening point

we can get some indication of the likely time to diabetes. Such a strategy could allow us to focus our intensive prospective study and offers a simple potential means to identify candidates for secondary prevention.

6.3.4 Metabolic markers

6.3.4.1 Introduction:

The immunological markers described above can identify those in whom the beta cells are under attack, but give no indication as to how advanced the process is. Many groups are therefore trying to identify the metabolic changes that occur as the total beta cell mass falls.

6.3.4.2 Glucose tolerance:

A slow clinical onset of Type 1 diabetes with progressive or intermittent hyperglycaemia over many months prior to diagnosis has been found in family members under prospective follow-up. Indeed, oral glucose tolerance tests diagnostic of diabetes or impaired glucose tolerance have been described up to 10 years prior to the development of symptoms. In the Bart's-Windsor Family Study, the average duration of specific symptoms preceding the clinical onset of diabetes in 13 secondary cases was four weeks, but a raised random glucose (> 97.5th centile) was found in 11 individuals between 26 and 364 weeks before insulin treatment. Three patients had a diabetic oral glucose tolerance test 16, 26 and 84 weeks before insulin and another 4 showed impaired glucose tolerance in tests between 16 to 78 weeks earlier (Tarn *et al.* 1987). Rosenbloom *et al.* were able to trace 105 of 140 siblings of children with Type 1 diabetes who had had oral glucose tolerance tests 10-12 years earlier and found that 5 out of 6 who had developed diabetes had impaired glucose tolerance on the original test between 3 months and 7 years before diagnosis (Rosenbloom *et al.* 1982). Using results from 5 intensively studied first degree relatives with ICA > 40 JDF units, the Joslin group described a progressive rise in fasting and 60 min plasma glucose levels in the IVGTT during the 18 months prior to overt diabetes. A fasting plasma glucose > 108 mg/dl (5.9 mmol/l) had a positive predictive value of 100% for overt diabetes within 18 months. The 60 minute glucose value did not discriminate so well (Bleich *et al.* 1990).

6.3.4.3 The intravenous glucose tolerance test

The intravenous glucose tolerance test (IVGTT) was first used in 1923 (Jorgensen *et al.* 1923), and its main justification was as a means of overcoming variable intestinal absorption of glucose (Lundbaek, 1972). It also provides a means of evaluating the various functional pools of insulin within the pancreatic islet. An abrupt increase in plasma glucose level stimulates the immediate release of a readily available stored pool of insulin. This has been termed the first phase or acute insulin release. Characteristically there is a rapid rise in the plasma insulin level with a peak within 5 minutes and subsequent return to pre-stimulated levels. A second phase or late insulin response occurs if elevation in plasma glucose is sustained and is thought to be related to insulin synthesis (Lerner *et al.* 1971). The insulin response to an intravenous glucose load as measured in the IVGTT is the most widely used measure of beta cell function used in the prediction of Type 1 diabetes, and it is the first phase response (FPIR) that has been found to be most useful.

Natural history of changes prior to Type 1 diabetes: A progressive decline in first phase insulin secretion prior to diabetes has been shown in prospective twin and family studies (Srikanta *et al.* 1983b; Chase *et al.* 1987; Vialettes *et al.* 1988). It was initially suggested, on the basis of pooled data, that this decline was linear, allowing an individual's time to diabetes to be estimated after two tests (Srikanta *et al.* 1984).

(a) Limitations

Variability and reproducibility: Unfortunately the test has proved highly variable, both within and between subjects. Smith *et al.* repeated the test in 8 normal subjects and found a minimum coefficient of variation between subjects of 58% and within subjects of 22% (Smith *et al.* 1988a). Similar results have been reported from other centres (Bardet *et al.* 1989; McCulloch *et al.* 1993), though one group achieved lower intra-subject coefficients of variation (median 6.7, range 1.7-18.8%) in 10 normal subjects (Rayman *et al.* 1990).

Changes in puberty: There are also major changes in the insulin response during

puberty, with a significant progressive rise through the stages of puberty amounting to a 65% rise between Tanner stage 1 and Stages 4 and 5 (Smith *et al.* 1988c) and a decline from puberty until the third decade after which it appears to remain constant (Smith *et al.* 1988b). This physiological variation makes interpretation of changes within the normal range extremely difficult and has limited the application of the IVGTT in individual subjects.

(b) Standardization of the intravenous glucose tolerance test for use in prediction of insulin dependent diabetes

Introduction

Prediction of insulin dependent diabetes (Type 1 diabetes) is currently based on measurement of circulating autoantibodies such as islet cell antibodies (ICA) and insulin autoantibodies (IAA), together with assessment of the first phase insulin response to glucose in the intravenous glucose tolerance test (IVGTT). Standards for ICA and IAA assay have recently been established, but no agreed standard procedure has been established for the IVGTT, even though it has been in use for many years. In view of its importance in the prediction of Type 1 diabetes, and the need for comparable methodology for the conduct of multicentre trials of intervention therapy (American Diabetes Association, 1990), this study set out to assess the comparability of intravenous glucose tolerance tests performed in different centres around the world, and to develop a consensus protocol suitable for general use. The standard protocol was drawn up and agreed by an ICARUS (Islet Cell Antibody Register Users Study) working group.

Methods

Postal questionnaires (see appendix) were sent to twelve centres which have published or presented work on the use of metabolic tests in the prediction of Type 1 diabetes. Five were in North America, 5 in Europe and 2 in Australasia (Table 6.15). The questionnaires covered details of procedure prior to testing, dose and rate of glucose infusion and timing of samples. Sample handling and glucose and insulin assays were also compared, with emphasis on quality assurance.

Table 6.15 Centres included in the survey of IVGTT protocols**EUROPE**

St Bartholomew's Hospital, London.
 Department of Paediatrics, University of Oulu, Finland
 Hopital Saint-Vincent, Paris
 Diabetes Research Institute, University of Dusseldorf
 Hospital Clinico, Barcelona

N. AMERICA

Barbara Davis Center for Childhood Diabetes, Denver, Colorado
 Joslin Diabetes Center, Boston, Mass.
 University of Florida College of Medicine, Gainesville, Florida
 Childrens Hospital, Pittsburgh, Philadelphia
 Veterans Administration Medical Center, Seattle, Washington

AUSTRALASIA

Royal Melbourne Hospital, Melbourne, Victoria
 University of Auckland, New Zealand

Results

All 12 centres responded to the questionnaire.

Preparation: 4 centres gave no dietary advice for the days prior to testing, 1 asked subjects to ensure a 'generous' carbohydrate intake on the previous evening and 7 specified a high carbohydrate intake ('high' or 150-300g) for 3 days before the test. All required a fast of 8-12 hours, and the test was started between 0700 and 1000 in all centres.

Glucose infusion:

(i) *dose:* 11/12 centres used a dose of 0.5g/kg. Four applied no upper limit to the dose, 5 gave a maximum dose of 25-50g. One centre gave 0.3g/kg up to a maximum of 25g.

(ii) *concentration:* The final concentration of glucose infused varied between 20 and 66%.

(iii) *method of infusion:* 8/12 centres gave a timed infusion of glucose using a manually driven syringe. A syringe pump was used in 2 centres, and glucose was delivered by gravity infusion in the remaining 2 centres.

(iv) *duration of infusion:* Nine different ranges for accepted infusion time were in use in the 12 centres, varying from 20-40 seconds to 2-6 minutes. Four centres stipulated an infusion time of 2-4 minutes.

Sampling:

(i) *designation of time zero*: 1 centre designated the beginning, 1 the middle, and 10 the end of the infusion as time zero.

(ii) *sampling times*: Figure 6.11 shows the potential range of time lapse between the start of the glucose infusion and the '+1 minute' sample. This ranged from 1 to 7 minutes between centres. For example, the topmost 2 centres in the figure consider time = 0 as the end of the glucose infusion, and allow a time-range of 2-4 minutes for the infusion. In consequence, the "+1 minute" sample could represent a time point anywhere between 3-5 minutes from the start of the infusion, as shown by the solid bar.

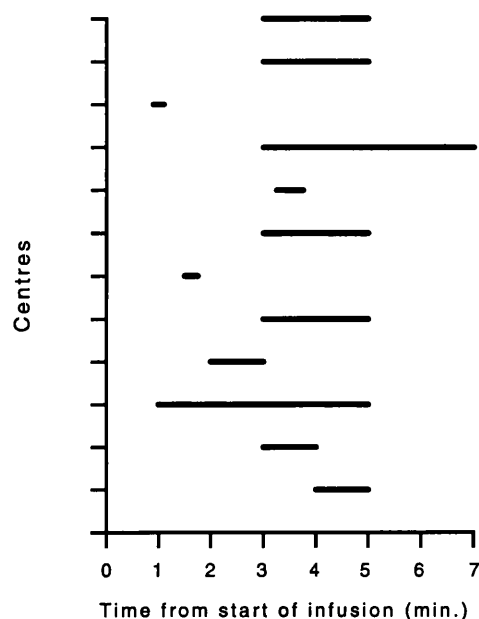


Figure 6.11 The potential variation in timing of the "+1 minute" sample, with reference to the start of the glucose infusion.

The number of samples taken over the first 10 minutes, excluding baseline samples, ranged from 4 to 7.

(iii) *cannulae*: A single cannula was

inserted and used for both glucose infusion and sampling in 8/12 centres; the remainder used 2 cannulae.

(iv) *arterialization*: Only 1 centre attempted to 'arterialize' the venous blood by warming the hand using a heated pad.

Glucose assay:

Glucose was estimated on plasma or serum samples in 7/11 centres providing this information. The remainder measured whole blood glucose.

Insulin assay:

Insulin was measured by radioimmunoassay, using a variety of commercial kits and customized assays. All centres used some form of quality assurance for their

assay. This was both external and internal in 10 centres and internal only in 2. The range of insulin concentrations covered by the quality assurance material is shown in Table 6.16.

Table 6.16 The insulin concentrations of quality assurance material used by each centre

Centre	Lowest QC material (mU/l)	Highest QC material (mU/l)
1.	16	95
2.	9.3	30.8
3.	6.1	41
4.	12.5	43.5
5.	8	136
6.	9	87.5
7.	4	75
8.	14.2	32.4
9.	40	40
10.	8	72
11.	29	78
12.	Not given	

Discussion

The IVGTT was first used in 1923 by Jorgensen and Plum, (Jorgensen *et al.* 1923), and developed by Lundbaek who argued in 1962 that it was the test of choice for the diagnosis of diabetes, since variable gastro-intestinal absorption complicates interpretation of the oral glucose tolerance test (OGTT) (Lundbaek, 1972). It has however been used primarily as a research tool, which may explain why it has never been standardized.

Our survey has confirmed that methodology for performance of the IVGTT varies very widely, so that data cannot usefully be compared or pooled until the test has been standardized. As a result an expert group appointed by ICARUS has developed a consensus protocol (Table 6.17). Before consensus could be reached, a number of important factors had to be considered.

Table 6.17 The ICARUS intravenous glucose tolerance test protocol

Preparation: As recommended by the NDDG for oral glucose tolerance tests; i.e. 3 days of unrestricted diet (containing at least 150g CHO) and normal physical activity (National Diabetes Data Group 1979). Unusual physical exertion should be avoided for one day prior to the test. The test should be deferred if the subject has intercurrent illness.

Fast: At least 10 hours but not more than 16 hours. Water is permitted during this period but the subject should not smoke.

Time of starting test (glucose infusion): 0730-1000

Glucose dose: 0.5g/kg up to 35g maximum

Glucose concentration infused: 25%

Infusion: Manual or pump driven syringe, timed to ensure steady rate of infusion.

Duration of infusion: 3 minutes \pm 15 seconds

Time zero: end of infusion.

Minimum samples to be collected: 2 baseline samples 5 minutes apart (the latter taken immediately prior to the glucose infusion) and +1, +3, +5, +10 minutes after the end of the infusion.

Cannula: A single forearm vein cannula may be used, but should be flushed with saline after the glucose is infused. The deadspace should be cleared before samples are drawn.

Previous diet affects insulin responses to the OGTT, and glucose tolerance deteriorates if less than 125g of carbohydrate is taken daily before the test (West, 1978). A minimum of 150g carbohydrate per day for three days is therefore proposed for the IVGTT. Physical fitness may affect insulin responses to the IVGTT, and unusual exertion should be avoided prior to the test. Both the OGTT and IVGTT show diurnal variation of glucose tolerance and insulin response (Bown *et al.* 1967; Carroll *et al.* 1973), and all tests should begin at the same time of day.

High-risk individuals, many of whom will be children, often need repeated testing. The test should therefore be as simple and acceptable as possible. Use of a single catheter to infuse glucose and withdraw samples does risk contamination of samples with glucose but this can be overcome by flushing the line carefully after the glucose is given. The test should also be as brief as possible. There is no

evidence to suggest that the second phase response is useful in the prediction of Type 1 diabetes, so that only the first phase response needs to be considered. The sum of the insulin concentrations 1 and 3 minutes after the glucose bolus has been the measure of first phase insulin release most widely used in the prediction of Type 1 diabetes, but the 0-10 minute incremental insulin area may be more reproducible, at least in normal subjects (Smith *et al.* 1988a). It is also possible that reproducibility may differ between ICA positive subjects and controls. We have therefore suggested that a minimum of 4 samples should be taken over the first 10 minutes to allow various measures of response to be evaluated.

The IVGTT has poor reproducibility (Smith *et al.* 1988a), but lower intra-individual coefficients of variation have been reported when the hand has been placed in a water bath at a temperature of 43°C for 10 minutes before and during the test (Rayman *et al.* 1990) or, more simply, under a 60°C thermostatically controlled heating pad (McCulloch *et al.* 1990). Since this would add somewhat to the complexity of the test, the value of "arterializing" venous blood and the stringency with which this should be performed needs to be confirmed.

Both the dose and the rate of glucose infusion affect the magnitude of the acute (first phase) insulin response (FPIR). A linear relationship between FPIR and glucose is observed with doses ranging from 0.5g to 20g, but higher doses do not increase the response (Lerner *et al.* 1971). Timing is also important and comparison of the response to 20g of glucose given over 0.3, 3, 6 and 12 minutes showed that peak insulin and incremental 0-9 minute insulin area fall significantly with slower rates of infusion. Responses did however reach a plateau at infusion rates over 7g/min (Chen *et al.* 1976). Rate of infusion may be more important than dose, since doses of 5, 10, and 20gm glucose produced similar insulin responses when given at the same slow rate of infusion (Chen *et al.* 1976). The conclusion of Chen and Porte was that: "For practical purposes, an IVGTT might best be performed using a maximal rate ($\geq 7\text{g/min}$), and a maximal dose ($\geq 20\text{g}$)".

In our survey most centres used a maximum dose well over 20g, and a maximum

dose of 35 gm was agreed, with a 3 minute infusion period, since this procedure will produce maximal stimulation with minimal deviation from established protocols. One advantage of the slower rate of glucose infusion is that it is more comfortable for the recipient, but rapid rates may generate a more reproducible insulin response, and this possibility is currently under investigation. One of the most striking differences we revealed was the differing designation of "time zero" and standardization is clearly essential.

Insulin assays must also be comparable. A standard textbook of clinical chemistry states that "The concentration of analyte in different control materials should be in the normal and abnormal ranges, corresponding to the concentrations that are critical in the medical interpretation of the test results" (Tietz, 1987). Precise determination of the first phase response requires accurate measurement of both basal and stimulated levels. The assay must therefore be precise at physiological fasting insulin concentrations. Since changes in first phase response at the lower end of the range are of greatest clinical interest, insulin assays should perform well at the lower end of the stimulated range. Wide variations were revealed by our survey in the lowest quality control material used, and we would suggest that, as a minimum, one control should be within the normal range for fasting insulin (around 5-10 mU/L) and another in the range of low stimulated responses (around 20-30 mU/l). Each assay should also be monitored by an external proficiency program; such programs are essential for quality maintenance.

There have been remarkably few attempts to establish a scientific basis for optimum performance of the IVGTT, even in normal subjects. The protocol we have proposed is essentially a pragmatic, rather than scientific, resolution of existing differences between centres, and focuses on the first phase response, since this appears to be of greatest value for the prediction of Type 1 diabetes. It will be necessary to evaluate this protocol, in normal and high risk subjects, both in terms of its reproducibility and its ability to stimulate a maximal response. A large pool of data from the healthy population will also be needed for comparison with data derived from high risk individuals. There are a number of unresolved

issues. Should basal insulin concentrations be subtracted when calculating insulin responses? Can interpretation of insulin secretion data be improved by considering insulin sensitivity? Can the prediction of Type 1 diabetes be improved by taking account of the effects of age, pubertal status and body mass index on first phase response? Can other metabolic tests be used to complement the IVGTT? Collaboration of many centres around the world will allow rapid and full evaluation of the role of the IVGTT in the prediction of Type 1 diabetes, and will standardize measurement of the first phase insulin response for use in prospective trials of intervention.

(c) First phase insulin secretion as a predictor of Type 1 diabetes

Loss of first phase insulin secretion (usually defined as below the first centile) is claimed to be highly predictive of imminent Type 1 diabetes in subjects with detectable ICA. The largest published series comes from Denver where 1169 family members were screened for ICA and 66/71 of those with detectable ICA had an IVGTT. 7/16 subjects with response below the first centile needed insulin within 2 years. The test was found to be most useful in predicting diabetes in children; 7/10 below the first centile (1'+3' insulin < 46 mU/l) developed Type 1 diabetes within 2 years. The positive predictive value of the test could be improved by using a lower threshold (25 mU/l); 7/7 children below this threshold developed Type 1 diabetes within one year, 5/7 within one month (Chase *et al.* 1987). In another study strongly ICA positive relatives in this category developed diabetes at the rate of 0.48 per subject-year of follow-up, compared with only 0.05 per subject-year in those with first phase insulin release above this level. The mean time to development of overt diabetes from the time of the first test found to be below the first centile was 656 days with a range from 71 to 1,543 days (Vardi *et al.* 1991). All the relatives with loss of FPIR developed diabetes. The short time lapse between loss of first phase response and the need for insulin treatment implies that the beta cell mass is only slightly greater than at diagnosis. This suggests that the benefit from intervening at this stage rather than at clinical presentation will always be marginal.

(d) Intravenous glucose tolerance tests in ICA positive family members in the Bart's-Windsor and Bart's-Oxford Family Studies

Aims

The aim of this study was to investigate the prognostic significance of acute insulin response to intravenous glucose in high risk subjects identified from the Bart's-Windsor and Bart's-Oxford family studies.

This involved

- (i) continuing and extending the cross-sectional study started by Dr Anne Tarn
- (ii) undertaking longitudinal studies to determine whether the predictive value of the test could be improved by repeated testing.

Subjects and methods

The subjects were all first degree relatives recruited to the Bart's-Windsor or Bart's-Oxford Family studies as described above. Forty-three subjects had at least one IVGTT and their clinical details are summarized in Table 6.18. Six of these family members have subsequently developed Type 1 diabetes. Another was found to have impaired glucose tolerance 26 weeks after the test (2 hour blood glucose 7.1 mmol/l), diabetes was diagnosed 34 months later (random glucose 21.3 mmol/l) but she remains non-insulin requiring after a further 12 months. 20 individuals have had multiple tests (range 2 - 5), including 2 who became diabetic. No subjects were taking any medication other than the combined oral contraceptive pill.

Table 6.18 Subject characteristics

Sex:	21 female:22 male
Age at first test:	median 22.3 yr (range 10.8 - 53 yr)
Peak ICA (JDFu)	
4-19	17
20-79	9
≥ 80	17
Follow-up from first test	median 4.2 yr (range 0.5 - 7.3 yr)

Methods: The majority of the tests were undertaken before the ICARUS standardized protocol was developed. They were therefore performed according to the original protocol used in the family studies. Studies were performed between 0730 and 1000 after an overnight fast. 18G cannulae were introduced into antecubital fossa veins in each arm after local infiltration with 1% lignocaine. Basal samples were taken after a 20 minute rest period. 0.5g/kg body weight (maximum 25g) 50% dextrose was infused at 20g/min via a Sage syringe pump (model 351, Arnold R Horwell Ltd, London). Zero time was taken as the midpoint of the infusion and samples were taken for estimation of blood glucose and insulin at -15,-5,1,3,5,7,10,15,20,30,45 and 60 minutes. Since 1990, tests have been shortened and sampling was stopped at 10 minutes in tests performed after that time. Details of insulin and glucose assays are given in the appendix.

Statistical analysis: The acute (first phase) insulin response to intravenous glucose was calculated as the 0-10 min area under the curve using trapezoidal integration. Insulin responses in different groups were compared by the Mann-Whitney test. Survival curves were compared by Logrank test.

Results

The first phase insulin responses in the initial IVGTT of each subject are shown in Figure 6.12. The median response was lower in those who developed IDDM than in those who did not (140.2 (36.5 - 266.3) vs 445 (67.9-1540), $p = 0.0009$) but there was overlap between the groups. Three whose response was essentially flat with initial FPIR less than $85 \text{ mU.l}^{-1} \cdot 10 \text{ min}^{-1}$ (the first centile derived from 50 normal controls previously studied in our laboratory) remain non-diabetic 2.0, 5.7 and 6.3 years later, a fourth has developed non-insulin requiring diabetes as described above.

Survival curves according to the quartile of the FPIR are shown in Figure 6.13. The cumulative risk of IDDM within 7 years was 52% in those with the lowest response, 12% in those whose response was between the the 25th and 50th percentile for

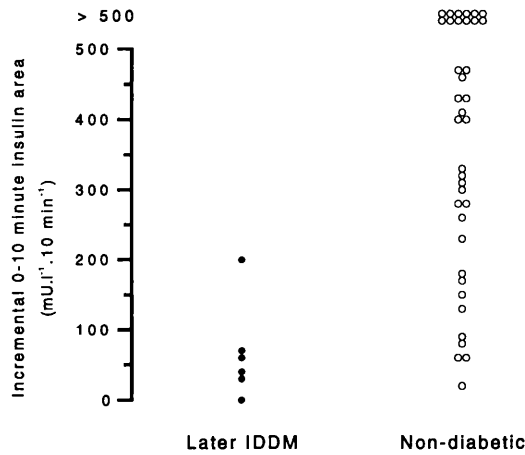


Figure 6.12 First phase insulin response in the initial test

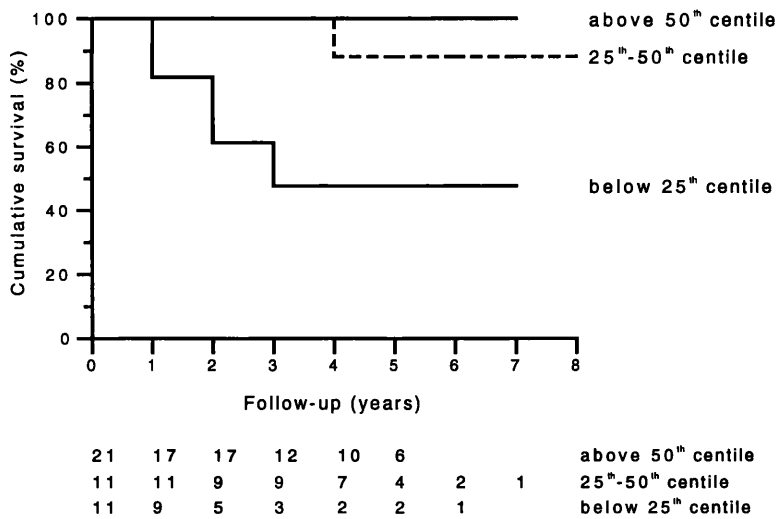


Figure 6.13 Cumulative risk of Type 1 diabetes after 7 years by first phase insulin response

the group. None of those whose response was above the median developed IDDM (chi-square = 14.09, 2df, $p < 0.001$; chi-square = 4.28, $p < 0.05$ between lowest 2 groups).

The results of serial tests in the 2 individuals who developed IDDM are shown in figure 6.14. The first phase response was persistently low from 2 years to 8 months prior to starting insulin in one case, and fell progressively over 4 years in the other. The FPIR in the 18 subjects who have remained non-diabetic in figure 6.15. No clear pattern is discernable. One of the 2 family members whose FPIR was below the first centile on the initial test has had 5 tests over 4.4 years in which the response has been similar; the other had a similar response on the second test but the FPIR rose to 120-150 $\text{mU.l}^{-1} \cdot 10 \text{ min}^{-1}$ on 2 subsequent tests.

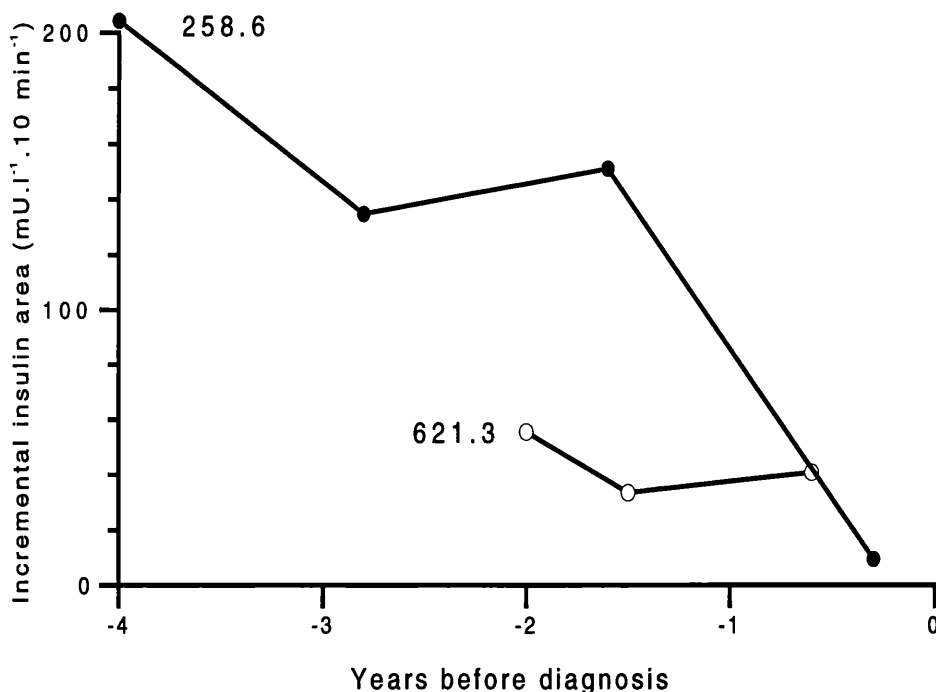


Figure 6.14 The first phase insulin response in serial IVGTTs in 2 family members who developed Type 1 diabetes during the study

Discussion

The findings of this study are similar to those of other groups (Chase *et al.* 1987; Vardi *et al.* 1991) that loss of first phase insulin response is associated with high risk of development of diabetes in the near future. The median time to diagnosis in those with FPIR less than the first centile at any time during follow-up was 1.1 years, confirming that this change is probably a late occurrence in the prodrome of

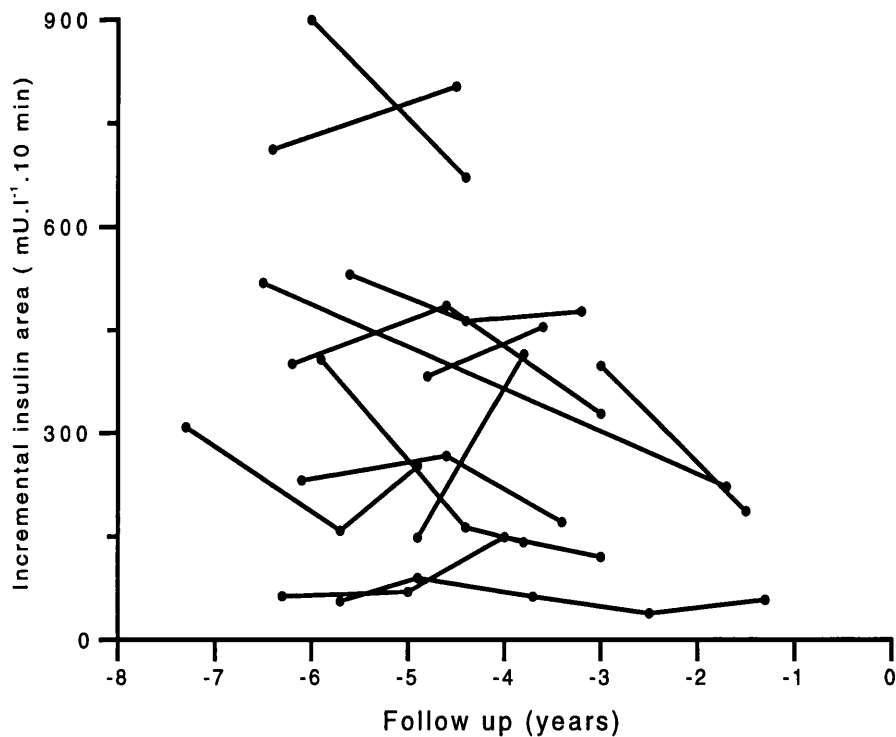


Figure 6.15 The first phase insulin response in serial IVGTTs in ICA positive family members who have not developed Type 1 diabetes

Type 1 diabetes. There is a highly significant statistical difference between the survival experience of those with low, moderate and high insulin release.

Even within this relatively small group of family members however, we have found exceptions. Individuals with low first phase response on initial testing have not developed diabetes within more than 5 years follow-up, and there was considerable overlap between those who became diabetic and those who did not. Seven family members who had FPIR below the first centile on their initial test have been followed up for more than 4 years. Four have progressed to Type 1 diabetes, 1 has non-insulin requiring diabetes and 2 remain non-diabetic. This gives a positive predictive value for diabetes of only 70% and for insulin requirement of 57% over this time period. Other investigators have reported higher levels, up to 90% within 3 years (Vardi *et al.* 1991), but in all the studies (including our own) the numbers are small and the 95% confidence intervals wide. In the largest study from Denver, the 95% confidence intervals for the development of Type 1 diabetes within 24 months were 31-89%.

There are other possible explanations for the lower positive predictive value in our study population. Six of 43 subjects in our study developed diabetes, compared with 18 of 35 in that reported by Vardi *et al.* (Vardi *et al.* 1991). We have included individuals with all levels of ICA detectable in our assay, that is greater than 4 JDF units. Others have included only family members with ICA \geq 40 JDF units whose overall risk of disease is considerably higher. The observed difference in predictive value may merely be a reflection of Bayes' theorem whereby the positive predictive value of a test depends on the overall prevalence of disease in the population in which it is applied. Further support for this interpretation comes from a study in which both ICA positive and ICA negative family members were tested. Eighteen out of 150 individuals (12%) had an FPIR below the first centile and only 2 of these became diabetic (Violettes *et al.* 1988). This difference is an important consideration if results derived from testing only individuals with high levels of ICA are to be extrapolated to identify high risk subjects in other populations, using different ICA thresholds. It has particular relevance in the design of intervention studies (see below). Large groups of individuals with comparable pretest risk (based on immune markers for example) need to be tested before we can be certain of the prognostic significance of first phase insulin responses. Use of a standardized protocol for the IVGTT and collaboration within bodies such as ICARUS should facilitate this.

6.3.4.4 Potential early metabolic markers

(a) Alternative secretagogues

Insulin secretion can also be stimulated by a number of other secretagogues and in newly diagnosed diabetes a 'hierarchy' of response has been described; IV arginine > IV glucagon > oral glucose > IV tolbutamide > IV glucose (Ganda *et al.* 1984). The slope of glucose potentiation, making use of a combination of secretagogues (arginine and glucose), attempts to improve on the predictive power of either test alone. It has been reported to provide the most sensitive measure of loss of beta cell function in animal models (Ward *et al.* 1988) and in humans (Johnston *et al.* 1987) but it is cumbersome to perform and has not been much

used outside the centre where it was originally described.

(b) Secretion of other insulin-molecules Specific assays for proinsulin and its derivatives have recently been developed and altered patterns of basal and stimulated secretion of proinsulin have been found in high risks groups (Heaton *et al.* 1988). This includes the Bart's-Oxford and Bart's-Windsor family study in which specific two-site immuno-radiometric assays were used to measure insulin, intact proinsulin and 32-33 split proinsulin in samples from intravenous glucose tolerance tests performed in 20 ICA positive family members and 20 age-sex matched controls. Fifteen of the family members had normal glucose tolerance, 2 had impaired glucose tolerance and 1 was diagnosed diabetic on the day of testing. Insulin responses, measured as peak/basal ratios were lower in subjects with IGT or diabetes but higher in unaffected ICA positive subjects compared with matched controls. Unaffected ICA positive family members also had higher basal and peak 32-33 split proinsulin than their controls but lower intact basal proinsulin levels. We concluded that release of insulin falls only in the final stages of the diabetes prodrome but that patterns of release of proinsulin and its derivatives may be altered in the earlier stages of beta cell attack (Clark *et al.* 1989).

(c) Patterns of basal insulin secretion in islet cell antibody positive non-diabetic family members:

Introduction

Prospective study of individuals developing Type 1 (insulin-dependent) diabetes mellitus has identified a long prodrome characterized by the appearance of islet cell antibodies (ICA) and other immunological abnormalities (Bosi *et al.* 1990; Colman *et al.* 1988).

Recognized metabolic changes include diminished insulin responses to intravenous glucose, impaired glucose tolerance and subclinical hyperglycaemia, all of which may precede the onset of clinical symptoms by months or years (Srikanta *et al.* 1983a; Rosenbloom *et al.* 1982; Tarn *et al.* 1987). Even so, they reflect a relatively late stage in the disease process, and there is a need for earlier and more

sensitive markers to identify pre-diabetic individuals at a time when more functioning beta cells might potentially be preserved.

In healthy individuals blood glucose is tightly regulated, a process that requires precise control of insulin secretion. Basal insulin levels show a regular oscillation with a period of about 13 minutes with synchronous oscillation of C-peptide indicating pulsatile insulin secretion (Lang *et al.* 1990; Goodner *et al.* 1977). These short-term oscillations occur within the longer cycles (approximately 40 minutes) of a feedback loop and may improve homeostasis by increasing sensitivity to changes in glucose. Glucose levels are more stable in normal subjects with regular insulin oscillations than in those in whom oscillatory patterns have been altered by truncal vagotomy or removal of the head of the pancreas (Matthews *et al.* 1983a). Alterations in pulsatile insulin secretion have been reported consistently in states of abnormal glucose tolerance in man and other animals (Matthews *et al.* 1981; Goodner *et al.* 1989). These include patients with non-insulin dependent diabetes and their first degree relatives with even minimal impairment of glucose tolerance (O'Rahilly *et al.* 1988). In these subjects the normal relationship between basal glucose and insulin peaks is disturbed and glucose levels vary more widely (Lang *et al.* 1990; Matthews *et al.* 1983a). Data from several research groups also show that pulsatile delivery of insulin improves the efficiency of its action (Matthews *et al.* 1983b, Paolisso *et al.* 1987).

We hypothesized that oscillatory insulin secretion might be affected at an early stage of the Type 1 diabetes prodrome, and therefore investigated a population of ICA positive family members to establish the presence and prognostic significance of such an abnormality. The study was carried out in collaboration with Dr David Matthews.

Subjects and methods

Nine individuals with peak ICA greater than 4 JDF units, 1 parent and 8 siblings of children with Type 1 diabetes, and 9 ICA negative control subjects with no family history of diabetes were studied. Controls were matched for age, sex and weight

(see Table 6.19). All individuals in both groups had normal fasting blood glucose levels. Five of the ICA positive group had oral glucose tolerance tests in the year preceding the study; four were normal and one showed impaired glucose tolerance (WHO criteria), with a two hour whole blood glucose of 6.7 mmol/l (subject 7).

All the ICA positive individuals were participants in the Bart's-Windsor or Bart's-Oxford family studies. At the time this study was undertaken a total of 16 subjects under follow-up in the family studies fulfilled the entry criteria of age >16 years, with ICA >4 JDF units on at least three occasions during the previous 5 years. The median duration of known ICA positivity was 7.8 yrs (range 2.1-11). Two ICA positive individuals and their controls were taking low dose combined oral contraceptives; none were taking other medication. Informed consent was obtained from all subjects and the study was approved by the City and Hackney District Health Authority Ethical Committee.

Methods: All studies were performed at 9 am. Subjects attended the hospital after an overnight fast, having previously been on a normal diet with no carbohydrate restriction. A 16 gauge double-lumen catheter was introduced into a forearm vein under local anaesthesia for continuous blood sampling. Heparin was delivered to the tip via the outer lumen giving a final sample concentration of 80 units/ml. The hand was warmed to arterialize the sampled blood. Venous samples were taken continuously, collected into 1 minute aliquots, for 110-120 minutes by peristaltic pump at a rate of 1.3 ml/minute, following a 30 minute rest after cannulation. Samples were centrifuged at 4° C within 10 minutes and stored at -20° C immediately after separation.

After the period of continuous sampling, 25g dextrose as a 50% solution was given into a second cannula at a rate of 20g/minute using a syringe pump (Sage syringe pump, model 351, Arnold R. Horwell Ltd, London, England). Samples were taken at baseline and 1, 3, 5, 7, 10, 15, 20, 30, 45 and 60 minutes after the midpoint of the glucose infusion.

Assays: Details of insulin, glucose and ICA assays are given in the appendix.

Statistical Methods:

Time series analysis:

Three minute moving averages: Individual data sets were averaged using a 3-point moving average on the duplicates ($n = 6$ for each point estimate) which reduces the size of fluctuations of short duration due to assay and experimental noise and increases the precision of the estimate by $\sqrt{6}$.

Stationarisation: Data were stationarised by spline detrending for autocorrelation and by differencing about the mean (which is equivalent to examining the first derivative of the data) for Fourier transform analysis. These methods eliminate long term trends before undertaking time series analysis (TSA).

Two types of TSA were used:

(i) Fourier transformation.

Fourier transforms dissect oscillatory data into all their attributes of frequency (Matthews, 1988b). Any complex signal can thus be described in terms of its dominant and sub-dominant harmonics. Fourier transforms have the advantage of providing a complete description of oscillations and can be represented graphically as a power spectrum (i.e. power at all the relevant frequencies or periods). The major peak in spectral power corresponds to the dominant frequency of oscillation. This method of analysis, in contrast to autocorrelation, will detect irregular as well as regular pulsation. Fourier transform analyses were pooled for ICA positive relatives and controls and a value for the mean and standard error of the oscillatory power was obtained at each frequency. These values can be regarded as normally distributed and their variance can be used to assess the standard error of the mean. Significance between peaks can thus be represented by error bars or formally assessed by Student's t-test.

(ii) Autocorrelation.

Autocorrelation was used to identify significant regular oscillation in plasma insulin

levels. Correlation coefficients were calculated between the original plot of plasma insulin against time and sequential 'copies' of the data generated by moving the original data by increments of one minute. The initial correlation is, by definition, +1 and the correlation coefficient (r) then falls to reach a nadir when the data are 180 degrees out of phase. The correlation coefficients were plotted against time delay (lag time) to produce correlograms (Matthews, 1988b). The period of oscillation is defined as the lag time to the first significant maxima of the r -value following an initial trough. Autocorrelograms were pooled by Z-transformation of the r -values (which renders them normally distributed). The significance was assessed from Fisher's values. (Pearson *et al.* 1958; Matthews, 1988b).

Amplitude of oscillation: The mean total amplitude of oscillation for each data array was calculated as the root-mean square of the detrended data set $\times 2\sqrt{2}$ (Matthews *et al.* 1983).

Statistical analysis: Standard deviation has been used as a measure of dispersion in anthropometric data; otherwise standard errors are quoted. Comparisons between groups were made using parametric methods (unpaired t-test).

The acute (first phase) and late (second phase) insulin responses to the IVGTT were calculated as the incremental 0-10 minute and 10-60 minute areas under the curve using trapezoidal integration. The responses were non-normal in distribution and standard errors were therefore derived after log transformation. The glucose disposal rate (Kg) was expressed as the slope of the semi-logarithmic decline of blood glucose over the 10 to 30 minutes following glucose infusion (Lundbaek, 1972). Homeostasis model assessment (HOMA) was used to estimate insulin resistance and beta cell function from fasting plasma glucose and insulin concentrations (Matthews *et al.* 1985).

Results

Fasting glucose and insulin levels: Fasting blood glucose was similar in ICA positive subjects and controls (4.0 ± 0.14 vs. 4.0 ± 0.11 mmol/L, $p = 0.8$), as was

fasting plasma insulin (5.4 ± 0.52 vs. 6.4 ± 0.6 mU/L, $p = 0.25$).

Acute insulin responses: First phase incremental insulin areas after glucose did not differ between ICA positive and control groups (243 (198 - 299) vs. 329 (285 - 380)) mU/L/10 min respectively, $p = 0.25$). All were above the first centile compared with a group of 50 non-diabetic ICA negative controls previously studied in our laboratory.

Late insulin response, glucose disposal and estimated beta cell function and insulin resistance: There were no differences in mean late insulin responses (524 (428-641) vs. 752 (640-883) mU/L/50 min, $p=0.19$), glucose disposal rates (1.53 ± 0.14 vs 1.53 ± 0.18 %/minute, $p=0.95$) or homeostasis model assessment derived estimates of beta cell function (121 ± 8 vs $130 \pm 6\%$, $p=0.4$) or insulin resistance (81 ± 9 vs. $67 \pm 6\%$, $p=0.22$) between the ICA positive and control group

Table 6.19 Clinical characteristics, fasting glucose and insulin and acute insulin response in nine ICA positive first-degree relatives of children with Type 1 diabetes and nine control subjects

	Age	Sex	Body mass index (kg/m ²)	Peak ICA (JDF units)	Fasting blood glucose (mmol/L)	Fasting plasma insulin (mU/l)	1st phase insulin (incremental 0-10 min area: mU.l ⁻¹ .10 min ⁻¹)	2nd phase insulin (incremental 10-60 minute area: mU.l ⁻¹ .50 min ⁻¹)	Glucose disposal rate (%/min)	HOMA estimate β -cell function (%)	HOMA estimate insulin resistance Ψ
ICA POSITIVE											
1.	44	M	24	>80	3.6	3.1	89	199	0.98	105	134
2.	26	F	20	>80	3.9	4.8	511	1388	1.56	116	85
3.	35	M	20	80	3.2	4.5	196	353	1.56	169	97
4.	19	F	21	52	4.1	7.5	560	*	*	139	54
5.	24	M	24	30	3.9	5.5	259	608	1.54	127	74
6.	39	F	23	16	4.3	6.3	429	739	2.15	113	62
7.	24	F	24	15	4.6	7.8	156	650	1.0	113	49
8.	25	M	23	15	4.2	4.0	213	448	1.94	89	98
9.	20	M	25	7	3.9	5.1	163	440	1.51	120	79
	28.4±8.8 [*]		22.7±1.9 [*]		4.0±0.14	5.4±0.52	243 (198-229)	524 (428-641)	1.53±0.14	121±8	81±9
CONTROLS											
1.	43	M	25	*	4.6	9.9	185	750	1.02	133	39
2.	23	F	19	*	3.9	8.6	374	1817	1.09	168	47
3.	33	M	20	*	3.6	4.5	349	885	1.6	132	93
4.	21	F	22	*	4.0	6.5	481	*	*	133	62
5.	24	M	24	*	3.8	5.4	351	633	2.06	132	76
6.	37	F	22	*	4.5	5.6	317	680	0.98	95	69
7.	21	F	21	*	4.0	6.1	155	620	1.58	128	66
8.	24	M	27	*	3.9	6	623	625	2.43	134	68
9.	20	M	26	*	3.8	4.6	364	504	1.5	119	89
	27.3±8.3		22.9±2.8		4.0±0.3	6.4±1.8	329 (285-380)	752 (640-883)	1.53±0.18	130±6	67±6

^{*} Mean ± standard deviation, remainder expressed as mean ± standard error
HOMA, homeostasis model assessment

Total oscillatory activity:

Figure 6.16 shows the pooled Fourier transformation analyses for ICA positive subjects and controls. In both groups the dominant spectral power was seen at periods between 12 and 15 minutes with no difference between the peak spectral power in the two groups (mean absolute spectral power 6.0 ± 1.0 vs. 8.5 ± 1.7 for ICA positive and control groups respectively).

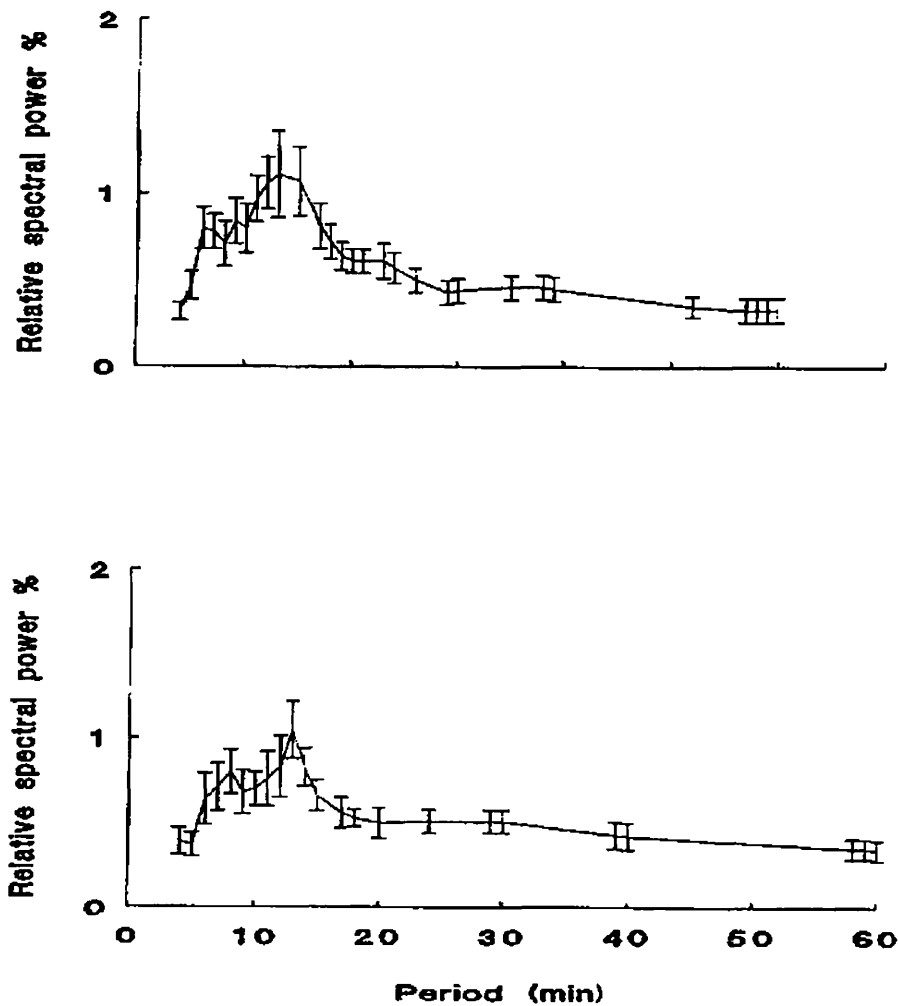


Figure 6.16 Pooled power spectra of Fourier transforms of basal insulin secretion in controls (upper panel) and ICA positive subjects (lower panel). Spectral power in absolute units. Bar \pm S.E.M.

Regular oscillatory activity:

Controls:

Pooling of autocorrelation data showed a significant peak of regular oscillation at 13 minutes ($p < 0.0001$). The correlation coefficient fell from +1 to a nadir below zero after a lag-time of 6 minutes, rising again to a peak above the level of significance after 13 minutes (Fig 2). Significant positive peaks of autocorrelation at 12 to 14 minutes following an earlier nadir were detected in 6/9 controls on individual correlograms (subjects 2, 4, 5, 6, 7 and 8). (Figure 6.18)

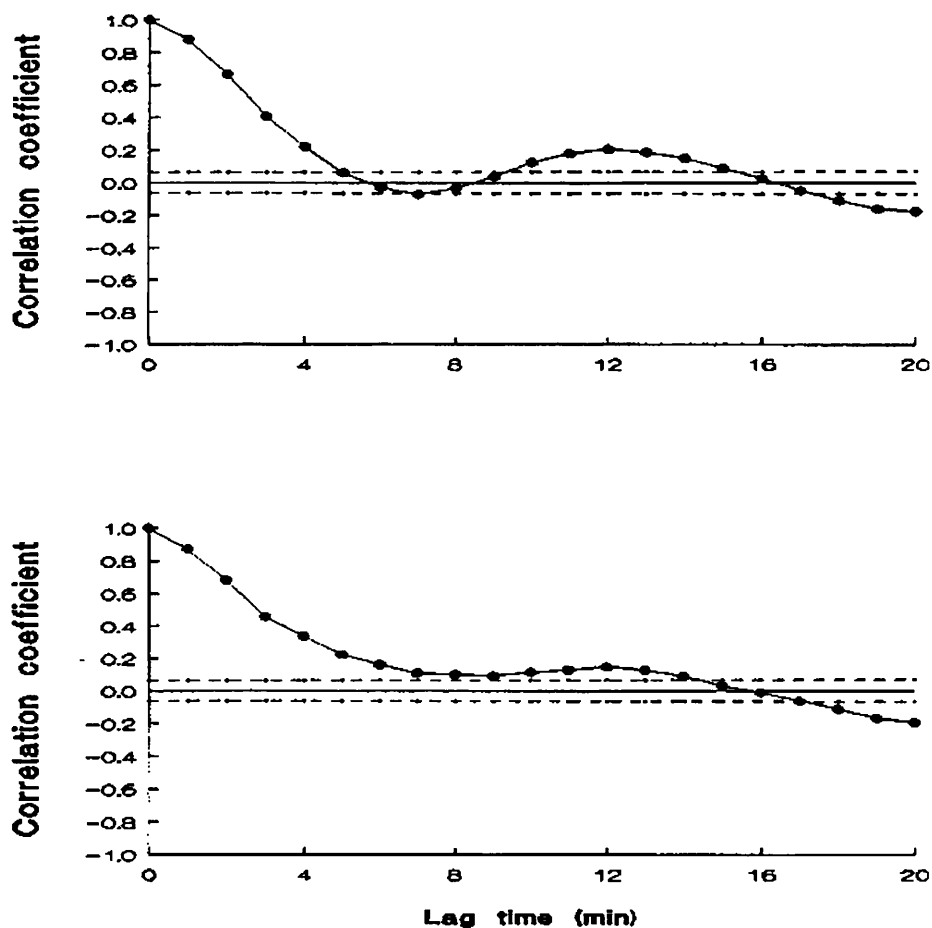


Figure 6.17 Pooled autocorrelograms of basal insulin secretion from control (upper panel) and ICA positive subjects (lower panel). Dotted line at $p=0.051$
Regular oscillation is demonstrated in controls by the trough below the significance limit followed by a positive peak above the significance limit ($p < 0.0001$ after Fisher's Z-transformation) in correlation coefficient.

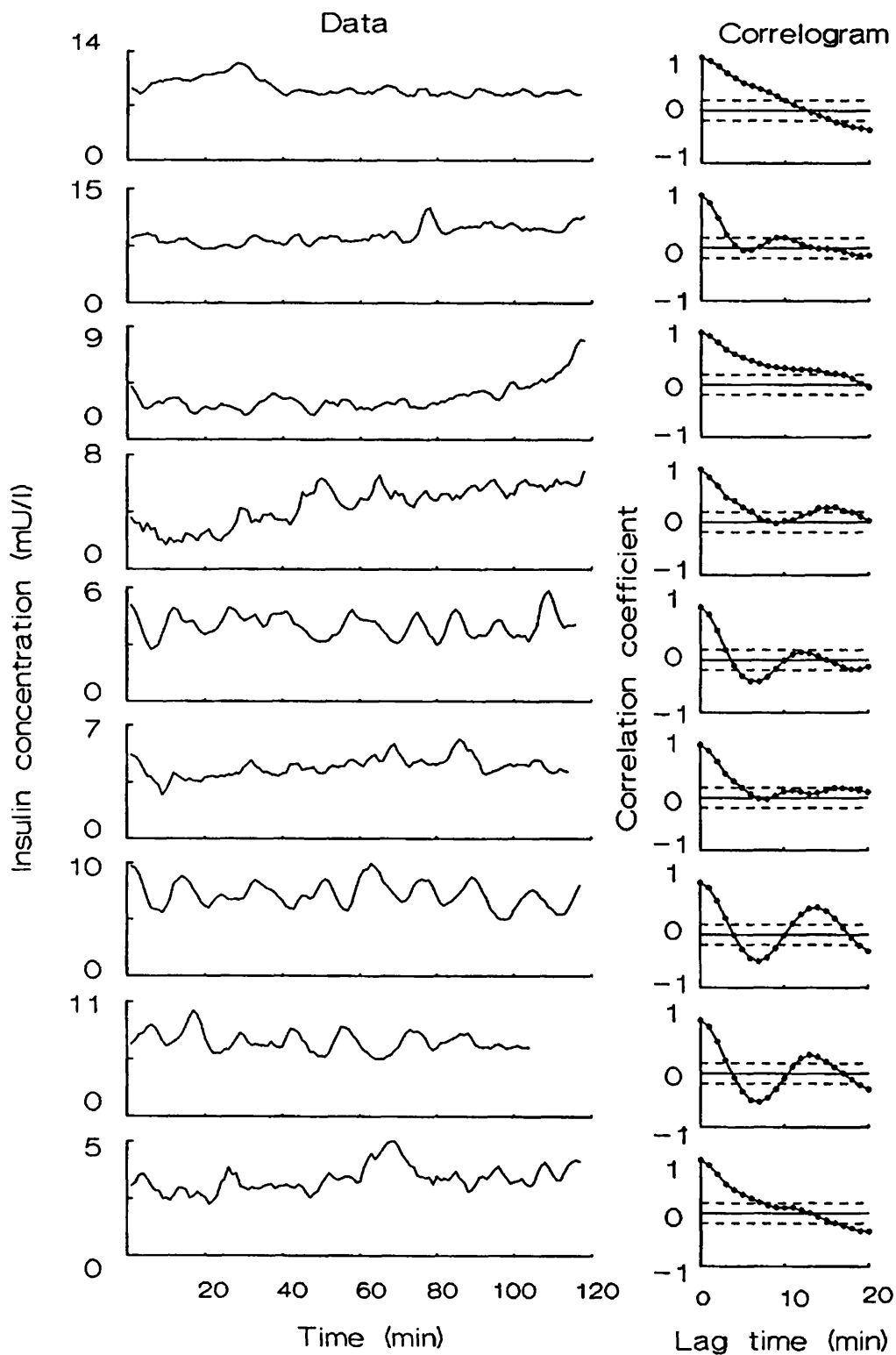


Figure 6.18 Three-minute moving average of plasma insulin (left panels) and individual autocorrelograms (right panels) in control positive subjects 1-9 (from top down)

ICA positive subjects:

There was no evidence of regular oscillation when autocorrelation data from ICA positive subjects were pooled. The correlation coefficient fell gradually, with no clear nadir below the level of significance or significant positive peak. (Fig. 2). 7/9 ICA positive subjects showed no significant regular oscillatory activity on individual correlograms (subjects 1, 2, 4, 5, 6, 7 and 9). (Figure 6.19)

Amplitude of oscillation: The mean amplitude of oscillation was similar in ICA positive subjects and controls (1.4 ± 0.2 vs. 1.1 ± 0.2 mU/L, $p=0.4$).

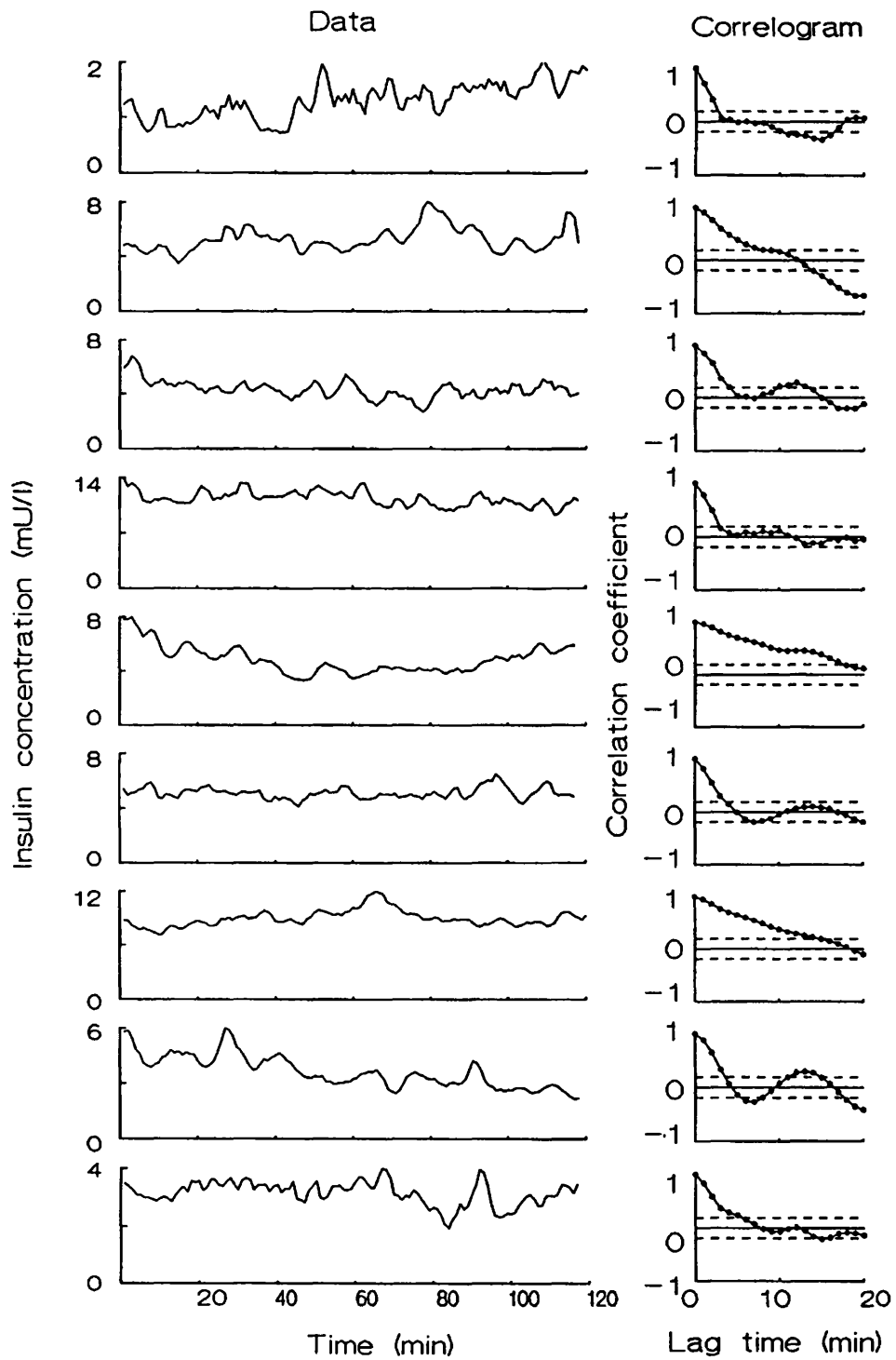


Figure 6.19 Three-minute moving average of plasma insulin (left panels) and individual autocorrelograms (right panels) in ICA positive subjects 1-9 (from top down)

Discussion

We have confirmed that regular oscillations of basal plasma insulin with a period of about 13 minutes can be demonstrated in healthy subjects (Lang *et al.* 1990; Hansen *et al.* 1982), and have shown that this pattern is generally altered in ICA positive individuals. These retained dominant oscillatory activity with a period of 12-13 minutes, as shown by Fourier transformation, but autocorrelation showed loss of regular pulsation, indicating that in this group secretion is either completely irregular or composed of short, intermittent periods of regular pulsation. We found no differences in the amplitude of oscillation in the two groups. This is in contrast with the observation that the amplitude of oscillation is reduced in the streptozocin-treated baboon, although this did not occur until the fasting glucose had begun to rise (Goodner *et al.* 1989), whereas in our study all subjects had a fasting glucose below 4.6 mmol/l.

The oscillatory patterns that we describe are derived from pooled data from carefully chosen groups, which amplifies the relatively subtle abnormalities and it is important to stress that these findings are from pooled data, and have no time-series characteristics which are consistently discernable in individuals. The data do show that *as a group* the ICA positive individuals have abnormal insulin secretion qualitatively different from control subjects. Since the ICA positive subjects are all under long-term prospective review, this should in time allow the prognostic utility of these patterns of secretion to be assessed. Abnormalities of basal insulin secretion might also be present in ICA negative relatives, and further studies would be need to exclude this possibility.

The earliest metabolic abnormality preceding the onset of Type 1 diabetes described hitherto has been impairment of the first phase insulin response to intravenous glucose (Srikanta *et al.* 1983a). This abnormality is however of confirmed predictive value only when the response has fallen below the first centile, corresponding to an advanced stage of beta cell destruction. Its predictive value is also limited by the marked variability of response within the normal range (Smith *et al.* 1988a) and the effect of physiological changes such as puberty (Smith

et al. 1988c). Attenuation of the first phase insulin response before the onset of Type 1 diabetes cannot simply be due to beta cell loss, since insulin responses to secretagogues other than glucose persist, implying an additional 'functional' beta cell deficit in glucose stimulated insulin release (Ganda *et al.* 1984). Furthermore, C-peptide secretion rises during the remission or 'honeymoon' period following diagnosis, and there is currently no evidence that this is due to beta cell regeneration (Block *et al.* 1973). These observations suggest that derangement of the mechanisms that control secretion, whether altered beta cell recognition of the glucose stimulus, or an abnormal response to it, may be an important component of the early metabolic disorder.

Pulsatile secretion of insulin may be regulated by an intrinsic pancreatic pacemaker modulated by extra-pancreatic factors (Matthews *et al.* 1983a; Lefebvre *et al.* 1984). This is influenced by levels of both insulin and glucose and can be altered by infusion of either (Matthews *et al.* 1983a). These factors cannot have been of importance in our study since basal levels of insulin and glucose were similar in both groups. Periodic insulin secretion requires coordination of the output from individual islets by a neural or paracrine network (Matthews *et al.* 1987). In Type 1 diabetes the pattern of autoimmune attack appears to be random, and at the time of diagnosis normal islets are interspersed with islets in which beta cells are scanty or absent (Foulis *et al.* 1984). It is as yet unclear whether loss of regular oscillation reflects actual disruption of neural or paracrine pathways within the pancreas, developing in parallel with beta cell destruction, or whether the effect is due simply to cumulative beta cell loss.

Loss of regular pulsation of basal insulin secretion could have important direct effects upon glucose homeostasis. In the first place, secretion of insulin may itself be modulated by fluctuation in pancreatic arterial insulin levels. In the anaesthetized rat, continuous intraportal infusion of insulin attenuates glucose stimulated insulin release whereas pulsatile infusion enhances it (Stapelfeldt *et al.* 1984). Loss of pulsatility might therefore be a factor in the loss of the first phase insulin response to intravenous glucose in ICA positive individuals though there is,

as yet, no evidence that there is a causative link. Second, insulin action on the liver is enhanced by variation in portal insulin levels, and pulsatile delivery of exogenous insulin has a greater hypoglycaemic effect in man than continuous infusion. Pulsed delivery allows equivalent stimulation of peripheral glucose utilization and suppression of hepatic glucose output to be achieved with lower doses of insulin, in both Type 1 diabetic patients and normal subjects (Matthews *et al.* 1983a; Bratusch-Marrain *et al.* 1986; Schmitz *et al.* 1986). In other words, loss of pulsatility results in insulin resistance, and this has been a consistent finding in other situations in which loss of regular insulin pulsatility has been reported, for example obesity and old age (Lang *et al.* 1990; Hansen *et al.* 1982; Matthews *et al.* 1988a).

The practical value of loss of regular oscillation in basal insulin levels as a marker of future diabetes is limited, in part because relatively little is known of the variability of this phenomenon in the normal population. It is clear that some normal subjects do not have regular oscillation, demonstrable by autocorrelation, and that some of the ICA positive group do. Some 10-50% of normal subjects have shown no significant peaks on autocorrelation in previous studies (Lang *et al.* 1990; Matthews *et al.* 1983a; O'Rahilly *et al.* 1988), and this was the case with three of the nine normal controls in the present study. There is thus no absolute diagnostic significance to individual observations. Sequential testing in normal or high risk individuals could improve the specificity of changes in oscillatory patterns. That two of the ICA positive subjects should have regular oscillations may be a significant finding in itself - we do not yet know whether those studied will develop diabetes and longitudinal studies would clearly be important to examine this hypothesis. It may be that retained regular oscillatory activity in an ICA positive individual is a sign of normal beta cell function, indicating that early progression to diabetes is unlikely. This is the case with relatives of patients with Type 2 (non insulin dependent) diabetes (O'Rahilly *et al.* 1988), and regular oscillation has not been found in any individual who has subsequently developed diabetes.

We have shown that pulsatile insulin secretion may be deranged in ICA positive individuals with normal insulin responses to intravenous glucose and normal beta

cell function and insulin sensitivity using simple modelling techniques. This change, which may be secondary to other subtle secretory abnormalities as yet unidentified, may represent an early detectable metabolic abnormality prior to the onset of Type 1 diabetes. The altered pattern of basal insulin secretion may in turn have direct functional consequences in terms of beta cell secretion and hepatic glucose sensitivity. Loss of first phase insulin secretion represents a late metabolic abnormality associated with extensive beta cell loss. Better understanding of early metabolic abnormalities such as loss of regular oscillation of basal insulin secretion may in time allow accurate prediction and intervention earlier in the prodrome of Type 1 diabetes, at a time when more beta cells are viable.

6.4 CONCLUSION

An individual's probability of developing diabetes can be set out in a series of steps known as a decision tree. This illustrates that we are dealing with a complex and evolving body of information and that risk cannot be assigned merely on the basis of a single test. Probability is refined at each successive step, and the outcome of each 'branching' is determined by all the previous steps, so that the overall risk is built up layer by layer. The first major determinant of risk is family history, and, within our region the risk that a sibling of a child with Type 1 diabetes will also develop the disease within 5 years is 3.0%. The addition of detectable ICA (≥ 4 JDF units) raises the cumulative risk to 18%). Specificity of prediction can be increased by taking titre of ICA into account; if ICA are ≥ 20 JDF units the risk is 37% (*Section 6.3.3.2 (b)*). Alternatively specificity can be improved by the addition of other autoantibodies, either alone or in combination (*Section 6.3.3.6*) or by the addition of metabolic tests, particularly the first phase insulin response (*Section 6.3.4.3 (d)*). Figure 6.20 summarizes our findings for these markers in the Bart's-Windsor and Bart's-Oxford family studies.

Family studies have therefore made it possible to use genetic and immunological markers to identify which individuals within the family are at highest risk of developing Type 1 diabetes but our powers of prediction are subject to several limitations. Thus, while risk can be quantified to a certain extent, it is not possible

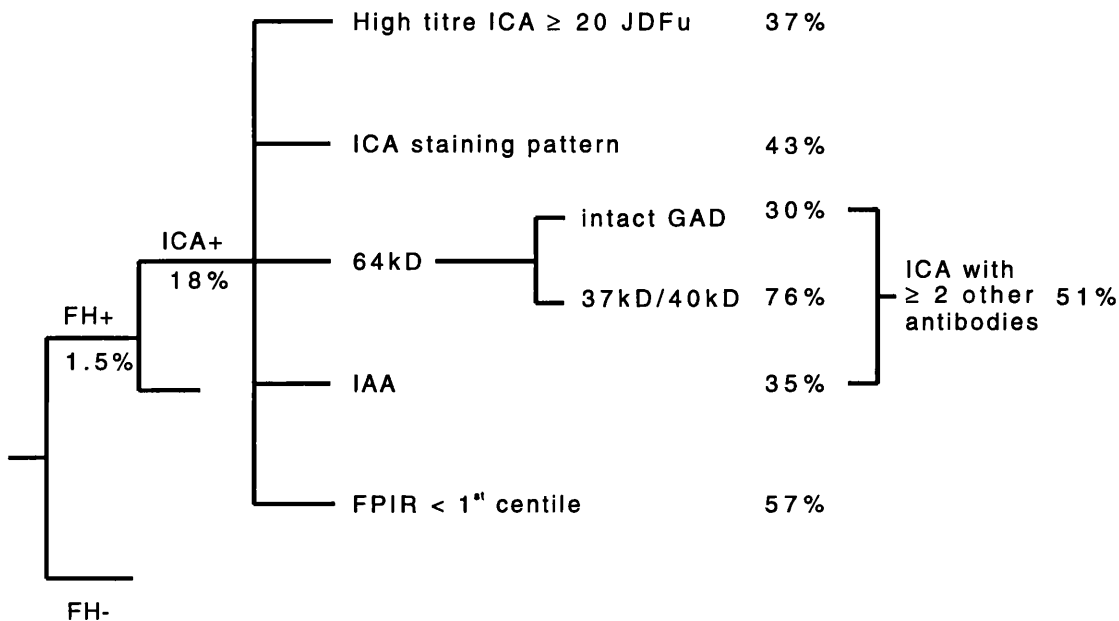


Figure 6.20 The Decision Tree illustrating the cumulative risk of Type 1 diabetes within 5 years as found in the Bart's-Oxford family study

to be certain if or when the clinical onset of Type 1 diabetes will occur, even with high titre ICA. In the context of family studies, only a minority of those who develop diabetes will be found to have very high titre ICA on first screening. For example 6/17 subjects (35%) who developed Type 1 diabetes in the Bart's Windsor Family Study had ICA > 40 on entry to the study. This means that, even in this high risk group, high titre ICA has low sensitivity as a marker of risk. The small numbers of subjects studied prospectively in any one centre mean that the confidence intervals around any risk estimate are very wide. This problem will only be resolved by

recruiting larger populations and by pooling data. Most studies have been clinic-based rather than population-based and are therefore potentially subject to sampling bias.

We know enough about the prodrome to consider trials of intervention prior to the clinical onset of Type 1 diabetes, but can consider intervention only in a small minority of those who will develop Type 1 diabetes and only in the final stages of the disease process. All that has been learnt over the last decade will have to be applied to ensure that the right agents are tested and that appropriate, properly designed studies are undertaken, but there remains a need to extend the proportion of those destined to develop Type 1 diabetes who might benefit from these interventions.

CHAPTER 7 - DESIGNING AN INTERVENTION STUDY IN FIRST DEGREE RELATIVES

7.1 INTRODUCTION

I have used data derived from the Bart's-Windsor and Bart's-Oxford family studies in the design of the European Nicotinamide Diabetes Intervention Trial (ENDIT), a proposed multicentre trial of nicotinamide in high risk subjects. I describe here the issues that need to be considered in planning such a trial. Data from the family studies have been used to estimate the necessary sample size (*Section 7.5.3*) and to devise a suitable screening strategy (*Section 7.5.4*).

7.2 RATIONALE FOR INTERVENTION STUDIES

Changes in humoral and cell-mediated immunity are detectable several years before the clinical onset of Type 1 diabetes (Gorsuch *et al.* 1981; Eisenbarth, 1986). This suggests that beta cell destruction is slow and that it may be possible to intervene during this prodrome to halt the process. In view of the evidence cited above, it would be reasonable to suppose that immunotherapy, acting at any stage of the process of beta cell destruction, might influence the progression of beta cell damage in Type 1 diabetes. Human studies of immune intervention have to date concentrated on the attempt to prolong beta cell function in recently diagnosed patients requiring insulin therapy. Two prospective randomized placebo controlled double blind studies have demonstrated that Cyclosporin A can protect beta cell function to the extent that some 25% of patients on Cyclosporin are able to control their diabetes on diet alone one year after diagnosis, as against 5 -10% of controls (Bougneres *et al.* 1988; Canadian-European Randomized Control Trial Group, 1988). Unfortunately this benefit does not persist and the high rate of complications has limited this approach to therapy. Azathioprine and prednisolone have also been shown to have a similar although less dramatic effect in recently diagnosed patients (Silverstein *et al.* 1988). In all of these studies the remission rate was highest in those with the 'least severe' disease (shorter duration of symptoms, less weight loss and higher bicarbonate levels), in whom beta cell destruction was probably less advanced. Since the great majority of beta cells appear to have

been destroyed by the time of clinical presentation, it seems reasonable to conclude that earlier intervention, at a stage when the beta cell mass is relatively intact, could produce a more lasting benefit. This was the view of an Expert Committee of the American Diabetes Association (American Diabetes Association, 1990), who concluded that "sufficient data exist to warrant intervention studies for the prevention of Type I diabetes".

Animal studies in two models with a spontaneous autoimmune type of diabetes, the BB rat and the NOD mouse, support this argument. In these animal models early treatment has been attempted with a variety of forms of immune intervention, some of which appear to confer lasting protection against the development of diabetes.

These findings, taken together with our improved ability to predict diabetes in humans, suggest that intervention trials should now be undertaken in the prediabetic period, provided these can be seen to offer a reasonable ratio of benefit to risk.

7.3 TYPES OF PREVENTION

Possible strategies for the prevention of Type 1 diabetes can be classified according to the stage of the disease process at which intervention occurs. Intervening to avoid initiation of the autoimmune process can be termed *primary prevention*, slowing or stopping an established destructive process as *secondary prevention*, and treatment given after clinical onset of Type 1 diabetes as *tertiary prevention*. The former is obviously the ultimate goal but, as discussed below, seems likely to be the last to be achieved.

7.3.1 Towards primary prevention

Aetiology: The aetiology of Type 1 diabetes involves an interaction of both genetic susceptibility and environmental factors. Siblings of children with Type 1 diabetes are 15 times more likely to develop Type 1 diabetes than children from the general population (Bingley *et al.* 1993a), suggesting a major genetic effect, yet the

concordance rate for Type 1 diabetes in monozygotic, genetically identical twins is only 30-50% (Barnett *et al.* 1981). As discussed above (*Section 4.5*), the incidence of Type 1 diabetes is rising sharply in many populations, equivalent to a doubling in 20 to 30 years in several European countries; more rapidly than could be explained by alterations in the population gene pool.

Genetics: Some 60% of the genetic susceptibility to insulin-dependent diabetes is estimated to be associated with genes in the Major Histocompatibility Complex (MHC) on the short arm of chromosome 6 (Rotter *et al.* 1984). The genetic susceptibility to Type 1 diabetes is discussed more fully in section 6.2 but, despite some enthusiasm in the late 1980's that the gene determining the amino acid at position 57 of the DQB chain might be *the* diabetes gene (Todd *et al.* 1987), it is becoming increasingly apparent that the issue cannot be reduced to such simplicity. The effect of this gene is modulated by many other genes both within and beyond the MHC genes. The amino acid at position 52 of the DQ α chain also exerts an effect (Khalil *et al.* 1990); the whole HLA haplotype affects the absolute risk associated with particular DQB1 and DQA1 types (Tienari *et al.* 1992), and the discrepancy between the concordance between monozygotic twins and HLA-identical siblings implicates non-MHC genes in determining overall genetic susceptibility. Studies in the NOD mouse have identified non-MHC genes on several chromosomes that modulate the major effect of MHC genes; for example a gene on chromosome 3 (analogous to human chromosome 1 or 4) determines frequency and severity of insulinitis and another, on chromosome 11 (analogous to chromosome 17 in humans) may influence the progression from insulinitis to overt diabetes in this animal model (Todd *et al.* 1991). It seems probable that the situation in humans will prove at least as complex and the possibility of using genetic manipulation in such a polygenic disorder is remote.

Environmental factors: There is a considerable body of indirect evidence that environmental factors do play a role in the aetiology of Type 1 diabetes but their identity remains elusive (*Section 4.6*). The environment is easy to manipulate in animal models and is affected by, for example, diet, temperature and exposure to

infection (Scott *et al.* 1988; Williams *et al.* 1990; Mordes *et al.* 1987). Certain environmental agents have been shown to produce disease in humans. Ingestion of the rat poison 'Vacor' results in an insulin-dependent form of diabetes (Prosser *et al.* 1978); maternal consumption of nitrosamine-rich foods such as smoked mutton around the time of conception is associated with an increased incidence of Type 1 diabetes in the offspring (Helgasson *et al.* 1981), and intrauterine infection with the rubella virus increases the risk of subsequent Type 1 diabetes (Ginsberg-Fellner *et al.* 1985). Our current understanding of the disease process suggests that environmental exposure is probably relevant many years before clinical presentation and that it may well be a 'hit and run' phenomenon. The subsequent autoimmune destruction could be an independent self-perpetuating process that continues long after the environmental agent has gone. It is also likely that only a minority of those in whom the process is started will develop disease. These factors all act to 'dampen' differences between patients and controls and mitigate against easy identification of the relevant factors.

Currently, the main contenders for a role as environmental determinants of Type 1 diabetes are infection or dietary factors.

(a) *Infective agents*: The rising incidence of Type 1 diabetes, a correlation between mean yearly temperature and the incidence of disease (Diabetes Epidemiology Research International Group, 1988) and the inverse relation between population density and disease found in some studies (Patterson *et al.* 1988) would all be consistent with a infective aetiology. The major problem at present is lack of a plausible candidate agent. A number of viruses can damage beta cells in vitro or in experimental animals, or have been implicated in cases of Type 1 diabetes in humans. These include mumps, coxsackie B (Yoon *et al.* 1979; Barrett-Connor, 1985), rubella (Ginsberg-Fellner *et al.* 1985), and cytomegalovirus (CMV) (Ward *et al.* 1979; Jenson *et al.* 1980; Pak *et al.* 1988). The long incubation of the disease however makes it necessary to look back over many years to early childhood, or perhaps even in utero. Viral infections also have a separate role in precipitating clinical onset of diabetes and evidence of such infections can complicate the picture. The idea that viral infection might be the initial trigger for

the autoimmune process remains theoretically attractive, particularly since vaccination is probably the simplest imaginable means of primary prevention, but no progress can be made in this direction until the responsible agent is known.

(b) Dietary factors

Diet is another factor that varies radically between countries and also changes over time. Alterations in diet have a profound effect on incidence in animal models (Scott *et al.* 1988; Daneman *et al.* 1987). These observations have motivated a search for dietary causes of Type 1 diabetes. One of these, cows' milk protein, has been the subject of considerable attention in recent years and its relevance is soon to be tested in the first primary prevention trial in man.

The cows' milk protein hypothesis: Alteration of the protein components of laboratory chow can alter the incidence of diabetes in the NOD mouse and BB rat models. Animals reared on a diet free of cow's milk for the first 3 months of life do not develop diabetes (Elliott *et al.* 1984). There is epidemiological evidence to support a role for these proteins in the aetiology of Type 1 diabetes. National *per capita* cow's milk consumption correlates with the incidence of diabetes (Dahl-Jorgansen *et al.* 1991), and the risk of Type 1 diabetes has been shown to be significantly lower in children who were exclusively breast fed for 3 months (Virtanen *et al.* 1991). Antibodies to bovine serum albumin were higher in children with newly diagnosed Type 1 diabetes than in normal children (Karjalainen *et al.* 1992) and it has been suggested that an albumin peptide containing 17 amino acids (ABBOS) may be the reactive epitope. Antibodies to this peptide cross-react with a beta cell specific surface antigen (p69) and an elegant hypothesis has been put forward whereby early exposure to bovine serum albumin, at a stage when it is able to cross the immature gut wall, triggers an immune response against the ABBOS peptide in genetically susceptible individuals. The p69 antigen is only expressed on the cell surface when it is induced by gamma-interferon during unrelated infectious events, but when these occur, the immune system is primed to attack the epitope that this antigen shares with the ABBOS protein. Delaying exposure to cow's milk until the gut is mature and therefore impermeable to large

peptides such as this means that anti-ABBOS immunity (and therefore anti-p69 immunity) should be prevented. This hypothesis is soon to be tested in a study in which genetically susceptible neonates will be randomized to receive supplementary feeds with either normal formula or with formula modified to remove cows' milk protein for the first 9 months of life (Skyler *et al.* 1993).

7.3.2 Secondary prevention

Intervention to slow or stop an established process of beta cell destruction is beginning to appear realistic and trials of a number of agents designed to prevent or delay the clinical onset of Type 1 diabetes in high risk subjects are already starting (Skyler *et al.* 1993).

Using our current understanding of the disease process, Harrison and colleagues have produced a useful theoretical classification of potential points of intervention.

Table 7.1: Categories of intervention in Type 1 diabetes (Harrison *et al.* 1990)

I. Nonspecific immunosuppression

eg Glucocorticoids, cytotoxic agents, anti-lymphocyte globulin or monoclonal antibody and total lymphoid or pancreatic irradiation.

II Nonspecific immunomodulation

eg plasma exchange, white cell transfusion, gamma-globulin, interferon- α or levamisole.

iii Semispecific immunotherapy

(targeted against particular components of the immune response)

eg anti-IL2 receptor monoclonal antibodies against activated T cells; Cyclosporin A against CD4+ helper-inducer cells; silica and methimazole against macrophages; Monoclonal antibodies against IL1, TNF or interferon gamma.

IV Specific immunotherapy

eg vaccination with disease/autoantigen-specific inactivated T cell clones or T cell receptors; anti-idiotypic monoclonal antibodies to T cell receptors; oral tolerization

V Anti-inflammatory agents

eg Gold; anti-malarials such as hydroxychloroquine; cyclooxygenase inhibitors; and anti-free radical agents such as superoxide dismutase, nicotinamide and vitamin E.

Vi Beta cell modification

eg insulin and maintenance of euglycaemia.

The majority of these do not have immediate therapeutic potential in humans but the list does draw attention to the diversity of approaches that could be used (Table 7.1) (Harrison *et al.* 1990).

Nonspecific immunosuppression: Glucocorticoids were used in open pilot studies in newly diagnosed diabetes with apparent benefit (Elliott *et al.* 1981). Their use has been limited by their adverse metabolic effects but these studies did stimulate controlled trials of other non-specific immunosuppressive agents.

In the first randomized controlled trial of azathioprine alone, remission occurred in 7/13 adults with newly diagnosed Type 1 diabetes compared with 1/11 controls (Harrison *et al.* 1985). In the trial in children, however, no patients had complete remission though fasting C-peptide was significantly higher in the treated group at 3 and 6 months.

One trial combined azathioprine with initial short-term prednisolone. After 12 months 10/20 treated patients and 3/20 control patients were in partial remission (Silverstein *et al.* 1988). Results were better in older patients and in those in whom the total lymphocyte count fell below the normal range.

Cyclosporin A represents an example of semispecific immunotherapy blocking the early stages of the immune response, in particular activation of CD4 positive helper/inducer T cells. Trials with this drug first showed that it was possible to alter the course of diabetes in humans, albeit transiently. In the first French controlled trial in adults with newly diagnosed Type 1 diabetes, 24% of the cyclosporin treated group vs. 6% of the control group were in complete remission at 9 months. The result was significantly better in those with trough levels of at least 300 ng/ml (Feutren *et al.* 1986). The Canadian-European trial confirmed these findings, and also showed that the results were better in those with a shorter duration of symptoms, less weight loss, higher C-peptide secretion and absence of ketoacidosis at diagnosis. The rate of non-insulin-requiring remission was also higher at both 6 and 12 months in adults than in children. These observations

suggest that, as would be predicted from its mode of action, the drug is more effective if given earlier in the disease process and less effective in the more rapid destructive process that occurs in children (Canadian-European Randomized Control Trial Group, 1988).

The use of cyclosporin prior to clinical onset is limited by its toxicity, particularly nephrotoxicity. This is dose related and usually reversible but most clinicians would feel that the risk-benefit ratio is too unfavourable to embark on long term treatment in healthy subjects, most of whom will be children and some of whom might never develop Type 1 diabetes even without intervention.

An alternative, more futuristic approach is to use 'magic bullets' whereby highly toxic agents are carried to the attacking immune cells by attaching them to antibodies against markers of T cell activation. In pilot studies, ricin A-chain immunoconjugated with anti-CD5 monoclonal antibodies and IL-2 conjugated with diphtheria toxin, given at the time of diagnosis, have both been reported to improve glycaemic control (Skyler *et al.* 1991; Boitard *et al.* 1992).

Specific immunotherapy. The overall aim of many investigators is to develop interventions that target disease- or antigen-specific immune changes. It may, for example, be possible to vaccinate with disease-specific T cells or perhaps with receptors that recognize disease-specific antigens. If T cell receptors can be characterized, it may be possible to target 'magic bullets' against them using anticonotypic immunoglobulins.

Oral tolerization is a form of specific immunotherapy that has been found to be helpful in some autoimmune conditions. This is based on the observation that oral ingestion of soluble antigen can induce a state of immunological hypo-responsiveness or tolerance while parenteral administration causes sensitization. Most work has been done in animal models of autoimmune disease such as collagen-induced arthritis and experimental allergic encephalomyelitis (EAE) in which oral administration of the antigen (collagen and myelin basic protein (MBP)

respectively) before conventional challenge reduces the incidence and severity of disease. There is also some reduction in severity of relapse if MBP is given after clinical onset of chronic relapsing EAE, an animal model of multiple sclerosis (Thompson *et al.* 1990).

Insulin, given orally to young NOD mice reduces insulinitis and delays onset of disease (Zhong *et al.* 1991). There is some evidence that administration of fragments of autoantigens that are not themselves autoimmunogenic may achieve the same degree of tolerance without risking sensitization and exacerbation of pre-existing disease. Thus, in pre-Type 1 diabetes, it may be preferable to give an antigen such as insulin that is unlikely to be the primary sensitizing antigen, and digestion of this peptide may even be beneficial. This theory is prompting a proposed multicentre trial of oral insulin therapy in family members of children with Type 1 diabetes.

Nicotinamide (nicotinic acid amide or niacinamide) is a water-soluble group B vitamin, derived from nicotinic acid. Its effect in prevention of toxin-induced models of diabetes has been known for more than 40 years (Lazarow, 1947; Dulin *et al.* 1969). It is also effective in preventing spontaneous diabetes in the NOD mouse, inhibits transplant allograft insulinitis, another animal model of immune beta cell damage,

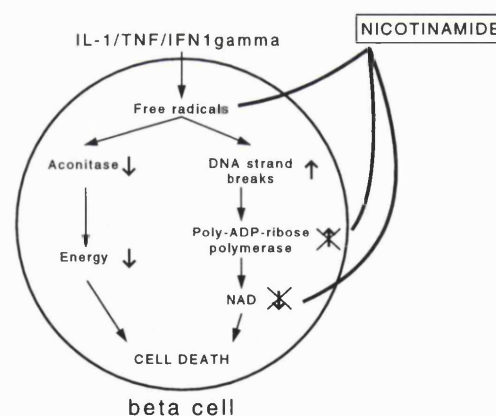


Figure 7.1 The effects of cytokines on the beta cell and proposed actions of nicotinamide

preserves residual beta cells in partially pancreatectomized rats, and promotes the growth of cultured human islet cells. It appears to act in the final stage of beta cell damage, preventing the cytotoxic effects of cytokines. There are two proposed

mechanisms for its action: (1) that it acts as a free radical scavenger to reduce DNA damage, and (2) that it restores the islet cell content of NAD towards normal by elevating intracellular NAD, and by inhibiting poly ADP-ribose polymerase, a major route of NAD metabolism (Pociot *et al.* 1993). Further effects of nicotinamide that have been demonstrated recently include suppression of MHC Class II antigen expression on murine islet cells (Yamada *et al.* 1982), implying that nicotinamide might reduce presentation of autoantigens to helper T cells by islet beta cells in human Type I diabetes. It has also been shown to inhibit macrophage-mediated cytotoxicity directed against rat islet cells (Kroncke *et al.* 1991). There is therefore evidence to suggest that nicotinamide could offer a protective effect to human beta cells, particularly if the drug were given at an early stage of the disease process.

Nicotinamide has been tried in humans with recently diagnosed Type 1 diabetes. The results have been variable but increased serum C-peptide concentrations and a longer remission period have been reported in treated individuals (Vague *et al.* 1987; Mendola *et al.* 1989), and similar effects were found in a longitudinal study of patients with established diabetes (Vague *et al.* 1989). Pilot studies of its ability to prevent Type 1 diabetes in first degree relatives have also been performed. Eight untreated historical controls aged under 16 and high ICA levels were compared with another group of 14 children receiving nicotinamide (Elliott *et al.* 1991). Four out of 8 historical controls developed diabetes within one year, as against 0 of 9 in the treatment group. By 2 years, 7 of 8 in the untreated group and none of 6 on nicotinamide had progressed to diabetes. Subsequently, after 4 years of follow-up 7 out of 14 children on nicotinamide have developed diabetes, as had the last historical control (Eisenbarth *et al.* 1993).

In contrast, nicotinamide was ineffective in 3 first degree relatives with very low first phase insulin response (FPIR)(Dumont Herskowitz *et al.* 1989). These data suggest that nicotinamide may not work if the treatment begins at a time when FPIR is effectively lost. This observation needs to be confirmed, but suggests that nicotinamide should be most effective if started during the early phase of beta cell destruction.

The toxicity of nicotinamide is low (Hoffer, 1969) even at the pharmacological doses (100-150 times the recommended daily intake) used in pilot studies. Concerns about the toxicity of nicotinamide arise from its potential to increase mutation and perhaps induce islet tumours. Tumours have been reported in rats and mice given nicotinamide together with streptozocin or alloxan (Rakieten *et al.* 1971; Kazumi *et al.* 1980; Yamagami *et al.* 1985) but not in mice treated with nicotinamide alone (Toth, 1983). One of the actions of nicotinamide is inhibition of poly(ADP-ribose) polymerase, an enzyme involved in repair of damaged DNA; another is replenishment of intracellular DNA. The likelihood that partial inhibition of poly(ADP-ribose) polymerase will be detrimental is likely to depend on the balance of these two actions and the degree of DNA damage. This issue was reviewed at a recent workshop and it was generally felt that, at a dose of $1.2\text{g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$, nicotinamide would probably supply NAD without critical inhibition of DNA repair (Pociot *et al.* 1993). The treatment has proved acceptable in large, open studies in New Zealand. Many investigators feel that a double-blind, randomized controlled trial of its efficacy in delaying or preventing the onset of Type 1 diabetes is indicated (Chase *et al.* 1992).

Insulin therapy has been shown to delay the onset of insulinitis in both the BB rat and NOD mouse and to prevent the adoptive transfer of diabetes in the NOD mouse (Gottfredsen *et al.* 1985; Vlahos *et al.* 1991; Atkinson *et al.* 1990). Proposed mechanisms are immune modulation (Peakman *et al.* 1990), tolerance induction, or beta cell rest, whereby reduced insulin secretion causes less antigen expression rendering the cells less susceptible to immune attack. Intensive insulin treatment (including 2 weeks of intravenous treatment given via biostator) at the time of diagnosis was associated with lower glycated haemoglobin levels and higher stimulated c-peptide after 12 months than in controls (Shah *et al.* 1989).

A pilot trial of pre-emptive insulin treatment given to 7 subjects predicted to have more than 90% risk of developing diabetes within 3 years has produced promising results (Keller *et al.* 1993). This is prompting a multicentre study in first degree relatives in the United States (Skyler *et al.* 1993).

7.4 THE CHOICE OF AGENT FOR HUMAN TRIALS:

In moving to intervention trials in humans the risk-benefit ratio of the treatment becomes of paramount importance; the participants will be young and healthy, and almost all the agents tested in newly diagnosed diabetes are associated with appreciable morbidity. In an ideal world one would balance a known risk of diabetes and its complications against the known short and long-term morbidity of the treatment for each individual. In real life, however, none of these risks can be precisely quantified and the decision has to be based on very much less satisfactory data.

Most of the agents that are listed in the above Table 7.1 are still in the early stages of development and are not yet suitable for human use so that, realistically, at present the possibilities for large scale testing are limited to nonspecific immunosuppressants such as azathioprine or glucocorticoids, semi-specific immunosuppressants including cyclosporin A, agents enhancing beta cell defences such as nicotinamide, or pre-emptive insulin therapy. Specific therapy such as avoidance of a putative primary antigen, bovine serum albumin and oral tolerization with insulin or GAD are recent additions to this list.

The characteristics of a suitable agent for testing should be defined in advance and the possibilities reviewed with these in mind:

- (a) Efficacy in animal models
- (b) Efficacy in humans after diagnosis
- (c) Detrimental metabolic effects
- (d) Toxicity

The accuracy of prediction and the acceptable degree of risk associated with treatment are inextricably linked. A clinician would probably not be prepared to consider, for example, toxic immunosuppressive treatment unless very certain that the patient would develop Type 1 diabetes, while a simple treatment such as avoidance of cow's milk protein for the first 3-4 months of life may be applicable in those whose risk of Type 1 diabetes might be much lower.

A *decision tree* can be used to summarize current thinking on intervention studies in family members. Figure 7.2 shows the levels of risk at which investigators are happy to proceed with trials of different agents. The trial of avoidance of cows' milk is being undertaken at the level of genetic risk only; the first study is planned to be undertaken in newborn offspring and children of patients with Type 1 diabetes and all familial cases of Type 1 diabetes could potentially benefit from the therapy. The nicotinamide study is being carried out in family members with ICA ≥ 20 JDF units who have an intermediate risk of 35% of Type 1 diabetes within 5 years. In our

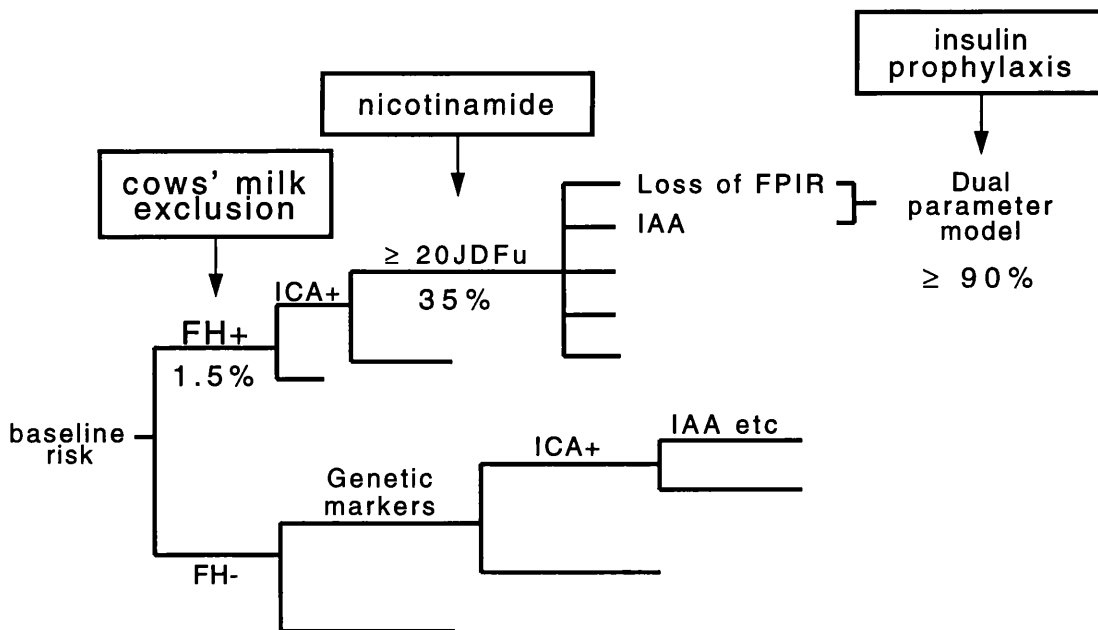


Figure 7.2 The Decision tree representation of prediction of Type 1 diabetes with intervention trials planned in 1993

experience, this would include some 70% of future cases of Type 1 diabetes in family members. The pre-emptive insulin study is being limited to those with ICA ≥ 40 JDF units and loss of FPIR and/or high levels of IAA who are estimated to have 90% risk of Type 1 diabetes within 3 years using the dual parameter model (Colman *et al.* 1988) but who probably only account for some 15% of future cases of Type 1 diabetes in family members (Bingley *et al.* 1993b).

7.5 THE DESIGNING OF THE EUROPEAN NICOTINAMIDE DIABETES INTERVENTION TRIAL (ENDIT):

7.5.1 Introduction

In this section I have illustrated how data derived from the prospective family studies have been used in the design of a European multi-centre intervention trial. At the time that this thesis was written, the trial had not yet started but the screening phase was well advanced and the treatment phase in the final stages of planning. The overall planning of the trial was the work of many people but I was primarily responsible for details of study design and methods. I have therefore included this application of my work within this thesis.

7.5.2 General considerations

It is essential that trials are double-blind placebo controlled. The early cyclosporin trials relied on historical controls to assess spontaneous remission and the rate was found to substantially different when a placebo controlled trial was undertaken (Assan *et al.* 1985; Feutren *et al.* 1986).

7.5.3 Sample Size

The sample size required to undertake a trial is calculated from the formula:

The required number of patients in each treatment group

$$= \frac{P1 \times (100-P1) + P2 \times (100-P2)}{(P2-P1)^2} \times f(\alpha, \beta)$$

where:

P1 = expected percent developing diabetes in control group

P2 = percent developing diabetes in treatment group in event of clinically relevant

effect (eg 50% of proportion in control group)

$f(\alpha, \beta)$ = function determined by required power $(1 - \beta)$ to detect effect at significance α (Pocock, 1983).

This calculation requires that a number of criteria must be defined:

The length of trial: A choice can be made between a relatively short, very large scale trial or a smaller, much longer one. Very large numbers will be needed for a short trial, but on the other hand maintaining enthusiasm and compliance will be difficult if the trial is too long. A 5 year trial has been chosen as providing the best balance of these factors. It has the added advantage of being able to provide good estimates of cumulative risk for this period corroborated by data from a number of independent studies.

Estimated risk of developing diabetes over the period of the study: The figures used are those derived from the combined results of the Bart's-Windsor and Bart's-Oxford family studies. The Bart's-Windsor Study showed that 37% of non-diabetic relatives with ICA ≥ 20 JDF units progressed to diabetes within 5 years (*Section 6.3.3.2*). Combined analysis of both groups showed a slightly lower rate equivalent to 35% at 5 years. This later more conservative estimate has been used in designing this trial. The threshold of 20 JDF units has been chosen because we have previously shown that this provides the best balance of sensitivity and positive predictive value (*Section 6.3.3.2 (b)*, Figure 6.3).

The likely efficacy of the treatment and what would be considered a clinically useful effect: A 50% reduction in the development of diabetes was initially chosen as the minimum clinically useful effect. This is, however, perhaps optimistic and it was later decided that the study should aim to have the power to detect a 40% treatment effect.

The required power of the trial to detect a significant effect: Such trials will be very difficult and expensive to run and it is therefore most important that they have

adequate power to avoid type II errors missing important true effects. This makes it appropriate to aim for 80-90% power to detect a statistically significant effect.

Combining these factors the **null hypothesis** to be tested is that nicotinamide cannot achieve a 40% reduction in the rate of progression to Type 1 diabetes in first degree relatives with ICA \geq 20 JDF units over the 5 year study period. The study should have 90% power to detect such a difference.

The **sample size required** to undertake this study is a minimum of 422 subjects (211 in each group).

Table 7.2 uses the risk estimates derived from the Bart's-Windsor Family Study to illustrate the effect of varying the threshold of ICA and the length of the trial on the size of the sample needed to undertake the trial.

Table 7.2: Subjects needed in each group to achieve 90% power to detect a 40% treatment effect with varying ICA threshold and length of trial (*based on risk estimates from the BWFS alone*).

Duration (years)	ICA threshold (JDF units)		
	4	20	80
1	5161	2011	786
3	961	436	195
5	495	195	121
7	349	81	38
9	174	55	16

7.5.4 The size of population to be screened:

The size of the population to be screened to recruit this number of people with ICA \geq 20 JDF units requires knowledge of

The likely acceptance rate: The overall acceptance rate reflects first, the proportion who would consent to be screened and, second, the proportion of those eligible for inclusion that would agree to participate. There are no directly comparable data but experience with the family study population confirms that relatives are a highly

motivated group. For example, in the Bart's-Windsor study, which requires regular blood tests and has offered no form of intervention, 88% of families continued to participate after 10 years (Bonifacio *et al.* 1990). Given the high level of motivation due to direct personal experience of diabetes and the low rate of side effects associated with nicotinamide, we assume that 80% of entrants will complete the study period. This means that 528 recruits need to be identified.

Numbers of relatives recruited for each proband: The Bart's-Oxford Study screened all first degree relatives of patients diagnosed under the age of 21. This study obtained blood samples from 2.7 family members , 1.7 parents and 1.2 siblings for each proband, so that just under 3 relatives will be available for study for each index case we identify. This rate may differ between countries participating in ENDIT.

The prevalence of ICA in family members: This was assessed from the results of initial screening in the Bart's-Oxford family study and is shown in Table 7.3

Table 7.3: The prevalence of ICA in family members in the Bart's-Oxford family study recruited up 31.12.90

JDF units	Parents	Siblings
1-4	15 (1.6%)	16 (2.9%)
4-9	26 (2.7%)	13 (2.3%)
10-19	12 (1.3%)	10 (1.8%)
20-39	6 (0.6%)	5 (0.9%)
40-79	1 (0.1%)	3 (0.5%)
≥ 80	1 (0.1%)	6 (1.1%)
Total ≥ 4 JDFu	46 (4.8%)	37 (6.6%)

The distribution of ICA by age in siblings is shown in Table 7.4

Table 7.4: The distribution of ICA in siblings in the Bart's-Oxford Family Study by age at entry

ICA (JDFu)	Age (years)				
	0-4	5-9	10-14	15-19	20-24
≤4	36	120	152	149	51
5-9	2	4	2	5	1
10-19		6	4		
20-39		2	2	1	
40-79		1	1	1	
≥ 80	1	2	3		
Total					
≥ 4 JDFu (%)	3 (7.6)	9 (7.0)	14 (8.4)	11 (6.9)	1 (1.9)
Total					
≥ 20 JDFu (%)	1 (2.6)	5 (3.9)	6 (3.6)	2 (1.3)	0

The reproducibility of ICA testing in family members: The number of family members that have to be screened and the screening strategy is also influenced by the reproducibility of ICA testing. This covers not only the reproducibility of the test and true biological variability in ICA levels, but also other discrepancies (for example clerical errors) that may occur when screening is extended to a large scale exercise involving many levels of data and sample handling. In order to evaluate the scale of all these problems together, I compared the results of all the first and second ICA results in the Bart's-Oxford family study. Table 7.5 summarizes the results of this exercise.

Table 7.5: A comparison of the ICA results of first and second samples from 1263 family members in the Bart's-Oxford Family Study (*Figures shown represent number of individuals with ICA in each class*)

ICA on second sample	ICA on first sample (JDFu)						
	0	1-4	5-9	10-19	20-39	40-79	≥80
0	1114	18	20	5			
1-4	16	3	5	4			
5-9	16	3	7	2			
10-19	8	3	2	3	2		
20-39	1		3	2	5		
40-79			3	2	3	2	3
≥80	2					2	4

Therefore the overall prevalence of ICA ≥ 4 JDF units on both of the first 2 tests performed was 45/1263 (3.6%), 18/804 (2.2%) in parents and 24/459 (5.2%) in

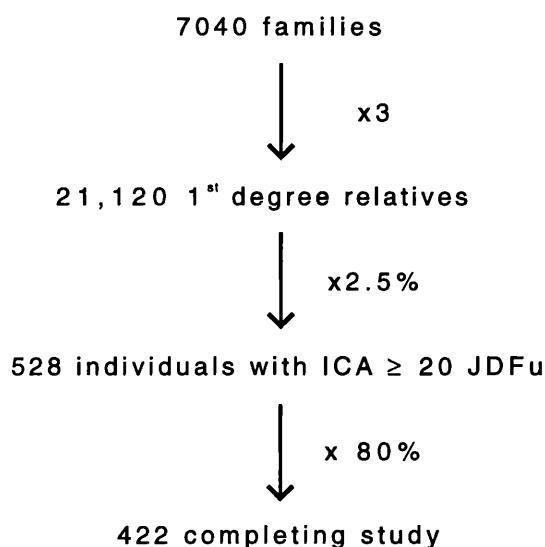
siblings. The prevalence of a combination of ICA ≥ 20 JDFu in one of these samples with ≥ 4 JDFu in the other was 31/1263 (2.5%), 11/804 (1.4%) in parents and 20/459 (4.4%) in siblings.

The prevalence of ICA in family members is an important consideration in the design of intervention studies. The feasibility of such studies will be largely determined by the size of screening exercise required to identify the study subjects. The yield of eligible subjects would be greatest if only siblings were studied but we have shown that, in practice, the mean number of unaffected siblings per proband is less than one so that more than 3 times as many probands would have to be identified if this strategy were adopted.

A further consideration is the number of tests per individual that should be used. The Bart's-Oxford family study has allowed the likely efficacy of the whole screening exercise to be tested, including not only the assay itself but also the data and sample collection and handling system. The results of the first and second ICA measurement were compared in 1263 family members. As expected, the intra-subject variability around the threshold of detection was high; 25/40 samples with ICA 5-9 JDFu had ICA below the detection threshold on re-testing and 19/28 with ICA 5-9 on second testing had undetectable ICA on first testing. ICA negativity was confirmed on retesting in 97.8% of samples. 3/1203 (0.08%) of samples that were ICA negative on first testing were found to have ICA ≥ 20 JDF on the second sample. This suggests that, if subjects with ICA ≥ 20 JDF units are required for an intervention study, subjects who are ICA negative on initial screening do not need to be re-tested. ICA ≥ 20 JDF units was confirmed on second testing in 19/21 family members, the others had ICA between 10 and 19 on retesting. The 'grey area' in terms of screening for high risk subjects is therefore those with ICA between 5 and 19 JDF units on initial screening. 10/58 of these family members with ICA between 5 and 19 JDF units on initial testing had ICA ≥ 20 JDF units on second testing. Our risk estimations are based on peak ICA and a single ICA estimation would miss 10/31 family members with ICA ≥ 20 JDF units.

The size of population to be screened for ENDIT

Figure 7.3 shows how these assumptions have been used to determine the size of population to be screened for ENDIT. We need to screen 21,120 first degree relatives to identify 528 individuals with ≥ 20 JDF units. This corresponds to approximately 7,040 families of children with IDDM.



7.5.5 Using different levels of risk:

An alternative study design could involve recruiting only subjects with a much higher risk of developing diabetes. For example those with $ICA \geq 20$ JDF units and loss of the first phase insulin response in the IVGTT who have a 90% risk of progressing to diabetes within 5 years. A

Figure 7.3 *The population to be screened for ENDIT*

minimum of 56 subjects (28 in each group) are required to detect a 40% treatment effect within this time. Less than a third of those with $ICA \geq 20$ JDF units have shown to have loss of the FPIR. These estimates are almost identical with those from the Joslin group (Ziegler *et al.* 1990). Using the assumptions given above 8,400 individuals would need to be screened to identify 70 with loss of FPIR. Alternatively, a sample size of 600 (300 in each group) would be needed if the trial were to be undertaken using a threshold of 4 JDF units. 750 individuals would have to be identified to allow for drop-outs and this would correspond to a screening population of 13,670. Using such a low threshold would mean that even if the treatment were 100% effective in preventing diabetes, 60% of those treated would probably never have developed diabetes even without intervention.

7.6 CONCLUSION

This chapter sums up the progress that has been made in research in pre-Type 1 diabetes. Some twenty years has been spent describing the disease process, the long prodrome, the genetics and epidemiology of the disease and markers of susceptibility. In the 1990's all these disciplines are coming together and we are beginning to apply these findings. In this chapter I have described how our understanding of the disease prodrome has been used to identify agents that show sufficient promise as potential preventative therapies to warrant large scale testing. I have then shown how the results of research in the prediction of Type 1 diabetes can and must be applied in the design of intervention trials to ensure rigorous testing of these agents.

CHAPTER 8 - PREDICTION OF TYPE 1 DIABETES IN THE GENERAL POPULATION

8.1 INTRODUCTION

Only about one in eight children who develop diabetes have an affected first degree relative (Bloom *et al.* 1975; Dahlquist *et al.* 1985) and if intervention strategies are to have a significant impact on the overall incidence of Type 1 diabetes, the disease must be predicted and prevented in the general population. Before the findings of family studies are to be extrapolated to this group the potential problems need to be considered. Are 'familial' and 'sporadic' Type 1 diabetes the same disease? What is the prognostic significance of available markers when applied to this group? In this chapter I have (i) reviewed the available literature on prediction of Type 1 diabetes in the children with no family history of the disease (ii) described my own study of the prevalence and prognostic significance of islet cell antibodies in schoolchildren in the Oxford region (*Section 8.4*) and (iii) suggested a possible strategy for screening for high risk of Type 1 diabetes in the general population.

8.2 'FAMILIAL' VS 'SPORADIC' TYPE 1 DIABETES

8.2.1 Genetic and clinical features

There is a theoretical problem with extrapolating the findings of family studies to the general population in that the disease might be heterogeneous, with distinct 'familial' and 'sporadic' forms. There is, however, no direct evidence to support this argument. Anderson and colleagues examined 25 simplex, 42 multiplex and 10 multigenerational families and found no differences in HLA associations (A,B,C and DR), family history of diabetes or thyroid disease, or antibody positivity between these groups (Anderson *et al.* 1983).

8.2.2 Immunological features:

ICA appear to be present before diagnosis in children with no family history of diabetes just as in those with a family history of the disease. There have now been several studies of ICA in schoolchildren (see below) and in each of these there

have been children with high titres of ICA who subsequently develop diabetes (Bruining *et al.* 1989; Maclaren *et al.* 1985; Karjalainen, 1990). IAA and autoantibodies to the 64kD islet antigen have also been described (Maclaren *et al.* 1985; Baekkeskov *et al.* 1989).

8.3 EXISTING STUDIES OF ICA IN THE GENERAL CHILDHOOD POPULATION

Several studies have been undertaken to determine the prevalence of ICA in normal school children (Table 8.1). The prevalence of ICA has varied between 0.26 and 4.1% but as can be seen from the table, comparisons cannot be made between these studies because of differences in age range and particularly in ICA threshold. Many of the studies were undertaken before ICA measurement was standardized and several studies did not even quote a ICA detection threshold. The numbers of ICA positive subjects have been small and prospective follow-up limited. This constraint will be common to all studies of ICA in children from the general population; even if the prevalence of ICA is 4.2%, many thousands of children will have to be recruited for screening and followed for at least 10 years if rigorous prospective study is to be performed. There is therefore a need for an indirect approach, maximizing the yield from a smaller study. This has been the rationale behind the study that we have undertaken in the Oxford region.

Table 8.1 Studies of the prevalence of ICA in the general child population

Country	age range (mean)	n	ICA threshold (JDFu)	ICA positive (%)	Follow-up	∩IDDM	Reference
Japan	7-18	473	N/G	0.4	-		(Notsu <i>et al.</i> 1985)
Florida,USA	5-15 (11.6)	6450	N/G	0.62	3 years	2/11 ICA>20	(Maclaren <i>et al.</i> 1985,1990)
Catalonia	14-17	2291	N/G (4?)	0.26	-		(Bergua <i>et al.</i> 1987)
The Netherlands	5-19	2805	N/G	0.28	10 years	4/8 ICA+,3/2797 ICA-	(Bruining <i>et al.</i> 1990)
Sweden	0-15 (10)	321	N/G (4?)	2.8	8-31 months	2/9 ICA+,0/312ICA-	(Landin-Olsson <i>et al.</i> 1989)
Finland	3-18 (10.7)	1212	5 JDFu	4.1	8 year	3/50 ICA+,0/1162 ICA-	(Karjalainen 1990)
France	6-17	7740	2.5 JDFu	1.7	-		(Levy-Marchal <i>et al.</i> 1991)

8.4 THE PREVALENCE OF IMMUNOLOGICAL MARKERS OF RISK IN SCHOOLCHILDREN IN THE OXFORD REGION

Introduction

The aim of this study was to determine the prevalence of immunological markers of risk for the development of Type 1 diabetes in a population of children with no family history of diabetes and to establish a cohort of high risk subjects for prospective study. A further aim was to compare the prevalence of ICA and the risk of progression to diabetes in schoolchildren and age-matched siblings from within the same region.

Subjects and Methods

Subjects

Healthy schoolchildren: 2925 healthy schoolchildren aged 9-13 years were recruited from 12 Middle Schools in Oxford and Windsor. Details of age and sex were obtained from the school and checked with the children, who were also asked whether they had a parent or sibling on insulin. Ethnicity (classified as European, Indian subcontinent, Afro-Caribbean or other) was determined by observation and, where necessary, by asking the child where his/her parents originated.

Siblings of children with Type 1 diabetes: 274 siblings of children with Type 1 diabetes resident in the same region, non-diabetic and aged 9-13 years at the time of entry to the Bart's-Windsor (105 children) or Bart's-Oxford Family Study (169 children) were compared with the schoolchildren.

Clinical characteristics of the study subjects are shown in Table 8.2

Methods

Sample collection:

(a) Schoolchildren: Venepuncture was performed at the schools after the application of Lignocaine/Prilocaine cream (EMLA Cream, Astra Pharmaceuticals Ltd, Kings Langley, England). Samples were taken between 0930 and 1530 hrs.

Table 8.2 Clinical characteristics of schoolchildren and siblings

	Schoolchildren	Siblings
n	2925	274
Age (mean(range))	11.35 (9.0-13.8) (SD 1.1)	11.58 (9.0-14.0) (SD 1.5)
Male:female ratio	1.07	1.03
Ethnic origin	88% Europid 8% Indian subcontinent 2% Afro-Caribbean 1% other	99% Europid
Affected 1st degree relative	1.8%	100%
Date of sampling	1989-90	1979-90

5-10ml venous blood samples were collected into plain tubes and separated and frozen on dry ice within 60 minutes. Blood for random glucose estimation was collected into tubes containing fluoride oxalate and plasma was separated and frozen on dry ice. Serum and plasma samples were stored at -20°C until assayed. (b) Siblings: Venepuncture was performed on home visits. The results of the first sample taken from each child were used in this study. Serum samples were stored at -20°C. Initial screening for ICA was performed within 6 months of samples being taken but ICA in positive samples taken before 1987 were retrospectively quantified in JDF units.

Follow-up: The parents of all schoolchildren with detectable ICA were contacted by post and the children were invited to give a second venous blood sample 7-14 months after the initial screening and follow-up continued. Since all cases of newly diagnosed diabetes in children resident in the Oxford region are notified to the Bart's-Oxford study as part of a continuing incidence survey (*Section 3.3*), we are also in a position to identify children without detectable ICA who develop Type 1 diabetes.

Ethical approval: Approval for the study was granted by the Central Oxford and East Berkshire Health Authority Research Ethics Committees, and the Oxfordshire and Berkshire County Council Education Departments. Written consent was obtained from parents of all children.

Laboratory methods

Islet cell antibodies and insulin autoantibodies: Serum samples from both schoolchildren and siblings were tested in the same assay. Insulin antibody positivity was defined as more than 3 standard deviations above the population mean. Assay details are given in the appendix.

Estimation of risk of developing diabetes: The cumulative incidence of Type 1 diabetes between age 10 and 20 in the Oxford region was used to estimate the risk of the schoolchildren developing diabetes before age 20. This was calculated from the age-specific rates of Type 1 diabetes in the region in 1985-86 (*Section 3.3*) and the Registrar General's mid-year estimates of population (Office of Population Censuses and Surveys, unpublished). The risk of development of diabetes between age 10 and 20 in siblings was estimated from life tables derived from data from 1003 siblings in the Bart's-Windsor and Bart's-Oxford Family.

Statistical methods: Confidence intervals were calculated for a Poisson distributed variable. Differences between recruited and non-recruited children were assessed by chi-squared testing.

The expected effect of differences in disease prevalence on the positive predictive value of islet cell antibodies was based on the mathematical formula derived from Bayes' theorem of conditional probabilities (Fletcher *et al.* 1988).

$$\text{Positive predictive value} = \frac{\text{Sensitivity} \times \text{Prevalence}}{(\text{Sensitivity} \times \text{Prevalence}) + (1 - \text{Prevalence})(1 - \text{Specificity})}$$

This equation was applied to the published figures for sensitivity and specificity of ICA in siblings in the Bart's-Windsor Family Study (*Section 6.3.3.2 (b)*) which were 91% and 98% respectively for ICA \geq 4 JDF units and 73% and 99.7% for ICA \geq 20 JDF units.

Results

Recruitment: The overall recruitment rate in schoolchildren was 66%, varying between 57-72% in different schools. There were no differences in age and sex between children who did and did not volunteer for venesection in a sample of 3 randomly selected schools (total roll 1089).

Prevalence of islet cell antibodies: Islet cell antibodies \geq 4 JDF units were found in 82/2908 schoolchildren (2.8%, 95% confidence interval 2.3-3.5%) and 24 (0.8%, 0.5-1.2%) of these had ICA \geq 20 JDF units.

Only 2 of the 82 schoolchildren with ICA \geq 4 JDF units had an affected first degree relative and if those were excluded, the prevalence of ICA was 80/2856 (2.8%) \geq 4 JDF units and 24/2856 (0.8%) \geq 20 JDF units. In contrast, 17 of 274 (6.2%, 3.6-9.9%) siblings of children with diabetes had ICA \geq 4 JDF units on initial testing, and 6/274 (2.2%, 0.8-4.8%) had greater than 20 JDF units.

The prevalence of ICA \geq 4 JDF units did not differ significantly between male and female schoolchildren (2.2% vs. 3.4%). ICA \geq 20 JDF units were found in 1.1% (0.6-1.7) males and 0.6% (0.3-1.1) females (p =NS). Six of 334 (1.8%, 0.7-3.9%) non-Europid children had ICA \geq 4 JDF.

Follow-up of children with islet cell antibodies: To date, second samples have been obtained from 31/82 children with ICA \geq 4 JDF units. Overall, 20/31 have detectable ICA on repeat testing. All 9 who had ICA \geq 20 JDF units on initial testing had ICA \geq 15 JDF units on the second test, and 10/11 children in whom ICA was $<$ 4 JDF units on repeat testing had had ICA between 4 and 10 JDF units on initial testing.

Two schoolchildren, (A 13 year old girl with ICA \geq 80 JDF units and an 11 year old boy with ICA 10 JDF units) have subsequently developed Type 1 diabetes, both within 3 months of screening. The first child had no family history of diabetes; the father of the second had insulin-treated diabetes diagnosed at age 35.

Prevalence of insulin autoantibodies: Insulin antibody levels were \geq 3 standard deviations above the population mean in 2/78 (2.6%) schoolchildren and 2/15 (13.3%) siblings with ICA \geq 4 JDF units ($p=0.1$).

Random plasma glucose values: The mean random plasma glucose from 2866 schoolchildren was 5.44 mmol/l (standard deviation 0.81).

Risk of developing diabetes: The cumulative incidence of Type 1 diabetes between age 10 and 20 in the general population Oxford region is 0.21%. In life table analyses, the projected risk of a sibling of a child with Type 1 diabetes developing the disease between the same ages is 2.8%.

Expected effect of differences in overall risk of disease: The calculated positive predictive value of ICA \geq 4 JDF units and \geq 20 JDF units in the 2 populations is shown in Table 8.3.

Discussion

Our study revealed a high prevalence of ICA in healthy schoolchildren aged 9 - 13 years in the UK, 2.8% of whom had detectable antibody levels. Most other studies have reported much lower rates, ranging from 0.24% in the Netherlands (Bruining *et al.* 1989), 0.3% in Spain (Bergua *et al.* 1987), Japan (Notsu *et al.* 1985) and Florida (Maclaren *et al.* 1985) to 1.6% in France (Levy-Marchal *et al.* 1992). Only Sweden (3.0%) (Landin-Olsson *et al.* 1989) and Finland (4.1%) (Karjalainen, 1990) have reported higher rates, but these countries have an incidence of Type 1 diabetes 1.5 - 2.5 times higher than that in England. These studies may not be strictly comparable, however, because of differences in age range and techniques for ICA measurement (Bonifacio *et al.* 1990a).

Prognostic significance

Previous studies have suggested that the prevalence of ICA within a given population exceeds the anticipated cumulative incidence of Type 1 diabetes (Bruining *et al.* 1989; Maclaren *et al.* 1985; Karjalainen, 1990). We have confirmed this. Siblings of children with Type 1 diabetes are 13 times more likely to develop Type 1 diabetes between the age of 10 and 20 than children in the general population, yet have only twice the prevalence of ICA, whether at low or high titer. This implies that the predictive power of ICA in the general population is about 6 times less than in family members. Since 89% of siblings with ICA ≥ 20 JDF units in the Bart's-Windsor Family study developed Type 1 diabetes within 10 years (Bonifacio *et al.* 1990b), this implies that some 15% of children with no family history of diabetes and ICA ≥ 20 JDF units could be expected to develop diabetes before age 20. Further, since a population based study has suggested that only some 40% of cases of Type 1 diabetes are diagnosed after that age (Laakso *et al.* 1985), it seems likely that at least 70% of children reaching puberty with high titers of ICA never will develop Type 1 diabetes.

This empirical observation could have been predicted on theoretical grounds. The two groups have widely differing risks of diabetes, and the positive predictive value of a test depends on the prevalence of disease in the population tested. Markers with a high positive predictive value in high risk populations are less useful in

Table 8.3 Expected effect of differences in overall risk of disease

	Schoolchildren	Siblings
Risk of IDDM	0.21%	2.8%
Calculated positive predictive value of ICA ≥ 4 JDF units	9%	57%
Calculated positive predictive value of ICA ≥ 20 JDF units	34%	88%

populations with low disease prevalence, and even highly specific tests generate a high proportion of false positives when applied to a low risk population. The effect of differences in disease prevalence can be calculated from the formula given above. In the present study, the 13-fold difference in risk of Type 1 diabetes between siblings and schoolchildren would be expected to result in a 6-fold reduction in the positive predictive value of ICA ≥ 4 JDF units and at least a 2 to 3-fold fall in that of ICA ≥ 20 JDF units between the two populations (Table 8.3).

The limited prognostic value of ICA is intrinsic to any predictive test applied to a population with a low disease prevalence. This constraint cannot be overcome by improving the sensitivity and specificity of the test, unless the improbable goal of 100% specificity could be achieved. For example, even a test with 95% sensitivity and 99.8% specificity would only have a positive predictive value of 50% in a population with a predicted disease prevalence of 0.21%.

A screening strategy using multiple markers

Genetic markers are subject to the same limitations. High sensitivity is relatively easy to achieve but current markers are so prevalent in the general population as to have low specificity. For example, in a recent Finnish study there was no aspartate residue at position 57 on either DQB chain in 74.5% of patients with Type 1 diabetes and 18% of controls, giving a relative risk for this genotype combination of 29 but an lifetime absolute risk of only 2%. A panel of 4 sequence-specific oligonucleotide probes was then used to identify susceptible and protective DQ alleles, thus achieving the best segregation of patients and controls yet described in Type 1 diabetes. DQw8, in the absence of a protective DQ allele, was found in 82% of patients as against 3% of controls, giving a lifetime absolute risk *in that population* of 13.7% (Reijonen *et al.* 1991). While this is inadequate as a basis for intervention, a strategy combining genetic and immune markers in series would allow much higher levels of risk to be defined within the general population.

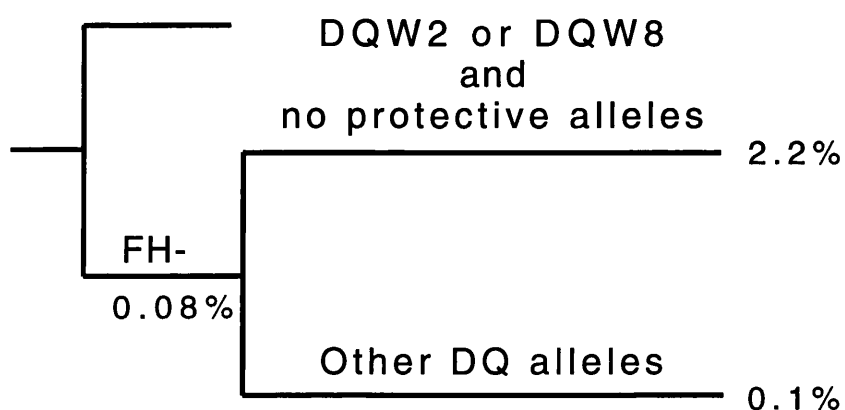


Figure 8.1

A theoretical approach using DQ alleles and ICA to predict IDDM in the general child population. The annual incidence of Type 1 diabetes in our region is 16/100,000 to age 20. A child has 0.08% risk of developing Type 1 diabetes with 5 years. In the Finnish population DQW8 with no protective DQ alleles is 27 times more common in Type 1 diabetes than in controls. If the combination achieved the same discrimination in our population, this combination would confer a 2.2% risk of Type 1 diabetes within 5 years. ICA ≥ 4 JDFu elevates the calculated risk to 35%.

In this approach the first test is used to identify a subgroup at increased risk, to which the second test can then be applied. The positive predictive value (PPV) of a test applied to a population of given disease prevalence can be given by the formula given above. Assuming that the sensitivity and specificity of ICA are similar in the general population and family members, the expected positive predictive

value of ICA ≥ 4 JDF units for developing Type 1 diabetes can be estimated using the sensitivity (88%) and specificity (96%) observed for the same assay in the Bart's-Windsor Family study. In schoolchildren this gives an expected positive predictive value of 2% within 5 years. If, however, ICA testing were confined to those already identified as genetically susceptible by DQ typing (again applying the Finnish oligonucleotide screen) the expected positive predictive value would rise to 35%. If subjects with this susceptible DQ combination and ICA ≥ 4 JDF units were followed for 10 years, almost 60% would be expected to develop Type 1 diabetes - a level of prediction equivalent to that achieved by ICA ≥ 20 JDF units in family members. This approach obviously needs testing, bearing in mind that the genetic marker can only be applied within the population in which it was developed. Moreover, any genetic screening method will only be useful if it can achieve a level of discrimination comparable to that reported in the Finnish study. Finally, it can be noted that tests which enhance the predictive power of ICA in family members could then be applied in similar fashion to ICA positive genetically susceptible individuals, and have the potential to reach equally high levels of specificity. A combined strategy using genetic markers, a number of humoral immune markers of risk and perhaps metabolic testing therefore promises precise prediction of Type 1 diabetes even in the absence of a family history of the disease.

Incident cases of childhood diabetes represent the tip of an iceberg of susceptibility. Only a very small proportion of those carrying known genetic susceptibility alleles within the general population are likely to develop Type 1 diabetes, and our study suggests that only a minority of those with high titers of ICA, the best-validated marker of risk, can be expected to progress to beta cell failure. This implies that the immune attack must pass through several stages, each perhaps involving multiple genetic or environmental determinants, before diabetes can develop. For example, the development of diabetes in the NOD mouse model of Type 1 diabetes is influenced by genes in the MHC complex, but genes on other chromosomes have been shown to control the intensity and rate of progress of insulinitis (Todd *et al.* 1991). The timing of exposure to environmental precipitants may be critical, and is most commonly thought to occur in utero or the

early post-natal period. Improved understanding of the natural history of ICA positivity, particularly in its early stages, may help to direct the search for environmental agents, and indeed it would seem logical to examine the role of such agents within the population who develop ICA, rather than within the subgroup which develops diabetes. Finally, as and when safe and effective forms of immune intervention become available, we can look forward to the day when population screening will form the basis for programmes to eradicate childhood diabetes.

CHAPTER 9 - CONCLUSION

9.1 Towards prediction of Type 1 diabetes

The term prediction, as applied to diabetes, implies a degree of inevitability that can, at present, only be achieved in a small segment of the population at increased risk of progression to Type 1 diabetes. It therefore seems more accurate and more honest to talk in terms of risk assessment rather than prediction and risk reduction rather than prevention. In this thesis I have outlined a decision tree analysis by which the probability of a given outcome is reached by series of steps, each

The Decision Tree

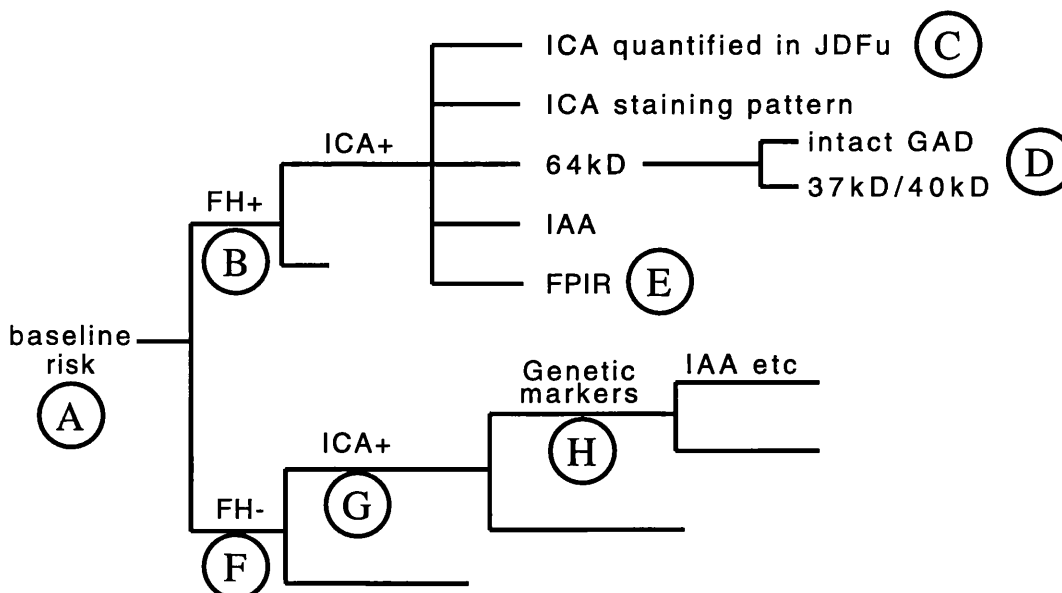


Figure 9.1 A Decision Tree representation of the work covered in the thesis

dependent on that which went before.

The work described in this thesis has allowed us to reach a clearer definition of a number of steps in this process, and this can be illustrated using the structure given in Figure 9.1.

(A): Baseline Risk. The incidence survey I carried out in the Oxford Region has produced some of the best validated data available on a population in the UK (Bingley *et al.* 1989a). This rate is substantially higher than those previously reported in this country, and confirms the impression of a rising incidence of the disease, as noted in many European countries (Bingley *et al.* 1989b). Our group later joined EURODIAB, a concerted action programme of the EC, and the protocol used in the Oxford Region formed the basis of that adopted for the study as a whole. This in turn has allowed accurate, comparable and up to date figures to be derived over much of Europe (Green *et al.* 1992b).

(B): Risk in Family Members. The population based design of the Bart's-Oxford Family Study has allowed overall risk in family members to be determined, with results comparable to those of previous studies (eg Tarn *et al.* 1988). More precise risk estimates could however be developed, showing, for example, that a sibling in the Oxford region has 13 times the risk of a child with no family history.

(C): Risk associated with ICA positivity. Quantitative measurement of ICA in terms of JDF units became possible in the course of this work, and I was therefore able to contribute towards assessment of the prognostic significance of ICA in family members in quantitative terms, taking advantage of the long follow-up provided by the Bart's-Windsor Family study, with sequential sera available for periods of up to 14 years in some individuals. This work emphasised the key influence of ICA titre in risk assessment (Bonifacio *et al.* 1990b), but the increased specificity reached at higher titres was balanced by a corresponding loss of sensitivity. I therefore went on to examine the possibility of combined analysis of ICA with other humoral markers.

(D): Risk associated with other humoral markers. The prognostic significance of autoantibodies to insulin, GAD and tryptic fragments of 64kD islet antigens were assessed (D). This analysis has shown that the sensitivity of screening could be considerably enhanced, without loss of specificity, by a combined marker approach. Thus, for example, a single blood test at initial screening examined for the 4 markers mentioned above could identify 88% of future cases of diabetes over the course of 10 years (Bingley *et al.* 1993c).

(E): Metabolic testing: I have evaluated the predictive value of the first phase insulin response (FPIR) to the IVGTT in a sample of our population (E), and contributed to a parallel study in ICA positive individuals with a background of other endocrine disease (Polyendocrine Study) (Wagner *et al.* submitted), and have considered the interaction of its predictive value with ICA level, pointing out the relevance of Bayes' theorem in this context.

Further, I have surveyed practice for performance of the IVGTT in 12 centres involved in screening for prediabetes and have shown wide divergence among them. This prompted development of the ICARUS consensus protocol, which is now standard at most centres round the world (Bingley *et al.* 1992a). I have since collaborated in a comparison of the reproducibility and variability of this protocol with others that involves different rates of glucose injection (McCulloch *et al.* 1993).

I have also used the Family Study to assess patterns of basal insulin secretion (Bingley *et al.* 1992b) and basal and stimulated secretion of other insulin-like molecules (Clark *et al.* 1989) as potential early metabolic markers of Type 1 diabetes.

(F): Prediction in the background Population: The first step towards this, accurate estimation of background risk, has been described above. This has allowed me to estimate the likelihood of Type 1 diabetes developing between the ages of 10 and 20 years in the population of the Oxford region, and hence the

prognostic significance of ICA in this population.

(G): The prevalence of ICA in the general childhood population: this has been measured in a population of 2,925 healthy schoolchildren, and a strikingly high prevalence was noted with a positive result in one child in 30. The prognostic significance of this observation would normally depend upon many years of prospective follow up, but an interim estimate was possible because I had access to the large family study population. Comparison with 274 age matched siblings showed that siblings were twice as likely to be ICA positive (at any titre), but were 15 times more likely to develop diabetes (E). The inference, that ICA are considerably less predictive of diabetes in the general population, accords well with the theoretical estimate I derived from Bayes' theorem (Bingley *et al.* 1993a).

(H): Genetic studies: The limited prognostic significance of ICA in this group can to a large extent be overcome by a combined analysis using genetic markers. This concept has now been described in 2 publications (Bingley *et al.* 1993a, Bingley *et al.* 1993b), and genetic testing for DQ haplotypes has now started in our children with ICA, allowing the model I have proposed, combining immune and genetic markers, to be tested prospectively.

9.2 Towards prevention

Work undertaken at the laboratory bench or in animal models of spontaneous diabetes has resulted in a number of models of beta cell damage, and a plethora of possible strategies of intervention up to and including possible 'magic bullets' capable of selective eradication of cells mediating the autoimmune attack on the beta cells. Basic science has thus opened up a range of exciting possibilities for the prevention of Type 1 diabetes. Clinical application of these new developments is more mundane, and rests upon familiar considerations of risk assessment, risk reduction and trial design. It is clearly unethical to start a clinical trial that lacks the statistical power to answer the question it sets out to address, yet small studies using cyclosporin, azathioprine, nicotinamide, insulin and other agents have already begun in ICA positive relatives and schoolchildren. Brief consideration of statistical

principles is sufficient to demonstrate that this approach can only succeed with agents that are nearly 100% effective in disease prevention. At present, it seems unlikely that we have access to such a "penicillin of prediabetes", and the studies described are likely not only to be fruitless, but also potentially harmful in directing attention away from agents with a useful though less dramatic margin of benefit.

How should we proceed? The first step should be to define risk as accurately as possible, and much of the work described in this thesis has been directed to that aim. I have pointed out that high levels of risk can be identified, but that "inevitable" progression can only be identified very close to the time of clinical onset. To intervene at a time when large numbers of beta cells are still viable therefore implies intervention in groups in whom diabetes is not inevitable, i.e. treatment directed towards groups in which some individuals would not, untreated, have developed diabetes in any case. Safety is therefore of prime importance when considering any such form of intervention.

The next step is to review the efficacy of those treatments considered acceptably safe. One major problem is that we lack an effective end-point other than the development of diabetes itself; no effective surrogate has as yet been identified. This limitation can, to some extent, be overcome by pilot studies in individuals in whom progression seems almost inevitable, identified for example by the "dual parameter" model of Jackson and Eisenbarth (Colman *et al.* 1988a), but, as pointed out above, this approach is likely to reject forms of intervention which are effective at earlier stages of the disease process, or only in subgroups of those at risk. At present, the combined criteria of relative safety and possible efficacy limit major clinical trials of secondary intervention to two agents, insulin or nicotinamide. It should also be remembered that it is just as important to be certain that an agent is ineffective, to avoid unhelpful and possibly harmful long term treatment, as it is to be sure that another does reduce progression to insulin dependent diabetes.

I have used the knowledge gained from family studies to design an intervention study using nicotinamide. This, the ENDIT trial, is shortly to begin in 20 European

countries and in Canada. The balance of likely risks and benefits have meant that the trial is being undertaken in family members with ICA \geq 20 JDF units. This seems appropriate for an agent as acceptable and apparently safe as nicotinamide, while in contrast trials of injected insulin are less acceptable to patients and are therefore taking place at a later stage of the disease process when FPIR is already lost. Other agents will come with different risk-benefit ratios; understanding of the full spectrum of risk assessment in pre-Type 1 diabetes will allow rational trial design for these too. In time, the same skills will, we hope, be needed when treatments tested in family members can be used to reduce the frequency of Type 1 diabetes in the population at large.

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APPENDIX 1

LABORATORY METHODS

These laboratory methods were used in studies in both the Bart's-Windsor and Bart's-Oxford Family studies (chapters 5-8) and to the study of prevalence of immune markers in schoolchildren (chapter 9).

Islet cell antibody determination:

This has been performed by Marion Shattock in the Departments of Immunology, at the Middlesex and University College Medical School and the London Hospital Medical College. Venous blood samples were separated after overnight storage at 4°, sera were stored at -20°. Undiluted sera were screened for conventional ICA-IgG by means of indirect immunofluorescence on 4µm cryostat sections of blood group O human pancreas using fluoresceinated rabbit anti-human IgG {74}. Samples were initially scored as negative, weak positive, positive or strongly positive. Samples scoring weak positive or greater were converted to JDF units. Samples collected before 1987 were converted to JDF units retrospectively; later samples were measured in JDF units at the time of first testing. Positive samples were titred by doubling dilutions in phosphate-buffered saline on tissue obtained from a single pancreas under standard incubation conditions (Bottazo *et al.* 1986a). Local standard sera calibrated to 2,4,8,16,32 and 80 JDF units were included in each assay. Endpoint titres were converted to JDF units (Bonifacio *et al.* 1988). The coefficients of variation between assays for control sera with 8, 32 and 80 JDF units tested in thirteen consecutive assays were 11%, 7% and 6% respectively, when expressed geometrically (SD log₂ JDF units/ mean log₂ JDF units). The threshold of ICA detection was 4 JDF units.

ICA selectivity: These studies were performed by Dr Maria Fonte and Dr Jessica McNally, Department of Immunology, London Hospital Medical College. Blocking experiments were performed on samples with ICA ≥ 20 JDF units using rat brain homogenate prepared by homogenization of Wistar Firth rat brain in 1 mmol/l 2-aminoethylisothiuronium bromide, 0.2 mmol/l pyridoxal phosphate, 1 mmol/l

EDTA, 1 mmol/l benzamidine, 25 mmol/l potassium phosphate, pH7.0 (homogenization buffer) and centrifugation at 100,000g for 30 min. Sera (10 μ l) were preincubated overnight at 4°C with 40 μ l of either rat brain homogenate or homogenate buffer. Each was titred to end point in PBS and tested in the ICA assay. ICA was classified as blocked if the end point titre in sample preincubated with rat brain homogenate was 2 or more doubling dilutions less than that preincubated with homogenization buffer only.

Insulin autoantibodies:

Insulin autoantibodies were measured by Hilary Gillmor, Dr Riccardo Bonifacio and Gary Chusney in the Department of Diabetes and Metabolism, St Bartholomew's Hospital. Insulin autoantibodies were assayed using a modification of the methods described by Palmer and Kurtz (Palmer *et al.* 1983, Kurtz *et al.* 1988). Sera were extracted using acid washed, Dextran coated charcoal to remove endogenous insulin. Serum was then incubated at 4°C with 40 mM phosphate buffer and radio-labelled human insulin (Amersham International, England), with and without cold insulin (Actrapid, Novo-Nordisk,). The immunoglobulin fraction was precipitated using polyethylene glycol 6000 and washed. The specific binding was calculated by subtracting the counts in the presence of cold insulin from the counts without the cold insulin. Results were expressed as % displaced binding. Insulin antibody positivity was defined as more than 3 standard deviations above the population mean. The coefficients of variation between assays for control sera with standard deviation scores 1.65, 4.4, 12.9, 38.2 and 193.4 were 46%, 25%, 26%, 27% and 9.5% respectively.

Glutamic acid decarboxylase antibodies: Antibodies to GAD in sera were measured by Dr Michael Christie, Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford by determining the enzyme activity immunoprecipitated by sera from a soluble extract of rat brain. Rat brain homogenate prepared by homogenization of Wistar Firth rat brain in a 10-fold volume of homogenization buffer (1 mM 2-aminoethylisothiuronium hydrobromide, 0.2 mM pyridoxal phosphate, 1 mM EDTA, 1 mM benzamidine, 25 mmol/l

potassium phosphate, pH7.0) and centrifugation at 100,000g for 30 min. The supernatant was diluted to a GAD activity of $5 \times 10^{-8} \text{ mmol.ml}^{-1}.16\text{h}^{-1}$ in homogenization buffer and immunoprecipitated with sera. Aliquots of homogenates and sera (12.5 μl) were incubated for 5 hours on ice then transferred to protein A sepharose pellets. These Protein A sepharose immunoprecipitates were incubated at 37°C with 25 μl of 5 mmol/l L-glutamic acid and 0.125 μCi [$1\text{-}^{14}\text{C}$] L-glutamic acid in homogenization buffer. $^{14}\text{CO}_2$ released during the reaction was absorbed onto filter paper soaked with 50 μl of 1 mmol/l hyamine hydroxide in methanol and quantified by liquid scintillation spectrometry. Enzyme activity in immunoprecipitates was calculated relative to that immunoprecipitated by a standard antibody-positive reference serum included in each assay. Test sera were regarded as positive if the relative activity exceeded 2 SD of the activity in sera from a group of control individuals.

Antibodies to islet antigen tryptic fragments: Antibodies to 37,000-Mr and 40,000-Mr tryptic fragments were measured by Dr Michael Christie, Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford by immunoprecipitation of ^{35}S -methionine labelled HIT cells.

Preparation of islet extracts: Labelled islets were homogenized on ice by 20 passes of a motor-driven Teflon/glass homogenizer in 1 ml of homogenization buffer (0.25 M sucrose, 10 mM Hepes (pH 7.4), L-methionine, 10 mM benzamidine, 0.1 mM *p*-chloromercuribenzenesulphonic acid and aprotinin), centrifuged at 100,000g for 30 min at 4°C, resuspended in 200 μl of homogenization buffer and respun at 4°C for 15 minutes. The pellet was resuspended in 2% (wt/vol) Triton X-114 detergent and subjected to temperature-induced phase separation. The detergent phase of the fractionation procedure was used in subsequent immunoprecipitations.

For trypsin treatments, the particulate fraction was resuspended in 200 μl of Hepes buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mM methionine, 10 mM benzamidine, 0.5% aprotinin) and was centrifuged at 100,000 g for 15 min. The pellet was resuspended in 400 μl of Hepes buffer, centrifuged at 10,000 g for 15 minutes. The pellet was resuspended in 10 mM Hepes (pH 7.4), 150 mM NaCl,

0.1% trypsin (wt/vol) was added and the suspension was incubated for 15 min on ice. Digestion was stopped by the addition of an equal volume of Hepes buffer with aprotinin and the suspension was centrifuged at 10,000 *g* for 15 minutes at 4°C to remove particulate material.

Immunoprecipitation of islet cell antigens by sera: Protease or detergent-solubilized extracts of islets were initially pre-cleared by incubation with 50 µl of normal human serum, negative for antibodies to the 64 kD antigen, for 2 hours at 4°C followed by binding to 100 µl of protein A-Sepharose (Pharmacia, Baie d'Urfe, Quebec) for 45 min at 4°C. A second preclearance was performed with 50 µl of serum for 18 hours at 4°C and 100 µl of protein A-Sepharose for 45 min. Extracts were incubated with 25 µl of test serum for 5 hours at 4°C and immune complexes were isolated on 50µl of protein A-Sepharose. Immunoprecipitates were washed five times in 1 ml of 10mM Hepes (pH7.4), 155 mM NaCl, 0.5% Triton X-114, 10 mM benzamidine, 0.5 mg/ml BSA, and once in 1 ml of water. Elution and electrophoresis on 10% SDS-polyacrylamide gels were performed as described (ref). Radioactive polypeptides immunoprecipitated were visualized by autoradiography. Antigens precipitated by individual sera were quantified by densitometric scanning of autoradiograms and standardized by comparison with immunoprecipitates from the standard human diabetic serum run in each experiment.

Blood glucose:

Intravenous glucose tolerance tests and oral glucose tolerance tests (*Section 6.3.4*): In all studies venous blood was collected into fluoride-oxalate and analysed within 4 hours. Whole blood was assayed hours by a glucose oxidase method using a YSI model 23AM glucose analyzer (Yellow Springs Instrument Co., Ohio, U.S.A.) The interassay coefficient of variation was 5.5% at 2.2 mmol/L and 2.7% at 11.6 mmol/L.

Schoolchildren study (*Section 8.4*): Samples were collected into fluoride oxalate and spun, separated and the plasma frozen on dry ice immediately. Plasma was stored at -20°C until assayed. Plasma glucose was measured by the hexokinase/glucose-6-dehydrogenase method using an autoanalyser (CPA

biochemical analyser, Coulter Electronics Inc, Florida, USA). The inter-assay c.v. was 4.6% at 2.5 mmol/l and 4.0% at 15 mmol/l.

Plasma insulin:

Intravenous glucose tolerance tests: Immunoreactive insulin was assayed by Hilary Gillmor and Alistair Williams, Department of Diabetes and Metabolism, St Bartholomew's Hospital. It was determined using a double-antibody radioimmunoassay with guinea-pig anti-human-insulin first antibody (Immunodiagnostic Systems Ltd, Washington, Tyne and Wear) and sheep anti-guinea pig FC (International Laboratory Systems, London) as the second antibody. ¹²⁵I labelled human insulin (specific activity 2000 ci/mmol; Amersham International, Bucks.) was used as tracer with human insulin standards (Novo Biolabs, Bagsvaerd, Denmark).

Patterns of basal insulin secretion: In order to optimize the performance of the assay at fasting insulin levels a slightly modified insulin assay was used. This was developed and performed by Alistair Williams, Department of Diabetes and Metabolism, St Bartholomew's Hospital. All samples from each study were assayed in duplicate in the same assay. An in-house guinea-pig anti-human-insulin first antibody was used. Sheep anti-guinea pig FC (ILS., London, England) was used as the second antibody as above. ¹²⁵I labelled human insulin (specific activity 1500 ci/mmol; Medgenix, Fleurus, Belgium) was used as tracer with human insulin standards (Novo Biolabs, Bagsvaerd, Denmark). The median intra-assay coefficient of variation of the duplicates for the 18 assays at mean insulin concentration 5.7 mU/L was 5.8% (range 3.6-18.8%).

APPENDIX 2

QUESTIONNAIRE
METABOLIC TESTING IN PRE-DIABETES

[A] DEFINITION OF DIABETES**1. Oral glucose tolerance test:**Diagnostic criteria used NDDG/WHO/OtherIf other: dose of glucose (g) _____ diagnostic criteria used _____**2. End-point of follow-up of pre-diabetics:**

<i>(ie which end point</i>	Diabetic OGTT	___
<i>do you use in risk</i>	Onset of symptoms	___
<i>calculation?)</i>	Start of insulin therapy	___
	Other (please specify)	___

[B] INTRAVENOUS GLUCOSE TOLERANCE TEST PROCEDURE**1. Diet prior to test:**Normal/SpecialIf special, what and for how long? __________**2. Fasting prior to test:**Duration _____**3. Time of day of starting test:**Accepted range _____**4. Glucose infusion:**Concentration of dextrose used: _____If diluted: diluent used _____final concentration _____Dose: g/kg _____maximum dose (g) _____

5. **Method of infusion:**
- | | |
|-------------------------|-----|
| gravity driven infusion | ___ |
| manually driven syringe | ___ |
| pump | ___ |
| other (please specify) | ___ |

If pump use, what type? _____

- Timing:
- | | |
|----------------------------|--------|
| Is infusion timed | yes/no |
| Duration | ___ |
| Range of duration accepted | ___ |

6. Timing of samples:

'Run in' time between cannulation and 1st baseline samples ___

- What is taken as time zero?
- | | |
|------------------------|-----|
| start of infusion | ___ |
| midpoint of infusion | ___ |
| end of infusion | ___ |
| other (please specify) | ___ |

Times samples taken (minutes) _____

7. Sampling:

Number of cannulae inserted one/two

- Sites
- | | |
|------------------------|-----|
| antecubital fossa | ___ |
| hand | ___ |
| other (please specify) | ___ |

Samples taken through same cannula as glucose is given? yes/no

Any attempt made to 'arterialize' blood? yes/no

If so what and what PaO₂ is achieved? _____

2. Glucose Assay:

Sample assayed whole blood/plasma

Method used glucose oxidase _____
hexokinase _____
other (please specify) _____

9. Insulin Assay:

Aprotinin (Trasylol) added to samples yes/no

If test performed away from your laboratory:

maximum delay before samples are separated _____

separated samples transported frozen/unfrozen

usual delay before reaching your laboratory _____

Assay commercial kit (please name) _____
customized assay _____

Quality assurance internal/external/both

Source of quality control material:

internal: _____

external: _____

Insulin concentrations of quality control material: _____

Inter-assay coefficient of variation at each level

Insulin concentration	C.V. (%)
_____	_____
_____	_____
_____	_____
_____	_____

Name: _____

Address: _____

Telephone No. _____

Fax No. _____

Please return to Edwin Gale,
Department of Diabetes and Metabolism,
St Bartholomew's Hospital,
London EC4A 7BE,
England.