

**Early onset of diabetes in the proband is the major determinant of risk in HLA  
DR3-DQ2/DR4-DQ8 siblings**

**Running title:** Identifying children at very high genetic risk of type 1 diabetes

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**ABSTRACT**

Islet autoimmunity is initiated in infancy, and primary prevention trials require children at high genetic risk to be identified before autoantibodies appear. To inform screening strategies, we evaluated risks of autoimmunity and diabetes associated with HLA DR3-DQ2/DR4-DQ8 in UK families. Extended HLA haplotypes were determined in 2,134 siblings from the Bart's-Oxford Study followed to median age 22 years. Risks of diabetes and islet autoimmunity ( $\geq 2$  antibodies) were estimated by survival analysis. Of 138 informative DR3-DQ2/DR4-DQ8 siblings, 63% shared both haplotypes with their diabetic proband, 29% shared one and 8% neither. In HLA-identical DR3-DQ2/DR4-DQ8 siblings, the cumulative risk of diabetes by age 15 was 17% (vs. 6% in those sharing one haplotype or none,  $p=0.095$ ). Risk varied, however, with diabetes age-at-onset in the proband; the cumulative risk of autoimmunity and/or diabetes by age 15 was 61% in siblings of probands diagnosed below age 10 years compared with only 4.7% in those diagnosed after age 10 years ( $p<0.001$ ), and proband age-at-diagnosis, but not HLA haplotype sharing, was an independent determinant of sibling risk. This suggests that non-HLA genes or epigenetic/environmental factors that accelerate progression of type 1 diabetes in the proband impact strongly on risk in siblings.

## INTRODUCTION

Type 1 diabetes results from autoimmune destruction of insulin-producing beta cells, a process that begins early in life. Islet autoantibodies are detectable by the age of 5 years in children who go on to develop diabetes, in most by the age of 2 years and autoantibodies to insulin (generally the first to appear) have been detected as early as 6-12 months (1; 2). Established islet autoimmunity is difficult to modulate and strategies aimed at primary prevention before initiation of autoimmunity are therefore needed. Trials of potential interventions at this stage will have to recruit children on the basis of genetic risk alone.

Recent whole genome studies have resulted in an explosion of information regarding genetic susceptibility to type 1 diabetes, but the human leucocyte antigen (HLA) region remains the most important genetic determinant. Over 90% of children with type 1 diabetes carry the HLA class II haplotypes *DRB1\*03-DQB1\*02:01* (DR3-DQ2) and/or *DRB1\*04-DQB1\*03:02* (DR4-DQ8), and the highest risk DR3-DQ2/DR4-DQ8 diplotype is present in 50% of very early onset cases (3). We and others have previously shown that the age of diagnosis of children with type 1 diabetes and the number of HLA haplotypes they share, are determinants of risk of diabetes in their siblings (3; 4). HLA class II determined risk can also be modulated by other factors close to, or within, the HLA region as well as by non-HLA genes (5).

Current trials to prevent initiation of islet autoimmunity use HLA-based risk assessment to identify individuals eligible for inclusion, but employ different

strategies to assign risk depending on the potential toxicity of the planned intervention (6; 7). The international Trial to Reduce IDDM in the Genetically at Risk (TRIGR) combined HLA-determined genetic susceptibility with a first degree family history of type 1 diabetes to identify children with an estimated 15.4% risk of type 1 diabetes by age 10 suitable for a dietary modification trial (8). In the Finnish Diabetes Prediction and Prevention (DIPP study), high risk HLA combinations identified children with no family history of type 1 diabetes who had 36.4% (95% CI 20.0-52.8) risk of developing two or more antibodies and 2.4% risk of diabetes within 5 years (9).

Other primary prevention trials are seeking to identify infants at much higher genetic risk. A study of 48 families with siblings matched for the high-risk DR3-DQ2/DR4-DQ8 genotype participating in the Diabetes Autoimmunity Study in the Young (DAISY) prospective study from birth reported a dramatically increased risk of islet autoimmunity and diabetes in 29 siblings who shared both extended high-risk haplotypes identical by descent (IBD) with the diabetic proband. In this group, the estimated risk of islet autoimmunity was 63% (95% CI 44-85) by age 7 and risk of diabetes was 55% (95% CI 30-80) by age 12 (10). This compares with 7% risk of diabetes by age 12 in HLA DR/DQ identical siblings who were not identical by descent. This strategy for identifying a subgroup of DR3-DQ2/DR4-DQ8 siblings at 'extreme risk' based on IBD haplotypes has been used to recruit children for pre-POINT – a clinical trial assessing the efficacy of oral insulin given very early in life (7).

More recently however, a smaller study in 14 HLA DR3-DQ2/DR4-DQ8-IBD siblings in the Dutch Kolibrie cohort failed to replicate this extreme risk as only 2 of the 14 developed type 1 diabetes by the age of 15 (11). The aims of our study were therefore to evaluate the risks of autoimmunity and diabetes associated with the extended DR3-DQ2/DR4-DQ8 diplotype in a larger group of UK families with over 25 years follow-up in the population-based Bart's-Oxford (BOX) study of childhood diabetes and to identify other factors that might contribute to the discrepant results of previous studies.

## RESEARCH DESIGN AND METHODS

### Subjects

The participants in this study were siblings of probands who had been notified to the BOX study which, since 1985, has recruited more than 95% of the families of children who have developed type 1 diabetes before the age of 21 years in the former Oxford Health Authority Region, United Kingdom (12). The background population is 95% Caucasian and the remainder originate mainly from the Indian subcontinent (data from Office of Population Censuses and Surveys for 1991). All cases of type 1 diabetes were referred by diabetes specialists based on diagnosis according to World Health Organization criteria (13) and a clinical requirement for insulin treatment from diagnosis. Patients with secondary diabetes, known genetic subtypes including Maturity Onset Diabetes of the Young, or clinical type 2 diabetes were not included in the study.

2,134 siblings from 1,745 families were recruited to the study between 1985 and 2002. HLA genotype data were available on 1,440 (67%) siblings. Of these, 191 had the high risk HLA *DR3/DR4* genotype. Haplotype sharing was uncertain in 53 proband/sibling pairs. 138 siblings were therefore included in the analysis (Figure 1).

### Clinical data and follow-up

Clinical data and family history of diabetes, including diabetes type and age of diagnosis were collected by interview at study entry. The median time between diagnosis of the proband and enrolment of siblings was 0.2 years (range 0.1-2.5). Of

138 siblings, seven had developed diabetes prior to study entry. Families were followed by annual visit, telephone call or postal questionnaire, and the diabetes status of every family member was ascertained on each occasion. A further 12 siblings developed diabetes during follow-up. Serum samples for islet autoantibody determination were collected routinely from consenting unaffected siblings at study entry with repeat sampling if any family member was positive for one or more autoantibody. At least one sample was tested for autoantibodies in 94 of 131 siblings (72%) who were non-diabetic at study entry. The first available sample was measured in each sibling and all those that were found to be islet autoantibody positive in this study were identified on the first sample. The median age at first sample collection was 12.9 years (IQR 8.1-16.4), at last sample collection 13.2 years (IQR 8.4-16.8) and at last contact 25.4 years (IQR 20.6-29.9).

### **HLA Class I and II Genotyping**

HLA class II *DRB1* and *DQB1* and HLA-class I *A* and *B* genotyping was carried out by polymerase chain reaction using a DYNAL Reli SSO system (Invitrogen DYNAL, UK). The type 1 diabetes associated haplotype *HLA-DRB1\*04-DQB1\*03:02* was abbreviated to DR4-DQ8 and *HLA-DRB1\*03-DQB1\*02:01* abbreviated to DR3-DQ2. Identical by descent (IBD) siblings were recognized by analysing transmission of extended haplotypes from HLA class II to HLA class IA from both parents to affected and unaffected children (10). This will be described as extended HLA identity.

### **Islet autoantibodies**

Samples from all participants were tested for ICA and autoantibodies to glutamate decarboxylase (GAD), islet antigen-2 (IA-2), and insulin as previously described (14). Data on antibodies to zinc transporter 8 (ZnT8) were not available in this cohort. Samples were judged antibody positive if levels were at, or above, the 97.5th percentile of levels in a control population of 2,860 schoolchildren from the same region (15). The ICA assay was shown to have 81% sensitivity with 86% specificity in the First Immunology of Diabetes Society (IDS) Combined Antibody Workshop (16). Radiobinding assays for autoantibodies to GAD, IA-2, and insulin, had 91% sensitivity with 99% specificity, and 74% sensitivity with 99% specificity, and 58% sensitivity with 99% specificity, respectively in the first Diabetes Antibody Standardization Proficiency Evaluation (17).

### **Data analysis**

Risks of diabetes and autoimmunity in siblings were estimated by survival analysis using the log-rank test to compare groups based on HLA sharing and age of diagnosis of diabetes in the proband. Islet autoimmunity was defined as  $\geq 2$  autoantibodies above the 97.5<sup>th</sup> centile. Cumulative risks were calculated for clinical diagnosis of diabetes and for a composite endpoint of diabetes and/or islet autoimmunity. For the analyses of diabetes risk, time to event was defined from birth to diabetes development or date of last contact, and for risk of diabetes and/or autoimmunity time to event was defined as birth to diabetes/first detection of two or more antibodies or date of last sample. Time-to-event outcomes for combined predictive markers (HLA haplotype sharing and age of diagnosis of the diabetic proband) were analyzed using Cox proportional hazards models. As samples for autoantibody testing were not available from birth in this cohort, the cumulative risks of positivity for two or more

islet autoantibodies were calculated using the following assumptions: (i) that all siblings who were antibody negative had not previously been positive for two or more antibodies, (ii) that all siblings with at least two autoantibodies remained in this category until diagnosis of diabetes or the date of last contact (end of follow-up), (iii) the date of collection of the first antibody positive sample was used as the initiation of islet autoimmunity. The risk of the composite end-point of diabetes and/autoimmunity could not be calculated in 30 individuals for whom no serum sample was available and who remained non-diabetic at last contact. A total of 108 individuals were therefore included in that analysis.

## RESULTS

Of 138 informative HLA DR3-DQ2/DR4-DQ8 siblings, 87 (63%) shared both haplotypes with their diabetic proband, 40 (29%) shared one haplotype and 11 (8%) shared neither. Of the siblings analysed, 69 (50%) were from families in which the diabetic proband was below age 10 years at the time of diagnosis (median 5.4 years, IQR 2.45-7.65). and the remaining 69 from families in which the proband was diagnosed after this age (median 13.4 years, IQR 11.4-15.2). The analysis included 34 siblings from multiplex families in which more than one first degree relative had type 1 diabetes. The characteristics of the cohort are shown in Table 1.

### **Cumulative Risk of Diabetes by Extended HLA identity and Age at Diagnosis of the Proband**

By the time of analysis, 19 of the 138 DR3-DQ2/DR4-DQ8 siblings had developed type 1 diabetes. Of these, 15 (11%) were HLA identical, 3 (2%) were haplo-identical and 1 (1%) did not share either high risk haplotype with the proband; In 16 diabetic siblings, diabetes had been diagnosed in the proband before age 10 years, and in 3 siblings the proband had been diagnosed after age 10.

The cumulative risk of diabetes by age 15 was 16.6% (95% CI 8.6-24.6) in the HLA-identical siblings, compared with 6% (95% CI 0-12.4) in those sharing one haplotype or none (  $p=0.095$ ), and was 22.5% (95% CI 12.5-32.5) in the siblings of probands diagnosed below age 10, compared with 3% (95% CI 0-6.8) in those whose proband

was diagnosed after age 10 ( $p=0.001$ ). Among the 34 siblings from multiplex families the risk of diabetes was 13.4% (95% CI 6.9-19.9).

As shown in Figure 2(a), the effect of extended HLA haplotypes on cumulative risk of diabetes varied with the age of onset of type 1 diabetes in the proband. Among the 50 HLA-identical DR3-DQ2/DR4-DQ8 siblings from families in which the proband was diagnosed before age 10, the cumulative risk of diabetes by age 15 was 25% (95% CI 13-38 ) compared with 5.4% (95% CI 0 - 13) in the 37 siblings from families in which the proband was diagnosed after age 10 ( $p=0.009$ ).

#### **Cumulative Risk of Diabetes and/or Islet Autoimmunity**

Of the 108 siblings included in this analysis, 8 had progressed to the combined endpoint of islet autoimmunity ( $\geq 2$  antibodies) and/or diabetes by age 5, 17 by age 10 and 23 by age 15. The overall cumulative risk of islet autoimmunity and/or diabetes by age 15 was 26% (95% CI 16-35%); 31% (18-44) in HLA-identical siblings, compared with 18% (4.5-31) in those sharing one haplotype or none ( $p=0.113$ ). In 53 siblings from families in which the proband was diagnosed before age 10, the risk of islet autoimmunity and/or diabetes was 52% (33-70%) by age 15. This compared with 8.6% (0-17) risk by age 15 in 55 siblings from families in which the proband was diagnosed after age 10 ( $p<0.001$ ).

As shown in Figure 2(b), the age of onset of type 1 diabetes in the proband modulated the combined risk of autoimmunity and/or diabetes: among the 36 HLA-identical DR3-DQ2/DR4-DQ8 siblings from families in which the proband was diagnosed before age 10, the cumulative risk of autoimmunity and/or diabetes by age 15 was

61% (95% CI 13-38 ) compared with 4.7% (95% CI 0 - 16 ) in the 29 siblings from families in which the proband was diagnosed after age 10 ( $p < 0.0001$ ).

Cox regression analysis showed that the age of diabetes diagnosis of the proband was an independent determinant of risk of diabetes and of the combined endpoint of autoimmunity and/or diabetes but HLA haplotype sharing was not. Coming from a family in which the proband was diagnosed before age 10 was associated with 5.9-fold increased risk of diabetes (95% CI 1.7-20.5,  $p = 0.006$ ) and 7.4-fold increased risk (95% CI 2.7 - 20.3,  $p < 0.001$ ) of the combined endpoint, while being HLA identical with the proband was associated with hazard ratio 1.9 (0.6 – 5.7,  $p = 0.265$ ) for development of diabetes and hazard ratio 1.6 (0.6 - 3.8,  $p = 0.322$ ) for the combined endpoint.

## DISCUSSION

In this study, we examined the risk of diabetes and islet autoimmunity in HLA DR3-DQ2/DR4-DQ8 siblings of children with type 1 diabetes in relation to the number of extended HLA haplotypes shared with the proband and to the age at which the proband's diabetes was diagnosed. We observed that increased risk was concentrated in the sub-group of siblings who were HLA-identical to the proband (sharing both haplotypes) compared with siblings who shared one haplotype or none and have shown for the first time that risk is higher in the DR3-DQ2/DR4-DQ8 siblings of children whose diabetes was diagnosed at a young age and that this factor is the dominant determinant.

The existing literature in this area has yielded conflicting results. Using a similar approach to that we have adopted, a study of 48 families from the DAISY cohort, reported that HLA DR3-DQ2/DR4-DQ8 siblings IBD with the proband for both extended HLA haplotypes had a 55% (95% CI 30-80) risk of diabetes by age 12 (10). The same study also found a 63% (95% CI 41-84) risk of autoimmunity by age 7 and 85% (95% CI 61-100) by age 15 in this group, compared with 20% (95% CI 0-42) by age 15 for those who shared only one haplotypes or none. More recently, however, a smaller study of 14 DR3-DQ2/DR4-DQ8 siblings sharing the IBD haplotypes failed to replicate these findings after up to 12 years follow-up (11).

Our study of 138 informative families under regular follow-up represents the largest study of IBD extended HLA haplotypes and sibling risk to date. As reported in the DAISY cohort, we found that HLA-identical DR3-DQ2/DR4-DQ8 siblings were at increased risk but the magnitude of risk was less. The estimates of diabetes/autoimmunity by age 15 were similarly high in both studies despite different definitions of islet autoimmunity; the DAISY study used single islet autoantibody positivity while the BOX study used  $\geq 2$  autoantibodies, a more accurate predictor of diabetes.

We have shown that this risk was concentrated in siblings of probands diagnosed under the age of 10 years, while there was little association with the degree of haplotype sharing in siblings of children diagnosed after age 10. The influence of age-of-onset of the proband on first degree relative risk has been described previously; the risk of progression to diabetes in monozygotic twins of probands diagnosed before 15 years of age was almost twice that in twins of probands diagnosed after age 25, and twins of patients diagnosed below age 10 were estimated to have a 50% risk of diabetes within 6 years (18). Although the investigators noted that this increased risk did not appear to be HLA-related the frequency of the high risk HLA DR2-DQ2/DR4-DQ8 diplotype was relatively low; only 15 of 79 twin pairs (19%) diagnosed before the age of 25 years were positive for HLA DR2-DQ2/DR4-DQ8 compared with 5 of 21 twin pairs (24%) diagnosed later. We also previously showed that the overall risk of diabetes was higher in siblings of probands diagnosed with type 1 diabetes very early in life (3). Given the high frequency of the HLA genotype DR3-DQ2/DR4-DQ8 we observed in early onset type 1 diabetes, as have

others (19), our interpretation was that increased HLA susceptibility in siblings of early-onset probands was the dominant mediator of increased diabetes risk. Our current study disproves this.

Rapid progression resulting in early onset of type 1 diabetes is associated with less preserved beta cell function (20) suggesting a more aggressive autoimmune response. Most genetic studies of early onset to date have focused on the HLA class I and II particularly DR3/4-DQ8, *A\*24:02*, *B\*18:01* and *B\*39:06* (21-23) as well as class I C/NK cell receptor interactions (24) yet our study strongly indicates that important factors outside the HLA modulate risk. These are likely to include other genes but also potentially other epigenetic and environmental modulators. Our data support a recent study from the Nationwide Italian Twin Study highlighting the importance of exposures in fetal or neonatal life over genetics on concordance rates (25). There is increasing focus on maternal and intrauterine conditions that modulate genetic risk of type 1 diabetes (26). Potential factors include viral infections (27), maternally-derived microbiome (28), weight gain in early life (29) and maternal/fetal cell transfer in pregnancy (30). Further studies focused on the determinants of early onset autoimmune diabetes are required.

A limitation of our study is that islet autoantibody testing did not start in infancy and was not carried out at defined intervals in all families. We therefore had to make a number of assumptions including use of the date of the first islet autoantibody positive sample as the date of antibody appearance, although antibodies may have been present for many years. This assumption is likely to have resulted in underestimation of the risk of islet autoantibody positivity in younger siblings. In

addition, we may have missed transient antibody positivity that would have been detected in a prospective study from birth. A further consideration is that data on antibodies for zinc transporter 8 were not available in this cohort. Our estimates therefore probably represent the lower limits of the true risk of islet autoimmunity.

This study represents an important clarification of increased risk of diabetes in IBD siblings of children with type 1 diabetes with long term follow up. It highlights the importance of integrating clinical data such as age at onset of the proband and family history in estimating individual risk. It also offers a simple way of stratifying risk in *DR3-DQ2/DR4-DQ2* siblings to identify those at extremely high genetic risk for future therapeutic trials.

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Professor Bingley is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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RJA, KMG, AW and IW researched data and co-ordinated sample collection and analysis, PJB carried out the statistical analysis and all authors contributed to discussion. KMG and PJB wrote the manuscript. All authors reviewed, edited, and discussed the manuscript.

**Table 1:** The characteristics of the cohort

	<b>HLA identical (87)</b>	<b>Haplo-identical (40)</b>	<b>Non-identical (11)</b>
<b>Gender (M:F)</b>	37:50	26:14	2:9
<b>Age at last contact (median, IQR)</b>	22.3 yr (15.6-28.2)	23.6 yr (19.5-30.5)	26 yr (21.0-28.4)
<b>Age of diagnosis of the proband</b>	9.2 yr (3.9-13.3)	11 yr (6.1-13.8)	13.3 yr (10.5-13.5)
<b>Diabetes (sibling)</b>	15	3	1
<b>Age at diagnosis of sibling</b>	5.7 (3.3-10.9)	6.6 (4.7-7.5)	1.8
<b>Multiplex family (2 or more affected first degree relatives)</b>	19	9	6

**Figure legends**

**Figure 1:** Characteristics of participants included in the study

**Figure 2:** Cumulative risks of (a) diabetes and (b) diabetes and/or islet autoimmunity in DR4-DQ8/DR3 siblings subdivided by HLA-sharing with the proband and age-at diagnosis of the proband. Survival curves are presented for HLA identical siblings of probands diagnosed before (solid line) and after age 10 years (dotted line) and haplo or non-identical siblings of probands diagnosed before (dashed line) or after (dash-dot line) age 10 years.

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Siblings in the BOX study

2134



Genotype available

1440



HLA-DR3/DR4-DQ8

191



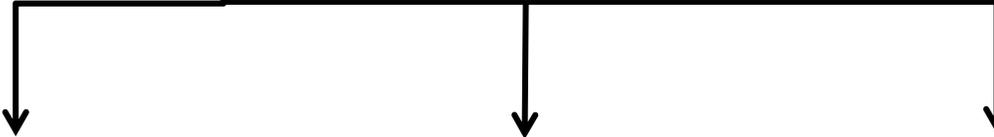
Haplotype sharing uncertain

- incomplete family samples (30)
- homozygous parents (23)



Included in the diabetes end-point analysis

138



HLA identical  
to proband

87



HLA  
haplo-identical

40



HLA  
non-identical

11



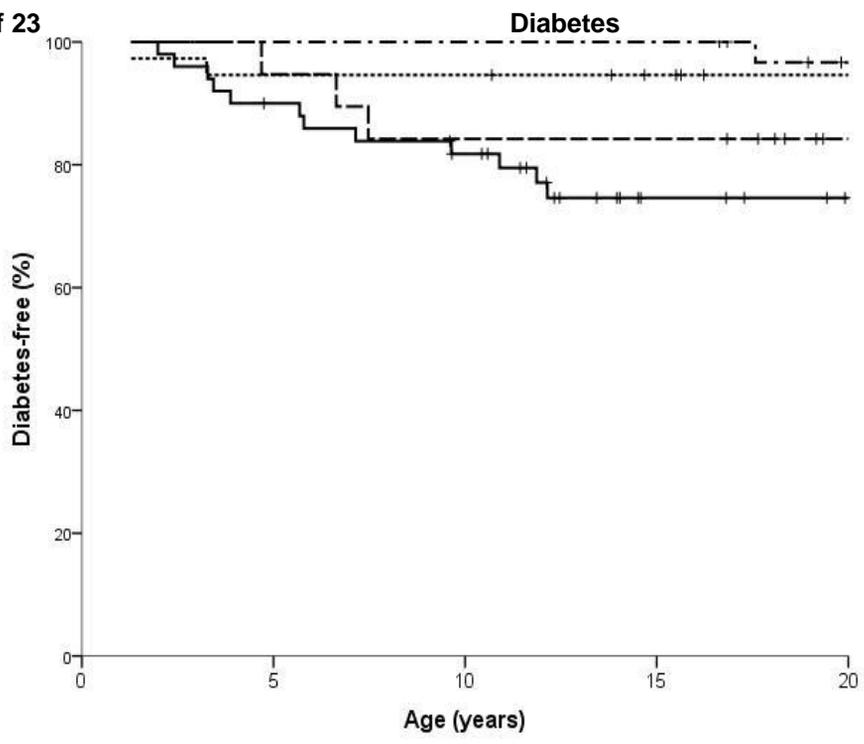
End-point not available

30

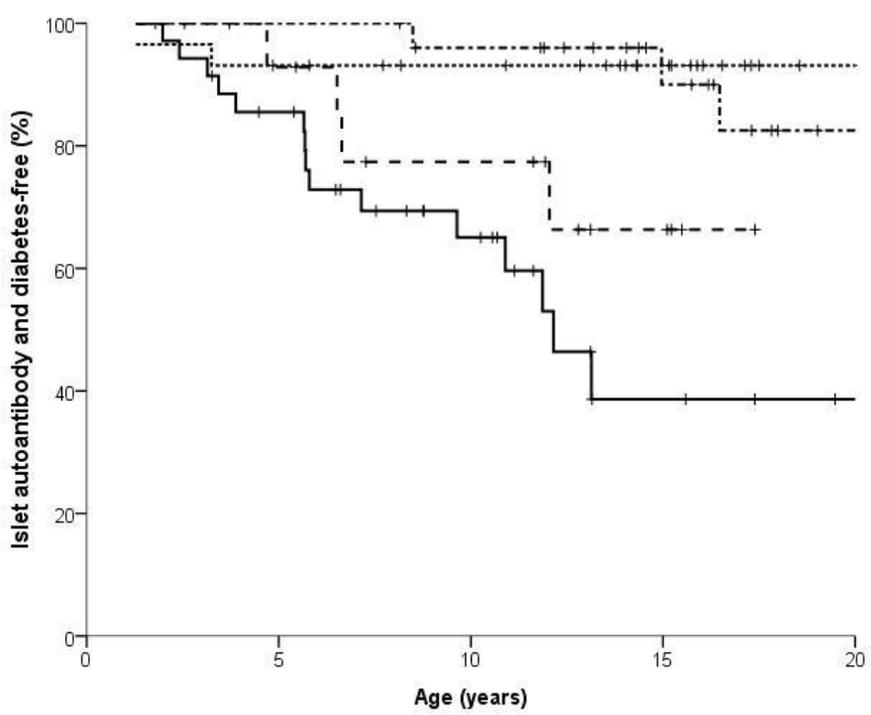


Included in the diabetes and islet autoimmunity end-point analysis

108



50	45	38	23	HLA-identical & proband diagnosis <age 10
37	35	35	32	HLA-identical & proband diagnosis ≥ age 10
19	18	16	16	Haplo- /non -identical & proband diagnosis < age 10
32	29	29	29	Haplo- /non -identical & proband diagnosis ≥ age 10



36	28	15	4	HLA-identical & proband diagnosis <age 10
29	26	23	16	HLA-identical & proband diagnosis ≥ age 10
17	13	9	4	Haplo- /non -identical & proband diagnosis < age 10
26	24	24	15	Haplo- /non -identical & proband diagnosis ≥ age 10