High Familial Risk and Genetic Susceptibility in Early Onset Childhood Diabetes

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Early onset type 1 diabetes is associated with rapid β-cell failure and high levels of HLA-mediated genetic susceptibility. We examined familial risk of disease in relation to age at onset in 1,299 families participating in the Bart's Oxford population-based family study of type 1 diabetes. Risk of diabetes was estimated by survival analysis in 1,430 siblings and 2,419 parents and related to age at onset in the proband. Unaffected relatives at high risk were identified by measurement of islet autoantibodies, and HLA class II genotyping was performed in probands where DNA was available (573 children). The cumulative risk of diabetes by age 20 years was 11.7% in siblings of probands diagnosed before age 5 years, compared with 3.6% for ages 5–9 years and 2.3% for ages 10–14 years ($P < 0.0001$). In parents, the cumulative risk by age 40 years was 5.9% if the proband was diagnosed before age 5 years, compared with 3.7% for ages 5–9 years and 3.7% for ages 10–14 years ($P = 0.04$). Of 1,169 unaffected siblings tested at study entry, 7.3% had two or more autoantibody markers if the proband was diagnosed before age 5 years, compared with 2.2 and 2.4%, respectively, for ages 5–9 and 10–14 years ($P = 0.002$). The frequency of the highest risk genotype decreased with increasing age at onset. Of children diagnosed before age 5 years, 52% were heterozygous for HLA DRB1*03-DQA1*0501-DQB1*02/DRB1*04-DQA1*0301-DQB1*0302 compared with 32% and 33%, respectively, for children diagnosed at ages 5–9 and 10–14 years ($P < 0.001$). Diabetes with onset before age 5 years is therefore a marker of high familial risk. *Diabetes* 51:210–214, 2002

The incidence of childhood-onset diabetes is increasing across Europe, most rapidly in those with onset before the age of 5 years (1). Early onset diabetes results from an aggressive disease process characterized by high genetic risk (2), high titers of islet autoantibodies (3), severe metabolic decompensation at presentation, and rapid failure of C-peptide secretion after diagnosis (2). Children who develop diabetes at a very young age may therefore hold important clues for our understanding of the etiology and changing natural history of type 1 diabetes.

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IAA, insulin autoantibody; ICA, islet cell antibody; IDS, Immunology of Diabetes Society; JDF, Juvenile Diabetes Foundation; PCR, polymerase chain reaction.

In type 1 diabetes, early onset suggests a major role for genetic factors, with a strong inverse association between age at diagnosis and prevalence of HLA alleles conferring susceptibility (4). High genetic susceptibility in the proband implies increased genetic susceptibility and disease risk in their parents and siblings. This has been confirmed by many family studies (5). This risk is highest in monozygotic twins (6), followed by HLA-identical and then HLA-haploidentical siblings (7). Because genetic risk is inversely correlated with age at onset, it might be anticipated that this would also apply to familial risk, and some evidence of this exists. For example, monozygotic twins are at highest risk when the co-twin was diagnosed under the age of 5 years (8). Some studies have reported that early onset in a child increases risk in parents and siblings (9,10), but others have produced conflicting findings (11).

We therefore set out to examine the relation between age at diagnosis in the proband and risk of diabetes in their parents and siblings, using both diabetes development and the prevalence of autoantibody markers of risk as our end points. Our aim was to clarify the relation between proband age and familial risk, because this may be of practical value in screening programs and could improve our understanding of the emerging epidemic of type 1 diabetes in the very young.

RESEARCH DESIGN AND METHODS

Study population. The Bart's-Oxford prospective family study has recruited parents and siblings of patients with type 1 diabetes diagnosed before age 15 years from within the Oxford Regional Health Authority area in England since 1985 (12,13). The overall levels of case ascertainment estimated from independent survey of general practitioners in the region are >95%, and 89% of eligible families have participated in the prospective study. This report is based on the study population as of 1 March 2000, by which date 1,299 families had been recruited. Details of diabetes in all first-degree relatives were collected by interview. Venous blood samples were taken from all consenting family members and analyzed for islet cell antibodies (ICAs) and antibodies to GAD and protein tyrosine phosphatase IA-2. Insulin autoantibodies (IAAs) were also measured in samples from nondiabetic family members (14). Families were followed by annual visit or telephone call, and the diabetes status of every family member was ascertained on each occasion. The study cohort comprised the families of 298 children diagnosed before age 5 years, 442 diagnosed between the ages of 5 and 10 years, and 559 diagnosed between ages 10 and 15 years and included 3,878 first-degree relatives (1,430 siblings and 2,448 parents). A total of 5 mothers had a history of gestational diabetes, 21 parents were classified as having type 2 diabetes, and 3 parents had insufficient data available for classification. These 29 individuals were therefore excluded, and analysis was based on the remaining 2,419 parents. Subject characteristics are given in Table 1. The median duration of follow-up for unaffected siblings and parents was 6.8 and 5.5 years, respectively.

Serum samples collected at study entry were available from 1,169 of the 1,430 nondiabetic siblings included in the analysis (median age 11 years) and 2,130 of the 2,328 nondiabetic parents (1,165 mothers and 965 fathers, median age 38 years). Of these, 19 siblings and 12 parents developed type 1 diabetes during follow-up.

DNA samples for HLA class II typing were available from 573 of the

TABLE 1
Subject characteristics

	Siblings	Parents
Nondiabetic		
n	1,377	2,313
Age at time of analysis	16.8 (0.23–40)	44 (23–94)
Male:female	656:721	1,092:1,221
Type 1 diabetes		
n	53	106
Age at diagnosis	6.7 (0.5–17)	21 (1–56)
Male:female	24:29	70:36
Diabetes on study entry	27	91
Diabetes onset during follow-up	26	15

Data are *n* and median (range).

diabetic probands in this cohort. This included 157 diagnosed before 5 years of age (86 male and 71 female), 201 diagnosed between 5 and 9 years of age (102 male and 99 female), and 215 diagnosed between 10 and 14 years of age (115 male and 100 female).

Islet autoantibody assays. ICAs and antibodies to GAD and the protein tyrosine phosphatase IA-2 were measured in all samples. As IAAs are generally the first antibody marker to appear and may be found in isolation in young children, these were tested in samples from all children aged <10 years (15). Any samples in which levels of one or more of the other markers were greater than or equal to the 97.5th percentile of 2,860 schoolchildren control subjects were also tested for IAA.

ICAs. ICAs were measured in undiluted sera by indirect immunofluorescence as previously described (16). End point titers of test samples were converted to Juvenile Diabetes Foundation (JDF) units by comparison with a standard curve of $\log_2$ JDF units versus $\log_2$ of end point titer of the standard sera. The threshold of detection was 4 JDF units. The interassay coefficient of variation was 10% at 13 JDF units and 4.3% at 80 JDF units. The assay achieved 78.4% sensitivity with 98% specificity in the First Immunology of Diabetes Society (IDS) Combined Antibody Workshop (17).

GAD and IA-2 antibodies. Antibodies to in vitro–translated [^{35}S]GAD$_{65}$ and [^{35}S]PTP-IA-2$_{ic}$ were measured by immunoassay as previously described (18). The interassay coefficient of variation of the GAD antibody assay was 17% for samples that had 1.5 units and 9% for samples with 17 units of antibody. The interassay coefficient of variation of the IA-2 antibody assay was 15% for samples with 1 unit and 21% for samples with 9 units of antibody. The GAD antibody assay achieved 91% sensitivity with 99% specificity and the IA-2 antibody assay 74.4% sensitivity with 99% specificity in the First IDS Combined Antibody Workshop (17).

IAAs. Antibodies to ^{125}I-labeled insulin were measured as previously described, using a format similar to that used to measure antibodies to GAD and IA-2 (19). The two-stage assay involved initial screening for insulin binding followed by a competition assay in samples with insulin binding above the 95th percentile of the schoolchildren. Immune complexes were isolated with protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) and expressed in arbitrary units derived from a standard curve. The assay achieved 58% sensitivity with 99% specificity on the samples included in the First IDS Combined Antibody Workshop (17).

Genetic analysis. HLA genotyping was carried out on DNA from blood or mouth swab samples. Details of DNA extraction methods and HLA class II analysis have been previously published (20). Briefly, mouth swab extractions were carried out using a guanadinium chloride/phenol chloroform method. DNA was extracted from blood using a salting-out method (21). DNA quantitation was achieved using OD_{260} readings for blood DNA and picoGreen analysis for mouth swab DNA. Low yield DNA samples from mouth swabs underwent whole genome amplification by primer extension preamplification. HLA analysis was carried out by polymerase chain reaction (PCR) using sequence-specific primers (22). PCR products were separated on 2% agarose containing 0.002% ethidium bromide and visualized on a dual-intensity gel documentation system (Ultra Violet Products, Cambridge, U.K.).

Statistical methods. Individuals were defined as having type 1 diabetes if they fulfilled one or more of the following criteria: permanent insulin treatment within 6 months of diagnosis, presence of one (or more) islet autoantibodies at study entry, or diagnosis before age 30 years with permanent insulin treatment. Patients were classified as having type 2 diabetes if they were antibody negative and diet or tablet treated or if they were insulin treated and antibody negative in samples collected within 6 months of diagnosis.

Life table analysis was used to estimate cumulative risk of development of diabetes in siblings and parents (23). The end of follow-up for each family member was defined as the age at diagnosis of diabetes or the age at last contact. Survival experience in sibings and parents subdivided according to age at diagnosis of the proband was compared using the Wilcoxon statistic. The prevalence of antibody markers in subgroups of relatives was compared using the χ^2 test. Haplotypes in diabetic probands were subdivided according to age at onset, and differences in genotype according to age at onset were analyzed by χ^2 analysis.

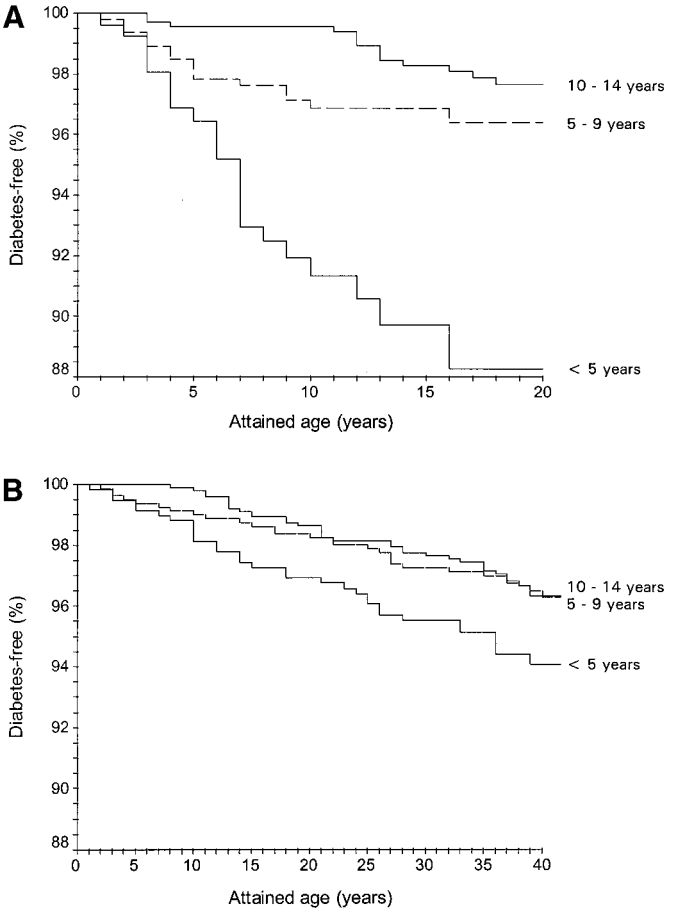

FIG. 1. The cumulative risk of diabetes in siblings (*A*) and parents (*B*) subdivided according to the age at diagnosis of the proband.

RESULTS

Diabetes in siblings and parents. At the time of analysis, 53 (3.7%) of 1,430 siblings and 106 (4.4%) of 2,419 parents had developed type 1 diabetes by median age 16.5 years (range 25–40) and age 44 years (range 1–94), respectively. Of the siblings, 27 (1.9%) were diagnosed before study entry and 26 (1.8%) during follow-up. Of the parents, 91 (3.7%) were diagnosed before study entry and 15 (0.6%) during follow-up. The median age at diagnosis of the siblings was 6.7 years (range 0.5–17) and of the parents was 21 years (range 1–56). In siblings, the overall risk of diabetes by age 20 was 4.3% (95% CI 3.1–5.4). In parents, the overall risk by age 40 was 4.2% (3.4–5.0). The risk in fathers was 5.7% (4.3–7.0) compared with 2.8% (1.9–3.8) in mothers ($P = 0.009$).

Figure 1 shows the survival curves for diabetes development in siblings and parents subdivided according to age at diagnosis of the proband. Siblings of a proband diagnosed before age 5 years had a cumulative risk of

TABLE 2
The prevalence of IAAs greater than or equal to the 97.5th percentile of schoolchildren control subjects in unaffected siblings and parents

Antibody markers	Age at diagnosis of the proband		
	<5 years	5–9 years	10–14 years
Siblings			
n	178	368	623
None	141 (79)	332 (90)	537 (86)
1	24 (13)	28 (8)	71 (11)
2	4 (2.2)	5 (1.4)	6 (1.0)
3	7 (3.9)	1 (0.3)	6 (1.0)
4	2 (1.1)	2 (0.5)	3 (0.5)
Parents			
n	515	702	913
None	456 (89)	631 (89)	815 (89)
1	48 (9)	60 (9)	81 (9)
2	4 (0.8)	6 (0.9)	13 (1.4)
3	7 (1.4)	4 (0.6)	3 (0.3)
4		1 (0.1)	1 (0.1)

Data are *n* (%).

diabetes by age 20 years of 11.7% (95% CI 6.7–16.8), compared with 3.6% (1.8–5.4) for diagnosis from 5–9 years and 2.3% (1.1–3.5) for 10–14 years ($P < 0.0001$). In parents, the cumulative risk by age 40 years was 5.9% (3.9–7.9) if the proband was diagnosed before age 5 years, compared with 3.7% (2.3–5.0) for diagnosis from 5 to 9 years and 3.7% (2.5–4.8) for 10–14 years ($P = 0.04$). In fathers, the cumulative risks of diabetes by age 40 years in these three groups were 8.5% (5.1–11.9), 5.0% (2.8–7.1), and 4.5% (2.7–6.4), respectively ($P = 0.05$), and in mothers were 3.3% (1.1–5.4), 2.4% (1.0–3.9) and 2.8% (1.4–4.2), respectively ($P = 0.7$).

Islet autoantibodies in unaffected relatives. Of 1,169 unaffected siblings tested at study entry, 159 had at least one islet autoantibody with levels above the 97.5th percentile of schoolchildren control subjects. The relation between the number of antibodies and age at onset of the proband is shown in Table 2. Detection of multiple antibodies in siblings was strongly associated with the age at onset of the proband. Two or more markers greater than or equal to the 97.5th percentile were found in 13 of 178 (7.3%) siblings of probands diagnosed before 5 years of age, 8 of 368 (2.2%) where the proband was 5–9 years of age, and 15 of 623 (2.4%) where the proband was 10–14 years of age ($P = 0.002$). Of those who remained nondiabetic at the time of analysis, two or more markers were detected in 7 of 168 (4.2%) siblings of probands diagnosed before 5 years of age, 7 of 366 (1.9%) where the proband was 5–9 years of age, and 8 of 615 (1.3%) where the proband was 10–14 years of age ($P = 0.06$). Of 2,130 unaffected parents tested, 228 had at least one antibody with levels greater than or equal to the 97.5th percentile of control subjects. A total of 189 had one marker, and 39 had two or more antibody markers above this level (Table 2). There was no association between the number of antibodies detected in the parent and the age at onset of the proband.

HLA analysis of probands. The frequency of the highest risk genotype decreased with increasing age at onset. The details are outlined in Table 3. Of 157 children diagnosed before the age of 5 years, 52% were heterozygous for HLA DRB1*03-DQA1*0301-DQB1*02/DRB1*04-DQA1*0501-DQB1*0302, compared with 32% of those diagnosed at 5–9 years and 33% of those diagnosed at 10–14 years ($P < 0.001$). The frequency of the other HLA class II genotypes was similar in the three age groups.

TABLE 3
Genotyping results

DRB1-DQA1-DQB1	Age at diagnosis of the proband		
	<5 years	5–9 years	10–14 years
n	157	201	215
03-0501-02/04-0301-0302	81 (52)	64 (32)	72 (33)
04-0301-0302/04-0301-0302	12 (8)	20 (10)	14 (7)
04-0301-0302/X	30 (19)	51 (25)	56 (26)
03-0501-02/03-0501-02	8 (5)	18 (9)	26 (12)
03-0501-02/X	22 (14)	38 (19)	40 (19)
X/X	4 (2)	10 (5)	7 (3)

Data are *n* (%).

DISCUSSION

This study has demonstrated that the risk of diabetes in relatives is strongly influenced by the age at diagnosis of the proband. Siblings of children diagnosed before the age of 5 years had a three- to fivefold greater cumulative risk of diabetes by age 20 years than siblings of children diagnosed between 5 and 15 years of age, and their parents were also more likely to develop diabetes. Previous studies have produced conflicting results. An analysis of 194 families of individuals with type 1 diabetes in Wisconsin found that risk was highest in siblings of those diagnosed before the age of 10 years (24), as did a clinic-based study of 493 families in Minnesota (9). In contrast, the Pittsburgh family study (25) found that risk was not increased in siblings of children diagnosed before age 5 years. The EURODIAB study (1) compared the prevalence of diabetes in relatives by age at diagnosis of 3,960 children with newly diagnosed type 1 diabetes in 16 European countries and found an increased prevalence of diabetes in the parents of children diagnosed before age 5 years, particularly fathers, but no difference in the prevalence of diabetes in their siblings. However, the EURODIAB study analyzed family history at the time of diagnosis, whereas our study included up to 15 years of follow-up, during which the number of affected siblings almost doubled.

We extended this observation by examination of islet autoantibodies in unaffected relatives, thus identifying those at the highest risk of subsequent progression to diabetes. This showed a similar trend, with 4.2% of siblings of children aged <5 years at diagnosis having two or more islet autoantibodies, compared with 1.9 and 1.3% of siblings of those diagnosed at ages 5–9 and 10–14 years, respectively ($P = 0.06$). Siblings of children diagnosed before age 5 years therefore have a higher prevalence of both overt and preclinical type 1 diabetes than older children.

These findings in siblings should be interpreted with some caution because affected family members tend to develop type 1 diabetes at around the same age (25). Early onset in the proband might therefore be a marker of early

onset in relatives, rather than a marker of increased risk over a lifetime. This possibility cannot be excluded but would not be consistent with the increased prevalence of autoantibodies we observed in siblings of early onset cases. Furthermore, age-clustering cannot explain differences in parental risk that persisted up to age 40 years. We therefore interpret our results as indicating an overall increase in risk of diabetes in the relatives of early onset patients, rather than early harvesting of susceptible individuals.

Our observation that risk to parents is associated with age of disease onset in the proband confirms several previous studies (8,11,26) and once again shows that fathers of children with diabetes are more often affected than mothers. In addition, as in the EURODIAB study (11), the effect of early onset in the child on parental risk was largely confined to fathers, whereas maternal risk varied little between onset age groups.

Analysis of the HLA class II genes DRB1, DQA1, and DQB1 in 573 probands from this cohort showed a significant increase in the number of HLA DRB1*03-DQA1*0301-DQB1*02/DRB1*04-DQA1*0501-DQB1*0302 heterozygotes in those diagnosed under the age of 5 years compared with older age groups. This confirms previous reports that young age at onset is associated with a greater genetic load (4) and correlates well with the results of a Finnish study (2) in which the same age groups were analyzed. The high proportion of DRB1 *03/*04 heterozygotes indicates that both parents carry at least one susceptibility haplotype for type 1 diabetes in many families of those diagnosed under the age of 5 years. In this situation, the siblings have a three in four chance of having at least one susceptibility haplotype for type 1 diabetes in common with the proband and at least a one in four chance of being heterozygous for DRB1 *03/*04. Their predicted risk of diabetes would therefore be greater than that of siblings of a proband with only one high-risk allele. The difference in sibling risk observed in our study could therefore be explained in terms of transmission of high-risk HLA alleles, although a role for non-HLA genes cannot be excluded.

Our study was performed in a population with an increasing incidence of childhood diabetes, particularly marked in the very young. The cumulative risk of diabetes by the age of 5 years in the Oxford region has doubled since the study began in 1985 (13). A rapid increase in a genetically stable population implies that environmental agents encountered in early life must play an important role in disease pathogenesis. Early onset diabetes is characterized by high genetic susceptibility, and we confirmed a high prevalence of the highest risk genotype HLA DRB1*03-DQA1*0301-DQB1*02/DRB1*04-DQA1*0501-DQB1*0302 in those diagnosed under the age of 5 years. Susceptibility alleles are therefore over-represented in these families, and we have shown that this is associated with considerably increased risk of progression to diabetes. Taken together, these observations suggest that a changing environment is interacting with the highest risk HLA susceptibility alleles and that each new case in early childhood represents another high-risk family. There is an urgent need to understand the forces that are driving this harmful interaction.

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REFERENCES

1. EURODIAB ACE Study Group: Variation and trends in incidence of childhood diabetes in Europe. *Lancet* 355:873–876, 2000
2. Komulainen J, Kulmala P, Savola K, Lounamaa R, Ilonen J, Reijonen H, Knip M, Åkerblom HK, the Childhood Diabetes in Finland (DIME) Study Group: Clinical, autoimmune, and genetic characteristics of very young children with type 1 diabetes. *Diabetes Care* 22:1950–1955, 1999
3. Vandewalle CL, Decraene T, Schuit FC, De Leeuw IH, Pipeleers DG, Gorus FK, the Belgian Diabetes Registry: Insulin antibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical onset of type 1 (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. *Diabetologia* 36:1155–1162, 1993
4. Caillat-Zucman S, Garchon H-J, Timsit J, Assan R, Boitard C, Djilali-Saiah I, Bougneres P, Bach JF: Age-dependent HLA genetic heterogeneity of type 1 insulin-dependent diabetes mellitus. *J Clin Invest* 90:2242–2250, 1992
5. Risch N: Assessing the role of HLA-linked and unlinked determinants of disease. *Am J Hum Genet* 40:1–14, 1987
6. Kyvik KO, Green A, Beck-Nielsen H: Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins. *BMJ* 311:913–917, 1995
7. Gorsuch AN, Spencer KM, Lister J, Wolf E, Bottazzo GF, Cudworth AG: Can future type 1 diabetes be predicted? A study in families of affected children. *Diabetes* 31:862–866, 1982
8. Kumar D, Gemayel NS, Deapen D, Kapadia D, Yamashita PH, Lee M, Dwyer JH, Roy-Burman P, Bray GA, Mack TM: North-American twins with IDDM: Genetic, etiological, and clinical significance of disease concordance according to age, zygosity, and the interval after diagnosis in first twin. *Diabetes* 42:1351–1363, 1993
9. Chern MM, Anderson VE, Barbosa J: Empirical risk of insulin-dependent diabetes (IDD) in sibs: further definition of genetic heterogeneity. *Diabetes* 31:1115–1118, 1982
10. Tillil H, Köbberling J: Age-corrected empirical genetic risk estimates for first degree relatives of IDDM patients. *Diabetes* 36:93–99, 1987
11. EURODIAB ACE Study Group: Familial risk of type 1 diabetes in European children. *Diabetologia* 41:1151–1156, 1998
12. Bingley PJ, Gale EAM: The incidence of insulin-dependent diabetes in England: a study in the Oxford region 1985–6. *BMJ* 298:558–560, 1989
13. Gardner SG, Bingley PJ, Sawtell PA, Weeks S, Gale EAM, the Bart's-Oxford Study Group: Rising incidence of insulin dependent diabetes in children aged under 5 years in the Oxford Region: time trend analysis. *BMJ* 315:713–717, 1997
14. Bingley PJ, Williams AJK, Gale EAM: Optimized autoantibody-based risk assessment in family members. *Diabetes Care* 22:1796–1801, 1999
15. Ziegler AG, Hummel M, Schenker M, Bonifacio E: Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: The 2-year analysis of the German BABYDIAB study. *Diabetes* 48:460–468, 1999
16. Bingley PJ, Bonifacio E, Shattock M, Gillmor HA, Sawtell PA, Dunger DB, Scott RDM, Bottazzo GF, Gale EAM: Can islet cell antibodies predict IDDM in the general population? *Diabetes Care* 16:45–50, 1993
17. Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ, Eisenbarth GS, participating laboratories: Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: combinatorial islet autoantibody workshop. *Diabetes* 47:1857–1866, 1998
18. Bingley PJ, Bonifacio E, Williams AJK, Genovese S, Bottazzo GF, Gale EAM: Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. *Diabetes* 46:1701–1710, 1997
19. Williams AJK, Bingley PJ, Bonifacio E, Palmer JP, Gale EAM: A novel micro-assay for insulin autoantibodies. *J Autoimmun* 10:473–478, 1997
20. Gillespie KM, Valovin SJ, Saunby J, Hunter KM, Savage DA, Middleton D,

Todd JA, Bingley PJ, Gale EAM: HLA class II typing of whole genome amplified mouth swab DNA. *Tissue Antigens* 56:530–538, 2000
21. Miller SA, Dykes D, Polesky H: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215–1218, 1998
22. Bunce M, O'Neill CM, Barnado MCNM, Krausa P, Morris PJ, Welsh KI: Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 and DQB1 by PCR with 144 primer mixes utilising sequence-specific primers (PCR-SSP). *Tissue Antigens* 46:355–367, 1995
23. Armitage P, Berry G: *Statistical Methods in Medical Research*. 2nd Ed. Oxford, U.K., Blackwell Scientific Publications, 1987
24. Allen C, Palta M, D'Alessio DJ: Risk of diabetes in siblings and other relatives of IDDM subjects. *Diabetes* 40:831–836, 1991
25. Wagener DK, Sacks JM, LaPorte RE, MacGregor JM: The Pittsburgh Study of insulin-dependent diabetes: risk for diabetes among relatives of IDDM. *Diabetes* 31:136–144, 1982
26. Metcalfe MA, Baum JD: Family characteristics and insulin-dependent diabetes. *Arch Dis Child* 67:731–736, 1992