



# Genetic Contribution to the Divergence in Type 1 Diabetes Risk Between Children From the General Population and Children From Affected Families

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The risk for autoimmunity and subsequently type 1 diabetes is 10-fold higher in children with a first-degree family history of type 1 diabetes (FDR children) than in children in the general population (GP children). We analyzed children with high-risk HLA genotypes ( $n = 4,573$ ) in the longitudinal TEDDY birth cohort to determine how much of the divergent risk is attributable to genetic enrichment in affected families. Enrichment for susceptible genotypes of multiple type 1 diabetes-associated genes and a novel risk gene, *BTNL2*, was identified in FDR children compared with GP children. After correction for genetic enrichment, the risks in the FDR and GP children converged but were not identical for multiple islet autoantibodies (hazard ratio [HR] 2.26 [95% CI 1.6–3.02]) and for diabetes (HR 2.92 [95% CI 2.05–4.16]). Convergence varied depending upon the degree of genetic susceptibility. Risks were similar in the highest genetic susceptibility group for multiple islet autoantibodies (14.3% vs .12.7%) and diabetes (4.8% vs. 4.1%) and were up to 5.8-fold divergent for children in the lowest genetic susceptibility group, decreasing

incrementally in GP children but not in FDR children. These findings suggest that additional factors enriched within affected families preferentially increase the risk of autoimmunity and type 1 diabetes in lower genetic susceptibility strata.

Type 1 diabetes has a presymptomatic phase that often starts with the appearance of autoantibodies to pancreatic islet cell antigens (1). The development of islet autoantibodies is strongly influenced by HLA DR and DQ genotypes, with smaller contributions from many other genes (2–4). Children with a first-degree family history of type 1 diabetes (FDR children) have a higher risk of developing islet autoantibodies and diabetes than the risk in children from the general population without a family history of the disease (GP children) (5–7). Enrichment of type 1 diabetes-susceptibility genotypes of HLA and other genes is likely to contribute to the inflated risk in FDR children. Understanding the genetic differences

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\*A list of members of the TEDDY Study Group can be found in Supplementary Data.

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and their contributions to the divergent risks between GP and FDR children could provide paradigms to identify novel genetic and environmental factors that modify risk and to identify GP children whose a priori risk of developing type 1 diabetes is similar to that of FDR children.

The Environmental Determinants of Diabetes in the Young (TEDDY) study has screened >400,000 newborns to identify and recruit >8,000 with high-risk HLA genotypes into a follow-up cohort that monitors the development of islet autoantibodies and diabetes (8,9). This cohort includes FDR and GP children, providing a rare opportunity to examine the excess risk of developing islet autoantibodies and diabetes in affected families. The children in the TEDDY study have been extensively genotyped (4). This allowed us to calculate genetic risk scores representing cumulative genetic susceptibility (10–12) and has enabled us to investigate which genes, beyond the known susceptibility regions, may contribute to risk. Here, we address whether the increased risk in FDR children is accounted for by enrichment of genetic susceptibility in TEDDY children with the highest-risk HLA genotypes (*DR3/DR4-DQ8* heterozygotes and *DR4-DQ8/DR4-DQ8* homozygotes). Using this approach, we could show enrichment of genetic susceptibility for multiple known risk genes and a novel risk gene. Matching FDR and GP children for genetic risk abrogated the excess risk in FDR children in the highest genetic risk stratum but not in the lower genetic risk strata. These findings provide evidence that additional factors preferentially contribute to type 1 diabetes risk in children without a full complement of genetic susceptibility.

## RESEARCH DESIGN AND METHODS

The TEDDY study screened 424,788 newborns for type 1 diabetes-associated HLA genotypes between 2004 and 2010, of whom 8,676 children were enrolled and followed in six centers located in the U.S., Finland, Germany, and Sweden (Supplementary Fig. 1). Detailed information on the study design, eligibility, and methods has previously been published (3,8,9). Here, we used data as of 30 June 2017. Written informed consent was obtained for all participants from a parent or primary caretaker, separately, for genetic screening and for participation in prospective follow-up. The study was approved by local institutional review boards and is monitored by the External Evaluation Committee formed by the National Institutes of Health.

The datasets generated and analyzed during the current study are available in the National Institute of Diabetes and Digestive and Kidney Diseases Central Repository at [www.niddkrepository.org/studies/teddy](http://www.niddkrepository.org/studies/teddy). TEDDY ImmunoChip SNP (single nucleotide polymorphism) data that support the findings of this study have been deposited in the National Center for Biotechnology Information Database of Genotypes and Phenotypes (dbGaP) with the primary accession code phs001037.v1.p1.

## Genotyping and Genetic Risk Score

The HLA genotypes were confirmed by the central HLA Laboratory at Roche Molecular Systems (Oakland, CA) for enrolled subjects. The present report includes 4,572 TEDDY children with the *DR3-DQA1\*05:01-DQB1\*02:01/DR4-DQA1\*03:0X-DQB1\*03:02* genotype (HLA *DR3/DR4-DQ8*) or the *DR4-DQA1\*03:0X-DQB1\*03:02/DR4-DQA1\*03:0X-DQB1\*03:02* genotype (HLA *DR4-DQ8/DR4-DQ8*), if at least one sample was obtained after birth. SNP analysis was performed using the Illumina ImmunoChip (13). The genetic risk score was calculated from 40 SNPs similar to the previously described merged genetic score (12), except the value of the HLA *DR-DQ* genotype (3.98 for *DR3/DR4-DQ8* or 3.15 for *DR4-DQ8/DR4-DQ8*) was not included in the score.

## TEDDY Study Outcomes

Blood samples were obtained every 3 months until age 4 years and biannually thereafter for the analysis of islet autoantibodies (insulin autoantibodies [IAA], GAD autoantibodies [GADA], and insulinoma antigen-2 autoantibodies). All radiobinding assays were performed as previously described (13,14). A positive outcome was defined as positive in both reference laboratories on two or more consecutive visits. The date of seroconversion (time to first autoantibody) was defined as the date of drawing the first of the two consecutive positive samples. The presence of multiple islet autoantibodies was defined as the presence of at least two islet autoantibodies. Diabetes was diagnosed according to American Diabetes Association criteria.

## Statistics

The risks of developing one or more islet autoantibodies, multiple autoantibodies, and diabetes in FDR and GP children were assessed using Kaplan-Meier plots, and groups were compared using the log-rank test. Hazard ratios (HRs) were computed using Cox proportional hazards model. Genotype frequencies between GP and FDR children were compared using  $\chi^2$  tests with Bonferroni correction for multiple testing. All analyses were carried out with R 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria), using the GWASTools, haplo.stats, qqman, and survminer packages.

## RESULTS

### Risk of Developing Islet Autoantibodies and Diabetes According to HLA Genotype and First-degree Relative Status

Of the TEDDY children with at least one follow-up sample ( $n = 7,894$ ), 3,035 (38.4%) had the HLA *DR3/DR4-DQ8* genotype and 1,537 (19.5%) had the HLA *DR4-DQ8/DR4-DQ8* genotype. Of these 4,572 children, 423 (9.3%) were FDR children and 4,149 were GP children (Table 1 and Supplementary Fig. 1). One or more islet autoantibodies developed in 500 (10.9%) children, of whom 324 (7.1%) had multiple islet autoantibodies. Diabetes was diagnosed in 192 (4.2%) children.

**Table 1—Study characteristics by first-degree relative status**

| Variable                         | FDR children<br>(n = 423) | GP children<br>(n = 4,149) |
|----------------------------------|---------------------------|----------------------------|
| Males                            | 200 (47.3)                | 2,082 (50.2)               |
| HLA genotype                     |                           |                            |
| <i>DR3/4-DQ8</i>                 | 280 (66.2)                | 2,755 (66.4)               |
| <i>DR4-DQ8/DR4-DQ8</i>           | 143 (33.8)                | 1,394 (33.6)               |
| Country                          |                           |                            |
| U.S.                             | 194 (45.9)                | 1,750 (42.2)               |
| Finland                          | 51 (12.1)                 | 792 (19.1)                 |
| Germany                          | 92 (21.7)                 | 209 (5.0)                  |
| Sweden                           | 86 (20.3)                 | 1,398 (33.7)               |
| First-degree relative with T1D   |                           |                            |
| None                             | 0 (0.0)                   | 4,149 (100.0)              |
| Mother                           | 146 (34.5)                | 0 (0.0)                    |
| Father                           | 180 (42.6)                | 0 (0.0)                    |
| Sibling                          | 79 (18.7)                 | 0 (0.0)                    |
| Multiplex                        | 18 (4.3)                  | 0 (0.0)                    |
| Outcome events                   |                           |                            |
| One or more islet autoantibodies | 85 (20.1)                 | 415 (10.0)                 |
| Multiple islet autoantibodies    | 69 (16.3)                 | 255 (6.1)                  |
| First-appearing IAA              | 51 (12.1)                 | 227 (5.5)                  |
| First-appearing GADA             | 46 (10.9)                 | 250 (6.0)                  |
| Diabetes                         | 47 (11.1)                 | 145 (3.5)                  |
| Genetic risk score available     | 408 (96.5)                | 4,006 (96.6)               |
| Data are n (%).                  |                           |                            |

Matching for HLA *DR-DQ* provided an estimate of the excess risk of developing islet autoantibodies or diabetes in FDR children that was due to factors other than enrichment for these genotypes (Fig. 1). Matching for HLA genotypes was sufficient to reduce the >10-fold excess risk usually observed in children from affected families to <3-fold. The cumulative risk by 6 years of age in HLA *DR3/DR4-DQ8* FDR children was 20.5% (95% CI 15.4–25.4%) for one or more islet autoantibodies, 17.0% (12.1–21.5%) for multiple islet autoantibodies, and 6.8% (3.8–9.7%) for diabetes compared with 10.0% (8.8–12.2%;  $P < 0.0001$ ), 6.4% (5.4–7.4%;  $P < 0.0001$ ), and 2.7% (2.0–3.3%;  $P < 0.0001$ ), respectively, in GP children with these genotypes (Fig. 1A, C, and E). Similar differences were observed in the HLA *DR4-DQ8/DR4-DQ8* children (Fig. 1B, D, and F). A first-degree family history of type 1 diabetes was associated with an increased incidence of islet autoimmunity in the first 3 years of life in children with the high-risk HLA genotypes (Supplementary Fig. 2A). This was similar if the outcome was defined as the detection of IAA before other autoantibodies or GADA as the first islet autoantibodies (Supplementary Fig. 2B).

#### ***DRB1\*04* Allele Subtype Enrichment in Children From Affected Families**

The risk for type 1 diabetes is influenced by the HLA *DRB1\*04* allele (15,16). We therefore searched for FDR enrichment of *DRB1\*04* subtypes (Table 2). The high-risk *DRB1\*04:01* allele was more frequent in the FDR children than in the GP children ( $P < 0.0001$ ) for children with

either the *DR3/4-DQ8* ( $P = 0.0067$ ) or *DR4-DQ8/DR4-DQ8* ( $P = 0.0005$ ) genotypes. In contrast, the lower-risk *DRB1\*04:04* ( $P < 0.0001$ ) and *DRB1\*04:07* ( $P = 0.035$ ) alleles were less frequent in the FDR children than in GP children. There were no differences in the frequencies of the *DRB1\*04:02* or the *DRB1\*04:05* alleles between the FDR children and the GP children. The remaining *DRB1\*04* alleles were infrequent in the study population and were not considered.

#### **Genetic Risk Scores in FDR and GP Children**

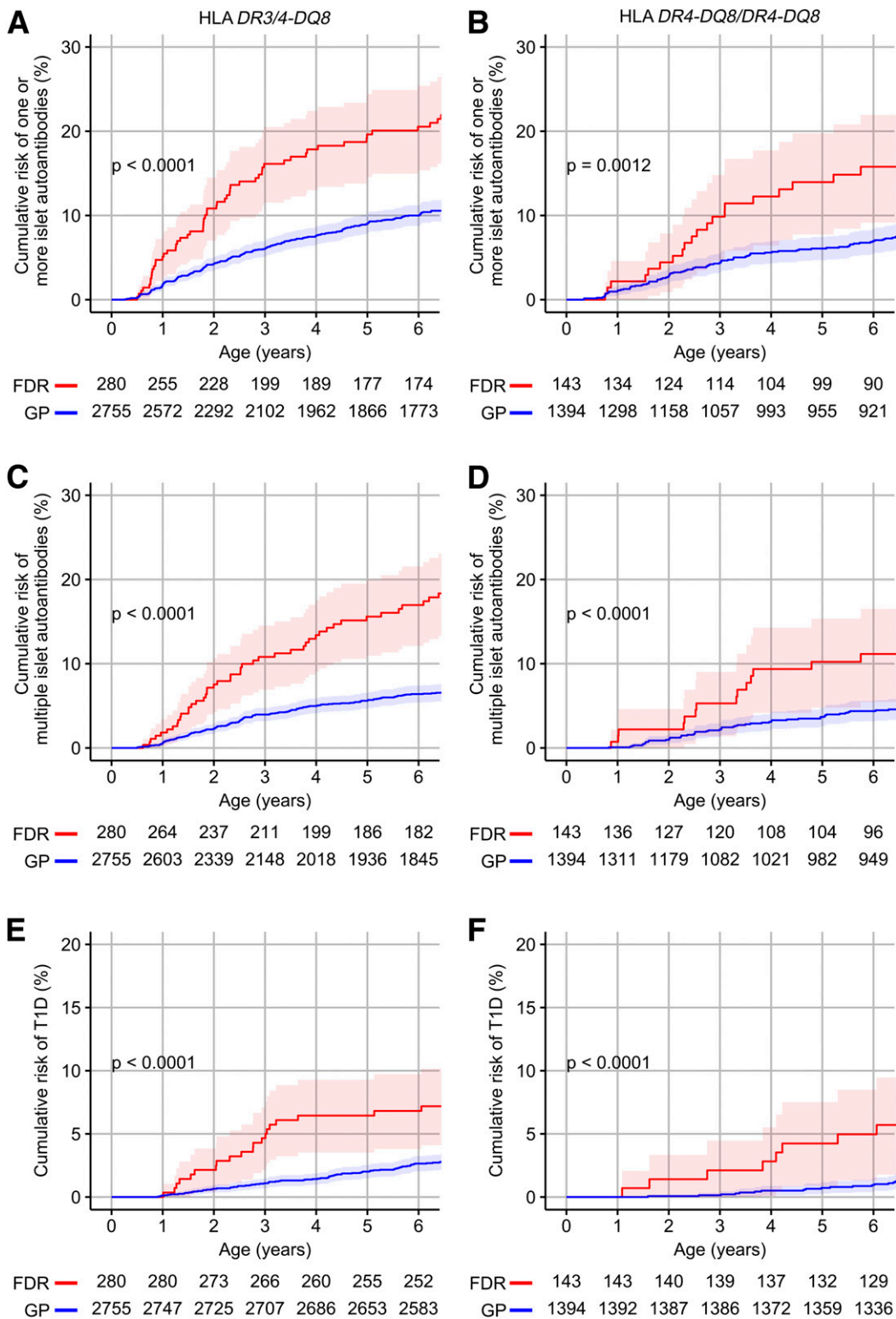
The additional non-HLA *DR-DQ* gene risk was expressed as a genetic risk score from 40 of the non-HLA *DR-DQ* SNPs (12). Genetic risk scores were higher in the FDR children (median 10.3 [interquartile range 9.7–11.0]) than in the GP children (10.1 [9.4–10.7];  $P < 0.0001$ ) (Fig. 2A). Enrichment of risk genotypes reached significance for six of the 40 SNPs (Supplementary Table 1). Similar genetic risk scores were observed in the multiple islet autoantibody-positive FDR children (10.4 [10.0–11.3]) and GP children (10.5 [10.0–11.1];  $P = 0.97$ ) (Fig. 2B).

#### **Additional Genes With Allele Enrichment in FDR Children**

HLA *DR-DQ* susceptible genotypes have additional variants in genes that are in linkage disequilibrium (LD) with HLA *DR-DQ* (17). We reasoned that the frequencies of susceptibility genotypes of such genes may be increased in the FDR children and could account for some of the excess risk in these children. With the rare opportunity to compare a large number of GP and FDR children matched for HLA *DR-DQ* genotype in the TEDDY study, we examined 6,097 SNPs with minor allele frequencies >5% on the short arm of chromosome 6, containing the HLA genes (6p21.32). SNPs rs3763305 ( $P = 7.27 \times 10^{-7}$ ) and rs3817964 ( $P = 8.26 \times 10^{-7}$ ) were enriched in the FDR children (Supplementary Fig. 3A). Both SNPs are intronic variants of *BTNL2* and are in complete LD ( $r^2 = 1$ ). The *BTNL2* gene is located close to the HLA *DRA/HLA DRB5/HLA DRB1* cluster and HLA *DQB1*. Extension of the analysis to all 111,069 ImmunoChip SNPs that passed quality control filters identified rs7735139 (intronic SNP in *ITGA1* [integrin subunit  $\alpha$  1, 5q11.2]) with allelic enrichment in the FDR children ( $P = 4.34 \times 10^{-8}$ ) (Supplementary Fig. 3B).

#### **Genetic Contribution to the Additional Risk for Islet Autoantibodies and Diabetes in FDR Children**

Cox proportional hazards models were used to assess the development of one or more islet autoantibodies, multiple islet autoantibodies, and diabetes (Table 3). Model 1 examined the excess risk conferred by a first-degree family history of type 1 diabetes adjusted for HLA genotype (*DR3/4-DQ8* vs. *DR4-DQ8/DR4-DQ8*), sex, and country of origin. HRs in the FDR children were 2.12 (95% CI 1.65–2.72) for one or more islet autoantibodies, 2.77 (95% CI 2.09–3.68) for multiple



**Figure 1**—Cumulative risks of islet autoantibodies and diabetes. Kaplan-Meier curves for the risk of one or more islet autoantibodies (A and B), multiple islet autoantibodies (C and D), and diabetes (E and F) in FDR children (red) and in GP children (blue), stratified into children with the HLA DR3/DR4-DQ8 (A, C, and E) or HLA DR4-DQ8/DR4-DQ8 (B, D, and F) genotypes. Shaded areas represent the 95% CI. Numbers represent children at risk. P values were calculated using log-rank tests.

**Table 2—Allelic enrichment of *DRB1\*04* subtypes in FDR children**

| DR4 subtype       | FDR children (alleles, n = 535) | GP children (alleles, n = 5,415) | P*      |
|-------------------|---------------------------------|----------------------------------|---------|
| <i>DRB1*04:01</i> | 329 (60.15)                     | 2,788 (51.49)                    | <0.0001 |
| <i>DRB1*04:02</i> | 33 (6.03)                       | 291 (5.37)                       | 0.42    |
| <i>DRB1*04:04</i> | 137 (25.05)                     | 1,928 (35.60)                    | <0.0001 |
| <i>DRB1*04:05</i> | 23 (4.20)                       | 226 (4.17)                       | 0.91    |
| <i>DRB1*04:06</i> | 1 (0.18)                        | 1 (0.02)                         | 0.17    |
| <i>DRB1*04:07</i> | 7 (1.28)                        | 153 (2.83)                       | 0.035   |
| <i>DRB1*04:08</i> | 4 (0.73)                        | 25 (0.46)                        | 0.33    |
| <i>DRB1*04:10</i> | 0 (0.00)                        | 1 (0.02)                         | 1       |
| <i>DRB1*04:11</i> | 1 (0.18)                        | 1 (0.02)                         | 0.17    |
| <i>DRB1*04:13</i> | 0 (0.00)                        | 1 (0.02)                         | 1       |

Data are n (%). Each *DRB1\*04* allele was counted separately (once for children with the *DR3/DR4-DQ8* genotype and twice for children with the *DR4-DQ8/DR4-DQ8* genotype). *DRB1\*04* subtype information was missing in 77 *DR3/DR4-DQ8* and 35 *DR4-DQ8/DR4-DQ8* children. Children with the nonrisk *DRB1\*04:03* allele were excluded a priori from the TEDDY study unless they had a first-degree relative with type 1 diabetes, and the 12 occurrences of this allele in FDR children were therefore not considered. \*P values were calculated using Fisher test.

islet autoantibodies, and 3.69 (95% CI 2.60–5.23) for diabetes.

For determination of whether the genetic factors that were enriched in FDR children contributed to the excess risk in FDR children, model 2 added the non-HLA *DR-DQ* genetic risk score, *DRB1\*04* subtype (enriched, *DR3/DRB1\*04:01* and *DRB1\*04:01/DR\*04:xx*, where *\*04:xx* is neither *\*04:04* nor *\*04:07*, vs. nonenriched, other genotypes), the *BTNL2* SNP rs3763305 (enriched, GG, vs. nonenriched, GA/AA genotypes), and the *ITGA1* SNP rs7735139 (enriched, GG, vs. nonenriched, GA/AA genotypes). HRs for FDR children were reduced to 1.82 (95% CI 1.42–2.35) for one or more islet autoantibodies, 2.26 (95% CI 1.70–3.02) for multiple islet autoantibodies, and 2.92

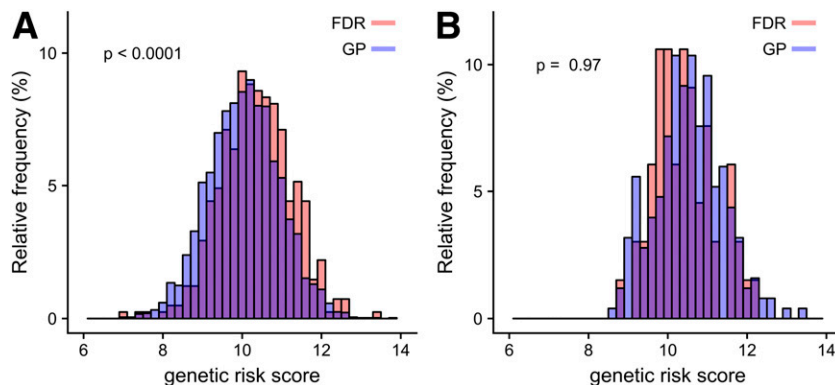
(95% CI 2.05–4.16) for diabetes. The enriched *DRB1\*04:01* subtype (HR 1.48 [95% CI 1.08–2.01];  $P = 0.014$ ) and the non-HLA *DR-DQ* genetic risk score (HR 1.66 [95% CI 1.47–1.88];  $P < 0.0001$ ) contributed to the risk of multiple islet autoantibodies. Similar HRs for these variables were also observed for the risk of one or more islet autoantibodies and diabetes, some of which reached significance. The *BTNL2* SNP rs3763305 GG genotype conferred additional risk for diabetes (HR 1.80 [95% CI 1.11–2.93];  $P = 0.017$ ) (Table 3), and this additional risk was also observed when the analysis was restricted to children with an HLA *DR3/4-DQ8* genotype (HR 1.92 [95% CI 1.11–3.35];  $P = 0.021$ ) (Supplementary Table 2). The *ITGA1* SNP was not associated with risk.

**Association Between *DRB1\*04* Subtypes and Islet Autoantibodies or Diabetes**

To further assess whether the *DRB1\*04:01* allele was associated with increased risk compared with the other *DRB1\*04* alleles in the TEDDY children, we removed the confounder of family history and performed a Kaplan-Meier analysis in the GP children. Among the 1,876 children with the *DRB1\*04:01* allele, the cumulative risks at 6 years old were 11.4% (95% CI 9.8–12.9%) for one or more islet autoantibodies, 7.7% (6.3–9.0%) for multiple islet autoantibodies, and 2.9% (2.1–3.6%) for diabetes compared with 6.8% (5.7–8.0%;  $P < 0.0001$ ), 4.0% (3.1–4.9%;  $P < 0.0001$ ), and 1.4% (0.9–1.9%;  $P < 0.0001$ ), respectively, among 2,176 children without *DRB1\*04:01* (Supplementary Fig. 4A, C, and E). These differences remained when the analysis was limited to GP children with the HLA *DR3/DR4-DQ8* genotype (Supplementary Fig. 4B, D, and F).

**Association Between *BTNL2* Genotypes and Islet Autoantibodies or Diabetes**

Variants in the *BTNL2* gene have not been implicated as independent genetic risk factors for type 1 diabetes previously, likely due to the extensive LD in the MHC region



**Figure 2—A:** Distribution of non-HLA *DR-DQ* genetic risk scores in all 4,414 *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* children stratified into FDR children (red) and GP children (blue). **B:** Distribution of non-HLA *DR-DQ* genetic risk scores in 317 *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* children who developed multiple islet autoantibodies. P values were calculated using the two-sided Mann-Whitney U test.

**Table 3—Cox proportional hazards models for developing islet autoantibodies and diabetes in FDR children compared with GP children (reference)**

|                            | One or more islet autoantibodies |         |                  |         |                  |         | Multiple islet autoantibodies |         |                  |         |                  |         | Diabetes         |        |                  |         |  |  |
|----------------------------|----------------------------------|---------|------------------|---------|------------------|---------|-------------------------------|---------|------------------|---------|------------------|---------|------------------|--------|------------------|---------|--|--|
|                            | Model 1*                         |         | Model 2*         |         | Model 1*         |         | Model 2*                      |         | Model 1*         |         | Model 2*         |         | Model 1*         |        | Model 2*         |         |  |  |
|                            | HR (95% CI)                      | P       | HR (95% CI)      | P       | HR (95% CI)      | P       | HR (95% CI)                   | P       | HR (95% CI)      | P       | HR (95% CI)      | P       | HR (95% CI)      | P      | HR (95% CI)      | P       |  |  |
| FDR child                  | 2.12 (1.65–2.72)                 | <0.0001 | 1.82 (1.42–2.35) | <0.0001 | 2.77 (2.09–3.68) | <0.0001 | 2.26 (1.70–3.02)              | <0.0001 | 3.69 (2.60–5.23) | <0.0001 | 2.92 (2.05–4.16) | <0.0001 | 1.38 (0.93–2.04) | 0.1052 | 1.67 (1.42–1.96) | <0.0001 |  |  |
| <i>DRB1*04:01/x†</i>       |                                  |         | 1.42 (1.10–1.83) | 0.0075  |                  |         | 1.48 (1.08–2.01)              | 0.014   |                  |         |                  |         |                  |        |                  |         |  |  |
| Genetic risk score‡        |                                  |         | 1.48 (1.34–1.64) | <0.0001 |                  |         | 1.66 (1.47–1.88)              | <0.0001 |                  |         |                  |         |                  |        |                  |         |  |  |
| <i>BTNL2</i> rs3763305     |                                  |         | 1.05 (0.79–1.40) | 0.73    |                  |         | 1.39 (0.96–2.00)              | 0.080   |                  |         |                  |         |                  |        |                  |         |  |  |
| GG§                        |                                  |         | 1.18 (0.86–1.63) | 0.30    |                  |         | 1.06 (0.70–1.60)              | 0.77    |                  |         |                  |         |                  |        |                  |         |  |  |
| <i>ITGA1</i> rs7735139 GG§ |                                  |         |                  |         |                  |         |                               |         |                  |         |                  |         |                  |        |                  |         |  |  |

\*Model 1 and 2 are adjusted for sex, country (reference: U.S.), and HLA genotype (reference: *DR4-DQ8/DR4-DQ8*). †Reference: *DRB1* without 04:01 or 04:01/04:04 and 04:01/04:07. ‡Per unit increase. §Reference: GAA genotype.

and inadequate sample size. The *BTNL2* rs3763305 GG genotype distribution was increased in children who developed one or more islet autoantibodies ( $P < 0.0001$ ), multiple islet autoantibodies ( $P < 0.0001$ ), or diabetes ( $P < 0.0001$ ) compared with children who remained islet autoantibody negative. These associations were observed separately for children with HLA *DR3/4-DQ8* or *DR4-DQ8/DR4-DQ8* (Table 4). The association between the *BTNL2* rs3763305 GG genotype and type 1 diabetes was also validated in the Type 1 Diabetes Genetics Consortium (T1DGC), using a nested case-control comparison after stratification for HLA *DR3/4-DQ8* (Supplementary Table 3).

The ImmunoChip contained 88 SNPs that were genotyped within *BTNL2*, including 34 that passed all quality-control metrics. The ImmunoChip genotypes were used to define haplotypes using the R package haplo.stats (Supplementary Table 4). The risk associated with the four most frequent *BTNL2* genotypes among children with *DR3/DR4-DQ8* and the four most frequent genotypes among children with *DR4-DQ8/DR4-DQ8* was stratified by the presence of haplotype 28, which uniquely contained an A allele at *BTNL2* rs3763305 and a T allele at *BTNL2* rs3817964 (Supplementary Fig. 5).

*BTNL2* lies close to the HLA *DRB5/HLA DRB6/HLA DRB1* protein-coding genes in a region of high LD. HLA *DR3* was in nearly complete LD with the rs3763305 G allele: the *BTNL2* rs3763305 GG genotype was identified in 1,608 (99.3%) of 1,619 children who had the HLA *DR3/DR3* genotype (Supplementary Table 5). We next examined the second *BTNL2* rs3763305 allele in children with *DR3/DR4-DQ8*. The *BTNL2* rs3763305 G allele was in nearly complete LD with *DRB1\*04:01* (allele frequency 99.5%), *DRB1\*04:02* (99.4%), and *DRB1\*04:05* (100.0%), whereas the *BTNL2* rs3763305 A allele was associated with *DRB1\*0404* (39.2%) and *DRB1\*0407* (34.4%) ( $P < 0.0001$ ). These associations were confirmed in a separate cohort of 149 children with type 1 diabetes and the *DR3/DR4-DQ8* genotype from Bavaria, Germany (Supplementary Table 6). The *BTNL2* rs3763305 A allele was also observed together with the protective HLA *DRB1\*04:03* allele in five (56%) of nine informative genotypes and with the protective HLA *DRB1\*13:01* allele in 10 (25%) of 40 informative genotypes but not with other HLA *DRB1* alleles in the German cohort (data not shown).

The *DRB1\*04:04* allele together with either the *BTNL2* rs3763305 G or the *BTNL2* rs3763305 A allele was relatively frequent in the TEDDY children and, therefore, provided an opportunity to determine whether the *BTNL2* gene conferred an independent risk to the HLA *DR4* subtype. Risks associated with *BTNL2* genotypes were examined in children with the *DR3/DRB1\*04:04-DQ8* or *DRB1\*04:04-DQ8/DRB1\*04:04-DQ8* genotypes. The cumulative risks at 6 years of age in children with the *BTNL2* rs3763305 GG genotype were 9.8% (95% CI 5.6–13.8%) for one or more islet autoantibodies, 6.3%

**Table 4—*BTNL2* SNP genotype frequencies in relation to the development of islet autoantibodies and diabetes among TEDDY children with HLA *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* and available genotype information**

|  | Islet autoantibody negative | One or more islet autoantibodies | Multiple islet autoantibodies | Diabetes   |
|--|-----------------------------|----------------------------------|-------------------------------|------------|
| HLA <i>DR3/4-DQ8</i> ( $n = 3,024$ ), <i>BTNL2</i> SNP rs3763305       |                             |                                  |                               |            |
| GG   | 1,839 (69.1)                | 272 (75.3)                       | 190 (79.8)                    | 120 (82.8) |
| GA   | 823 (30.9)                  | 89 (24.7)                        | 48 (20.2)                     | 25 (17.2)  |
| AA   | 1 (0.0)                     | 0 (0.0)                          | 0 (0.0)                       | 0 (0.0)    |
| <i>P</i> *   |                             | 0.030                            | 0.0007                        | 0.0007     |
| HLA <i>DR4-DQ8/DR4-DQ8</i> ( $n = 1,532$ ), <i>BTNL2</i> SNP rs3763305 |                             |                                  |                               |            |
| GG   | 692 (49.7)                  | 89 (64.0)                        | 63 (73.3)                     | 36 (76.6)  |
| GA   | 571 (41.0)                  | 44 (31.7)                        | 21 (24.4)                     | 10 (21.3)  |
| AA   | 130 (9.3)                   | 6 (4.3)                          | 2 (2.3)                       | 1 (2.1)    |
| <i>P</i> *   |                             | 0.0033                           | <0.0001                       | 0.0010     |

Data are  $n$  (%). \*Children who remained islet autoantibody negative were compared with children who developed one or more islet autoantibodies, multiple islet autoantibodies, and diabetes using Fisher test.

(2.9–9.6%) for multiple islet autoantibodies, and 3.7% (1.3–6.0%) for diabetes compared with 8.0% (6.2–9.8%;  $P = 0.46$ ), 4.7% (3.3–6.2%;  $P = 0.096$ ), and 1.6% (0.8–6.0%;  $P = 0.0048$ ) in the children with the GA or AA genotypes (Fig. 3). Cox proportional hazards models adjusted for *DR3/DRB1\*04:04-DQ8* or *DRB1\*04:04-DQ8/DRB1\*04:04-DQ8* genotype replicated the additional risk for diabetes conferred by the *BTNL2* rs3763305 GG genotype ( $P = 0.0086$ ) (Supplementary Table 7).

The additional risk conferred by the *BTNL2* rs3763305 GG genotype may be due to a specific association with specific *DRB1\*04:04* subtypes. We, therefore, examined the relationship between the *BTNL2* rs3763305 alleles and *DRB1\*04:04* subtypes in the German cohort of patients who had been HLA genotyped by sequencing of HLA *DRB1* exon 2, which harbors variations in all 12 subtypes of *DRB1\*04:04*. All subjects with *DRB1\*04:04* had the *DRB1\*04:04:01* allele regardless of whether the *BTNL2* rs3763305 was A or G, indicating that the *BTNL2* rs3763305 A allele does not appear to mark a subtype of *DRB1\*04:04*.

Finally, we examined the effect of *BTNL2* knockdown on in vitro immune responses (Supplementary Fig. 6). Compared with nontargeting siRNA control treated dendritic cells, *BTNL2*-targeted siRNA-treated dendritic cells had increased naïve alloreactive CD4<sup>+</sup> T-cell activation ( $P = 0.031$ ) but not memory antigen-specific CD4<sup>+</sup> T-cell activation ( $P = 0.43$ ).

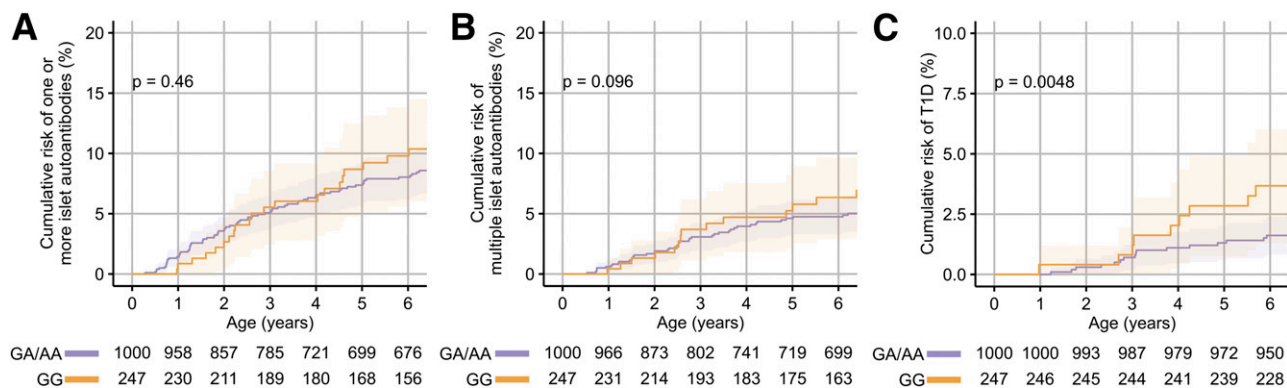
#### Risk Excess in FDR Children After Stratification by Genetic Risk

We asked whether the observed enrichment of type 1 diabetes genetic susceptibility in the FDR children could account for their excess risk. Four risk strata were defined by HLA *DRB1\*04* subtype and genetic risk score. With these strata, we could discriminate the risk of developing islet autoantibodies and T1D in

the GP children (Fig. 4A, C, and E). A similar stratification in the GP children was observed if the strata were defined by the children's *BTNL2* genotype and genetic risk score (Supplementary Fig. 7). In contrast to the GP children, a discrimination of risk in the FDR children was only achieved in the lowest risk stratum (Fig. 4B, D, and F). Comparing FDR children and GP children showed complete convergence of the risks of developing islet autoantibodies and diabetes in the highest-risk stratum and divergence of risk in the lower-risk strata (Supplementary Table 8). The fold difference in risk for multiple islet autoantibodies between the FDR and GP children was 1.1 in the highest-risk stratum (14.3% vs. 12.7%), 1.9 in the second risk stratum (17% vs. 9%), 3.3 in the third risk stratum (14.8% vs. 4.5%), and 5.8 in the lowest-risk stratum (9.2% vs. 1.6%).

#### Risk in FDR Children Is Modified by Maternal, Paternal, and Sibling Type 1 Diabetes

Risk divergence at lower genetic susceptibility strata implied that additional factors that influence risk may act differently depending on the a priori genetic susceptibility. One factor that is known to affect risk in FDR children is maternal type 1 diabetes (18). The HRs for one or more islet autoantibodies (2.37 [95% CI 1.71–3.28]), multiple islet autoantibodies (2.89 [95% CI 2.00–4.17]), and diabetes (3.06 [95% CI 1.89–4.94]), were increased compared with GP children if the first-degree relative index case was a father. The HRs were also increased if the first-degree relative was a sibling. By contrast, if the first-degree relative index case was the mother, the HR was not increased for one or more islet autoantibodies (0.85 [0.50–1.44]), multiple islet autoantibodies (0.97 [95% CI 0.52–1.7]), and diabetes (1.39 [95% CI 0.67–2.87]) (Supplementary Table 9). This relative protection conferred by maternal type 1 diabetes versus paternal or sibling type 1 diabetes was observed in the higher risk strata (Supplementary Fig. 8).



**Figure 3**—The modification of risk by the *BTNL2* SNP rs3763305 on the development of one or more islet autoantibodies (A), multiple islet autoantibodies (B), and diabetes (C) in children with the *DR3/DRB1\*04:04-DQ8* or *DRB1\*04:04-DQ8/DRB1\*04:04-DQ8* genotypes. Risks are shown for the GG genotype versus the GA or AA genotypes at rs3763305. *P* values were calculated using log-rank tests.

## DISCUSSION

We found that the excess risk for islet autoantibodies and diabetes in FDR children could be abrogated by accounting for an increased load of type 1 diabetes susceptibility alleles at multiple loci, including a novel susceptibility region marked by SNPs within the *BTNL2* gene. The risk converged when children were matched at the highest genetic susceptibility and became increasingly divergent as genetic susceptibility was attenuated. These data suggest that additional factors shared within families may modify type 1 diabetes risk heterogeneously and are dependent upon a priori genetic susceptibility.

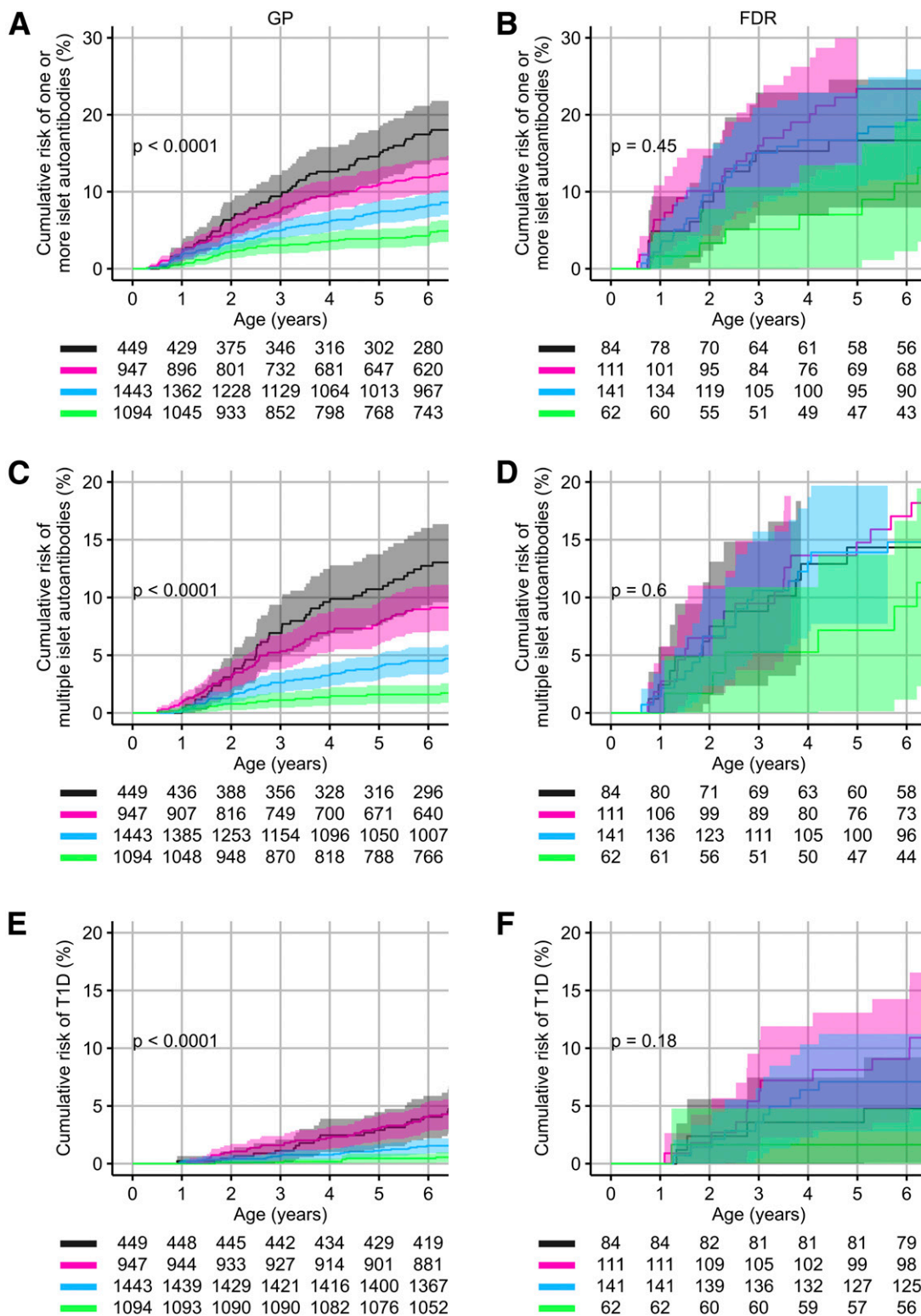
The study was performed in a large number of FDR and GP children of mainly European descent who were matched for the two highest-risk HLA class II genotypes. This unique cohort allowed us to assess the contributions of other genetic factors. After selection by HLA genotype, the excess risk for islet autoantibodies and diabetes was approximately two- to threefold higher in FDR children, which is markedly less than the >10-fold excess observed without HLA selection. Enrichment of genetic susceptibility was observed for HLA *DR4* subtypes and by an increased genetic risk score for non-HLA loci. The addition of these genetic markers further reduced the excess risk in FDR children, but the adjusted HRs remained >2 for the development of multiple islet autoantibodies or diabetes. Remarkably, this excess risk was heterogeneous and depended on the a priori genetic susceptibility. A limitation of our study is that we could not examine children with other HLA genotypes and, therefore, cannot assess whether the divergence continues in children with HLA genotypes associated with moderate or low risk. Although TEDDY is a unique study with unprecedented numbers of FDR and GP children for comparisons, the findings require further validation, especially in different ethnic populations.

The excess risk that remained unaccounted for by susceptibility genes in families is likely due to further genetic enrichment, including rare variants that may be

more frequent in familial cases, or other factors, such as a shared environment. The study provided the opportunity to search for additional genetic factors that may contribute to risk by exploring genes with allelic enrichment in FDR children. A limitation of this approach is that, despite the size of the TEDDY study, there was relatively little power to find these genes across the whole genome, particularly for genes with low minor allele frequencies.

We were successful in finding an enrichment of alleles for two additional genes. One of the genes with allelic enrichment in the FDR children, *BTNL2*, lies within the HLA class II region. SNPs within *BTNL2* were previously shown to be associated with other HLA DR-linked diseases, but in almost all cases, including type 1 diabetes, the risk was attributed to LD with HLA DR (19). Our study, which included >3,000 children with the HLA *DR3/DR4-DQ8* genotype and >1,500 with the *DR4-DQ8/DR4-DQ8* genotype, had sufficient power to adequately test the independent contribution of *BTNL2*. The G allele of the SNP rs3763305 increased the risk for type 1 diabetes, with an HR of ~1.7 in these HLA-selected children. Although our analyses also controlled for the HLA *DRB1\*04* subtype, we cannot exclude the possibility that the *BTNL2* SNP marks HLA *DR4* extended haplotypes. However, there was an association between the nonsusceptible *BTNL2* allele and *DRB1\*04* subtypes that are protective or confer relatively low risk. It is, therefore, equally possible that some of the associations between *DRB1\*04* subtype and type 1 diabetes risk are due to variation in *BTNL2* rather than or in addition to HLA DR. *BTNL2* is a negative regulator of immunity that is expressed on antigen-presenting cells and affects the generation, proliferation, and function of regulatory T cells (20–22) and, as shown here, activation of naïve CD4<sup>+</sup> T cells. It was demonstrated that *BTNL2* SNPs confer risk for sarcoidosis (23), a T cell-related inflammatory disease, independently of HLA DR (24), and influence antibody responses to dietary antigens (25). A relationship between the minor allele of the *BTNL2* rs3763305 genotype and *BTNL2* transcriptomic expression has been





**Figure 4**—Risk of developing islet autoantibodies and diabetes in FDR children (B, D, and F) and in GP children (A, B, and C) according to genetic susceptibility strata based on HLA *DRB1\*04* subtype and genetic risk score. Risks are shown for the development of one or more islet autoantibodies (A and B), multiple islet autoantibodies (C and D), and diabetes (E and F). All of the children had the *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* genotype. Genetic susceptibility strata were defined as 1) high-risk *DRB1\*04* subtype (*DR3/DRB1\*04:01* or *DRB1\*04:01-DQ8/DR4* without 04:04 or 04:07) and GRS in the upper quartile (gray), 2) high-risk *DRB1\*04* subtype and GRS in the second quartile or lower-risk *DRB1\*04* subtype and GRS in the upper quartile (pink), 3) high-risk *DRB1\*04* subtype and GRS in the lower 50th centile or lower-risk *DRB1\*04* subtype and GRS in the second quartile (light blue), and 4) lower-risk *DRB1\*04* subtype and GRS in the lower 50th centile (green). The strata appear in this order from top to bottom in the risk tables. P values were calculated across all strata using log-rank tests.

reported (26). Further studies are required to determine whether there are functional differences between *BTNL2* genotypes that may be relevant to type 1 diabetes susceptibility.

The remaining increased risk for islet autoimmunity and type 1 diabetes in FDR children after accounting for genetic load implies that other factors, which are shared or enriched within affected families, contribute to the child's risk. It is known that a family history of type 1 diabetes is associated with changes in parental practices in an effort to reduce the risk in their unaffected children. It is likely that such practices are more frequent in the children of affected families, and it seems possible that some of these practices may be associated with increased risk. It is also possible that family members more often share infections or diet that increase the risk for islet autoimmunity. One enriched factor in the FDR children is maternal type 1 diabetes, which is known to offer relative protection (18). Indeed, unlike children whose father or sibling had type 1 diabetes, there was no excess risk in children whose mother had type 1 diabetes compared with GP children in the Cox proportional hazards model. The relative protection conferred by maternal compared with paternal type 1 diabetes was pronounced in the higher genetic susceptibility strata, suggesting that maternal type 1 diabetes harbors a dominantly protective environment in the presence of enriched genetic susceptibility. These data also suggest that the shared environment of siblings and fathers with type 1 diabetes may be a source from which to identify environmental risk factors.

In summary, we have shown that the increased risk of developing islet autoimmunity in FDR children is largely due to an excess load of genetic susceptibility, we identified a potential novel gene that confers risk for islet autoimmunity, and we have shown that accounting for the excess genetic susceptibility leads to convergence in high-risk strata and divergence in lower-risk strata for the risk of developing islet autoantibodies and diabetes between FDR children and GP children. These findings stress that environmental risk factors of disease will likely exert different effects in a gene-dependent manner and that searching for these factors may require genetic stratification. Of practical relevance, the study showed that it is possible to identify GP children whose risk for islet autoantibodies and type 1 diabetes is as high as that in the highest-risk FDR children.

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