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An exploration of G1b1 Homologue Antibody Levels in Children at Increased Risk for Type 1 Diabetes mellitus

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Abstract

Aims—To determine whether G1b1 homologue antibodies are associated with islet autoimmunity (IA) in children at increased risk for type 1 diabetes (T1D), and to investigate their relation with putative environmental correlates of T1D.

Methods—We selected a sample from the Diabetes Autoimmunity Study in the Young (DAISY), a prospective study of children at increased risk for T1D. Cases were those who were positive for insulin, glutamic acid decarboxylase (GAD), or insulinoma-associated antigen-2 (IA-2) autoantibodies on two consecutive visits and either diagnosed with diabetes mellitus or still autoantibody positive when selected. Controls were from the same increased risk group, of similar age as the cases but negative for autoantibodies. Sera from 91 IA cases and 82 controls were analyzed in a blinded manner for immunoglobulin G (IgG) antibodies to G1b1 homologue by ELISA.

Results—Adjusting for family history of T1D and HLA-DR4 positivity, G1b1 homologue antibodies were not associated with IA case status (OR: 1.01, 95% CI: 0.99 – 1.03). Adjusting for age, family history of T1D, and HLA-DR4 positivity, G1b1 homologue antibody levels were inversely associated with breast-feeding duration (beta = -0.08, p = 0.001) and directly associated with current intake of foods containing gluten (beta = 0.24, p = 0.007) in IA cases but not in controls. Zonulin, a biomarker of gut permeability, was directly associated with G1b1 homologue antibody levels in cases (beta = 0.73, p = 0.003) but not in controls.

Conclusion—Differences in correlates of G1b1 antibodies in IA cases and controls suggest an underlying difference in mucosal immune response.

Keywords

Type 1 diabetes mellitus; G1b1 homologue; Zonulin; Islet Autoimmunity

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Introduction

Type 1 diabetes mellitus (T1D) is a deficiency of the endocrine pancreas that results in aberrations in glucose metabolism; it is preceded by a period of islet autoimmunity (IA) that lasts for varying amounts of time. A well documented genetic component to this disease exists (1) but the environmental etiologies need to be definitively characterized.

The gastrointestinal immune system may have etiologic importance in this disease process, as infant diet and current dietary habits may affect the behavior of the gut immune system. Wheat has been investigated for its role in the pathogenesis of IA and T1D, because of its known role in the etiology of celiac disease, an autoimmune disease that frequently occurs with T1D (2), and because timing of cereal introduction in the infant diet has been associated with IA in children at increased risk of T1D(3;4). We previously found that exposure to cereals during months 4 – 6 in infancy was associated with a lower risk of IA compared with first cereal exposure either before or after this time period. We also reported that concurrent breast feeding at the time of cereal introduction was associated with a decreased IA risk (3). Similarly, Ziegler et al found that older age at introduction to foods containing gluten was associated with a decreased risk of IA(4). One mechanistic explanation for these findings may be that early life feeding practices modify the mucosal immune system and/or gut permeability. Specifically, breast feeding has been shown to stimulate gut closure (5;6) and gluten (found in cereals) has been shown to initiate an inflammatory response that could result in compromised intestinal barrier function in many disease conditions including celiac disease, rheumatoid arthritis, dermatitis herpetiformis, and autism (7–9).

In an effort to further understand the role of wheat in the pathogenesis of IA and T1D, we measured antibodies to a wheat storage globulin homologue of Glb1, which is a non-gluten component of the wheat protein matrix. This protein was originally identified by screening a wheat cDNA library using serum from diabetic rats. Researchers found a correlation between the reactivity of IgG antibodies against this protein and islet inflammation and damage (10). Two-dimensional Western blot analysis of a protein extract from wheat gluten, which contains Glb1 homologue as a trace contaminant, demonstrated that Glb1 homologue antibodies were present in sera pooled from recently diagnosed diabetic patients but were absent in non diabetic controls (10). Moreover, Glb1 homologue antibody levels were elevated in a patient with both type 1 diabetes mellitus and celiac disease (11). To date this biomarker has not been investigated during the autoimmune period.

Zonulin reportedly modulates the integrity of intestinal tight junctions, and may be a marker of gut permeability. In animal studies, researchers found that diabetes-prone rats have higher levels of zonulin than their wild-type counterparts (12;13). A study in human subjects found that zonulin was elevated in T1D patients compared to their first degree relatives and this elevation correlated with increased gut permeability (14).

We tested the association between antibodies to Glb1 homologue and IA status; and further explored potential correlates of Glb1 homologue antibodies, specifically dietary factors, such as gluten consumption at the time of blood collection, breast feeding duration, age at introduction to cereals, and age at introduction to cow's milk based products, and serum zonulin levels. The rationale for this approach was that these diet variables have been studied for their potential role in the development of diabetes mellitus (whether a risk factor or protective) and some have been identified as potential modifiers of gut permeability (5;6;10).

Methods

Study population

The Diabetes Autoimmunity Study in the Young (DAISY) is a prospective study of more than 2300 children at increased risk for developing type 1 diabetes mellitus (15). Children are identified as high diabetes risk if 1) they have a first degree relative with type 1A diabetes mellitus, or 2) they were screened and found to have diabetes-susceptibility alleles in the HLA region. First degree relatives were identified and recruited between birth and eight years of age through the Barbara Davis Center for Childhood Diabetes in Denver, Colorado, other diabetes care clinics and the Colorado IDDM Registry (16). The second group consists of babies born at St. Josephs Hospital in Denver, Colorado, which is representative of the general population of the Denver Metropolitan Area, who were screened for high risk HLA alleles. The details of the newborn screening (15) and follow up (3) have been published elsewhere. Cord blood is sent to Roche Molecular Systems, Inc, Alameda, CA for PCR-based HLA class II typing. Recruitment for this study began in January of 1994 and includes data collected through November 2006. Written informed consent was obtained from the parents of study participants. The Colorado Multiple Institutional Review Board approved all study protocols. From this population, IA cases and autoantibody negative controls were selected for this study.

Infant diet measurement

For those children followed from birth, infant diet data were collected every three months from ages 3 to 15 months. The interviews and questionnaires captured infant feeding practices including duration of breast feeding, age at the time of introduction to cereals, and age at introduction to cow's milk containing products. Mothers of children enrolled after the age of 15 months completed a similarly structured questionnaire at the time of enrollment.

Dietary measurement

Childhood diet is assessed beginning at the age of two years using a semi-quantitative food frequency questionnaire (FFQ) (17;18). The FFQ is administered once a year and asks parents to report the average food consumption over the course of the previous year of their child. It is a structured response survey regarding how often the child consumes commonly sized portions (example: 1 slice of bread), with the response options ranging from 'never' to '6+ servings per day'. The FFQ has been validated against multiple 24 hour recalls (19) and has been found to correlate with micronutrient (20) and fatty acid (21) biomarkers in our population. Only the dietary data regarding the time period around the study visit at which the serum sample was collected are included in the analyses.

Daily servings of foods containing gluten were calculated based on the reported intake of 25 food items that contain gluten. We summed the number of reported servings of the following foods listed on the FFQ: 1. brownies; 2. cake, home baked; 3. cake, ready made; 4. sweet roll, coffee cake, or other pastry, home baked; 5. sweet roll, coffee cake, or other pastry, ready made; 6. pie, home made; 7. pie, ready made; 8. cookies, home baked; 9. cookies, ready made; 10. doughnuts; 11. cold breakfast cereal; 12. cooked breakfast cereal, eg: oatmeal; 13. white bread; 14. dark bread; 15. English muffins, bagels, or rolls; 16. muffins or biscuits; 17. pasta eg: spaghetti, noodles, etc.; 18. pancakes or waffles; 19. crackers, triscuits, or wheat thins; 20. pizza; 21. beef, pork, or lamb as a sandwich; 22. hamburgers; 23. hot dogs; 24. bran added to food; 25. wheat germ. We were not able to quantify gluten contained in foods as a palatability enhancer or stabilizer.

Measurement of Islet Autoantibodies

All children who were recruited at birth were tested at 9 months, 15 months, 24 months, and annually thereafter for antibodies to pancreatic islet antigens. Children who were recruited after birth had their blood first drawn at enrollment and then annually thereafter. Children who tested positive for any of the 3 autoantibodies were placed on an accelerated schedule on which they returned for a blood draw every 3 to 6 months for the duration of the study. Individuals who were negative for the autoantibodies remained on the aforementioned clinic visit schedule. Glutamic acid decarboxylase 65 (GAD) autoantibodies and insulinoma-associated antigen-2 autoantibodies (IA-2) were measured with a combined radiobinding assay as previously described (22). In brief, the sera were incubated with 3-H labeled GAD65 and 35-S label ICA512 and then precipitated with protein A Sepharose (Amersham, Little Chalfont, England). The assay was performed on a 96-well filtration plate (Fisher Scientific, Loughborough, England) and radioactivity was counted on a Topcount 96-well plate beta counter (PerkinElmer Life Sciences, Wilmington, Delaware). The antibody levels were expressed as an index. The interassay coefficients of variation (n=50) are 10% and 5% for GAD and IA-2, respectively. The upper limits of normal controls (0.032 for GAD and 0.049 for insulinoma-associated antigen-2 autoantibodies) were established as the 99th percentile in 198 healthy controls. In the most recent Diabetes Autoantibody Standardization Program workshop (2005), the sensitivity and specificity were 76% and 99%, respectively, for GAD, and 66% and 100%, respectively, for insulinoma-associated antigen-2 autoantibodies.

Insulin autoantibody was measured by a micro-insulin autoantibody assay as described previously (23). Briefly, ¹²⁵I-labeled human insulin (Amersham) was incubated with patient serum with and without cold human insulin, and immune complex was precipitated with protein A and G Sepharose. The assay was performed on a 96-well filtration plate and radioactivity was counted on a Topcount 96-well plate beta counter. An index was calculated on delta counts per minute between wells without and with cold human insulin, with a positivity criterion of 0.010, which was the 99th percentile of 106 normal controls. The interassay coefficient of variation is 20% (n=100) at low-positive levels. In the most recent Diabetes Autoantibody Standardization Program workshop (2005), the sensitivity and specificity for insulin autoantibody were 58% and 99%, respectively.

Case/Control definition

Both cases and controls were selected from the larger DAISY cohort which is comprised of children at increased risk for T1D. In order to be included as a case, a DAISY child must have tested positive for at least 1 autoantibody on at least 2 consecutive visits less than 6 months apart and either diagnosed with diabetes mellitus or still autoantibody positive at the time of case selection. Ninety-one DAISY children met this case definition and had an available serum sample at the second of their first two consecutively positive visits. The controls were selected from DAISY children who had never had an autoantibody positive visit, and were frequency matched by age to the case samples that correspond to the second of the two autoantibody positive visits required to meet the case definition.

Glb1 homologue antibody measurement

The previous non-quantitative 2D Western analysis(10) indicated the presence of antibodies binding denatured Glb1 homologue, a trace contaminant of wheat gluten, in pooled sera from newly diagnosed children but not in controls. To measure Glb1 homologue antibodies in individual sera, we developed a sensitive quantitative capture ELISA that measures binding to purified recombinant Glb1 homologue. Anti – Glb1 immunoglobulin G was measured in serum at the Ottawa Health Research Institute (OHRI). A modified capture ELISA was established using purified Glb1 to measure IgG-Glb1 antibodies in serum of cases and controls. Analyses were performed blinded without knowledge of case or control status. To reduce the background

and improve the sensitivity of the assay, human serum albumin was used to prevent non-specific binding of sera to the plate and the secondary antibody was absorbed by G1b1 to reduce non-specific signal. The assay was performed as follows: 96 well flat-bottom plates (Nunc MaxiSorp Greiner's Cat No. 439454) were coated with 50 μ l of 0.5 μ g/well recombinant G1b1 (expressed and purified in OHRI) in coating buffer (Phosphate buffer pH 9.6 NaHCO₃/Na₂CO₃) for 90 min at 37°C. Plates were washed twice with ELISA washing buffer (PBS containing 0.05% Tween20, Sigma). Plates were blocked with 200 μ l of blocking buffer (5% human serum albumin in PBS) (Albumin 25% solution, DIN 02223708, Bayer Corporation, Elkhart, IN) for 1 hour at 37°C or 4°C overnight. After removing blocking buffer, 100 μ l of control or test sample were diluted 1:20 in dilution buffer (5% human serum albumin in PBS containing 0.05% Tween 20) and added to each duplicate well. ELISA dilution buffer was used in duplicate wells as a blank. Plates were incubated overnight at 4°C. The next day, plates were washed 6 times with washing buffer with at least three 5–10 min incubation intervals. At least one hour before starting the washing process, the secondary antibody (Anti-human IgG A0170 conjugated with horse radish peroxidase (HRP) (Sigma, Oakville, ON, Canada) was diluted 1:20,000 in dilution buffer and absorbed with G1b1 1 μ g/ml for 1 hour with continuous shaking at room temperature to block non-specific binding. 100 μ l of pre-absorbed secondary antibody was added to each well and incubated for 90 min at room temperature with slight shaking. The plates were washed and incubated with 100 μ l/well 3, 3', 5, 5' tetramethylbenzidine (TMB) peroxidase substrate (BD Opt EIA Cat No. 555214) for 10–20 min at room temperature. The reaction was stopped using 100 μ l/well of 1M H₂SO₄. The plates were evaluated at 450 nm using a Multiscan Ascent plate reader. To standardize the results, arbitrary units were calculated in relation to the serum value of an individual who displayed high levels of G1b1 homologue antibody. The antibody level in this serum was considered as 100 arbitrary units and used as a standard to calculate the value of other samples in the assay. Two other sera with low and intermediate absorbance were used as internal controls in each assay. Sera with different values for G1b1 homologue antibodies were evaluated to develop a standard curve. The optical density (OD) for low arbitrary values was indeed low and that of the serum assigned 100 arbitrary units displayed an OD of 1.04. Two arbitrary units corresponded to 0.015 OD. In order to determine the repeatability of the assay, 124 samples were re-assayed. The intra-class correlation between these sample sets was 0.75.

Serum zonulin measurement by sandwich ELISA

Zonulin sandwich ELISA was performed as previously described (15,25). Detailed description of the structural and functional characterization of zonulin has been previously described (A. Fasano, personal communication). To define the intra-assay precision of the ELISA-sandwich method, the coefficient of variation (CV) was calculated using double replicates from two samples with different concentrations of zonulin, on three consecutive days. The coefficient of variation of the intra-assay test was 4.2% at day 1, 3.3% at day 2 and 2.9% at day 3. Zonulin was expressed as ng/mg total protein detected in the tested samples. DAISY samples were compared against a standard zonulin curve using spectrophotometry.

Statistical analysis

All analyses used Statistical Analysis Software version 9.2 (SAS institute, Cary, North Carolina).

To explore the association between G1b1 homologue antibody levels and IA case status, we performed univariate and multivariate logistic regression. G1b1 homologue antibody levels were analyzed as a continuous variable, because there is no predefined dichotomization level for this novel biomarker, and we maximize power by analyzing it continuously.

In order to explore the correlates of G1b1 homologue antibodies, univariate and multivariate linear regression analyses were performed, with G1b1 antibody levels as the outcome. Because G1b1 homologue antibodies was not normally distributed, we natural log transformed the variable, in order to satisfy the conditions of the linear regression analyses.

When exploring risk factors for IA case status, age at cereal introduction was considered in three categories: 0 to 3 months of age, 4–6 months of age (reference group), and 7 months of age and older in order to be consistent with the non linear association with IA that was reported in Norris *et al.* (3). When exploring correlates of G1b1 homologue antibody levels, we examined age at cereal introduction as a continuous variable to maximize power.

We did not have dietary information on all of the cases and controls for the time period proximal to the serum sample that was tested for G1b1, because either the sample was obtained before we started collecting FFQs in 1997, or the child was too young (< 1 year of age) for the FFQ protocol, or the family did not submit an FFQ. Therefore, the sample size for the analysis of current intake of foods containing gluten consists of 47 cases and 46 controls.

Zonulin levels were not available on 43 controls; therefore, the dataset for the analysis of zonulin consisted of 91 cases and 39 controls.

Results

G1b1 and IA case status

Cases were more likely to have a first degree relative with T1D and to be positive for the HLA-DR4 allele than controls (Table 1). Adjusting for family history of T1D and HLA-DR4 positivity, G1b1 homologue antibodies were not elevated in children with IA compared to children who were IA antibody negative (adjusted OR: 1.01, 95% CI: 0.99 – 1.03).

Correlates of G1b1 homologue antibody levels

Using linear regression and natural log transformed G1b1 homologue antibody levels as the outcome, we examined associations between G1b1 homologue antibody levels and the correlates listed in Table 2. Given that case status was a selection variable in our analysis population, this variable was included in all multivariate models of potential predictors of G1b1 homologue antibody, as a main effect variable and in interaction terms. We detected significant interactions between case status and daily servings of gluten ($p_{\text{interaction}}$: 0.003, not reported in a table), and duration of breast feeding ($p_{\text{interaction}}$: 0.001, not reported in a table). The significance of the interaction terms suggested that the results should be presented separately in cases and controls. Therefore, to present the adjusted correlates of G1b1 homologue antibody levels, we used a single adjusted model that included age, HLA-DR4 allele positivity, having a first degree relative with T1D, IA case status, daily servings of foods containing gluten, breast feeding duration, and the interaction terms of daily servings of foods containing gluten by IA case status and breast feeding duration by IA case status. Beta estimates and p-values for the gluten and breast-feeding variables were calculated from the interaction terms; and the beta estimates and p-values for the remaining variables were obtained from the single model, thus they are identical in cases and controls (as presented in Table 2). The r^2 of the base model with just age, HLA-DR4 allele positivity, and having a first degree relative with T1D as correlates of G1b1 homologue antibodies was 0.05, whereas the r^2 of the full model with gluten intake and breast-feeding duration (and their corresponding interaction terms with case status) was 0.29; suggesting that these variables explain a substantial amount of the variability in G1b1 homologue antibody levels.

Figures 1 and 2 graphically display the interactions between case status and gluten intake and breast feeding duration respectively, using adjusted estimates (interaction p: 0.003 and 0.001,

respectively). In cases, every 1 serving increase in daily servings of foods containing gluten is associated with a 24% increase in G1b1 antibodies (Figure 1), adjusting for age, having a first degree relative with T1D, HLA-DR4 allele positivity, and breast-feeding duration. Likewise, in cases, every additional month of breast feeding is associated with an 8% decrease in G1b1 homologue antibody levels (Figure 2), adjusting for age, having a first degree relative with T1D, HLA-DR4 allele positivity, and daily servings of foods containing gluten.

Figure 3 demonstrates that the association between zonulin and G1b1 antibody levels is different between cases and controls. Adjusting for age, having a first degree relative with T1D, and HLA-DR4 allele positivity, zonulin levels are significantly positively associated with G1b1 homologue antibody levels in cases ($\beta = 0.73$, $p = 0.003$), but not in controls ($p = 0.66$). Because zonulin is hypothesized to be regulated by gluten exposure, we examined whether intake of foods containing gluten was associated with zonulin levels (natural log transformed) as the outcome. Gluten exposure was marginally associated with increased zonulin levels in cases ($\beta: 0.27$, $p = 0.08$) but not in controls ($\beta: -0.007$, $p = 0.54$).

The longitudinal nature of DAISY afforded us the opportunity to assess the current autoantibody status of the cases included in these analyses. In the time between their selection as an IA case for this study and their most recent follow up visit, 10 subjects became negative for autoantibodies, and 81 either became diabetic or remained autoantibody positive. Because the stated purpose of this paper was to analyze their G1b1 at the time they became autoantibody positive, all of the statistical modeling included above does not make a distinction concerning current autoantibody status. The mean G1b1 homologue antibody level for those children who subsequently became negative for their antibodies is 3.20 (sd: 3.36) and for children who became diabetic or remained autoantibody positive the mean G1b1 homologue antibody level was 13.88 (sd: 23.75). This difference was not statistically significant ($p = 0.22$, adjusting for age, having a first degree relative with T1D, and being HLA-DR4 positive) likely due to low power.

Because age was found to be a marginally significant predictor of G1b1 homologue antibody levels in the population (Table 2), we examined whether G1b1 homologue antibody levels differed by age within individuals, by obtaining the earliest serum sample available on our IA cases (prior to IA positivity), measuring G1b1 homologue antibody levels at this younger age, and comparing this to the level at the age when they were IA positive. For clarity of presentation, we graphed children whose G1b1 homologue antibody levels were higher at the second measurement separately (Figure 4a) from those whose levels were the same or lower at the second measurement (Figure 4b). These figures suggest heterogeneity in the relationship between G1b1 homologue antibody levels and age in IA cases. While G1b1 levels increased prior to IA positivity in the majority of cases (Figure 4a), there were also a number of IA cases in which G1b1 homologue antibody levels either stayed the same or decreased prior to IA positivity (Figure 4b).

Discussion

The immunologic response to environmental stimuli such as gluten by individuals susceptible to T1D is poorly defined and heterogeneous.(24) Our data suggest that G1b1 homologue antibody levels did not differ between cases of IA at initial autoantibody positivity and age-similar controls at increased risk for T1D. However, our subsequent exploratory analyses have provided intriguing data that offer hypothesis-generating insights into the role of diet and the gut in type 1 diabetes.

Our data did not support our hypothesis that G1b1 homologue antibody levels would differ between cases of IA at initial autoantibody positivity and similarly aged controls. MacFarlane

et al.(10) used pooled sera from Finnish children aged ~ 10 years to probe 2D immunoblots of wheat proteins and found that recently diagnosed diabetic patients had increased Glb1 homologue antibody levels compared to their non-diabetic counterparts. In addition, this previous non-quantitative 2D Western blot analysis indicated the presence or absence of antibodies binding denatured Glb1 homologue, a trace contaminant of wheat gluten. To measure Glb1 homologue antibodies in individual sera, we developed a more sensitive quantitative capture ELISA that measures binding to purified recombinant Glb1 homologue used in the currently study. Differences in definition of outcome, approach (pooled vs individual sera), study populations and assay likely explain the seemingly contrasting results between MacFarlane *et al* (10) and the current study. However, the MacFarlane *et al* finding, along with our finding that wheat intake is associated with Glb1 homologue antibody levels measured with a more sensitive ELISA, may provide indirect evidence of increased immune reactivity among a subset of IA positive and diabetic cases. This could be one explanation of may provide insight into why Glb1 homologue antibody is not a correlate of case status; if IA subjects are prone to a non-specific hyper-reactivity to all what proteins (or conceivably all dietary proteins) then antibodies to Glb1 homologue would only provide a partial picture of a larger scale response to dietary antigens. Further characterization of response to a variety of dietary antigens over time is needed to explore this hypothesis further.

When interpreting these results it is important to consider that the controls used in our study were selected from a high risk population (ie DAISY) and that while this makes the controls truly comparable to those who developed IA within DAISY, there is the potential that given their increased risk, the controls may eventually develop IA and T1D. It is becoming clear that T1D can develop by several pathways in different individuals. The data in Figures 4a and b support the notion that heterogeneity is present among IA cases, perhaps suggesting that the etiologic pathway in which Glb1 homologue antibodies are involved is responsible for part, but not all, of the risk of islet autoimmunity or that this antibody may be an important etiologic marker for some children and not for others.

In our study population, zonulin levels were not different between cases of IA at initial autoantibody positivity and similarly aged controls, which is not consistent with a previous report showing children with T1D had higher levels of zonulin than healthy controls (14). However, differences between the previous and the current study in terms of case definition (T1D vs IA) and control population (healthy vs increased risk) may explain the differing findings.

Our finding that Glb1 homologue antibody levels appear different by whether or not the cases subsequently lost their islet autoantibodies is intriguing. These differences, although not significant, may reflect a heretofore unmeasured proclivity among those children who are persistently autoantibody positive compared to those who revert from autoantibody positivity. In order to study this further, it will be important to analyze Glb1 antibodies longitudinally in the autoimmune period to characterize how they change in comparison to the change in autoantibody positivity.

The correlates (e.g. breast-feeding duration, current gluten intake, zonulin) of Glb1 homologue antibody levels that we found in cases but not in controls are another potentially interesting finding with several plausible underlying mechanisms, including inflammation and gut permeability, which has been shown to be increased in newly diagnosed diabetic patients (25–28). These results may indicate that, for a given daily amount of gluten or a given duration of breast feeding that cases respond with Glb1 homologue antibodies to a greater degree than controls, perhaps due to an underlying predisposition influenced by either genetic or environmental factors early in life.

This study is an initial exploration of the associations between Glb1 homologue antibody levels and islet autoimmunity. While levels do not differ between cases and controls, some of the findings are supportive of the idea that there is a great deal of etiologic heterogeneity in IA and T1D, and that there may be a subset of children who developed autoimmunity where the Glb1 homologue antibody response may be important in the pathogenesis of IA. In spite of the caveats for this study, the results provide compelling evidence that further research is warranted.

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Figure 1.

Association between daily servings of foods containing gluten and Glib1 homologue antibody levels in IA cases and controls. • represents unadjusted, untransformed data points in arbitrary units for the cases ($n = 47$) in the left panel, • represents unadjusted, untransformed data points in arbitrary units for controls ($n = 46$) in the right panel. The lines are the adjusted regression line for cases and controls, respectively. The regression lines were generated from the significant interaction term ($p = 0.003$) and have been adjusted for age, having a first degree relative with T1D, and having a HLA-DR4 allele using natural log transformed Glib1 homologue antibody as the outcome which was then back transformed for graphing purposes.^a

Figure 2.

Association between the duration of breast feeding (in months) and Glib1 homologue antibody levels in IA cases and controls. • represents unadjusted, untransformed data points in arbitrary units for the cases ($n = 47$) in the left panel, • represents unadjusted, untransformed data points in arbitrary units for controls ($n = 46$) in the right panel. The lines are the adjusted regression line for cases and controls, respectively. The regression lines were generated from the significant interaction term ($p = 0.001$) and have been adjusted for age, having a first degree relative with T1D, and having a HLA-DR4 allele using natural log transformed Glib1 homologue antibody as the outcome which was then back transformed for graphing purposes.*

Figure 3.

Association between zonulin levels and G1b1 homologue antibody levels in IA cases and controls. • represents unadjusted, untransformed data points in arbitrary units for the cases (n = 91) in the left panel, • represents unadjusted, untransformed data points in arbitrary units for controls (n = 39) in the right panel. The lines are the adjusted regression line for cases and controls, respectively. Both regression lines were adjusted for age, having a first degree relative with T1D, and having a HLA-DR4 allele using natural log transformed G1b1 homologue antibody as the outcome which was then back transformed for graphing purposes.

Figure 4.

Figure 4a and b. G1b1 homologue antibody levels among 63 IA cases who had an early, pre-IA positivity, sample and a sample at the time of initial IA positivity, for G1b1 homologue antibody measurement. For each case, the G1b1 homologue antibody level at the early pre-positive visit and the level at the IA positive visit are indicated by the same symbol, with a line connecting the two for ease of comparison. G1b1 values are natural log transformed. For clarity of presentation, we divided our study population into IA cases ($n=39$) whose G1b1 homologue antibody levels increased between the first and second measures (Figure 4a) and those IA cases ($n=24$) whose G1b1 homologue antibody levels remained the same or decreased between the first and second measures (Figure 4b).

Table 1

Description of characteristics and their association with IA positivity. The Diabetes Autoimmunity Study in the Young Nested Case-Control Study.

Variable	Cases (n=91)	Controls (n=82)	Unadjusted			Adjusted		
			Odds Ratio	95% C.I.		Odds Ratio	95% C.I.	
Glb1 mean(sd)	12.71(22.67)	8.90(14.78)	1.01	0.99 – 1.03		1.01	0.99 – 1.03	
Zonulin mean(sd) ^a	0.54(0.88)	0.57(0.67)	0.66	0.23 – 1.94		c		
Age mean(sd)	4.83(3.53)	4.65(3.13)	1.02	0.93 – 1.11		c		
First degree relative with T1D n(%)	64(70)	26(32)	1.28	2.67 – 9.75		5.74	2.91 – 11.44	
Female n(%)	50(55)	40(49)	1.28	0.70 – 2.33		c		
Maternal education ≥ 12 years n(%)	72(80)	61(75)	1.31	0.64 – 2.70		c		
HLA-DR4 allele n(%)	75(67)	55(82)	2.30	1.13 – 4.68		3.07	1.38 – 6.83	
Non-Hispanic white n(%)	81(89)	66(80)	1.96	0.84 – 4.61		c		
Daily servings of foods containing gluten mean(sd) ^b	3.73(1.68)	4.25(2.25)	0.87	0.70 – 1.08		c		
Breast feeding duration in months mean (sd)	7.33(7.31)	6.62(5.98)	1.02	1.02 – 1.06		c		
Age in months at introduction to cow's milk mean(sd)	4.55(3.94)	3.70(3.48)	0.98	0.98 – 1.16		c		
Cereal introduction n(%)	17(19)	11(14)	1.65	0.71 – 3.80				
	4–6 months	64(79)	1.00	referent		c		
	7 months and older	6(7)	2.31	0.83 – 6.47				

^a Due to missing data, analyses of zonulin included 91 cases and 39 controls.

^b Due to missing data, analyses regarding daily servings of foods containing gluten included 47 cases and 46 controls.

^c These variables were not included in the final logistic regression model.

Table 2

Correlates of G1b1 antibody levels in IA cases and autoantibody negative controls in the Diabetes Autoimmunity Study in the Young Nested Case-Control Study

Variable	Cases (n = 47)			Controls (n = 46)		
	Unadjusted Beta Estimate	p-value	Adjusted ^a Beta Estimate	Unadjusted Beta Estimate	p-value	Adjusted ^a Beta Estimate
Age	-0.12	0.07	-0.08	-0.05	0.50	-0.08
HLA-DR4 allele	-1.50	0.002	-0.43	-0.22	0.51	-0.43
First degree relative with T1D	-0.05	0.91	0.30	0.81	0.01	0.30
Non-Hispanic white	-0.23	0.73	<i>b</i>	0.31	0.52	<i>b</i>
Daily servings of foods containing gluten	0.24	0.04	0.24	0.04	0.62	-0.03
Age in months at first cereal introduction	-0.16	0.12	<i>b</i>	-0.03	0.83	<i>b</i>
Age in months at cow's milk introduction	-0.08	0.12	<i>b</i>	0.02	0.67	<i>b</i>
Breast feeding duration in months	-0.08	0.003	-0.08	0.04	0.16	0.02

^aThe final adjusted model included both the case status*daily servings of foods containing gluten and case status*breast-feeding duration interaction terms.

^bThese variables were not included in the final model. Maternal education did not differ between cases and controls in this analysis so it was not eligible for inclusion in the adjusted model.