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Phospholipid Levels at Seroconversion Are Associated With Resolution of Persistent Islet Autoimmunity: The Diabetes Autoimmunity Study in the Young

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Reversion of islet autoimmunity (IA) may point to mechanisms that prevent IA progression. We followed 199 individuals who developed IA during the Diabetes Autoimmunity Study in the Young. Untargeted metabolomics was performed in serum samples following IA. Cox proportional hazards models were used to test whether the metabolites (2,487) predicted IA reversion: two or more consecutive visits negative for all autoantibodies. We conducted a principal components analysis (PCA) of the top metabolites; |hazard ratio (HR) >1.25| and nominal $P < 0.01$. Phosphatidylcholine (16:0_18:1(9Z)) was the strongest individual metabolite (HR per 1 SD 2.16, false discovery rate (FDR)-adjusted $P = 0.0037$). Enrichment analysis identified four clusters (FDR $P < 0.10$) characterized by an overabundance of sphingomyelin (d40:0), phosphatidylcholine (16:0_18:1(9Z)), phosphatidylcholine (30:0), and L-decanoylcarnitine. Overall, 63 metabolites met the criteria for inclusion in the PCA. PC1 (HR 1.4, $P < 0.0001$), PC2 (HR 0.85, $P = 0.0185$), and PC4 (HR 1.28, $P = 0.0103$) were associated with IA reversion. Given the potential influence of diet on the metabolome, we investigated whether nutrients were correlated with PCs. We identified 20 nutrients that were correlated with the PCs ($P < 0.05$). Total sugar intake was the top nutrient. Overall, we identified an association between phosphatidylcholine, sphingomyelin, and carnitine levels and reversion of IA.

Type 1 diabetes (T1D) is an autoimmune disorder with a strong underlying genetic component (1). However, the incidence of T1D has increased (2,3) at a rate that suggests nongenetic factors such as environmental or lifestyle factors may play a relevant role in the pathogenesis of the disease (4). International birth cohort studies have yielded a long list of potential causative risk factors related to nutrients/dietary factors (5–7), exposure to viruses (8), and changes in body dimensions (9). However, many of the risk factors have not been consistently replicated across studies. This demonstrates the need for novel methods for understanding the complex etiology of T1D.

The presence of autoantibodies, termed islet autoimmunity (IA), is the best marker of T1D risk as well as the underlying autoimmune disease process (10). However, progression from IA to T1D is variable. A subset of individuals with persistent IA revert to an autoantibody-negative state (11). Among 596 individuals enrolled in The Environmental Determinants of Diabetes in the Young (TEDDY), risk of T1D in individuals who no longer produced autoantibodies was markedly reduced relative to risk in individuals who continued to produce autoantibodies (hazard ratio [HR] 0.14, 95% CI 0.04–0.59) (11).

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Reversion of IA is appealing as a potential marker of reduced T1D risk. Understanding mechanisms associated with reversion may help guide development of interventions that can slow or even prevent progression of the critical islet autoimmune phase of the T1D disease process. Previous analyses have focused on association between reversion and nonmodifiable factors such as demographics. Biomarkers associated with IA reversion may provide a more complete understanding of mechanisms underlying the autoimmune process and, more importantly, may highlight modifiable risk factors. Metabolites represent biomarkers of exogenous and endogenous biological processes that can inform our understanding of complex diseases (12). Previous metabolomics studies in the area of T1D have focused on early life metabolites and subsequent onset of autoimmunity or T1D (13–18). The influence of metabolite levels at or after seroconversion to IA has not been well studied. Johnson et al. (19) examined metabolites at seroconversion associated with progression to T1D in order to develop nutrient patterns associated with progression. However, no previous studies have tested the association between metabolite levels and reversion of IA. The purpose of this study was to test the association between metabolomic markers and IA reversion. We also aimed to explore the association between metabolites and nutrient intake in order to understand potential dietary factors that influence the metabolite patterns.

RESEARCH DESIGN AND METHODS

Study Population

Subjects were identified from the Diabetes Autoimmunity Study in the Young (DAISY) cohort of 2,547 children at risk, which has previously been described (20,21). A total of 231 individuals developed IA and/or T1D between February 1994 and February 2019. Radioimmunoassays were used to test serum samples for autoantibodies to insulin (IAA), GAD65 (GADA), and IA-2 (IA-2A). Prior to 2010, GADA and IA-2A were tested with a combined radioassay. The National Institute of Diabetes and Digestive and Kidney Diseases harmonized assay was used to test for GADA and IA-2A after 2010 (22). Serum samples from individuals positive for GADA, IAA, or IA-2A were tested for zinc transporter 8 (ZnT8) autoantibodies following development and implementation of the ZnT8 assay (23).

IA was defined as the presence of one or more autoantibodies on at least two consecutive visits 3–6 months apart. Autoantibody levels were then tested every 3–6 months. Drawing from Vehik et al. (11), we defined reversion as two or more consecutive autoantibody-negative visits that occur after two or more consecutive autoantibody-positive visits. The date of reversion was defined as the first visit among two or more consecutive visits where the individual tested negative for all autoantibodies. In order to ensure that reversion was possible in all individuals included in the study, we queried the DAISY database to identify all subjects who underwent autoantibody testing on two or more consecutive visits and developed IA ($n = 211$). We excluded subjects who did not have autoantibody testing data available prior to the onset of IA ($n = 12$) as well as subjects missing metabolomics data available at the onset of IA ($n = 32$). Although rare (11), reversion is possible in subjects who present with multiple autoantibodies at the onset of IA. Therefore, we included all individuals who developed IA and underwent autoantibody testing during multiple consecutive study visits following their seroconversion visit. The Colorado Multiple Institutional Review Board approved all DAISY protocols (COMIRB 92-080). Informed consent and assent, if appropriate, were obtained from the parents/legal guardians of all children prior to participation in any research-related activities.

Metabolomics

The National Institutes of Health West Coast Metabolomics Center at the University of California, Davis, performed the metabolomics analysis in nonfasting plasma samples, measuring untargeted hydrophilic interaction liquid chromatography (HILIC), gas chromatography time of flight (GCTOF), and lipid panels. The blood samples were obtained from the first DAISY visit after the onset of IA (i.e., at the first visit at which autoantibodies were detected). Serum (30 μL) was extracted from frozen samples (−80°C) with custom modifications to established protocols (24). Internal standards were added for quality control assessments and retention time correction (25). Following extraction, the aqueous phase was split, dried, and resuspended in 1:1 acetonitrile:water. HILIC–quadrupole time of flight (QTOF) tandem mass spectrometry (MS/MS) (26) was used to analyze one polar aliquot, while GCTOF–mass spectrometry (MS) (27) was used to analyze the second aliquot. CSH-QTOF MS/MS was used to identify and quantify (relative) complex lipids and free fatty acids (25). BinBase (28) was used to process and annotate the GCTOF-MS data. MS-DIAL (29) was used to process and annotate the liquid chromatography, CSH-QTOF-MS, and HILIC-QTOF-MS data. LipidBlast (30) and MassBank of North America were also used to annotate the complex lipids (https://mona.fiehnlab.ucdavis.edu/). In the liquid chromatography data sets, MS-FLO was used to remove erroneous peaks (31). After data were collected, annotated, and postprocessed, they were normalized using the systematic error removal using random forest (SERRF) algorithm (32). A high number of metabolites estimated to be zero in samples (>25) was considered low abundance ($n = 2$), and these samples were excluded from analyses.

We Box-Cox transformed all metabolites that passed the initial quality control checks. We filtered the

transformed data based on the coefficient of variation (CV). For each panel, we dropped metabolites if the CV for individual metabolite was ±2 median absolute deviations away from the median panel-specific CV. For the GCTOF panel, we removed metabolites with a CV <9.51 or >27.07 (77). For the lipid panel, we removed metabolites with a CV >36.32 (123). For the HILIC panel, we removed metabolites with a CV >32.3 or <0.38 (144). In order to maximize sample size, we excluded metabolites that were missing in one or more subjects.

Nutrient Data

Diet was assessed with a validated semiquantitative food-frequency questionnaire (FFQ) (33–35). Prior to the age of 10 years, the FFQ is completed by the parent. After 10 years of age, adolescent subjects complete the Youth/Adolescent Questionnaire (YAQ). Both instruments are used to assess food intake during the previous calendar year. A high level of concordance between the two instruments has previously been observed within the DAISY population (34). The Channing Laboratory, Harvard, MA, calculated average daily nutrient intake values based on the dietary data reported on the FFQ and YAQ in the current study. Nutrients were reported in accordance with the USDA National Nutrient Database for Standard Reference. Subjects reporting unreasonable total calories (>5,000 kcal, $n = 1$) were excluded from the analysis. The nutrient data set includes 270 nutrients with complete annotation data. We excluded nutrients for the following reasons: nutrient not available for all subjects ($n = 106$ nutrients) and nutrient value coded as zero in >25% of subjects ($n = 16$ nutrients). We Box-Cox transformed all nutrients ($n = 148$) meeting the inclusion criteria. We used linear regression models to adjust all nutrients for total caloric intake and age. The age- and calorie-adjusted nutrients (residuals from the linear models) were used in subsequent analyses.

Statistical Analysis

Metabolites that passed the filtering criteria included metabolites from the HILIC (1,032 metabolites), lipid (1,163 metabolites), and GCTOF (286 metabolites) panels. Given that individuals with IA who develop T1D are no longer at risk for IA reversion, we modeled T1D onset as a competing risk. Cause-specific Cox proportional hazards regression analyses were used to test the association between each metabolite and the hazard of reversion. In the base (no metabolites) univariate analysis, we tested the association between subject demographics at the onset of IA and hazard of reversion. The following variables were adjusted for in subsequent models as potential confounding variables and/or significant precision variables: multiple autoantibodies at the seroconversion visit, high-risk HLA (DR3/4) genotype, non-Hispanic White ethnicity, age at initial seroconversion, and family history of T1D. Metabolites from all panels were standardized to facilitate a consistent interpretation of the effect size as the change in hazard of reversion per 1-SD increase in metabolite levels. False discovery rate (FDR)-adjusted P values were calculated for all individual metabolites according to methods described by Benjamini and Hochberg (36). Due to the overlapping nature of the untargeted panels, FDR-adjusted P values were calculated separately for each platform. Metabolites were considered significant individual predictors of reversion if the FDR-adjusted P value was <0.10. R, version 4.0.1, and SAS, version 9.4, were used for all statistical analyses.

Metabolite Set Enrichment Analysis

We also performed an enrichment analysis using ChemRICH (37). This online tool developed by the Fiehn Laboratory performs an enrichment analysis based on chemical structural similarity and chemical ontology. ChemRICH does not require a background database, allowing for identification of novel clusters of molecules that may not be annotated in existing databases. We input effect sizes (HRs), P values, chemical names, Simplified Molecular-Input Line-Entry System (SMILES) codes, and PubChem identifiers for all compounds with a known InChIKey identifier (423 metabolites). Based on the broad metabolomics coverage in our study, it was possible for known compounds to be measured on multiple platforms. Among duplicate InChIKeys, we selected the metabolites with the largest effect size for enrichment. We translated the InChIKeys into PubChem identifiers and SMILES codes using The Chemical Translation Service (CTS) (https://cts.fiehnlab.ucdavis.edu/) and PubChem Identifier Exchange Service (https://pubchem.ncbi.nlm.nih.gov/idexchange), respectively.

Metabolite Signatures

Metabolites from the individual metabolite analysis were considered candidates for a subsequent principal components analysis (PCA) if the nominal P value was <0.01 and the absolute value of the HR was >1.25. A PCA was then applied to the metabolite candidates. Elbow plots and corresponding proportion of variance explained by each PC were used to select PCs for subsequent analyses.

Metabolite PC and Nutrient Correlation

We tested the association between the top metabolite principal components (PCs) significantly associated with hazard of reversion and the nutrients (adjusted for age and calories) using multivariable linear regression models in a subset of the population with complete metabolite and nutrient data ($n = 132$). In these models, each calorie- and age-adjusted nutrient was modeled as the outcome variable and the PCs were modeled as the explanatory variables. Model fit was assessed based on the R^2 statistic. Next, we tested whether the top 20 nutrient candidates that were significantly correlated with PC1 or PC2 were associated with reversion of IA, adjusting for multiple autoantibodies

at the seroconversion visit, high-risk HLA (DR3/4) genotype, non-Hispanic White ethnicity, age at initial seroconversion, and family history of T1D. Nutrients were considered significant individual predictors of reversion if the FDR-adjusted *P* value was <0.10.

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Metabolites and Hazard of Reversion

Among the 199 subjects (see Table 1) meeting the inclusion criteria, 37% reverted to an autoantibody negative state. With stratification by first appearing autoantibody (Supplementary Table 1), reversion was most common among individuals who developed an IAA autoantibody at their first positive visit, followed by GADA, ZnT8 autoantibodies, and IA-2A. Reversion was least common among individuals who presented with multiple autoantibodies (Table 1 and Supplementary Table 1). After adjustment for multiple autoantibodies at the seroconversion visit, high-risk HLA (DR3/4) genotype, non-Hispanic White ethnicity, age at initial seroconversion, and family history of T1D, phosphatidylcholine (16:0_18:1(9Z)) (PubChem compound identifier [CID] 5497103) was the only known metabolite significant at an FDR-adjusted *P* value <0.10 (Table 2). We also identified six unknown metabolites that were significant based on the FDR-adjusted *P* value <0.10 (Supplementary Tables 2 and 3).

A total of 63 metabolites were identified as candidates in the preliminary analysis ($P < 0.01$ and $\exp(|\mathrm{Ln}(\mathrm{HR})|) > 1.25$) (Fig. 1); 12 represented known compounds (Table 2). Among candidate metabolites, 10 came from the GCTOF panel (10 of 286 [3.5% of metabolites on the panel]), 27 came from the HILIC panel (27 of 1,032 [2.6%]), and 26 came from the lipid panel (26 of 1,163 [2.2%]).

Enrichment Analysis

Individual metabolites tend to group together based on chemical similarity and ontology, allowing for a more complete biological interpretation of the metabolites (37). We used the ChemRICH algorithm to identify clusters of similar metabolites that were overrepresented in individuals who reverted. We input effect sizes (HRs), *P* values, and chemical identifiers for all known metabolites. We identified four clusters significantly (FDR-adjusted *P* value <0.10) enriched for compounds associated with hazard of reversion (Table 3). The top cluster of metabolites represents a novel grouping of metabolites that clustered together based chemical similarity. It is not named because it is not included in existing Medical Subject Heading (MeSH) databases.

Metabolite Signatures

Individual metabolite results are difficult to interpret because multiple metabolites are often interrelated. It may be more meaningful to consider patterns of multiple metabolites than any single metabolite. Therefore, we used a data reduction technique, PCA, to obtain metabolite signatures that represent all of the top 63 individual metabolites, known and unknown. The first four PCs were selected for subsequent testing based on the elbow plot and proportion of variance explained by the first four PCs (36%). In the Cox proportional hazards model, PC1, PC2, and PC4 were significantly associated with hazard of reversion, whereas PC3 was not (Table 2).

Metabolites Nutrient Correlation

With the PCA we identified three metabolite signatures related to reversion. The inclusion of known and unknown metabolites in these PC signatures makes the identification of potentially modifiable aspects of these signatures challenging. Diet, as represented by nutrients, is a strong potential environmental influence of metabolite levels that can be directly modified through dietary intervention. Therefore, we tested the association between nutrients, after adjusting for age and calories, and the three metabolite PCs (PC1, PC2, and PC4) that were significantly associated with the hazard of reversion. We identified 20 nutrients that were correlated with PC1 and/or PC2 (nominal model *P* value <0.05) (Table 4). Total sugar intake, positively associated with PC2, was the nutrient intake that was most strongly correlated with the metabolite PCs (nominal $P = 0.0065$, $R^2 = 0.06$). Figure 2 provides a visual representation of the

Table 1—Demographics and association with hazard of reversion

	Ever reverted, *n* = 74	Never reverted, *n* = 125	HR	Lower CI	Upper CI	*P*
Age at IA	7.4 ± 4.5	6.4 ± 4.9	1.05	1.01	1.10	0.0499
BMI *z* scores at onset of IA	0.2 ± 0.9	0.1 ± 1.1	1.05	0.82	1.34	0.6901
Multiple autoantibodies at IA	2 (2.7)	37 (29.6)	0.09	0.02	0.37	0.0008
DR3/4 high-risk genotype	16 (21.6)	52 (41.6)	0.50	0.29	0.87	0.0135
Non-Hispanic White	52 (70.3)	104 (83.2)	0.55	0.33	0.91	0.0196
First-degree relative with T1D	40 (54.1)	83 (66.4)	0.73	0.46	1.15	0.7280
Female sex	37 (50.0)	64 (51.2)	1.02	0.65	1.61	0.9234

Data are means ± SD or *n* (%) unless otherwise indicated.

Table 2—Known metabolites identified as candidates in reversion hazard analysis*

Metabolite	PubChem CID	HR†	Lower CI	Upper CI	Nominal *P*	FDR-adjusted *P*
Phosphatidylcholine (16:0_18:1(9Z))	5497103	2.16	1.56	2.98	<0.0001	0.0037
Sphingomyelin (d18:1_22:1)	52931203	0.54	0.38	0.77	0.0006	0.1223
Phosphatidylcholine (16:0_20:4)	10747814	1.75	1.27	2.41	0.0006	0.1223
Phosphatidylcholine (16:0_16:1)	6443788	1.65	1.23	2.20	0.0007	0.1848
Choline	305	0.60	0.44	0.81	0.0010	0.1917
(3S)-3-azaniumyl-4-hydroxy-4-oxobutanoate	44367445	1.80	1.24	2.60	0.0019	0.2270
Phosphatidylcholine (30:0)	129657	1.58	1.18	2.11	0.0020	0.2093
Sphingomyelin (d40:0)	44260132	1.64	1.16	2.31	0.0050	0.1223
Creatinine	588	1.47	1.11	1.96	0.0077	0.2652
Hydroxycarbamic acid	16639161	0.69	0.52	0.91	0.0084	0.2652
L-decanoylcarnitine	11953821	1.48	1.11	1.97	0.0080	0.3302
Decanoic acid	2969	0.69	0.52	0.92	0.0100	0.4148
Metabolite signatures						
PC1		1.40	1.24	1.59	<0.0001	
PC2		0.85	0.75	0.97	0.0178	
PC3		1.10	0.96	1.26	0.1696	
PC4		1.28	1.06	1.56	0.0103	

*All models are adjusted for multiple autoantibodies at the seroconversion visit, high-risk HLA (DR3/4) genotype, non-Hispanic White ethnicity, age at initial seroconversion, and family history of T1D. †HR for individual metabolites represents change in hazard per 1-SD increase in metabolite levels.

correlation between each nutrient (*y*-axis) and the three PCs (*x*-axis) significantly associated with reversion. We also tested whether the nutrient candidates were associated with hazard of reversion. Increasing zinc intake and increasing palmitic acid intake were significantly (FDR-adjusted $P < 0.10$) associated with increased hazard of reversion (Table 4).

DISCUSSION

IA is a dynamic predisease state. In this study of individuals who developed persistent IA during the prospective DAISY, 37% reverted to an autoantibody-negative state. We aimed to test the association between metabolite levels measured at the onset of IA and hazard of reversion. We identified 63 unique metabolite candidates measured at the onset of IA that were associated with subsequent reversion. Enrichment analysis of known compounds revealed four clusters of metabolites that collectively were different in subjects who did versus did not revert. Using a PCA of the known and unknown metabolite candidate metabolites, we identified three metabolite signatures associated with reversion.

Phosphatidylcholine (16:0_18:1(9Z)) (PubChem CID 5497103), positively associated with reversion, was the strongest individual candidate in our analysis. Three additional phosphatidylcholine candidates [phosphatidylcholine (16:0_20:4), PubChem CID 10747814; phosphatidylcholine (16:0_16:1), PubChem CID 6443788; and phosphatidylcholine (30:0), PubChem CID 129657] were also positively associated with reversion. We also identified two metabolite clusters enriched for compounds structurally related to phosphatidylcholines that tended to be increased in individuals who reverted. Choline, the first molecule in the phosphatidylcholine biosynthetic pathway was negatively associated with reversion. Therefore, it is possible that the reduced synthesis of phosphatidylcholine from choline may explain direction of association between choline and reversion (negative) relative to the phosphatidylcholine (positive) and reversion—a hypothesis that should be investigated in subsequent research.

Phosphatidylcholines represent the most abundant class of phospholipid, having numerous biological functions including anti-inflammatory (38) properties. Increased levels of phosphatidylcholines in plasma are associated with decreased likelihood of type 2 diabetes (39), an effect that may be mediated by insulin regulatory pathways due to the known inverse association between glycerophospholipids and both dysglycemia and insulin resistance (40). Inhibition of phosphatidylcholine synthesis has also been shown to lead to endoplasmic reticulum stress (41), a cellular response implicated in β-cell dysfunction and, subsequently, T1D onset (42). Together, the anti-inflammatory effects, endoplasmic reticulum stress-related properties, and insulin modulatory influence of phosphatidylcholine provide a potential mechanistic connection between phosphatidylcholine levels and IA reversion.

The leading metabolite from the top cluster was a sphingomyelin, sphingomyelin (d40:0) (CID 44260132). Sphingomyelins represent a type of sphingolipid, a class of molecules associated with pathologies including inflammatory and metabolic disorders (43). Sphingolipids have direct relevance to the T1D disease process based on the

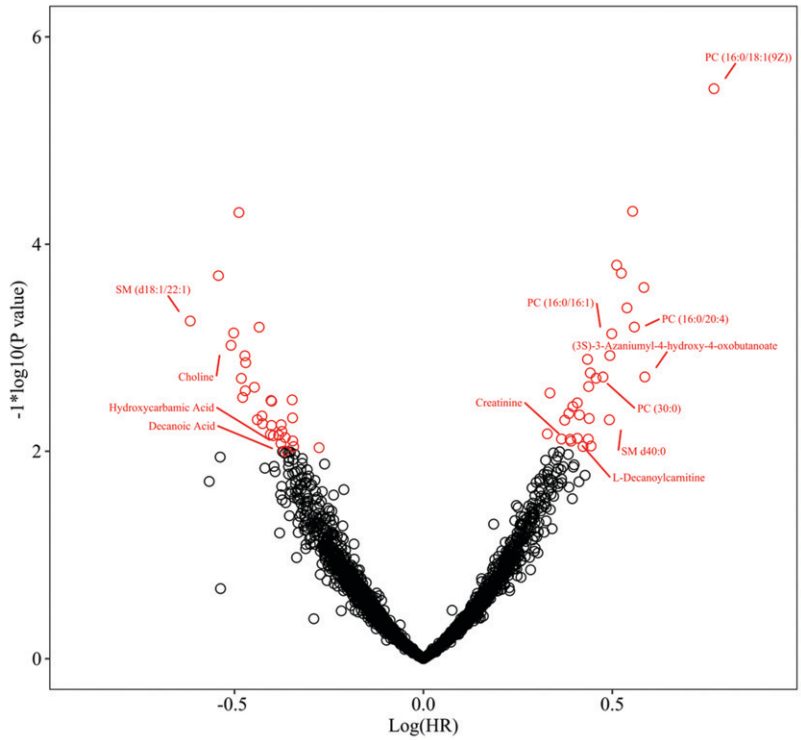

Figure 1—Volcano plot from individual metabolite analysis representing association between each metabolite and reversion. Presentation of log HRs (*x*-axis), representing the association between metabolite levels and hazard of reversion, and corresponding nominal *P* values (*y*-axis). All metabolites were standardized to facilitate interpretation. HRs represent the change in hazard of reversion per 1-SD increase in the level of the metabolite. The absolute values for all log hazard ratios were exponentiated (HR). Metabolites were considered hits if the hazard ratio was >1.25 (−0.2235 > *x*-axis > 0.2235) and the nominal *P* value <0.01 (*y*-axis >2). Hits are highlighted in red. Annotations are provided for all known hits (Table 2).

role of sphingolipid metabolism in regulation of proinsulin folding, insulin secretion, β-cell apoptosis, and the development of the diabetic inflammatory state (43). In a clinical study, sulfatide levels (an important sphingolipid) in pancreatic islet cells of individuals with newly diagnosed T1D were 23% lower than sulfatide levels in control participants (44). Sphingolipids likely act through several mechanisms. In mouse and rat models, sphingolipid levels were both increased (sphingosine-1-phosphate) and decreased (nervonic acid containing ceramide, sphingomyelin, and cerebrosides) in cases relative to control animals (45). This heterogeneity in effect was also present in our study, as these compounds were both positively [sphingomyelin (d40:0)] and negatively [sphingomyelin (d18:1_22:1)] associated with hazard of reversion.

Carnitine and related compounds were also identified in the enrichment analysis. L-carnitine plays an essential role in fatty acid oxidation by transporting fatty acids to the mitochondria (46). Acylcarnitine has been implicated in type 2 diabetes based on its association with insulin resistance (47). In a mouse model, dietary L-carnitine administration has been shown to have immunosuppressive effects on both

Table 3—Chemical set enrichment analysis (ChemRICH) summary of significantly enriched clusters

Cluster name	Cluster size	Nominal *P*	FDR-adjusted *P*	Key compound, CID	Altered metabolites	Metabolites that are increased	Metabolites that are decreased
New cluster	5	0.0002	0.0076	Sphingomyelin (d40:0), 44260132	3	3	0
Unsaturated phosphatidylcholines	81	0.0044	0.0840	Phosphatidylcholine (16:0_18:1(9Z)), 5497103	12	9	3
Saturated phosphatidylcholines	7	0.0060	0.0840	Phosphatidylcholine (30:0), 129657	2	2	0
Carnitine	5	0.00920	0.0960	L-decanoylcarnitine, 11953821	3	3	0

Table 4—Nutrient candidates correlated with one or more of the candidate metabolite PCs as well as association between nutrient candidates and hazard of reversion

		Nutrient vs. metabolite PCA			Nutrient vs. reversion analysis		
Nutrient candidate	Category	Model R^2	PC	β	*P*	HR	FDR-adjusted *P*
Total sugars, g	CHO	0.06	2	0.215	0.0065	0.85	0.4944
Lauric fatty acid, g	Fat	0.05	1	0.022	0.0171	1.13	0.5805
Zinc (wo), mg	Minerals	0.05	1	0.008	0.0197	1.54	0.0650
Saturated fat, g	Fat	0.05	1	0.035	0.0204	1.45	0.1187
Myristic fatty acid, g	Fat	0.04	1	0.037	0.0222	1.24	0.4823
Fructose, g	CHO	0.05	2	0.082	0.0246	0.74	0.2268
Animal fat, g	Fat	0.05	1	0.082	0.0269	1.42	0.1870
Folate, μg	Vitamins	0.05	2	−0.049	0.0272	1.01	0.9647
Capric fatty acid, g	Fat	0.04	1	0.019	0.0275	1.16	0.5635
Caproic fatty acid, g	Fat	0.04	1	0.020	0.0307	1.12	0.6544
Manganese, mg	Minerals	0.04	1	−0.017	0.0310	0.82	0.4823
Palmitic fatty acid, g	Fat	0.05	1	0.013	0.0333	1.53	0.0650
Butyric fatty acid, g	Fat	0.03	1	0.022	0.0368	1.15	0.5635
Myricetin, mg	Flavonoids	0.04	2	0.062	0.0377	1.01	0.9647
Eicosenoic fatty acid, g	Fat	0.03	2	−0.035	0.0377	1.16	0.5635
Vitamin C (wo), mg	Vitamins	0.05	2	0.061	0.0391	0.91	0.6556
Caprylic fatty acid, g	Fat	0.04	1	0.024	0.0425	1.11	0.6556
β-Cryptoxanthin, μg	Carotenoids	0.03	2	−0.327	0.0432	0.93	0.6958
Niacin, mg	Vitamins	0.03	2	−0.005	0.0467	1.05	0.8129
Sucrose, g	CHO	0.04	2	0.014	0.0484	0.79	0.2857

Table shows the direct association between the nutrient candidates and hazard of reversion in a subset of subjects with nutrient and metabolite data ($n = 132$). Model R^2: represents multiple correlations between calorie- and age-adjusted nutrient levels (as the outcome) and PC1, PC2, and PC4. PC: metabolite candidate PCs that the nutrient is most strongly associated with. β: PC-specific slope representing strength of association between nutrient and PC; describes direction of association. *P*: PC-specific *P* value (nominal *P* value). HR: represents change in hazard of reversion per 1-SD increase in the nutrient, with adjustment for multiple autoantibodies at the seroconversion visit, high-risk HLA (DR3/4) genotype, non-Hispanic White ethnicity, age at initial seroconversion, and family history of T1D. FDR-adjusted *P*: FDR-adjusted *P* value for nutrient hazard of reversion association, with adjustment for number of nutrient candidates tested (20 nutrients). CHO, carbohydrates; vitamin C (wo), vitamin C nutrient intake that excludes vitamins and supplements; zinc (wo), zinc nutrient intake that excludes vitamins and supplements.

the innate and adaptive immune systems in the context of another chronic autoimmune disorder, Crohn's disease (48).

Overall, the known metabolite results in the current study, overabundance of phospholipids and reversion, support previous associations between lipid metabolism and T1D etiology. Based on umbilical cord samples, La Torre et al. (16) observed an association between onset of T1D prior to 4 years of age and decreased levels of phospholipids (including phosphatidylcholine). Studies from another birth cohort study, the Finnish Type 1 Diabetes Prevention and Prediction Study (DIPP), confirmed the association between decreased phosphatidylcholine and sphingomyelin levels and T1D onset (13,15). Using longitudinal metabolite biomarkers in the TEDDY cohort, investigators found that decreased levels of the phospholipid phosphatidylethanolamine were present among individuals who become positive for IAA and GADA prior to seroconversion (18). Johnson et al. (19) also reported a significant inverse association between phosphatidylcholine levels in infancy and onset of multiple autoantibodies. la Marca et al. (49) reported that carnitine and acylcarnitine levels, essential for fatty acid metabolism, were significantly decreased among infants who developed T1D early in life. Decreased total and free carnitine levels have also been reported among individuals with T1D relative to controls (50). Lipid metabolic pathways have also been evaluated as a potential therapeutic target for treating and/or preventing T1D. In the NOD mice model, Holm et al. (44) demonstrated that intervention with fenofibrate, a drug that activates sulfatide biosynthesis, prevented diabetes and in diabetic mice reversed the disease process in 50% of the animals.

Our analysis of known metabolites highlights the importance of phosphatidylcholines, sphingomyelins, and carnitine levels in etiology of reversion. However, many metabolite candidates in the current study represent unknown compounds (see Supplementary Tables 2–4). We used a PCA to develop metabolite signatures representative of all the metabolite candidates. PC1, PC2, and PC4 were significantly associated with hazard of reversion. However, PCs lack a direct biologically accessible interpretation.

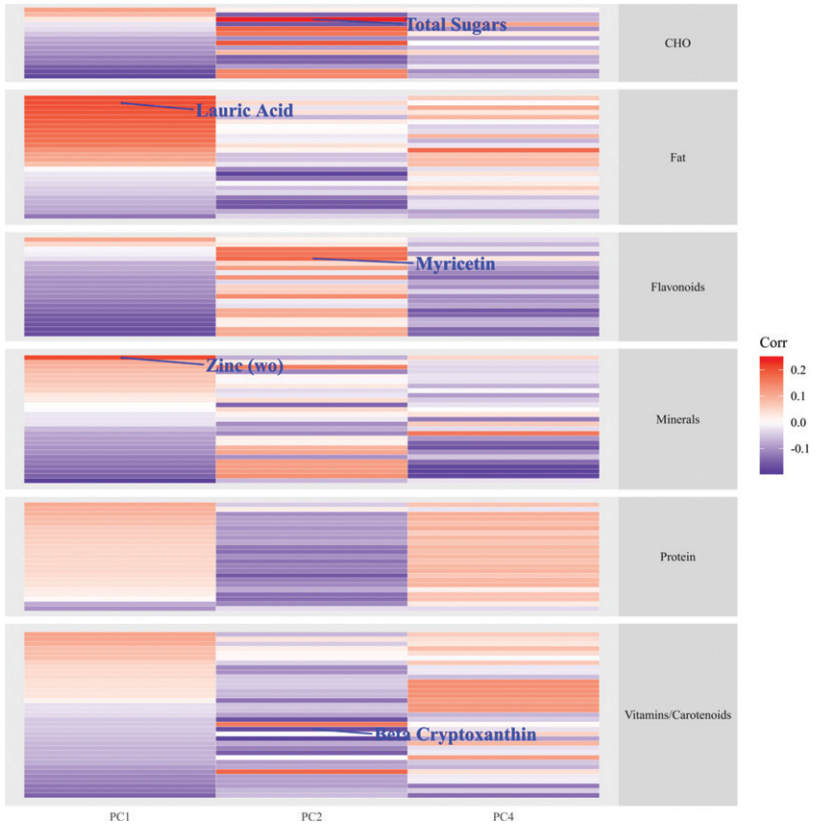

Figure 2—Heat maps representing correlation between PCs from PCA of known and unknown metabolite candidates and all nutrients; heat maps for each metabolite PC (*x*-axis) that was significantly associated with reversion. Each nutrient that passed the quality control filters is represented on the *y*-axis. Positive correlation is represented by the color red, and negative correlation is represented by the color blue. The top nutrient within each nutrient category in Table 4 is annotated based on the PC with which the nutrient is most strongly correlated. CHO, carbohydrates; Zinc (wo), zinc nutrient intake that excludes vitamins and supplements.

Therefore, we used correlation between PCs and nutrient data, a potential environmental influence of metabolite levels, to aid in the interpretation of our results. Nutrients are also appealing because they may be more directly modifiable than metabolites. We identified 20 nutrients that were correlated with PC1 or PC2 (model P value <0.05). Total sugar intake, positively associated with PC2, was the nutrient intake that was most strongly correlated with the metabolite PCs.

Previous research from DAISY has reported an association between increased sugar intake, both individually (HR 1.75 per SD of sugar) (6) and in combination with other nutrients (HR 3.17 per SD of nutrient pattern) (51), and increased risk of progression to T1D. However, sugar was not directly associated with reversion. Increased levels of zinc, commonly found in oysters, red meat, poultry, beans, and whole grains (52), were associated with increased likelihood of reversion. Previous ecological studies have identified some evidence of an association between decreased zinc levels in drinking water and increased incidence of T1D (53,54). Palmitic acid intake, a common saturated fatty acid found in palm oil, meat, and dairy products (55), was also positively associated with hazard of reversion. Although palmitic acid and a high-fat diet is typically associated with negative health effects (55) and poor glycemic control among individuals with T1D (56), endogenously formed branched fatty acid esters of hydroxy acids including palmitic acid 9 hydroxy stearic acid (9-PAHSA) have been shown to have positive effects on insulin sensitivity and glucose tolerance in a mouse model (57). However, it is not known whether 9-PAHSA plays a role in humans or whether dietary intake of palmitic acid is associated with 9-PAHSA. Overall, the associations between nutrients and reversion were modest. The metabolite PCs suggest that there is a more complex relationship between metabolite patterns and reversion that should be explored in subsequent studies.

Limitations

Metabolomics profiling was performed in nonfasting samples, which could influence metabolite levels. A subset of individuals who reverted (26%), including one of the two individuals who presented with multiple autoantibodies at the onset of IA, subsequently reseroconverted. However, <3% of individuals who reverted developed T1D.

Secondary seroconversion events were not included in the analysis because metabolomics data were not collected from all study visits. T1D and reversion are inherently related. Individuals who revert are at a lower risk for T1D, raising concerns that reversion may not be a unique outcome. Using a similar data set, Johnson et al. (51) identified 13 metabolite hits related to progression from IA to T1D. Only 3 of the 13 hits in the work of Johnson et al. (51) (<25%) were identified as hits in our reversion analysis, providing evidence that reversion is a novel end point. More importantly, reversion is more common and occurs earlier after IA onset relative to T1D, providing an alternative, novel end point for understanding the T1D disease process. The nutrient data were meant to augment the interpretation of the metabolite signatures. The model R^2 representing association between nutrients and metabolite PCs in the current study was small, <0.07, indicating that nutrients account for a small proportion of variance in metabolites. Although best in class, survey-based dietary data represent average nutrient intake, whereas metabolites represent a specific cross section in time. Temporal variability in metabolite levels may contribute to the low correlation between nutrient intake and metabolite levels. Additional research is needed to pursue other potentially stronger predictors of metabolite levels including underlying genetics, toxin exposure, medication usage, and ambient environmental factors.

Conclusion

T1D etiologic studies have predominantly focused on individuals who develop IA and/or T1D. Identifying the mechanisms that lead to IA reversion may guide development of novel interventions and may aid in the identification of individuals more amenable to intervention. The current study builds on previous literature by identifying an association between reversion of persistent IA and phos pholipid, sphingomyelin, and carnitine—candidates and pathways implicated in inflammation, endoplasmic reticulum stress, and insulin secretion. Metabolite patterns may have greater biological significance than single molecules. PCs from a PCA of all metabolite hits were strongly associated with reversion. This signature was correlated with sugar and fat intake. Additional work is needed to understand contribution of this signature to biological mechanisms underlying IA reversion and, more importantly, to identify modifiable environmental factors that drive this metabolite profile.

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