Cord Serum Lipidome in Prediction of Islet Autoimmunity and Type 1 Diabetes

Matej Orešić,1 Peddinti Gopalacharyulu,1 Juha Mykkänen,2,3 Niina Lietzen,1 Marjaana Mäkinen,2,3 Heli Nygren,1 Satu Simell,2,3 Ville Simell,2,3 Heikki Hyötyniemi,4,5 Riitta Veijola,6 Jorma Ilonen,7,8 Marko Sysi-Aho,1 Mikael Knip,9,10,11,12 Tuulia Hyötyläinen,1 and Olli Simell2,3

Previous studies show that children who later progress to type 1 diabetes (T1D) have decreased preautoimmune concentrations of multiple phospholipids as compared with nonprogressors. It is still unclear whether these changes associate with development of β-cell autoimmunity or specifically with clinical T1D. Here, we studied umbilical cord serum lipidome in infants who later developed T1D (N = 33); infants who developed three or four (N = 31) islet autoantibodies, two (N = 31) islet autoantibodies, or one (N = 48) islet autoantibody during the follow-up; and controls (N = 143) matched for sex, HLA-DQB1 genotype, city of birth, and period of birth. The analyses of serum molecular lipids were performed using the established lipidomics platform based on ultra-performance liquid chromatography coupled to mass spectrometry. We found that T1D progressors are characterized by a distinct cord blood lipidomic profile that includes reduced major choline-containing phospholipids, including sphingomyelins and phosphatidylcholines. A molecular signature was developed comprising seven lipids that predicted high risk for progression to T1D with an odds ratio of 5.94 (95% CI, 1.07–17.50). Reduction in choline-containing phospholipids in cord blood therefore is specifically associated with progression to T1D but not with development of β-cell autoimmunity in general. Diabetes 62:3268–3274, 2013

The incidence of inflammatory and autoimmune diseases, including type 1 diabetes (T1D), is increasing at an alarming rate (1,2). T1D often presents in early childhood and, although it currently cannot be prevented, preliminary results from the Trial to Reduce IDD in the Genetically at Risk (TRIGR) pilot study performed in Finland indicate that early dietary intervention reduces the cumulative incidence of β-cell autoimmunity by ~50% by the age of 10 years (3).

The impact of the environment on T1D pathogenesis is evident. Although ~70% of subjects with T1D have defined risk-associated genotypes at the HLA locus, only 3–7% of the carriers of such genetic risk markers develop the disease before adulthood (4). The environment may play a role not only postnatally but also during the prenatal and perinatal periods. In utero and early life conditions contribute to the development of many chronic diseases (5), as also implicated in T1D (6,7). For example, the period of pregnancy is associated with marked changes in gut microbiota that affect the metabolism of the host as well as that of the offspring (8).

It would be crucial to identify biomarkers of T1D risk that are sensitive to contributing genetic and environmental factors to facilitate the identification of at-risk children as early as possible. Metabolome is sensitive to many genetically relevant factors, including host genotype (9), gut microbiota (10), and immune system status (11,12). In our previous metabolomics investigation in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study, we observed that children who later progress to T1D are characterized by decreased amounts of choline-containing phospholipids already at birth, i.e., as measured in cord blood, independent of the strength of HLA risk (11). This finding reinforces the concept that events during gestation may contribute to the risk of T1D (6,7), although they do not yet answer whether the observed metabolic changes specifically associate with progression to T1D or more broadly with the development of β-cell autoimmunity.

Herein, we sought to validate the previous findings (11) in a different study group and to determine whether the metabolic profiles at birth are associated with development of β-cell autoimmunity later in life or specifically with progression to T1D. A comprehensive lipidomics (13) approach was applied to analyze molecular lipids in umbilical cord serum samples from the DIPP infants who, during the follow-up, developed a single autoantibody or multiple autoantibodies or progressed to T1D.

RESEARCH DESIGN AND METHODS

Study protocol and subjects. The subjects in this study were chosen from the ongoing prospective DIPP study (14) in which infants in three university hospitals in Finland (Turku, Tampere, and Oulu) are screened for T1D-associated HLA genetic risk alleles (15). Families of children recognized to have increased HLA-conferred risk for T1D are invited to join the study (14). According to the DIPP study protocol, the children are prospectively observed at 3- to 12-month intervals until age 15 years or until the development of clinical T1D. Levels of T1D-associated autoantibodies (islet cell antibodies [ICAs], insulin autoantibodies, IA-2 autoantibodies, and GAD autoantibodies) are determined from the serum samples taken at each follow-up visit (16,17).

This study included DIPP children born between 1994 and 2006 in Turku. According to the DIPP data collected by 31 March 2009, the following groups of children with available cord blood samples were included in the analyses: infants who later developed T1D-associated autoantibodies and progressed to T1D during the follow-up (progressors, N = 33), infants who later developed three or four autoantibodies but have remained clinically unaffected (N = 31), infants who later developed two autoantibodies but have remained clinically unaffected (N = 31), infants who later developed one autoantibody during the follow-up but have remained clinically healthy (N = 48), and clinically
unaffected healthy control children \((N = 143)\) without any autoantibodies matched for each child in the other study groups by sex, HLA-conferred T1D risk genotype, and date of birth. All control children were persistently autoantibody-negative from birth and for at least 12 months after the age when the matched case child developed clinical T1D or seroconverted to positivity for the last detected autoantibody during the follow-up. In total, cord serum samples from 286 infants were analyzed in this study (Table 1). Serum of all cord blood samples was isolated within 3 h after collection and stored at \(-75^\circ C\) until analyzed.

The Ethics Committee of the Hospital District of Southwest Finland approved the study, and written informed consent was obtained from all participating families.

**HLA genotyping.** The analysis of the cord blood HLA-DQB1 genotype was performed with time-resolved fluorometry-based assay using four lanthanide chelate–labeled sequence-specific oligonucleotide probes detecting DQB1*02, DQB1*03:01, DQB1*03:02, and DQB1*06:02/3 alleles (18). Children with T1D risk-associated DQB1*02/DQB1*03:02 or DQB1*03:02/x genotypes (with x representing lack of DQB1*02 and protection-associated DQB1*03:01, DQB1*06:02, and DQB1*06:03 alleles) were eligible for the follow-up program. Detection of β-cell autoimmunity. The ICAs were detected with the use of indirect immunofluorescence, whereas the three biochemical autoantibodies were quantified with the use of specific radiobinding assays (3). We used cutoff limits for positivity of 2.5 Juvenile Diabetes Foundation units for ICA, 3.48 relative units for insulin autoantibodies, 5.36 relative units for GAD autoantibodies, and 0.43 relative units for IA-2 autoantibodies. The disease sensitivity and specificity of the ICA assay were 98 and 99%, respectively, in the fourth round of the international workshops on standardization of the ICA assay. The disease sensitivity and specificity of the insulin autoantibodies assay were 58 and 100%, respectively, in the 2005 Diabetes Antibody Standardization Program (DASP) workshop. The corresponding characteristics of the GAD autoantibodies assay were 100% and 98%, respectively, in the 2005 Diabetes Antibody Standardization Program (DASP) workshop.

**TABLE 1**
Clinical characteristics of subjects and study groups

| Study groups | T1D progressors \((n = 33)\) | 3–4 Aabs \((n = 31)\) | 2 Aabs \((n = 31)\) | 1 Aab \((n = 48)\) | Matched controls* \((n = 143)\) |
|--------------|---------------------|----------------|----------------|----------------|----------------|
| Sex, \(n\)   |                     |                |                |                |                |
| Girls        | 16                  | 12             | 9              | 21             | 58             |
| Boys         | 17                  | 19             | 22             | 27             | 85             |
| High HLA risk, \(n^\dagger\) | 12           | 12             | 4              | 14             | 42             |
| Moderate HLA risk, \(n^\ddagger\) | 21         | 19             | 27             | 34             | 101            |
| Follow-up time (months) |           |                |                |                |                |
| Median       | 120                 | 105            | 107            | 114.5          | 112            |
| Range        | 46–175              | 37–172         | 56–165         | 51–168         | 38–174         |
| Age at seroconversion (months) |           |                |                |                |                |
| Median       | 24                  | 30             | 51             | 42             | —              |
| Range        | 6–116               | 9–103          | 6–128          | 9–127          |                |
| Children grouped by age at seroconversion, \(n\) |           |                |                |                |                |
| Early Aab\^*, <4 years | 23              | 20             | 15             | 25             |                |
| Late Aab\^*, >4 years | 10               | 11             | 16             | 23             |                |
| Diagnosis age (months) |           |                |                |                |                |
| Median       | 93                  |                |                |                |                |
| Range        | 19–173              |                |                |                |                |
| Season of birth, \(n^\$\) |           |                |                |                |                |
| Winter       | 9                   | 10             | 5              | 13             | 36             |
| Spring       | 15                  | 7              | 8              | 10             | 45             |
| Summer       | 3                   | 8              | 9              | 14             | 30             |
| Autumn       | 6                   | 6              | 9              | 11             | 32             |
| Season of diagnosis, \(n\) |           |                |                |                |                |
| Winter       | 13                  |                |                |                |                |
| Spring       | 8                   |                |                |                |                |
| Summer       | 8                   |                |                |                |                |
| Autumn       | 4                   |                |                |                |                |
| Mode of delivery, \(n\) |           |                |                |                |                |
| Vaginal      | 32                  | 27             | 22             | 43             | 115            |
| Cesarean delivery | 1          | 4              | 9              | 5              | 28             |
| Birth weight (kg) |           |                |                |                |                |
| Median       | 3.57                | 3.80           | 3.49           | 3.55           | 3.57           |
| Range        | 2.39–4.58            | 2.35–4.59      | 1.94–4.60      | 1.82–4.82      | 1.49–4.96      |
| Birth height (cm) |           |                |                |                |                |
| Median       | 51                  | 51             | 51             | 50             | 51             |
| Range        | 45–57               | 46–55          | 44–55          | 43–56          | 42–56          |
| Gestational age (weeks) |           |                |                |                |                |
| Median       | 39.7                | 39.4           | 39.9           | 39.4           | 39.9           |
| Range        | 35.0–42.0            | 34.4–42.4      | 33.9–42.3      | 33.0–42.4      | 30.0–42.7      |
| Age of mother (years) |           |                |                |                |                |
| Median       | 31.5                | 30.7           | 31.5           | 30.0           | 30.9           |
| Range        | 24.6–42.0            | 21.6–40.5      | 21.8–37.8      | 20.4–42.3      | 19.9–46.4      |

Aab, autoantibody; Aab\^*, autoantibody-positive. \(^\ast\) A clinically unaffected healthy child was selected and matched for each child in Aab\^* and T1D study groups. See matching criteria in **STUDY PROTOCOL AND SUBJECTS**. \(^\dagger\) HLA genotype = DQB1*02/DQB1*03:02. \(^\ddagger\) HLA genotype = DQB1*03:02/x, with x = anything except DQB1*02 *03:01, *06:023. \(^\$\) Seasons are defined as winter being December–February, spring being March–May, summer being June–August, and autumn being September–November.
The established lipidomics platform applied in this study covers the major serum lipid classes such as PCs, sphingomyelins (SMs), CERs, PEs, and TGs. However, because the mass spectrometry analysis is performed with electrospray ionization in positive ion mode, the chosen approach is not optimal for the detection of less abundant negatively charged serum lipids such as phosphatidylserines, phosphatidyleglycerols, and phosphatidylinositols.

**Statistical methods.** Data were analyzed using R software (21). Lipid concentration values were first log-transformed. Clustering analyses were performed using the MCLUST (22) method. In univariate analyses, the log fold changes for every pair of groups were computed as the differences of the mean intensity values between each case and the pooled control group. The 95% CI was computed based on the average SEs of intensity values in each group. Fold changes were obtained by transforming back the log fold changes. 95% CI was computed based on the average SEs of intensity values in each group. The upper phase was collected after centrifugation and 20 mL of the second internal standard mixture (LPC(16:1D3), PC(16:1/16:1-D6), and TG(16/0/16/16/0-13C3)) was added. The extracts (2.0 mL) were analyzed on Waters Q-ToF Premier mass spectrometer (Waters, Milford, MA) combined with an Acuity UPLC (Waters) using an Acuity UPLC BEH C18 2.1- × 100-mm column with 1.7-µm particles. The solvent system included 1% v/v methanol, 0.1% v/v HCOOH, and acetonitrile/isopropanol (1:1, 1% v/v methanol, 0.1% v/v HCOOH) in gradient elution mode with a flow rate of 0.4 mL/min. The lipid profiling was performed using electrospray ionization in positive mode at a mass range of charge/mass ratio 300–1,200 with scan duration of 0.2 s. The data processing using MZmine 2 (20) included detection and alignment of peaks, peak integration, normalization, and peak identification. Only the lipids detected in ≥60% of the samples after the MZmine 2 peak detection were retained for the data analysis. Lipids were identified using an internal spectral library or with tandem mass spectrometry (10). The data were normalized using one or more internal standards representative of each class of lipid present in the samples as previously described (19).

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**Cord serum lipidome in development of autoimmunity and progression to T1D.** The lipidomics data were first analyzed at the cluster level (Fig. 1 and Table 2). Pairwise correlation analysis showed that among the 10 LCs, only LC2 was significantly inversely correlated with the study groups (variable study group in Fig. L4; i.e., low lipid concentrations associate with increased risk of T1D). This

# Table 2

| Name | General LC description | Most abundant representative lipids | Case group vs. controls (P) |
|------|------------------------|-------------------------------------|-----------------------------|
| LC1  | 4 Unknown lipids       | PC(36:4), PC(16:0/18:1), PC(34:2), PC(36:5), PC(38:5) | 0.484 0.656 0.569 0.142† |
| LC2  | 49 Major phospholipids | PC(36:4), PC(16:0/18:1), PC(34:2), PC(36:5), PC(38:5), SM(d18:1/24:1), SM(d18:1/16:0), PC(18:1/20:4), PC(38:5e) | 0.032‡ 0.527 0.301 0.963 |
| LC3  | 14 LPCs                | LPC(16:0), LPC(18:1), LPC(18:2), PC(22:6) | 0.694 0.569 0.730 0.821 |
| LC4  | 10 Minor phospholipids | PC(30:3), PC(33:1), SM(d18:1/23:0) | 0.380 0.309 0.473 0.587 |
| LC5  | 10 Primarily unknown   | PC(34:1e), SM(18:1/20:4) | 0.750 0.710 0.257 0.666 |
| LC6  | 17 PUFA containing phospholipids | PC(34:1e), PC(38:6), PC(38:4) | 0.776 0.504 0.173 0.446 |
| LC7  | 7 Minor SMs            | SM(d18:1/20:4), SM(d18:1/18:0) | 0.092‡ 0.752 0.048‡ 0.577 |
| LC8  | 16 Major TGs containing SFAs and MUFAs | TG(16/0/18/18:1), TG(16/0/16/18:1), TG(16/0/16/0/18:1) | 0.726 0.720 0.425 0.794 |
| LC9  | 23 Major TGs containing MUFAs and PUFAs | TG(16/0/18/2/18:1), TG(16/0/18/2/18:2) | 0.757 0.379 0.803 0.602 |
| LC10 | 9 Long-chain TGs containing PUFAs | TG(56:7),TG(56:8), TG(58:8) | 0.812 0.032‡ 0.420 0.264 |
association is specific for T1D progressors, whereas no significant changes were observed in other study groups (Fig. 1B). None of the LCs were correlated with HLA-associated T1D risk (variable HLA risk in Fig. 1A). A pattern similar to that of the cluster level was observed at the molecular lipid level. The top 12 ranked lipids based on odds of progression to T1D were from LC2 (Table 3), including the most abundant SMs and PCs, such as SM(d18:1/16:0), SM(d18:1/16:0), PC(16:0/18:1), and PC(18:0/18:1).

Because it has been previously documented that age at seroconversion for islet autoantibody positivity is associated with different metabolic profiles before and after seroconversion (12), we also investigated whether age at seroconversion is associated with the cord serum lipidomic profile. When age at seroconversion was treated as a continuous variable, no significant associations were found for any of the LCs (Fig. 1A). When dividing the T1D progressors into two groups by age at seroconversion (early autoantibody-positive at age younger than 4 years and late autoantibody-positive at age earlier than 4 years), *P < 0.05. Aab, autoantibody; Aab+, antibody-positive during the follow-up.

We next sought to predict development of β-cell autoimmunity using the data from infants in the one autoantibody group and their matched controls. The best model was developed on the data from T1D progressors and their matched controls. The best model was developed based on the data from T1D progressors and their matched controls. The best model was derived from logistic regression analysis by combining seven lipid metabolites (Fig. 2A). Tests for goodness-of-fit revealed no evidence for the lack of fit of the model (P = 0.43), indicating that it was well-calibrated. The model had an AUC of 0.71 (95% CI, 0.53–0.85) and an OR of 5.94 (95% CI, 1.07–17.50) when using a different set of controls to test the model (those matched to the three or four autoantibodies group, two autoantibodies group, and one autoantibody group), the OR was 2.71 (95% CI, 1.08–6.79). The prediction of T1D was poor in the three or four autoantibodies group, two autoantibodies group, and one autoantibody group, with the ratio of percent predicted T1D cases in each group compared with percent predicted cases in pooled controls from the three groups being 1.00, 0.84, and 0.96, respectively.

Among other clinical variables, gestational age was positively associated with several LCs including all three TG clusters (Fig. 1A). At the individual lipid level, the strongest associations were observed for TGs from LC9 and LC10, i.e., TGs that contain essential fatty acids. Out of 30 top-ranked lipids according to the P value (null hypothesis [H0: R = 1]), 19 were from LC9 and 6 were from LC10 (P < 10−8 for all of them). None of these top-ranked lipids were significantly associated with age at diagnosis or age at initial seroconversion. These findings are in agreement with previous studies in which the fatty acid composition of TG fractions was assessed in pregnant women and infants (28,29).

Feasibility of predicting autoimmunity and progression to T1D at birth. To assess the feasibility of predicting T1D, we performed a model selection in multiple cross-validation runs as described. The diagnostic model was developed based on the data from T1D progressors and their matched controls. The best model derived from logistic regression analysis was obtained by combining seven lipid metabolites (Fig. 2A). Tests for goodness-of-fit revealed no evidence for the lack of fit of the model (P = 0.43), indicating that it was well-calibrated. The model had an AUC of 0.71 (95% CI, 0.53–0.85) and an OR of 5.94 (95% CI, 1.07–17.50). When using a different set of controls to test the model (those matched to the three or four autoantibodies group, two autoantibodies group, and one autoantibody group), the OR was 2.71 (95% CI, 1.08–6.79). The prediction of T1D was poor in the three or four autoantibodies group, two autoantibodies group, and one autoantibody group, with the ratio of percent predicted T1D cases in each group compared with percent predicted cases in pooled controls from the three groups being 1.00, 0.84, and 0.96, respectively.

Next, we sought to predict development of β-cell autoimmunity. Among the different case groups, seroconverters for one autoantibody were least likely to develop T1D (16,30) and thus independent from T1D progression. Therefore, we developed a model to predict exclusively β-cell autoimmunity using the data from infants in the one autoantibody group and their matched controls. The best model was
**DISCUSSION**

Our study confirms previous findings (11) that progression to TID is associated with decreased concentrations of major choline-containing phospholipids (SMs and PCs) in cord blood. Here, we also show that the reduction in phospholipids is specifically associated with progression to TID but not with β-cell autoimmunity in general and is predominantly seen in TID children who seroconverted before age 4 years.

Low PC and SM levels in TID progressors cannot be explained by genetics alone because the subjects in our study were matched with the controls for the single most important gene locus, HLA-DQB1. Choline is an essential nutrient that is mainly found in plasma and not in free-form as part of the head groups of SMs and PCs. Choline is in particular high demand during pregnancy as a substrate for building cellular membranes because of rapid fetal tissue expansion and for increased production of lipoproteins (31). Furthermore, choline is a major donor of methyl groups needed for DNA methylation and therefore is essential for developmental processes, including genomic imprinting and the maintenance of genome stability (31,32). For example, maternal choline intake during pregnancy alters the epigenetic state of fetal cortisol-regulating genes (31,32). In a recent study of pregnant women, it has been shown that increased dietary choline intake during pregnancy increases its utilization as a methyl donor in both maternal and fetal compartments (34). Although dietary supplementation mainly has been considered as a way to increase the levels of choline, other factors affecting the choline metabolism such as gut microbiota (35) also may need to be obtained by combining seven lipid metabolites (all of the lipids were different from the ones included in the lipid signature to predict TID; Fig. 2B). Tests for goodness-of-fit revealed no evidence for the lack of fit of the model ($P = 0.63$), indicating that it was well-calibrated. The model had an AUC of 0.70 (95% CI 0.57–0.63), indicating that it was well-calibrated. The model had an AUC of 0.70 (95% CI 0.57–0.63), indicating that it was well-calibrated. The model had an AUC of 0.70 (95% CI 0.57–0.63), indicating that it was well-calibrated.
Lipidomics technology has been maturing rapidly, but also for the risk of multifactorial chronic diseases. Beyond T1D because they suggest that lipidomics, or more broadly metabolomics, may be useful in newborn screening not only for the risk of in-born errors of metabolism but also for the risk of multifactorial chronic diseases.

Our findings may have wider clinical significance beyond T1D because they suggest that lipidomics, or more broadly metabolomics, may be useful in newborn screening not only for the risk of in-born errors of metabolism but also for the risk of multifactorial chronic diseases. Lipidomics technology has been maturing rapidly, and translation of findings such as ours in the current study into an inexpensive assay applicable in the health care setting is already feasible.

In conclusion, our study suggests that decreased choline-containing phospholipids in umbilical cord blood are predictors of T1D but not of β-cell autoimmunity per se. The lipidomic profiles associated with progression to T1D may have diagnostic value and may help identify infants at risk for development of T1D. Our findings primarily implicate altered choline metabolism in pregnancy as a contributing factor behind the observed lipidomic profiles in cord sera of T1D progressors. Future studies focusing on metabolism and genomic imprinting during pregnancy and neonatal life will be important to disclose the character of gestational events contributing to reduced phospholipid levels in relation to the development of T1D in the offspring.

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M.O. was responsible for the study concept and design, analyzed and interpreted data, drafted the manuscript, critically revised the manuscript for important intellectual content, and supervised the study. P.G. and J.M. analyzed and interpreted data. N.L., M.M., H.N., S.S., and V.S. were responsible for acquisition of data. H.H. and R.V. were responsible for critical revision of the manuscript for important intellectual content. J.I. and M.K. were responsible for acquisition of data and critical revision of the manuscript for important intellectual content. M.S.-A. analyzed and interpreted data, was responsible for acquisition of data and critical revision of the manuscript for important intellectual content. T.H. was responsible for acquisition of data and critical revision of the manuscript for important intellectual content and supervised the study. M.O. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIG. 2. Feasibility of cord serum lipids to predict T1D and β-cell autoimmunity in later life. A: Model to predict T1D based on cord serum concentrations of seven lipid metabolites [SM(d18:1/24:1), SM(d18:0/20:0), PC(18:1/20:4), PC(18:0/22:4), PC(38:2), PE(38:2), TG(14:0/16:0/16:0)]. Samples from all T1D progressors and their matched controls were used to develop the model and calculate its characteristics. B: Model to predict β-cell autoimmunity for single autoantibody based on cord serum concentrations of seven lipid metabolites [LPC(22:6), PC(16:0e/16:0), PC(p16:0/16:0), TG(18:0/18:1/18:1), TG(16:0/18:2/20:4), as well as two unidentified lipids from clusters 1 and 5, respectively]. Samples from the one autoantibody group and their matched controls were used to develop the model and calculate its characteristics. Aab, autoantibody.
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