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Metabolite Profiling of LADA Challenges the View of a Metabolically Distinct Subtype

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Latent autoimmune diabetes in adults (LADA) usually refers to GAD65 autoantibodies (GADAb)–positive diabetes with onset after 35 years of age and no insulin treatment within the first 6 months after diagnosis. However, it is not always easy to distinguish LADA from type 1 or type 2 diabetes. In this study, we examined whether metabolite profiling could help to distinguish LADA (*n* = 50) from type 1 diabetes (*n* = 50) and type 2 diabetes (*n* = 50). Of 123 identified metabolites, 99 differed between the diabetes types. However, no unique metabolite profile could be identified for any of the types. Instead, the metabolome varied along a C-peptide–driven continuum from type 1 diabetes via LADA to type 2 diabetes. LADA was more similar to type 2 diabetes than to type 1 diabetes. In a principal component analysis, LADA patients overlapping with type 1 diabetes progressed faster to insulin therapy than those overlapping with type 2 diabetes. In conclusion, we could not find any unique metabolite profile distinguishing LADA from type 1 and type 2 diabetes. Rather, LADA was metabolically an intermediate of type 1 and type 2 diabetes, with those patients closer to the former showing a faster progression to insulin therapy than those closer to the latter.

Diabetes is currently divided into two main types. Type 2 diabetes (T2D) is characterized by insulin resistance and features of the metabolic syndrome but usually includes preserved β-cell function. Type 1 diabetes (T1D), with onset at an early age, develops as a consequence of autoimmune destruction of the insulin-producing β-cells. There is, however, also an intermediate form, called latent autoimmune diabetes in adults (LADA), which, despite similarities with T1D, shows a slower progression toward insulin requirement (1,2). The frequency of LADA is ∼10% of patients with diabetes (3–5). LADA is usually diagnosed at an older age compared with T1D, and affected individuals therefore often present with features of the metabolic syndrome, making it difficult to distinguish LADA from T2D (3). Studies aimed at dissecting the genetic causes of diabetes have shown that LADA shares genetic features with both T1D and T2D (6). LADA is usually defined as non–insulin-requiring diabetes for 6 months after diagnosis, with onset in adult life (>35 years of age) with autoantibodies to GAD65 (GADAb). However, because the decision to initiate insulin therapy is very subjective, it has been suggested that the criteria of lack of insulin treatment for the first 6 months after diagnosis should be excluded from the definition (6). Instead, we propose to add the criterion of C-peptide level of >0.3 nmol/L because absolute insulin deficiency is not a characteristic of LADA.

In the current study, we examined whether LADA would be characterized by a unique metabolic profile distinct from T1D and T2D.

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See accompanying article, p. 801.

RESEARCH DESIGN AND METHODS

Study Population

The ANDIS (All New Diabetics In Scania) project (http://andis.ludc.med.lu.se/) was launched in 2008 aiming at recruiting all incidence cases of diabetes regardless of age at diagnosis within Scania County in southern Sweden. As of January 2014, when samples were collected for this study, 8,722 subjects, all >18 years of age, were included in ANDIS. Of these subjects, 7,701 received a diagnosis of T2D, 193 received a diagnosis of T1D, and 454 received a diagnosis of LADA. Another 581 subjects <18 years of age, the majority of whom had received a diagnosis of T1D, were collected as part of the Better Diabetes Diagnosis (BBD) study. The ANDIS register is linked to the national drug prescription registry. Fasted blood samples were collected shortly after diagnosis (on average, 76 days) for analysis of GADAb (GADAb ELISA Kit; RSR Ltd, Cardiff, U.K.), C-peptide (Immulite; Siemens Healthcare, Deerfield, IL), HbA_{1c} (VARIANT TURBO; Bio-Rad, Hercules, CA), and blood glucose. Blood plasma was stored at −80°C. One hundred fifty subjects were randomly selected, with equal numbers of subjects having LADA, T1D, and T2D. T1D at <18 years of age was excluded because of expected large differences in the metabolite profile between these young individuals and the older individuals in whom LADA or T2D was diagnosed. Additionally, patients with secondary diabetes due to pancreatic disease were also excluded from the study. LADA was defined here as subject age >35 years, GADAb positivity, and C-peptide concentration >0.3 nmol/L. Patients were considered to have T1D if they were GADAb positive and had a C-peptide concentration <0.3 nmol/L. The remaining patients were considered to have T2D. Patients with T1D were younger and had lower BMI values ($P < 0.01$), lower C-peptide concentrations ($P < 0.01$), and higher HbA_{1c} levels ($P < 0.01$) than patients with T2D and LADA (Table 1). LADA patients had lower body weight ($P < 0.05$), age at onset ($P < 0.01$), BMI ($P < 0.01$), and C-peptide levels ($P < 0.001$) but higher GADAb levels ($P < 0.01$) than patients with T2D. The study was approved by the local ethics committee. All study subjects gave their written informed consent.

Sample Preparation

Metabolites were extracted from 40 μL of plasma in methanol/water (80:20, volume for volume), as previously described in detail (7,8), but with the inclusion of Val-Tyr-Val, leucine-enkephalin, and reserpine at 0.81 ng/μL as internal standards. After centrifugation, the supernatant was divided into two aliquots for analysis by gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) and ultra–high-performance liquid chromatography/quadrupole-TOF-MS (UHPLC/QTOF-MS).

Metabolite Profiling by GC/TOF-MS

Samples for GC/TOF-MS analysis were dried by vacuum centrifugation, methoximated, and trimethylsilylated, as previously described in detail (8,9). Metabolite derivatives were analyzed on a 30-m DB-5MS Ultra Inert column (Agilent J&W, Folsom, CA) using a 6890N Gas Chromatograph (Agilent Technologies, Atlanta, GA) connected to a Pegasus III TOF electron impact MS (LECO, St. Joseph, MI), as previously described in detail (8,9). Samples were analyzed in randomized order. In addition to the samples and sample blanks, which were prepared in parallel with the samples, a homologous series of n-alkanes was also analyzed to calculate retention indexes. Data were acquired using ChromaTOF Software (LECO), exported as NetCDF files, and processed by hierarchical multivariate curve resolution in MATLAB 7.0 (MathWorks, Natick, MA) (10). The identification of metabolites was performed using in-house–developed databases.

Table 1—Subject characteristics by diabetes type

	T2D	T1D	LADA	T2D vs. T1D	T2D vs. LADA	T1D vs. LADA
Ketoacidosis†	1/31	28/16	5/29	***	NS	***
Blood glucose, mmol/L	10.78 ± 0.75	25.19 ± 1.28	14.29 ± 1.36	***	NS	***
HbA_{1c}, % (mmol/mol)	7.02 (53) ± 0.32 (3.5)	11.34 (100) ± 0.44 (4.8)	7.98 (64) ± 0.40 (4.4)	***	NS	***
Sex§	27/23	40/10	23/26	*	NS	**
Gestational diabetes mellitus†	2/15	0/8	2/20	NS	NS	NS
Length, cm	169.06 ± 1.61	179.52 ± 1.03	170.17 ± 1.58	***	NS	***
Weight, kg	90.17 ± 2.36	67.62 ± 1.69	82.23 ± 2.81	***	*	***
Family history†	28/19	27/21	28/17	NS	NS	NS
Debut age, years	63.74 ± 1.89	24.04 ± 0.62	55.94 ± 1.82	***	**	***
BMI, kg/m^2	31.52 ± 0.71	20.98 ± 0.50	28.30 ± 0.86	***	**	***
GADAb, log nmol/L	0.076 ± 0.043	2.28 ± 0.090	2.24 ± 0.080	***	***	NS
C-peptide, nmol/L	1.33 ± 0.068	0.14 ± 0.009	0.78 ± 0.063	***	***	***

Data are expressed as *n* or the mean ± SEM. Differences between groups were assessed by ANOVA followed by post hoc Tukey test. NS, not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; †Yes/no; §Male/female.

Metabolite Profiling by UHPLC/QTOF-MS

Samples for UHPLC/QTOF-MS were dried by vacuum centrifugation and dissolved in 40 μL of methanol/water (1:1, volume for volume). Metabolites were analyzed on a 10-cm ACQUITY UPLC CSH C18 column (Waters, Milford, MA) using a VanGuard precolumn (Waters) on an 1290 Infinity UPLC (Agilent Technologies, Santa Clara, CA) connected to a 6550 iFunnel Q-TOF (Agilent) in positive and negative electrospray ionization (ESI) mode (ESI^+ and ESI^-, respectively). A 2-μL sample was injected and chromatographed as previously described in detail (11). The mass spectrometer was operated in full scan mode with a mass/charge ratio from 50 to 1,800, a capillary voltage of 3.5 kV, and a fragmentor voltage of 175 V. The drying gas flow rate was set to 14 L/min at 200°C, with a nebulizer pressure of 35 psi. Samples were analyzed in randomized order. A quality control sample created from a mixture of all samples was analyzed eight times prior to the first sample injection and then after every 10th injection. Extraction blanks were analyzed after the final sample injection. Data were acquired using MassHunter B.06.00 Build 6.0.633.0 (Agilent). Metabolites were identified by tandem MS using MassHunter METLIN Metabolite PCDL (Agilent) and the injection of pure standards, when available. Batch-recursive feature extraction was performed using MassHunter Profinder B.06.00 Build 6.0625.0 (Agilent). Data were further filtered using Mass Profiler Professional 12.6.1-Build 196252 (Agilent) and exported as csv files for statistical analysis.

Data Treatment and Statistical Analysis

Peak areas were normalized to internal standards, as previously described in detail (8,9,12). Metabolites with a percentage relative SD of >40% in the quality control samples were excluded, and the remaining data were log2 transformed. Metabolites with >20% missing values were excluded, and missing values for unidentified metabolites in <20% of samples were imputed by a nearest neighbor (KNN) procedure (pamr package, R). All identified metabolites were manually reintegrated. Principal component analysis (PCA) and orthogonal projections to latent structures-discriminant analysis (OPLS-DA) were performed on mean centered and unit variance scaled data in Simca P+ 12.0 (Umetrics, Umeå, Sweden). Differences between groups were assessed by ANOVA followed by adjustment for multiple comparisons using the false discovery rate method and post hoc Tukey test in R. Linear models were calculated by lmFit (Limma, R). One LADA sample was removed from both the GC/TOF-MS and the UHPLC/QTOF-MS data sets, and two T1D samples were excluded from the UHPLC/QTOF-MS data because of extraction and analysis faults, respectively.

RESULTS

Targeted Analysis of the Low–Molecular Weight Metabolome

First, we examined whether any unique low–molecular weight metabolite could distinguish LADA from T1D and T2D. In total, 64 metabolites were measured. Two OPLS-DA models were calculated to compare the metabolome of T2D and LADA to the metabolome of T1D. Thereby, the analysis was focused on variation in the metabolome that can be explained by the diabetes type, followed by a rotation of the model to achieve class separation along the predictive component, and by this means the interpretation of the results was facilitated (13). Both models yielded a good classification of the samples based on the diabetes type (R^2 = 0.804, Q^2 = 0.689, cross validated [CV] ANOVA, $P < 10^{-20}$; and R^2 = 0.777, Q^2 = 0.521, CV ANOVA, $P < 10^{-9}$, respectively) (Fig. 1*A* and *B*). However, OPLS-DA failed to classify LADA and T2D (R^2 = 0.182, $Q^2 < 0.1$). The loadings from these models, scaled as correlations, were combined in a shared and unique structures plot (SUS-plot) (Fig. 1*C*) (14). The SUS-plot enables a comparison of the outcomes of multiple classification models using a common reference, in this case T1D. In this plot, differences that are shared between classes (found close to the diagonal) or are unique to a specific class (outside the diagonal) can be identified. The vast majority of metabolites ended up along the diagonal (R^2 = 0.79, $P < 10^{-25}$), suggesting a shared metabolite profile between LADA and T2D. Of the 64 low–molecular weight metabolites, 51 differed between the subgroups of diabetes (ANOVA, $q < 0.05$) (Table 2). The highest levels were generally seen in patients with T2D, followed by those with LADA and T1D. Only erythritol levels were higher in patients with T1D than in those with LADA ($P < 0.01$) and T2D ($P < 0.001$). With respect to succinate, fumarate, and glycerol 3-phosphate, the levels in patients with LADA were more similar to those with T1D than to those with T2D. These metabolites are implicated in mitochondrial metabolism and shuttling (MetaboAnalyst 3.0, Kyoto Encyclopedia of Genes and Genomes, Krebs cycle pathway; Fisher exact test, $P < 0.05$). None of the metabolites was uniquely altered in patients with LADA compared with those with T2D.

Untargeted Analysis of the Lipophilic Metabolome

Since no low–molecular weight metabolite was found to uniquely distinguish LADA from the other two diabetes types, we continued to examine the lipophilic and high–molecular weight metabolome by UHPLC/QTOF-MS. Data derived from both ESI^+ and ESI^- were combined to provide a total of 1,204 putative metabolites. Again, OPLS-DA revealed a clear difference between patients with T2D and T1D (R^2 = 0.829 Q^2 = 0.755, CV ANOVA, $P < 10^{-24}$), and between those with LADA and T1D (R^2 = 0.887, Q^2 = 0.583, CV ANOVA, $P < 10^{-12}$), but failed to distinguish between patients with T2D and LADA (R^2 = 0.491, $Q^2 < 0.1$) (Fig. 2*A* and *B*). A SUS-plot showed that the majority of metabolites followed a diagonal from the lower left to the upper right quadrant (R^2 = 0.81, $P < 10^{-25}$), supporting a shared metabolic milieu among the subgroups of diabetes (Fig. 2*C*). Of the 1,204 molecular features, 106 could be identified by tandem MS. After the removal of duplicates, 59 unique metabolites remained.

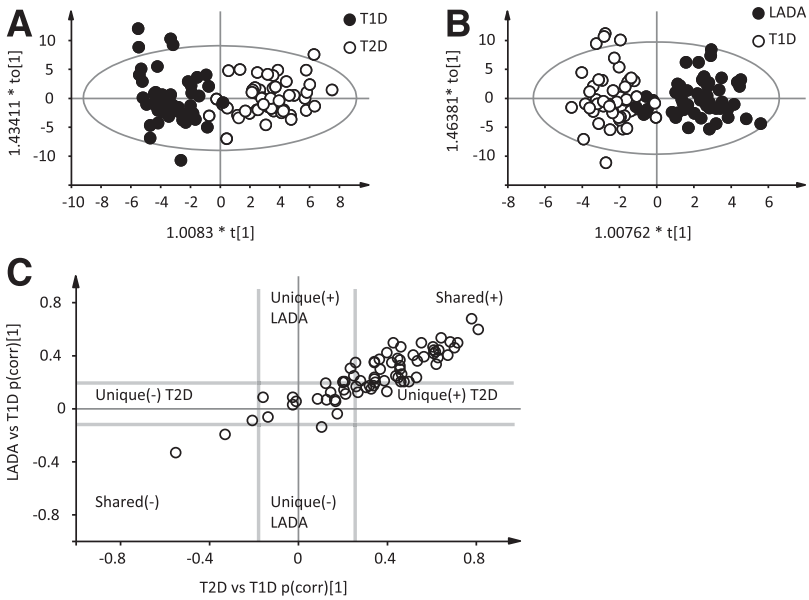

Figure 1—Analysis of low–molecular weight metabolites suggests a metabolic continuum extending from T1D via LADA to T2D. OPLS-DA accurately classifies T2D and T1D (*A*) and LADA and T1D (*B*) based on the low–molecular weight metabolome. *C*: Loadings, scaled as correlations, from the OPLS-DA models combined in a SUS-plot reveals differences between LADA and T1D and differences between T2D and T1D to be shared. Hence, none of the low–molecular weight metabolites uniquely distinguish between the diabetes types. p(corr)[1], loadings for the predictive component scaled as correlations; t[1], predictive component; to[1], the first orthogonal component. T1D, n = 50; T2D, n = 50; and LADA, n = 49.

Of these, 46 differed among the groups (ANOVA, $q <$ 0.05) (Table 3).

Next, we combined metabolites identified by UHPLC/QTOF-MS with those identified by GC/MS, yielding a data set of 123 metabolites, and examined the relation between the metabolic continuum and C-peptide, blood glucose, and GADAb levels using linear models. As for the SUS-plots, two diabetes class models were calculated, using dummy-coded variables and with T1D as a common reference. The strongest association was obtained for C-peptide levels (β = 0.90, $P = 2.8 \times 10^{-64}$ and β = 0.79, $P = 2.0 \times 10^{-52}$, for models of LADA and T2D, respectively, vs. T1D).

Prediction of Time to Insulin

A PCA calculated on the 123 identified metabolites showed that T1D and T2D were well separated along the first principal component, with LADA in between (Fig. 3*A*). The scores for LADA along the first principal component differed from the scores for both T1D ($P <$ 0.001) and T2D ($P < 0.01$), being more similar to the latter ($\Delta score_{LADA\text{-}T1D}$ = 5.5 and $\Delta score_{LADA\text{-}T2D}$ = 3.1). As expected, the scores associated strongly with blood glucose, HbA_{1c}, BMI, and C-peptide levels ($P < 0.001$). A PCA calculated on C-peptide, HbA_{1c}, and GADAb levels reveals a similar pattern (Fig. 3*B*). The scores for LADA differed from the scores for both of the other two types ($P < 0.001$), but were more similar to the score for T1D ($\Delta score_{LADA\text{-}T1D}$ = 1.1 and $\Delta score_{LADA\text{-}T2D}$ = 2.0).

There was, however, a significant overlap among the three types of diabetes. We further examined LADA patients who deviated >1 SD from the center of the LADA cluster. Six LADA patients were found outside −1 SD, suggesting that they had a metabolome more similar to that of T1D. Of these, five patients required insulin treatment within 2 years after diagnosis. Five LADA patients were found outside +1 SD, suggesting a more T2D-like metabolome. Of these, four patients could be treated without insulin for >2 years. Hence, insulin treatment within 2 years tended to be more prevalent among LADA patients showing a more T1D-like metabolome (Fisher exact test, P = 0.081). Whereas the PCA-derived scores differed strongly between these two groups ($P = 10^{-8}$), neither fasting glucose (P = 0.068) and C-peptide (P = 0.19) nor GADAb (P = 0.77) levels differed. However, patients requiring insulin treatment within 2 years had a lower age at diagnosis (49 ± 4 vs. 61 ± 3 years, $P < 0.05$). Levels of 69 metabolites differed between the two groups of LADA patients (Supplementary Table 1), and among these patients the levels of aromatic amino acids phenylalanine, tyrosine, and tryptophane ($q < 0.01$) and the branched-chain amino acids valine ($q < 0.01$), isoleucine ($q < 0.01$), and leucine ($q < 0.05$) were higher in those who could be managed without insulin for >2 years. Insulin treatment was required in 54% of LADA patients, but in only 8% of T2D patients, at 3 years after diagnosis.

DISCUSSION

The identification of biomarkers distinguishing diabetes subtypes from each other could have great clinical value. LADA, which is associated with a faster progression to insulin replacement therapy compared with T2D, may be

Table 2—Low–molecular weight metabolites differing in levels between LADA, T1D, and T2D

Metabolite	*q*	*P*	LADA vs. T1D	LADA vs. T2D	T1D vs. T2D	T1D	LADA	T2D
Alanine	5.86E-18	8.02E-20	****	*	****	1 ± 0.040	1.41 ± 0.042	1.60 ± 0.036
Uric acid	5.03E-15	1.38E-16	****	**	****	1 ± 0.077	1.66 ± 0.079	2.13 ± 0.082
Tyrosine	8.06E-12	3.31E-13	****	**	****	1 ± 0.047	1.33 ± 0.047	1.64 ± 0.065
Isocitrate	7.55E-11	4.13E-12	****	*	****	1 ± 0.067	1.30 ± 0.050	1.57 ± 0.052
C16:0	1.10E-10	7.53E-12	****	NS	****	1 ± 0.065	1.45 ± 0.077	1.76 ± 0.087
Myo-inositol	1.48E-10	1.42E-11	****	**	****	1 ± 0.038	1.26 ± 0.043	1.45 ± 0.045
Phenylalanine	1.48E-10	1.42E-11	****	**	****	1 ± 0.040	1.35 ± 0.067	1.56 ± 0.058
C18:0	2.51E-10	2.76E-11	****	NS	****	1 ± 0.033	1.18 ± 0.029	1.26 ± 0.030
Lysine	7.09E-10	8.74E-11	****	*	****	1 ± 0.045	1.25 ± 0.036	1.45 ± 0.044
Ornithine2	1.94E-09	2.66E-10	****	NS	****	1 ± 0.068	1.37 ± 0.057	1.55 ± 0.056
Anthranilic acid	2.16E-09	3.26E-10	****	NS	****	1 ± 0.059	1.80 ± 0.133	1.96 ± 0.154
C22:6	1.09E-08	1.79E-09	****	NS	****	1 ± 0.090	1.69 ± 0.147	1.96 ± 0.124
C17/0	1.57E-08	2.80E-09	****	NS	****	1 ± 0.068	1.38 ± 0.071	1.58 ± 0.076
Quinic acid	4.12E-08	7.91E-09	****	NS	****	1 ± 0.201	2.69 ± 0.328	2.62 ± 0.376
Citric acid	1.02E-07	2.10E-08	***	*	****	1 ± 0.042	1.29 ± 0.067	1.47 ± 0.058
2-hydroxyglutarate	1.99E-07	4.35E-08	****	NS	****	1 ± 0.027	1.24 ± 0.049	1.32 ± 0.044
Pyroglutamic acid	2.42E-07	5.64E-08	***	NS	****	1 ± 0.024	1.15 ± 0.025	1.22 ± 0.030
C18:1	3.88E-07	9.56E-08	***	NS	****	1 ± 0.092	1.29 ± 0.066	1.51 ± 0.071
Cholesterol	6.97E-07	1.81E-07	***	NS	****	1 ± 0.039	1.14 ± 0.043	1.36 ± 0.048
Hypoxanthine	1.30E-06	3.56E-07	***	NS	****	1 ± 0.148	1.89 ± 0.249	2.28 ± 0.218
Proline	1.66E-06	4.78E-07	***	NS	****	1 ± 0.059	1.34 ± 0.069	1.44 ± 0.052
C14:0	5.66E-06	1.71E-06	**	NS	****	1 ± 0.151	1.28 ± 0.114	1.53 ± 0.094
Glycerol 2-phosphate	6.68E-06	2.10E-06	*	*	****	1 ± 0.049	1.13 ± 0.041	1.34 ± 0.040
Malic acid	7.27E-06	2.39E-06	*	*	****	1 ± 0.051	1.28 ± 0.090	1.57 ± 0.088
C20:4	9.67E-06	3.31E-06	*	*	****	1 ± 0.053	1.15 ± 0.045	1.39 ± 0.062
Homocysteine	1.05E-05	3.75E-06	**	NS	****	1 ± 0.056	1.23 ± 0.057	1.44 ± 0.072
Cysteine	1.14E-05	4.23E-06	***	NS	****	1 ± 0.095	1.44 ± 0.133	1.56 ± 0.112
Fumaric acid	1.64E-05	6.29E-06	NS	*	****	1 ± 0.039	1.15 ± 0.053	1.36 ± 0.059
C18:2	1.68E-05	6.69E-06	**	NS	****	1 ± 0.074	1.21 ± 0.062	1.37 ± 0.058
Aspartic acid	2.18E-05	8.96E-06	*	NS	****	1 ± 0.070	1.28 ± 0.075	1.55 ± 0.084
Histidine	3.19E-05	1.37E-05	***	NS	****	1 ± 0.046	1.19 ± 0.033	1.27 ± 0.047
Succinic acid	3.19E-05	1.40E-05	NS	*	****	1 ± 0.034	1.11 ± 0.038	1.32 ± 0.057
Erythritol	4.99E-05	2.26E-05	**	NS	****	1 ± 0.039	0.81 ± 0.043	0.73 ± 0.037
Taurine	9.78E-05	4.56E-05	**	NS	****	1 ± 0.049	1.33 ± 0.073	1.35 ± 0.058
Glycerol	1.75E-04	8.38E-05	*	NS	****	1 ± 0.083	1.19 ± 0.080	1.43 ± 0.077
Tryptophane	1.97E-04	9.73E-05	**	NS	****	1 ± 0.048	1.18 ± 0.042	1.29 ± 0.048
Arginine product	2.18E-04	1.11E-04	**	NS	***	1 ± 0.045	1.20 ± 0.042	1.24 ± 0.047
Ornithine 1	3.96E-04	2.06E-04	**	NS	***	1 ± 0.045	1.19 ± 0.038	1.21 ± 0.044
Methyl malate	6.65E-04	3.55E-04	NS	NS	***	1 ± 0.173	1.27 ± 0.170	1.83 ± 0.255
Glutamic acid	6.86E-04	3.76E-04	NS	NS	***	1 ± 0.099	1.29 ± 0.111	1.62 ± 0.135
Threonine	9.42E-04	5.29E-04	*	NS	***	1 ± 0.039	1.13 ± 0.035	1.23 ± 0.044
Creatinine	1.38E-03	7.94E-04	**	NS	**	1 ± 0.050	1.20 ± 0.048	1.25 ± 0.057
Beta-alanine	1.65E-03	9.97E-04	NS	NS	***	1 ± 0.060	1.16 ± 0.061	1.35 ± 0.073
Valine	1.65E-03	9.90E-04	*	NS	**	1 ± 0.058	1.14 ± 0.043	1.18 ± 0.036
Glyceric acid	1.80E-03	1.11E-03	NS	NS	***	1 ± 0.054	1.18 ± 0.064	1.28 ± 0.050
Xylose	2.66E-03	1.68E-03	NS	NS	**	1 ± 0.072	1.64 ± 0.223	2.17 ± 0.327

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Table 2—Continued

Metabolite	*q*	*P*	LADA vs. T1D	LADA vs. T2D	T1D vs. T2D	T1D	LADA	T2D
Leucine	3.56E-03	2.29E-03	*	NS	**	1 ± 0.048	1.11 ± 0.038	1.17 ± 0.036
Asparagine	3.94E-03	2.59E-03	*	NS	**	1 ± 0.076	1.25 ± 0.066	1.32 ± 0.079
Isoleucine	6.39E-03	4.29E-03	NS	NS	*	1 ± 0.064	1.12 ± 0.051	1.13 ± 0.039
Indole 3-acetic acid	9.31E-03	6.38E-03	*	NS	**	1 ± 0.083	1.48 ± 0.221	1.53 ± 0.154
Hippuric acid 1	2.14E-02	1.49E-02	*	NS	*	1 ± 0.137	1.29 ± 0.133	1.34 ± 0.159
Glycine	3.39E-02	2.42E-02	NS	NS	*	1 ± 0.031	1.09 ± 0.025	1.09 ± 0.026
Hippuric acid 2	3.74E-02	2.71E-02	NS	NS	*	1 ± 0.153	1.40 ± 0.143	1.51 ± 0.191
Glycerol 3-phosphate	4.20E-02	3.16E-02	NS	*	NS	1 ± 0.042	0.96 ± 0.037	1.10 ± 0.040
Xylitol	6.03E-02	4.62E-02	NS	NS	NS	1 ± 0.082	1.08 ± 0.070	1.13 ± 0.064

Data are expressed as the mean fold to T1D ± SEM. Differences were assessed by ANOVA followed by post hoc Tukey test on log2-transformed data. *P* values were corrected for multiple comparisons using the false discovery rate method. When more than one derivative of the same metabolite are detected, this is indicated by a number after the name. NS, not significant. *$P/q < 0.05$; **$P/q < 0.01$; ***$P/q < 0.001$; ****$P/q < 0.0001$.

difficult to distinguish from T2D at diagnosis (3). This is largely due to the heterogeneity of LADA, combining different metabolic (3) and genetic (6) features of T1D and T2D. In this study, we examined whether metabolite profiling could identify metabolites with improved capability of distinguishing LADA from T1D and T2D.

LADA was found to be a metabolic intermediate of T1D and T2D, overlapping significantly with both of these types. Hence, the metabolome mirrors the clinical heterogeneity. Consequently, no unique metabolic marker could be identified that had the capacity of distinguishing between LADA and the other diabetes types. Instead, all three diabetes types were found along a metabolic continuum, extending from T1D via LADA to T2D. Plasma C-peptide levels was found to be the strongest determinant of the metabolite profile. This is in line with insulin regulating the metabolism of all macronutrients. All metabolites except for three showed lower levels in patients with T1D than in those with LADA and T2D.

The two strongest discriminants between diabetes types were alanine and uric acid. Alanine is a central component of the Cahill cycle, connecting tissue proteolysis to hepatic gluconeogenesis and the urea cycle. Uric acid has been suggested to reflect tissue ATP depletion (15).

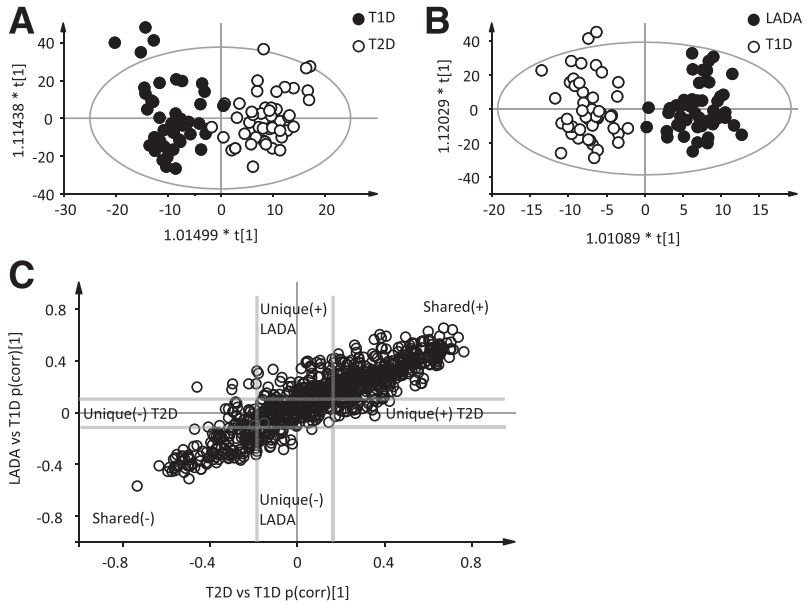

Figure 2—Analysis of lipophilic and high–molecular weight metabolites suggests a metabolic continuum extending from T1D via LADA to T2D: a similar analysis, as depicted for the low–molecular weight metabolome in Fig. 1, was conducted for the lipophilic and high–molecular weight metabolome. This analysis revealed a perfect classification of T2D and T1D (*A*) and LADA and T1D (*B*). *C*: The SUS-plot derived from these models revealed that LADA and T2D share differences with T1D, with no unique metabolites associated with either LADA or T2D. Abbreviations are as explained in Fig. 1. T1D, *n* = 48; T2D, *n* = 50; and LADA, *n* = 49.

Table 3—Lipophilic and high–molecular weight metabolites differing in levels between LADA, T1D, and T2D

Metabolite	*q*	*P*	LADA vs. T1D	LADA vs. T2D	T1D vs. T2D	T1D	LADA	T2D
LPC(20:3)	1.44E-12	2.44E-14	****	NS	****	1 ± 0.093	1.82 ± 0.105	2.13 ± 0.104
LPC(16:1)	3.07E-12	1.04E-13	****	NS	****	1 ± 0.073	1.98 ± 0.170	2.59 ± 0.211
Phe/Phe	7.83E-12	3.98E-13	****	*	****	1 ± 0.072	1.71 ± 0.105	2.22 ± 0.154
LPC(20:5)	1.11E-10	7.52E-12	****	NS	****	1 ± 0.129	2.67 ± 0.346	2.89 ± 0.282
LPC(14:0)	2.63E-10	2.23E-11	****	NS	****	1 ± 0.083	1.96 ± 0.181	2.43 ± 0.231
PC(36:5)	3.07E-10	3.13E-11	****	NS	****	1 ± 0.092	1.83 ± 0.129	2.07 ± 0.113
PC(40:6)	4.18E-08	5.48E-09	****	NS	****	1 ± 0.091	1.77 ± 0.168	1.88 ± 0.123
Uric acid	1.39E-07	2.13E-08	***	NS	****	1 ± 0.103	1.67 ± 0.144	2.20 ± 0.159
LPC(20:2)	1.40E-07	2.38E-08	****	NS	****	1 ± 0.073	1.49 ± 0.085	1.58 ± 0.076
LPC(17:1)	3.47E-07	6.47E-08	****	NS	****	1 ± 0.061	1.67 ± 0.157	1.71 ± 0.126
LPC(20:4)	5.78E-07	1.18E-07	***	NS	****	1 ± 0.065	1.35 ± 0.073	1.49 ± 0.058
C14:1 carnitine	8.25E-07	1.82E-07	****	NS	****	1 ± 0.176	1.97 ± 0.239	2.92 ± 0.421
Tyrosine	9.54E-07	2.26E-07	**	NS	****	1 ± 0.064	1.26 ± 0.054	1.51 ± 0.067
C16 carnitine	1.79E-06	4.55E-07	***	NS	****	1 ± 0.123	1.75 ± 0.181	2.36 ± 0.245
Hypoxanthine	2.26E-06	6.14E-07	***	NS	****	1 ± 0.313	3.26 ± 0.775	4.80 ± 0.924
PC(34:3)	2.44E-06	7.02E-07	****	NS	****	1 ± 0.057	1.37 ± 0.064	1.45 ± 0.065
LPC(16:0)	2.82E-06	8.62E-07	***	NS	****	1 ± 0.033	1.14 ± 0.026	1.20 ± 0.021
C14 carnitine	5.72E-06	1.84E-06	***	NS	****	1 ± 0.149	2.17 ± 0.275	3.19 ± 0.439
C18:1 carnitine	1.58E-05	5.36E-06	**	NS	****	1 ± 0.136	1.62 ± 0.171	2.12 ± 0.218
PC(38:6)	3.30E-05	1.18E-05	***	NS	****	1 ± 0.070	1.35 ± 0.067	1.36 ± 0.050
C8 carnitine	4.97E-05	1.85E-05	***	NS	****	1 ± 0.171	2.05 ± 0.270	2.75 ± 0.432
C12 carnitine	8.76E-05	3.45E-05	***	NS	****	1 ± 0.137	1.90 ± 0.240	2.52 ± 0.336
Prothionamide	8.76E-05	3.56E-05	**	NS	****	1 ± 0.201	3.47 ± 0.549	4.48 ± 0.618
LPC(15:0)	8.93E-05	3.78E-05	**	NS	****	1 ± 0.072	1.40 ± 0.114	1.52 ± 0.097
Cinnamic acid	1.10E-04	4.86E-05	*	NS	****	1 ± 0.065	1.25 ± 0.054	1.53 ± 0.077
C18 carnitine	2.05E-04	9.37E-05	**	NS	****	1 ± 0.100	1.37 ± 0.136	1.57 ± 0.126
LPC(18:0)	2.82E-04	1.34E-04	**	NS	***	1 ± 0.040	1.15 ± 0.034	1.20 ± 0.029
C10 carnitine	4.27E-04	2.10E-04	**	NS	***	1 ± 0.218	2.28 ± 0.460	3.06 ± 0.512
Inosine	6.32E-04	3.21E-04	NS	NS	***	1 ± 0.321	2.65 ± 0.582	3.77 ± 0.665
PC(36:1)	7.00E-04	3.68E-04	NS	NS	***	1 ± 0.069	0.78 ± 0.053	0.64 ± 0.042
C5 carnitine	1.00E-03	5.45E-04	*	NS	***	1 ± 0.103	1.55 ± 0.148	1.79 ± 0.215
LPC(18:1)	1.32E-03	7.37E-04	*	NS	***	1 ± 0.039	1.12 ± 0.030	1.15 ± 0.023
C18:2 carnitine	1.89E-03	1.09E-03	*	NS	***	1 ± 0.132	1.39 ± 0.156	1.87 ± 0.220
C8:1 carnitine	2.19E-03	1.30E-03	**	NS	****	1 ± 0.136	1.62 ± 0.171	2.12 ± 0.218
Leu/Ile	3.62E-03	2.21E-03	NS	NS	NS	1 ± 0.121	1.22 ± 0.183	1.87 ± 0.248
3-amino 2-naphtoic acid	5.74E-03	3.60E-03	NS	NS	**	1 ± 0.047	1.11 ± 0.039	1.23 ± 0.048
PC(32:1)	1.01E-02	6.48E-03	NS	NS	**	1 ± 0.097	1.46 ± 0.169	1.80 ± 0.192
Tyr/Val	1.02E-02	6.73E-03	NS	NS	**	1 ± 0.083	1.86 ± 0.157	2.10 ± 0.149
LPC(17:0)	1.13E-02	7.68E-03	*	NS	*	1 ± 0.067	1.24 ± 0.082	1.24 ± 0.062
C10:2 carnitine	1.42E-02	9.88E-03	NS	NS	**	1 ± 0.076	1.13 ± 0.071	1.42 ± 0.162
C6 carnitine	2.22E-02	1.58E-02	NS	*	NS	1 ± 0.040	0.88 ± 0.031	1.04 ± 0.046
Pipecolic acid	3.68E-02	2.68E-02	*	NS	NS	1 ± 0.059	0.68 ± 0.042	0.60 ± 0.035
Glu/Phe	4.00E-02	2.98E-02	NS	NS	NS	1 ± 0.096	1.04 ± 0.090	1.25 ± 0.092
4-Acetamidobutanoate	4.03E-02	3.07E-02	NS	NS	*	1 ± 0.199	1.91 ± 0.484	2.52 ± 0.503
Betaine	4.47E-02	3.56E-02	NS	NS	***	1 ± 0.052	1.14 ± 0.046	1.27 ± 0.056

Data are expressed as the mean fold to T1D ± SEM. Differences were assessed by ANOVA followed by post hoc Tukey test on log2-transformed data. *P* values were corrected for multiple comparison using the false discovery rate method. NS, not significant. *$P/q < 0.05$; **$P/q < 0.01$; ***$P/q < 0.001$; ****$P/q < 0.0001$.

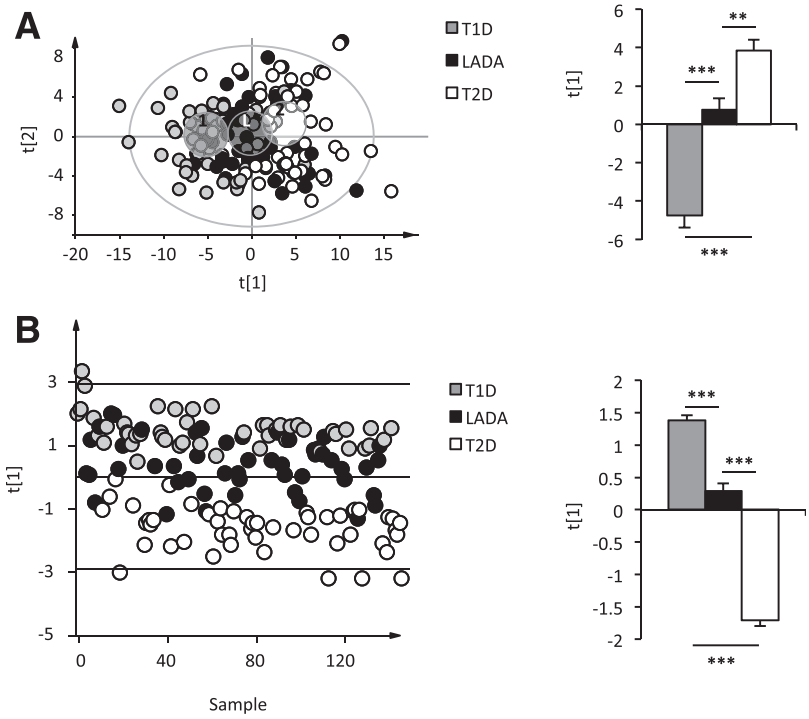

Figure 3—LADA is a metabolic intermediate of T1D and T2D: *A*: The score scatter plot from a PCA calculated on the 123 identified metabolites reveals LADA to cluster in between T1D and T2D, with a significant overlap with the T1D and T2D clusters. *B*: Score scatter plot from a PCA calculated on C-peptide, HbA_{1c}, and GADAb levels. L, average LADA score; t[1] and t[2], first and second principal component, respectively. 1, average T1D score; 2, average T2D score. Average scores ± SEM are shown to the right of the score plots. Differences were examined by ANOVA followed by post hoc Tukey test. $^{**}P < 0.01$, $^{***}P < 0.001$. T1D, $n = 48$; T2D, $n = 50$; and LADA, $n = 49$.

However, ATP depletion is expected to be highest in T1D, which suggests that this association may not apply to all diseases. Other metabolites discriminating between diabetes types included branched-chain and aromatic amino acids, which previously were associated with insulin resistance (16) and increased future risk of cardiovascular disease (CVD) (17) and T2D (18). Notably, these metabolites were also elevated in LADA patients with a slower progression to insulin replacement therapy. Levels of cysteine were higher in patients with T2D and LADA than in those with T1D. Cysteine levels have been associated with elevated BMI (19) and increased CVD risk (20). Acylcarnitines, long and intermediate chains in particular, also showed large differences between diabetes types. These intermediates, suggested to be formed as a result of an imbalance between lipolysis and Krebs cycle activity (21), are associated with increased insulin resistance and T2D (22) and have previously been associated with future CVD (17).

In conclusion, we could not find any unique metabolite signature capable of distinguishing between LADA and T2D. Rather, the metabolome showed a strong association with C-peptide levels. Hence, the metabolome of LADA was an intermediate of that observed in T1D and T2D, with significant overlap with both of these diabetes types, but more similar to the latter. Hence, our data support that T1D and T2D represent extremes on a continuum (23). LADA patients showing a higher metabolic resemblance to T2D patients had a slower progression to insulin therapy than those showing a higher metabolic resemblance to T1D, despite having similar C-peptide levels. Overall, our data challenge the view of metabolically distinct diabetes subtypes.

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Author Contributions. M.A.-M. and A.A. performed metabolite profiling and analyzed the data. P.St. assisted in analysis of the data. A.H.R. and L.G. headed the ANDIS study and assisted in the study design. P.Sp. conceived the study, assisted in analysis of the data, and wrote the first draft of the manuscript. All authors contributed to writing the final version of the manuscript. P.Sp. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Groop LC, Bottazzo GF, Doniach D. Islet cell antibodies identify latent type I diabetes in patients aged 35-75 years at diagnosis. Diabetes 1986;35:237–241
2. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. Diabetes 1993;42:359–362
3. Hawa MI, Kolb H, Schloot N, et al.; Action LADA consortium. Adult-onset autoimmune diabetes in Europe is prevalent with a broad clinical phenotype: Action LADA 7. Diabetes Care 2013;36:908–913
4. Turner R, Stratton I, Horton V, et al.; UK Prospective Diabetes Study Group. UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. Lancet 1997;350:1288–1293
5. Tuomi T, Carlsson A, Li H, et al. Clinical and genetic characteristics of type 2 diabetes with and without GAD antibodies. Diabetes 1999;48:150–157
6. Cervin C, Lyssenko V, Bakhtadze E, et al. Genetic similarities between latent autoimmune diabetes in adults, type 1 diabetes, and type 2 diabetes. Diabetes 2008;57:1433–1437
7. Danielsson APH, Moritz T, Mulder H, Spégel P. Development of a gas chromatography/mass spectrometry based metabolomics protocol by means of statistical experimental design. Metabolomics 2012;8:50–63
8. Spégel P, Ekholm E, Tuomi T, Groop L, Mulder H, Filipsson K. Metabolite profiling reveals normal metabolic control in carriers of mutations in the glucokinase gene (MODY2). Diabetes 2013;62:653–661
9. Spégel P, Danielsson APH, Bacos K, et al. Metabolomics analysis of a human oral glucose tolerance test reveals fatty acids as reliable indicators of regulated metabolism. Metabolomics 2010;6:56–66
10. Jonsson P, Johansson ES, Wuolikainen A, et al. Predictive metabolite profiling applying hierarchical multivariate curve resolution to GC-MS data—a potential tool for multi-parametric diagnosis. J Proteome Res 2006;5:1407–1414
11. Wuolikainen A, Jonsson P, Ahnlund M, et al. Multi-platform mass spectrometry analysis of the CSF and plasma metabolomes of rigorously matched amyotrophic lateral sclerosis, Parkinson's disease and control subjects. Mol Biosyst 2016;12:1287–1298
12. Chorell E, Moritz T, Branth S, Antti H, Svensson MB. Predictive metabolomics evaluation of nutrition-modulated metabolic stress responses in human blood serum during the early recovery phase of strenuous physical exercise. J Proteome Res 2009;8:2966–2977
13. Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). J Chemom 2002;16:119–128
14. Wiklund S, Johansson E, Sjöström L, et al. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. Anal Chem 2008;80:115–122
15. Fox IH. Metabolic basis for disorders of purine nucleotide degradation. Metabolism 1981;30:616–634
16. Newgard CB, An J, Bain JR, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 2009;9:311–326
17. Shah SH, Sun JL, Stevens RD, et al. Baseline metabolomic profiles predict cardiovascular events in patients at risk for coronary artery disease. Am Heart J 2012;163:844–850.e1
18. Wang TJ, Larson MG, Vasan RS, et al. Metabolite profiles and the risk of developing diabetes. Nat Med 2011;17:448–453
19. El-Khairy L, Ueland PM, Nygård O, Refsum H, Vollset SE. Lifestyle and cardiovascular disease risk factors as determinants of total cysteine in plasma: the Hordaland Homocysteine Study. Am J Clin Nutr 1999;70:1016–1024
20. Ozkan Y, Ozkan E, Simşek B. Plasma total homocysteine and cysteine levels as cardiovascular risk factors in coronary heart disease. Int J Cardiol 2002;82:269–277
21. Koves TR, Ussher JR, Noland RC, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab 2008;7:45–56
22. Mihalik SJ, Goodpaster BH, Kelley DE, et al. Increased levels of plasma acylcarnitines in obesity and type 2 diabetes and identification of a marker of glucolipotoxicity. Obesity (Silver Spring) 2010;18:1695–1700
23. Tuomi T, Santoro N, Caprio S, Cai M, Weng J, Groop L. The many faces of diabetes: a disease with increasing heterogeneity. Lancet 2014;383:1084–1094