Exocrine Pancreatic Enzymes Are a Serological Biomarker for Type 1 Diabetes Staging and Pancreas Size

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Exocrine pancreas abnormalities are increasingly recognized as features of type 1 diabetes. We previously reported reduced serum trypsinogen levels and in a separate study, smaller pancreata at and before disease onset. We hypothesized that three pancreas enzymes (amylase, lipase, and trypsinogen) might serve as serological biomarkers of pancreas volume and risk for type 1 diabetes. Amylase, lipase, and trypsinogen were measured from two independent cohorts, together comprising 800 serum samples from single-autoantibody–positive (1AAb$^+$) and multiple-AAb$^+$ (≥2AAb$^+$) subjects, individuals with recent-onset or established type 1 diabetes, their AAb-negative (AAb$^-$) first-degree relatives, and AAb$^-$ control subjects. Lipase and trypsinogen were significantly reduced in ≥2AAb$^+$, recent-onset, and established type 1 diabetes subjects versus control subjects and 1AAb$^+$, while amylase was reduced only in established type 1 diabetes. Logistic regression models demonstrated trypsinogen plus lipase (area under the receiver operating characteristic curve [AUROC] = 81.4%) performed equivalently to all three enzymes (AUROC = 81.4%) in categorizing ≥2AAb$^+$ versus 1AAb$^+$ subjects. For cohort 2 (n = 246), linear regression demonstrated lipase and trypsinogen levels could individually and collectively serve as indicators of BMI-normalized relative pancreas volume (RPV_{BMI}, $P < 0.001$), previously measured by MRI. Serum lipase and trypsinogen levels together provide the most sensitive serological biomarker of RPV_{BMI} and may improve disease staging in pretype 1 diabetes.

Early reports, predating even the discovery of insulin, noted a small pancreas size in patients with type 1 diabetes (1), but this finding was generally presumed to be a consequence of insulinopenia and loss of paracrine insulin trophic signaling (2,3). Indeed, well-known type 1 diabetes-associated comorbidities, such as arteriosclerosis, microvascular disease, and neuropathy, could ostensibly contribute to reduced pancreas mass in subjects with established disease (1,4–6). Hence, the vast majority of investigations related to type 1 diabetes pathophysiology have focused on loss of functional β-cell mass, autoimmune features, and genetic susceptibility (7). However, over the course of the past decade, studies of individuals with type 1 diabetes, including those with recent-onset disease, revealed small pancreas size (weight or volume) (8–12), increased immune cell infiltration of the exocrine pancreas (13–15), greater C4d complement deposition in exocrine pancreatic ducts and blood vessels (16), and morphological alterations within the acinar tissue, including reduced acinar cell numbers (17) and fewer peri-islet amylase-negative cell clusters (18). Importantly, a number of these exocrine pancreas alterations were also observed in individuals without diabetes but with type 1 diabetes-predictive islet autoantibodies (AAbs) (11,19). These findings have renewed interest in the exocrine pancreas as a potential target or potentiating factor during type 1 diabetes pathogenesis involving β-cell failure and autoimmunity.

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We previously evaluated serum levels of the exocrine pancreatic enzyme, trypsinogen, from banked samples in our University of Florida (UF) Diabetes Institute Study Bank (UFDI-SB) (20). Although trypsinogen values generally remained in the low range of normal, they were significantly reduced in individuals with recent-onset type 1 diabetes and in subjects with two or more islet AAbs ($\geq$2AAb$^+$) compared with AAb-negative (AAb$^-$) first-degree relatives (FDR)of a type 1 diabetes proband and unrelated AAb$^-$ control subjects. We also recently conducted a cross-sectional clinical trial of pancreas volume using MRI wherein we demonstrated a reduced BMI-normalized relative pancreas volume (pancreas volume divided by BMI; RPV_{BMI}) not only in subjects with recent-onset type 1 diabetes but also in their FDRs (with or without AAbs) compared with AAb$^-$ control subjects without a family history of type 1 diabetes (11). In this cohort, serum trypsinogen levels were once again significantly lower in patients with recent-onset type 1 diabetes compared with AAb$^-$ control subjects and FDR (11). A recent meta-analysis of 20 independent studies suggested that low serum levels of trypsin and two additional exocrine pancreas enzymes, namely amylase and lipase, may affect patients with type 1 and type 2 diabetes (21). Herein, we sought to further characterize the role of the exocrine pancreas in type 1 diabetes pathogenesis by determining whether amylase and lipase were altered in various stages of type 1 diabetes progression and, secondarily, whether these enzymes, individually or collectively with trypsinogen, are indicative of exocrine pancreas volume.

RESEARCH DESIGN AND METHODS

Study Approval

Cohort 1 subjects were recruited from outpatient clinics at UF Health (Gainesville, FL), Nemours Children's Hospital (Orlando, FL), or Emory University with written informed consent (and assent in the case of minors) received before inclusion in the study, as approved by the Institutional Review Boards (IRBs) at each institution. Cohort 2 subjects provided written informed consent (and assent in the case of minors) before being enrolled from UF Health outpatient clinics and the National Institutes of Health (NIH) Type 1 Diabetes TrialNet network in a cross-sectional clinical trial (NCT02234947) approved by the UF IRB, as previously described (11). Participants in both cohorts were assigned identification numbers, and the data were deidentified. All studies reported herein were conducted in accordance with IRB-approved protocols, federal guidelines, and the Declaration of Helsinki.

Subject Enrollment, Serum Collection, and Islet AAb Testing

For cohort 1, blood samples were collected from consented participants at random (i.e., unknown time of day or prandial state) by routine venipuncture into Vacutainer tubes containing clot activator and gel, and serum was separated via centrifugation and stored at −20°C in the UFDI-SB. Sera were screened for type 1 diabetes-associated AAbs against GAD 65 antibody (GADA), insulinoma-associated protein-2 (IA-2A), and zinc transporter-8 (ZnT8A) using Islet Autoantibody Standardization Program (IASP) validated, commercially available ELISA kits (Kronus, Star, ID) according to modified protocols, as previously described (22). Cohort 1 sera (N = 554) were then selected from the UFDI-SB as follows: samples from AAb$^+$ subjects without diabetes (81 single-AAb positive [1AAb$^+$] and 50 $\geq$2AAb$^+$), individuals with recent-onset type 1 diabetes (duration $\leq$1 year, n = 112), subjects with established type 1 diabetes (duration >1 year, n = 75), AAb$^-$ FDR (n = 112), and AAb$^-$ control subjects (n = 124) in an attempt to balance age and sex distribution across clinical groups. Cohort 1 subjects were aged 2.7–70 years, with detailed demographics provided in Table 1. Of these, trypsinogen levels were previously reported for 377 individuals (20) (Supplementary Fig. 2). In this previous publication (20), recent-onset type 1 diabetes was defined as duration <3 months, but here we raised the cutoff to 1 year for consistency with the cohort 2 enrollment criteria (described below and as previously reported [11]). Importantly, this only resulted in reclassification of eight subjects and did not significantly impact our findings.

Blood samples were collected from consented cohort 2 subjects (N = 246; aged 8–70 years; clinical trial identifier: NCT02234947) by venipuncture in the morning while fasting, and serum was stored at −20°C. GADA, IA-2A, ZnT8A, and insulin autoantibodies were measured from serum by radioimmunoassay (23) at the University of Colorado at Denver, and subjects were classified as AAb$^-$ control subjects (n = 52), AAb$^-$ FDR (n = 63), 1AAb$^+$ FDR (n = 35), $\geq$2AAb$^+$ FDR (n = 39), recent-onset type 1 diabetes (duration $\leq$1 year, n = 56), and established type 1 diabetes (duration >1 year, n = 1). Of these, 229 subjects had pancreas volume by MRI and trypsinogen levels previously reported (11) (Supplementary Fig. 2). Cohort 2 demographics are provided in Table 2. Serum amylase and lipase levels were not previously reported for any subject in cohort 1 or cohort 2.

Amylase and Lipase Assays

Amylase and lipase were quantified from all 800 cohort 1 and cohort 2 serum samples under blinded conditions at UF Health Pathology Laboratories (Gainesville, FL) via standard clinical laboratory assays involving direct enzymatic colorimetric analysis (Beckman Coulter, Brea, CA) (24) and enzyme-coupled colorimetric methods (25), respectively. The assay-specific normal serum reference ranges were 29–103 units/L for amylase and 11–82 units/L for lipase.

Trypsinogen and Trypsin Assays

For a subset of cohort 1 and cohort 2 samples, serum trypsinogen was measured at ARUP Laboratories (Salt Lake City, UT; reference range of 10.0–57.0 ng/mL), as previously reported (11,20). For cohort 1 and cohort 2 subjects evaluated thereafter, serum trypsin was instead measured at ARUP via radioimmunoassay due to discontinuation of the trypsinogen assay; hence, trypsinogen levels were inferred according to calculations provided by ARUP.

Table 1—Demographic information for cohort 1

Characteristic	AAb$^-$ control n = 124	AAb$^-$ FDR n = 112	1 AAb$^+$ n = 81	≥2 AAb$^+$ n = 50	Recent-onset T1D n = 112	Established T1D n = 75
Sex						
Female	55 (44)	51 (46)	52 (64)	27 (54)	42 (38)	43 (57)
Male	69 (56)	61 (54)	29 (36)	23 (46)	70 (62)	32 (43)
Race						
American Indian/Alaska Native	0 (0)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Asian	5 (4)	0 (0)	1 (1)	1 (2)	1 (0)	0 (0)
Black/African American	23 (19)	18 (16)	15 (19)	6 (12)	10 (9)	10 (13)
Caucasian	92 (74)	91 (81)	60 (74)	39 (78)	95 (85)	62 (83)
Native Hawaiian or other Pacific Islander	1 (1)	1 (1)	2 (2)	0 (0)	0 (0)	0 (0)
More than one race	1 (1)	0 (0)	2 (2)	4 (8)	1 (1)	3 (4)
Unknown/not reported	2 (2)	1 (1)	1 (1)	0 (0)	5 (5)	0 (0)
Ethnicity						
Hispanic	18 (15)	23 (21)	15 (19)	8 (16)	16 (14)	10 (13)
Non-Hispanic	99 (80)	82 (73)	61 (75)	39 (78)	82 (74)	65 (87)
Unknown/not reported	7 (6)	7 (6)	5 (6)	3 (6)	13 (12)	0 (0)
Age (years)	20.7 ± 12.3	24.2 ± 13.2	27.6 ± 14.4	21.2 ± 13.1	15.2 ± 10.2	26.6 ± 12.0
Height (inches)*	52.5 ± 8.5	61.1 ± 8.4	61.1 ± 7.7	62.5 ± 7.7	61.3 ± 8.3	65.9 ± 5.8
Weight (lb)*	120 ± 54.3	145 ± 64.2	162 ± 64.1	128 ± 52.0	121 ± 52.7	161 ± 40.4
CDC-standardized BMI-for-age percentile*	30.7 ± 24.7	45.0 ± 29.2	51.2 ± 28.7	40.1 ± 28.8	52.3 ± 32.7	38.4 ± 28.0
T1D duration (years)	NA	NA	NA	NA	0.12 ± 0.1	12.8 ± 11.5
AAb distribution	NA	NA	64 GADA$^+$, 5 IA-2A$^+$, 12 ZnT8A$^+$	4 IA-2A$^+$ ZnT8A$^+$, 6 GADA$^+$ IA-2A$^+$, 18 GADA$^+$ ZnT8A$^+$, 22 GADA$^+$ IA-2A$^+$ ZnT8A$^+$	NA	NA

Data are presented as n (%) or as mean ± SD. NA, not applicable; T1D, type 1 diabetes. *Height, weight, and CDC-standardized BMI-for-age percentile data are not available for all subjects in this study because the provision of such information is voluntary.

Pancreas Volume Assessment

Pancreas volume was measured by 1.5T pancreatic MRI and normalized against BMI (RPV_{BMI}), as previously reported (11).

Statistics

Statistical analyses were conducted using R v3.6.3 with a two-sided significance level of $\alpha = 0.05$ and graphed via the yarrr v0.1.5 package in R. For cohort 1 and cohort 2 combined, serum amylase, lipase, and trypsinogen levels were log-transformed to better meet the assumption of normality, with mean levels compared by one-way ANCOVA adjusted to account for age, sex, race, cohort, and Centers for Disease Control and Prevention (CDC)-standardized BMI-for-age percentile with a post hoc Tukey-Kramer adjustment to control for multiple testing via the TukeyHSD function in R. BMI was standardized for adults using the 2015–2016 National Health and Nutrition Examination Survey data based on 5,337 adults with measured BMIs (removing pregnant women) and for adolescents (age <20) using the get_BMI_percentile function in the R package PAutilities v0.3.1. Because height and weight were optional and self-reported for UFDI-SB participants, BMI could not be calculated for 117 cohort 1 subjects. Hence, 21 AAb$^-$ control subjects, 25 AAb$^-$ FDR, 24 1AAb$^+$, 15 ≥2AAb$^+$, 20 subjects with recent-onset type 1 diabetes, and 12 with established type 1 diabetes were excluded from the ANCOVA model but are included in scatter plots of the raw data (Fig. 2A–C).

Principal component analysis (PCA) was performed using the combined cohort 1 and cohort 2 data to examine the joint relationship of the enzymes with disease status wherein component 1 was correlated with trypsinogen, lipase, and amylase levels, whereas component 2 was correlated only with amylase levels.

Logistic regression models, including age, sex, BMI percentile, and cohort, and each enzyme alone, pairs of enzymes, or all three enzymes together as covariates, were used to classify subjects as having low risk (defined as AAb$^-$ or 1AAb$^+$) versus stage 1–3 type 1 diabetes (i.e., ≥2AAb$^+$, recent-onset and established type

Table 2—Demographic information for cohort 2

Characteristic	AAb$^-$ control n = 52	AAb$^-$ FDR n = 63	1 AAb$^+$ n = 35	≥2 AAb$^+$ n = 39	Recent-onset T1D n = 56	Established T1D n = 1
Sex						
Female	32 (62)	39 (62)	22 (63)	16 (41)	26 (46)	0 (0)
Male	20 (38)	24 (38)	13 (37)	23 (59)	30 (54)	1 (100)
Race						
American Indian/Alaska Native	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Asian	1 (2)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)
Black/African American	6 (12)	0 (0)	1 (3)	3 (8)	2 (4)	0 (0)
Caucasian	36 (69)	62 (98)	31 (89)	34 (87)	51 (91)	1 (100)
Native Hawaiian or other Pacific Islander	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
More than one race	2 (4)	0 (0)	0 (0)	2 (5)	1 (2)	0 (0)
Unknown/not reported	6 (12)	1 (2)	3 (9)	0 (0)	1 (2)	0 (0)
Ethnicity						
Hispanic	7 (13)	8 (13)	5 (14)	4 (10)	5 (9)	0 (0)
Non-Hispanic	44 (85)	54 (86)	29 (83)	35 (90)	50 (89)	0 (0)
Unknown/not reported	1 (2)	1 (2)	1 (3)	0 (0)	1 (2)	1 (100)
Age (years)	18.4 ± 6.6	21.3 ± 12.0	27.9 ± 13.9	17.7 ± 13.3	16.8 ± 9.4	17.9
Height (inches)	63.9 ± 5.7	63.4 ± 5.0	64.0 ± 6.0	60.8 ± 8.1	63.4 ± 5.7	67.0
Weight (lb)	134 ± 40.5	137 ± 49.0	163 ± 66.3	111 ± 50.0	132 ± 51.6	114
CDC-standardized BMI-for-age percentile	25.9 ± 21.4	47.9 ± 27.9	52.1 ± 28.9	44.2 ± 36.1	35.4 ± 26.0	3.1 ± 0
T1D duration (years)	NA	NA	NA	NA	0.5 ± 0.3	2.0
AAb distribution	NA	NA	27 GADA$^+$, 1 IA-2A$^+$, 1 ZnT8A$^+$, 6 IAA	2 IA-2A$^+$ ZnT8A$^+$, 2 IAA$^+$ ZnT8A$^+$, 5 IAA$^+$ GADA$^+$, 3 GADA$^+$ IA-2A$^+$, 3 GADA$^+$ ZnT8A$^+$, 6 GADA$^+$ IA-2A$^+$ ZnT8A$^+$, 5 GADA$^+$ IAA$^+$ ZnT8A$^+$, 14 GADA$^+$ IA-2A$^+$ ZnT8A$^+$ IAA	NA	NA

Data are presented as n (%) or as mean ± SD. IAA, insulin autoantibodies; NA, not applicable; T1D, type 1 diabetes.

1 diabetes, according to consensus in the field for staging type 1 diabetes progression [26]) or as 1AAb$^+$ versus ≥2AAb$^+$. For logistic regression, the caret v6.0–85 and pROC v1.16.1 R packages were used to perform a k-fold cross validation with k = 10 and in reporting area under the receiver operating characteristic curve (AUROC) values.

For cohort 2, linear regression models included age, sex, race, as well as amylase, lipase, or trypsinogen as covariates with RPV_{BMI} as the outcome variable. For a combined enzyme linear regression model, we again included age, sex, and race as covariates, but due to multicollinearity among the enzymes, we performed a PCA on cohort 2 subjects and used principle component 1 (PC1; relative contribution of trypsinogen, −0.71; lipase, −0.71) as the predictor.

Data and Resource Availability

All data generated and analyzed during this study are included in the manuscript and its Supplementary Material. Raw data are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

We first performed internal quality control assessments of exocrine enzyme stability in frozen serum samples. As expected, lipase and amylase levels measured from 42 randomly selected sera were consistent across technical replicates in two separate runs performed 7 months apart (lipase $R^2 = 0.98$, amylase $R^2 = 0.89$) (Supplementary Fig. 1). Next, we measured serum levels of three exocrine pancreas enzymes (trypsinogen, amylase, and lipase) for six groups (AAb$^-$ control subjects, AAb$^-$ FDR, 1AAb$^+$, ≥2AAb$^+$, recent-onset type 1 diabetes, and established type 1 diabetes) from two clinical cohorts (Supplementary Fig. 2). In contrast with a previous report (27), trypsinogen ($R^2 = 0.31$, $P < 0.001$) (Fig. 1*A*) and lipase levels ($R^2 = 0.11$, $P < 0.001$) (Fig. 1*B*) were significantly correlated with age at the time of blood draw among AAb$^-$ subjects, but amylase levels were not ($R^2 = -0.002$, $P > 0.05$) (Fig. 1*C*). Among individuals with recent-onset type 1 diabetes (duration ≤1 year), trypsinogen levels were significantly associated with age at disease onset, although the model fit was quite poor

($R^2 = 0.06$, $P < 0.01$) (Fig. 1*D*); lipase and amylase levels were not associated with age at disease onset (Fig. 1*E* and *F*). Finally, among AAb^- subjects, serum lipase ($P < 0.05$) and amylase ($P < 0.0001$) levels differed significantly in subjects grouped according to self-reported race, whereas trypsinogen levels did not (Supplementary Fig. 3). Importantly, this study was not designed to comprehensively evaluate differences in exocrine pancreas enzyme levels across racial groups, but the data are presented to characterize factors for consideration in our downstream analyses.

Hence, for our study, serum trypsinogen, amylase, and lipase data from cohorts 1 and 2 were combined and log-transformed to approximate a normal distribution, with ANCOVA used to account for significant differences between groups with regard to the covariates age ($P < 0.001$, one-way ANOVA), sex ($P < 0.01$, χ^2 test), race ($P < 0.01$, χ^2 test), BMI ($P < 0.03$, one-way ANOVA), and cohort ($P < 0.001$, χ^2 test), with a post hoc Tukey-Kramer adjustment to control for multiple testing. We first confirmed our prior observations (11,20) of reduced serum trypsinogen levels in $\geq 2AAb^+$ ($P < 0.05$) with recent-onset type 1 diabetes ($P < 0.001$) and established type 1 diabetes groups ($P < 0.001$) compared with AAb^- control, AAb^- FDR, and $1AAb^+$ groups (Fig. 2*A*). Serum lipase levels were also significantly lower for established type 1 diabetes, recent-onset type 1 diabetes, and $\geq 2AAb^+$ subjects compared with AAb^- control subjects ($P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively) and $1AAb^+$ subjects ($P < 0.001$, all) (Fig. 2*B*); although compared with trypsinogen (coefficient of variation = 0.59), lipase levels were more variable (coefficient of variation = 0.72). In contrast, serum amylase levels were significantly lower only for individuals with established type 1 diabetes compared with AAb^- FDR ($P < 0.01$) and control subjects ($P < 0.05$) (Fig. 2*C*).

We previously reported that serum trypsinogen levels were within the clinical reference range for the vast majority of subjects despite being lower, on average, in groups with and at risk for type 1 diabetes (20). In this study, we confirmed this original observation, with 93.4% (746 of 800) of subjects having trypsinogen within the normal range (Fig. 2*D*). We further noted that serum levels of lipase and amylase were also within the clinical reference range for most of the subjects examined (87.8% [684 of 800]) and 76.3% [596 of 800], respectively) (Fig. 2*E* and *F*). However, established type 1 diabetes, recent-onset type 1 diabetes, and $\geq 2AAb^+$ (binned together as stage 1–3 type 1 diabetes [26]) had significantly greater proportions of individuals with clinically low levels of trypsinogen ($P < 0.001$), lipase ($P < 0.001$), and amylase ($P < 0.002$) compared with $1AAb^+$, AAb^- FDR, and AAb^- control subjects (binned together as "low risk" for type 1 diabetes; χ^2 test) (Supplementary Fig. 4).

We next used PCA to assess whether trypsinogen, lipase, and amylase levels collectively differed according to type 1 diabetes disease and AAb risk status (Fig. 2*G*). Specifically, for cohorts 1 and 2 combined, subjects were again binned as having stage 1–3 type 1 diabetes versus low risk for the disease. The stage 1–3 type 1 diabetes group had a significantly smaller mean principle component 1 (PC1) score (which positively correlated with amylase, lipase, and trypsinogen levels; $P < 0.001$) and a slightly higher principle component 2 (PC2) score (which positively correlated with amylase only; $P = 0.052$) compared with the low-risk group (Fig. 2*G*). Hence, PCA results were primarily driven by lipase and trypsinogen.

We next fit logistic regression models with age, sex, BMI percentile, and cohort as covariates to determine whether amylase, lipase, and/or trypsinogen were individually or collectively predictive of subject status as low-risk versus stage 1–3 type 1 diabetes. For models having only one or no enzymes, the trypsinogen AUROC was 73.7%, the lipase AUROC was 69.5%, the amylase AUROC was 64.8%, and the no-enzymes AUROC was 61.1% (Fig. 3*A*). The AUROC for the full model with all three exocrine pancreas enzymes was 75.6%, indicating that the combination of serum amylase, lipase, and trypsinogen may yield the greatest performance for categorizing subjects as having low risk, which includes $1AAb^+$ subjects (i.e., prestage 1 type 1 diabetes [26]) versus stage 1–3 type 1 diabetes. However, a two-enzyme model containing trypsinogen plus lipase performed comparably (AUROC = 75.0%) (Fig. 3*B*). To further evaluate the utility of exocrine pancreas enzymes as a biomarker in pretype 1 diabetes, we fit similar logistic regression models to determine whether amylase, lipase, and/or trypsinogen were predictive of subject status as $\geq 2AAb^+$ versus $1AAb^+$. Amylase alone (AUROC = 74.0%) was similar to the no-enzymes model (AUROC = 72.6%). Interestingly, lipase alone (AUROC = 81.1%) outperformed trypsinogen alone (AUROC = 78.7%), and trypsinogen plus lipase performed equivalently to the three-enzyme model (AUROC = 81.4%).

Pancreatic volume was previously measured by MRI and normalized against BMI as published for 229 of 246 cohort 2 subjects (11). Here we assessed whether levels of the three exocrine enzymes were reflective of RPV_{BMI}. We first fit linear regression models incorporating age, sex, race, and enzyme level as covariates with RPV_{BMI} as the outcome variable for amylase, lipase, and trypsinogen alone. The three enzymes were each significantly predictive of pancreas volume, with amylase having the smallest effect (adjusted $R^2 = 0.12$, $P < 0.05$) (Fig. 4*A*) compared with lipase (adjusted $R^2 = 0.18$, $P < 0.001$) (Fig. 4*B*) and trypsinogen (adjusted $R^2 = 0.23$, $P < 0.001$) (Fig. 4*C*). Taken together with findings from Figs. 2 and 3, we elected to exclude amylase from our combinatorial analysis and instead assessed trypsinogen plus lipase as potential biomarkers of RPV_{BMI}. Owing to multicollinearity, we could not generate a linear regression model that included both enzymes as covariates. To address this, we first performed a PCA on cohort 2 subjects wherein lipase and trypsinogen contributed to the first principle component (PC1 loadings: trypsinogen = −0.71 and lipase = −0.71). PC1 was then used as the predictor variable in our linear regression

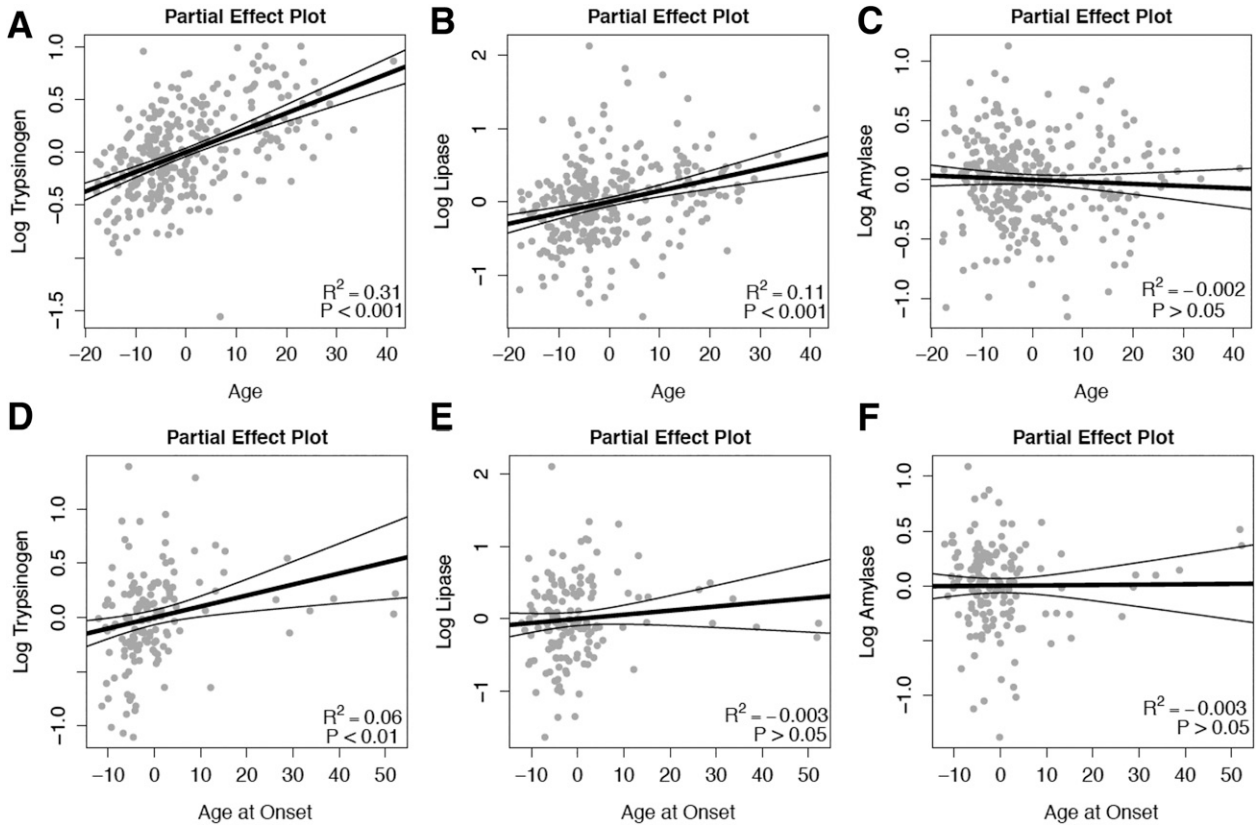

Figure 1—Exocrine pancreatic enzyme levels vs. age. Partial effect plots show the relationship (adjusted R^2 and P value) between age at blood draw and serum levels of trypsinogen (*A*), lipase (*B*), or amylase (*C*) in islet AAb$^-$ subjects, as well as the relationship (adjusted R^2 and P value) between age at diagnosis and serum levels of trypsinogen (*D*), lipase (*E*), or amylase (*F*) in individuals with new-onset type 1 diabetes, while accounting for sex, BMI, race, and cohort. Trypsinogen ($R^2 = 0.31$, $P < 0.001$) and lipase ($R^2 = 0.11$, $P < 0.001$) were significantly correlated with age at draw, while amylase was not ($R^2 = -0.002$, $P > 0.05$). Trypsinogen ($R^2 = 0.06$, $P < 0.01$), lipase ($R^2 = -0.003$, $P > 0.05$), and amylase ($R^2 = -0.003$, $P > 0.05$) were not significantly correlated with age at type 1 diabetes onset. Log-transformed data are shown as scatter plots plus trend lines with 95% CIs.

model, alongside age, sex, and race. Indeed, PC1 was significantly associated with RPV_{BMI} (adjusted $R^2 = 0.22$, $P < 0.001$).

DISCUSSION

In clinical medicine, elevated serum levels of amylase, lipase, and trypsinogen are indicators of acute/chronic pancreatitis, whereas reduced enzyme levels can indicate pancreatic insufficiency (28–30). More than four decades ago, a series of studies noted serum trypsin, amylase, and lipase levels were reduced in patients with long-standing type 1 diabetes (31–34). At that time, these enzymes were not evaluated at earlier stages in the disease process, and their decline was presumed to occur secondarily to loss of insulin secretion. More recently, however, new-onset and pretype 1 diabetes have been associated with smaller pancreas weight/volume, exocrine pancreas inflammation, reduced acinar cell numbers, and changes in enzyme expression patterns within the exocrine pancreas tissue (9,10,14–19), reigniting interest in the acinar pancreatic tissue as a possible early target or potentiator of the disease pathogenesis.

Here, we confirm our prior observation that serum trypsinogen is reduced not only in individuals with new-onset or established type 1 diabetes but also on ≥2AAb$^+$ subjects (20) who are at high risk (~100%) for diabetes progression within 10 years (26). We similarly observed serum lipase levels to be significantly reduced in ≥2AAb$^+$ subjects and patients with type 1 diabetes, whereas amylase levels were only reduced in subjects with established disease. "Cohort" was a significant factor for all three enzymes and was thus included as a study-level covariate, allowing for pooled analysis in our ANCOVA model (35). As potential contributors to the observed differences between the two cohorts, cohort 1 serum samples were collected under an unknown prandial state at unspecified time of day, whereas cohort 2 samples were collected under fasting conditions in the morning. However, amylase and lipase from frozen samples have demonstrated remarkable stability in the circulation during oral glucose tolerance tests as well as after consumption of low-, medium-, and high-fat meals in patients with type 2 diabetes and in individuals without diabetes (36). Similarly, in a separate study, serum concentrations of immunoreactive trypsin

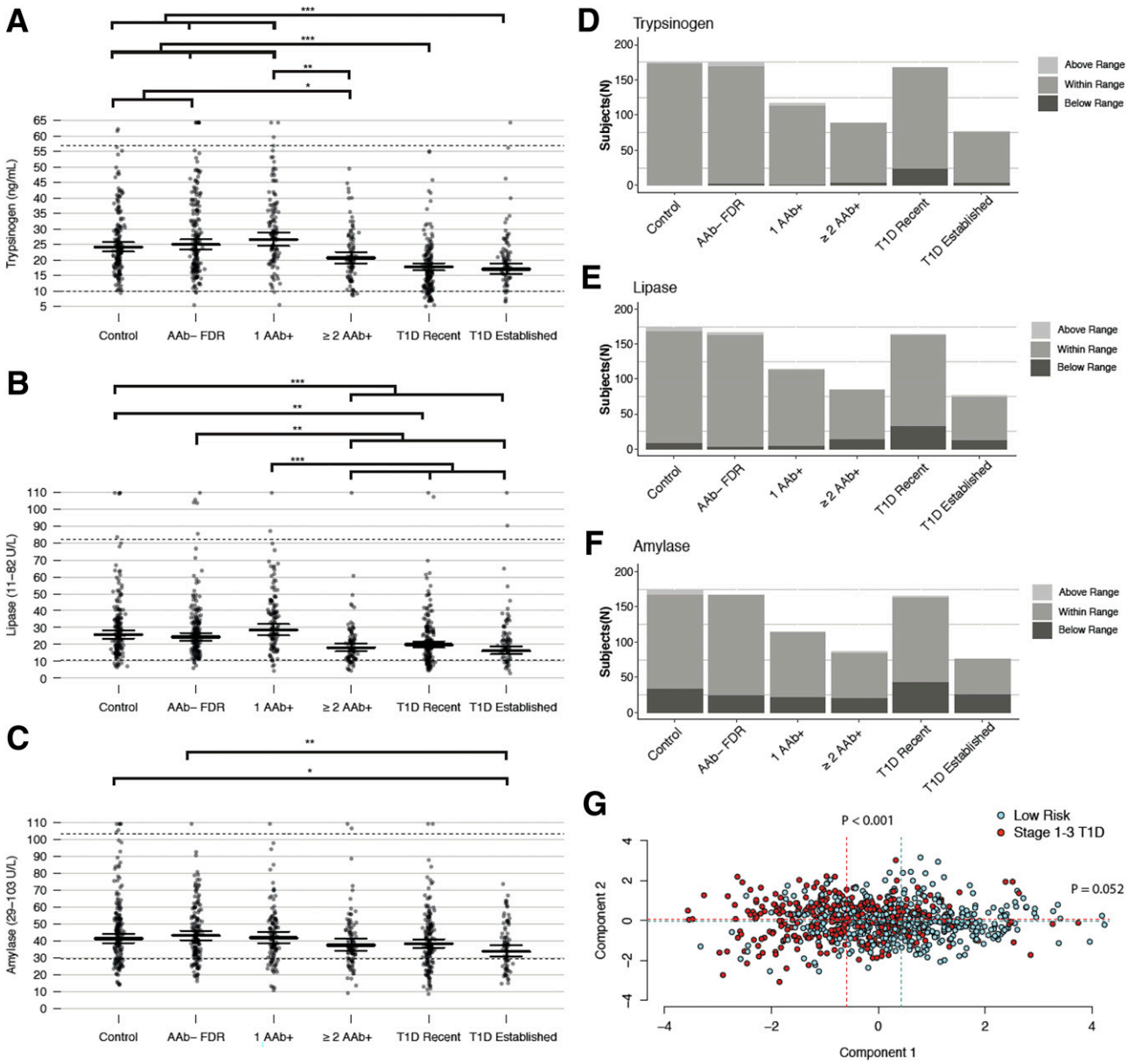

Figure 2—Exocrine pancreatic enzyme levels pre- and posttype 1 diabetes (T1D) onset. Serum trypsinogen (*A*), lipase (*B*), and amylase (*C*) levels in controls, AAb^- FDR, subjects with $1AAb^+$, subjects with $\geq 2AAb^+$, subjects with recent-onset T1D (T1D Recent), and subjects with established T1D (T1D Established). Log-transformed data were converted via back transformation for graphical presentation such that individual dots are unadjusted raw data. Black lines are median (95% CI) which enable simultaneous visualization of data adjusted for age, sex, race, and CDC-standardized BMI-for-age percentile; one-way ANCOVA. Data are shown in the scatter plots for all subjects, including those where BMI data were missing, which excluded them from analysis by ANCOVA. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Dotted horizontal lines show the clinical reference range. Subjects who had serum levels of trypsinogen (*D*), lipase (*E*), or amylase (*F*) below, within, or above the clinical reference range are shown. Data were analyzed by χ^2 test (trypsinogen, $P < 0.001$; lipase, $P < 0.001$; and amylase, $P < 0.02$). *G*: The first two PCs assigned to each subject are displayed. Component 1 increases with larger values of the three exocrine pancreatic enzymes (amylase, lipase, and trypsinogen). Component 2 increases with larger values of amylase alone. Light blue circles: AAb^- control subjects, AAb^- FDR, and $1AAb^+$ subjects; red circles: $\geq 2AAb^+$ subjects and patients with recent-onset or established T1D. The *P* value is <0.001 for the mean difference in component 1 for low risk vs. stage 1–3 T1D is <0.001. The *P* value is 0.052 for the mean difference in component 2 for low risk vs. stage 1–3 T1D.

did not fluctuate significantly after oral glucose, protein meal, or mixed meal tests in "normal" subjects (37), implying that pancreatic exocrine enzymes may provide a robust biomarker that is not easily swayed by prandial status. Importantly, serum levels of amylase and lipase have also demonstrated little longitudinal fluctuation over the course of 32–56 weeks in subjects with type 2 diabetes and/or obesity (38), although to our knowledge, similar studies have not been reported in subjects with or at-risk for type 1 diabetes.

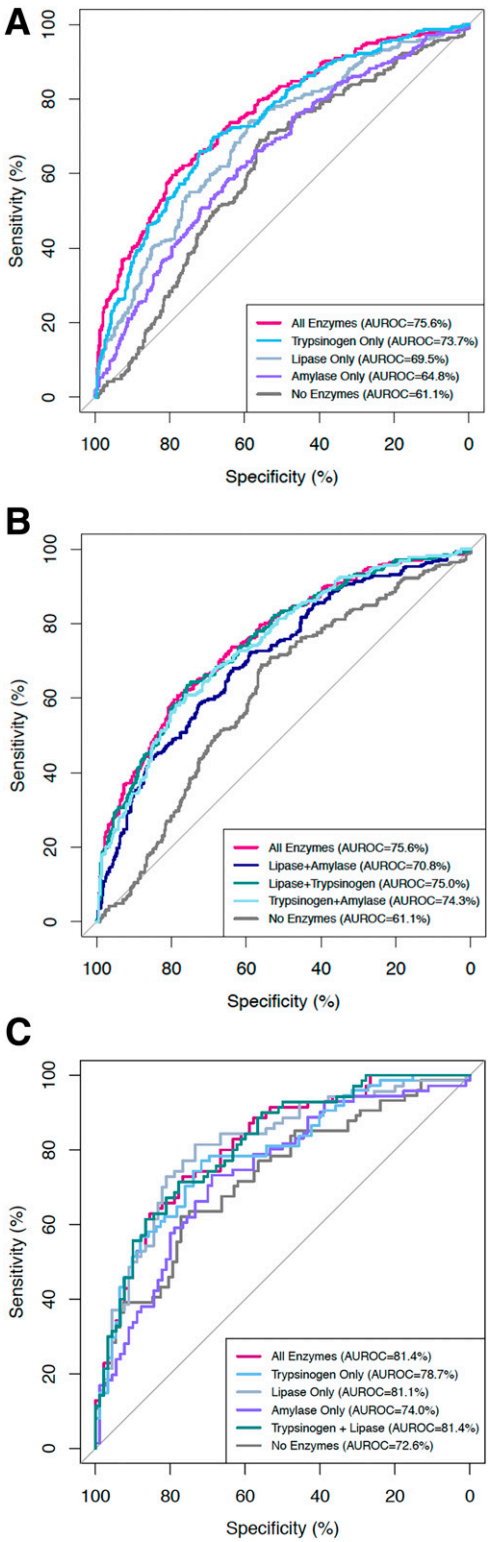

Figure 3—Receiver operating characteristic (ROC) curve showing sensitivity and specificity for classifying subjects according to type 1 diabetes (T1D) risk or status. Logistic regression models categorizing subjects as low risk (AAb^- and $1AAb^+$) vs. stage 1–3 T1D ($\geq 2AAb^+$, recent-onset, and established T1D) are shown for no enzymes, amylase, lipase, trypsinogen, or all three enzymes as covariates (*A*), or for no enzymes, trypsinogen + amylase, lipase + trypsinogen, lipase + amylase, or all three enzymes (as indicated on the graph) (*B*) with age, sex, BMI percentile, and cohort 1 or 2 in our study included as additional covariates. Logistic regression models categorizing subjects as $1AAb^+$ vs. $\geq 2AAb^+$ are shown for no enzymes, amylase, lipase, trypsinogen, lipase + trypsinogen, or all three enzymes as covariates (as indicated on the graph), with age, sex, BMI percentile, and cohort 1 or 2 in our study included as additional covariates (*C*). A repeated k-fold cross-validation was performed (k = 10), and the AUROC was calculated for each model (as indicated on the graph).

Trypsinogen, lipase, and amylase levels were within the normal range for most of the subjects in both cohorts. Interestingly however, for each of the three enzymes, serum levels were below the reference range in a significantly greater proportion of individuals with ≥2AAb, recent-onset type 1 diabetes, and established type 1 diabetes (considered together as stage 1–3 type 1 diabetes [26]) compared subjects with low risk for type 1 diabetes (defined as AAb^- control subjects, AAb^- FDR, and $1AAb^+$), yet with unknown clinical relevance. Given the overall small proportion of subjects with values below normal and the overlap between clinical groups, it is possible that the fold change over time in longitudinal measurements might be of even greater value in pretype 1 diabetes.

A PCA wherein PC1 correlated with all three exocrine pancreas enzymes, whereas PC2 correlated with amylase only, demonstrated significantly different PC1 but not PC2 values for subjects with stage 1–3 type 1 diabetes versus low-risk subjects, supporting the collective use of lipase and trypsinogen levels as an additional disease biomarker. Logistic regression modeling further demonstrated that serum levels of all three enzymes together provide the best performance (AUROC = 75.6%) for discriminating subjects as stage 1–3 type 1 diabetes versus low risk, but trypsinogen plus lipase (AUROC = 75.0%) performed nearly as well as the three enzymes combined. Importantly, a logistic regression model containing trypsinogen plus lipase (AUROC = 81.4%) was similarly able to differentiate $1AAb^+$ from $\geq 2AAb^+$ subjects, further supporting the need to longitudinally evaluate these two enzymes as a biomarker for disease staging and progression in pretype 1 diabetes.

While Fig. 2 based on ANCOVA models shows similar differences for trypsinogen and lipase across the six clinical groups, with trypsinogen and lipase being highly correlated, lipase alone has a lower correct classification for stage 1–3 type 1 diabetes (AUROC = 69.5%) but a high correct classification for $\geq 2AAb^+$ (AUROC = 81.1%) revealing some differences in the models. Indeed, lipase levels were more variable compared with trypsinogen, which could be a contributing factor. Although longitudinal studies are needed to further address the ability of pancreatic exocrine enzymes to predict disease stage and progression, our data are in line with a recent report from the Environmental Determinants of Islet Autoimmunity (ENDIA) study demonstrating that fecal elastase-1 levels decline longitudinally in at-risk children who eventually

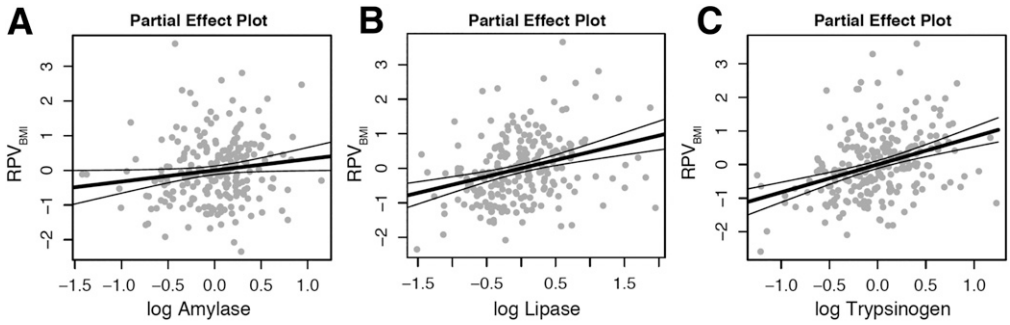

Figure 4—Partial effect plots for each single-enzyme linear regression model. The relationship with RPV_{BMI} is shown for amylase (*A*), lipase (*B*), and trypsinogen (*C*) after controlling for the other covariates in the models (age, sex, race, and levels of the other two enzymes). Amylase had the smallest effect (adjusted $R^2 = 0.12$, $P < 0.05$) compared with lipase (adjusted $R^2 = 0.18$, $P < 0.001$) and trypsinogen (adjusted $R^2 = 0.23$, $P < 0.001$).

develop islet autoimmunity and type 1 diabetes (39). Serological markers are likely of greater clinical utility given historical challenges surrounding patient compliance with fecal tests (40).

Finally, we explored whether amylase, lipase, and trypsinogen could serve as biomarkers of pancreas size. We previously reported that pancreas volume by MRI is reduced in subjects with pretype 1 diabetes (i.e., $\geq$2AAb$^+$), in those with recent-onset disease, as well as in 1AAb$^+$ and AAb$^-$ FDR subjects compared with unrelated AAb$^-$ control subjects (19). Here, we uncovered significant associations between RPV_{BMI} and serum levels of each enzyme individually, with the lipase and trypsinogen having the largest effect size, suggesting these two enzymes could together serve as a noninvasive biomarker of pancreas size, eliminating the need for MRI, which is expensive and time consuming. Cohort 2 did not include a sufficient number of subjects with established type 1 diabetes to assess RPV_{BMI} over disease duration. Hence, the association linking exocrine pancreas enzyme levels in serum with pancreatic volume is the subject of ongoing longitudinal studies.

In a recent report, we histologically evaluated the temporal expression of amylase, lipase, and trypsinogen in human organ donor pancreas tissue from subjects with type 1 diabetes and control subjects, noting the presence of peri-islet amylase-negative cell clusters, which stained positive for lipase and trypsinogen, scattered throughout the exocrine pancreas from control subjects older than 2 years of age (18). Interestingly, type 1 diabetes pancreas tissues contained fewer amylase-negative cell clusters compared with tissue from age- and sex-matched control subjects. Moreover, Wright et al. (17) recently reported that type 1 diabetes pancreata contain fewer acinar cells. Hence, we expect that alterations to the exocrine tissue architecture and/or function may underscore the serological findings presented herein; however, the pathogenic mechanisms potentially linking these observations—reduced pancreas mass/volume (9–11), increased exocrine pancreas inflammation (14–16), fewer acinar cells (17), fewer peri-islet amylase-negative cell clusters (18), and reduced serum levels of trypsinogen (20) and lipase during and throughout type 1 diabetes pathogenesis—remain to be elucidated.

Amylase and lipase levels can be elevated in up to 25% of patients with diabetic ketoacidosis (DKA) upon copresentation with acute pancreatitis (41); however, none of the individuals enrolled in our study were known to be in DKA at the time of the blood draw. Moreover, a recent study of children with recent-onset type 1 diabetes noted simultaneous presentation of pancreatitis to be rare, while pancreatic amylase values fell below normal range in 62% of their cohort (42), in agreement with our findings reported here. Interestingly, children enrolled in the Type 1 Diabetes Prediction and Prevention (DIPP) study in Finland showed lower levels of elastase, another pancreatic enzyme measured from stool samples, but only at onset of diabetes (43). Thus, combinations of pancreatic exocrine enzymes may provide a better biomarker, along with AAb, for diabetes progression. Decreased pancreatic enzyme levels have also been observed in subjects with type 2 diabetes and may be part of several pathological changes within the exocrine pancreas, including mild-to-marked interacinar fibrosis, minimal inflammatory infiltrates, a lack of ductal abnormalities, and arterial hyalinization (44). This is in contrast with fulminant type 1 diabetes, where serum amylase and lipase levels are typically elevated and bodyweight-normalized RPVs are comparable to control subjects (45,46). Hence, when evaluating amylase, lipase, and trypsinogen levels as a biomarker for type 1 diabetes and pancreatic organ volume, these values must be considered within the context of the full clinical picture.

Taken together, the data presented herein provide further documentation for pathophysiological perturbations affecting the exocrine portions of the pancreas in subjects with and at risk for developing type 1 diabetes, although it remains unclear whether these are a cause or effect of the disease pathogenesis. As a noninvasive biomarker of pancreas size and type 1 diabetes disease staging, we expect lipase and trypsinogen levels may improve our ability to predict or monitor type 1 diabetes progression in longitudinal studies of FDR of patients with

type 1 diabetes and individuals with stage 1–2 disease ($\geq$2AAb$^+$, with or without dysglycemia) using well-established clinical assays that could be easily incorporated into routine care. To fully address the utility of exocrine pancreas enzymes in improving precise disease staging will require longitudinal studies, ideally with comparisons against other established markers of type 1 diabetes risk, such as the Diabetes Prevention Trial-Type 1 Risk Score (DPTRS) (47) or Index60 (48). Finally, we anticipate that once validated in a longitudinal setting, serum levels of trypsinogen and lipase combined with other known and emerging markers of type 1 diabetes risk (e.g., islet AAb (26), genetic risk score [49–52], and IGFs [53]) may improve our ability to stage disease onset and potentially identify disease endotypes (54), ideally without the need for glucose tolerance tests or pancreatic MRI. The data reported here suggest amylase will be less informative. As such, efforts are currently ongoing to develop and refine combinatorial type 1 diabetes risk scores (55), which could have direct implications for informing clinical trial design (in terms of subject enrollment and end point criteria) and eventually influence a precision medicine approach to type 1 diabetes management in the clinical setting.

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