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Exocrine Proteins Including Trypsin(ogen) as a Key Biomarker in Type 1 Diabetes

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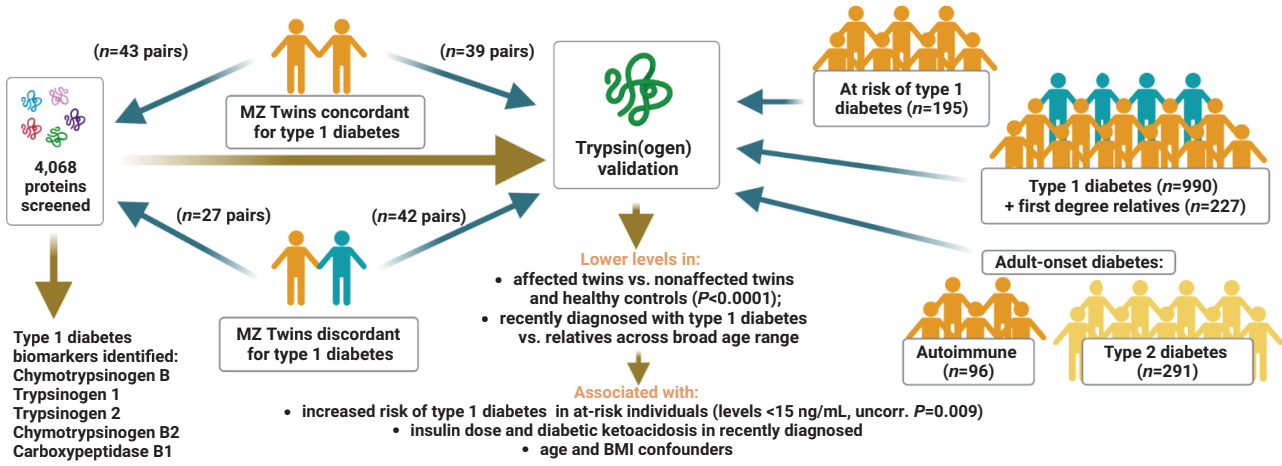
Exocrine proteins, including trypsin(ogen), as key biomarkers in type 1 diabetes. MZ, monozygotic; uncorr., uncorrected.



ARTICLE HIGHLIGHTS

- This study describes the first agnostic proteomics screen in well-characterized monozygotic twins discordant for type 1 diabetes.
- The results identify exocrine biomarkers as the top five hits.
- Validation of the proteomic data in >1,000 samples from well-characterized individuals with autoimmune diabetes show that trypsin(ogen) levels are decreased before, at, and after diagnosis of diabetes.

Exocrine Proteins Including Trypsin(ogen) as a Key Biomarker in Type 1 Diabetes

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OBJECTIVE

Proteomic profiling can identify useful biomarkers. Monozygotic (MZ) twins discordant for a condition represent an ideal test population. We aimed to investigate and validate proteomic profiling in twins with type 1 diabetes and in other well-characterized cohorts.

RESEARCH DESIGN AND METHODS

A broad, multiplex analysis of 4,068 proteins in serum samples from MZ twins concordant (*n* = 43) and discordant (*n* = 27) for type 1 diabetes identified major differences that were subsequently validated by a trypsin(ogen) assay in MZ pairs concordant (*n* = 39) and discordant (*n* = 42) for type 1 diabetes, individuals at risk for (*n* = 195) and with (*n* = 990) type 1 diabetes, as well as individuals with non–insulin-requiring adult-onset diabetes diagnosed as either autoimmune (*n* = 96) or type 2 (*n* = 291).

RESULTS

Proteomic analysis identified major differences between exocrine enzyme levels in discordant MZ twin pairs despite a strong correlation between twins, whether concordant or discordant for type 1 diabetes ($P < 0.01$ for both). In validation experiments, trypsin(ogen) levels were lower in twins with diabetes than in the co-twin without diabetes ($P < 0.0001$) and healthy control participants ($P < 0.0001$). In recently diagnosed participants, trypsin(ogen) levels were lower than in control participants across a broad age range. In at-risk relatives, levels <15 ng/mL were associated with an increased risk of progression (uncorrected $P = 0.009$). Multiple linear regression in recently diagnosed participants showed that trypsin(ogen) levels were associated with insulin dose and diabetic ketoacidosis, while age and BMI were confounders.

CONCLUSIONS

Type 1 diabetes is associated with altered exocrine function, even before onset. Twin data suggest roles for genetic and nongenetically determined factors. Exocrine/endocrine interactions are important underinvestigated factors in type 1 diabetes.

The natural history of type 1 diabetes is increasingly well understood, with approximately one-half of risk attributed to genetics and one-half to unidentified environmental factors (1). The classical approach to examining genetic risk is through monozygotic (MZ) and dizygotic twin studies, which help to disentangle the role of

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**A complete list of Action LADA Consortium and BOX Study Group members are provided in the supplementary material online.*

ORIGINAL ARTICLE

genetic susceptibility to complex conditions. Disease-discordant MZ twins are also ideal for hypothesis-free comparison of omics profiles, including proteomics.

Proteomic approaches have the advantage of capturing complex disease interactions at the cell and tissue level and, therefore, have the potential to identify sensitive and specific biomarkers of disease (2). The aim of this study was to investigate serum proteomics in well-characterized MZ twin pairs concordant and discordant for type 1 diabetes and to validate potential biomarkers using specific commercial assays in concordant and discordant twin pairs compared with healthy age- and sex-matched control participants. If validated in twins, subsequent analysis would expand to well-characterized cases of childhood and adult-onset type 1 diabetes at different disease stages compared with age- and sex-matched, nonaffected first-degree relatives (FDRs) as control participants and those with initially non–insulin-requiring adult-onset diabetes with diabetes-associated autoimmunity or without autoimmunity (presumed type 2 diabetes). Unexpectedly, our agnostic approach to identify proteomic biomarkers of type 1 diabetes highlighted the importance of markers of the exocrine rather than endocrine pancreas, with subsequent experiments confirming a key role for trypsin(ogen) as a predictive biomarker of type 1 diabetes, likely reflecting exocrine deficiency.

RESEARCH DESIGN AND METHODS

Study Populations

Descriptions of the populations examined in this study are included in the Supplementary Material. Characteristics of the clinical cohorts examined in the validation study are shown in Table 1.

Laboratory Procedures

SomaLogic Proteomic Profiling

Serum samples from 70 MZ twin pairs concordant (n = 43) and discordant (n = 27) for type 1 diabetes were analyzed on the SomaLogic Platform Version 4 based on synthetic slow off-rate–modified aptamers that bind to specific analytes to allow for noncompeting measurement of 4,068 human proteins in a multiplex assay (3). Briefly, samples were run at three different dilutions to ensure that each protein was analyzed on a linear portion of its concentration curve. For readout, SOMAmer reagents were hybridized on a chip and quantified by fluorescence.

Validation of Proteomic Results—Trypsin(ogen) Assay

Serum was used to detect serum trypsin(ogen) levels in 2 μL duplicates using the Delfia Neonatal IRT kit (PerkinElmer) according to the manufacturer's instructions and optimized in-house to detect levels <12.5 ng/mL. The assay measures a mixture of different forms of trypsin/trypsinogen. We therefore describe the outcome of these assays as trypsin(ogen) levels. Subsequent time-resolved fluorescence was read on a Victor 2 fluorometer using Wallac 3.0 software with Europium detection settings.

Trypsin(ogen) stability was evaluated in sera from After Diabetes Diagnosis Research Support System-2 (ADDRESS-2) participants (n = 60 serum samples, 120 aliquots). All serum samples were initially defrosted and stored at −20°C. One aliquot per participant was thawed only once; the other aliquot was freeze-thawed up to seven times.

Long-Standing British Diabetic Twin and Control Cohort

Trypsin(ogen) was subsequently measured in 39 pairs of twins concordant for type 1 diabetes (50% islet autoantibody positive [Aab^+]; 36 male; median age at sampling 27.6 years [range 5.5–53.7 years]; median time since diagnosis 13.7 years [range −8.6 to 38.1 years]). In addition, 42 discordant twin pairs were analyzed (median age at sampling 28.0 years [range 7.0–68.7 years]; median time since diagnosis 10.8 years [range 0.1–37.9 years]). Of these 84 individuals from discordant twin pairs, 30% were Aab^+ and 44 were male. Both twin cohorts were compared with 39 healthy control participants (random blood glucose <7.0 mmol/L at sampling; 100% Aab^-; 19 males; median age 29.7 years [range 13.0–65.0 years]).

Recent-Onset Type 1 Diabetes—ADDRESS-2

Trypsin(ogen) levels were investigated in ADDRESS-2 participants (n = 932) with recent-onset type 1 diabetes (85% Aab^+; 554 male; median age at sampling 21.8 years [range 5.1–66.4 years]; median time since diagnosis 0.2 years [range 0.0–0.9 years]). Sera from 169 siblings without type 1 diabetes were tested as control samples (100% Aab^-; 88 males; median age at sampling 15.6 years [range 5.1–58.5 years]). Data were available on ethnicity and clinical characteristics, including BMI, diabetic ketoacidosis (DKA), total insulin dose within 24 h of sampling, HbA_{1c}, and other autoimmune conditions.

At Risk—Bart's Oxford Study and European Nicotinamide Diabetes Intervention Trial

Serum trypsin(ogen) was measured in single and multiple Aab^+ FDRs at risk for developing type 1 diabetes (N = 195) from the Bart's Oxford (BOX) study (n = 82; 46 male; median age at sampling 19.6 years [range 5.5–69.3 years]; median follow-up 11.7 years [range 0.0–32.0]) and European Nicotinamide Diabetes Intervention Trial (ENDIT) (n = 113; 63 male; median age at sampling 12.7 years [range 3.5–44.3 years]; median follow-up 3.8 years [range 0.1–5.3 years]) cohorts.

Within and More Than Two Years Postdiagnosis—BOX Study Participants and Aab^- FDRs

Serum trypsin(ogen) levels were also measured in BOX study participants with type 1 diabetes within 2 years of diagnosis (n = 27; 96% Aab^+; 12 male; median age at sampling 10.7 years [range 2.1–37.1 years]; median time since diagnosis 0.9 years [range −0.1 to 1.7 years]) compared with participants with trypsin (ogen) measured >2 years postdiagnosis (n = 31; 87% Aab^+; 16 male; median age at sampling 16.6 years [range 6.4–23.9 years]; median time since diagnosis 5.2 years [range 3.0–14.9 years]). Both cohorts were appropriately age and sex matched to Aab^- FDRs.

Adult-Onset Diabetes: Autoimmune Diabetes and Type 2 Diabetes

Serum trypsin(ogen) was evaluated in 96 participants with autoimmune diabetes of adults (ADA) (48 male; median age at sampling 53.4 years [range 31.3–70.5 years]; median age at diagnosis 51.8 years [range 30.0–68.3 years]; median duration of diabetes 1.0 years [range −2.2 to 5.0 years]), and all were Aab^+, including 18 who were multiple Aab^+. Serum samples from 291 patients with type 2 diabetes were also analyzed (175 male; median age at sampling 51.5 years [range 48.0–54.0 years]; age at diagnosis 49.3 years [range 42.3–56.8 years]; median time since diagnosis 1.9 years [range −1.1 to 6.2 years]).

Table 1—Characteristics of cohorts used to validate trypsin(ogen) as a biomarker in type 1 diabetes

	Twin study			ADDRESS-2		ENDIT	BOX					Action LADA	
								Within 2 years of diagnosis		>2 years after diagnosis			
Study subset	Concordant	Discordant	Control	Case	Control	At risk	At risk	Case	Control	Case	Control	ADA	Type 2 diabetes
Participants, *n*	78	84	39	932	169	113	82	27	27	31	31	96	291
Sex, *n*													
Male	36	44	19	554	88	63	46	12	12	16	16	48	175
Female	42	40	20	378	81	50	36	15	15	15	15	48	116
Participants with diabetes, *n* (%)	78 (100)	42 (50)	0 (0)	932 (100)	0 (0)	56 (50)	41 (51)	27 (100)	NA	31 (100)	0 (0)	96 (100)	291 (100)
Caucasian, *n* (%)	78 (100)	84 (100)	NA	872 (94)	159 (94)	NA	NA	NA	NA	NA	NA	89 (93)	269 (92)
Age >16 years at sampling, *n* (%)	56 (72)	58 (70)	35 (90)	604 (65)	82 (49)	38 (34)	50 (61)	6 (22)	6 (22)	18 (58)	18 (58)	96 (100)	291 (100)
Age at sampling, years													
Median	27.6	28.0	29.7	21.8	15.6	12.7	19.6	10.7	11.0	16.6	16.2	53.4	51.5
Range	5.5 to 53.7	7.0 to 68.7	13.0 to 65.0	5.1 to 66.4	5.1 to 58.5	3.5 to 44.3	5.5 to 69.3	2.1 to 37.1	2.5 to 37.0	6.4 to 23.9	5.8 to 24.0	31.3 to 70.5	48.0 to 54.0
Time since diagnosis, years													
Median	13.7	10.8	NA	0.2	NA	1.54	8.3	0.9	NA	5.2	NA	1.0	1.9
Range	−8.6 to 38.1	0.1 to 37.9	NA	0.0 to 0.9	NA	0.1 to 5.3	0.5 to 25.1	−0.1 to 1.7	NA	3.0 to 14.9	NA	−2.2 to 5.0	−1.1 to 6.2
Aab$^+$, *n* (%)	39 (50)	25 (30)	0 (0)	790 (85)	0 (0)	113 (100)	82 (100)	26 (96)	0 (0)	27 (87)	0 (0)	96 (100)	18 (6)
IRT, ng/mL													
Median	6.6	8.9	13.1	11.4	13.9	13.0	18.2	12.2	20.9	13.5	23.7	15.4	15.4
Range	2.0 to 24.5	0.0 to 33.9	0.0 to 46.1	2.1 to 123.8	5.6 to 101.5	4.9 to 58.3	7.3 to 77.8	7.5 to 29.9	7.9 to 44.9	5.6 to 29.7	10.1 to 46.6	3.9 to 78.5	2.6 to 104.8
BMI, kg/m^2													
Median	22.4	23.1	22.5	22.1	21.2	NA	NA	NA	NA	NA	NA	29.1	30.4
Range	13.4 to 36.8	13.2 to 42.3	16.8 to 37.3	8.3 to 52.2	12.1 to 39.9	NA	NA	NA	NA	NA	NA	18.5 to 59.5	19.8 to 59.4
Follow-up, years													
Median	NA	NA	NA	NA	NA	3.8	11.7	NA	NA	NA	NA	NA	NA
Range	NA	NA	NA	NA	NA	0.1 to 5.3	0.0 to 32.0	NA	NA	NA	NA	NA	NA

IRT, immunoreactive trypsin(ogen); NA, not available.

Statistical Analysis

Proteomic Analysis

A paired *t* test with Bonferroni correction was used to test whether proteomic levels were the same between participants with type 1 diabetes and their discordant twins and between twins concordant for type 1 diabetes. For all cohorts, a Shapiro-Wilk normality test was used to assess distribution.

Trypsin(ogen) Stability, Agreement, and Levels Between Twin Pairs and Participants Before and After Diagnosis

A Wilcoxon matched pairs test was used to test whether single and multiple freeze-thaw cycles affected trypsin(ogen) levels, trypsin(ogen) levels were the same between twin pairs, and if trypsin(ogen) levels were the same in BOX study participants within 2 years of diagnosis and 2 years after diagnosis and in appropriate control groups. Bland-Altman analysis was used to assess the limits of agreement between trypsin(ogen) levels in serum aliquots thawed once versus multiple freeze-thaw cycles.

Trypsin(ogen) Levels Between Groups

A Mann-Whitney *U* test was used to determine whether trypsin(ogen) varied between twins without diabetes and control participants, between twins with diabetes and control participants, and between each age-group of ADDRESS-2 participants and siblings.

Relationship Between Trypsin(ogen) and Clinical Characteristics

Pearson and Spearman rank correlation tests were used to examine the relationship between trypsin(ogen) levels and age at sampling, age at diagnosis, time since diagnosis, and BMI in twin pairs and control participants and in at-risk FDRs, patients with type 1 diabetes, FDRs without diabetes, and participants with ADA and type 2 diabetes. All univariate analyses were performed using GraphPad Prism 6.0 software.

A nested linear mixed model was used to compare trypsin(ogen) levels between participants (recent onset) and related siblings from ADDRESS-2, adjusted for sex, ethnicity, age at sampling, and BMI. In participants, simple linear regression was used to analyze the relationship between trypsin(ogen) levels and sex, ethnicity, age at sampling, BMI, siblings with type 1 diabetes, DKA, total insulin dose in a 24-h period closest to sampling, HbA_{1c} closest to the sampling date, islet Aab (GAD antibody [GADA], IA-2 antigen [IA-2A], zinc transporter 8 autoantibody) levels, and Aab positivity and levels associated with other autoimmune diseases, including celiac disease and hypothyroidism. Four multivariable linear regression models were considered using SPSS version 27 software. These models included variables not related to diabetes (model A), diabetes-related covariates only (model B), and variables from models A and B combined (model C). Backward stepwise reduction was performed using standardized β-slopes; *P* values and clinical judgment were used to define the most clinically relevant and statistically justified model (model D). In all models, trypsin(ogen) data were $\log_2$-transformed to account for a positively skewed distribution. Assumptions of the models were checked using variance inflation factor tolerance and multicollinearity diagnostics, plotting of predicted and standardized residuals, and visual checking of partial regression plots for each variable of interest. Overall model fit was assessed using R^2.

Trypsin(ogen) Level and Risk of Progression to Type 1 Diabetes

The effect of trypsin(ogen) level on progression to diabetes in at-risk FDRs was investigated using a cumulative hazard plot, Mantel-Cox test, and Cox regression with SPSS version 27 software.

Data and Resource Availability

The study protocol and statistical analysis plan for this project are available on request from the corresponding authors. Deidentified individual participant data that underlie the results reported in this article will be made available.

RESULTS

Proteomic Profiling

There was a strong correlation for most analytes in MZ twin pairs either discordant (n = 27 pairs) or concordant (n = 43 pairs) for type 1 diabetes. Some 630 metabolites were identified with a false discovery rate $P < 0.05$. After Bonferroni correction, eight analytes were significantly different between participants with type 1 diabetes and their discordant co-twin as well as matched control participants. Of these, the top five were related to pancreatic exocrine function (Supplementary Table 1). In order of significance, the five proteins were chymotrypsinogen B ($P = 2.01^{E-09}$), trypsin(ogen) 1 ($P = 1.68^{E-8}$), trypsin(ogen) 2 ($P = 1.91^{E-7}$), chymotrypsinogen B2 ($P = 3.67^{E-07}$), and carboxypeptidase B1 ($P = 1.38^{E-06}$) (Table 2). Serum trypsin(ogen) 1 was then validated in well-characterized cohorts.

Validation of the Trypsin(ogen) Proteomic Data Using an Adapted Commercial Assay

Trypsin(ogen) Was Stable for Up to Seven Freeze-Thaw Cycles

Trypsin(ogen) was chosen for validation because an assay was already established in our laboratory that required only 4 μL of serum per individual. Trypsinogen levels in all cohorts and control participants are shown in Supplementary

Table 2—SomaLogic proteomic profiling

SOMAmer reagent	*P*	$\log_2$ change	False discovery rate *P*	Bonferroni-corrected *P*	Rank	Target
CTRB.5671.1.3.	2.01^{E-09}	−0.122	8.17^{E-08}	8.17^{E-06}	1	Chymotrypsinogen B
PRSS1.3049.61.2	1.68^{E-08}	−0.104	3.42^{E-05}	6.84^{E-05}	2	Trypsin(ogen) 1
PRSS2.5034.79.1	1.91^{E-07}	−0.104	2.58^{E-04}	7.75^{E-04}	3	Trypsin(ogen) 2
CTRB2.5648.28.3	3.67^{E-07}	−0.135	3.73^{E-04}	1.49^{E-03}	4	Chymotrypsinogen B2
CPB1.6356.3.3	1.38^{E-06}	−0.08	1.12^{E-03}	5.59^{E-03}	5	Carboxypeptidase B1

Shown are results from paired *t* tests comparing MZ twins discordant for type 1 diabetes (n = 43 pairs). Of 4,068 human proteins, the top five differences in discordant MZ twins are shown.

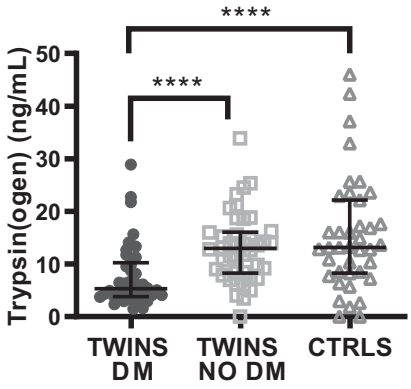

Figure 1—Trypsin(ogen) levels in twins discordant for type 1 diabetes (TWINS DM) (n = 42) were significantly reduced compared with twins without type 1 diabetes (TWINS NO DM) (n = 42) and age-matched healthy control participants (CTRLS) (n = 39). ****$P < 0.0001$ by Wilcoxon matched pair test and Mann-Whitney U test, respectively. The median trypsin(ogen) level was lower in TWINS NO DM compared with CTRLS, but not significantly (P = 0.5088 by Mann-Whitney U test). $P < 0.05$ was considered significant.

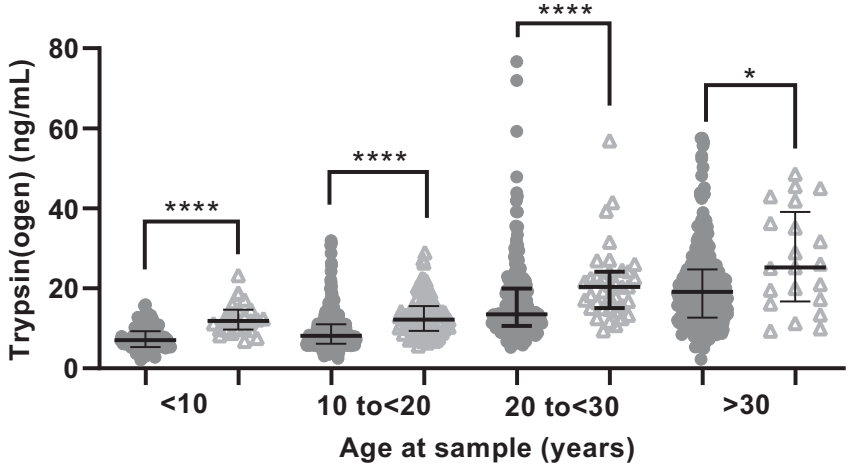

Figure 2—Trypsin(ogen) levels in individuals with type 1 diabetes (n = 932) (dark gray circles) and their siblings without diabetes (n = 169) (light gray triangles) grouped by age at sampling, tested close to diagnosis. $P < 0.05$ was considered significant. *P = 0.0158, ****$P < 0.0001$.

Fig. 1. Trypsin(ogen) was stable for up to seven rounds of freeze-thawing compared with serum replicates stored at −20°C and thawed only once (Wilcoxon P = 0.3817; Spearman r = 0.9463 [$P < 0.0001$]; bias 0.02452 [SD 2.55; 95% confidence limits of agreement −4.976, 5.025]) (Supplementary Fig. 2).

Trypsin(ogen) Levels Were Decreased in Twins With Type 1 Diabetes

In twins discordant for diabetes (n = 42 pairs), trypsin(ogen) was reduced in twins with type 1 diabetes compared with their disconcordant co-twin and age-matched healthy control participants (n = 39, both $P < 0.0001$), while there was no difference between twins without type 1 diabetes and control participants (P = 0.5088) (Fig. 1). Trypsin(ogen) levels in concordant twins were similar compared with discordant twins (P = 0.5222), and the difference was significant between concordant twins and control participants ($P < 0.0001$). There was a positive correlation between MZ twins, both those concordant and discordant for the disease (r = 0.43 [P = 0.0061] and 0.43 [P = 0.0045], respectively). In twins with type 1 diabetes from discordant pairs, trypsin(ogen) levels were related to age at diagnosis (r = 0.46, P = 0.0022) but not age at sampling. In the concordant twin pairs, selecting the second twin diagnosed from each pair, levels were also related to age at diagnosis (r = 0.473, P = 0.002), and trypsin(ogen) levels positively correlated with age at diagnosis in the same twins when diagnosed at age <30 years (r = 0.35, P = 0.030). In discordant twin pairs, trypsin(ogen) levels increased with age at sampling in the twins without diabetes (r = 0.35, P = 0.024) but not in their co-twin with diabetes (r = 0.25, P = 0.11).

Trypsin(ogen) Levels Were Decreased in Participants With Type 1 Diabetes Compared With Aab⁻ Age- and Sex-Matched FDRs

Trypsin(ogen) levels were measured in sera from BOX study participants within 2 years from diagnosis of type 1 diabetes, participants >2 years postdiagnosis, and age-matched Aab⁻ healthy control participants. In both groups, trypsin(ogen) was significantly lower than in age- and sex-matched islet Aab⁻ FDRs ($P < 0.0001$). No difference was observed between the two affected groups (P = 0.8406).

Age and BMI Were Major Determinants of Trypsin(ogen) Levels.

Trypsin(ogen) levels were lower in ADDRESS-2 participants up to the age of 30 years compared with age-matched Aab⁻ FDRs grouped by age at sampling (Fig. 2). Simple linear regression (Supplementary Fig. 3) showed an increase in trypsin(ogen) with age for both participants and FDRs.

Nested linear mixed model analysis within families from ADDRESS-2 (participants with recent-onset type 1 diabetes and their siblings [n = 112]) with two to four relatives per group revealed that trypsin(ogen) levels varied in different families (model equation constant $P < 0.001$), but the association with other variables did not differ between families. In all families, participants had significantly lower trypsin(ogen) levels than siblings. Age at sampling, BMI, height, and body weight were significantly associated with trypsin(ogen) levels ($P < 0.001$, P = 0.015, P = 0.001, and P = 0.010, respectively).

Four multiple linear regression models were used to analyze the data available in ADDRESS-2 participants with recent-onset type 1 diabetes. Model A (nondiabetes-related traits in participants [n = 931]) showed BMI (B = 0.006 [95% CI 0.003–0.009]; β = 0.111; $P < 0.0001$) and age at sampling (B = 0.011 [95% CI 0.010–0.012]; β = 0.550; $P < 0.0001$) were positively associated with trypsin(ogen) levels (Supplementary Table 2).

Model B (diabetes-related variables in participants [n = 752]) showed that trypsin(ogen) was most strongly negatively associated with time since diagnosis (B = −0.023 [95% CI −0.034 to −0.012]; β = −0.154; $P < 0.0001$). GADA (B <0.0001 [95% CI <0.0001 to <0.0001]; β = 0.163; $P < 0.0001$), total insulin dose 24 h closest to trypsin(ogen) sampling date (B = 0.002 [95% CI 0.001–0.003]; β = 0.132; $P < 0.0001$), and DKA (B = 0.061 [95% CI 0.025–0.097]; β = 0.132; P = 0.001) were positively associated with trypsin(ogen) levels in participants (Supplementary Table 2).

Model C (all available variables in participants [n = 917]) showed that trypsin

(ogen) was positively associated with age at sampling (B = 0.010 [95% CI 0.009–0.012]; β = 0.540; $P < 0.0001$), DKA (B = 0.054 [95% CI 0.025–0.084]; β = 0.103; $P < 0.0001$) and BMI (B = 0.005 [95% CI 0.001–0.009]; β = 0.097; $P = 0.006$), while time since diagnosis (B = −0.015 [95% CI −0.024 to −0.006]; β = −0.101; $P = 0.001$) showed the strongest negative association (Supplementary Table 2). Model D (after the model reduction) showed that the strongest associations of trypsin(ogen) were age at sampling (B = 0.010 [95% CI 0.009–0.011]; β = 0515; $P < 0.0001$), BMI (B = 0.006 [95% CI 0.003–0.009]; β = 0.113; $P < 0.0001$), and DKA (B = 0.058 [95% CI 0.031–0.085]; β = 0.110; $P < 0.0001$). Time since diagnosis (B = −0.015; [95% CI −0.022 to −0.007]; β = −0.098; $P < 0.0001$) and IA-2A (B <0.001 [95% CI <0.0001 to <0.0001]; β = −0.058; $P = 0.030$) showed the strongest negative association with trypsin(ogen) (Supplementary Table 2).

Low Trypsin(ogen) Levels Were Associated With a Modest Increased Risk of Progression to Type 1 Diabetes

The outcome of the Cox proportional hazard model is shown on Supplementary Table 3. Cumulative hazard analysis in the cohort ($n = 195$) of individuals at risk for developing type 1 diabetes from the ENDIT study ($n = 113$) and at-risk FDRs from the BOX study ($n = 82$) showed that trypsinogen levels <15 ng/mL were associated with an increased risk of progression ($P = 0.009$) (Fig. 3). When adjusted for sex, age at sampling, each islet Aab level, and multiple Aab status, trypsin (ogen) did not independently predict risk of progression to type 1 diabetes ($P = 0.138$).

Low Trypsin(ogen) Levels Were Not Associated With Non–insulin-Requiring Adult-Onset Diabetes

There was no difference in serum trypsin(ogen) levels between participants with ADA and type 2 diabetes, the former with ($n = 96$) and the latter without ($n = 291$) diabetes-associated Aabs (Supplementary Fig. 1). Even participants with ADA with multiple Aab^+ had similar trypsin(ogen) levels to participants with type 2 diabetes. As with control participants, in type 2 diabetes, trypsin(ogen) levels correlated with age at sampling ($r = 0.12$, $P = 0.05$). However, in participants with ADA, there was no association between age at sampling or age at diagnosis and trypsin(ogen) levels, irrespective of whether they had one or multiple Aab^+ or were diagnosed above or below the median age.

CONCLUSIONS

In MZ twins discordant for diabetes, SomaScan analysis for >4,000 proteins identified pancreatic exocrine enzymes, rather than the expected endocrine proteins, as major biomarkers associated with type 1 diabetes. Levels of all five pancreatic exocrine enzymes identified were lower in twins with type 1 diabetes than their co-twin without diabetes. This difference was subsequently validated for serum trypsin(ogen) using a separate assay optimized to detect lower levels of trypsin(ogen), which confirmed decreased serum trypsin(ogen) levels in individuals with recently diagnosed type 1 diabetes at a range of ages at diagnosis. Analysis of the large ADDRESS-2 cohort, in which samples were taken from participants close to diagnosis and from FDRs, suggested relationships between trypsin(ogen) and islet Aabs (GADA and IA-2A), as well as DKA, insulin dose, and time since diagnosis, but not sex or ethnicity.

The strength of this study is that we used an agnostic approach to identify a deficiency of pancreatic exocrine enzymes in MZ twin pairs discordant for type 1 diabetes. Additionally, we confirmed that trypsin(ogen) levels were robust in samples that were freeze-thawed in multiple cycles. Access to a series of large, well-described diabetes cohorts enabled us to examine in greater detail the relationship between low serum trypsin(ogen) and type 1 diabetes, including the ability of low levels to antedate the disease. Clinical data available in the ADDRESS-2 study allowed us to further examine covariates such as insulin dose, DKA, presence of other autoimmune diseases, and individual islet Aab levels in association with trypsin(ogen) levels, which was not possible in previous studies (4).

Weaknesses, however, include the variability in clinical data available on the different cohorts and that detailed multiple linear regression analysis could not be conducted on all data sets. Samples analyzed in this study were randomly collected, with an unknown prandial state; however, historical studies in healthy individuals have suggested that this should not have affected trypsin(ogen) levels (5,6). Our cohorts included mostly Caucasians; therefore, the association between trypsin(ogen) and ethnicity should be further investigated in a more diverse data set.

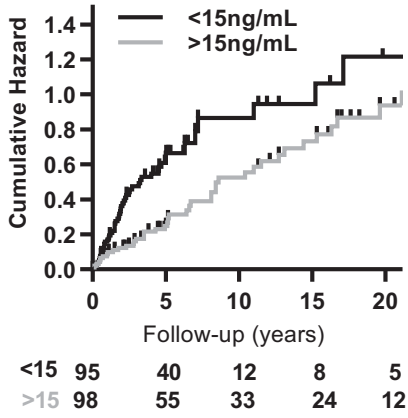

Figure 3—Hazard function plot for follow-up to development of type 1 diabetes in FDRs at risk with confirmed single or multiple islet Aabs ($n = 195$), including the ENDIT cohort ($n = 113$) and BOX cohort ($n = 82$). Trypsin(ogen) levels <15 ng/mL were associated with an increased risk of progression to type 1 diabetes ($P = 0.009$). Data were truncated at 20 years follow-up because of the small number of participants remaining. The *x*-axis shows follow-up (years), and below the *x*-axis, the number of participants remaining in the study with trypsin(ogen) levels <15 ng/mL and >15 ng/mL is shown.

Our results extend intriguing evidence from other studies that type 1 diabetes, considered a disease of the endocrine pancreas, is actually a disease of both the exocrine and endocrine pancreas and that changes occur before diagnosis (7–9). We found that age at sampling and BMI are major determinants of trypsin(ogen) levels in individuals with newly established type 1 diabetes. This finding echoes those of studies by Li et al. (8), Ross et al., (7) and an earlier study by Saisho et al. (10) using computed tomography scans in individuals with type 2 diabetes and control participants, demonstrating a steady increase in pancreatic size up to the age of ∼30 years.

Trypsin(ogen) levels in twins with long-standing type 1 diabetes were positively correlated with age at diagnosis but not with disease duration or age at sampling. In contrast, their co-twin without diabetes showed increasing levels, which were correlated with age, as was the case with control participants. By implication, the normal increase in serum trypsin(ogen)

with age is blunted in patients with type 1 diabetes over time, which could explain the reported widening difference with age in serum trypsin(ogen) between individuals with and without type 1 diabetes (8). In our population-based cohort with recently diagnosed type 1 diabetes, trypsin(ogen) levels were consistently lower than age-matched control participants across a broad age range up to diagnosis at age 30 years. In participants diagnosed at older ages, the relationship was less clear both in classic type 1 diabetes and in initially non–insulin-requiring autoimmune diabetes, even when participants with the latter had two diabetes-associated Aabs. Larger numbers of participants with adult-onset type 1 diabetes and type 2 diabetes and matched control participants are required for future studies.

Decreased trypsin(ogen) levels in type 1 diabetes appear to reflect decreasing pancreatic function/mass, suggesting that the level of serum trypsin(ogen) is a robust biomarker of pancreatic atrophy and reflects pancreas exocrine function, although the underlying pathogenesis is not understood (11–14). The correlation for trypsin(ogen) in MZ twins, even when discordant for type 1 diabetes, implies that shared gene-environment factors are important in determining the trypsin(ogen) levels. Genes associated with pancreatic disease have been implicated in type 1 diabetes (15,16). High-probability type 1 diabetes risk variants map to novel exocrine-specific *cis*-response elements, including in *CFTR* where the potential causal variant rs7795896 localizes to a ductal-specific distal *cis*-response element, reducing transcription factor binding, enhancer activity, and *CFTR* expression in ductal cells (15) and, thereby, potentially promoting inflammation and immune effector cell infiltration. Additionally, in a recent Mendelian randomization study, increased serum expression of chymotrypsinogen isoenzyme (*CTBR1*) was associated with a decreased risk of type 1 diabetes (16). Campbell-Thompson et al. (17) showed that pancreas volume is reduced in FDRs of patients with type 1 diabetes, highlighting the importance of genetics in determining pancreatic mass. In our study, however, trypsin(ogen) levels were not significantly lower in the twins without diabetes compared with age-matched control participants, also emphasizing the importance of nongenetic effects.

The residual difference in trypsin(ogen) level in discordant MZ twins did not increase with disease duration, and the difference in trypsin(ogen) level is evident at diagnosis, implying that it is unlikely to be secondary to either clinical type 1 diabetes or insulin therapy. In line with this finding, we found that trypsin(ogen) levels $<$15 ng/mL in participants without diabetes at risk for type 1 diabetes were associated with a modest increase in risk of progression to type 1 diabetes, although this effect diminished after adjusting for age, sex, and islet Aab status. A larger analysis in at-risk individuals is warranted. Studies in the Environmental Determinants of Islet Autoimmunity (ENDIA) cohort of fecal elastase showed that levels decreased over time in progressors compared with nonprogressors (18).

Pancreas size is already reduced by 25% at diagnosis, even in patients with type 1 diabetes at a very young age (12,19). Decreased pancreatic mass in long-standing type 1 diabetes implies a causative role for lack of the trophic effects of insulin (8,12,18,19). A recent study showed that diabetic acinar cells were similar in size but fewer in number compared with those in pancreata from donors without diabetes, accounting for the difference in pancreas size (8). The exocrine pancreas was thought to be functionally homogeneous, but studies focusing on acinar cells by immunohistochemistry identified amylase-negative clusters in control tissues, which were positive for trypsinogen (20). However, the patchy amylase-negative clusters were observed in paraffin-embedded tissue (20) and not replicated in frozen tissue (21). Furthermore, in type 1 diabetes, there is evidence of both innate and adaptive immune cell infiltration of exocrine tissue, with the majority of T cells found as peri-insulitis rather than within islets (22). It follows that genetic effects might predispose to low-grade inflammation promoting peri-insulitis and, thereby, initiating an autoimmune process. Reduction in acinar cell trypsin(ogen) production could be secondary to reduced acinar trophic effects either by insulin or immune-associated factors.

The results presented here raise a query about whether individuals with long-standing type 1 diabetes develop clinically relevant pancreatic exocrine insufficiency (PEI). In 2015, Piciucchi et al. (23) reported that PEI prevalence in type 1 diabetes is estimated to reach ~25–74%. PEI is associated with high insulin requirement, poor glycemic control, and long-standing diabetes (24). However, PEI is understudied in patients with type 1 diabetes because it is not routinely measured (25). Where it is studied, data are often cross sectional, and given the effects of age and BMI, longitudinal studies are warranted. Trypsin(ogen) measurement can be used among other noninvasive tests, such as fecal elastase, serum amylase, and lipase, to detect PEI. The fecal elastase test is most commonly used to detect PEI; therefore, the data on trypsin(ogen) levels in mild and severe PEI are scarce. Capurso et al. (24) referred to trypsinogen levels as being highly sensitive for advanced PEI ($<$20 ng/mL) and having low sensitivity in mild PEI (trypsin[ogen] levels between 20 and 29 ng/mL). We have observed some interassay variability in measuring trypsin(ogen); therefore, interlaboratory variability is also likely. These levels should thus be treated with some caution.

In conclusion, we present the first proteomic analysis in a cohort of well-characterized twins showing exocrine proteins as important biomarkers for type 1 diabetes, with evidence for both genetic and nongenetic determinants. Subsequent validation studies in large well-characterized patient, at-risk, and control populations confirmed the importance of serum trypsin(ogen) before and after onset of type 1 diabetes. Moving forward, measures of pancreatic mass should be included in prediction and progression studies.

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Duality of Interest. SomaLogic performed the SomaScan assays at its own cost. No other potential conflicts of interest relevant to this article were reported.

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