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`Exocrine proteins including Trypsin(ogen) as a key biomarker in type 1 diabetes

Running title: Proteomics, type 1 diabetes and the exocrine pancreas

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*Twitter handle:* Proteomic analysis in identical twins discordant for type 1 diabetes highlights the importance of exocrine pancreas biomarkers

### *Abstract*

#### *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 characterised cohorts.

#### *Research Design and Methods*

A broad, multiplex analysis of 4068 proteins in sera from MZ twins concordant (n=43) and discordant for type 1 diabetes (n=27) identified major differences which 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 (n=195) and with type 1 diabetes (n=990), as well as 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 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 compared with non-diabetic co-twins ( $p < 0.0001$ ) and healthy controls ( $p < 0.0001$ ). In recently-diagnosed cases, trypsin(ogen) levels were lower than in controls across a broad age range. In at-risk relatives, levels  $< 15$  ng/ml were associated with increased risk of progression (uncorr.  $p = 0.009$ ). Multiple linear regression in recently-diagnosed cases 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 non-genetically determined factors. Exocrine/endocrine interactions are important under-investigated factors in type 1 diabetes.

*Article highlights:* This study describes

- the first agnostic proteomics screen in well characterised monozygotic twins discordant for type 1 diabetes

- results identify exocrine biomarkers as the top five “hits”
- validation of the proteomic data in >1000 samples from well characterised individuals with autoimmune diabetes showing that trypsin(ogen) levels are decreased before, at and after diagnosis of diabetes

### *Introduction*

The natural history of type 1 diabetes is increasingly well understood with approximately half of risk attributed to genetics and half to unidentified environmental factors (1). The classical approach to examining genetic risk is through monozygotic and dizygotic twin studies which help disentangle the role of genetic susceptibility to complex conditions. Disease-discordant identical twins are also ideal cohorts 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-characterised monozygotic (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-gender-matched controls. If validated in twins, subsequent analysis would expand to well characterised cases of childhood and adult-onset type 1 diabetes at different disease stages, compared with age-and-gender-matched, non-affected first-degree relatives (FDR) as well as participants with initially non-insulin requiring adult-onset diabetes with diabetes-associated autoimmunity (ADA) 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 Supplementary data. Characteristics of 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 for type 1 diabetes (n=27) were analysed on a SomaLogic Platform Version 4, based on synthetic Slow Off-Rate Modified Aptamers that bind to specific analytes allowing for non-competing measurement of 4068 human proteins in a multiplex assay (3).

Briefly, samples were run at three different dilutions to ensure that each protein was analysed 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 2µl duplicates using the Delfia Neonatal IRT kit following the manufacturer's instructions (PerkinElmer) and optimised in house to detect levels <12.5ng/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 fluoro-meter using Wallac 3.0 software with Europium detection settings.

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

#### Longstanding British Diabetic Twin and Control cohort

Trypsin(ogen) was subsequently measured in 39 pairs of twins concordant for type 1 diabetes [50% were islet autoantibody positive (Aab+ve), 35 male; median age-at-sample 27.6 years (range: 5.5-53.7); median time since diagnosis 13.7 years (range: -8.6 – 38.1)]. In addition, 42 discordant twin pairs were analysed [median age-at-sample [28.0 years (range 7.0-68.7), median time from diagnosis: 10.8 years (range 0.1-37.9)]. Of these 84 individuals from discordant twin pairs, 30% were Aab+ve and 44 were male. Both twin cohorts were compared with 39 healthy control subjects [random blood glucose <7.0 mM at sampling, 100% were islet-autoantibody negative (Aab-ve), 19 males; median age 29.7 years (range 13.0-65.0)].

#### 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% were Aab+ve and 554 were male [median age-at-sample 21.8 years (range 5.1-66.4); median time from diagnosis 0.2 years (range 0.0-0.9)]. Sera from 169 non-affected siblings were tested as controls [100% Aab-ve, 88 males; median age-at-sample 15.6 years (range 5.1-58.5)]. Data were available on ethnicity and clinical characteristics including BMI, diabetic ketoacidosis (DKA), total insulin dose within 24 hours of sampling, HbA1c and other autoimmune conditions.

#### At risk – BOX and ENDIT

Serum trypsin(ogen) was measured in single and multiple islet autoantibody positive FDR at-risk of developing type 1 diabetes (n=195 in total) from BOX [n=82; 46 male; age-at-sample median 19.6 years (range 5.5 to 69.3); median follow-up 11.7 years (range 0.0 to 32.0)] and ENDIT [n=113; 63 male; age-at-sample 12.7 years (range 3.5 to 44.3), median follow-up 3.8 years (range 0.1-5.3)] cohorts.

#### Within and >2 years post-diagnosis - BOX Study cases and Aab- FDR

Serum trypsin(ogen) levels were also measured in BOX study cases with type 1 diabetes within 2 years of diagnosis [n=27, 96% Aab+ve, 12 male; age-at-sample 10.7 years (range 2.1 to 37.1); time since diagnosis 0.9 years (range -0.1 to 1.7) compared with participants with trypsin(ogen) measured more than two years post-diagnosis including 87% who were Aab+ve and 16 were male [n=31; age-at-sample



16.6 years (range 6.4 to 23.9); time since diagnosis 5.2 years (range 3.0 to 14.9).

Both cohorts were appropriately age-and-gender matched to Aab-ve FDRs.

Adult-onset Diabetes: autoimmune diabetes (ADA) and type 2 diabetes

Serum trypsin(ogen) was evaluated in 96 participants diagnosed with adult-onset autoimmune diabetes (ADA) [48 male; median age-at-sample 53.4 years (range 31.3 to 70.5); median age at diagnosis 51.8 years (range 30.0 to 68.3); median duration of diabetes 1.0 years (range -2.2 to 5.0)], all were Aab+ve, including 18 multiple Aab+ve. Sera from 291 patients with type 2 diabetes were measured [175 male; median age-at-sample 51.5 years (range 48.0 to 54.0); age at diagnosis 49.3 years (range 42.3 – 56.8); median time since diagnosis 1.9 years (range -1.1 – 6.2 years)].

### *Statistical analysis*

### *Proteomic analysis*

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

### *Trypsinogen stability, agreement and levels between twin pairs and cases before and after diagnosis*

The Wilcoxon matched-pairs test was used to test if single and multiple freeze-thaw cycles affected trypsin(ogen) levels; if trypsin(ogen) levels were the same between

twin pairs; if trypsin(ogen) levels were the same in BOX participants within 2 years of diagnosis and 2 years after diagnosis and in appropriate control groups. A 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.

#### *Trypsinogen levels between groups*

The Mann-Whitney U-test was used to determine whether trypsin(ogen) varied between non-diabetic twins and controls; between diabetic twins and controls and between each age group of ADDRESS-2 cases and siblings.

#### *Relationship between Trypsinogen and clinical characteristics*

Pearson's and Spearman's rank correlation tests were used to examine the relationship between trypsin(ogen) levels and age-at-sample, age at diagnosis, time since diagnosis and BMI in twin pairs and controls, and in at-risk FDRs, patients with type 1 diabetes, non-affected FDRs, ADA and subjects with type 2 diabetes. All univariate analysis were performed using PRISM (v6.0).

A nested linear mixed model was used to compare trypsin(ogen) levels between cases (recent-onset) and related siblings from ADDRESS-2 adjusted for sex, ethnicity, age-at-sample and BMI. In cases, simple linear regression was used to analyse the relationship between trypsinogen levels and sex, ethnicity, age-at-sample, BMI, siblings with type 1 diabetes, diabetic ketoacidosis (DKA), total insulin dose in a 24-hour period closest to sample, HbA1c closest to sampling date, islet autoantibody (GADA, IA2A, ZnT8A) levels; and autoantibody positivity and levels associated with other autoimmune diseases including celiac disease and hypothyroidism. Four multivariable linear regression models were considered using

SPSS (v27): variables not-related to diabetes (Model A); diabetes-related covariates only (Model B); variables from models A and B combined (Model C). Backward stepwise reduction was performed using standardised beta slopes, p-values, and clinical judgement was used to define the most clinically relevant and statistically justified model (Model D). In all models, trypsin(ogen) data were log-transformed ( $\log_2$ ) to account for a positively skewed distribution. Assumptions of the models were checked using VIF 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  $R^2$ .

#### *Trypsinogen level and risk of progression to type 1 diabetes*

The effect of trypsin(ogen) level on progression to diabetes in at-risk FDR was investigated using a cumulative hazard plot, Mantel-Cox test and Cox regression (SPSS v27).

## *Results*

### *Proteomic profiling*

There was a strong correlation for most analytes between MZ twin pairs either discordant (n=27 pairs) or concordant (n=43 pairs) for T1D. Some 630 metabolites were identified with a false discovery rate  $p < 0.05$ . After Bonferroni correction, eight analytes were significantly different between affected and non-affected discordant co-twins, as well as matched controls. Of these, the top 5 related to pancreatic exocrine function (Supplementary Table 1). In order of significance, the five proteins were chymotrypsinogen B ( $p = 2.01 \times 10^{-9}$ ), followed by 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-characterised cohorts.

#### *Validation of the trypsin(ogen) proteomic data using an adapted commercial assay*

Trypsin(ogen) was stable for up to 7 freezing/thawing cycles.

Trypsin(ogen) was chosen for validation because an assay was already established in our laboratory which required only 4 $\mu$ l of serum per individual. Trypsinogen levels in all cohorts and controls are shown in Supplementary Figure 1.

Trypsin(ogen) was stable for up to seven rounds of freeze/thawing, when compared with serum replicates stored at -20°C and thawed only-once [ $p=0.3817$ ;  $R=0.9463$ ,  $p<0.0001$ ; 0.02452, SD of bias 2.55 (from -4.976 to 5.025)] Supplementary Figure 2.

Trypsin(ogen) levels are decreased in twins with type 1 diabetes.

In twins discordant for diabetes ( $n=42$  pairs), trypsin(ogen) was reduced in affected compared with non-affected twins and with age-matched healthy controls ( $n=39$ ), ( $p<0.0001$ ;  $p<0.0001$  respectively), while there was no difference between non-affected twins and controls ( $p=0.5088$ ) (Figure 1). Trypsin(ogen) levels in concordant twins were similar when compared to affected discordant twins ( $p=0.5222$ ), the difference was significant when concordant twins were compared to controls ( $p<0.0001$ ). There was a positive correlation between MZ twins, both those

concordant and discordant for the disease ( $r=0.43$ ,  $p=0.0061$  and  $r=0.43$ ,  $p=0.0045$  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 affected twins when diagnosed less than age 30 years ( $r=0.35$ ,  $p=0.030$ ). In discordant twin pairs, trypsin(ogen) levels increased with age at sampling in the non-diabetic co-twins ( $r=0.35$ ,  $p=0.024$ ), but not in their affected twins ( $r=0.25$ ,  $p=0.11$ ).

Trypsin(ogen) levels are decreased in type 1 diabetes cases when compared with islet antibody negative age and gender matched FDRs

Trypsin(ogen) levels were measured in sera from BOX study participants within 2 years from diagnosis of type 1 diabetes, cases >2 years post-diagnosis and in sera of age-matched autoantibody negative healthy controls. In both groups, trypsin(ogen) was significantly lower than in age and gender-matched islet antibody negative 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 cases up to the age of 30 years, when compared with age-matched Aab-ve FDR, grouped by age-at-sample (Figure

2). Simple linear regression (Supplementary Figure 3) showed an increase in trypsin(ogen) with age for both cases and FDR.

Nested linear mixed model analysis within families from ADDRESS-2 (recent-onset type 1 diabetes cases and 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, cases had significantly lower trypsin(ogen) than siblings. Age-at-sample, BMI, height and body weight were significantly associated with trypsin(ogen) levels ( $p < 0.001$ ,  $p = 0.015$ ,  $p = 0.001$ ,  $p = 0.010$ , respectively).

Four multiple linear regression models were used to analyse the data available in ADDRESS-2 recent-onset cases.

Model A (non-diabetes related traits in cases, n=931) showed BMI ( $B = 0.006$ , 95% CI from 0.003 to 0.009;  $\beta = 0.111$   $p < 0.0001$ ) and age-at-sample ( $B = 0.011$ ; 95% CI from 0.010 to 0.012;  $\beta = 0.550$ ;  $p < 0.0001$ ) were positively associated with trypsin(ogen) levels (Supplementary Table 2).

Model B (diabetes-related variables in cases, n=752) showed that trypsin(ogen) was most strongly negatively associated with time from diagnosis ( $B = -0.023$ , 95% CI from -0.034 to -0.012;  $\beta = -0.154$ ;  $p < 0.0001$ ). GADA ( $B < 0.0001$ , CI from  $< 0.0001$  to  $< 0.0001$ ;  $\beta = 0.163$ ;  $p < 0.0001$ ), total insulin dose 24 hrs closest to trypsin(ogen) sample date ( $B = 0.002$ , CI from 0.001 to 0.003;  $\beta = 0.132$ ;  $p < 0.0001$ ) and DKA ( $B = 0.061$ , CI from 0.025 to 0.097;  $\beta = 0.132$ ;  $p = 0.001$ ) were positively associated with trypsin(ogen) levels in cases (Supplementary Table 2).

Model C (all available variables in cases, n=917) showed that trypsin(ogen) was positively associated with age-at-sample (B=0.010, CI from 0.009 to 0.012;  $\beta=0.540$ ;  $p<0.0001$ ), DKA (B=0.054, CI from 0.025 to 0.084;  $\beta=0.103$ ;  $p<0.0001$ ) and BMI (B=0.005, CI from 0.001 to 0.009;  $\beta=0.097$ ;  $p=0.006$ ); while time-from-diagnosis (B=-0.015, CI from -0.024 to -0.006;  $\beta=-0.101$ ;  $p=0.001$ ) showed the strongest negative association (Supplementary Table 2).

Model D (after the model reduction) showed that the strongest association of trypsin(ogen) were with age-at-sample (B=0.010, CI from 0.009 to 0.011;  $\beta=0.515$ ;  $p<0.0001$ ), BMI (B=0.006, CI from 0.003 to 0.009;  $\beta=0.113$ ;  $p<0.0001$ ) and DKA (B=0.058, CI from 0.031 to 0.085;  $\beta=0.110$ ;  $p<0.0001$ ). Time from diagnosis (B=-0.015, CI from -0.022 to -0.007;  $\beta=-0.098$ ;  $p<0.0001$ ) and IA-2A (B<0.001, CI from <0.0001 to <0.0001;  $\beta=-0.058$ ;  $p=0.030$ ) 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 of developing T1D from the ENDIT study (n=113) and “at risk” FDR from the BOX study (n=82) showed that trypsinogen levels lower than 15ng/ml are associated with an increased risk of progression ( $p=0.009$ , Figure 3). When adjusted for sex, age-at-

sample, each islet antibody level and multiple antibody status, trypsinogen did not independently predict risk of progression to T1D ( $p=0.138$ ).

Low trypsin(ogen) levels were not associated with non-insulin requiring adult-onset diabetes

There was no difference in serum trypsin(ogen) levels between cases with ADA and type 2 diabetes, the former with ( $n=96$ ) and the latter without ( $n=291$ ) diabetes-associated autoantibodies (Supplementary Figure 1); even autoimmune ADA cases with mAab+ had similar trypsin(ogen) levels to cases with type 2 diabetes. As with controls, in type 2 diabetes trypsin(ogen) levels correlated with age at sampling ( $r=0.12$ ,  $p=0.05$ ). However, in cases with ADA, there was no association between age-at-sample or age at diagnosis and trypsin(ogen) levels irrespective of whether they had one or mAab+ or were diagnosed above or below the median age.

### *Conclusions*

In MZ twins discordant for diabetes, Somascan analysis for over 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 in their



non-diabetic co-twins. This difference was subsequently validated for serum trypsin(ogen) using a separate assay optimised to detect lower levels of trypsin(ogen), which confirmed decreased serum trypsin(ogen) in recently diagnosed type 1 diabetes cases at a range of ages at diagnosis. Analysis of the large ADDRESS-2 cohort, in which samples were taken from cases close to diagnosis and from first degree relatives, suggested relationships between trypsin(ogen) and islet autoantibodies (GADA and IA-2A) as well as DKA, insulin dose and time from 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 between 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 autoantibody 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 datasets. Samples analysed in this study were randomly collected, with an unknown prandial state however historical studies in healthy individuals suggest 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 dataset.

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-sample and BMI are major determinants of trypsin(ogen) levels in cases with newly established type 1 diabetes. This echoes the studies of Li *et al.* 2017 and Ross *et al.* 2021 and an earlier study by Saisho *et al.* 2007 using CT-scans in individuals with type 2 diabetes and controls, demonstrating a steady increase in pancreatic size up to approximately the age of 30 years (7,8,10).

Trypsin(ogen) levels in twins with longstanding type 1 diabetes were positively correlated with age at diagnosis but not with disease duration or age at sampling. In contrast, their non-diabetic co-twins showed increasing levels, which were correlated with age as was the case with control subjects. 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 type 1 diabetes cases and controls (8). In our population-based cohort with recently diagnosed type 1 diabetes, trypsin(ogen) levels were consistently lower than age-matched controls across a broad age range up to diagnosis at age 30 years. In cases diagnosed at older ages, the relationship was less clear both in classic type 1 diabetes as well as in cases with initially non-insulin requiring autoimmune diabetes,

even when the latter had two diabetes-associated autoantibodies. Larger numbers of adult-onset type 1 diabetes, type 2 diabetes and matched controls 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) between 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 localises to a ductal-specific distal cis-response element reducing transcription factor binding, enhancer activity and *CFTR* expression in ductal cells (15), thereby potentially promoting inflammation and immune effector cell infiltration. In addition, 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 and colleagues showed that pancreas volume is reduced in first degree relatives of patients with type 1 diabetes (17) highlighting the importance of genetics in determining pancreatic mass. In our study however, trypsin(ogen) levels were not significantly lower in the non-diabetic twins compared with age-matched controls also emphasising the importance of non-genetic effects.

The residual difference in trypsin(ogen) level between 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, we found that trypsin(ogen) levels <15ng/ml in non-diabetic subjects at risk of 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 autoantibody status. A larger analysis in “at risk” individuals is warranted. Studies in the ENDIA cohort of faecal elastase (FE-1) showed that levels decreased over time in progressors compared with non-progressors (18).

Pancreas size is already reduced by 25% at diagnosis even in type 1 diabetes cases at a very young age (12,19). Decreased pancreatic mass in longstanding 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 pancreases from non-diabetic donors 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). Further, 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-

insulinitis, rather than within islets (22). It follows that genetic effects might predispose to low-grade inflammation promoting peri-insulinitis 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 longstanding type 1 diabetes develop clinically relevant pancreatic exocrine insufficiency (PEI). In 2015 Piciocchi et al. reported that PEI prevalence in type 1 diabetes is estimated to reach approximately 25 - 74% (23). PEI is associated with high insulin requirement, poor glycaemic control and longstanding diabetes(24). However, PEI is understudied in type 1 diabetes patients, because 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 non-invasive tests such as faecal elastase, serum amylase and lipase to detect PEI. The Faecal elastase test is most commonly used to detect PEI, therefore the data on trypsin(ogen) levels in mild and severe PEI is scarce. Capurso and colleagues (24) in the recent review referred to trypsinogen levels being highly sensitive for advanced PEI (<20ng/ml) and having low sensitivity in mild PEI (trypsin(ogen) levels between 20 and 29 ng/ml). We have observed some inter-assay variability in measuring trypsin(ogen) and therefore inter-laboratory variability is also likely. These levels should therefore be treated with some caution.

In conclusion, we present the first proteomic analysis in a cohort of well characterised twins showing exocrine proteins as important biomarkers for type 1 diabetes with evidence for both genetic and non-genetic determinants. Subsequent validation studies in large well characterised 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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RDL, KG, and AJKW (deceased) conceived the study. LB, AEL, and TV led the analysis with input from TP, MT, KC, SJ, SW, MF, DJ, KP, the BOX study Group and Action LADA Consortium members. Details of Study Group Members are shown in Online Appendix 1. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. LB, TV, AEL, RDL and KG wrote the first draft of the manuscript. All authors provided input on interpretation of results. All authors revised the manuscript critically for important intellectual content and read and approved the final manuscript. Professor Kathleen Gillespie is the guarantor of this work and takes full responsibility for the work as a whole. No potential conflicts of interest relevant to this article were reported.

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#### Declaration of Interests

The authors declare no conflict of interest.

#### Data Sharing

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

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**Maintext Tables and figures**

**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	
Study subset	CONCORD ANT	DISCORD ANT	CONT ROLS	CASES	CONTR OLS	AT RISK		within 2 years of DX		>2 years post DX		ADA	T2D
								CASES	CONTROLS	CASES	CONTROLS		
<b>Total n</b>	78	84	39	932	169	113	82	27	27	31	31	96	291
<b>Males/Females</b>	35/42	44/40	19/20	554/378	88/81	63/50	46/36	12/15	12/15	16/15	16/15	48/48	175/116
<b>Cases n (%)</b>	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)
<b>Caucasian n (%)</b>	78 (100)	84 (100)	NA	872 (94)	159 (94)	NA	NA	NA	NA	NA	NA	89 (93)	269 (92)
<b>Age &gt;16 years at sample n (%)</b>	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)
<b>Age at sample median years</b>	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
<b>(range)</b>	(5.5 - 53.7)	(7.0 - 68.7)	(13.0 - 65.0)	(5.1 - 66.4)	(5.1 - 58.5)	(3.5 - 44.3)	(5.5 - 69.3)	(2.1 - 37.1)	(2.5 - 37.0)	(6.4 - 23.9)	(5.8 - 24.0)	(31.3 - 70.5)	(48.0 - 54.0)
<b>Time since diagnosis median years</b>	13.7	10.8	NA	0.2	NA	1.54	8.3	0.9	NA	5.2	NA	1.0	1.9
<b>(range)</b>	(-8.6 - 38.1)	(0.1 - 37.9)	NA	(0.0 - 0.9)	NA	(0.1 - 5.3)	(0.5 - 25.1)	(-0.1 - 1.7)	NA	(3.0 - 14.9)	NA	(-2.2 - 5.0)	(-1.1 - 6.2)
<b>Aab positive n(%)</b>	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)
<b>IRT (ng/ml) median</b>	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
<b>(range)</b>	(2.0 - 24.5)	(0.0 - 33.9)	(0.0 - 46.1)	(2.1 - 123.8)	(5.6 - 101.5)	(4.9 - 58.3)	(7.3 - 77.8)	(7.5 - 29.9)	(7.9 - 44.9)	(5.6 - 29.7)	(10.1 - 46.6)	(3.9 - 78.5)	(2.6 - 104.8)
<b>BMI median</b>	22.4	23.1	22.5	22.1	21.2	NA	NA	NA	NA	NA	NA	29.1	30.4
<b>(range)</b>	(13.4 - 36.8)	(13.2 - 42.3)	(16.8 - 37.3)	(8.3 - 52.2)	(12.1 - 39.9)	NA	NA	NA	NA	NA	NA	(18.5 - 59.5)	(19.8 - 59.4)
<b>Follow up years</b>	NA	NA	NA	NA	NA	3.8	11.7	NA	NA	NA	NA	NA	NA
<b>(range)</b>	NA	NA	NA	NA	NA	(0.1 - 5.3)	(0.0 - 32.0)	NA	NA	NA	NA	NA	NA

**Table 2:** SomaLogic proteomic profiling: paired T-tests comparing affected and non-affected identical twins (n=43 pairs). Of 4068 human proteins, the top five differences between discordant monozygotic twins were Chmotrypsinogen B ( $p=2.01E-09$ ), followed by trypsin(ogen) 1 ( $p=1.68E-8$ ), trypsin(ogen) 2 ( $p=1.91E-7$ ), chymotrypsinogen B2 ( $p= 3.67E-07$ ) and carboxypeptidase B1 ( $p=1.38E-06$ ).

SOMAmer Reagent	p.value	Log2 fold change	FDR	p. bonferroni	rank	Target
CTRB.5671.1.3.	2.01E-09	-0.122	8.17E-08	8.17E-06	1	Chymotrypsinogen B
PRSS1.3049.61.2	1.68E-08	-0.104	3.42E-05	6.84E-05	2	Trypsin(ogen) 1
PRSS2.5034.79.1	1.91E-07	-0.104	2.58E-04	7.75E-04	3	Trypsin(ogen) 2
CTRB2.5648.28.3	3.67E-07	-0.135	3.73E-04	1.49E-03	4	Chymotrypsinogen B2
CPB1.6356.3.3	1.38E-06	-0.08	1.12E-03	5.59E-03	5	Carboxypeptidase B1

Figure Legends:

Figure 1. Trypsin(ogen) levels in twins discordant for type 1 diabetes affected (TWINS DM, n=42), were significantly reduced when compared to non-affected (TWINS NO DM, n=42, \*\*\*\* $p<0.0001$ ; Wilcoxon-Matched pair test) and to age-matched healthy controls



(n=39, \*\*\*\*p<0.0001; Mann-Whitney test). The median trypsin(ogen) level was lower in non-affected twins when compared with controls, but not significantly (p=0.5088; Mann-Whitney test). P<0.05 was considered as significant.

Figure 2. Trypsin(ogen) levels in individuals with T1D (n=932, dark grey circles) and their diabetes-free siblings (n=169, light grey triangles) grouped by age-at-sample, tested close to diagnosis. P < 0.05 was considered significant (\*p=0.0158, \*\*\*\*p<0.0001).

Figure 3. Hazard-function plot for follow-up to development of type 1 diabetes in FDR at risk with confirmed single or multiple islet autoantibodies (n=195), including ENDIT cohort (n=113) and BOX cohort (n=82). Trypsin(ogen) levels <15 ng/ml (black line) were associated with an increased risk of progression to type 1 diabetes (p=0.009). Data were truncated at 20 years follow-up due to the small number of participants remaining. The X axis shows follow-up (years) and below number of participants remaining in study with trypsin(ogen) levels <15ng/ml and >15ng/ml.