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Temporal Expression Profiling of Plasma Proteins Reveals Oxidative Stress in Early Stages of Type 1 Diabetes Progression

Chih-Wei Liu^a, Lisa Bramer^b, Bobbie-Jo Webb-Robertson^b, Kathleen Waugh^c, Marian J. Rewers^c, and Qibin Zhang^{a,d,*}

^aCenter for Translational Biomedical Research, University of North Carolina at Greensboro, North Carolina Research Campus, Kannapolis, North Carolina

^bApplied Statistics & Computational Modeling, Pacific Northwest National Laboratory, Richland, Washington

^cBarbara Davis Center for Diabetes, University of Colorado School of Medicine, Aurora, Colorado

^dDepartment of Chemistry & Biochemistry, University of North Carolina at Greensboro, Greensboro, North Carolina

Abstract

Blood markers other than islet autoantibodies are greatly needed to indicate the pancreatic beta cell destruction process as early as possible, and more accurately reflect the progression of Type 1 Diabetes Mellitus (T1D). To this end, a longitudinal proteomic profiling of human plasma using TMT-10plex-based LC-MS/MS analysis was performed to track temporal proteomic changes of T1D patients ($n = 11$) across 9 serial time points, spanning the period of T1D natural progression, in comparison with those of the matching healthy controls ($n = 10$). To our knowledge, the current study represents the largest (>2000 proteins measured) longitudinal expression profiles of human plasma proteome in T1D research. By applying statistical trend analysis on the temporal expression patterns between T1D and controls, and Benjamini-Hochberg procedure for multiple-testing correction, 13 protein groups were regarded as having statistically significant differences during the entire follow-up period. Moreover, 16 protein groups, which play pivotal roles in response to oxidative stress, have consistently abnormal expression trend before seroconversion to islet autoimmunity. Importantly, the expression trends of two key reactive oxygen species-decomposing enzymes, Catalase and Superoxide dismutase were verified independently by ELISA.

Graphical abstract

* Correspondence to: Qibin Zhang, PhD, UNCG Center for Translational Biomedical Research, 500 Laureate Way Suite 4226, Kannapolis, NC 28081; q_zhang2@uncg.edu.

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Keywords

Temporal proteome change; Type 1 Diabetes; TMT10; pediatric plasma proteome; longitudinal profiling; oxidative stress

INTRODUCTION

T1D is an autoimmune chronic disorder resulted from the progressive destruction and dysfunction of insulin-producing β -cells in the pancreatic islets, which further results in severe insulin deficiency, hyperglycemia, and secondary complications [1–3]. Clinically, seroconversion to islet cell autoantibodies, including autoantibodies to insulin (IAA), glutamic acid decarboxylase 65 (GAD65A), protein tyrosine phosphates IA2 (IA2A) and zinc cation efflux transporter 8 (ZnT8A), is used to predict the risk of developing this disease. In particular, the presence of multiple autoantibodies reveals high risk of T1D [4–6]. However, not all islet autoantibody-positive subjects progress to T1D and independent biomarkers are needed to elucidate the etiology of this disease [7], monitor β -cell destruction and accurately stage the progression of pre-clinical T1D for timely therapeutic intervention.

Cross-sectional characterization of serum proteome at diagnosis of T1D has suggested associations with proteins involved in inflammation and immune response [8–10]. However, samples used in these studies were collected at or shortly after the clinical diagnosis of T1D, reflecting late stages of β -cell destruction with hyperglycemia. In contrast, prospective cohorts allow profiling of serum/plasma proteome as children develop islet autoantibodies and progress to T1D [11].

We investigated whether the temporal expression trends of plasma proteins are able to differentiate T1D patients from healthy controls in the early stage of disease, multiple time point samples collected prior to T1D onset are necessary to accurately track the temporal changes in proteome. To this end, longitudinally collected plasma from subjects enrolled in the Diabetes Autoimmunity Study in the Young (DAISY) prospective cohort [12, 13] were analyzed by quantitative proteomics, 9 time points per subject were selected, with the time points covering from birth to development of islet autoimmunity and overt T1D. Tandem mass tag (TMT)-based isobaric labeling quantitation approach [14–17] was applied to the serial 9 time point samples that can be quantified in parallel, and increased time points in a longitudinal study helped improve the statistical power needed to identify the expression patterns/trends significantly variant between the T1D progressors and healthy controls. To

our knowledge, this study presented the most comprehensive profiling in temporal proteome changes in T1D research, and pattern recognition was being applied to differentiate the temporal changes of proteins correlated to disease progression from those in age-sex matched healthy controls. Importantly, the temporal expression trends of proteins observed by mass spectrometry were consistently verified by antibody-based ELISA platform. Our results provide a promising list of protein markers that temporally dysregulate before appearance of islet autoimmunity, and those proteins have a role in response to oxidative stress.

EXPERIMENTAL SECTION

Study Population and Design

This is a nested case-control study. Participants were selected from the DAISY cohort, and they were identified with T1D susceptible HLA-DR/DQ alleles through genotyping at birth and followed prospectively. The details of screening [12] and follow-up [13] have been published previously. Informed consent was obtained from the parents of each study subject. The Colorado Multiple Institutional Review Board approved all study protocols.

Islet autoantibodies (to IAA, GADA, IA-2A, and ZnT8A) were measured in the laboratory of Dr. George Eisenbarth at the Barbara Davis Center in Denver at 9, 15 and 24 months and yearly thereafter [13]. Children positive for any autoantibody were followed in 3–6 month intervals for autoantibodies, hemoglobin A_{1c}, and random blood glucose until diagnosis of diabetes. Islet autoimmunity (IA) is a pre-T1D phenotype defined here as the presence of one or more of the autoantibodies on at least two consecutive visits 3–6 months apart or development of T1D within 6 months after a positive autoantibody test. Diabetes was diagnosed using the American Diabetes Association criteria [18]. Venous non-fasting blood samples were collected at each study visit and plasma was separated and stored at –80 °C. All positive autoantibody values and 5% of negative ones were confirmed by blind duplicate retesting.

Archived plasma samples from 11 T1D and 10 healthy subjects enrolled in DAISY were selected for this study. A series of 9 plasma samples were selected from each subject in the T1D group to represent different phases of disease progression, i.e. from autoantibody negativity to seroconversion and the ultimate T1D diagnosis. Healthy individual samples were matched on age, sex and sampling frequency to the T1D group except having permanent autoantibody negativity during the entire follow-up period and designated as NP group in this study. Frozen plasma samples were transferred to proteomics measurement laboratories for sample processing and measurement. In total, 99 and 90 plasma samples from the T1D and control groups, respectively, were used in this MS-based proteomics profiling study and they were processed according to the experimental strategy (Fig. 1) [17].

Proteomics sample preparation

Unless otherwise specified, all reagents and chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO). The kit for BCA protein assay was obtained from

ThermoFisher Scientific (Rockford, IL). Sequencing-grade trypsin was purchased from Promega (Madison WI). All solvents used were HPLC-grade.

All samples were prepared in the same batch by the same researcher to ensure minimal variations in sample preparation. The detailed experiments for sample preparation have been published previously [17]. Briefly, 5 mg plasma proteins from each sample were depleted to remove the top 14 most abundant proteins using an MARS Human-14 column (Agilent Technologies), and the flow-through fractions (depleted plasma) were collected and concentrated. The depleted plasma proteins were denatured, reduced, alkylated, and digested as previously described [10], and the C₁₈ SPE desalted peptide solution was completely dried before isobaric labeling. Aliquots of each individual sample from all healthy subjects were pooled to create the common reference sample used for healthy control group, to be used in subsequent TMT labeling as reference channel. The 9 serial samples from each subject and the common reference sample were included in one TMT10 labeling experiment set as shown in Fig. 1. This labeling strategy avoids the potential missing value issues resulted from data-dependent MS/MS acquisitions [19] because all samples from the same subject were included in the same labeling experiment. The same pooling and labeling strategy was applied to T1D patient samples (sample labels in Supplementary material Tables S1 and S2). After labeling, the 10 labeled samples in each TMT labeling experiment set were pooled, concentrated and fractionated using high-pH RPLC on an XBridge C₁₈ analytical column (particle size of 5 μ m, 250 $\times$ 4.6 mm, Waters). Mobile phases A and B consisted of 10 mM ammonium formate in water (pH 10) and 90% CH₃CN (pH 10), respectively. In total, 24 fractions were generated [16, 20], dried, stored at -80 °C, and reconstituted in 0.1% FA until LC-MS/MS analysis.

Quantitative LC-MS/MS analysis

LC-MS/MS analysis was conducted using an UltiMate 3000 RSLCnano system coupled to a Q Exactive HF mass spectrometer through an EASY-Spray ion source (ThermoFisher Scientific). Peptides were separated on a PepMap C₁₈ analytical column (2 μ m particle, 50 cm $\times$ 75 μ m i.d.). A binary solvent system consisting of 0.1% FA in ddH₂O (solvent A) and 0.1% FA in CH₃CN (solvent B) was used to separate peptides at a flow rate of 250 nL min⁻¹. LC separation was performed using the following gradient setting: held at 4% B for 3 min, from 4% to 8% B in 0.1 min, 8% to 40% B in 90 min, 40% to 90% B in 0.1 min, held at 90% B for 10 min, 90% to 4% B in 0.1 min, and held at 4% B for 17 min for re-equilibrating column.

MS data was acquired in profile mode using a data-dependent top 15 method and resolution for full scans (m/z 400–1950) was set to 120,000 at m/z 200 with maximum fill time of 50 ms. Precursors were isolated with a window of 1.4 m/z [21] and fragmented with HCD fragmentation with normalized collision energy of 32. Resolution for MS/MS spectrum was set to 60,000 at m/z 200 with maximum fill time of 100 ms. AGC target for full scan and MS/MS scan was 3e6 and 1e5, respectively. Precursor ions with unassigned, single, seven, and higher charge states were excluded, and dynamic exclusion time was set to 20 s.

Database search

All raw files obtained from LC-MS/MS analyses were analyzed using MaxQuant software version 1.5.3.30, and searched against Swiss-Prot human protein database (91,960 protein entries, 02/17/2016 release) using the built-in Andromeda search algorithm. Semispecific Trypsin/P was selected as the enzyme. Cysteine carbamidomethylation and TMT-10plex labeled N-terminus and lysine were set as fixed modifications. Methionine oxidation was set as variable modifications. FDR was set at 1% for proteins and peptides level identification using decoy database, and precursor intensity fraction was set to >0.75 [22]. Other parameters were used as default settings for Orbitrap-type data. The search results in proteinGroups.txt generated by MaxQuant were processed in Perseus software version 1.5.1.6 [23]. Identified protein is represented as protein group in the database search output when the search algorithm identifies a cluster of proteins with high sequence similarity or protein isoforms, which cannot be further differentiated on the basis of shared peptides. The potential contaminants (manually selected for contaminants with no protein names), reverse hits and proteins only identified by modification site were excluded from the identification list. Furthermore, a filtering criterion was set to keep the identified proteins with the quantified values of all ten reporter ions (no missing value) in at least one individual. The longitudinal expression data of this confident protein list was used for statistical trend analysis. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [24] partner repository with the dataset identifier PXD007884.

Statistical trend analysis

An evaluation of Body Mass Index (BMI) was initially performed to determine if changes over time in BMI were different for the two groups. This was performed by fitting a mixed effects model with BMI as the dependent variable that included a fixed effect to account for different mean intercepts, slopes and quadratic terms (where applicable) for age and the two groups.

Quantified protein data based on the reporter ion intensity was normalized to the reference sample, $\log_2$ transformed, and median centered,[25] i.e. bring the median intensity values of each sample to the same level to correct for the slight variations in the amount of sample used for each labeling channel. To complete the statistical trend analysis and ensure valid results, data was further filtered to include only protein groups that had at least 2 subjects from one of the two groups (healthy subjects and T1D patients) with at least 7 time points without missing values.

Two mixed effect linear models were fit to the normalized log abundance data to determine whether the trend is linear or quadratic: a quadratic model with random quadratic, linear, and intercept terms (Eq. 1), and a linear model with random linear and intercept terms (Eq. 2). Age was used as the fixed effect in these models to examine trends in abundance over time, while controlling for age difference between subjects and random effects for each subject were included to account for the subject variability and non-independent nature of the data over time.

$$y_i = \beta_0 + \beta_1 x_i + \beta_2 x_i^2 + \tau_{1,i} + \tau_{2,i} x_i + \tau_{3,i} x_i^2 \quad (\text{Eq. 1})$$

$$\tau_{1,i} \sim N(0, \sigma_c), \tau_{2,i} \sim N(0, \sigma_s), \tau_{3,i} \sim N(0, \sigma_q)$$

$$y_i = \beta_0 + \beta_1 x_i + \tau_{1,i} + \tau_{2,i} x_i \quad (\text{Eq. 2})$$

$$\tau_{1,i} \sim N(0, \sigma_c), \tau_{2,i} \sim N(0, \sigma_s)$$

Likelihood ratio tests were conducted to test for significant quadratic and linear trends and p values were recorded. An $\alpha = 0.05$ level of significance was used to determine if a linear or quadratic model was significant, and if both were significant the lower model (linear) was used. The difference in trends between control and T1D groups for each protein was statistically evaluated by also fitting a mixed effects linear or quadratic model that included a fixed effect for age, group and the interaction between age and group and a random effect for subject (Eq. 3). This model was either linear or quadratic based on the results from the likelihood ratio test (Eq. 1 and 2).

$$y_i = \beta_0 + \beta_1 x_i + \beta_2 g_i + \beta_3 x_i g_i + \tau_{1,i} + \tau_{2,i} x_i \quad (\text{Eq. 3})$$

$$\tau_{1,i} \sim N(0, \sigma_c), \tau_{2,i} \sim N(0, \sigma_s)$$

where g_i is an indicator variable that takes a value of 0 if the subject is from the control group and a 1 otherwise. All models were fit in R (version 3.3.0) using the lme4 package. [26] The statistical significance of the linear and quadratic models, as relevant, were captured as p-values and q-values, where q-values were based on a standard Benjamini-Hochberg correction using the p.adjust function in R.

Verification of expression patterns using ELISA

ELISA kits for Superoxide dismutase [Cu-Zn] (SOD1, #ab202410, intra-assay coefficient of variation (CV) 4.2%) and Catalase (CAT, #ab171572, intra-assay CV 3.0%) were purchased from Abcam (Cambridge, MA). The plasma samples from each time point of selected subjects were diluted with dilution factors of 1:2 and 1:200 for SOD1, and CAT, respectively, and measured according to manufacturer's protocol. Duplicated wells were used to average the absorbance values (except for SOD1 measurement owing to the limited samples) and the concentration of target proteins in the plasma were calculated based on the calibration curve ($R^2 = 0.9603$ (4-Parameter logistic fit) and 0.9996 (linear fit) for SOD1 and CAT, respectively.) and dilution factor after subtraction of background.

RESULTS

To explore the temporal changes of protein expressions during the natural history of T1D, plasma samples prospectively collected from 21 individuals, including healthy subjects ($n = 10$, without T1D, persistent autoantibody negative) and T1D patients ($n = 11$) were selected and comprehensively analyzed in this quantitative proteomics study. For healthy subjects, the 9 time points were selected at almost identical sampling ages; for T1D patients, samples were selected to have the time point of seroconversion to multiple islet autoantibodies in the middle of the time series, aiming for equal number of samples before and after seroconversion, including both the earliest time point as possible and the time point of clinical T1D diagnosis (Fig. 2). Details of the clinical metadata for each sample and each subject are listed in Supplementary material Tables S1 and S2.

No association between BMI and T1D progression in DAISY cohort subjects analyzed in this study

“Accelerator hypothesis” proposed that a higher BMI is associated with a younger age at diagnosis of T1D, and this hypothesis was evaluated in small (<200 patients) [27, 28] and large (9,248 patients) [29] cohorts enrolled in UK, Germany, and Austria. Another large cohort study with 3,203 patients in a Mediterranean area indicated that no differences between T1D patients and healthy controls in terms of BMI at diagnosis and age at diagnosis [30]. Therefore, the BMI effect in diagnosis of T1D is inconclusive, depending on the environments/areas and studying cohorts. The effects of BMI has also been evaluated in the DAISY cohort in the past, and we did not observe a strong association between BMI and the age at T1D diagnosis [31–33], although a greater height growth velocity may be involved in the progression from genetic susceptibility to autoimmunity and then to T1D in pre-pubertal children [32]. We therefore conducted a statistical test to investigate whether there is an BMI effect for the subjects selected in this study, i.e. the T1D patients ($n = 11$) and healthy controls ($n = 10$) analyzed in the current proteomics study. A mixed-effects linear model was fit to the BMI data for all individuals, and p-values of 0.8857 and 0.5135 were obtained in quadratic and linear models, respectively, indicating no difference between groups in terms of BMI evolution trend.

Comprehensive identification and quantification of plasma proteins

In total, 2235 protein groups were identified and relatively quantified at 1% FDR in the plasma proteome using MaxQuant after strictly filtering the data matrix as described in the method section (Supplementary material Table S3). Among those protein groups, 2037 were identified by at least two “razor + unique” peptides and one unique peptide, and razor peptides are non-unique peptides assigned to the protein group with the most other peptides. Among them, 871 protein groups were commonly identified in all 210 samples, while 91 and 98 protein groups were exclusively identified in healthy subjects and T1D patients, respectively (Fig. 3A).

Furthermore, all 108 proteins mentioned in the recent longitudinal serum study by Moulder et al. [11] were quantified in this study. Proteins reported there belong to the high abundant proteins identified in this study (Supplementary material Fig. S1), and one fifth of those

proteins are complement factors, components or subunits that abundantly exist in the human plasma [34]. In addition, we compared our plasma proteome data with two large-scale proteomic analyses of cultured human islet cells by Schrimpe-Rutledge et al. [35] and of human pancreatic tissues by Liu et al. [16]. Venn diagram shows there are 1095 proteins in common among those three datasets (Fig. 3B), and 1335 and 1286 proteins were overlap between the current data with Liu et al. and with Schrimpe-Rutledge et al., respectively. Next, Uhlén et al. recently established a comprehensive tissue-based map of the human proteome and reported 37 pancreas-enriched genes (at least five-fold higher mRNA levels in pancreas as compared to all other tissues) [36]. Among those 37 pancreas-enriched genes, we identified 10 (including PRSS1, CTRB2, SPINK1, CPA1, CPA2, CPB1, PNLIP, SYCN, CELA3A and AMY2A) proteins, directly from our global plasma proteomics data in spite of the high complexity of plasma proteome.

Temporal expression patterns of plasma proteins variant between T1D and healthy controls

Recently, using the same proteomics workflow we demonstrated the temporal profiles of plasma proteome during childhood development [17], and the temporal patterns of plasma proteins obtained by TMT-10plex based quantitative proteomics were independently validated by ELISA, indicating the consistency between MS and ELISA data in both the expression patterns and relative abundance of the proteins. In that study, 970 plasma proteins were reported to have the age-dependent expression trends [17], which demonstrated the importance of longitudinal profiling study to identify the potential biomarkers specific to childhood diseases. Therefore, in the current study we implemented the same experimental design to reveal the plasma proteins with different temporal expression patterns between T1D and healthy controls.

Statistical modeling was applied to fit the temporal changes of each protein within the same subject, and statistical p-values were obtained to evaluate the confidence of fit to a linear or quadratic model. Statistical comparisons were also made between children who developed T1D and healthy subjects who kept persistent autoantibody negativity, and p-values were obtained to determine whether there are significant differences between two group trends based on each model (Supplementary material Table S3). All of the expression patterns/trends were uploaded online and can be visualized using Trelliscope (https://ascm.shinyapps.io/Zhang_2Group_Trend_Analysis/), which allows rapid exploration of the expression trends for each protein between two groups [37]. A brief introduction to access this online resource was provided in the Supplementary Material.

Table 1 summarized the number of protein groups in each trend category between healthy subjects and T1D patients, and majority of the protein groups have the temporal expression trend of flat, i.e. age-independent expressions. In comparison with the number of protein groups with same trend between groups, many of protein groups in the T1D group clearly have different expression trends from healthy subjects. Statistical trend analysis was therefore performed to identify protein groups with significant expression patterns ($p < 0.05$) between control and T1D groups. In total, 67 protein groups have p-values < 0.05 in both linear and quadratic models, while 61 and 39 protein groups have statistical significance in

linear and quadratic modes, respectively. Next, we applied Benjamini-Hochberg procedure to compute FDRs based on the p-value distributions in linear and quadratic models, separately (Supplementary material Table S3) [38]. Because this is a proteomics discovery study aiming to find interesting temporal changes between the T1D and healthy controls for further validation, we set a FDR of 10% ($q < 0.1$) for this multiple testing correction. The 13 protein groups identified in all 210 plasma samples without any missing value satisfied this FDR criterion, and those protein groups were confidently identified by at least 6 unique peptides (Table 2).

Those protein groups were further categorized into 2 clusters based on the difference of expression trends after (A cluster) and before (B cluster) age 7 (the average seroconversion age to persistent islet autoimmunity in the 11 T1D patients is 6.6). The protein groups in A cluster have reverse expression trends between T1D and healthy controls after seroconversion; in contrast, protein groups in B cluster have different patterns before seroconversion. Oresic et al. showed that longitudinal profiles of plasma metabolites and lipids change before seroconversion to islet autoimmunity but partially normalize afterwards [39]. Interestingly, FABP4 (Fatty acid-binding protein, adipocyte, $p = 0.00421$; $q = 0.0953$), a lipid transport protein, has the expression pattern of “flat” in the control group versus “decrease-then-increase” in T1D patients (see Trelliscope for corresponding plot). The temporal profile changes of this protein, in part, correspond to the lipid profile changes observed by Oresic et al. [39]. PLA2G7 has unique patterns of “Curved-increase-then-flat” versus “Flat-then-curved-increase” in healthy and T1D groups, respectively (see Trelliscope for corresponding plot). The roles of PLA2G7 has been extensively studied in cardiovascular development and diseases owing to the positive or negative regulations in oxidative stress and inflammation processes [40]. Because of the importance of PLA2G7 in inflammation, the association of enzyme activity of PLA2G7 in serum/plasma has been evaluated in adult patients with T1D, and the positive associations between T1D and PLA2G7 activity have been reported by Gomes et al. [41, 42]. PLA2G7 expressions in the latter follow-up period in our study may partially confirm the findings reported from adult T1D patients in Gomes et al., and both studies indicates the potential roles of PLA2G7 in T1D, especially in oxidative stress and inflammation.

Unique and consistent expression patterns of protein groups with roles in response to oxidative stress

Glutamate–cysteine ligase catalytic subunit (GCLC), one of protein groups (with $p < 0.05$ and $q < 0.1$) listed in Table 2, is a rate limiting enzyme in glutathione synthesis, and glutathione is a well-known antioxidant that protects cells from oxidative stress [43]. The trend of “Flat-then-curved-increase” in healthy subjects had obvious difference from T1D group before seroconversion, which had the continuous trend of “increase” and “spike” expression patterns at different stages of T1D progression (see Trelliscope for corresponding plot). Significantly, the similar expression patterns were also found in Catalase (CAT, $p = 0.03663$; $q = 0.26739$), and the spike expression patterns were clearly observed around the seroconversion event (Fig. 4A and B). The corresponding ELISA data verified the expression trends of CAT are as consistent as observed in MS data (Fig. 4C). CAT involves in decomposition of hydrogen peroxide to protect cells/tissues from oxidative stress [44],

and Lei et al. demonstrated that overexpression of CAT aggravated the onset of T1D in NOD mice [45]. Because of the consistent expression trends in GCLC and CAT and their roles in oxidative stress, we manually checked the protein groups with similar trends as GCLC/CAT, and summarized in Table 3. Those protein groups were identified in all 210 plasma samples without any missing quantitative values and confidently identified by at least 8 unique peptides. Those 16 protein groups have been reported for their potential roles in oxidative stress (see the references cited in Table 3), for example, Peroxiredoxin-1 and -2 (PRDX1 and PRDX2), as well as Superoxide dismutase [Cu-Zn] (SOD1, $p = 0.09901$; $q = 0.35545$) are well-known proteins involve in antioxidant and oxidative stress. Fig. 4D–F shows the expression patterns of SOD1 observed by MS and consistent results verified by ELISA. Taken together, the consistent expression patterns of the proteins involved in oxidative stress and, their higher expressions before seroconversion in T1D group strongly indicate oxidative stress exists in the early stage of T1D progression, prior to earliest sign of islet autoimmunity. The panel of those plasma protein markers identified in this study warrant further investigation for their potential roles in regulation of islet autoimmunity and T1D onset.

DISCUSSION

Comparison with previous longitudinal proteomic studies of human blood samples

In the first prospectively longitudinal study of the proteome in pre-T1D, Moulder et al. [11] studied serial serum samples collected from birth to T1D diagnosis in children at high genetic risk participating in the Finnish DIPP cohort. In total, 658 proteins were identified at 5% FDR and 220 proteins were quantified in all patients/controls. Fifteen proteins showed different expressions between case and control, and 5 out of them demonstrated differences before seroconversion and also throughout the entire follow-up period to diagnosis of T1D. Compared with that study, in which iTRAQ-8plex and label free quantification were respectively used to profile the proteomic changes of 13 and 6 T1D-control pairs with average 7 time points, we had 4 times of more proteins identified and quantified than in their study. To our knowledge, the current study represents the largest longitudinal expression profiles of human plasma proteome in T1D research to date. Moreover, the number of 2037 protein groups identified by at least two razor + unique peptides and one unique peptide is also higher than the results from any independent study summarized in a recent review for plasma proteome [46].

Moulder et al. [11] compared their data with the potential T1D serum markers from Zhi et al. [9] and Zhang et al. [10], and reported the expression patterns for 9 out of 32 proteins (Supplementary material Table S4); it is of note that the expression trends of all 32 proteins were observed in the present study. Similar to the observation of Moulder et al., age-associated increase of Beta-Ala-His dipeptidase was also observed by us in both the T1D and control groups. For some proteins (Apolipoprotein A-IV and N-acetylmuramoyl-L-alanine amidase) with decrease trends in T1D group reported by Moulder et al., we observed the trends of decrease and flat in healthy control and T1D groups, respectively, but without statistical significance between groups. For Complement C4-B, Moulder et al. observed the increase trend in healthy control, and in our data we detected the flat trend in both groups. In

addition, for Insulin-like growth factor-binding protein 2, Moulder et al. reported the decrease trend in T1D group, and we observed the same trend of decrease in T1D group and the trend of curved-decrease-then-flat in healthy control with statistical significance in quadratic model ($p = 0.019$). The advantage and importance of 9 time points per individual analyzed in this study is that it can provide higher statistical power in identifying proteins with different temporal expression trends.

Statistical trend analysis for a longitudinal study

While a longitudinal study could adopt a case-control paired study design where the ratio between case and control is compared longitudinally to establish the temporal change profiles, this requires very strict pairing between case and control and large sample size because the inter individual variability may be higher than the small variations at the initial stage of the pathological process.[47] For this reason, we applied a different strategy that the protein abundances were primarily compared longitudinally within the same subject, i.e. each subject serves as one's own reference to establish the temporal trend/pattern using TMT-10plex labeling based quantification strategy, which allows to simultaneously relatively quantify the samples from 9 time points while one TMT labeling channel is being used as common reference for between-subject comparison [17, 48]. Moreover, a well-designed control cohort provides a reference baseline for each protein to distinguish the temporal expression changes in T1D on the basis of disease pathology rather than the age-dependent variations of human physiology [17].

Oxidative stress in Type 1 diabetes

Oxidative stress is well characterized in the development of diabetes complications [49] and beta-cell dysfunction [50], because of the low expression and activity of antioxidant enzymes in beta-cells. Oxidative stress is mainly induced by reactive oxygen and nitrogen species (ROS and RNS); in general, ROS is generated in the mitochondrial respiratory chains, and the generated superoxide is converted to less active H_2O_2 and neutral H_2O via the sequential actions of SOD1 and CAT and glutathione peroxidases. It is of note that a certain amount of ROS/RNS is necessary for beta-cell normal function such as stimulation of insulin secretion in glucose responsiveness at low concentrations of H_2O_2 (see review by Drews et al. [50]). However, oxidative stress could predispose onset of T1D as indicated by Matteucci et al. [51], who reported that inside plasma and red blood cells, levels of malondialdehyde – the oxidative stress markers significantly elevated for the nondiabetic siblings of T1D patients. In this longitudinal proteomics study, we found the abnormal expression patterns of those 16 proteins with roles in response to oxidative stress before seroconversion to islet autoimmunity (Table 3), which may suggest that oxidative stress involves in early stage of T1D progression.

In summary, a very comprehensive longitudinal profiling of human plasma proteome was demonstrated here using a multiplexed TMT-10plex-based LC-MS/MS platform to identify potential protein markers to T1D progression. Age-dependent variations in plasma proteome were eliminated by including a well-designed healthy control group for analysis, so the temporal changes as a result of T1D natural progression can be clearly distinguished. Statistical curve modeling was applied to differentiate the expression patterns/trends

between healthy subjects and T1D patients, and Benjamini-Hochberg procedure was used for multiple testing corrections, which resulted in 13 protein groups ($p < 0.05$, $q < 0.1$) having statistical significance in expression trends between the two groups during the entire follow-up period. Moreover, unique and consistent expression patterns were found in a panel of protein groups with roles in response to oxidative stress, and those protein groups clearly showed abnormal expression even before seroconversion to islet autoimmunity. Importantly, the temporal expression trends of key enzymes against oxidative stress, CAT and SOD1, were verified independently by ELISA. The significance of those promising potential markers deserves more attention. Validation studies using large scale and independent cohorts are planned for the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biological Significance

The temporal changes of >2000 plasma proteins (at least quantified in two subjects), spanning the entire period of T1D natural progression were provided to the research community. Oxidative stress related proteins have consistently different dysregulated patterns in T1D group than in age-sex matched healthy controls, even prior to appearance of islet autoantibodies – the earliest sign of islet autoimmunity and pancreatic beta cell stress.

Highlights

- >2000 plasma proteins were longitudinally measured during development of T1D.
- Oxidative stress related proteins dysregulate before islet autoimmunity.
- Temporal changes of plasma proteome as reference to childhood disease research.
- TMT-10plex quantitative proteomics improved proteome coverage and data quality.

Fig. 1.

Schematic representation of experimental workflow. The plasma samples of 9 serial time points from each subject were longitudinally collected and immunodepleted by removing the top 14 abundant proteins prior to tryptic digestion. The peptides of serial 9 time points plus one common reference were labeled by TMT-10plex, and the high-pH RPLC was used to fractionate the pooled TMT-labeled peptide mixtures before nanoLC-MS/MS analysis. All of raw files were searched together using MaxQuant for protein identification and quantification. The quantification data matrix was further used for statistical trend analysis and visualization by Trelliscope. Finally, ELISA experiments were conducted for trend verifications. This modified workflow was adopted from Liu et al. [17] with permission.

Fig. 2. The sampling time points in healthy subjects (NP, $n = 10$) and T1D patients (TD, $n = 11$). The filled circles/squares/triangles indicate the sampling time points. Large filled squares and triangles indicate the seroconversion time and T1D clinical diagnosis, respectively.

Fig. 3.

(A) Summary and Venn diagram of plasma proteins identified in healthy subjects (NP) and T1D patients (T1D). (B) Overlap comparison between the proteins identified in this study, in cultured human islet cells by Schrimpe-Rutledge et al. [35], and in human pancreatic tissues by Liu et al. [16]. Venn diagram was calculated based on the reported gene name.

Fig. 4.

The temporal expression patterns of protein CAT (A–C) and SOD1 (D–F) observed by LC-MS/MS analysis from healthy subjects (NP, A and D) and T1D patients (T1D, B and E) as well as their corresponding verification by ELISA (C and F). The dots and triangles indicate the sampling time points from female and male, respectively, and the lines with different colors indicate each individual subject. X- and Y-axis are the real age and protein relative abundance (Log 2 scale), respectively. CAT: Catalase; SOD1: Superoxide dismutase [Cu-

Zn]. Pearson Correlation values between ELISA and LC-MS data (CAT: 0.92 for NP, 0.98 for T1D; SOD1: 0.87 for NP, 0.89 for T1D).

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Temporal Expression Patterns Categorized in this Study (identified in at least five individuals in one group)

Table 1

Trend category	Trend subcategory	Graphic trend	Number of protein groups in NP group	Number of protein groups in T1D group	Number of protein groups with same trend between groups
Increase			236	197	96
Curved increase	Curved-increase-then-flat		15	3	1
	Flat-then-curved-increase		76	6	3
Increase-then-decrease			155	85	15
Decrease			257	229	129
Curved decrease	Curved-decrease-then-flat		67	27	27
	Flat-then-curved-decrease		7	3	1
Decrease-then-increase			158	182	72
Flat			613	895	413

Table 2

Protein groups with statistically significant (p-value <0.05 in linear or quadratic models, and Benjamini-Hochberg FDR <0.1) trend changes between T1D and healthy subjects (identified in all 210 plasma samples)

Cluster	Uniprot IDs	Protein names	Gene names	Unique peptides	Sequence coverage [%]	Trend in NP	Trend subcategory in NP	Trend in T1D	Trend subcategory in T1D	High_Degree_Sig_Model	Sig_Quad_Sig_pval	Adj_Sig_Quad_Diff_pval	Functional Annotations
A	O15031	Plexin-B2	PLXNB2	35	24.8	Flat		Decrease-then-increase		Quadratic	0.00296	0.08100	Cell-cell signaling, differentiation, axon guidance
	P15090	Fatty acid-binding protein, adipocyte	FABP4	6	40.9	Flat		Decrease-then-increase		Quadratic	0.00421	0.09530	Lipid transport
	P10768	S-formylglutathione hydrolase	ESD	11	46.5	Increase		Increase-then-decrease		Quadratic	0.00187	0.06780	Detoxification
	P16152	Carbonyl reductase [NADPH] 1	CBR1	12	45.8	Increase		Increase-then-decrease		Quadratic	0.00013	0.04188	Arachidonic acid and glutathione metabolisms
	P20618	Proteasome subunit beta type-1	PSMB1	13	54.4	Increase		Increase-then-decrease		Quadratic	0.00165	0.06780	Proteasome
	Q03154	Aminoacylase-1	ACY1	15	46.3	Decrease		Decrease-then-increase		Quadratic	0.00412	0.09530	Amino acids metabolism
	P23471	Receptor-type tyrosine-protein phosphatase zeta	PTPRZ1	21	9.2	Decrease		Decrease-then-increase		Quadratic	0.00117	0.06780	Dephosphorylation
	P30086	Phosphatidylethanolamine-binding protein 1	PEBP1	8	44.4	Curved increase	Flat-then-curved-increase	Increase		Quadratic	0.00245	0.07317	Phospholipids binding protein, serine protease inhibitor
	P48506	Glutamate-cysteine ligase catalytic subunit	GCLC	20	35.8	Curved increase	Flat-then-curved-increase	Increase		Quadratic	0.00233	0.07284	Glutathione biosynthesis
	Q14574	Desmocollin-3	DSC3	6	6.7	Decrease-then-increase		Decrease		Quadratic	0.00153	0.06780	Cell-cell adhesion
B	P17174	Aspartate aminotransferase, cytoplasmic	GOT1	23	63.9	Decrease-then-increase		Decrease		Quadratic	0.00191	0.06780	Glutamate biosynt hesis
	Q6UW P8	Suprabasin	SBSN	6	37.3	Decrease-then-increase		Flat		Quadratic	0.00198	0.06780	Unknown
	Q13093	Platelet-activating factor acetylhydrolase	PLA2 G7	26	49	Curved increase	Curved-increase-then-flat	Curved increase	Flat-then-curved-increase	Quadratic	0.00124	0.06780	Modulates the action of platelet activating factor

High_Degree_Sig_Model: The highest degree significant model for at least one of the groups, this is the model fit to test for differences between groups

Sig_Quad_Diff_pval: p-value for test of significant difference in quadratic trend between two groups

Adj_Sig_Quad_Diff_pval: adjusted p-value in quadratic mode obtained from Benjamini-Hochberg multiple testing corrections

Functional Annotations: the functional annotations were obtained from UniProt database (<http://www.uniprot.org/>)

Table 3

Oxidative stress related protein groups with different expression trends between T1D and healthy subjects (identified in all 210 plasma samples) before seroconversion to islet autoimmunity.

Uniprot IDs	Protein names	Gene names	Unique peptides	Sequence coverage [%]	Trend subcategory in NP	Trend in T1D	Sig_Quad_Diff_pval	Adj_Sig_Quad_Diff_pval	Functional Annotations	Oxidation stress related reports
P48506	Glutamate–cysteine ligase catalytic subunit	GCLC	20	35.8	Flat-then-curved-increase	Increase	0.00233	0.07284	Glutathione biosynthesis	Lu et al. [43]
P30086	Phosphatidylethanolamine-binding protein 1	PEBP1	8	44.4	Flat-then-curved-increase	Increase	0.00245	0.07317	Phospholipids binding protein, serine protease inhibitor	Albano et al. [52]
P13798	Acylamino-acid-releasing enzyme	APEH	14	28	Flat-then-curved-increase	Increase	0.00489	0.10049	N-acetylated amino acid generation	Yoshioka et al. [53]
P07738	Bisphosphoglycerate mutase	BPGM	12	48.6	Flat-then-curved-increase	Increase	0.00907	0.13816	Glycolysis / Gluconeogenesis	Kaminsky et al. [54]
P00568	Adenylate kinase isoenzyme 1	AK1	14	65.2	Flat-then-curved-increase	Increase	0.01117	0.14973	Cellular energy homeostasis, adenine nucleotide metabolism	Kang et al. [55]
P04040	Catalase	CAT	31	45.7	Flat-then-curved-increase	Increase	0.03663	0.26739	Antioxidation, promote immune cell growth	Chelikani et al. [44]
Q06830	Peroxioredoxin-1	PRDX1	13	53.3	Flat-then-curved-increase	Increase	0.05863	0.32233	Antioxidation	O’Leary et al. [56]
P13716	Delta-aminolevulinic acid dehydratase	ALAD	20	53.3	Flat-then-curved-increase	Increase	0.05938	0.32233	Tetrapyrroles biosynthesis	Goncalves et al. [57]
Q86VP6	Cullin-associated NEDD8-dissociated protein 1	CAND1	27	27.2	Flat-then-curved-increase	Increase	0.06737	0.33062	Protein ubiquitination	Villeneuve et al. [58]
P30043	Flavin reductase (NADPH)	BLVRB	8	55.8	Flat-then-curved-increase	Increase	0.07990	0.33062	Riboflavin metabolism	Hertzberger et al. [59]
P00918	Carbonic anhydrase 2	CA2	18	65.4	Flat-then-curved-increase	Increase	0.09656	0.34858	Reversible hydration of carbon dioxide	Iuchi et al. [60]
P00441	Superoxide dismutase [Cu-Zn]	SOD1	9	46.1	Flat-then-curved-increase	Increase	0.09901	0.35545	Detoxification of reactive oxygen species	Stralin et al. [61]
P00352	Retinal dehydrogenase 1	ALDH1A1	16	36.7	Flat-then-curved-increase	Increase	0.13675	0.42187	Retinol metabolism	Salzano et al. [62]
P32119	Peroxioredoxin-2	PRDX2	17	63.6	Flat-then-curved-increase	Increase	0.15075	0.43759	Antioxidation	Rinalducci et al. [63]
Q13228	Selenium-binding protein 1	SELENBP1	24	62.3	Flat-then-curved-increase	Increase	0.15119	0.43759	Sensing of reactive xenobiotics in the cytoplasm	Fang et al. [64]
P00915	Carbonic anhydrase 1	CA1	26	72.8	Flat-then-curved-increase	Increase	0.27979	0.56044	Reversible hydration of carbon dioxide	Cordoba et al. [65]

Sig_Quad_Diff_pval: p-value for test of significant difference in quadratic trend between two groups
Adj_Sig_Quad_Diff_pval: adjusted p-value in quadratic mode obtained from Benjamini-Hochberg multiple testing corrections
Functional Annotations: the functional annotations were obtained from UniProt database (<http://www.uniprot.org/>)