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Pancreatic tissue proteomics unveils key proteins, pathways, and networks associated with type 1 diabetes

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Abstract

Purpose: Type 1 diabetes (T1D) is characterized by autoimmune mediated self-destruction of the pancreatic islet beta cells and the resultant insulin deficiency. However, little is known about the underlying molecular pathogenesis at the pancreatic tissue level given the limited availability of clinical specimens.

Experimental design: We performed quantitative proteomic studies on age-matched T1D and healthy cadaveric pancreatic tissues (n = 18 each) using TMT 10plex-based isobaric labeling and BoxCar-based label-free LC-MS/MS approaches. ELISA was used to validate the differentially expressed proteins (DEPs).

Results: Overall, the two quantitative proteomics approaches identified 8,824 proteins, of which 261 were DEPs. KEGG pathway and functional network analyses of the DEPs revealed dysregulations to pancreatic exocrine function, complement coagulation cascades, and ECM receptor interaction pathways in T1D. A selected list of the DEPs associated with pathways, subnetworks, and plasma proteome of T1D were validated using ELISA.

Conclusions and clinical relevance: Integrating labeling and label-free approaches improved the confidence in quantitative profiling of pancreatic tissue proteome, which furthers our understanding of the dysregulated pathways and functional subnetworks associated with T1D pathogenesis and may aid to develop diagnostic and therapeutic strategies for T1D.

Graphical Abstract

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Conflicts of Interest Statement

The authors have no conflict of interest to declare.

Keywords

pancreatic tissue proteome; TMT labeling; Boxcar label free quantitation; nPOD; ECM-receptor interaction pathway

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease caused by progressive destruction of insulin producing pancreatic beta cells due to insulinitis [1]. T1D mainly affects children and young adults but can occur at any age. Currently, autoantibodies of insulin, GAD65, IA-2, and ZnT8 are useful markers for T1D diagnosis upon patients detected with hyperglycemia [2]. However, a better understanding of disease pathogenesis at the molecular level is still needed to develop diagnostic tools and administer therapeutics in the early phase to prevent onset of the disease.

Insulinitis and immune-mediated destruction of pancreatic beta cells has long been established with T1D pathogenesis; however, increasing evidence also point to abnormalities of the exocrine pancreas in the pathology of T1D [3, 4]. For example, a recent study found significant decrease in the levels of elastase, a serine protease marker for pancreas exocrine function, in T1D children but not in autoantibody positive cases relative to controls, suggesting impaired exocrine function in T1D pancreas [5]. This is in line with previous findings of pancreatic exocrine deficiency in 25–74% of T1D patients [6], and the association of T1D patients with pancreas volume and size reduction [7, 8]. In addition, autoantibodies of exocrine carbonic anhydrase and lactoferrin are observed in exocrine pancreas [9] and infiltration of immune cells can also occur in exocrine pancreas, which indicates the existence of inflammation in the exocrine pancreas is similar to what observed in the endocrine pancreas [10]. These studies suggest that the entire pancreas, not just the endocrine islets is affected in T1D. Therefore, exploring the novel mechanistic roles associated with T1D is essential in the context of whole pancreas. Proteomic profiling of the T1D pancreatic tissue specimens has the potential to uncover the pathological changes within pancreatic islets and the surrounding exocrine tissues. However, due to limitations in samples availability, prior human pancreatic tissue proteome studies including our own have used five T1D and five healthy control tissues to explore the T1D proteomes [11, 12]. Increasing the number of T1D tissue specimens is essential to improve the confidence in disease-associated changes of the proteome, which in turn can enhance the specificity and sensitivity of the marker proteins as well as to better elucidate the T1D pathogenic mechanism.

Recent developments in mass spectrometry and other quantitative proteomics tools advanced proteome-wide profiling of a variety of sample specimens, including cell, tissue and biofluid in relevance to T1D and its comorbidities [11–13, 14]. In particular, the BoxCar label-free quantitative approach has been developed to increase the quality of MS1 signals [15]. Isobaric labeling followed by MS2 reporter ion comparison, on the other hand, is a more popular approach in quantitative proteomics as it has advantages in multiplexing and reduction of analytical variability when compared with label-free approach. By using TMT 10plex labeling approach, our previous T1D pancreatic tissue proteomics study highlighted the association of immune response, viral infection, and apoptosis with the T1D pathology [11], while others using label-free proteomics revealed the pancreatic tissue proteins involved in inflammatory, metabolic, and autoimmunity pathways in T1D and T2D [12]. Given that these two pancreatic tissue proteomic studies used different approaches in quantitation, it is desirable to apply labeling and label-free quantitative proteomics to the same samples to further improve the accuracy in proteome wide quantitation. To overcome limitations in sample size and single quantitative proteomics approach in previous studies, 18 T1D and 18 healthy control pancreatic tissues were profiled in the present work by BoxCar label-free and TMT-labeling quantitative proteomic approaches. The comparable results between label-free and labeling quantitative experiments demonstrated, with more confidence, differentially regulated proteins, dysregulated pathways, altered functional subnetworks associated with T1D. Some of the key proteins, which could serve as potential biomarkers for T1D were also validated using ELISA.

2. Materials and Methods

2.1. Pancreatic tissue collection from the nPOD repository

Cadaveric pancreatic tissues from the head region (PanHead) were obtained from the Network for Pancreatic Organ Donors with Diabetes (nPOD) repository. The nPOD usually accepts organs when processing is possible within 24 h of cold ischemia time [16]. All tissue dissection procedures were conducted by the nPOD Organ Processing and Pathology Core in accordance with federal guidelines for organ donation and the University of Florida Institutional Review Board (IRB). The nPOD provided information regarding the organ donors such as the case identification number, disease condition, tissue integrity, clinical parameters, tissue histopathological contents and serum immunological testing data (Supplementary Table S1). All work studied here was approved by the IRB of the University of North Carolina at Greensboro.

2.2. Processing tissue specimens

Tissues with approximately 25 mg from each sample were processed. In order to remove high abundance proteins contaminated by blood on the tissue sample, the samples were washed with 0.9% NaCl and PBS three times. Lysis buffer containing 5% SDS and 100 mM Tris-HCl pH 8.0 was added to the washed samples followed by intermitted sonication for 1 min using ultra-sonic probe homogenizer (QSONICA Q55, NY). After applying heat at 95°C for 20 min, protein concentration was estimated using reducing agent compatible BCA assay kit (Thermo Fisher).

2.3. S-Trap-based protein digestion

Proteomic samples were prepared using S-trap mini columns (Protifi, NY) [17]. Briefly, 300 µg of proteins from each sample were reduced and alkylated by incubating with 10 mM dithiothreitol (Sigma-Aldrich) for 30 min at 37 °C and 40 mM iodoacetamide (Sigma-Aldrich) for 1 hr at room temperature, respectively. Then 12% aqueous phosphoric acid was added into the sample with a 1:10 ratio followed by adding S-Trap binding buffer with a 1:7 ratio (90% MeOH, 100 mM triethylammonium bicarbonate (TEAB), pH7.1). Translucent protein samples were transferred into S-Trap column and were cleaned four times using 450 µl of binding buffer per each washing. Finally, 3 µg of sequencing-grade trypsin and 100 mM TEAB buffer were added and reloaded to the top of the column. It was allowed to digest for 2 hours at 47 °C. Eluted peptides were separated into two portions, one for label-free quantification and the other for TMT-labeling quantification. (Supplementary Fig. 1)

2.4. Fractionation and library construction for BoxCar label-free quantitative proteomics

To build the label-free quantitation (LFQ) spectrum library using BoxCar acquisition method, library sample was prepared by pooling 20 µg of peptides from each sample. Using StageTip-based high-pH fractionation [18], the peptides were separated into 16 fractions. After drying, the fractionated peptide samples were reconstituted in 0.1% formic acid (FA) with 3% acetonitrile (ACN) at a final concentration of 0.4 µg / µL for further analysis. About 50 µg of the pooled peptides were used for QC samples.

2.5. TMT 10plex labeling and fractionation

For TMT experiment, first 10 µg of each peptide sample was pooled for the reference peptide channel. Ten TMT 10plex reagents (Thermo Fisher) with different mass of reporter ions were labeled on N-terminus or amine group of Lysine amino acid, and the last channel of TMT-131 was used for reference peptides to normalize experimental batch effects. After quenching of excess TMT reagent using 5 % ammonium hydroxide (NH₄OH), all channels in a batch were pooled in a tube followed by cleaning up salts using C18 column (Isolute-C18, Biotage). Eluted peptides were fractionated on HPLC (Ultimate 3000, Thermo Fisher) coupled with C18 column (ZORBAX 300Extend-C18, Agilent). Mobile phases were composed of 2% ACN, 15 mM NH₄OH, pH 10 for Buffer A and 90% ACN, 15 mM NH₄OH, pH 10 for Buffer B. Sample separation was accomplished using the following linear gradient: from 0% to 7% B in 2 min, from 7% to 45% B in 78 min, from 45% to 80% B in 5 min, and held at 80% B for an additional 10 min. Separated samples were collected in a 96 deep well plate and further concatenated into 24 fractions. The fractionated peptides were dried down and reconstituted in 0.1% FA with 3% ACN at a final concentration of 0.1 µg/µL for further analysis.

2.6. LC-MS/MS analysis

Prepared peptide samples were injected and loaded onto trap column (Acclaim PepMap™ 100, 2 cm × 75 µm, Thermo fisher) and analytical column (PepMap™ RSLC C18, 50 cm × 75 µm, Thermo Fisher) followed by separation using low pH mobile phases (A: 0.1% FA in water; B: 0.1% FA in ACN) on Easy-nLC 1000 (Thermo Fisher). Peptide separation was

carried out with a linear gradient of 32% buffer B in 100 min and in 107 min for LFQ and TMT analysis, respectively, at a flow rate of 300 nL/min.

BoxCar acquisition method was activated on a Q-Exactive HF mass spectrometer (Thermo Fisher) under MaxQuant live environment [19]. Briefly, two BoxCar scans were set up with 12 boxes in each scanning. For the full scan, 250 ms injection time and 3e6 AGC target were used as well as a 120k resolution at scan range from 350 to 1,650 m/z. For the BoxCar scan, 1e6 AGC target was set to fill up with precursor ions ranging from 400 to 1,200 m/z. Top 5 precursor ions were fragmented under normalized collision energy of 27 with exclusion time of 20 sec. Injection time, resolution, and AGC target were configured as 25 ms, 8,000, and 1e5, respectively. For TMT labeled peptide analysis, the peptides were analyzed on the same instruments as used in BoxCar experiment but with different method parameters under XCalibur. One full MS scan (resolution 60k at m/z 200) followed by 20 MS/MS scan (resolution 45k at 200 m/z), 3e6 and 1e5 AGC target for full MS and MS/MS, dynamic exclusion of 30 s, and normalized collision energy of 32.

2.7. Data analysis

Raw instrument files of two datasets obtained from BoxCar LFQ and TMT labeling approach were processed using MaxQuant (ver.1.6.3.3) with a human UniProt database (05/10/2019, 73,910 entries) [20]. Carbamidomethyl on cysteines or oxidation of methionine residues and protein N-terminal acetylation were applied for fixed or variable modifications, respectively. In the case of BoxCar method, match between runs (MBRs) was applied with a parameter of 1 min for matching time window and 10 min for alignment time windows; in addition, a peptide spectrum library corresponding to 5526 proteins was used which was constructed in house after peptide level fractionation (see 2.4). In the case of TMT data search, searches were configured with fixed modifications for the TMT reagents on lysine and N-termini. Correction factors of each 10-channel reagent provided by the manufacturer were input to revise reporter ion intensities. In order to minimize the influence of the co-eluting peptides in quantification, precursor intensity fraction (PIF) was set as 0.5. The other parameters were set as default in MaxQuant for processing Orbitrap-type data.

LFQ intensity data matrix calculated by MaxQuant was used for BoxCar based quantification of proteins. For TMT experiment, corrected reporter ion intensities obtained after MaxQuant analysis were normalized with two procedures to handle the multiplicity of the data. Total sum normalization within each TMT experiment was first performed to correct for small differences in protein assay results and pipetting errors. To correct the batch effects, intensities of the pooled reference channel were used to normalize reporter ion intensities of proteins between different TMT experiments. Intensity of all pooled proteins measured between all TMT runs were averaged and used to compute adjustment factors (Supplementary Fig. 2). The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE^[21] partner repository with the dataset identifier PXD019324.

2.8. Statistics and bioinformatic approaches for data analysis

Normalized datasets were further analyzed using Perseus software (ver. 1.6.6.0) [22]. After removing reverse hits and potential contaminants, the protein intensities were log2 transformed and processed using various built-in statistical functions of Perseus. For statistical analysis between T1D and healthy group, student's t-test was performed with permutation-based FDR cut off 0.05 and S0 of 0.02.

DAVID (Version 6.8, <https://david.ncifcrf.gov/summary.jsp>) [23] and PANTHER (Version 14.1, <http://pantherdb.org/geneListAnalysis.do>) tools [24] were used to annotate gene ontology terms and pathways. The 8824 proteins identified in the pancreatic tissue were used as “background” for GO enrichment of significantly expressed proteins. Color mapping for KEGG pathways based on intensity was performed using Pathview (<https://pathview.uncc.edu/>) [25]. Visualization of protein-protein network model was drawn using Cytoscape software (Version 3.7.1) based on STRING database. Functional clustering in the network model was enriched by BiNGO app manually installed in Cytoscape [26].

2.9. ELISA-based verification of candidate proteins

ELISA kits were purchased from multiple vendors (INS from ALPCO, NH; C8A, C9, PNLIP, and VTN from LSBio, WA and COL6A3, CPB1, and PRSS3 from MyBioSource, CA). ELISA assays were carried out by following the manufacturer's protocols. The OD values of the samples in 96 well plates were obtained using Synergy 2 plate reader (Biotek). Expression levels of candidate proteins in healthy and T1D pancreatic tissue lysate were determined by 4 or 5-parameteric regression algorithm. The statistical significant difference of candidate proteins between T1D and healthy specimens was determined by Welch's t-test analysis.

3. Results

3.1. Comprehensive proteomic analysis of healthy and T1D pancreatic specimens

In large scale proteomic studies, reducing the biological variability, improving the quantitation reproducibility, analysis of large biological cohorts, and selection of well-matched disease and controls are critical in uncovering the underlying biology of the disease. In this study, 18 T1D and 18 healthy subjects that were relatively well-matched in age, gender, and BMI factors were selected. However, as expected, these two groups showed significant difference in the levels of HbA1c and C-peptide (Table 1). As shown in the schematic workflow of the study (Figure 1), the proteomes of 18 T1D and 18 healthy pancreatic head tissue specimens were analyzed individually using BoxCar label-free quantitation method (BLFQ) and also in multiplexed fashion using TMT 10plex labeling approach (TMT), with four labeling experiments performed in the latter case and samples within each labeling experiment pooled and fractionated prior to LC-MS/MS analysis. In total, 8824 proteins were identified, of which 5110 and 7963 proteins were identified by BLFQ and TMT approaches, respectively with less than 1% of protein and peptide level FDR (Fig. 2A, supplementary Table S2). The majority (83.1%) of 5110 proteins identified in BLFQ approach were also identified in TMT approach. 3767 proteins were uniquely identified in the TMT approach, which is attributed to a broader coverage of low abundance

proteins resulted from the extensive fractionation of samples at peptide level (Fig. 2B). The results from these two workflows demonstrated the comprehensiveness in coverage of pancreatic tissue proteome.

3.2. Comparison of the qualitative and quantitative results obtained from the BLFQ approach and the TMT approach

To understand whether there are biases toward proteomic coverage by these two approaches, the identified proteins were subjected to Gene Ontology (GO) enrichment analysis using DAVID database and the GO terms were ranked based on the percentage of identified proteins (Fig. 2C). Interestingly, the top 10 terms in biological processes, cellular components, and molecular functional categories showed similar percentage of proteins for both approaches. The cell-cell adhesion process was the most enriched term in both datasets in the category of biological process (Fig. 2C, upper panel). Although most of the cellular components showed a similar percentage of proteins, about 10% more extracellular proteins were identified in BLFQ compared to the TMT approach (Fig. 2C middle panel). In the category of molecular functions, more than 80% of the identified proteins were linked with protein and RNA binding in both approaches (Fig. 2C, lower panel). Next, we performed GO enrichment analysis using PANTHER. In contrast to DAVID, PANTHER uses the parent terms in enrichment analysis. PANTHER analysis revealed that percentages of proteins identified in both approaches were involved in similar molecular functions, cellular components, and biological processes (Supplementary Fig. 3). These qualitative results indicate that, although different number of proteins were uncovered in these two approaches, there are no apparent biases in the breadth of the proteomic coverage.

Next, quantitative comparison was performed on the 3097 proteins identified in at least 70% of T1D or healthy controls in the BLFQ dataset and 5396 proteins identified without missing values in all samples in TMT dataset, respectively. The intensity values of each dataset were subjected to width adjustment normalization ahead of investigating the differentially regulated proteins (DEPs) [27]. We observed highly reproducible correlations between healthy and T1D samples in both BoxCar and TMT approaches (Supplementary Fig. 4). However, two T1D pancreatic tissue samples, which showed poor correlation with other samples because of predominance of fat tissue, were excluded from further analyses (Supplementary Fig. 4). The DEPs in each dataset were identified by means of student's t-test (permutation-based FDR < 0.05, S0 = 0.02). As a result, 174 and 245 DEPs were identified in BLFQ and TMT datasets, respectively, with 83 DEPs overlapped between the two datasets. Among them, 86 and 151 proteins were upregulated, 88 and 94 proteins were downregulated in T1D in BLFQ and TMT datasets, respectively. The DEPs identified in BLFQ and TMT approaches were subjected to KEGG pathway analysis to compare the quantitative results and the altered pathways are shown in Fig 2D. The results showed that, common to both approaches, proteins involved in pancreatic secretion, and protein digestion and absorption pathways were significantly downregulated, and proteins involved in complement cascade, ECM-receptor interaction, focal adhesion, PI3K-Akt, and systemic lupus erythematosus pathways were upregulated.

3.3. Integration of BLFQ and TMT datasets improved the differentiation of healthy and T1D specimens

To find out if the quantitative results between T1D and healthy control subjects is consistent between these two quantification approaches, we compared the fold changes as measured by Log2 ratios of proteins that were quantified in both BLFQ and TMT approaches. Overall, a good correlation ($R = 0.86$) was obtained for all proteins commonly quantified in BLFQ and TMT (Fig. 3A). In particular for the 83 DEPs that were overlapped between BLFQ and TMT, we found that they showed similar patterns of upregulation and downregulation in both BLFQ and TMT datasets (Supplementary Fig. 5A). In addition, a very good correlation ($R = 0.94$) was obtained as well (Supplementary Fig. 5B). Therefore, an attempt was made to integrate the BLFQ and TMT datasets for further downstream analysis and data interpretation.

The two datasets were integrated by averaging the width adjustment normalized intensity values (supplementary Table S2). Student t-test statistics (permutation-based FDR < 0.05 , $S_0 = 0.02$) were then performed which resulted in identification of 261 DEPs between T1D and healthy controls (Fig. 3B, supplementary Table S3). We first checked the regulation status of those 83 DEPs that were identified in both BLFQ and TMT datasets before data integration. It showed that data integration did not change the regulation status for all of the 83 DEPs. In particular, 42 DEPs showed upregulation before data integration were significantly upregulated in integrated dataset. Similarly, 41 DEPs showed downregulation before data integration were still significantly downregulated after data integration. The 261 DEPs include insulin that showed a significant down regulation in T1D subjects. In addition, phosphofructokinase 2 (PFKFB2), which is known to be rapidly degraded in the absence of insulin, was also significantly decreased in T1D. On the other hand, alpha cell specific protein, glucagon and housekeeping protein, GAPDH and actin did not show significant changes between T1D and healthy groups as expected (Fig. 3B and Supplementary Fig. 6). More importantly, as shown in the heatmap of Fig. 3C, the integrated quantitative data set has improved differentiation of disease and control groups when compared with either BoxCar or TMT 10plex datasets alone (Supplementary Fig. 7), suggesting the need of data integration and the complementary nature of the BLFQ and TMT quantitative proteomics approaches.

3.4. Comparison of our data with transcriptomic atlas and plasma proteome reveals pancreas cell-type specific and plasma-specific DEPs in T1D

The pancreatic tissue consists of endocrine and exocrine compartments with diverse cell types. Mauro et al. provided a single-cell transcriptome atlas of the human pancreas that described five cell-types for endocrine and four cell-types for exocrine compartments [28]; however, up to date there is no similar cell-type specific pancreatic proteome database available as a reference. Therefore, we compared our quantitative proteomics data with the single-cell transcriptome atlas, which enabled us to classify 193, 368, 30, 12, 22, 298, 554, 73, and 124 proteins as specific for alpha, beta, delta, epsilon, PP, acinar, duct, endothelial, and mesenchyme cell-types, respectively. Among them, 13 beta cell- and 32 acinar cell-type specific proteins were significantly decreased, respectively in T1D tissue (Fig. 4A). On the other hand, 33 duct and 12 mesenchyme cell-type specific proteins of the exocrine system

were significantly upregulated in T1D tissues (Fig. 4A). Although other cell-type specific proteins showed differential regulation in T1D, they were lower in number (i.e., <5 proteins) and therefore not shown. The downregulation of beta and acinar cell-type specific proteins is predictable as T1D is associated with the destruction of the beta cells and exocrine atrophy. These cell-type specific results provide potential candidates to understand the T1D pathogenesis.

Next, to see if the DEPs can be detectable in human plasma, we compared the DEPs identified in this study with the T1D disease-related plasma proteome dataset from our previous study [14], which included >2,000 plasma proteins. Out of 261 DEPs, 117 proteins were detected in that plasma proteome dataset [14], which suggests that they may serve as potential plasma biomarker candidates for T1D disease (Fig. 4B).

3.5. Pathway and Network analyses reveal the signaling pathways and functional networks dysregulated in T1D

To assess the altered pathways in T1D, we performed the pathway enrichment analysis of 261 DEPs identified between T1D and healthy controls. The upregulated and downregulated proteins were subjected to DAVID database and the output results were listed in supplementary Table S4. DAVID analysis identified several dysregulated pathways in T1D including the downregulation of digestive system pathways (e.g., pancreatic secretion, and protein digestion and absorption) and upregulation of complement and coagulation cascades, ECM-receptor interaction, and PI3K-Akt signaling pathways, each with at least 10 proteins (supplementary Table S4). Among them, we illustrated complement and coagulation cascades (Fig. 5A), ECM-receptor interaction (Fig. 5B), and pancreatic secretion (Fig. 5C) that were most significantly enriched in T1D. In particular, the proteins associated with complement cascade were mainly upregulated in T1D when compared with coagulation cascade (Fig. 5A), which is in line with complement cascade's association with B cell receptor signaling pathway activation, immune response, and cell lysis. These results not only revealed the known complement cascade and pancreatic secretion pathways but also highlighted the novel molecular evidence for the increased activity of ECM-receptor interaction pathway in T1D.

Next, we focused on the network analysis of STRING database-validated protein-protein interactions of 261 DEPs identified in T1D. This analysis revealed three distinct subnetworks based on biological functions – the upregulated proteins in T1D were associated with complement activation and cell adhesion whereas the downregulated proteins were involved in digestion and proteolysis (Fig. 6). In particular, most of the proteins associated with these three subnetworks were identified in T1D plasma proteome showed in Figure 4B, which were marked with red text in Fig. 6. In addition, cell-type specific proteins were identified, which were upregulated in complement activation and cell adhesion subnetworks but downregulated in digestion and proteolysis subnetwork. Specifically, 15 acinar cell-type specific proteins and 8 duct cell-type specific proteins were identified in three subnetworks (Fig. 6). Although acinar cell-type specific proteins were downregulated, the duct cell-type specific proteins were significantly upregulated in T1D, and 7 proteins of them were observed in plasma dataset as well (SERPING1, C6, C1QA,

C1QC, LAMA3, LAMA5, and CD44). Interestingly, the biological functions of the identified subnetwork groups (Fig. 6) are in consistent with the three pathways that were significantly dysregulated in T1D (Fig. 5), suggesting their potential roles in T1D pathology.

3.6. ELISA-based validation confirms the key protein alterations in T1D

We selected 8 proteins from the DEPs associated with T1D for further validation, which includes the beta cell specific insulin that is downregulated in T1D and 7 proteins detectable in T1D plasma proteome [14]. Our ELISA results confirmed the downregulation of insulin in T1D group as compared to control group (Fig. 7A). Three proteins (CPB1, PRSS3, PNLIP) were acinar cell-type specific and were significantly downregulated in T1D, similarly as observed in MS-based proteomics data (Fig. 7B–D). These three proteins were associated with pancreatic secretion pathway (Fig. 5C) and digestion and proteolysis subnetwork (Fig. 6). Three other proteins (C8A, C9, and VTN) were associated with complement cascade pathway (Fig. 5A) and complement activation subnetwork (Fig. 6) – ELISA results confirmed the statistical significant upregulation of C8A and C9 proteins but not VTN ($p = 0.0949$) (Fig. 7E–H). We also confirmed the upregulation of COL6A3 involved in ECM-receptor interaction pathway (Fig. 5B), and cell adhesion subnetwork (Fig. 6). Overall, among the proteins that we validated using ELISA, most of them agree with what we observed in LC-MS based proteomics analysis.

4. Discussion

Research in T1D has been extensively focused on pancreatic islet beta cells and related autoimmunity. Examining the molecular level changes in T1D pancreas, where both the pathologic islets and surrounding environment coexist, has garnered less attention. Large-scale proteomics studies at the pancreatic tissue level can provide proteome-wide systemic changes accompanying T1D development. However, cadaveric sample availability is the major limiting factor for these studies. Collecting T1D pancreatic tissues with similar age, gender, and BMI is an even more difficult task, as differences in these factors could result in age and gender-related differential proteins, as well as proteins confounded by underlying pathological conditions related to obesity. In this study, 18 T1D and 18 healthy controls relatively well-matched in age, gender, and BMI were obtained from the nPOD (Table 1) and were processed for quantitative proteomic analysis to understand the T1D pathogenesis.

We analyzed the T1D and healthy control specimens using both BLFQ and TMT quantitative proteomic approaches. This resulted in a comprehensive proteomic dataset with 8824 identified proteins (Fig. 2A) at 1% protein and peptide FDR level. In comparison, our previous proteomic study identified 5357 proteins using five healthy and five T1D tissue specimens [11], and another study which analyzed nondiabetic, autoantibody positive, T1D and T2D proteomes identified 1167 proteins [12]. In the present study, similar qualitative and quantitative results were observed between BLFQ and TMT approaches in the context of GO terms and KEGG pathways (Figs. 2C and 2D). In addition, the fold changes of all quantified proteins in both BLFQ and TMT approaches showed a very good correlation (Fig. 3). Therefore, BLFQ and TMT datasets were merged, and 261 DEPs were identified between T1D and healthy controls. Merging of datasets increased the confidence of the

quantitative results and the DEPs clearly distinguished the T1D and healthy controls as two groups (Fig. 3C). To the best of our knowledge, this is the first study to integrate BLFQ and TMT approaches in a proteomics study.

The DEPs identified were subjected to pathway and functional network analyses, which resulted in identification of dysregulated pathways and altered functional networks in T1D (Figs. 5 and 6, Supplementary Table S4). In particular, activation of complement cascade and ECM-receptor interaction pathway and downregulation of pancreatic secretion pathway were observed in T1D. It is known that complement cascade activates proteolytic process in blood plasma against pathogens. Interestingly, a higher level of complement protein, C4d has been reported in T1D relative to T2D, autoantibody positive, and autoantibody negative pancreatic tissues [29], and the authors also observed, by using immunostaining approach, localization of C4d in blood vessel endothelium and exocrine ducts of pancreatic tissues, suggesting the existence of complement activation in T1D. Our network analysis showed the significant upregulation of a subnetwork associated with complement activation (Fig. 6). Several complement proteins of complement cascade (C1QA, C1QB, C1QC, C1R, C1S, C3, C4B, C5, C5AR1, C6, C7, C8A, C8B, C8G, and C9) identified in this study were shown with their regulation status in T1D (Fig. 5A). In particular, C5-C9 proteins were upregulated in T1D pancreatic tissue. These proteins are also major components of the membrane attack complex of complement cascade. However, future studies are needed to investigate the colocalization C5-9 proteins to confirm the formation of membrane attack complex in T1D pancreatic tissue by immunohistochemistry. Further, we also identified the upregulation of protein inhibitors, Clusterin (CLU) and Vitronectin (VTN) of the membrane attack complex in our study (Fig. 5A). Therefore, exploring the functional dynamics of the membrane attack complex would be an interesting subject in the future.

Extracellular matrix (ECM) composition could play important roles in T1D as the immune cells migrate through ECM to reach the islets. ECM is mainly made up of collagens and laminins where its interaction with cells is mediated through integrins and proteoglycans. The basement membrane of islets contains collagen type IV, laminins, and heparin sulfate proteoglycans [30]. Loss of basement membrane has been observed at the sites of leukocytes migration into the T1D islets. However, regeneration of basement membrane in long term T1D can occur when inflammation is diminished. In addition, insulinitis association with the accumulation of hyaluronan has been reported in islets [30, 31]. Our results showed upregulation of proteins associated with ECM-receptor interaction pathway in T1D (Fig. 5B). These interactions regulate different biological processes such as cell adhesion, migration, differentiation, proliferation and apoptosis. Especially, our network analysis showed the upregulation of cell adhesion in T1D, where the subnetwork consists of collagens (COL6A1, COL6A2, COL6A3, COL4A1, and COL4A2), laminins (LAMA3 and LAMA5), ITGB5, CD44, LAMB2, and TNC (Fig. 6). Among these subnetwork proteins, CD44 is a known cell adhesion protein expressed on cell surface in different splice variant forms, which can inhibit insulin secretion in pancreatic beta cells (MIN6 cell line) by decreasing LAT1-mediated amino acid uptake [32]. CD44 expression on beta cells also increases the caspase-3 activity and makes cells susceptible to apoptosis in NOD mice [33]. The later study also proposed that CD44 interaction with hyaluronic acid potentially triggers apoptosis of beta cells. Our study is the first to provide molecular evidence for the

accumulation of ECM-receptor interaction pathway components. Future studies are warranted to explore the roles of high glucose, inflammatory cytokines release, or ER stress on accumulating ECM-receptor components, and to investigate whether the upregulation is specific to exocrine, endocrine, or both compartments, which could help to elucidate the role of ECM-receptor interaction pathway in T1D pathogenesis.

Although pancreatic secretion of digestive enzymes has been well-defined in a healthy state, our knowledge on other pancreatic exocrine functional alterations in T1D condition remains elusive. Most studies in the past mainly emphasized the insulin deficiency in T1D which reflects the endocrine function. However, our previous pancreatic tissue proteomic study revealed dysregulation of exocrine proteins in T1D [11]. A recent study found significant decrease in elastase levels in T1D compared with controls, suggesting the impaired pancreas function [5]. In agreement, the present work found the downregulation of chymotrypsin-like elastase family members, CELA2A, CELA3A, and CELA3B in T1D (Fig. 5C and 6). In addition, the abnormalities in histology, anatomy, and function of exocrine pancreas have also been well-documented [3]. By using T1D and control groups with relatively well-matched age, gender, and BMI factors, a progressive reduction in pancreatic volume and microstructural changes have been observed during the first year after T1D diagnosis [8]. Our study also found significant downregulation of amylases, proteases, and lipases associated with acinar cell secretion pathway in T1D pancreatic tissues relative to healthy controls (Fig. 5C). In support, the functional network analysis showed significant downregulation of proteins involved in digestion and proteolysis in T1D (Fig. 6). These results suggest that T1D disease is not only restricted to beta cell dysfunction but also associated with exocrine changes. The decrease in exocrine digestive enzymes secretion in T1D could be due to abnormal function of acinar cells, inflammation during insulinitis, or pancreatic anatomical changes such as reduction in pancreas volume, which needs further investigation.

Scarcity of human T1D pancreatic tissue is a limiting factor in the investigation of the pathogenic mechanism of T1D. To this end, the nPOD has set up a biobank to provide these precious specimen for basic biomedical research, including limited number of flash-frozen pancreatic tissue sections for proteomic studies [16]. Despite this, it is of note that for the 18 healthy and 18 T1D tissue samples used in this study, two of the T1D tissue samples were determined as outliers because of their large fat content. In the present study, we identified 261 DEPs using 18 healthy and 16 T1D pancreatic tissue specimens. These 261 DEPs were compared with our previous study in which we used 5 healthy and 5 T1D tissues [11]. We found that 47 of 261 DEPs with significant differential regulation in our previous study (Permutation-based FDR < 0.05, and $S_0 = 0.02$). This could be due to gender and race differences between healthy and T1D sample selection in our previous study, which were minimized in the present study (Supplementary Table S1). Other contributing factors might include the pancreas tissue heterogeneity resulted from differences in diabetes duration among T1D patients. In this regard, duration of T1D ranged from newly diagnosed to >20 years in the samples used in this study. While this suggests that T1D stage-specific or T1D duration-specific pancreatic tissue proteomics analysis may reduce the differences between the DEPs identified in different studies, it likely will be very challenging to obtain enough specimens matched in T1D duration from organ donors. An alternative, however, is to

validate the DEPs using large number of T1D plasma samples longitudinally collected during T1D progression to confirm the specificity of candidate tissue markers.

5. Conclusions

In this study, healthy and T1D cadaveric pancreatic tissue specimens relatively well-matched in terms of age, gender, and BMI factors were obtained from the nPOD and processed for BoxCar label-free and TMT 10plex labeling quantitative proteomic analysis. We demonstrated in this study, for the first time, that BoxCar and TMT 10plex approaches revealed similar proteomic coverages in the context of GO terms and KEGG pathways, although more proteins were identified because of extensive peptide level sample fractionation in the latter. Importantly, the data integration of label and label-free approaches increased the confidence of quantitative proteomic results. Pathway and functional network analyses revealed biologically significant proteins associated with T1D. These proteins were mainly linked with complement activation, accumulation of ECM-receptor interaction pathway components, and decreased pancreatic secretion and digestion. Furthermore, some of the proteins associated with these altered pathways and functional subnetworks were validated using ELISA. Future studies on complement-mediated membrane attack complex function, ECM-receptor components accumulation, and validation of biomarker proteins using large biological cohorts would be helpful to further our understanding of T1D disease development and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of clinical significance

Type 1 Diabetes (T1D) is characterized by insulin deficiency as a result of insulinitis and immune-mediated destruction of pancreatic beta cells. In addition to the endocrine dysfunction, increasing evidence also point to abnormalities of the exocrine pancreas in the pathology of T1D. Unlike human islets, human pancreatic tissue from T1D patients provide the ideal specimen to examine the proteomic changes at both the endocrine and the exocrine levels and enable uncovering the molecular interactions between these two that contribute to the pathogenesis of T1D. To this end, we integrated label-free and isobaric labeling based-quantitative proteomics to investigate the pancreatic tissue proteome changes of T1D and healthy subjects. We identified dysregulated proteins important to pancreatic exocrine function, complement coagulation cascades, and ECM receptor interaction pathways in T1D. The results further our understanding of the dysregulated pathways and functional subnetworks associated with T1D pathogenesis and may aid to develop diagnostic and therapeutic strategies for this disorder.

Fig. 1. Schematic workflow of the study. Pancreatic tissue samples (18 healthy and 18 T1D) obtained from nPOD were lysed, S-trap digested, and analyzed by both BoxCar label-free and TMT 10plex labeling approaches. QE-HF raw files were processed using MaxQuant. Data analysis was performed using bioinformatic tools to interpret the biological significance.

Fig. 2. Qualitative and quantitative comparison of BoxCar and TMT 10plex results. (A) Overlap between the proteins identified in BoxCar and TMT 10plex approaches. (B) Assessment of low and high abundance proteins identified in BoxCar, TMT 10plex, or both approaches. X axis: log 10 of the summed ion intensities of protein. (C) Percentages of proteins identified in BoxCar and TMT 10plex approaches for the top 10 Gene Ontology terms in three categories, biological processes (top panel), cellular components (middle panel), and molecular functions (lower panel). (D) List of KEGG pathways significantly (Benjamini-Hochberg FDR < 0.05) associated with the DEPs identified in BoxCar and TMT 10plex approaches. The figure shows the pathways that were significantly regulated with at least 10% of DEPs in BoxCar or TMT approaches.

Fig. 3.

Integration of BoxCar and TMT 10plex datasets. (A) Correlation between the log2 fold changes of proteins quantified in both BoxCar and TMT 10plex approaches. P values of proteins were calculated from t-test between HC and T1D within each dataset. (B) Volcano plot shows the 261 DEPs identified ($S0 = 0.02$ and permutation-based $FDR < 0.05$) after integration of BoxCar and TMT 10plex datasets. (C) Hierarchical clustering analysis of the 261 DEPs identified between healthy controls and T1D patients.

Fig. 4. Integration of the DEPs identified between T1D and healthy controls with previously published resource databases. (A) Radar plot shows beta, acinar, duct, and mesenchyme cell type-specific DEPs identified in T1D based on a single-cell transcriptome atlas of the human pancreas [Ref 28]. (B) Venn diagram shows overlap between DEPs identified in this study and a T1D plasma proteome dataset [Ref 14].

Fig. 5. KEGG pathway analysis of the DEPs identified between T1D and healthy controls from the integrated dataset. Shown are the upregulation of coagulation and complement cascades (A) and ECM-receptor interaction pathways (B), and downregulation of pancreatic secretion pathway (C) in T1D. The scale bar represents the color intensity based on the normalized fold changes (between -1 and 1) of the quantified proteins including the DEPs.

Fig. 6.

Functional networks analysis of the DEPs identified between T1D and healthy controls. The upregulation of complement activation (A) and cell adhesion (B), and downregulation of digestion and proteolysis subnetworks in T1D are shown. The node boarder colors with yellow (acinar), blue (duct), green (epsilon), or pink (mesenchyme) represent the pancreatic cell-type specific proteins. The DEPs identified in previously published T1D plasma proteome [Ref 14] are shown with red color text. Node size is proportional to the significance of protein fold changes. The scale bar shows the color intensity with protein normalized fold changes between T1D patients and healthy controls.

Fig. 7.

ELISA validation of the DEPs identified in T1D. ELISA analysis confirmed the significant downregulation of INS, CPB1, PRSS3, and PNLIP ($p < 0.05$), and upregulation of COL6A3, C9, and C8A ($p < 0.05$) in T1D. VTN upregulation was not significant between T1D patients and healthy controls. The differential significance between healthy controls and T1D was determined by Welch's t-test.

Table 1.

Clinical details of nPOD pancreatic specimens used in this study (see Supplementary Table S1 for more details).

	Sex (n)	Age [*] (year)	Diabetes duration (year)	RIN	BMI [*] (kg/m ²)	C-pep (ng/ml)	HbA1c (mmol/mol)
Healthy	Male (13)	28.8 ± 11.5	-	4.3 ± 1.5	24.6 ± 5.5	9.9 ± 6.4	5.5 ± 0.4
	Female (5)	13.7 ± 3.2	-	5.1 ± 1.7	21.6 ± 3.2	16.5 ± 15.7	5.6 ± 0.4
T1D	Male (15)	27.2 ± 9.6	15.2 ± 9.5	5.2 ± 2.2	22.8 ± 3.8	0.1 ± 0.15	9.5 ± 2.6
	Female (3)	14.2 ± 2.7	5.7 ± 4.5	6.8 ± 0.2	23.1 ± 4.6	0.1 ± 0.02	9.7 ± 0.2

^{*} No statistical significant differences in age and BMI exists between healthy and T1D groups; *p* values for age: 0.92, 0.87 and 0.70, *P* values for BMI: 0.56, 0.71 and 0.35 for group, female and male of group, respectively. Student's T-test: two tailed, unequal variance between groups.