



Distinct secretion pattern of serum proinsulin in different types of diabetes

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Background: Latent autoimmune diabetes in adults (LADA) is characterized by autoimmunity, late-onset and intermediate beta-cell deprivation rate between type 2 diabetes mellitus (T2DM) and type 1 diabetes mellitus (T1DM). Herein, we investigated proinsulin (PI) secretion patterns and the endoplasmic reticulum (ER) dysfunction biomarker, PI-to-C-peptide (PI:CP) ratio, to elucidate beta-cell intrinsic pathogenesis mechanisms in different types of diabetes.

Methods: Total serum fasting PI (FPI) were measured in adult-onset and newly-diagnosed diabetes patients, including 60 T1DM, 60 LADA and 60 T2DM. Thirty of each type underwent mixed meal tolerance tests (MMTTs), and hence 120 min postprandial PI (PPI) were detected. PI:CP ratio = PI (pmol/L) ÷ CP (pmol/L) × 100%. PI-related measurements among types of diabetes were compared. Correlation between PI-related measurements and beta-cell autoimmunity were analyzed. The possibility of discriminating LADA from T1DM and T2DM with PI-related measurements were tested.

Results: FPI and PPI were significantly higher in LADA than T1DM ($P < 0.001$ for both comparisons), but lower than those in T2DM ($P < 0.001$ and $P = 0.026$, respectively). Fasting PI:CP ratio was significantly higher in T1DM than both LADA and T2DM (median 3.25% *vs.* 2.13% and 2.32%, $P = 0.011$ and $P = 0.017$, respectively). In LADA, positive autoantibody numbers increased by both fasting and postprandial PI:CP ratio ($P = 0.007$ and $P = 0.034$, respectively). Areas under receiver operation characteristic curves (AUC_{ROC}) of FPI and PPI for discriminating LADA from adult-onset T1DM were 0.751 ($P < 0.001$) and 0.838 ($P < 0.001$), respectively. Between LADA and T2DM, AUC_{ROC} of FPI and PPI were 0.685 ($P < 0.001$) and 0.741 ($P = 0.001$), respectively.

Conclusions: In the development of autoimmune diabetes, interplays between ER stress and beta-cell autoimmunity are potentially responsible for severer beta-cell destruction. PI-related measurements could help in differentiating LADA from adult-onset T1DM and T2DM.

Keywords: Latent autoimmune diabetes in adults (LADA); autoimmune diabetes; endoplasmic reticulum stress; pancreatic beta cells; proinsulin (PI)

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Introduction

Latent autoimmune diabetes in adults (LADA) stands for late-onset (later than 30-year-old) diabetes patients identified from type 2 diabetes mellitus (T2DM) by beta-cell autoimmunity, which is assessed by serum islet autoantibodies (1,2). LADA patients are independent from insulin treatment for at least 6 months after disease initiation, but would progress considerably faster towards total loss of pancreatic beta-cell than T2DM patients (3). Adult-onset type 1 diabetes mellitus (T1DM) and LADA patients composed of the whole adult-onset autoimmune diabetes population (4). When compared to classic T1DM, the genetic loads, beta-cell autoimmunity and beta-cell deprivation rate of adult-onset autoimmune diabetes was significantly lower in LADA patients (5-8), but studies on differences between adult-onset T1DM and LADA were scarce. Underneath mechanisms of a much slower beta-cell damage in LADA than adult-onset T1DM, especially on beta-cell intrinsic properties, were still largely unknown.

It is well-known that the development of T1DM is dominantly autoimmune-derived, whereas LADA, which accounts for a large amount in autoimmune diabetes, shares milder autoimmunity in the disease development (1,9,10). Yet, recent discoveries have elucidated alternative disruption of pancreatic beta-cells by non-autoimmune processes (11,12), especially pathological pathways intrinsic to beta-cells during the development of T1DM (13,14). Also, as a possible intermediate form between T1DM and T2DM, LADA shares features like insulin resistance with T2DM, through which overwhelmed beta-cells could suffer from excessive endoplasmic reticulum (ER) stress (12,15). The production of insulin in pancreatic beta-cells is tightly controlled by a series of organelles involving ER (16). Proinsulin (PI), the direct matrix of bio-reactive insulin, processed in ER, stands at the checkpoint of insulin production (17). PI has been largely proved to be actively participated in promoting autoimmunity (18,19). Meanwhile, disturbed beta-cells with dysfunctional ER would discharge improperly modified PI molecules and correspondingly decreased amount C-peptide (CP) and insulin, which provided a reasonable method for identifying ER stress, a sign of beta-cell disturbances, from peripheral blood by measuring serum PI to CP (PI:CP) or PI to insulin ratios (20). Studies on beta-cell lines and rodent models found that pathways involved in ER stress during PI processing were crucial for beta-cell function and survival (21,22) and early autoimmunity initiation (23).

Meanwhile, disproportionately elevated PI was a sensitive biomarker for ER dysfunction (24,25). Peripheral PI concentration in human was intimately related to beta-cell stress (26). In human T1DM patients, elevated circulating PI and PI:CP ratios, abnormal expression of ER stress markers were found in newly onsets (27,28). Scientists also adduced that increased PI:CP ratio was related to T1DM progression in first-degree relatives with positive serum islet autoantibodies (24,25,29,30). In T2DM, ER stress has also shown early participation in disease initiation (11,31). In long-established T1DM, PI appeared to be an effective measurement of residual beta-cell function in individuals with undetectable insulin and CP levels (32). Aforementioned evidence indicated that, either in T1DM or in T2DM, PI:CP ratio is an early trait and a sensitive biomarker of beta-cell destruction either due to intrinsic beta-cell dysfunction or autoimmune attack. Conclusively, results on the important role of ER stress in both T1DM and T2DM enlightened corresponding research on LADA patients, to elucidate insulin production changes in the development of LADA and to evaluate how beta-cell autoimmunity has participated in this process.

Herein, we designed a study in exploring beta-cell function and dysfunction in newly diagnosed and adult-onset diabetes patients, including T1DM, LADA and T2DM. In this study, we focused on PI-related measurements, namely fasting and postprandial PI and PI:CP ratio, and in this manner may we probe into the beta-cell intrinsic pathogenesis mechanisms of different types of diabetes, especially LADA.

Methods

Study subjects

Sixty T1DM, 60 LADA and 60 T2DM patients diagnosed within 2 years were recruited in the Second Xiangya Hospital of Central South University, Changsha, China. All subjects were diagnosed with diabetes after 18-year-old. Diabetes was diagnosed on the basis of the World Health Organization (WHO) criteria for diabetes mellitus [1999] (33). Both T1DM and T2DM were diagnosed according to the criteria of American Diabetes Association (ADA) (34). LADA patients were diagnosed according to the 2005 Immunology of Diabetes Society (IDS) criteria with (I) at least one positive serum islet autoantibody [glutamate decarboxylase autoantibody (GADA), protein tyrosine phosphatase autoantibody (IA-2A) and zinc transporter

8 autoantibody (ZnT8A)]; (II) hyperglycemia controlled with insulin-independent therapy for at least 6 months after disease onset; (III) disease onset at 30-year-old or older (35). Specific medical treatments, including glucagon-like peptide 1 (GLP-1) receptor agonists and dipeptidyl peptidase 4 (DDP-4) antagonists, pancreas or islet transplantation, metabolic surgeries and immunotherapy, could directly manipulate beta-cell function and change the production of either insulin, CP or PI. Therefore, patients underwent these prescriptions were excluded. Pancreatectomy, pancreatitis, and injuries or diseases (other than diabetes) adversely affect pancreas function or immune system could possibly manipulate the beta-cell performance, and hence were also listed in our exclusion criteria.

Anthropometric and biochemical measurements

Height, body weight, waist and hip circumferences and blood pressure were measured in a standardized procedure. Based on these measurements, body mass index (BMI) and waist-hip ratio (WHR) were calculated as follow: BMI (kg/m^2) = body weight [kilograms, kg] \div [height (meters, m)]² and WHR = Waist circumferences [meters, m] \div Hip circumferences [meters, m]. Fasting serum samples were obtained from all 180 patients. Among them, 30 T1DM, 30 LADA and 30 T2DM patients underwent mixed meal tolerance tests (MMTTs), and hence 120 min postprandial serum samples were collected. Total serum PI concentration, including fasting PI (FPI) and 120 min postprandial PI (PPI), were measured with STELLUX Proinsulin Chemiluminescence enzyme-linked immunosorbent assay (ELISA) kit (ALPCO) on serum samples stored at minus 80 degrees centigrade. Upon detection, all samples underwent less than two freeze/thaw cycles. Cross-reactivity with human CP and human insulin of this ELISA kit are reported to be lower than 0.01% and 0.1%, respectively. To obtain the concentration of samples with Chemiluminescence reads (relative light units, RLU) below the lower limit of quantitation (LLOQ), we calculated with a simple linear equation based on two points, namely point “zero” and “the lowest standard concentration on the standardized curve” of each ELISA plate. Following detection were conducted with serum samples upon collection: both fasting CP (FCP) and 120 min postprandial CP (PCP) levels were measured by a chemiluminescence kit (Adiva Centaur System, Siemens, Germany); fasting and postprandial PI:CP ratios were calculated as molar ratios of PI (pmol/L) to CP (pmol/

L) multiplied by 100%; fasting blood glucose (FBG), 120 min postprandial blood glucose (PBG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL) were measured by an automatic chemistry analyzer (Hitent 7170A, Japan); fasting venous blood collected in anti-coagulate tubes were used in hemoglobin A1c (HbA1c) detection, using automated liquid chromatography (Bio-Rad VARIANT-II Hemoglobin Testing System, United States); the detection of three serum islet autoantibodies, namely GADA, IA-2A and ZnT8A, were of Diabetes Antibody Standardization Program (DASP) standard and measured by radioligand binding assay in duplicate.

Statistical analyses

Chi-square tests were applied on categorical variables, and Fisher exact tests were used for crosstabs cells with less than five expected counts. For variables with more than 2 categories, Cramer's V value were calculated. All continuous variables, excepting BMI and WHR, are not normally distributed based on Shapiro-Wilk tests and Quantile-Quantile plots. Hence, the medians (interquartile ranges, IQRs) were calculated and compared among groups by Kruskal-Wallis tests with Bonferroni post hoc comparisons. For BMI and WHR, ANOVA tests were performed with Bonferroni post hoc comparisons. Age and BMI were not perfectly matched across groups, and data on these analyses were not adjusted for these two measurements and were therefore descriptive. Generalized linear model was applied to assess differences of PI-related parameters with skewed distribution in different groups adjusted for age, BMI, and diabetes duration. Receiver operating characteristic (ROC) curves were plotted and area under ROC curves (AUC_{ROC}) was calculated based on fasting PI-related measurements for all patients and 120 min postprandial PI-related measurements for patients underwent MMTTs. Spearman correlation analysis and Mantel-Haenszel Chi-Square tests were performed along with Spearman correlation covariates (r_s) and Goodman and Kruskal's gamma [G] values calculated to assess relationships between beta-cell autoimmunity and PI-related parameters.

All tests were considered significant with two-sided P values lower than 0.05. Statistical analysis was carried out with GraphPad Prism version 8.0.0 for Windows (GraphPad Software, California, USA) and IBM SPSS version 25.0 for Windows (IBM Corp, IBM SPSS Statistics, Armonk, NY, USA).

Results

PI-related measurements in LADA were of intermediate levels between T1DM and T2DM

Fasting measurements were compared among groups for all 180 patients, and 120 min postprandial measurements were compared among groups for 90 patients underwent MMTTs (*Table 1*). Anthropometric and biochemical characteristics showed in *Table 1* were compared among three types of diabetes without adjustments for unmatched parameters, namely age and BMI, and were therefore descriptive. There were no differences in diabetes duration or sex among groups. Age was significantly different among groups, and T1DM patients were younger than both LADA and T2DM patients ($P < 0.001$ for both comparisons). Under ANOVA tests, significant differences were presented among groups for both BMI and WHR (for both measurements $P < 0.001$, and post hoc comparisons showed $P = 0.003$ between T1DM and LADA, $P < 0.001$ between LADA and T2DM and $P < 0.001$ between T1DM and T2DM).

Glucose metabolism parameters measured at fasting states, including FBG and HbA1c, presented hyperglycemia of all three groups and revealed better glycemic control in T2DM than both T1DM and LADA. As expected, T1DM had the lowest levels of FCP and FPI, and these levels were significantly lower in LADA than in T2DM ($P < 0.001$ for all comparisons, *Figure 1A*). The median of FPI in T1DM was 2.58, and were 5.29 and 7.97 in LADA and T2DM, respectively. Medians of FPI:FCP ratio of T1DM, LADA and T2DM were 3.25%, 2.13% and 2.32%. FPI:FCP ratio was significantly different among groups as shown in *Figure 1B* ($P = 0.005$). Pairwise comparisons revealed higher FPI:FCP ratio in T1DM than LADA as well as in T1DM than T2DM ($P = 0.011$ and $P = 0.017$, respectively), but these differences were not sustained between LADA and T2DM. Among 90 patients underwent MMTTs, the distribution of PBG was only significant different between T1DM and T2DM ($P < 0.001$) but not between either T1DM and LADA or between T2DM and LADA. Median of PPI and delta PI were listed as follow: in T1DM, median PPI = 7.46 and median delta PI = 4.65, in LADA these two levels were 21.81 and 14.86 while in T2DM they were 37.61 and 27.67. As observed in comparisons on fasting parameters, the concentration of PCP, PPI, delta CP and delta PI were higher in LADA patients than T1DM but were lower than that in T2DM (*Figure 1C,D*). When it came to PPI:PCP ratio, the differences among three groups did not reach statistical significance ($P = 0.699$, *Figure 1E*).

Because all eight beta-cell function measurements, including FCP, FPI, FPI:FCP ratio, PCP, PPI, delta CP, delta PI and PPI:PCP ratio, showed skewed distributions, comparisons of these parameters among groups were adjusted using generalized linear models with log-linked gamma distribution, followed by least significance differences (LSD) tests for pairwise post-hoc comparisons (*Table 2*). After adjustments for (I) age; (II) age and BMI; (III) age, BMI and diabetes duration, differences among groups remained significant except for (I) in model 2 adjusting age among groups, the difference of FPI:FCP ratio was not significant between T1DM and LADA ($P > 0.05$) and (II) in model 3 adjusting age and BMI and model 4 adjusting age, BMI and diabetes duration, the differences of FPI were not significant between LADA and T2DM ($P > 0.05$).

PI secretion patterns were correlated to autoimmunity in autoimmune diabetes

Islet autoimmune antibodies characterized both T1DM and LADA. As indicated in previous studies, the number of autoantibodies represented the severity of beta-cell autoimmunity, especially in LADA (36). These autoantibodies were responsible for immune attacks targeting pancreatic beta-cells and subsequent beta-cell dysfunctions. Hence, correlations between autoimmune reactions and the secreting patterns of PI were measured to evaluate possible relationships between beta-cell dysfunction and autoimmunity. We have tested these relationships in LADA patients ($n = 60$), as well as the whole autoimmune diabetes population which encompasses T1DM and LADA ($n = 120$). For postprandial measurements, these relationships were tested on participants underwent MMTTs [LADA patients ($n = 30$), as well as the whole autoimmune population ($n = 60$)].

According to Spearman correlation analysis, among autoimmune diabetes patients, the autoantibody IA-2A titer was positively correlated with FPI:FCP ratio ($r_s = 0.202$, $P = 0.027$). Among LADA patients, $r_s = 0.429$, $r_s = 0.413$, respectively for correlations between IA-2A titer and PPI:PCP ratio and between ZnT8A titer and PPI:PCP ratio ($P = 0.018$, $P = 0.023$, respectively). Meanwhile, trends in autoimmune reactions by PI-related measurements quartile were observed. In these trend tests, we divided the whole autoimmune population (including T1DM and LADA patients) and LADA patients by either FPI or FPI:FCP ratio quartiles (by second, third and fourth quartile into Q1, Q2, Q3 and Q4). Ordinal to ordinal correlations

Table 1 Clinical characteristics of all participants with type 1 diabetes mellitus, LADA and type 2 diabetes mellitus

Variants	Diagnosis			P [†]	P [‡]	P [§]	P [¶]
	T1DM (median, IQR)	LADA (median, IQR)	T2DM (median IQR)				
All participants							
n	60	60	60				
Age (years)	36.79 (27.35, 42.57)	45.57 (37.74, 57.72)	46.08 (39.16, 55.19)	<0.001	<0.001	1.000	<0.001
Diabetes duration (months)	6.23 (2.14, 11.68)	3.90 (1.25, 9.97)	3.85 (1.39, 9.18)	n.s.	N/A	N/A	N/A
Female (%)	24 (40.00)	25 (41.70)	22 (36.70)	n.s.	N/A	N/A	N/A
BMI (kg/m ²)	20.54 (19.09, 22.11) 20.51 (2.36)	21.68 (19.98, 24.35) 22.20 (2.67)	24.56 (21.75, 26.60) 24.39 (3.16)	<0.001	0.003	<0.001	<0.001
WHR	0.83 (0.78, 0.87) 0.83 (0.06)	0.87 (0.82, 0.91) 0.87 (0.06)	0.91 (0.86, 0.98) 0.92 (0.07)	<0.001	0.003	<0.001	<0.001
SBP (mmHg)	116 (104, 125)	116 (109, 125)	130 (118, 140)	<0.001	n.s.	<0.001	<0.001
DBP (mmHg)	70 (65, 79)	73 (66, 81)	80 (75, 89)	<0.001	n.s.	<0.001	0.001
Autoantibody positivity (%)	41 (68.30)	60 (100.00)	N/A	<0.001	<0.001	N/A	N/A
Multiple autoantibodies (%)	17 (28.30)	21 (35.00)	N/A	n.s.	n.s.	N/A	N/A
HbA1c (%)	8.30 (6.90, 9.60)	8.60 (6.60, 10.90)	6.60 (6.00, 9.20)	0.005	n.s.	0.010	0.023
FBG (mmol/L)	7.28 (6.01, 9.61)	6.65 (5.64, 8.64)	6.18 (5.28, 7.32)	0.014	n.s.	n.s.	0.013
FCP (pmol/L)	63.70 (19.60, 150.60)	225.60 (145.30, 368.90)	424.30 (280.40, 562.00)	<0.001	<0.001	<0.001	<0.001
FPI (pmol/L)	2.58 (0.38, 4.34)	5.29 (2.78, 8.13)	7.97 (5.40, 13.57)	<0.001	<0.001	<0.001	<0.001
FPI:FCP ratio	3.25 (2.21, 5.55)	2.13 (1.46, 3.16)	2.32 (1.34, 3.42)	0.005	0.011	1.000	0.017
GADA titer	0.19 (0.01, 0.95)	0.70 (0.15, 1.58)	0.00 (-0.01, 0.00)	<0.001	0.011	n.s.	n.s.
IA-2A titer	0.00 (0.00, 0.19)	0.00 (0.00, 0.06)	0.00 (0.00, 0.00)	<0.001	n.s.	<0.001	<0.001
ZnT8A titer	0.00 (0.00, 0.01)	0.00 (0.00, 0.01)	0.00 (0.00, 0.00)	<0.001	n.s.	<0.001	<0.001
TG (mmol/L)	0.78 (0.62, 1.13)	0.99 (0.67, 1.52)	1.37 (0.91, 2.09)	<0.001	n.s.	0.020	0.001
TC (mmol/L)	4.29 (3.65, 4.67)	4.44 (3.73, 4.84)	4.56 (3.63, 5.15)	n.s.	N/A	N/A	N/A
HDL (mmol/L)	1.47 (1.29, 1.76)	1.19 (1.09, 1.36)	1.15 (0.97, 1.38)	<0.001	<0.001	n.s.	<0.001
LDL (mmol/L)	2.20 (1.91, 2.69)	2.67 (2.09, 3.01)	2.82 (2.14, 3.42)	0.012	n.s.	n.s.	0.011
Participants underwent MMTTs							
n	30	30	30				
PBG (mmol/L)	17.03 (13.80, 23.55)	14.63 (10.31, 16.91)	11.50 (7.37, 14.58)	<0.001	n.s.	n.s.	0.001
PCP (pmol/L)	266.60 (38.00, 444.80)	883.70 (596.50, 1,154.80)	1,344.30 (988.70, 1,813.80)	<0.001	<0.001	0.015	<0.001
delta CP (pmol/L)	132.30 (2.60, 353.20)	543.90 (357.80, 847.10)	962.70 (589.10, 1,285.80)	<0.001	<0.001	n.s.	<0.001
PPI (pmol/L)	7.46 (1.56, 12.55)	21.81 (13.98, 32.05)	37.61 (25.60, 65.69)	<0.001	<0.001	0.026	<0.001
delta PI (pmol/L)	4.65 (0.13, 9.13)	14.86 (6.06, 22.54)	27.67 (13.41, 51.31)	<0.001	0.001	0.045	<0.001

Table 1 (Continued)

Table 1 (Continued)

Variants	Diagnosis			P [†]	P [‡]	P [§]	P [¶]
	T1DM (median, IQR)	LADA (median, IQR)	T2DM (median IQR)				
PPI:PCP ratio	2.87 (1.70, 4.80)	2.41 (1.55, 4.07)	2.87 (1.78, 4.32)	n.s.	N/A	N/A	N/A

Values are presented as the median (IQR), excepting BMI and WHR which are reported in both median (IQR) and mean (SD). Comparisons were carried out with Kruskal-Wallis tests among groups with Bonferroni post hoc pairwise comparisons. P[†] values for comparisons among T1DM, LADA and T2DM, P[‡] for post hoc comparisons between T1DM and LADA, P[§] for post hoc comparisons between LADA and T2DM, P[¶] for post hoc comparisons between T1DM and T2DM. T1DM, type 1 diabetes mellitus; LADA, latent autoimmune diabetes in adults; T2DM, type 2 diabetes mellitus; MMTTs, mixed meal tolerance tests; IQR, interquartile range; BMI, body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; Multiple Autoantibodies, multiple autoantibody positivity; HbA1c, Hemoglobin A1c; FBG, fasting blood glucose; FCP, fasting C-peptide; FPI, fasting proinsulin; FPI:FCP ratio, fasting proinsulin to C-peptide ratio; PBG, 120 min postprandial blood glucose; PCP, 120 min postprandial C-peptide; delta CP, PCP – FCP; PPI, 120 min postprandial proinsulin; delta PI, PPI – FPI; PPI:PCP ratio, 120 min postprandial proinsulin to C-peptide ratio; TG, total cholesterol; TC, triglyceride; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; n.s., not significant; N/A, not applicable.

were tested by Goodman and Kruskal's gamma (G). In the whole autoimmune population, Mantel-Haenszel Chi-square tests showed a likelihood of increasing FPI from negative autoantibody to positive autoantibody ($n=30$ for each quartile, $P=0.003$, *Figure 2A*), and $G = 0.547$ for correlations between FPI quartiles and autoantibody positivity ($P=0.002$). Trend from zero autoantibody to three positive autoantibodies by FPI quartile were also revealed in the whole autoimmune population ($n=30$ for each quartile, $P=0.036$, *Figure 2B*), and $G = 0.226$ between FPI quartile and positive autoantibody numbers ($P=0.036$). In LADA, significant trends were observed from one positive autoantibody to three positive autoantibodies by FPI:FCP ratio quartile ($n=15$ for each quartile, $P=0.007$, *Figure 2C*), and $G = 0.447$ between FPI:FCP ratio quartile and positive autoantibody numbers ($P=0.010$). Autoimmunity increased by PPI:PCP ratio quartile reached statistical significance in positive autoantibody number in LADA patients (Q1 $n=8$, Q2 $n=7$, Q3 $n=8$, Q4 $n=7$, $P=0.034$, *Figure 2D*), and $G = 0.517$ for correlation between PPI:PCP ratio quartile and positive autoantibody number ($P=0.021$).

PI-related parameters could discriminate LADA from T1DM and T2DM

To evaluate the possibility of applying PI-related parameters in discriminating forms of diabetes, we plotted ROC curves of FPI and FPI:FCP ratio separately on all patients, and of PPI, delta PI, and PPI:PCP ratio separately on patients underwent MMTTs. These curves were established between T1DM and LADA, as well as between LADA and T2DM (*Figure 3*). The AUC_{ROC} of ROC curves built

between LADA and T1DM, as well as between LADA and T2DM for FPI, PPI, and delta PI reached 0.700, and we calculated cut-off values choosing Youden index as the technical criterion for these discriminating tests. ROC curves between T1DM and LADA (*Figure 3A*) demonstrated (I) FPI AUC_{ROC} for discriminating LADA and T1DM was 0.751 ($P<0.001$, cut-off =3.76, sensitivity =66.67%, specificity =71.67%), (II) delta PI AUC_{ROC} for discriminating LADA and T1DM was 0.793 ($P<0.001$, cut-off =11.06, sensitivity =66.67%, specificity =90.00%) and (III) PPI AUC_{ROC} for discriminating LADA and T1DM was 0.838 ($P<0.001$, cut-off =12.45, sensitivity =80.00%, specificity =76.67%). Between LADA and T2DM (*Figure 3B*), ROC curves revealed (I) FPI AUC_{ROC} for discriminating LADA and T2DM was 0.685 ($P<0.001$). For patients underwent MMTTs, (II) delta PI AUC_{ROC} for discriminating LADA and T2DM was 0.709 ($P=0.005$, cut-off =23.09, sensitivity =76.67%, specificity =60.00%) and (III) PPI AUC for discriminating LADA and T2DM was 0.741 ($P<0.01$, cut-off =35.21, sensitivity =86.67%, specificity =56.67%).

Discussion

In this cross-sectional study, we summarized PI secretion patterns among adult-onset T1DM, LADA and T2DM patients at their onsets. Specifically, LADA patients possess mid-way beta-cell properties between adult-onset T1DM and T2DM as measured by PI-related parameters. These parameters were applicable in discriminating LADA from adult-onset T1DM and LADA from T2DM. We also presented positive correlations between beta-cell

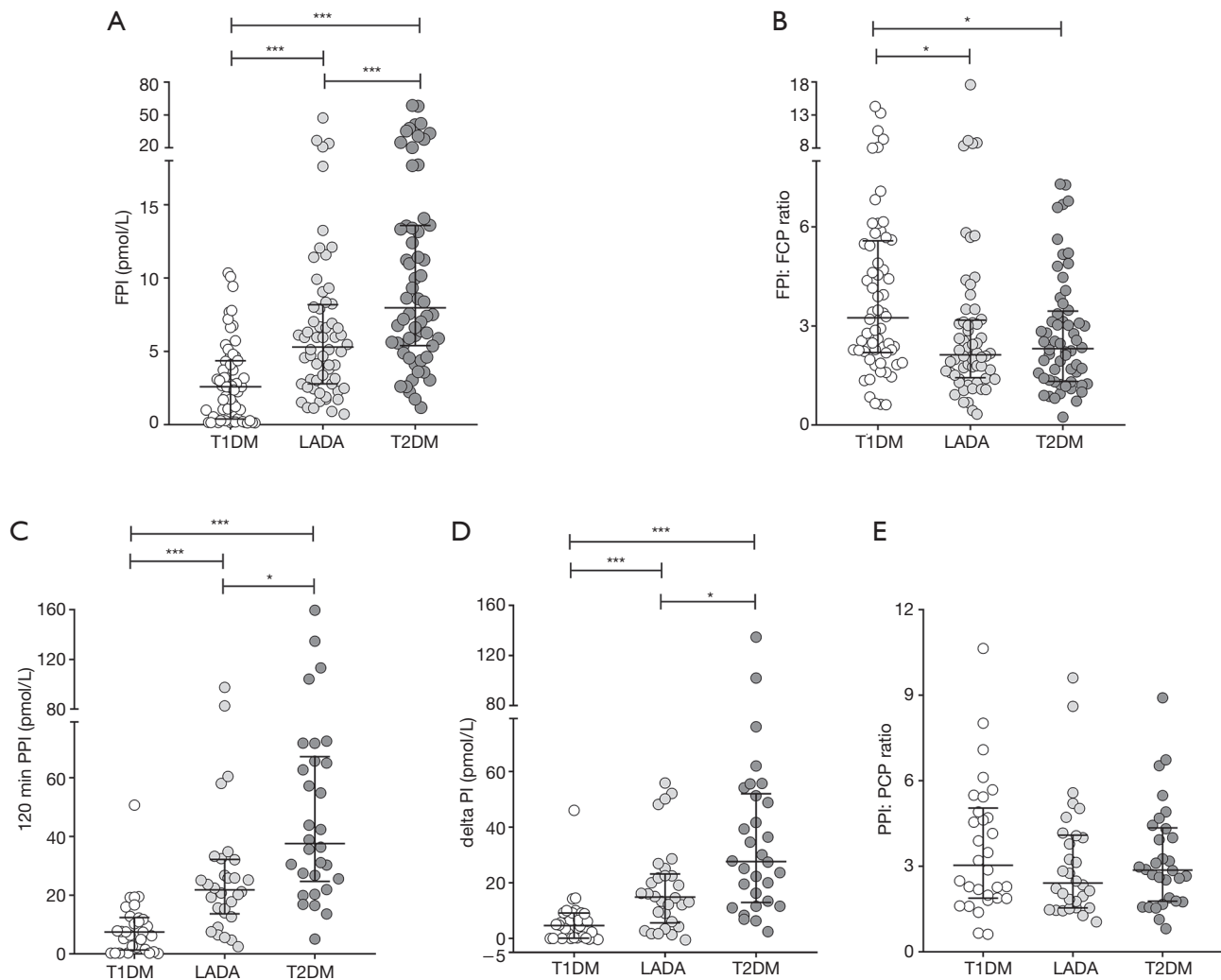


Figure 1 Scatterplots of fasting PI-related parameters for all 180 patients and 120 min postprandial PI-related parameters for 90 patients underwent MMTTs in T1DM, LADA and T2DM: (A) FPI; (B) FPI:FCP ratio; (C) PPI; (D) delta PI; (E) PPI:PCP ratio. Each point represents the PI-related parameters of one participant. Error bars depict median and IQRs. P values were calculated with Kruskal-Wallis tests with Bonferroni post hoc comparisons. *, P<0.05; ***, P<0.001. T1DM, type 1 diabetes mellitus; LADA, latent autoimmune diabetes in adults; T2DM, type 2 diabetes mellitus; IQR, interquartile range; FPI, fasting proinsulin; FPI:FCP ratio, fasting proinsulin to C-peptide ratio; PPI, 120 min postprandial proinsulin; delta PI, PPI – FPI; PPI:PCP ratio, 120 min postprandial proinsulin to C-peptide ratio.

Table 2 Beta-cell function measurements and adjusted comparisons between type 1 diabetes mellitus, LADA and type 2 diabetes mellitus

Models	Diagnosis			P value		
	T1DM (EMMs, 95% CIs)	LADA (EMMs, 95% CIs)	T2DM (EMMs, 95% CIs)	T1DM vs. LADA	T1DM vs. T2DM	LADA vs. T2DM
Model 1						
FCP	83.15 (65.54, 100.77)	272.35 (224.62, 320.08)	444.05 (390.02, 498.09)	*	*	*
FPI	2.89 (2.20, 3.59)	7.12 (5.18, 9.06)	13.12 (9.73, 16.52)	*	*	*

Table 2 (Continued)

Table 2 (Continued)

Models	Diagnosis			P value		
	T1DM (EMMs, 95% CIs)	LADA (EMMs, 95% CIs)	T2DM (EMMs, 95% CIs)	T1DM vs. LADA	T1DM vs. T2DM	LADA vs. T2DM
	FPI:FCP ratio	4.05 (3.31, 4.79)	3.00 (2.28, 3.72)	2.76 (2.30, 3.22)	*	*
PCP	278.66 (180.94, 376.39)	939.97 (724.50, 1,155.44)	1,506.08 (1,240.56, 1,771.60)	*	*	*
delta CP	188.99 (106.81, 271.17)	640.29 (457.11, 823.47)	1,042.18 (814.42, 1,269.93)	*	*	*
PPI	9.02 (5.26, 12.77)	26.62 (18.44, 34.81)	50.54 (36.71, 64.36)	*	*	*
delta PI	5.96 (2.67, 9.24)	18.17 (12.35, 24.00)	35.87 (24.83, 46.92)	*	*	*
PPI:PCP ratio	3.59 (2.62, 4.55)	3.16 (2.39, 3.93)	3.30 (2.61, 3.99)			
Model 2						
FCP	73.56 (59.66, 90.69)	286.92 (236.35, 348.32)	463.79 (382.70, 562.07)	*	*	*
FPI	2.61 (2.02, 3.37)	7.50 (5.90, 9.54)	13.53 (10.72, 17.09)	*	*	*
FPI:FCP ratio	3.95 (3.29, 4.74)	3.04 (2.55, 3.61)	2.79 (2.35, 3.31)		*	
PCP	250.24 (182.85, 342.47)	970.17 (728.23, 1,292.49)	1,590.00 (1,184.00, 2,135.22)	*	*	*
delta CP	209.71 (144.50, 310.81)	657.66 (476.97, 906.79)	1,094.47 (787.51, 1,521.08)	*	*	*
PPI	7.71 (5.40, 11.01)	27.80 (20.10, 38.44)	54.32 (38.94, 75.76)	*	*	*
delta PI	5.75 (3.79, 8.72)	19.63 (13.82, 27.86)	38.70 (27.18, 55.11)	*	*	*
PPI:PCP ratio	3.30 (2.62, 4.14)	3.24 (2.64, 3.99)	3.46 (2.79, 4.29)			
Model 3						
FCP	83.77 (67.00, 104.72)	279.50 (231.33, 337.66)	399.90 (325.79, 490.86)	*	*	*
FPI	3.17 (2.44, 4.12)	7.21 (5.75, 9.03)	9.92 (7.78, 12.66)	*	*	
FPI:FCP ratio	4.14 (3.41, 5.03)	3.06 (2.58, 3.64)	2.61 (2.17, 3.15)	*	*	
PCP	268.45 (188.05, 383.21)	949.65 (710.86, 1,268.66)	1,503.14 (1,092.77, 2,067.61)	*	*	*
delta CP	216.71 (137.63, 341.25)	652.67 (471.56, 903.34)	1,073.06 (753.03, 1,529.11)	*	*	*
PPI	8.54 (5.73, 12.74)	26.88 (19.40, 37.25)	49.82 (34.86, 71.19)	*	*	*
delta PI	5.98 (3.71, 9.66)	19.45 (13.65, 27.72)	37.72 (25.72, 55.30)	*	*	*
PPI:PCP ratio	3.44 (2.70, 4.39)	3.25 (2.64, 3.99)	3.29 (2.61, 4.14)			
Model 4						
FCP	81.54 (65.50, 101.50)	280.14 (232.68, 337.28)	396.50 (324.07, 485.12)	*	*	*
FPI	3.10 (2.38, 4.03)	7.30 (5.83, 9.15)	9.91 (7.77, 12.64)	*	*	
FPI:FCP ratio	4.19 (3.46, 5.07)	2.99 (2.52, 3.55)	2.60 (2.16, 3.13)	*	*	
PCP	248.26 (177.92, 346.41)	924.52 (703.84, 1,214.41)	1,474.51 (1,091.82, 1,991.32)	*	*	*
delta CP	200.08 (129.29, 309.63)	633.08 (462.87, 865.88)	1,072.96 (761.46, 1,511.89)	*	*	*
PPI	7.87 (5.37, 11.53)	26.58 (19.43, 36.36)	49.29 (34.90, 69.60)	*	*	*
delta PI	5.59 (-2.37, 13.55)	19.03 (12.23, 25.83)	35.38 (27.78, 42.99)	*	*	*
PPI:PCP ratio	3.41 (2.68, 4.34)	3.25 (2.65, 3.98)	3.30 (2.63, 4.14)			

Values are presented as the estimated marginal means (95% confidence intervals). For unadjusted model (model 1), values are presented as means (95% confidence intervals). Generalized linear models with log-linked gamma distribution were performed for eight beta-cell function parameters (FCP, FPI, FPI:FCP ratio, PCP, delta CP, PPI, delta PI and PPI:PCP ratio), and least significance difference (LSD) tests were used to compare parameters between diagnosis groups, namely T1DM, LADA, T2DM. Model 1, unadjusted model 2, model adjusted for age; model 3, model adjusted for age and BMI; model 4, model adjusted for age, BMI and diabetes duration. *, P<0.05. T1DM, type 1 diabetes mellitus; LADA, latent autoimmune diabetes in adults; T2DM, type 2 diabetes mellitus; EMMs, estimated marginal means; 95% CIs, 95% credential intervals; FCP, fasting C-peptide; FPI, fasting proinsulin; FPI:FCP ratio, fasting proinsulin to C-peptide ratio; PCP, 120 min postprandial C-peptide; delta CP, PCP – FCP; PPI, 120 min postprandial proinsulin; delta PI, PPI – FPI; PPI:PCP ratio, 120 min postprandial proinsulin to C-peptide ratio.

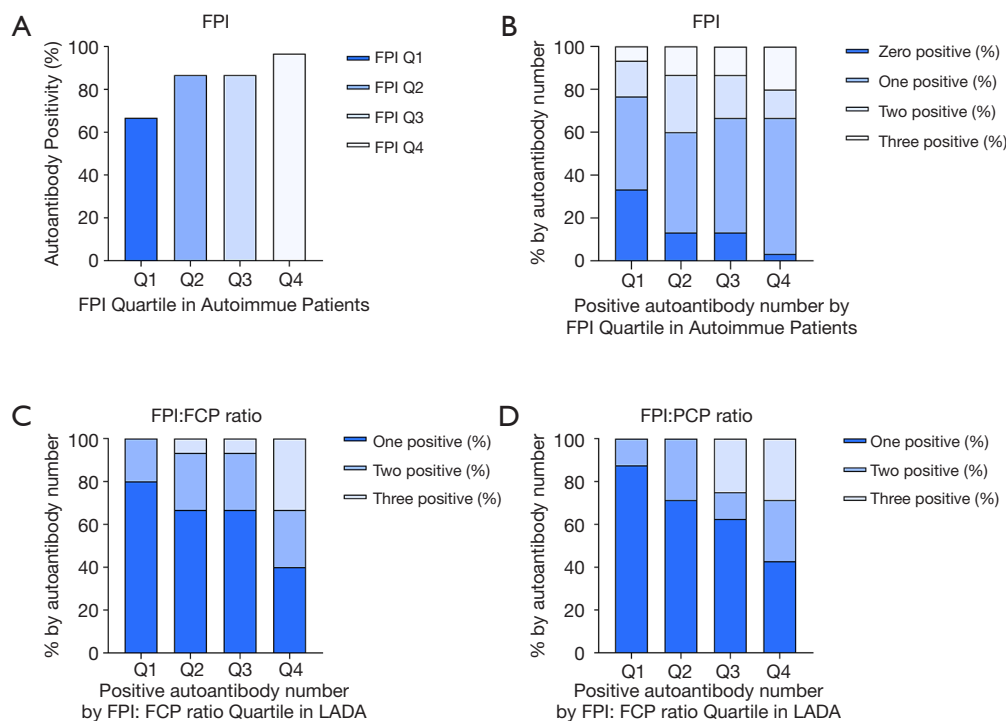


Figure 2 Trend tests performed in autoimmune diabetes patients and LADA patients for autoimmune reactions by quartile of PI-related parameters in autoimmune diabetes. Q1, Q2, Q3 and Q4 stand for four quartiles of PI-related parameters defined by the first, second and third quartile. (A) Percentage of individuals with positive autoantibody in the whole autoimmune population increased by FPI quartile ($P=0.003$); (B) percentage of individuals with zero, one, two or three positive autoantibodies in the whole autoimmune population increased by FPI quartile ($P=0.036$); (C) percentage of individuals with one, two or three positive autoantibodies in LADA increased by FPI:FCP ratio quartile ($P=0.007$); (D) percentage of individuals with one, two or three positive autoantibodies in LADA increased by PPI:PCP ratio quartile ($P=0.034$). LADA, latent autoimmune diabetes in adults; Autoimmune diabetes, LADA and type 1 diabetes mellitus; FPI, fasting proinsulin; FPI:FCP ratio, fasting proinsulin to C-peptide ratio; PPI:PCP ratio, 120 min postprandial proinsulin to C-peptide ratio.

autoimmunity and impaired beta-cell function among autoimmune diabetes, especially in LADA. These results indicated that beta-cell autoimmunity and beta-cell ER stress could possibly synergize beta-cell destructions.

Dysfunctional pancreatic beta-cell is the final denominator of all forms of diabetes. ER stress, which is efficiently and accurately measured by PI to insulin or PI to C-peptide ratios in peripheral blood of human, has been corroborated by an increasing number of studies to be involved in beta-cell intrinsic pathogenesis pathways related to the development of diabetes. Increased secretion of PI and the elevation of PI:CP ratio were considered signs of both early beta-cell intrinsic dysfunctionality among individuals about to progress T1DM and T2DM (24,31) and among T1DM patients at onset (27). In T1DM, ER stress intricately connected autoimmunity and inflammatory processes during beta-cell destruction (14).

Dysfunctional ER provides stressing environments for protein modification and could accelerate ER stress to a terminate extend, resulting in the generation of neo-antigens and activation of immune responses (37). In turn, autoimmunity, which is responsible for insulinitis, could add on inflammation and then further induce ER stress (38). In these regards, blockage of ER stress signaling pathways could reverse T1DM (39,40), and reducing ER stress by targeting key molecules in ER function has become a plausible way in relieving diabetes (22,41). When it comes to T2DM, in which insulin resistance has a dominant contribution, upregulated PI production could reveal poor beta-cell function (42), and attenuating ER stress could alleviate beta-cell damage. Considered as a milder form of autoimmune diabetes which shared T2DM traits, ER stress could be possibly involved in autoimmunity and inflammatory process in LADA. However, there

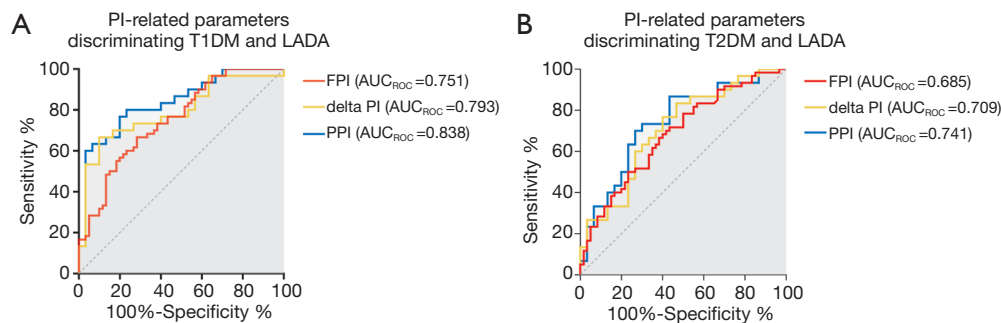


Figure 3 ROC curves in evaluating the application of proinsulin as a discriminating test for (A) LADA and adult-onset T1DM and (B) LADA and T2DM. Red lines stand for FPI, yellow lines stand for delta PI, and blue lines stand for PPI. T1DM, type 1 diabetes mellitus; LADA, latent autoimmune diabetes in adults; T2DM, type 2 diabetes mellitus; PI, proinsulin; FPI, fasting proinsulin; PPI, 120 min postprandial proinsulin; delta PI, PPI – FPI; ROC curves, receiver operation characteristic curves. AUC_{ROC}, area under the ROC curve.

has been few explorations concerning ER stress and PI-related measurements in LADA. A former study found increased PI:CP ratios and defective PI processing features in offspring of LADA patients with positive GADA (43). Hence, we, for the first time, performed this study to assess beta-cell dysfunctionality in LADA and to demonstrate differences among different types of diabetes.

We demonstrated intermediate levels of fasting PI, delta PI and postprandial PI in LADA patients between adult-onset T1DM and T2DM. Besides, fasting PI:CP ratio was significantly higher in patients in the T1DM group than either LADA or T2DM. These differences remained significant after adjustments for age, BMI and diabetes duration. Increasing PI levels from T1DM, through LADA, to T2DM observed in our study were in consistency with the increasing insulin secretion capacity in that order (6), and hence could be a revelation of remaining beta-cell function in each type. That is, T1DM patients possess the lowest residual beta-cell function, T2DM retained the highest, and LADA stood between them. When it comes to PI:CP ratio, the obviously higher ratio in T1DM than in LADA indicated severer disproportionated hyperproinsulinemia in T1DM. Possibly the already largely destructed beta-cell population in T1DM, owing to more intense genetic predispositions and abnormalities, higher levels of insulinitis, severer beta-cell autoimmunity and faster disease progression, could not resist the extremely uncontrolled hyperglycemia (5,8,12,44). Consequently, excessive requirements on insulin production resulted in extra ER stress and increased amounts of un-processed PI molecule, instead of insulin and CP, to be released into peripheral blood, and hence could account for the

disproportionated hyperproinsulinemia in T1DM. When it comes to LADA patients who have experienced slower less intense beta-cell destruction than T1DM, the beta-cell dysfunctionality was not as detrimental as it was in T1DM. But we could not credit similar pathogenetic mechanisms in both LADA and T2DM to the indiscrimination on PI:CP ratios between them. As demonstrated in the former study investigating PI secretion in LADA, it was under hyperglycemic clamp test, rather than oral glucose tolerance test, that the significantly increased PI and PI:CP ratios were observed between LADA offspring at high diabetes risks and individuals with normal glucose tolerance (43). Presumably, in LADA, an excessively intense beta-cell stress, which could have resulted from metabolic loads stronger than MMTTs, was required for uncovering the beta-cell dysfunctionality, but further explorations focusing the beta-cell stress loads are required to confirm the speculation. Recent studies also observed severer impaired PI processing in non-obese T2DM than obese T2DM, indicating still unknown ER dysfunction mechanisms in T2DM other than insulin resistance, which has long been proposed to be responsible for disproportionated hyperproinsulinemia in T2DM (45). Thus, these possible reasons could explain comparable PI:CP in LADA and T2DM.

We further carried out correlation analysis between beta-cell autoimmunity and PI secretion patterns, and positive correlations between IA-2A titers and FPI:FCP ratios, between IA-2A titers and PPI:PCP ratios and between ZnT8A and PPI:PCP ratios were observed among the whole autoimmune diabetes population, including T1DM and LADA. While in LADA patients, besides the aforementioned correlations between autoantibody

titers and PI:CP ratios, positive correlations were also observed between both fasting and postprandial PI:CP ratios and numbers of positive autoantibody. These results indicated a negative correlation between the intensity of autoimmunity and the capability of beta-cells in resisting stresses: the severer the immune responses, the heavier that beta-cell were stressed, and hence the production of PI could be adversely affected, to which the disproportionated hyperproinsulinemia was led. Also, both IA-2A and ZnT8A were pancreatic beta-cell-specific autoantibodies and were directly involved in beta-cell destructions, and hence possibly these autoantibodies could be sensitive biomarkers for beta-cell function and stress (46-49). Based on this, we may presume that PI secretion patterns in T1DM and LADA was intimately manipulated by immune attacks, but different levels of autoimmunity should be responsible for their differences in PI secretion patterns. Besides, these correlations indicated fundamentally different underneath mechanisms for beta-cell destructions in LADA when compared to T2DM.

In T1DM, former studies suggested synergized effects from both beta-cell autoimmunity and beta-cell intrinsic dysfunction (14). These two mechanisms were possibly connected and could have formed a vicious cycle, which actively involved in the destruction of pancreatic beta-cells: autoimmunity could promote inflammations which will further accelerate ER stress and successive pro-death processes (50-52), and ER stress were intimately involved in neo-antigen generation and self-recognition through posttranslational modifications, protein degeneration processes, assisted antigen presenting, etc. (53-56). These may account for the relationship between beta-cell autoimmunity and ER stress found in our study. But beta-cell autoimmunity is far more complicated than what we could observe from islet autoantibodies: there were possible fusional mechanisms for the pathogenesis of autoimmune diabetes through which beta-cells were attacked by synergized beta-cell failure from within beta-cells and immune responses from without. Intrinsic and eccentric retaliations supported each other and were both responsible for beta-cell death in autoimmune diabetes. Thus, future exploration need focuses on the crosstalk and contribution of both autoimmune attack and intrinsic beta-cell failure to elucidate the identity of autoimmune diabetes and to develop effective prevention and treatment.

Scientists have demonstrated significantly different PI:CP ratio in T1DM patients at onset than healthy control

subjects with normal glucose tolerance (27), indicating PI-related measurements could be applicable biomarkers for identifying diabetes patients and evaluating ER stress among them. To further evaluate the possibility of applying PI-related parameters in discriminating forms of diabetes, we performed AUC plots. Though AUC_{ROC} of PPI, delta PI and FPI were all reached or nearly reached 0.700, the greatest AUC_{ROC} was observed in PPI, indicating higher discrepancies between groups after meal. This was possibly resulted from different ER stress resulted from metabolism stress related to carbohydrates, proteins and fat loads (57,58). Thus, applying PI measurements on discriminating forms of diabetes was applicable, especially after meal loads, but whether a standard meal test is a requirement still need further investigation.

There were several limitations in our study. First, the cross-sectional design restricted the temporal and causal association assessments and the exact molecular mechanism underlying the beta-cell destruction remains unknown. Further, in this study, we did not include a healthy control group with normal glycemic control since former results have already proved different PI secretion pattern and ER stress in diabetes patients compared to healthy individuals. Also, since BMI and age were not perfectly matched among groups, we could not eliminate the influences on PI secretion from either age or BMI. Last, we did not assess possibly related ER stress protein expression and cytokine-related changes in this study, and thus further studies involving other ER stress markers and cytokines are warranted to further explore underneath mechanisms contributing to the different PI secretion patterns among types of diabetes.

Conclusions

To sum up, this study confirmed intermediate serum levels of PI in LADA between T1DM and T2DM, and the fasting PI:CP ratio in T1DM is significantly higher than LADA and T2DM. Positive correlations between PI secretion and islet autoantibody titers and numbers indicated that, in LADA, beta-cell function and ER stress is intimately manipulated by autoimmunity. Our results provided further evidence for the heterogeneity of diabetes, especially autoimmune diabetes. Significantly, we demonstrated that peripheral PI could be applied as a serum biomarker in discriminating different forms of diabetes, which may further assist in precision diagnosis for diabetes.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm.2020.03.189>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study, involving human subjects, human material and human data, was carried out in agreement with the guidelines in the Declaration of Helsinki and approved by the Human Ethics Committee of The Second Xiangya Hospital of Central South University, Changsha, China. All patients in this study have understood and signed the informed consent form. The study outcome will not affect the future management of patients. Patient data in this study were retrieved from the hospital medical record system and patient's personal data have been secured.

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