รูปแบบของโปรตีนในเลือด และ Potential Signaling Pathways ที่สัมพันธ์กับภาวะก่อนเกิดโรคเบาหวานและโรคเบาหวานชนิดที่ 2 ในกลุ่มผู้สูงอายุ

พิมพ์วรีญ์ ไตรสถิตวร[1] สิทธิรักษ์ รอยตระกูล[2] ศิรสา เรืองฤทธิ์ชาญกุล[3] เพียงพร เจริญวัฒน์[3] ปิยะมิตร ศรีธรา[3] จินตนา ศิริวราศัย[4,*]

[1] หลักสูตรวิทยาศาสตรมหาบัณฑิตสาขาวิชาโภชนศาสตร์ โครงการร่วมคณะแพทยศาสตร์โรงพยาบาลรามาธิบดี และสถาบันโภชนาการ มหาวิทยาลัยมหิดล

[2] ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

[3] ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

[4] กลุ่มสาขาวิชาโภชนศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

บทคัดย่อ

โรคเบาหวานชนิดที่ 2 เป็นหนึ่งในปัญหาสุขภาพระดับโลกและพบมากในกลุ่มผู้สูงอายุ ที่มีสาเหตุจากหลายปัจจัย การศึกษานี้มีวัตถุประสงค์ เพื่อศึกษาความแตกต่างของการแสดงออกของโปรตีนในเลือดกลุ่มผู้สูงอายุไทยที่ไม่มีและมีภาวะเบาหวาน โดยใช้ข้อมูลและตัวอย่างเลือดจากโครงการ EGAT1/5 (2012) ซึ่งเป็นกลุ่มศึกษาเพศชายที่มีอายุระหว่าง 60-80 ปี จำนวน 2 กลุ่ม ได้แก่กลุ่มควบคุมที่มีสุขภาพดีที่ไม่มีภาวะอ้วนลงพุง (จำนวน 9 คน) และกลุ่มภาวะก่อนเกิดโรคเบาหวานและโรคเบาหวานชนิดที่ 2 (จำนวน 35 คน) การวิเคราะห์รูปแบบของโปรตีนในเลือดใช้เครื่องมือ liquid chromatography-tandem mass spectrometry พบว่ามีโปรตีน จำนวน 19 ชนิดที่พบเฉพาะในกลุ่มภาวะก่อนเกิดโรคเบาหวานและโรคเบาหวานชนิดที่ 2 ที่มีการทำงานเกี่ยวข้องกับกระบวนการ glycosylation การขนส่งโปรตีนและไขมัน การทำงานของ growth factor และการตอบสนองต่อการอักเสบ ในการวิเคราะห์ปฏิสัมพันธ์ระหว่างโปรตีนที่มีการแสดงออกมากกว่า 1.5 เท่าของกลุ่มภาวะก่อนเกิดโรคเบาหวานและโรคเบาหวานชนิดที่ 2 เปรียบเทียบกับกลุ่มควบคุม พบว่า มีความสัมพันธ์กับกลไก PI3K-AKT signaling pathway ส่วนที่มีการแสดงออกของโปรตีนลดลงมากกว่า 1.5 เท่า มีความสัมพันธ์กับกลไก MAPK pathway ซึ่งทั้ง 2 กลไกมีบทบาทสำคัญกับการทำงานของเบต้าเซลล์ของตับอ่อนและการควบคุมการตอบสนองต่อการทำงานของอินซูลิน โดยสรุปพบว่าผลที่ได้จากการศึกษาทางด้านโปรตีโอมิกส์ สามารถนำไปสู่การอธิบายที่ชัดเจนในเรื่องของกลไกที่เกี่ยวข้องการกับโรคเบาหวานชนิดที่ ตลอดจนเป็นข้อมูลที่สำคัญสำหรับการนำไปสู่แนวทางการรักษาขั้นสูงสำหรับโรคเบาหวาน และโรคแทรกซ้อนในกลุ่มผู้ป่วยที่เป็นกลุ่มผู้สูงอายุ

คำสำคัญ: ภาวะก่อนเกิดโรคเบาหวาน โรคเบาหวานชนิดที่ 2 โปรตีโอมิกส์ ผู้สูงอายุ กลไกระดับโมเลกุล

รับบทความ: *11 พฤษภาคม 2566* ***แก้ไข:*** *4 มิถุนายน 2566* ***ตอบรับ:*** *6 มิถุนายน 2566*

[*]ผู้รับผิดชอบบทความ

จินตนา ศิรวราศัย

กลุ่มสาขาวิชาโภชนศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

อีเมล: jintana.sir@mahidol.ac.th

Serum Proteomes and Potential Signaling Pathways Linked to Prediabetes and T2DM in the Elderly

Pimvaree Tristitworn[1] Sittiruk Roytrakul[2] Sirasa Ruangritchankul[3] Piangporn Charernwat[3] Piyamitr Sritara[3] Jintana Sirivarasai [4,*]

[1] Master of Science Program in Nutrition, Faculty of Medicine Ramathibodi Hospital and Institute of Nutrition, Mahidol University, Bangkok, Thailand

[2] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani, Thailand

[3] Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

[4] Graduate Program in Nutrition, Faculty of Medicine Ramathibodi Hospital Mahidol University

Abstract

Type 2 diabetes mellitus (T2DM) is a global health problem and most common in the elderly population with various causes. The present study aimed to investigate the different expressions of serum proteins in Thai elderly population with and without diabetes. Data and serum were based on the EGAT cohort study (2012), with participants aged 60-80 years classified into two study groups; the control group (N=9) were healthy men without metabolic syndrome and the prediabetes/T2DM (N=35). Identification of serum proteome profile by liquid chromatography-tandem mass spectrometry was performed. Nineteen unique proteins found only in the prediabetes and T2DM involved in glycosylation and transport of proteins and lipids, growth factor activity, and inflammatory responses. Analysis of protein-protein interaction among proteins with relative fold change >1.5 in the prediabetes and T2DM compared to the control group showed association with PI3K-AKT signaling path for up-regulated proteins and MAPK pathway way for down-regulated proteins. Both pathways play role in pancreatic beta cell function and regulation of insulin response. In conclusion, overall findings can lead to elucidate pathways of T2DM and contribute to important information for advance therapeutic strategies for T2DM and its complications in elderly population.

Keywords: Prediabetes, T2DM, Proteomics, Elderly, Molecular mechanism

Received: *11 May 2023,* ***Revised:*** *4 June 2023,* ***Accepted:*** *6 June 2023*

[*]Corresponding author

Jintana Sirivarasai

Graduate Program in Nutrition, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Thailand.

Email: jintana.sir@mahidol.ac.th

Introduction

Type 2 diabetes mellitus (T2DM) is one of the most significant non-communicable diseases (NCDs), and is considered as a major health threat for humans globally. Current report from International Diabetes Federation in 2020 predicted prevalence trend of T2DM among people 20–79 year olds of T2DM from 10.5% (536.6 million) in 2021 to 12.2% (783.2 million) in 2045[1]. The prevalence of individuals over 65 years of age with T2DM in Thailand was 17.2% as reported by the InterASIA study in 2003[2]. In addition, a cross-sectional study among Thai elderly with age more than 65 years found that 76.4% of those patients experienced with poor glycemic control (HbA1c >7.5%)[3]. Key risk factors implicated in the pathogenesis of T2DM include genetic predisposition, overweight/obesity, metabolic syndrome (MetS), sedentary lifestyle, aging, unhealthy diet/poor nutritional status, low socioeconomic status, stress, anxiety, depression and certain medications[4].

Underlying mechanisms related to T2DM have been reported, including β cell dysfunction, abnormal of glucose uptake via insulin signaling pathway in muscle tissue, increased hepatic glucose production, insulin resistance caused by excessive free fatty acids, abnormal of glucagon metabolism, and dysregulation of glucose metabolism in kidney[5]. Previous analysis by microarray showed the significant networks of gene signatures associated with insulin resistance and T2DM such as JAK-STAT, MAPK, TGF, Toll-like receptor, p53 and mTOR, adipocytokine, FOXO, PPAR, P13-AKT, and triglyceride metabolic pathways[6].

Development of the application of multi-omic technologies aims to study health and disease in diverse populations and patients. Multiple key components of various metabolic and genetic diseases can be uncovered by proteomics research using both functional and structural methods. Mass spectrometry-based proteomic analysis is a rapid, high-throughput technologies and can be applied for multiple purposes. Serum proteomic study provides the identification of proteins with differential expressions among healthy and patients together with biomarker discovery, analysis of protein-protein or-small molecules interaction in target disease, and identification of posttranslational modifications[7].

Proteome-wide association study between prevalent and incident of MetS and proteins from two cohorts were carried out[8]. The results found novel protein associations with MetS, including neural cell adhesion

molecule L1-like protein (CHL1), complement factor I (CFI), GDNF family receptor alpha-1 (GFRA1), kallikrein-8 (KLK8), brevican core protein (BCAN), dickkopf-like protein 1 (DKKL1), netrin receptor (UNC5D), NTR domain-containing protein 2 (WFIKKN2), and endoplasmic reticulum protein 29 (ERP29)[8]. Proteomic data from the same cohorts related to incident of T2DM were aminoacylase-1, growth hormone receptor, and insulin-like growth factor–binding protein 2 [9]. Further analysis with causal inference method showed suggestive causal effects of T2DM on both cathepsin Z and renin[9]. According to MALDI-TOF mass spectrometry analysis of plasma samples from normal and T2D individuals, apolipoprotein A-I expression was shown to be decreased by 4.2-fold and galectin-1 expression to be elevated by 4.8-fold in diabetic samples[10]. Clinical study investigated the possible mechanisms underlying the effect of very low calorie diets (VLCD,~450 kcal/day) with and without exercise programs for 16 weeks and improvements in obese with T2DM patients (N=27)[11]. MS analysis found significant differential proteins in patients as potential disease state and intervention specific biomarkers, including fibrinogen, and transthyretin-associated with diabetes; complement C3- associated with obesity; and apolipoprotein A-IV-associated with dietary markers[11]. Therefore, this study aimed to identify protein profiles involving diabetes in individulas with prediabetes and diabetes and to provide insight into the mechanisms underlying disease-associated alterations which these researches were limited in Thai diabetes patients.

Materials and Methods

Study design and participants

This study was a part of the cohort study of the Electricity Generating Authority of Thailand (EGAT) with re-survey in 2012 (study population, aged ≥ 60 years). Survey data were collected by using a self-administered questionnaire, physical examination, electrocardiography, chest radiography, and blood analysis. The study details and protocols of the EGAT study cohort have been previously described[12]. Procedures for the large-scale metabolic profiling of serum/plasma in the EGAT cohort were designed for biological sample stability by controlling pre-analytical factors, including study design, sample collection, sample handling and storage, and sample preparation. All biomarker analyses were conducted in accordance with current best practices, which serious concerns in optimal collection and storage of plasma/serum samples include splitting samples into multiple aliquots for single use

to avoid multiple freeze–thaw cycles and storing them at −80 °C [13]. For this proteomic study, serum samples were analyzed in 2020 with two study groups. The control group (N=9) were male without metabolic syndrome (criteria for MetS : HbA1c < 5.7 %, BMI < 22.9 kg/m^2, waist, < 90 cm, SBP <130 mmHg, DBP < 80 mmHg, triglyceride < 150 mg/dL, total cholesterol < 200 mg/dL, LDL-C < 130 mg/dL, and HDL-C > 40 mg/dL) [14]. The prediabetes/T2DM groups were male with HbA1c > 5.7 % and dyslipidemia or hypertension (N=35). All participants in the prediabetes/T2DM groups were not diagnosis with diabetes and did not receive medical treatment. Clinical measurements, including systolic blood pressure (SBP), diastolic blood pressure (DBP), waist circumference (WC), weight, height and body mass index (BMI), were also performed by trained medical staff.

The present study was approved by Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (protocol number: COA. MURA 2020/1790 and 2022/374). All the participants were informed with respect to the objective, process, benefits and potential problems risks. Then, written informed consent was obtained before their participations.

Biochemical measurement

On the morning of the visit, 12 hours-fasting venous blood samples were collected with clotted blood, NaF and EDTA tubes for different target analyses. These serum samples were separated for subsequent analysis and stored frozen at −80 ◦C. Fasting plasma glucose, hemoglobin A1C (HbA1C), lipid profiles, liver and kidney functions were measured through automated methods (Cobas-Mira, Roche, Milan, Italy).

Protein quantitation and identification by LC-MS/MS

Determination of protein concentration by Lowry assay: The quantification of the protein samples was performed using the Lowry method. Protein samples and protein standards (0, 2, 4, 6, 8, 10 µg/ml BSA) were transferred into 96-well plates. Then, 200 µl of solution A (2.5% SDS, 2.5% Na_2CO_3, 0.2 N NaOH, 0.025% $CuSO_4$ and 0.05% tartaric acid) was added, and incubated at room temperature for 30 min. Then, 50 µl of solution B (20% Folin-Ciocalteu phenol reagent) was added, and incubated at room temperature for 30 min. The protein samples were measured at OD750 and compared with the standards to estimate the concentrations[15].

In-solution digestion: The 5 µg proteins were transferred into low binding -96well plates and incubated with 25 mM

NH_4HCO_3 at room temperature for 10 min. Then, 200 µl of acetonitrile (ACN) was added and incubated for 10 min with shaking. After ACN removal, the sample were incubated at °56C for 1 hr with 50 µl of 10 mM DTT in 10 mM NH_4HCO_3.Next, 50 µl of 100 mM iodoacetamide in 10 mM NH_4HCO_3 was added, and incubated for 1 hr in the dark. After that,10 µl of enzyme solution (10 ng/µl trypsin in 10 mM NH_4HCO_3) was added and incubated at °37C for 3 hr. The peptide solutions were dried at °40C and kept at -°20C until analysis[16].

LC-MS/MS analysis: The peptide samples were resuspended in %0.1 formic acid then mixed with a pipette 100 times and transferred into low-binding tubes. The samples were centrifuged at 8,000×g for 10 min and the peptide solution were transferred into vial tubes. Then, 4.5 µl of peptide sample was injected into a LC-MS/MS analyzer (HCT Ultra Discovery System mass spectrometer, Bruker, Germany). The values were normalized using a BSA external intensity control. Proteins identification and database analysis were conducted with various software. Proteins will be identified by using DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare). Consequently of protein identification, the data will be submitted by using the Mascot software (Matrix Science, London, UK) and will search against the NCBI database. Database interrogation is; taxonomy (*Homo sapiens*), enzyme (trypsin): variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopie); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (± 0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages[17]. MultiExperiment Viewer (Mev) software version 4.6.1 will be performed for changes in protein quantification between the control and experimental group. Then, Jvenn diagram will be applied for the comparison of protein expression in each study group[18]. Proteins will be identified and classified according to their functionally relate biological process, cellular component and molecular function by using Panther (http://www.pantherdb.org). Moreover, the identified proteins were submitted to The Search Tool for Interacting Chemicals (STITCH) (http://stitch.embl.de) at the same time for searching for interactions between proteins and small molecules[19]. Molecular-level functions of identified proteins were further analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [20].

Statistical analysis

Statistical analyses were performed with the SPSS for Windows software

package, version 25 (IBM Corp., Armonk, NY, USA). Continuous data are reported as means ± standard deviation. Comparisons of continuous data between two groups were analyzed by independent t-tests. All tests were two-tailed, and statistical significance was set at p-value < 0.05.

Results

General characteristics and biochemical parameters of the study population was described in Table 1. The age range for the control and prediabetes and T2DM groups was between 65 and 75 and there was no statistical difference in mean of age between both groups. FPG and HbA1C levels in prediabetes and T2DM groups were 98.15±28.20 mg/dL and 6.42±0.71%, respectively, significantly higher than in the non-diabetes control group (87.86 ±8.08 mg/dl and 5.01±0.23%, respectively). Prediabetes and T2DM group indicated significantly higher BMI (24.98±2.78 vs 21.52±1.68 kg/m^2) and waist circumference (88.63±7.45 vs 80.24±5.02 cm) than those in the control group (p values <0.05). Both SBP and DBP in prediabetes and T2DM group were statistically higher than the control group together with TC and LDL-C levels, all p values <0.05.

Table 1. General characteristics and biochemical parameters of the study population (Mean±SD)

Characteristics	Control group (N=9)	Prediabetes and T2DM group (N=35)
Age (years)	67.21±3.47	67.52±4.18
BMI (kg/m^2)	21.52±1.68	24.98±2.78[a]
Waist circumference (cm.)	80.24±5.02	88.63±7.45[a]
SBP (mmHg)	124.56±9.47	135.68±8.56[a]
DBP (mmHg)	72.58±5.09	79.05±7.85[a]
FPG (mg/dL)	87.86±8.08	98.15±28.20[a]
HbA1c (%)	5.01±0.23	6.42±0.71[a]
TG (mg/dL)	118.36±32.89	125.68±36.44
TC (mg/dL)	180.23±33.25	232.56±31.28
LDL-C (mg/dL)	121.09±5.69	147.33±26.47[a]
HDL-C (mg/dL)	62.35±4.47	61.55±8.09
Albumin (mg/dL)	4.32±0.47	4.62±0.31
ALT (U/L)	18.67±3.69	20.79±8.56
AST (U/L)	21.56±4.89	23.69±7.14
BUN (mg/dL)	11.98±3.02	13.89±3.08
Creatinine (mg/dL)	0.89 ±0.09	0.92±0.17

[a] Significant difference from the control group, p<0.05

Proteomic analysis in this study compared data between the control and prediabetes and T2DM groups. A Venn diagram displayed the unique and overlapping expressed proteins from the control and prediabetes and T2DM groups (Figure 1). From all proteins identified in this study (N=1171), only 1121 were common to both groups. Regarding the unique proteins, thirty-one and nineteen proteins were exclusively identified in the control and prediabetes and T2DM groups, respectively (Tables 2 and 3). All identified proteins were classified and categorized with different biological processes such as cellular process (28.90%), metabolic process (17.00%), biological regulation (16.20%), response to stimulus (9.00%), localization (7.10%), signaling (6.70%) and others (Figure 2).

List name and function of the identified unique proteins in the control group based on biological process were described in Table 2. These proteins included insulin-like growth factor-binding protein 2 (IGFBP2) related to regulation of insulin-like growth factor receptor signaling, pro-adrenomedullin (ADM) related to response to insulin, hypotensive effects, cadherin 6 (CDH6) and protocadherin gamma-C4 (PCDHGC4) related to cell adhesion, tyrosine-protein kinase Yes (YES) related to transmembrane receptor protein tyrosine kinase signaling pathway, phospholipase C epsilon 1 (PLCE1) related to small GTPase mediated signal transduction and others. In addition, identified proteins found only in the prediabetes and T2DM in our study were Nucleolar protein 8 (NOL8), Golgin A4 (GOLGA4) involved in glycosylation and transport of proteins and lipids in the secretory pathway; Fibroblast growth factor (FGF) involved in growth factor activity; Toll-like receptor 4 (TLR4), Proteoglycan 4 (PRG4) involved in immune and inflammatory response, and others, as shown in Table 3.

Protein-protein or protein–small molecules interactions of differential expression proteins between the control and prediabetes and T2DM groups with relative fold change > 1.5 were analyzed by STITCH program with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and presented in the Figures 2 and 3. The upregulated proteins (N=186) in prediabetes and T2DM group were performed to search the potential pathways. The results found PI3K-Akt signaling pathway associated with identified proteins (N=6) including Fibroblast growth factor receptor (FGFR2), Cyclin-dependent kinase 4 (CDK4), BRCA1 isoform PI21-Delta 2-21 (BRCA1), Pleckstrin homology domain-containing family E member 1 (PHLPP1), CREB-regulated transcription

coactivator 2 (CRTC2), and Glycogen synthase (GYS1) which all these proteins also linked with glucose, insulin receptor (INSR), insulin receptor substrate 1(IRS1), NF-kappa-B (NFKB1), as shown in Figure 2. For all downregulated proteins with relative fold change >1.5 (N=168), MAPK signaling pathway was discovered with association between five identified proteins, including Neurofibromatosis-related protein NF-1 (NF1),TGF-beta-activated kinase 1 and MAP3K7-binding protein 1 (TAB1 MAP3K7IP), protein phosphatase, Mg2+/Mn2+ dependent 1A (PPM1A), Voltage-dependent P/Q-type calcium channel subunit alpha (CACNA1A) and Voltage-dependent calcium channel subunit alpha-2/delta-4 (CACNA2D4). These proteins interacted with glucose, insulin INSR, IRS1, NFKB1and mitogen-activated protein kinase kinase kinase 7 (MAP3K7) (Figure 3).

Discussion

The prevalence of diabetes has become a growing epidemic worldwide in adults aged 18 years and older, as reported by World Health Organization with indicating impaired glucose tolerance and impaired fasting glycaemia are at high risk of progressing to T2DM[21]. In this study, elderly participants with abnormal of HbA1C level were classified into prediabetes and T2DM. Furthermore, we found that this study group had a mean BMI of more than 23.0 kg/m^2, indicating that they were in the overweight category according to the Asia-Pacific cutoff (23-24.9 kg/m^2)[22]. For the prevalence of MetS, it has been reported with age-dependent, with people over 65 at a higher risk for developing MetS due to excessive weight gain, hyperglycemia, insulin resistance and dyslipidemia[23]. Prediabetes and T2DM group in our study also had hypercholesterolemia, high LDL-C and blood pressure levels. Similar to previous study in Taiwanese aged over 65 years, that found the prevalence rates of LDL-C ⩾160 mg/dL, TG ⩾ 200 mg/dL and HDL-C ⩽35 mg/dL were 14.8%, 11.2%, 11.0% for male and 13.6%, 13.4%, 12.9% for female, respectively[24]. Increased age and obesity can lead to chronic hyperglycemia and development of T2DM due to glucotoxicity, lipotoxicity and/or β-cell senescence, subsequently with insulin resistance and the activation of pro-inflammatory and oxidative stress pathways[25]. Increased SBP and DBP after the age of 60 years may be associated with a variety of underlying mechanisms, including reduction in the vascular elasticity, neurohormonal and autonomic dysregulation, and aged-induced kidney function with impairment of the sodium/potassium and calcium adenosine

triphosphate pumps and promoting vasoconstriction and vascular resistance[26].

As T2DM is a complex disease, a comprehensive mechanistic understanding requires an integrated approach. In this study, we used proteomics to identify unique proteins and differential protein expression to obtain a more comprehensive view of the metabolic pathways involved in abdominal obesity, dyslipidemia and glucose metabolism between controls (non-diabetic and non-MetS) and prediabetes and T2DM groups. A Venn diagram based analysis showed unique proteins related to

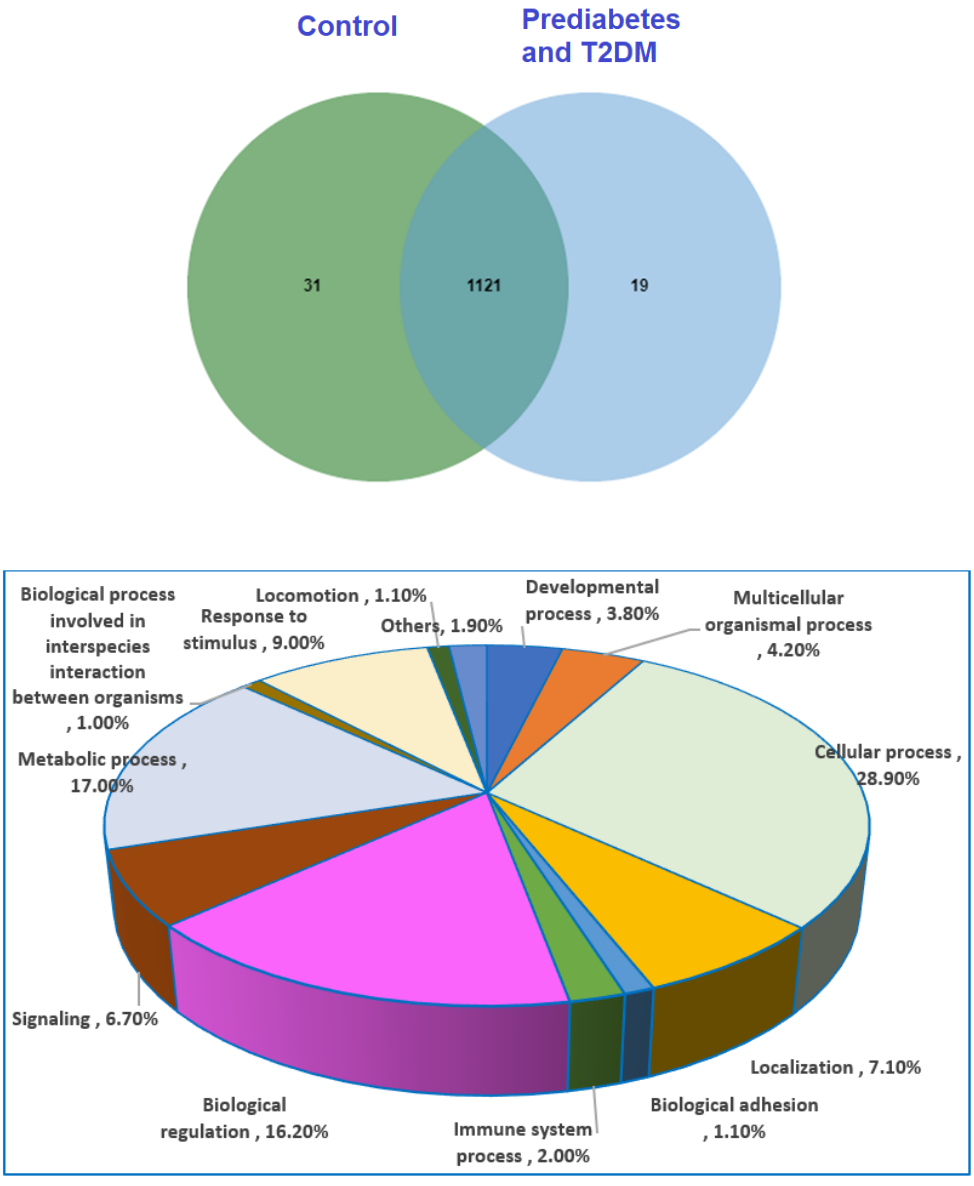

Figure 1. Venn diagram of the serum unique and overlapping proteins among the control and prediabetes and T2DM (A) and their functions of all identified proteins based on biological process (B)

Table 2. Unique proteins in the control group (N=31)

Protein ID	Protein names	Gene Names	Function: biological process)
D6RF27	THAP domain containing 6	THAP6	
A0A0U1RRG3	Fatty acid synthase	FASN	fatty acid biosynthetic process
A0A0U1RQX9	Glutaminyl-tRNA synthetase 1	QARS1	tRNA aminoacylation for protein translation
Q9Y653	Adhesion G-protein coupled receptor G1	ADGRG1	G protein-coupled receptor activity
Q5VXJ5	Synaptonemal complex protein 1	SYCP1	synaptonemal complex assembly
Q9BRG8	CLK2 protein	CLK2	protein phosphorylation
Q59FI2	protein-tyrosine-phosphatase		protein dephosphorylation
O60502	Protein O-GlcNAcase (OGA)	OGA	glycoprotein catabolic process
Q9Y5F7	Protocadherin gamma-C4 (PCDH-gamma-C4)	PCDHGC4	cell adhesion
Q9NR77	Peroxisomal membrane protein 2	PXMP2	peroxisomal membrane protein import
Q5JY88	Solute carrier family 25 member 14	SLC25A14	transfer of anions
I3L320	DnaJ heat shock protein family (Hsp40) member A2	DNAJA2	molecular chaperone activity
M0QZE2	Zinc finger protein 347	ZNF347	regulation of DNA-templated transcription
D6RGG3	Collagen type XII alpha 1 chain	COL12A1	organization of the collagen fibrils
A0A024RBI1	D-amino-acid oxidase isoform 1	DAO	D-amino acid catabolic process
O14646	Chromodomain-helicase-DNA-binding protein 1	CHD1	chromatin remodeling
B7Z493	FYVE, RhoGEF and PH domain containing 4	FGD4	guanyl-nucleotide exchange factor activity
Q5XPI4	E3 ubiquitin-protein ligase RNF123	RNF123	proteolysis involved in protein catabolic process
O94967	WD repeat-containing protein 47	WDR47	autophagy
P07947	Tyrosine-protein kinase Yes	YES	transmembrane receptor protein tyrosine kinase signaling pathway
H7C2X0	Eukaryotic translation initiation factor 2B subunit epsilon	EIF2B5	regulator for protein synthesis.
P18065	Insulin-like growth factor-binding protein 2	IGFBP2	regulation of insulin-like growth factor receptor signaling
E5RGQ6	Sorting nexin 16	SNX16	phosphatidylinositol binding
P35318	Pro-adrenomedullin	ADM	response to insulin, hypotensive effects
D6RF86	Cadherin 6	CDH6	cell adhesion via plasma membrane adhesion molecules
A0A075B6Z2	T cell receptor alpha joining 56	TRAJ56	recognizing fragments of antigen
Q59FG2	Low density lipoprotein-related protein 1	LRP1	endocytic receptor
H3BQA7	Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	OBSCN	Ca(2+)/calmodulin, and G protein-coupled signal transduction in the sarcomere.
A0A6Q8PHP9	Phospholipase C epsilon 1	PLCE1	small GTPase mediated signal transduction
Q86UY0	protein disulfide-isomerase	TXNDC5	protein disulfide isomerase activity
B4DMJ6	60S ribosomal protein L4		translation

Table 3. Unique proteins in prediabetes and T2DM group (N=19)

Protein ID	Protein names	Gene Names	Function: biological process)
Q9H857	5'-nucleotidase domain-containing protein 2	NT5DC2	5'-nucleotidase activity
A0A087X2D4	Aldehyde dehydrogenase 3 family member B1	ALDH3B1	cellular aldehyde metabolic process
A0A7U3JVZ5	Fibroblast growth factor (FGF)	FGF	growth factor activity
A0A024R250	Nucleolar protein 8	NOL8	RNA binding
E9PNZ4	Microtubule actin crosslinking factor 1	MACF1	intermediate filament cytoskeleton organization
O00206	Toll-like receptor 4	TLR4	Immune and inflammatory response
A0A024R930	Proteoglycan 4	PRG4	immune response
O75132	Zinc finger BED domain-containing protein 4	ZBED4	positive regulation of transcription by RNA polymerase II
A0A087WVA7	IQ motif containing with AAA domain 1 like	IQCA1L	ATP hydrolysis activity
A0A140VJM3	cGMP-dependent protein kinase	PRKG2	peptidyl-serine autophosphorylation, protein localization to plasma membrane
Q59EI3	2-oxoisovalerate dehydrogenase subunit alpha	BCKDHA	Transferase activity
C9JHJ5	Golgin A4	GOLGA4	glycosylation and transport of proteins and lipids in the secretory pathway
Q9NQR7	Coiled-coil domain-containing protein 177	CCDC177	regulation of alternative mRNA splicing
B2R9Y2	Coiled-coil domain-containing protein 55	CCDC55	regulation of alternative mRNA splicing
F5GZZ5	EPH receptor A4	EPHA4	protein-tyrosine kinase activity
D6R9D2	Glycoprotein M6A	GPM6A	calcium channel activity
D6REB4	Poly(A) binding protein interacting protein 1	PAIP1	RNA binding
Q6UWJ8	CD164 sialomucin-like 2 protein	CD164L2	cytoplasmic vesicles mediate vesicular transport
C9D7D0	Cellular tumor antigen p53	TP63	apoptotic process, cell cycle

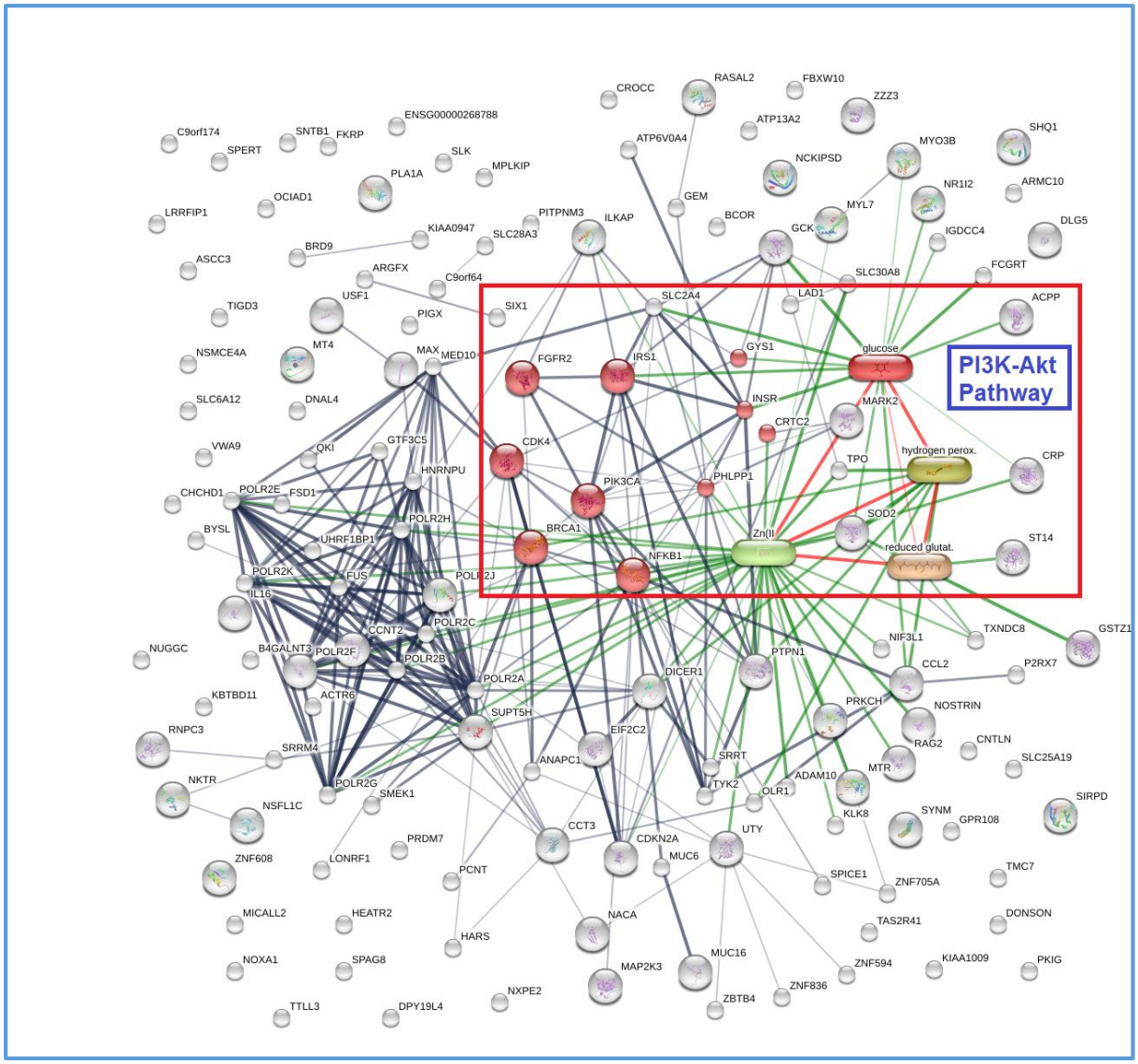

Figure 2. Interaction of upregulated proteins with relative fold change > 1.5 compared between prediabetes and T2DM group with the control group with PI3K-Akt signaling pathway. (Stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, small molecules-protein interactions in green and interactions between chemicals in red)

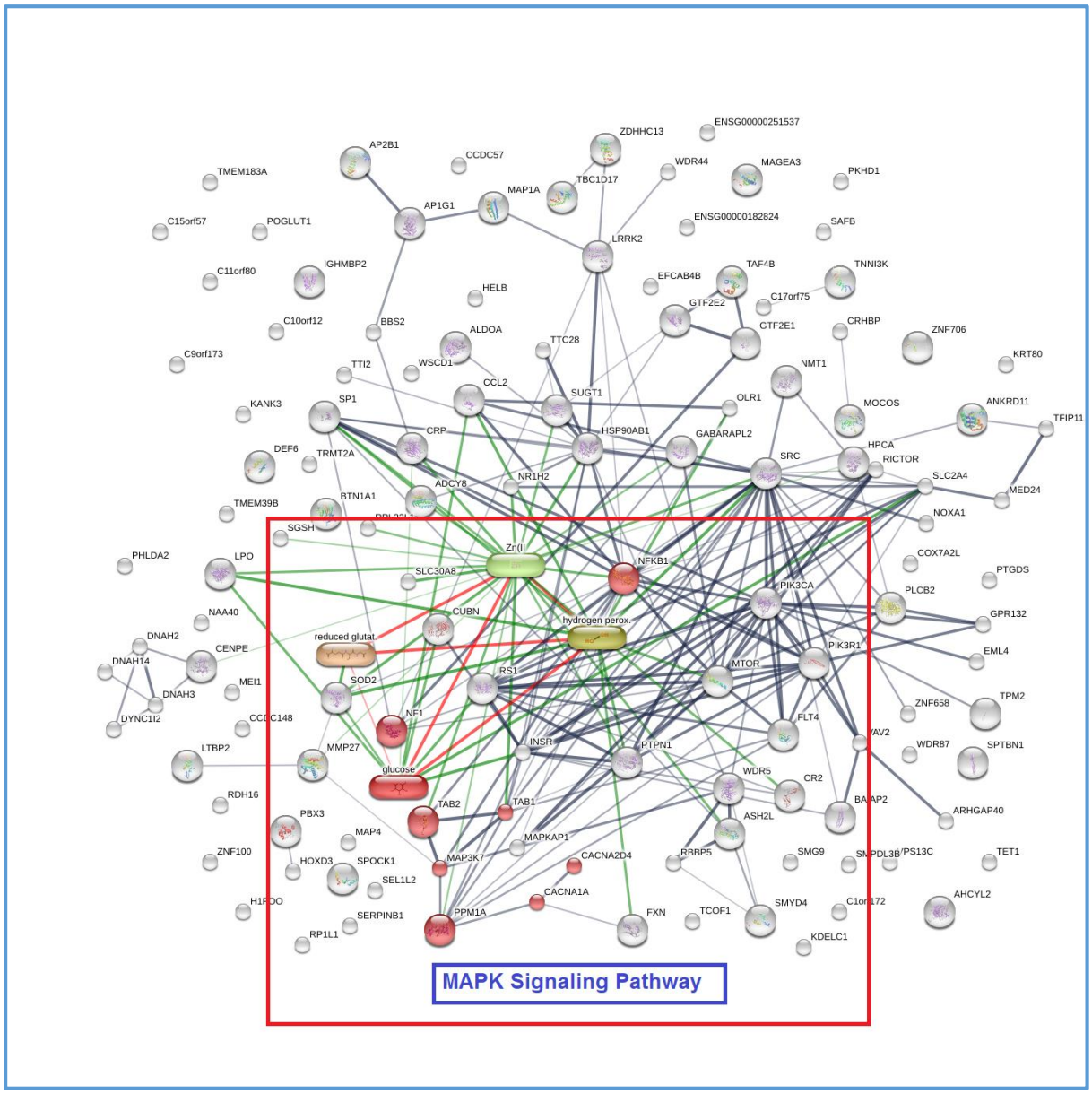

Figure 3. Interaction of downregulated proteins with relative fold change > 1.5 compared between prediabetes and T2DM group with the control group with MAPK signaling pathway. (Stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, small molecules-protein interactions in green and interactions between chemicals in red)

glucose and insulin metabolisms in the control group which some proteins have been published. IGFBP2 is one of proteins that bind insulin-like growth factors I and II (IGF-I and IGF-II). Previous studies provided role of IGFBP2 related to T2DM such as inhibition of adipogenesis and enhance long-term insulin sensitivity[27], regulation of hepatic glucose homeostasis[28], improvement of insulin resistance[29]. Increases in the levels of circulating IGFBP-2 were found to be significantly associated with a decreased risk of T2DM in the population-based study on IGFBP-2 and incident T2DM[30]. ADM is a peptide hormone and plays multiple roles in the regulation of hormonal secretion, glucose metabolism and inflammatory response. ADM regulates insulin balance and may participate in the development of diabetes[31].

The coordinated function of the pancreatic β cells is necessary for effective insulin secretion. Intercellular interactions are anticipated by signaling pathways that are mediated by junctional complex formation and cell adhesion molecule engagement[32]. In this study, we found CDH6 and PCDHGC4 in the non-diabetes group and both proteins are categorized in a large family of cadherin-related molecules. In vitro study found cadherin activation acted as an important signaling from cell-to-cell contact and targeted insulin secretion via regulation of actin cytoskeleton remodeling[33]. Tyrosine-protein kinase Yes is a Src Family Kinase (SFKs) and previous study has been explored function of this protein with potential regulation mechanisms of insulin secretion via granule mobilization/ replenishment and F-actin remodeling[34]. Another protein, PLCE1 is a phospholipase enzyme involving the hydrolysis of phosphatidylinositol-4,5-bisphosphate to generate two second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). In addition, an experimental study found association between an IP_3-dependent Ca^{2+} release and insulin on glucose transporter 4 (GLUT4) translo-cation and stimulation of glucose uptake[35].

For serum proteomic analysis in prediabetes and T2DM group, NOL8 is a nucleolar protein and binds to Ras-related GTP-binding proteins. The potential role of nucleolar protein, NOM1 nucleolar protein with MIF4G domain 1, with in the function of pancreatic islet β cells and insulin secretion were investigated and the findings suggested that NOM1 expression associated with pancreatic islet β cell apoptosis which was a pivotal role in diabetes[36]. TLR4 is a cell surface receptor, involves in modulating innate immunity and contributes to the development of insulin resistance and inflammation, especially in obese states[37]. Activation of

TLR4 directly promotes ROS generation via activation of NADPH oxidase and increased activities of pro-inflammatory kinases as well as indirectly influences on activation of cytokine signaling and inhibition of insulin signal transduction, primarily through IRS serine phosphorylation[37]. Another protein, PRG4 is a member of the proteoglycan family which can interact with extracellular matrix proteins, receptors or signaling molecules, resulting in a possible factor contributing to weight gain, dyslipidemia and insulin resistance[38]. Nahon et al., found lower glucose utilization by skeletal muscle, lower uptake of triglyceride-derived fatty acids and lower gene expression of inflammatory markers in Prg4 knockout (KO) mice and wild-type mice[39]. FGFs consists of 22 members of the FGF family and these proteins bind to FGF receptors (FGFRs) for regulation a crucial signalling pathways, including cellular proliferation, survival, migration, and differentiation. The FGF signal pathways are the RAS/MAP kinase pathway, PI3 kinase/AKT pathway, and PLCγ pathway[40]. Relationship between FGFs and development of diabetes has been proposed due to their functions in oxidative stress, immune inflammation, glucose and lipid metabolism and islet resistance[41]. In prediabetes and T2DM group we found GOLGA4 which is one of the golgins, a family of proteins localized to the Golgi. The Golgi apparatus (GA) is an important site of insulin processing and granule maturation. Possible link between GA organelle dysfunction and GA stress in the pancreatic β-cell can lead to diabetes [42]. An informatics-based approach to develop a transcriptional signature of β-cell GA using human islets from T1DM and T2DM found GA-associated genes are dysregulated in diabetes and identify putative markers of β-cell GA stress[42].

Further analysis to investigate potential mechanisms underlying prediabetes and T2DM in the study found two important signaling pathways; PI3K-AKT and MAPK (Figure 2 and 3). According to PI3K-AKT signaling pathway, upregulated protein in prediabetes and T2DM group related to this pathway included FGFR2, CDK4, BRCA1, PHLPP, CRTC2 and GYS1. PI3K/AKT signaling plays a central role in cellular physiology by mediating growth factor signals for critical cellular processes, such as glucose homeostasis, lipid metabolism, and protein synthesis. Their ligands included growth factors, cytokines and hormones, activate receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCR), activate PI3K. Insulin regulates skeletal muscle metabolism by promoting glucose transport, glycogen synthesis and protein synthesis through PI3K/AKT signaling pathway[43]. Experimental study showed that

mice with FGFR2 dysfunction were susceptible to insulin resistance and fat accumulation from a high fat diet which supportive role of FGFR in regulating glucose metabolism[44]. Previous study analyzed role of CDK4 in adipose tissue and found that insulin activated the CCND3-CDK4 complex, which in turn phosphorylated insulin receptor substrate 2 (IRS2) and conclude that CDK4 acted as a major regulator of insulin signaling in WAT[45]. For BRCA1, current study has been proposed that BRCA1 is an endocrine and metabolic regulator due to complex interactions between the insulin/insulin-like growth factor-1 (IGF1) signaling axis and BRCA1, subsequently development of metabolic disorders, including diabetes and the metabolic syndrome[46].

In prediabetes and T2DM group, interaction of downregulated proteins indicated their association with MAPK signaling pathways. MAPKs play an important role in specific intracellular signaling processes via interaction with MAPK-activated protein kinases (MAPKAPKs) [47]. In this study, we found MAP3K7-binding protein 1 (TAB1 MAP3K7IP), a regulator of the MAP kinase kinase kinase MAP3K7/TAK1, that mediated various intracellular signaling pathways-induced by TGF beta, interleukin 1, and WNT-1[47]. In addition, MAP kinase participated in TGF-β pathway and involved in pancreas development, β-cell proliferation, differentiation, and apoptosis. Sustained metabolic stress from insulin resistance and hyperglycemia resulted in β-cell failure, characterized by severe β-cell dysfunction and loss of β-cell mass[48]. Another protein, PPM1A also known as PP2Cα is a member of the Ser/Thr protein phosphatase family. It dephosphorylates, and negatively regulates the activities of, MAP kinases and MAP kinase kinases. PP-1G phosphorylation is mediated via a complex, cell type specific mechanism involving PI3-kinase/PKC/PKB and/or the ras/MAP kinase/Rsk kinase cascade[49]. Overexpressed wild-type (WT) PP2Cα by in 3T3-L1 adipocytes showed role of this enzyme on regulator of insulin sensitivity that acts through a direct activation of PI3K[50]. Both CACNA1A and CACNA2D4 are voltage-gated calcium (CaV) channels which they mediate in insulin secretion and play an important role in β-cell physiology and pathophysiology. Inappropriate regulation of β-cell CaV channels may cause beta-cell dysfunction and even death manifested in both T1DM and T2DM[51]. These identified proteins also associated with development of T2DM.

Through the use of the high throughput LC-MS-MS platform, we evaluated the associations of prediabetes and T2DM with a large number of proteins. Furthermore, identifying metabolic-

associated proteins in serum would be a less invasive, and cost-efficient method that may be more effectively applied for use as clinical biomarkers. However, a limitation of this study similar to other studies is the large dynamic range of protein concentrations in serum, this state may lead to the defect of a typical MS platform to detect low-abundance proteins[52].

Conclusion

Finding from our serum proteome analysis of prediabetes and T2DM provided data of known association and also reported unpublished candidate proteins. Especially, upregulated proteins involved in PI3K-AKT signaling pathway, including FGFR2, CDK4, BRCA1, PHLPP1, CRTC2 and GYS1 together with downregulated proteins, NF2, TAB1, PPMA1 CACNA1A and CACNA2D4 related to MAPK signaling pathway. These data potentially suggested a causal effect of T2DM and its complications and could be valuable targets for preventive strategies related to dietary, physical activity and behavioral modification and future therapeutic applications.

Acknowledgements

This study was supported by the grants from the Office of the Higher Education Commission (OHEC), the National Research Council of Thailand (NRCT) and the Thailand Research Fund (TRF). The authors wish to thank the EGAT and their people for participating and establishing this study. The authors would like to express thanks to Miss Sawanya Charoenlappanit and all research staffs at Proteomics Research Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC) for technical assistance in proteomic analysis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

1. Sun H, Saeedi P, Karuranga S, *et al*. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract* 2022; 183: 109119.
2. Aekplakorn W, Stolk RP, Neal B, *et al*. The prevalence and management of diabetes in Thai adults: the international collaborative study of cardiovascular disease in Asia. *Diabetes Care* 2003; 26(10): 2758-63.
3. Viengthong P, Bunloet A. Prevalence and associated factors of poor glycemic control among Type 2 Diabetes elderly patients in a community hospital, Khon Kaen Province. *SRIMEDJ* 2020; 35(4): 476-83.
4. Kyrou I, Tsigos C, Mavrogianni C, *et al*. Sociodemographic and lifestyle-related risk factors for identifying vulnerable groups for type 2 diabetes: a narrative review with

emphasis on data from Europe. *BMC Endocr Disord* 2020; 20(Suppl 1): 134.

5. Defronzo RA. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 2009; 58(4): 773-95.
6. Muhammad SA, Raza W, Nguyen T, *et al*. Cellular signaling pathways in insulin resistance-systems biology analyses of microarray dataset reveals new drug target gene signatures of type 2 diabetes mellitus. *Front Physiol* 2017; 8: 13.
7. Wright GL Jr, Semmes OJ. Proteomics in health and disease. *J Biomed Biotechnol* 2003; 2003(4): 215-16.
8. Elhadad MA, Wilson R, Zaghlool SB, *et al*. Metabolic syndrome and the plasma proteome: from association to causation. *Cardiovasc Diabetol* 2021; 20(1): 111.
9. Elhadad MA, Jonasson C, Huth C, *et al*. Deciphering the plasma proteome of type 2 diabetes. *Diabetes* 2020; 69(12): 2766-78.
10. Liu X, Feng Q, Chen Y, *et al*. Proteomics-based identification of differentially-expressed proteins including galectin-1 in the blood plasma of type 2 diabetic patients. *J Proteome Res* 2009; 8(3): 1255-62.
11. Sleddering MA, Markvoort AJ, Dharuri HK, *et al*. Proteomic analysis in type 2 diabetes patients before and after a very low calorie diet reveals potential disease state and intervention specific biomarkers. *PLoS One* 2014; 9(11): e112835.
12. Vathesatogkit P, Woodward, M, Tanomsup S, *et al*. Cohort profile: The electricity generating authority of Thailand study. *Int. J. Epidemiol* 2012: 41; 359-65.
13. Dunn WB, Broadhurst D, Begley P, *et al*. Human Serum Metabolome (HUSERMET) Consortium. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat Protoc* 2011; 6(7): 1060-83.
14. Huang PL. A comprehensive definition for metabolic syndrome. *Dis Model Mech* 2009; 2(5-6): 231-7.
15. Lowery OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193(1): 265-75.
16. Kaewseekhao B, Roytrakul S, Namwat W, et al. Responses of activated THP-1 cells to Mycobacterium Tuberculosis infection during isoniazid and rifampicin in vitro treatment. *Srinagarind Med J* 2015; 30(5): 422-31.
17. Johansson C, Samskog J, Sundström L, et al. Differential expression analysis of Escherichia coli proteins using a novel software for relative quantitation of LC-MS/MS data. *Proteomics* 2006; 6(16): 4475-85.
18. Bardou P, Mariette J, Escudié F, *et al*. jvenn: an interactive Venn diagram viewer. *BMC Bioinform* 2014; 15(1): 293.
19. Szklarczyk D, Santos A, von Mering C, *et al*. STITCH 5: augmenting protein-chemical interaction networks with tissue and affinity data. *Nucleic Acids Res* 2016; 44(D1): D380-4.
20. Kanehisa M, Furumichi M, Tanabe M, *et al*. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res* 2017; 45(D1): D353-61.
21. Global Burden of Disease Collaborative Network. Global Burden of Disease Study 2019. Results. Institute for Health Metrics and Evaluation, 2020. Available at https://vizhub.healthdata.org/gbd-results/, accessed on April 20, 2023.
22. Pan WH, Yeh WT. How to define obesity? Evidence-based multiple action points for public awareness, screening, and treatment: an extension of Asian-Pacific recommendations. *Asia Pac J Clin Nutr* 2008; 17(3): 370-74.

23. Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet* 2005; 365(9468): 1415-28.
24. Chang HY, Yeh WT, Chang YH, *et al*. Prevalence of dyslipidemia and mean blood lipid values in Taiwan: results from the Nutrition and Health Survey in Taiwan (NAHSIT, 1993-1996). *Chin J Physiol* 2002; 45(4): 187-97.
25. Bellary S, Kyrou I, Brown JE, et al. Type 2 diabetes mellitus in older adults: clinical considerations and management. *Nat Rev Endocrinol* 2021; 17(9): 534-48.
26. Oliveros E, Patel H, Kyung S, et al. Hypertension in older adults: Assessment, management, and challenges. *Clin Cardiol* 2020; 43(2): 99-107.
27. Russo VC, Azar WJ, Yau SW, *et al*. IGFBP-2: The dark horse in metabolism and cancer. *Cytokine Growth Factor Rev* 2015; 26: 329-46.
28. Li Z, Wu Z, Ren G, *et al*. Expression patterns of insulin-like growth factor system members and their correlations with growth and carcass traits in Landrace and Lantang pigs during postnatal development. *Mol Biol Rep* 2013; 40: 3569-76
29. Hedbacker K, Birsoy K, Wysocki RW, *et al*. Antidiabetic effects of IGFBP2, a leptin-regulated gene. *Cell Metab* 2010; 11(1): 11-22.
30. Rajpathak SN, He M, Sun Q, *et al*. Insulin-like growth factor axis and risk of type 2 diabetes in women. *Diabetes* 2012; 61(9): 2248-54.
31. Wong HK, Tang F, Cheung TT, *et al*. Adrenomedullin and diabetes. *World J Diabetes* 2014; 5(3): 364-71.
32. Wojtusciszyn A, Armanet M, Morel P, *et al*. Insulin secretion from human beta cells is heterogeneous and dependent on cell-to-cell contacts. *Diabetologia* 2008; 51: 1843-52
33. Parnaud G, Lavallard V, Bedat B, *et al*. Cadherin engagement improves insulin secretion of single human β-cells. *Diabetes* 2015; 64(3): 887-96.
34. Yoder SM, Dineen SL, Wang Z, *et al*. YES, a Src family kinase, is a proximal glucose-specific activator of cell division cycle control protein 42 (Cdc42) in pancreatic islet β cells. *J Biol Chem* 2014; 289(16): 11476-87.
35. Contreras-Ferrat A, Llanos P, Vásquez C, *et al*. Insulin elicits a ROS-activated and an IP_3-dependent Ca^{2+} release, which both impinge on GLUT4 translocation. *J Cell Sci* 2014; 127(Pt 9): 1911-23.
36. Yu L, Wang H, Guo Z, et al. Role of nucleolar protein NOM1 in pancreatic islet β cell apoptosis in diabetes. *Exp Ther Med* 2016; 12(4): 2275-80.
37. Kim JJ, Sears DD. TLR4 and insulin resistance. *Gastroenterol Res Pract* 2010; 2010: 212563.
38. Iozzo RV, Schaefer L. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biol* 2015; 42: 11-55.
39. Nahon JE, Hoekstra M, van Harmelen V, *et al*. Proteoglycan 4 deficiency protects against glucose intolerance and fatty liver disease in diet-induced obese mice. *Biochim Biophys Acta Mol Basis Dis* 2019; 1865(2): 494-501.
40. Yun YR, Won JE, Jeon E, *et al*. Fibroblast growth factors: biology, function, and application for tissue regeneration. *J Tissue Eng* 2010; 2010: 218142.
41. Deng J, Liu Y, Liu Y, et al. The multiple roles of fibroblast growth factor in diabetic nephropathy. *J Inflamm Res* 2021; 14: 5273-90.
42. Bone RN, Oyebamiji O, Talware S, *et al*. A computational approach for defining a signature of β-cell golgi stress in diabetes. *Diabetes* 2020: 2364-76.

43. Huang X, Liu G, Guo J, *et al*. The PI3K/AKT pathway in obesity and type 2 diabetes. *Int J Biol Sci* 2018; 14(11): 1483-96.
44. Liu S, Marcelin G, Blouet C, *et al*. A gut-brain axis regulating glucose metabolism mediated by bile acids and competitive fibroblast growth factor actions at the hypothalamus. *Mol Metab* 2018; 8: 37-50.
45. Lagarrigue S, Lopez-Mejia IC, Denechaud PD, *et al*. CDK4 is an essential insulin effector in adipocytes. *J Clin Invest* 2016; 126(1): 335-48.
46. Werner H. BRCA1: An endocrine and metabolic regulator. *Front Endocrinol (Lausanne)* 2022; 13: 844575.
47. Sidarala V, Kowluru A. The regulatory roles of mitogen-activated protein kinase (MAPK) pathways in health and diabetes: Lessons learned from the pancreatic β-cell. *Recent Pat Endocr Metab Immune Drug Discov* 2017; 10(2): 76-84.
48. Lee JH, Lee JH, Rane SG. TGF-β signaling in pancreatic islet β cell development and function. *Endocrinology* 2021; 162(3): bqaa233.
49. Li M, Xu X, Su Y, *et al*. A comprehensive overview of PPM1A: From structure to disease. *Exp Biol Med (Maywood)* 2022; 247(6): 453-61.
50. Yoshizaki T, Maegawa H, Egawa K, et al. Protein phosphatase-2C alpha as a positive regulator of insulin sensitivity through direct activation of phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. *J Biol Chem* 2004; 279(21): 22715-26.
51. Yang SN, Berggren PO. The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology. *Endocr Rev* 2006; 27(6): 621-76.
52. Tu C, Rudnick PA, Martinez MY, et al. Depletion of abundant plasma proteins and limitations of plasma proteomics. *J Proteome Res* 2010; 9(10): 4982-91.