การเปลี่ยนแปลงของระดับ CCR5 ในเลือดและโปรตีโอมิกส์ที่เกี่ยวข้องกับเมแทบอลิซึมส์ของคาร์โบไฮเดรตในกลุ่มคนที่มีภาวะไตรกลีเซอร์ไรด์สูงในเลือด

จินตนา ศิริวราศัย[1,*] พิสชา ชาญณงค์[2] สิทธิรักษ์ รอยตระกูล[3] ปิยะมิตร ศรีธรา[4]

[1] กลุ่มสาขาวิชาโภชนศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

[2] หลักสูตรวิทยาศาสตรมหาบัณฑิต สาขาวิชาโภชนศาสตร์ โครงการร่วมคณะแพทยศาสตร์โรงพยาบาลรามาธิบดี และสถาบันโภชนาการ มหาวิทยาลัยมหิดล

[3] ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

[4] ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

บทคัดย่อ

ภาวะไตรกลีเซอร์ไรด์สูงในเลือด ภาวะอ้วน และภาวะดื้ออินซูลิน เป็นตัวชี้วัดเพื่อทำนายความเสี่ยงต่อการเกิดโรคหลอดเลือดหัวใจ อย่างไรก็ตามปัจจุบันยังไม่สามารถอธิบายสาเหตุ และกลไกที่เกี่ยวข้องได้อย่างชัดเจน จึงเป็นที่มาของการศึกษาความสัมพันธ์ของโปรตีนในครั้งนี้ เพื่อบ่งชี้รูปแบบของโปรตีนในเลือดของกลุ่มควบคุมและกลุ่มคนที่มีภาวะไตรกลีเซอร์ไรด์สูงในเลือด โดยกลุ่มควบคุมเป็นเพศชายสุขภาพดี จำนวน 5 คน ส่วนกลุ่มที่มีระดับไตรกลีเซอร์ไรด์สูงในเลือด แบ่งเป็น 3 กลุ่ม (กลุ่มละ 6 คน) ได้แก่ระดับสูงเล็กน้อย (150–199 มิลลิกรัม/เดซิลิตร) ระดับสูง (200–499 มิลลิกรัม/เดซิลิตร) และระดับสูงมาก (≥ 500 มิลลิกรัม/เดซิลิตร) การวิเคราะห์โปรตีนในเลือดโดยใช้เครื่อง LC-MS/MS พบว่า มีปริมาณของโปรตีนที่เกี่ยวข้องกับ chemokine และ cytokine signaling pathway (6.5%) the integrin signaling pathway (6.1%) Wnt signaling pathway (4.2%) และอื่นๆ รวมถึงพบ protein interaction และ molecular network ที่สัมพันธ์กับภาวะไตรกลีเซอร์ไรด์สูงในเลือด การเปลี่ยนแปลงของระดับโปรตีน CCR5 ในเลือดมากกว่า 10 เท่า ที่สัมพันธ์กับภาวะไขมันในเลือดสูง ภาวะอ้วน ภาวะดื้ออินซูลินและการอักเสบ ผลการศึกษาโดยรวมสรุปได้ว่าปริมาณโปรตีนที่วิเคราะห์ได้สัมพันธ์กับระดับไตรกลีเซอร์ไรด์ในเลือด รวมถึงชนิดของโปรตีนที่ตรวจพบ มีทั้งกลุ่มที่ทราบหน้าที่และเป็นกลุ่มโปรตีนใหม่ที่มีบทบาทในเรื่องการควบคุมเมแทบอลิซึมของไขมันและการอักเสบ ทั้งนี้การศึกษาเรื่องของโปรตีนร่วมกับสารเมตาบอไลท์จะนำไปสู่ตัวชี้วัดทางชีวภาพใหม่ๆ รวมถึงกลไกที่เกี่ยวข้องกับสาเหตุของโรคเรื้อรังต่างๆ และแนวทางการรักษาในอนาคต

คำสำคัญ: โปรตีโอมิกส์ เมแทบอลิซึมส์ของคาร์โบไฮเดรต ภาวะไตรกลีเซอร์ไรด์สูงในเลือด

รับบทความ: *10 ธันวาคม 2565* ***แก้ไข:*** *23 ธันวาคม 2565* ***ตอบรับ:*** *25 ธันวาคม 2565*

[*] **ผู้รับผิดชอบบทความ**

จินตนา ศิรวราศัย

กลุ่มสาขาวิชาโภชนศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

อีเมล: jintana.sir@mahidol.ac.th

Alterations of Serum CCR5 and Carbohydrate Metabolic Proteomics in Hypertriglyceridemia

Jintana Sirivarasai[1,*] Pischa Channaron[2] Sittiruk Roytrakul[3] Piyamitr Sritara[4]

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

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

[3] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency

[4] Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University

Abstract

Hypertriglyceridemia, obesity, and insulin resistance are recognized as the predictive markers for coronary artery disease risk. However, until now their specific etiologies and mechanism of pathogenesis remain unclear. We therefore conducted a proteome association study to identify protein profiles between control and hypertriglyceridemia groups. There were 2 study groups; Control group (N=5) was healthy male and hypertriglyceridemia group were divided into 3 subgroups (N=6/subgroup) based on plasma triglyceride levels; (1) borderline TG level (150–199 mg/dL); (2) high TG level (200–499 mg/dL); and (3) very high TG level (≥ 500 mg/dL). LC-MS/MS-based serum proteomics analysis revealed chemokine and cytokine signaling pathway (6.5%), the integrin signaling pathway (6.1%), and Wnt signaling pathway (4.2%). Protein-protein interaction as well as molecular network related to triglyceride were observed. Relative fold change of serum CCR5 was found with possible association with dyslipidemia, obesity, insulin resistance, and inflammation. Overall findings indicated that relative abundance of the proteome varied with TG levels and untargeted serum proteomics detects comprehensive sets of both known and novel associated proteins likely reflecting regulation of lipid metabolism and inflammation. Future proteomics study with metabolomics may lead to new biomarkers and metabolic pathways underlying etiology and also may serve to identify new therapeutic targets.

Keywords: Proteomics, Carbohydrate metabolism, Hypertriglyceridemia

Received: *10 December 2022,* ***Revised:*** *23 December 2022,* ***Accepted:*** *25 December 2022*

[*]Corresponding author

Jintana Sirivarasai

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

Email: jintana.sir@mahidol.ac.th

Introduction

Lipid and carbohydrate metabolisms are very important in the development of metabolic diseases, such as obesity, type 2 diabetes mellitus, cardiovascular disease, non- alcoholic fatty liver disease and cancer[1]. Impaired lipid metabolism is the primary feature as dyslipidemia which hypertriglyceridemia is one of the most important one. This abnormal amount of lipids may cause by increased triglyceride (TG) production or decreased catabolism of TG- rich lipoproteins, and directly influences low density lipoprotein (LDL) and high density lipoprotein (HDL) composition and metabolism. Therefore, TGs play a central role in the pathogenesis of atherosclerosis and increasingly important in individuals with obesity and insulin resistance[2]. Obesity is the key biological features of interplay between glucose and lipid abnormal metabolisms that promote various metabolic disorders.

Predominantly, main functions of adipose tissue relate to synthesis and storage of triacylglycerol (TAG) in periods of energy excess and to lipolysis for releasing free fatty acids during periods of energy deprivation. In addition, this tissue also secretes adipokines that modulate various biological processes, including energy intake, glucose metabolism and immune function[3]. Various adipokines can exhibit either proinflammatory or anti-inflammatory properties, thereby mediating in inflammation and insulin resistance[3]. Obesity also exhibits adipocyte hypertrophy which releases pro- inflammatory adipokines such as adiponectin, leptin, tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1) etc. These adipokines are also involved in modulating insulin function[4]. MCP- 1 may secrete from hypertrophic adipocytes and induce the recruitment of monocytes to adipose tissue by interacting with C-C chemokine receptor (CCR), its receptor, and finally with contributing the recruitment of adipose tissue macrophages (ATMs)[5]. Particularly, the C-C chemokine receptor (CCR5) has been linked to obesity- induced adipose tissue inflammation and an effect on carbohydrate metabolism via insulin resistance by regulating both macrophage recruitment and M1/M2 status[6]. Moreover, obese with dyslipidemia exhibited a decreased production of adiponectin, lead to a reduced of glucose uptake in muscle cells and increase in glycogenolysis and gluconeogenesis through a cross talk of adiponectin with the insulin signaling pathway. These changes contribute to the development of obesity- induced insulin resistance[7]. Overall mechanisms highlighted closely link between obesity, dyslipidemia and abnormal of carbohydrate metabolism[8].

Proteins are complex molecules that are crucial to the structure, function, and regulation of cells. Numerous protein complexes discover multiple molecular functions, including transport across the cell membrane, regulation of the energy metabolism of the cell, activation of cell membrane receptors, and signal transduction. Proteomics is defined as the study of the proteome, protein- protein interaction, and innovative biomarkers related to human diseases by using high-throughput liquid chromatography tandem-mass spectrometry (LC-MS/MS)[9]. Previous proteomic analysis of the foam cells based on the stimulation of differentiated cells with hypertriglyceridemia- induced increased VLDL found 8 up- regulated proteins and 6 down- regulated proteins. They were involved in energy metabolism, oxidative stress, cell growth, differentiation and apoptosis[10]. The findings suggested that VLDL resulted in lipid accumulation and changes the characteristics of foam cells by altering the expression of various proteins such as adipose differentiation- related protein (ADRP), enolase, S100A11, heat shock protein 27[10]. Another study indicated plasma proteins correlated with triglyceride concentrations in 500 Nepalese children, including apoC-II/C-III/C-IV, cathelicidin antimicrobial peptide, proteoglycan 4, retinol- binding protein 4 (RBP4), apoE, anthrax toxin receptor (ANTXR) 2, neuropilin 1, and insulin- like growth factor binding protein 1, CETP and phospholipid transfer protein (PLTP) [11]. Based on literature review, no report among Thai individuals with dyslipidaemia have been performed with proteomic approach. This methodology is advantageous for the identification of biomarkers, altered pathways, functional alterations and mechanisms. Researches with this technique will lead to identify several differentially expressed proteins in high risk groups compared to healthy and the associated outcomes. The objective of this study was to compare the protein profiles of the hypertriglyceridemia and control groups in order to clarify the molecular mechanism underlying their potential chronic metabolic risks.

Materials and Methods

Participant selection and samples

The EGAT study began in Bangkok in 1985. This survey mainly covered details of established CVD risk factors in various settings, including nutrition and toxicology. Blood samples were stored for future use, e.g. in genetic and biomarker studies. Blood was collected in a fasting state at all survey visits to date. Laboratory tests for EGAT study subsequently carried out every time of cohort study included glucose, total cholesterol, low-density lipoprotein (LDL), high- density lipoprotein (HDL) and

triglycerides, creatinine, total protein, albumin, total bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), gamma-glutamyl transpeptidase (GGT) and a full blood count. Some special blood tests were added in certain surveys. In our study, all serums were obtained from the second survey of EGAT2 in 2013. Samples were classified into four groups by triglyceride levels with the criteria of NCEP ATP III[11]. The self-administered questionnaires were used for data collection including general demographic data, lifestyle factors related to metabolic risks (such as cigarette smoking, alcohol consumption, physical exercise, and sleep pattern, etc.), history, family history and use of medications. In addition, physical examination, anthropometric measurements and blood tests were also performed at the same day. For anthropometric measurements, trained staffs performed by using standardized methods, body weight and height are determined and BMI is calculated as weight (kg) divided by height squared (m^2).

Based on the procedure of EGAT cohort study, venous blood samples were collected in the morning after an overnight fast (12 hours). Following collection, serum were aliquoted into cryovials for long-term storage. In general, blood processing were performed as soon as possible, and samples were stored to avoid the degradation of any biomolecules. Serum samples were stored at −80°C for specific subsequent analysis with no freeze and thaw cycles. All samples were properly labeled, and their locations were saved in a database for future analysis. For this study, we selected data only triglyceride levels for analysis with proteomic analysis in 2017.

In this study, there were two study groups; 1) Control group (N=5) were male with mean age of 45.67±3.25 years with TG level of 123.56 ± 2.45 mg/dL, normal BMI(18.5–22.9 kg/m^2), no metabolic syndrome and other chronic disease, no smoking and drinking alcohol and 2) Hypertriglyceridemia group (male with mean age of 49.85 ±6.52 years) was divided into 3 subgroups (N=6/subgroup) based on plasma triglyceride levels; (1) borderline TG level (150–199 mg/dL) with BMI of 18.5–22.9 kg/m^2; (2) high TG level (200–499 mg/dL) with BMI of 18.5–22.9 kg/m^2; and (3) very high TG level (≥500 mg/dL) with BMI ≥ 25 kg/m^2. This study was approved by the Ethics Committee of Faculty of Medicine Ramathibodi Hospital, Mahidol University (MURA2017/115) and was performed according to the principles expressed in the Declaration of Helsinki. Participants will provide written informed consent to take part in the EGAT cohort study.

Sample preparation

The protein concentration of samples was determined by Bradford assay[13]. All the chemicals and solvents used were of analytical grade and were purchased from Sigma–Aldrich Corporation (USA), unless otherwise stated. Protein samples were separated by two- dimensional gel electrophoresis: sodium dodecyl- sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 10 µg of proteins were loaded per well in the pooled of both groups. The gels were then visualized with silver stain. All samples were subjected to the in-solution digestion followed by trypsin digestion[14]. Samples were subjected to the in- solution digestion. First, dissolved 4 µg of samples in 10 mM ammonium bicarbonate (AMBIC), added 5 mM dithiothreitol (DTT) in 10 mM AMBIC, incubated at 60 °C for 1 hour to eliminate disulfide bonds and then spun down. Added 15 mM Iodoacetamide (IAA) in 10 mM AMBIC at room temperature for 45 minutes in the dark. For digestion, samples were mixed with 50 ng/µl of sequencing grade trypsin (1:20 ratio) and incubated at 37 °C overnight. Prior to LC-MS/MS analysis, the digested samples must be purified by using C18 LTS Tip to remove buffer and salts. The C18 LTS tip (Thermo Fisher Scientific Inc, USA) was washed with 100% acetonitrile (ACN)/0.1% formic acid (FA) and equilibrated with 0. 1% FA. The sample/0. 1% FA was fully pipetting into C18 LTS Tip and out of tip 3- 4 times. Washed the tip with 5% ACN/0.1% FA to remove salts and contaminants. After that, Sample was eluted peptides in 70% ACN/ 0. 1% FA and dried at 44 °C. Tryptic peptides were protonated with 0. 1% FA before injection into the NanoAcquity system.

LC-MS/MS analysis

The peptide fraction was analyzed using an Ultimate3000 Nano/Capillary LC System (Dionex, UK) coupled to a Hybrid quadrupole Q- Tof impact II™ (Bruker Daltonics GmbH, Germany) equipped with a Nano captive spray ion source. The raw LC-MS/MS Spectra were carried out using Bruker CompassData Analysis 4.4 software (Bruker Daltonics GmbH, Germany)[15].

Data analysis

DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) was used for proteins quantitation[16-17]. Acquired LC- MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on the peptide ions signal intensities in MS mode. The analyzed MS/ MS data from DeCyderMS were submitted for a database search using the Mascot software (Matrix Science, London, UK)[18]. The data were searched against the

NCBI database for protein identification. Database interrogation was; taxonomy (*Homo sapiens*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); 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[16]. The maximum value of each group was used to determine the presence or absence of each identified protein. Peptide intensities from the LC-MS analyses were transformed and normalized by using a mean central tendency procedure. These datasets performed statistically significant proteins at $p<0.05$. All differentially expressed proteins were identified the identity by UniProt (http://www.uniprot.org/) and their unique proteins among different groups were analyzed by using jvenn and described as Venn diagram (http://jvenn.toulouse.inra.fr/app/example.html)[19]. Gene ontology annotation including biological process, molecular function, and cellular component was performed using Panther (http://www.pantherdb.org)[20]. The identified proteins were simultaneously submitted to The Search Tool for Interacting Chemicals (STITCH) (http://stitch.embl.de) version 5.0 to search for understanding of cellular functions and interactions between proteins and small molecules[21]. Molecular-level functions of our identified proteins were further analysed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Higher-level functions are represented by networks of molecular interactions, reactions and relations in the forms of KEGG pathway maps[22].

Results

The Bradford assay was used for determination of protein concentration which sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, as shown in Figure 1. The protein bands were separated on the basis of molecular weight (kDa) which bands of the pooled of control (healthy) and hypertriglyceridemia groups did not show any differences.

Each sample was subjected to the in-solution digestion and was identified by liquid chromatography- tandem mass spectrometry (LC-MS/MS). The results found 1,041 GI numbers of proteins (for GenInfo Identifier) from all groups and 706 of them were successfully identified by Uniprot. In addition, all proteins were classified by Panther program. Most proteins involved in the cellular process (26.8%) and metabolic process (22.9%), classified by the biological process (Figure 2A). Based on molecular function, 40.5% and 38.3% of proteins were related to the catalytic activity and were binding proteins,

respectively (Figure 2B). Most of the proteins were in the group of cell part (45.4%) and organelle (26.3%), respectively, classified by the cellular component (Figure 2C).

Proteins were classified by several pathways. Most of the proteins which were observed >4% involved in the inflammation mediated by chemokine and cytokine signaling pathway (6.5%), the integrin signaling pathway (6.1%), Wnt signaling pathway (4.2%). The pathways which were observed in ranges 3-4% including p53 pathway (3.7%), PDGF signaling pathway (3.3%), Nicotinic acetylcholine receptor signaling pathway (3.3%). For protein group with distributed by pathways about 2-3%, they were related to EGF receptor signaling pathway, Cytoskeletal regulation by Rho GTPase, cell cycle, hypoxia response via HIF activation, endothelin signaling pathway, cadherin signaling pathway, apoptosis signaling pathway and CCKR signaling map. Other pathways were demonstrated in Table 1.

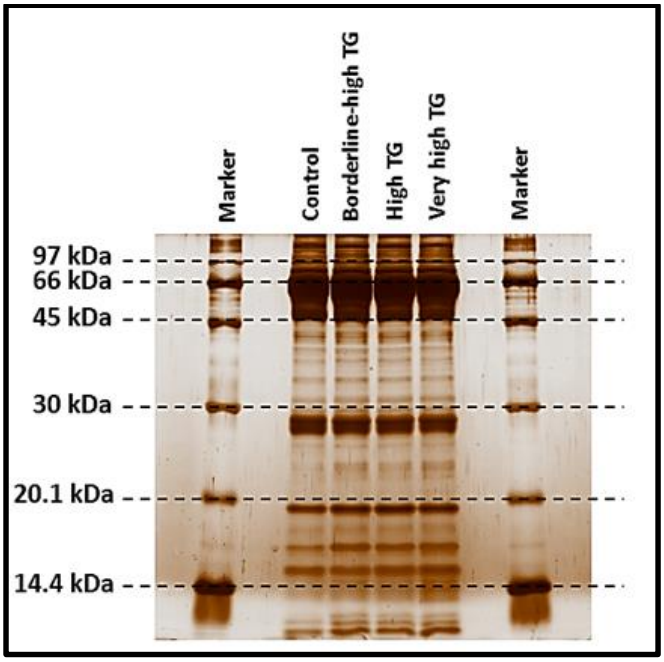

Figure 1. SDS-PAGE- pooled serum of control (healthy) and hypertriglyceridemia groups.

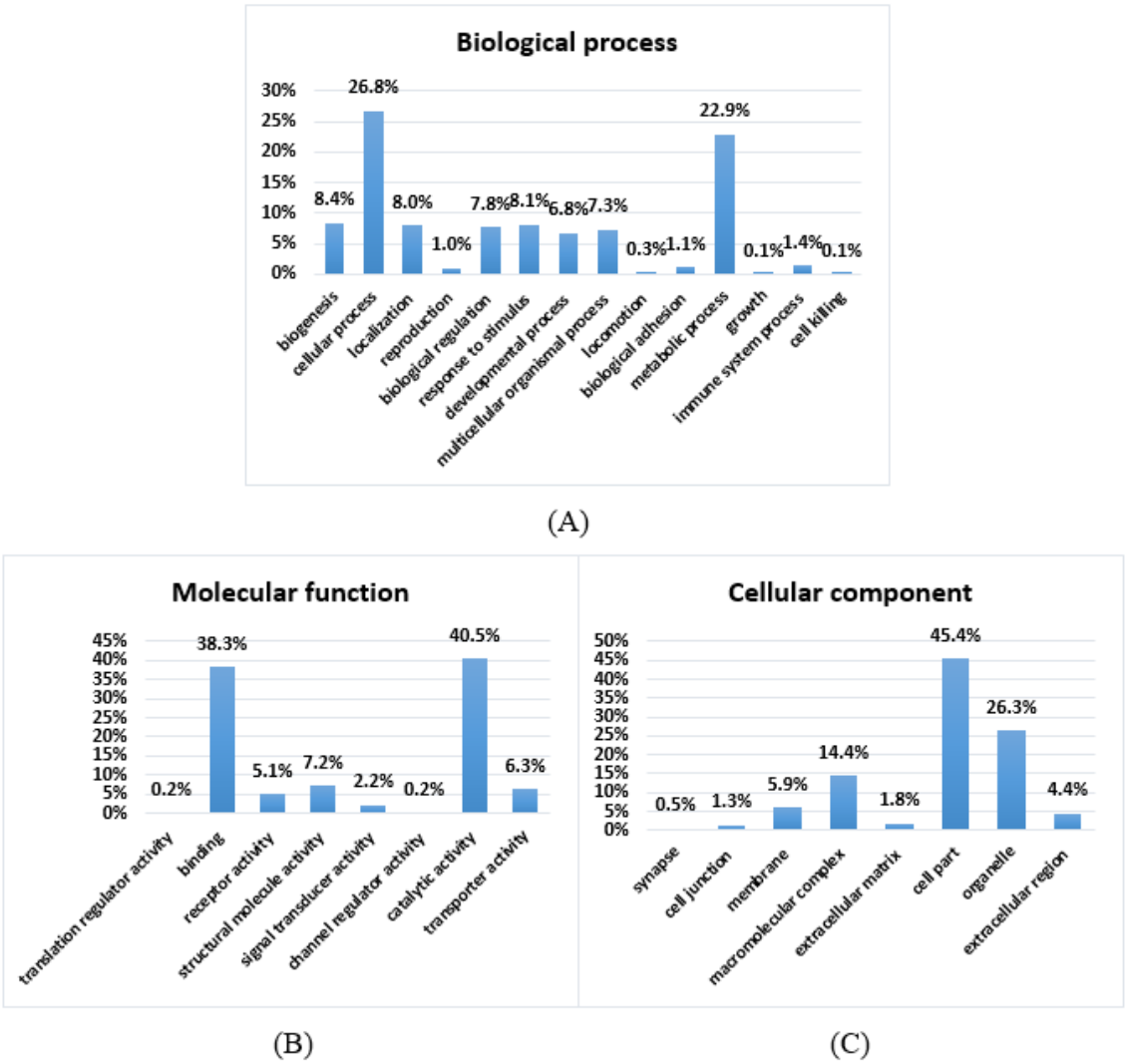

Figure 2. Classification of proteins by their functions in the categories of biological process (A), molecular function (B), and cellular component (C).

A Venny 2. 1 software was used to depict the comparison of protein lists between four groups. There were 992, 1,029, 1,017 and 1,011 proteins found in the control, borderline- high triglyceride, high triglyceride and very high triglyceride groups, respectively. 959 proteins were common to all four groups. In addition, we found 465 overlapping proteins among three groups of abnormal triglyceride levels. The identification and functions of proteins among three groups of abnormal triglyceride levels were presented in Table 2. The relative fold change of identified proteins with relative fold change more than 10 (log 2 values compared to control group) were described in the very high, high and borderline triglyceride levels. For very high triglyceride group, there were 11 significant proteins, including C-C chemokine receptor

type 5 (CCR-5), Fibronectin type III domain-containing protein 11 (FNDC11), Cyclin-dependent kinase inhibitor 1B (CDKN1B), Transcription factor SOX-13 (SOX13), Thiosulfate sulfurtrans-ferase/ rhodanese-like domain-containing protein 2 (TSTD2), Helicase-like transcription factor (HLTF), Myosin-binding protein C, slow-type (MYBPC1), Ras-specific guanine nucleotide-releasing factor (RALGPS2), Neural proliferation differentiation and control protein 1 (NPDC-1), Protein PRR14L (Proline rich 14-like protein), and Neuron navigator 3 (Pore membrane and/or filament-interacting-like protein 1) (NAV3). All proteins were interacted with various factors related hypertriglyceridemia including triglyceride, apolipoproteins, antioxidants (selenium, vitamin C and E), PUFA, fructose, glucose and drugs (fibrates, statin, niacin, and ezetimibe) by using STITCH database to explore their interaction (Figure 3).

We observed an important finding with interaction analysis which CCR5 protein (28.41 time of fold changes compare to control group) directly interacted with glucose and linked to triglyceride (triacylglycerol). Moreover this association was identified as carbohydrate metabolism by the KEGG pathway map. The other proteins such as RALGPS2, TSTD2, NPDC1, PRR14L and CDKN1B indirect interacted with HLTF and linked to glucose and triacylglycerol. Overall identified proteins among very high triglyceride group indicated possible association with hypertriglyceridemia.

Discussion

Pooled analysis of control and three hypertriglyceridemia groups were performed to lessen the inter-individual variability and verify the intergroup differences (Figure 1). The relative quantification of proteins was performed for comparing relative changes in protein abundance between control and hypertriglyceridemia groups. As obesity has been proposed as a strong predisposing risk factor for CHD, we aimed to analyse the lipid parameters as triglyceride level with focused on very high TG and obesity group. We discovered 11 differentially expressed proteins in the obese with very high TG level group, representing more than 10-fold changes in expression. There were NAV3, NPDC1, RALGPS2, PRR14L, TSTD2, FNDC11, CDKN1B, transcription factor SOX-13 (SOX13), myosin-binding protein C, slow-type (MYBPC1), C-C chemokine receptor type 5 (CCR5) and helicase-like transcription factor (HLTF) (Table 2). For the borderline TG and high TG groups, a large number of proteins related to various functions, including ATP binding, GTP binding, translation

regulation, cell-cycle regulation, lysosomal degradation, kinase modification, protein degradation were also described.

Table 1. Protein classification according to the pathway

Group by percentages	Pathways	Percentages
>4%	Inflammation mediated by chemokine and cytokine signaling pathway	6.5%
	Integrin signaling pathway	6.1%
	Wnt signaling pathway	4.2%
3-4%	p53 pathway	3.7%
	PDGF signaling pathway	3.3%
	Nicotinic acetylcholine receptor signaling pathway	3.3%
2-3%	EGF receptor signaling pathway	2.8%
	Cytoskeletal regulation by Rho GTPase	2.8%
	Cell cycle	2.3%
	Hypoxia response via HIF activation	1.9%
	Endothelin signaling pathway	1.9%
	Cadherin signaling pathway	1.9%
	Apoptosis signaling pathway	1.4%
	CCKR signaling map	1.4%
	Transcription regulation by ZIP transcription factor	1.4%
	T cell activation	1.4%
	Muscarinic acetylcholine receptor 1 and 3 signaling pathway	1.4%
	Interleukin signaling pathway	1.4%
	Insulin/IGF pathway-protein kinase B signaling cascade	1.4%
	Ras Pathway	1.4%
	General transcription regulation	1.4%
	Blood coagulation	1.4%
<1%	VEGF signaling pathway	0.9%
	Toll receptor signaling pathway	0.9%
	Tetrahydrofolate biosynthesis	0.9%
	Others	<0.9%

Table 2. Proteins identification and their functions according to three abnormal triglyceride groups

No.	Group	gi number	Protein names	Function	Fold changed		
					Borderline-high TG	High TG	Very high TG
1.	Very high TG group	gi|1705896	C-C chemokine receptor type 5 (CCR-5)	Binding, Receptor activity	-	-	28.41
2.	Very high TG group	gi|74761293	Fibronectin type III domain-containing protein 11 (FNDC11)	Transcription	-	-	20.45
3.	Very high TG group	gi|1168871	Cyclin-dependent kinase inhibitor 1B (CDKN1B)	Binding, Catalytic activity	-	-	27.21
4.	Very high TG group	gi|3982829	Transcription factor SOX-13 (SOX13)	Binding	-	-	13.21
5.	Very high TG group	gi|74745447	Thiosulfate sulfurtransferase /rhodanese-like domain-containing protein 2 (TSTD2)	Stress response	-	-	21.36
6.	Very high TG group	gi|60390864	Helicase-like transcription factor (HLTF)	Binding, Catalytic activity	-	-	27.42
7.	Very high TG group	gi|6166597	Myosin-binding protein C, slow-type (MYBPC1)	Binding, Structural molecule activity	-	-	14.65
8.	Very high TG group	gi|74750518	Ras-specific guanine nucleotide-releasing factor (RALGPS2)	Cytoskeletal organization, the stimulation of transcription in a Ras-independent fashion	-	-	22.31
9.	Very high TG group	gi|22261810	Neural proliferation differentiation and control protein 1 (NPDC-1)	Regulation of immune response, transcriptional regulation	-	-	23.09
10.	Very high TG group	gi|74746580	Protein PRR14L (Proline rich 14-like protein)	Phosphoprotein	-	-	23.27
11.	Very high TG group	gi|313104213	Neuron navigator 3 (Pore membrane and/or filament-interacting-like protein 1) (NAV3)	ATP binding, neuron regeneration, negative regulation of cell migration	-	-	16.54
12.	Borderline and high TG groups	gi|6226858	Translation initiation factor eIF-2B subunit beta (S20I15) (S20III15)	ATP binding, GTP binding, translation initiation factor activity	12.45	10.36	-
13.	Borderline and high TG groups	gi|118572229	ELM2 and SANT domain-containing protein 1 (MIDEAS)	Transcription factor activity, sequence-specific DNA binding	16.47	18.09	-
14.	Borderline and high TG groups	gi|22547207	Lysine-specific demethylase	Transcription regulation, DNA-binding	10.97	15.21	-

Table 2. Proteins identification and their functions according to three abnormal triglyceride groups (cont.)

No.	Group	gi number	Protein names	Function	Fold changed		
					Borderline-high TG	High TG	Very high TG
				Specific signal transduction responses in the cell, the regulation of cell cycle progression, actin cytoskeleton assembly, and for cell migration, cell adhesion and other functions	16.32	12.89	14.11
15.	Borderline, high, and very high TG groups	gi|1002635084	Serine/threonine-protein kinase N2 (PKN gamma) (Protein kinase C-like 2) (Protein-kinase C-related kinase 2)				
16.	Borderline, high, and very high TG groups	gi|119622900	Achaete-scute associated protein (HCG1648122)	Unknown	16.33	11.87	13.24
17.	Borderline, high, and very high TG groups	gi|21752137	cDNA FLJ35984 fis, clone TESTI2014097, highly similar to V_segment translation product	Unknown	19.23	12.36	14.25
18.	Borderline, high, and very high TG groups	gi|148887370	Dual specificity tyrosine-phosphorylation-regulated kinase 2 (EC 2.7.12.1)	The mitotic cell cycle, cell proliferation, apoptosis, organization of the cytoskeleton	16.32	17.25	17.99
19.	Borderline, high, and very high TG groups	gi|67476446	Coagulation factor IX (EC 3.4.21.22) (Christmas factor) (Plasma thromboplastin component) (PTC) [Cleaved into: Coagulation factor IXa light chain; Coagulation factor IXa heavy chain]	The intrinsic pathway of blood coagulation	12.03	15.21	14.09
20.	Borderline, high, and very high TG groups	gi|300669605	Apolipoprotein B-100 (Apo B-100) [Cleaved into: Apolipoprotein B-48 (Apo B-48)]	A recognition signal for the cellular binding and internalization of LDL particles by the apoB/E receptor	15.46	14.09	16.96
121.	Borderline, high, and very high TG groups	gi|121940572	Retrotransposon Gag-like protein 8B (Mammalian retrotransposon derived protein 8B)	Neofunctionalized retrotransposons genes	10.23	16.14	13.26
22.	Borderline, high, and very high TG groups	gi|2914206	Collagen alpha-1(II) chain (Alpha-1 type II collagen) [Cleaved into: Collagen alpha-1(II) chain; Chondrocalcin]	For linear growth and for the ability of cartilage to resist compressive forces.	12.07	14.12	11.78

Table 2. Proteins identification and their functions according to three abnormal triglyceride groups (cont.)

No.	Group	gi number	Protein names	Function	Fold changed		
					Borderline-high TG	High TG	Very high TG
23.	Borderline, high, and very high TG groups	gi|14916956	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial	ATP-binding, heme biosynthesis	16.35	15.23	16.98
24.	Borderline, high, and very high TG groups	gi|55977885	Zinc finger protein 219	Transcriptional regulation	14.23	12.55	17.96
25.	Borderline, high, and very high TG groups	gi|20141238	Beta-arrestin-1 (Arrestin beta-1)	Signal transduction inhibitor, protein transport, transcription regulation	11.35	17.63	13.87
26.	Borderline, high, and very high TG groups	gi|31615803	Polyubiquitin-C [Cleaved into: Ubiquitin]	ERAD (endoplasmic reticulum-associated degradation) and in cell-cycle regulation, lysosomal degradation, kinase modification, protein degradation via the proteasome	16.38	17.78	16.04
27.	Borderline, high, and very high TG groups	gi|296439369	Dedicator of cytokinesis protein 4	Regulation of adherens junction between cells, cell migration	13.25	12.99	15.74
28.	Borderline, high, and very high TG groups	gi|15214936	SLAMF1 protein (Signaling lymphocytic activation molecule)	Receptor activity, lymphocyte activation	14.23	10.96	13.25
29.	Borderline, high, and very high TG groups	gi|41018482	Zinc finger SWIM domain-containing protein 5	Zinc ion binding, regulation of transcription	13.25	18.23	14.94
30.	Borderline, high, and very high TG groups	gi|172046234	Putative Polycomb group protein ASXL3 (Additional sex combs-like protein 3)	DNA binding, regulation of transcription	10.78	12.36	14.01

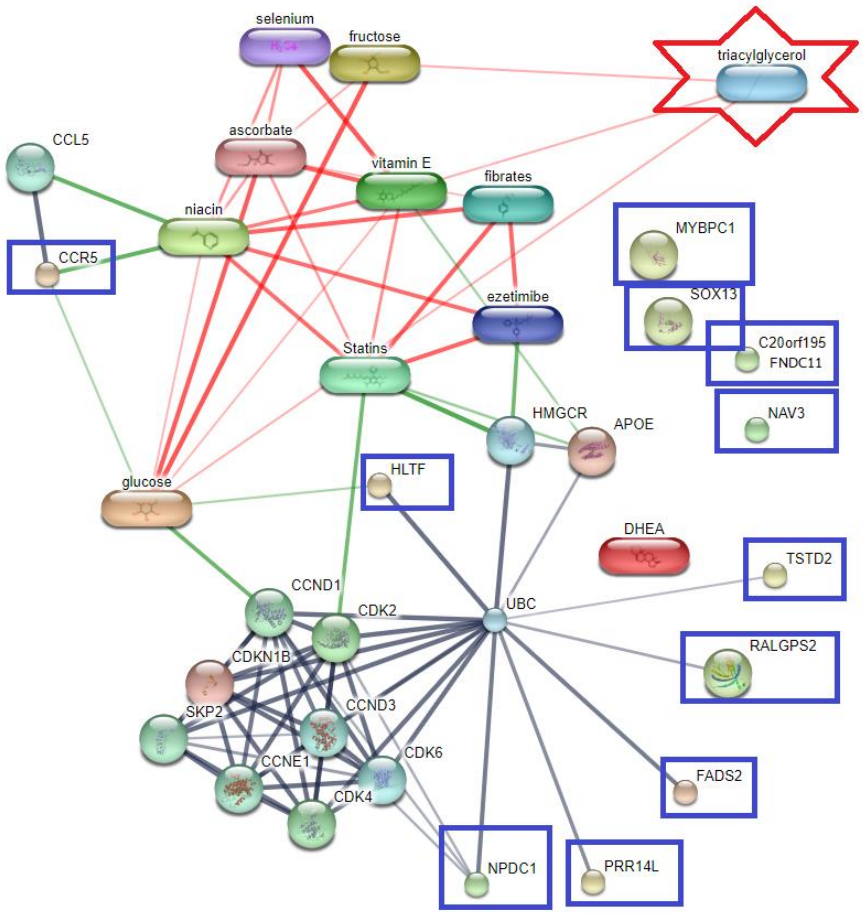

Figure 3. Interaction of all factors (triglyceride, apolipoproteins, antioxidants, DHEA, fructose, glucose and drugs) related to 11 identified proteins in the very high triglyceride groups. (Stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, chemical-protein interactions in green and interactions between chemicals in red.)

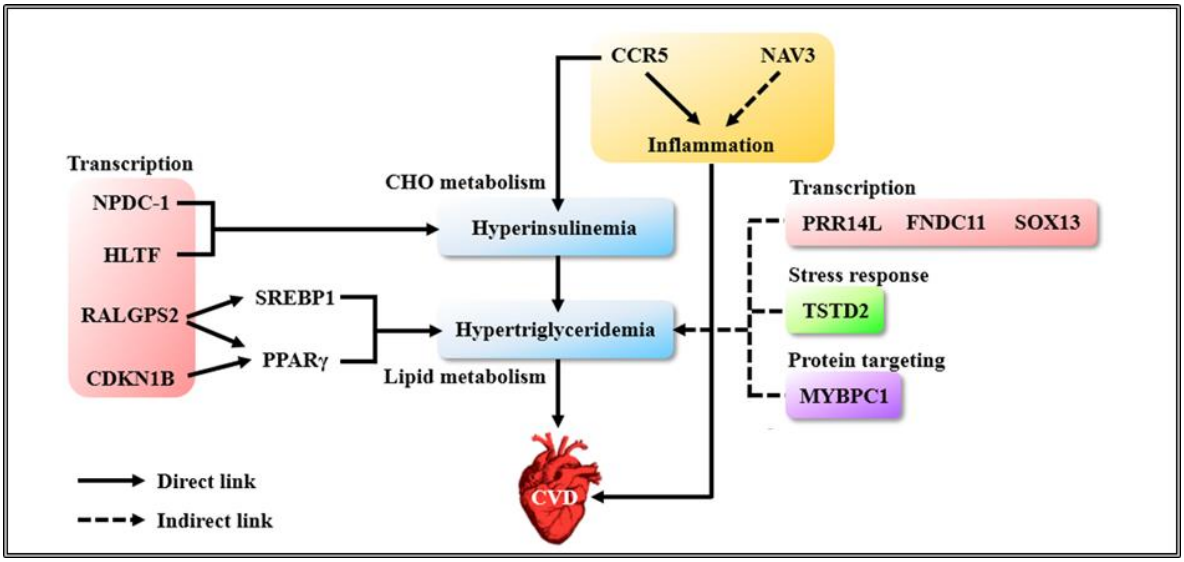

Figure 4. Schematic of association between identified proteins and CVD risk in hypertriglyceridemia

A group of potential factors, including apolipoprotein, antioxidants (selenium, vitamin C, and E), PUFA (DHA), fructose, glucose, and medications (statins, ezetimibe, fibrates, and niacin), were proposed into interaction analysis in order to better understand the relationship between proteins and factors related to hypertriglyceridemia [23-31]. Interaction between all identified proteins from very high TG group and potential factors has been described in Figure 3. Chemokines are small proteins and a family of cytokines which associated with allergic and autoimmune diseases. C-C chemokine receptor type 5 (CCR5), a chemokine receptor, found on the surface of white blood cells such as macrophages, T cells, dendritic cells and expressed in lymphoid organs[32]. Normally, the insulin sensitivity is maintained by alternatively activated non-inflammatory macrophages (M2 macrophages) which produce anti-inflammatory cytokines such as interleukin-4 (IL-4) and IL-10. In contrast, obesity can cause hypertrophic adipocytes. The monocyte chemoattractant proteins (MCP)-1 or CCL2 acts as a ligand by interacting with C-C motif chemokine receptor 2 (CCR2), leads to an accumulation of adipose tissue macrophages (ATMs). CCR5+ ATMs infiltrate and secrete pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6 and IL-1β which inhibit insulin function subsequently promote insulin resistance. An abnormal carbohydrate metabolism (hyperinsulinemia) resulting in increased hepatic de novo lipogenesis (DNL) and increased hepatic triglyceride synthesis. Hypertrophic adipocytes also secrete MCP-1 which promotes the infiltration of monocytes into adipose tissue and the secretion of those pro-inflammatory cytokines by classically activated pro-inflammatory macrophages (M1 macrophages), these can cause inflammation and lead to CVD[33]. Previous study reported that CCR5+ macrophages accumulated in WAT of obese mice due to the expression of CCR5 and its ligands was increased and was equal to CCR2 and its ligands in WAT of obese mice and high fat diet-induced obese mice[34]. The expression of CCR5 and its ligand also increased in obese individuals with insulin resistance and indicated that CCR5 could be the novel protein associated with obesity, insulin resistance and inflammation[3,34]. In addition, our results supported carbohydrate metabolic proteomics that partly indicated role of CCR5 with directly interaction with glucose. All these findings closely related to carbohydrate metabolism and the development of type 2 diabetes.

NPDC1 is a protein expressed primarily in brain and lung. NPDC1 interacts with the transcription factor E2F1

by reducing the binding of E2F1 to DNA and modulates its transcriptional activity[35]. E2F1 plays roles in the differentiation of many tissues. Pathways repressed E2F1 such as glucose oxidation, oxidative metabolism which related to cardiomyopathy or cardiac infarction. Moreover, it was found that the activity of E2F1 has increased in obesity which could contribute to the comorbidities of some conditions such as hyperinsulinemia, lipogenesis, and non-alcoholic fatty liver disease (NAFLD) [36]. It can be explained that abdominal adipose tissue release increased FFA which inhibit the uptake of glucose by muscle. In addition, excess FFA combined with angiotensin II impaired pancreatic function, resulting in insulin resistance. Increased circulating FFA and fasting plasma glucose levels delivered substrates for increased triglyceride synthesis by the liver, resulting in lipoproteins transporting more triglyceride and less HDL. Hyperinsulinemia leads to an increased in de novo lipogenesis, contributing to hypertriglyceridemia and NAFLD development[1].

Ras or Rheb (Ras homolog enriched in brain) is one of the subfamilies of small guanine nucleotide-binding proteins (G proteins) that function as transducers in signaling pathways for RALGPS2. The tuberous sclerosis complex (TSC) acts as a GTPase-activating protein (GAP) converts an active Rheb-GTP to an inactive Rheb-GDP. The active Rheb-GTP stimulates the mammalian target of rapamycin (mTOR) signaling pathways that associates with cell proliferation, growth and vascular endothelial growth factor (VEGF) transcriptional activation while the inactive Rheb-GDP has an inhibitory effect[37-38]. Interestingly, mTOR complex 1 (mTORC1) regulates the activity of sterol regulatory element-binding proteins 1 (SREBP1) and peroxisome proliferator-activated receptor-γ (PPARγ), the transcription factors that control the expression of genes encoding proteins especially fatty acid and cholesterol pathway genes, associated with increased cholesterol synthesis[39]. Increased VLDL causes an increase in LDL. Because LDL particles are responsible for delivering cholesterol to cells, high levels of LDL can raise total cholesterol levels. As a result, elevated cholesterol levels are associated with elevated VLDL-triglyceride levels. Moreover, IR is associated with increased secretion of very low density lipoproteins (VLDL) and increased plasma triglycerides[40].

CDKN1B binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1. CDK is a small protein involved in the cell cycle,

differentiation, apoptosis, transcription and neuronal functions. CDK binds with cyclin, a regulatory protein to phosphorylate proteins which are required to initiate cell cycle. This cell cycle regulator participates in the lipid metabolism as it regulates adipogenesis by modulating PPARγ. It also controls other metabolic processes such as glycolysis or mitochondrial activity related to obesity and type II diabetes [41]. In addition, previous work investigated the relationship between high-glucose (HG) culture, CpG methylation of genes involved in cell signaling pathways, and the regulation of carbohydrate and lipid metabolism in hepatocytes. DNA methylation of CDKN1B was found as directly related to carbohydrate, lipid, and energy metabolism through PI3K-Akt signaling pathway, cAMP signaling pathway, type II diabetes mellitus, and insulin secretion[42].

Another protein, HLTF is a transcription factor involved in many different pathways such as cell cycle, apoptosis, DNA repair, collagen biogenesis, angiogenesis, and contractile function[43]. Previous study reported that HLTF involved in the glucose metabolism by activating the transcription of glucose transporters included GLUT1 and GLUT9 which the protein products control glucose flux. In addition, the expression of GLUT1 was associated with cardiac glucose demands that increased in response to ATP demanding, leading to heart failure [44]. Bacos et al. demonstrated that knockdown of HLTF gene affected on clonal β-cells which can impair insulin secretion and increase T2DM risk. Moreover, HLTF may have ubiquitinylating properties which consistent to our results [45].

PRR14L is a proline-rich protein, binds to the Src homology 3 (SH3) domains resulting in phosphatidylinositol 3 kinase (PI3K) activation[46]. PI3K is a key molecule in the initiation of signal transduction pathways. Dysregulation of PI3K activity, and as aberrant PI3K signaling, lead to a broad range of human diseases, such as cancer, immune disorders, diabetes, and cardiovascular diseases. A growing number of studies have shown that PI3K and its signaling pathways play key roles in the pathophysiological process of atherosclerosis[47]. In addition, phosphatidylinositol-3 kinases (PI3K)-Protein kinase B (Akt)-mammalian target of rapamycin (mTOR) pathway plays an important role in the synthesis and secretion of triacylglycerol. The reduction of lipids accumulation induced-by inhibiting PI3K-Akt-mTOR pathway was closely linked to the decrease of lipogenesis, the increase of fatty acids oxidation, and the increase of

VLDL assembly and secretion in hepatocytes[48].

TSTD2, also known as a family of thiosulfate sulfurtransferase, is a mitochondrial enzyme which catalyzes the transfer of sulfur in several molecular pathways. There were data reported role of TST in sulfur and selenium metabolism, the regulation of respiratory function and the interaction with antioxidant systems. These evidences suggested TST to be a tightly regulated link between diverse mitochondrial processes. Consistent with the diversity of mechanisms, an imbalance in TST activity or availability may have implications for an equally diverse range of pathological processes[49]. However, researches related to TSTD2 in human metabolic diseases were limited.

Proposed association between our findings and hypertriglyceridemia with CVD risk were summarized in Figure 4. Functions of NPDC1, RALGPS2, CDKN1B, and HLTF were suggested with involvement in the transcription which NPDC1 and HLTF linked to hyperinsulinemia while RALGPS2 and CDKN1B linked to de novo lipogenesis and hypertriglyceridemia. Other proteins including NAV3, PRR14L, TSTD2, FNDC11, SOX13, and MYBPC1 may indirectly link to hypertriglyceridemia with possible their functions as inflammatory mediator of NAV3; transcription factors of PRR14L, FNDC11, and SOX13; stress response mediator of TSTD2 and protein targeting of MYBPC1.

A variety of identified and signaling pathways might be associated in the pathogenesis of other metabolic conditions or comorbidities. Because hypertriglyceridemia is a metabolic disease, further analysis determine other factors related to elevated triglyceride levels, including apolipoproteins will lead to an increase in our understanding of the etiology and underlying molecular events of abnormal of triglyceride metabolism. However, this is the preliminary results with small sample size. Larger sample size may be needed to confirm the current relationship. In addition, for confirmed the validation of selected proteins, further study may be performed with the western blotting or enzyme-linked immunosorbent assay (ELISA).

Conclusion

This is the first study that use LC-MS/MS to analyse the serum proteome profiles and classify the differential protein expression according to triglyceride levels among Thai males. These results may provide novel insights of proteins which associated with hypertriglyceridemia. The

present study exclusively found 11 unique proteins in very high TG group which CCR5 also found as a significant protein due to the highest relative fold change and potential mechanisms related to carbohydrate metabolism. Because this metabolism is the major regulator of storage and distribution of glucose to the peripheral tissues and, in particular, to glucose-dependent tissues through function of insulin. The interplay between abnormal of insulin function and hypertriglyceridemia was supported by insulin resistance, as manifested by a high triglyceride/HDL-C ratio, associated with adverse cardiovascular outcomes more than other lipid metrics, including LDL-C[50]. Furthermore, the identified proteins in our study increase the chance that quantifying the triglyceride-proteome could provide insights into the efficacy of lipid therapy and support as in identification of cardioprotective agents.

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 Mr. Yodying Yingchutrakul, Mr. Sucheewin Krobthong 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.

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