

Large-Scale Proteomic Analysis of Patients with Type 2 Diabetes Mellitus and Atherosclerosis Using a Label-Free LC-MS/MS Approach

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ABSTRACT

Objective: Type 2 diabetes mellitus (T2D) is a metabolic disease whose molecular events have not yet been fully clarified. However, next-generation powerful molecular approaches such as mass spectrometry (MS)-based proteomics holds promise. In this study, we aimed to reveal the protein profile of serum samples obtained from patients with T2D and atherosclerotic cardiovascular disease using the high-resolution liquid chromatography (LC)-MS/MS system.

Materials and Methods: Immune depletion was performed for the top 12 abundant proteins in 10 µl serum samples taken from individuals. Then, tryptic peptides were obtained from total proteins by applying a digestion protocol. Accordingly, reduction, alkylation, and digestion with trypsin enzyme were carried out, respectively. Tryptic peptides were analyzed in an ultra-high-pressure LC-MS/MS system with a label-free proteomic approach. The raw data were processed using the software program.

Results: LC-MS/MS analyses revealed 120 proteins with significant expression changes. Some of these proteins were associated with inflammation, lipid transport, and oxidative stress, which are known to play an important role in T2D and its complications.

Conclusion: As a result, LC-MS/MS analyses highlighted the proteins that will provide predictions in the treatment and course of T2D. We believe that validation of these proteins with targeted proteomic approaches in a larger sample in further studies will contribute to the development of clinically usable panels.

Keywords: Diabetes, proteomics, serum, mass spectrometry, atherosclerosis

INTRODUCTION

Type 2 diabetes mellitus (T2D) is a complex disease in which the individual's proteome changes depending on metabolic and functional disturbances, and the pathogenesis is not fully understood. However, proteomic approaches are becoming increasingly important in advancing our understanding of the etiology and pathology of T2D, and its complications. Liquid chromatography-mass spectrometry (LC-MS)-

based proteomic methods allow sensitive detection of altered expression of proteins in T2D-dependent disrupted signaling pathways (1,2). Various biological materials such as serum, plasma, and urine have been used in proteomic studies investigating the disease and its complications (3-5). Serum samples are of particular interest for biomarker discovery as they are easily obtainable biological materials and contain information on a large number of proteins in

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various tissues. However, highly abundant proteins such as albumin, immunoglobulins, haptoglobin, transferrin, and α -1-antitrypsin in serum samples constitute more than 80% of the total protein content. These abundant proteins prevent the detection of low-abundance proteins in LC-MS/MS analysis. Various techniques and methods continue to be developed to overcome this limitation. Immunodepletion, enrichment, and electrophoretic or chromatographic fractionation are some of the methods used (6).

To date, various serum, and plasma proteins that may be associated with T2D, its complications have been identified using LC-MS/MS-based proteomic approaches. A recent study reported 62 proteins, mostly immune-related, with altered expression in serum samples from T2D patients (3). In another study, a significant correlation was found between the levels of circulating APOC2, C4A, CXCL7, DOCK2, LBP, and VTDB proteins, the degree of coronary artery stenosis in cardiovascular patients with T2D (7). According to the results of LC-MS/MS analysis combined with gel methods, fibrinolysis, complement-dependent immune responses, and inflammation-related proteins were prominent in plasma samples of T2D and atherosclerotic patients (4).

Despite the advanced methods and techniques, the findings obtained in serum samples are still insufficient to explain the molecular mechanism of the disease, and new studies are needed. In this context, in this study, we aimed to reveal the protein profile of serum samples obtained from patients with T2D and atherosclerotic cardiovascular disease using the label-free proteomics approach.

MATERIALS AND METHODS

Case Information

Twelve patients with T2D and atherosclerosis (2 female and 10 male), and 9 control subjects (2 female and 7 male) were enrolled in the study. The study was approved by the Ethics Committee of Medipol University (24.10.2018/585), conducted by the principles of the Helsinki Declaration, and informed consent was obtained from each patient. Following an overnight fast, peripheral blood samples of the participants were collected into EDTA-free tubes. The serum samples were obtained by centrifugation for 10 minutes at 3000 rpm. The mean age of the patients was 53 years (45 to 60 years) and there was no statistically significant difference with the control group (mean age 52.4 ± 6.9 years).

Sample Preparation

Samples were prepared according to a label-free mass spectrometry-based protein quantification strategy. For the control group, three pools were generated from nine different healthy subjects before digestion. However, patient samples were prepared individually. According to the manufacturer's instructions, abundant proteins in serum were depleted with a Top 12 spin column. Briefly, 10 μ L of serum was added to each column and incubated at room temperature, with gentle end-over-end mixing. Then, the samples were centrifuged at

1000 \times g and the flow through were collected. The digestion protocol applied to obtain the tryptic peptide was similar to our previous study (8). An equal amount of protein for each sample was reduced with 20 mM DTT for 10 min at 95°C and then alkylated with 40 mM iodoacetamide for 20 min in the dark. Following the reduction and alkylation procedures, proteins were digested with trypsin at 37°C for 18 hours. Digested peptides were dried under a vacuum, and stored at -80°C.

LC-MS/MS Analysis and Data Processing

The peptide mixtures were separated by a reverse phase nano-flow liquid chromatography (Dionex UltiMate 3000, Thermo Fisher Scientific) and identified using high resolution mass spectrometry (Q-Exactive Plus Orbitrap, Thermo Fisher Scientific) equipped with an electrospray ionization source. The analyses were performed by selecting the previous nano-UPLC-ESI-MS/MS system parameters (8). For analysis, each sample was re-dissolved in 0.1% formic acid and then loaded onto a trap column. Following the trapping, peptide mixtures were eluted to a C18 analytical column and separated with a linear gradient of acetonitrile. The collected data range was 400–2000 m/z. MS raw files were processed with Proteome Discoverer (version 2.3; Thermo Fisher Scientific, Bremen, Germany). All data were searched against the human UniProt database (downloaded in Jan 2022) containing 203,711 protein sequences. For protein quantitation, the minora feature detector and precursor ions quantifier node was used in the workflow generated in the informatics program.

Statistical Analysis

To calculate the p value, a hypothesis test (ANOVA individual) based on the abundance of individual proteins and peptides was selected. Using cut-off criteria, a 1.5-fold increase, or a 0.65-fold decrease in expression was considered to be of biological importance. A p value < 0.05 was considered to be significant. Benjamini-Hochberg correction for multiple testing was applied to the p values. STRING database (<https://string-db.org/>), Cytoscape, and CytoHubba plugin were used for protein interaction networks and classifications.

RESULTS

Using a label-free quantitative proteomics workflow, we quantified 1,860 unique peptides mapped to 336 proteins in 12 patients with type 2 diabetes and 9 control samples (peptide and protein FDR 1%). As seen in Figure 1, this approach allowed the detection of 120 differentially expressed proteins (DEPs) including 50 proteins up-regulated (≥ 1.5 -fold) and 70 down-regulated (≤ 0.65 -fold) between T2D and the control group (adj. p-value < 0.05). These proteins are listed in Table 1 with more information. The protein interaction network constructed using 120 proteins showed that they are highly related proteins (Figure 2A). The top 10 hub genes were identified by CytoHubba. These hub proteins are given in Figure 2B. The 6 hub proteins were mostly associated with lipid transport. Apart from APOA2 (3.2-fold), lipid transporter proteins such as APOA1 (0.21-fold), APOA4 (0.08-fold), APOC3

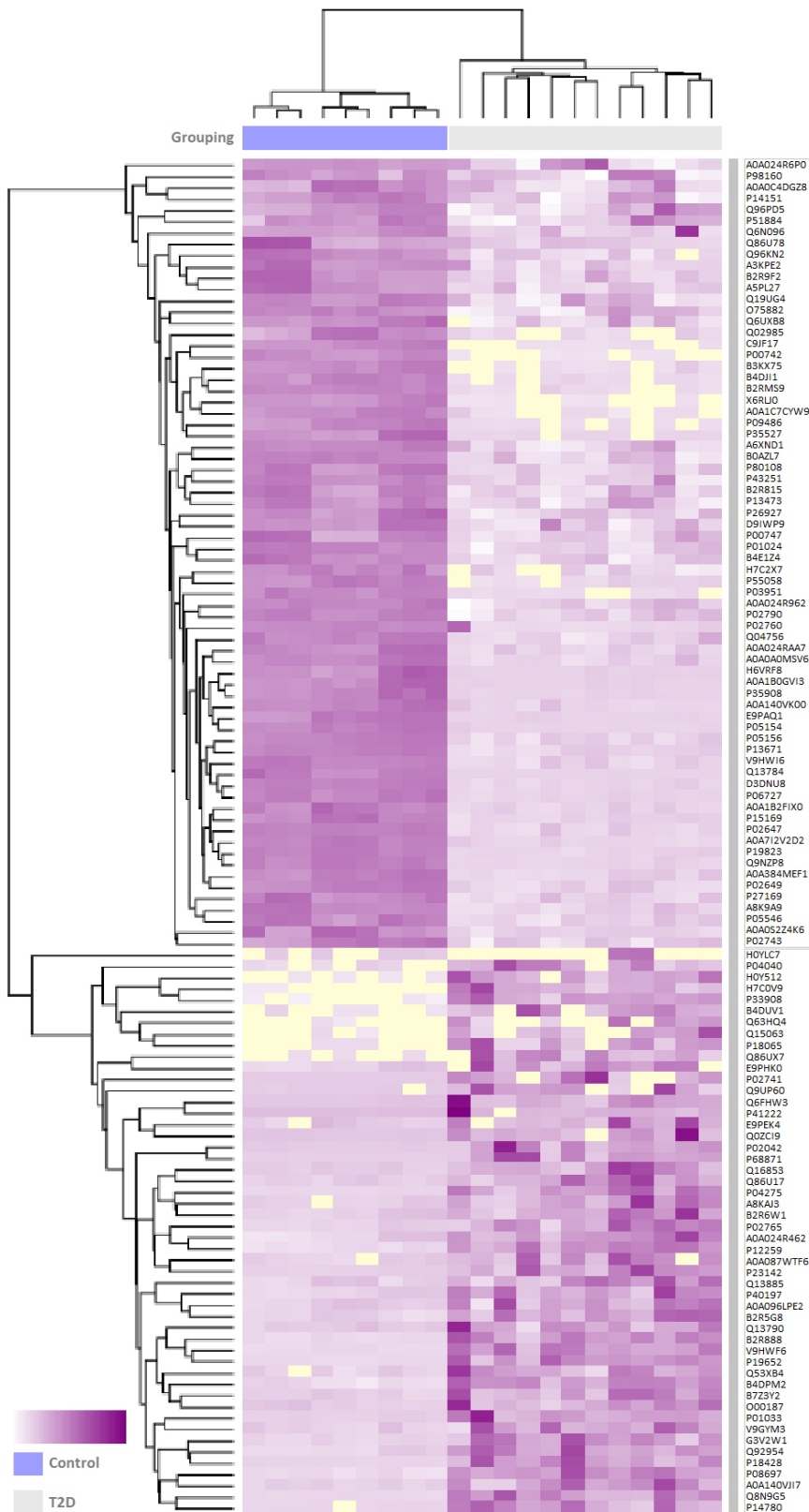


Figure 1. Hierarchical cluster analysis of the 120 DEPs. The proteins were clustered hierarchically by Manhattan correlation analysis. The greater the abundance of protein, the deeper the purple color. DEPs: Differentially expressed proteins.

(0.32-fold), APOD (0.03-fold), APOE (0.25-fold), APOH (0.37-fold), APOL1 (0.13-fold), and paraoxonase (PON)1 (0.27-fold) were significantly reduced ($p < 0.001$) (Figure 3). Moreover, the level of C-reactive protein (CRP) (36.5-fold) as an inflammation

marker was found to be higher compared to the control, whereas the level of adiponectin (0.21-fold), which is known to have an anti-inflammatory and antioxidant role, was found to be low ($p < 0.00001$). However, we observed upregulation

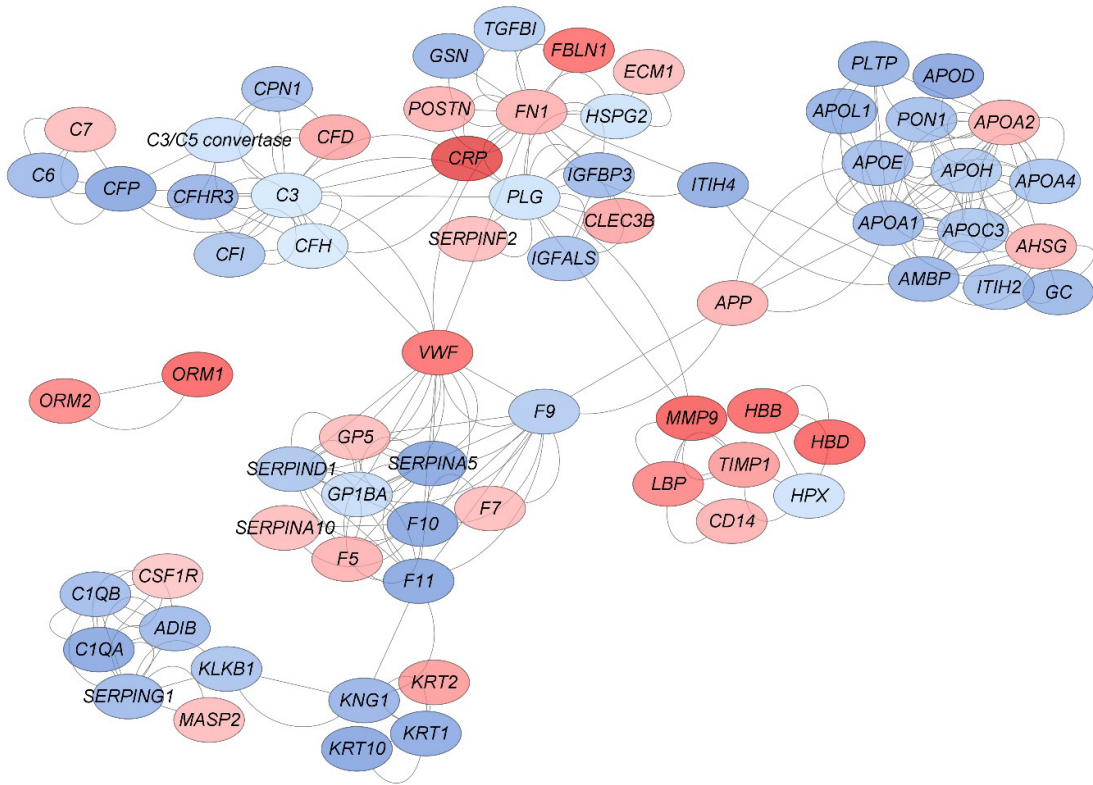


Figure 2A.

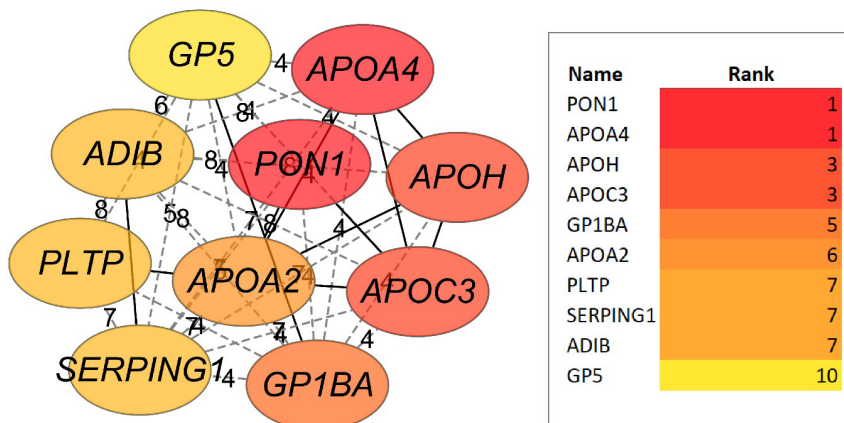


Figure 2B.

Figure 2. A. Protein interaction network of DEPs according to the STRING v11 database. B. The top 10 hub genes were identified by CytoHubba. The minimum required interaction score was selected with high confidence (0.900) and disconnected nodes were removed from the network. Average node degree: 2.1; avg. local clustering coefficient: 0.39; PPI enrichment p-value: $< 1.0e-16$. Blue indicates downregulated proteins, whereas red indicates upregulated proteins. The darker the color, the greater the difference in expression. DEPs: Differentially expressed proteins.

Figure 3A.

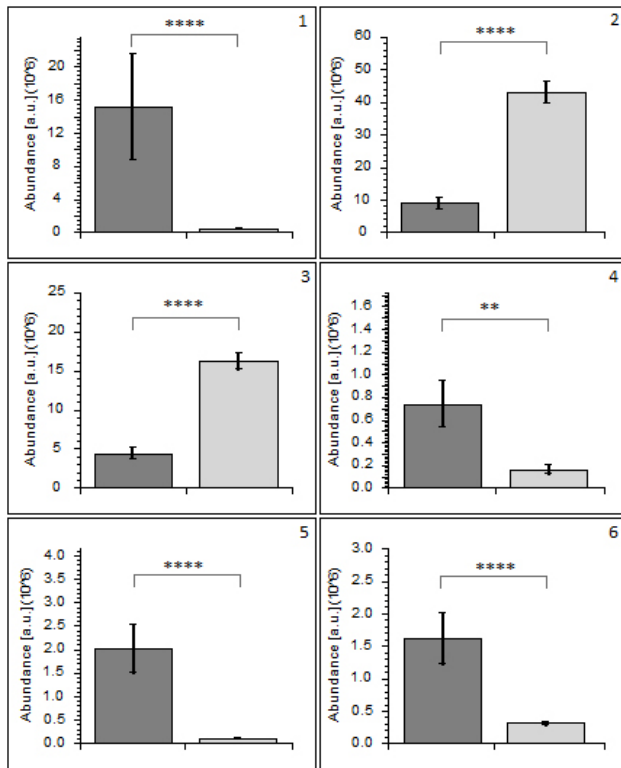


Figure 3B.

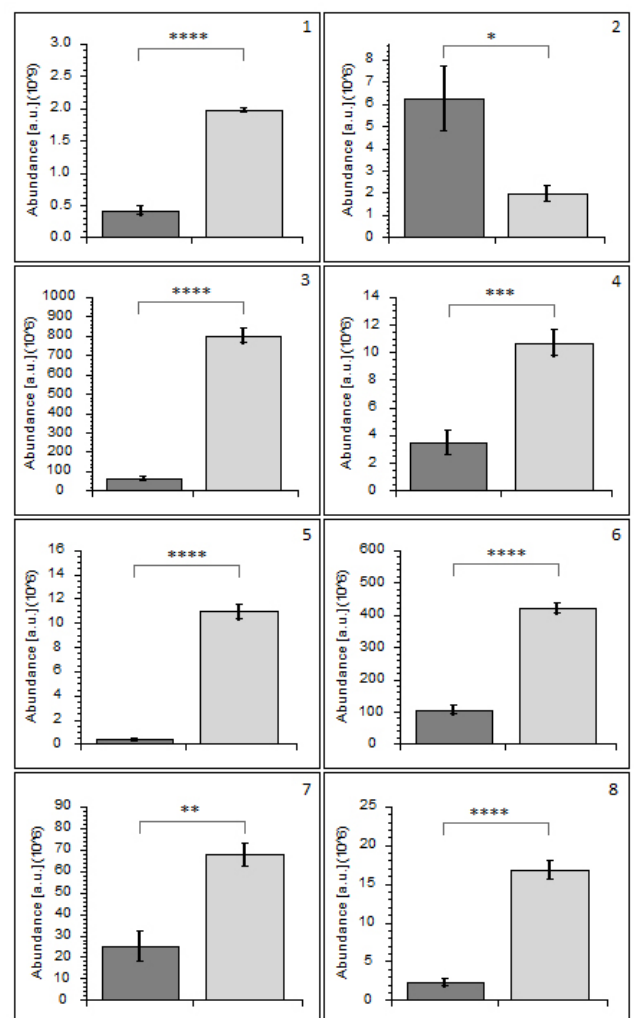


Figure 3. Bar chart reporting relative abundances of both six differentially expressed proteins (panel A: 1, CRP; 2, ADIP; 3, PON1; 4, CAT; 5, MMP9; 6, TIMP1) and eight proteins (panel B: 1, APOA1; 2, APOA2; 3, APOA4; 4, APOC3; 5, APOD, 6, APOE; 7, APOH; 8, APOL1), in patients and controls. All values are expressed as mean ± standard error of the mean (SEM). *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001. Dark grey indicates the patient group, whereas light grey indicates the control group.

of catalase (4.5-fold), a known antioxidant protein (p=0.0026). Other significantly upregulated DEPs in our proteomic data were matrix metalloproteinase (MMP) 9 (17.9-fold) and tissue inhibitor of metalloproteinases (TIMP) 1 (5.17-fold) (p<0.00001) (Figure 3B). These two proteins belong to the protease inhibitor family and both of them have a vital role in the degradation of extracellular matrix and cell signaling.

We also performed gene ontology (GO) enrichment analysis to elucidate differentially expressed proteins' biological role. A total of 103 serum proteins were annotated, accounting for 85.8% of the DEPs. The classifications of DEPs in the proteomes of samples from T2D patients are given in Table 2. A significant portion of the DEPs were extracellular regions, vesicles, exosomes, blood microparticles, and collagen-containing extracellular matrix proteins. According to molecular function,

the DEPs quantified are mainly involved in protein binding, ion binding, hydrolase activity, enzyme regulator activity, lipid binding, carbohydrate binding, and transporter activity. Interestingly, we found that the majority of proteins associated with lipid binding (F10, PON1, APOA1, APOC3, APOA4, SERPINA6, CPN1, AZGP1, SELL, APOH, APOD, APOE, APOL1, GC and PLTP), carbohydrate binding (ATRN, APCS, PGLYRP2, SERPIND1, SELL, APOH, KRT1, F11, APOE, KNG1, and SERPINA5) were down-regulated in T2D patients. In addition, the top 10 categories according to their significance are given in Table 2. Regarding the biological process, platelet degranulation, response to stress, complement activation, defense response, and regulated exocytosis were in the top 5 significantly enriched categories. Moreover, more than 50% of the proteins with increased expression were associated with response to stimulus and response to stress.

Table 1. List of significantly downregulated and upregulated proteins in patients with T2D and atherosclerotic cardiovascular disease relative to control, identified by LC-MS/MS analysis.

A-Downregulated proteins					
Accession	Gene name	Description	FC	adj. p-value	MW [kDa]
A0A384MEF1	GSN	Actin-depolymerizing factor	0.19	8.7E-11	85.6
A0A024RAA7	ADIB	Adiponectin	0.21	3.3E-08	25.8
Q86U78	Serpin A8	Angiotensin 1-10	0.55	0.000	53.1
Q13784	APOA4	APOA4 protein (Fragment)	0.01	0.000	28.1
P02647	APOA1	Apolipoprotein A-I	0.21	0.000	30.8
P06727	APOA4	Apolipoprotein A-IV	0.08	0.000	45.3
A3KPE2	APOC3	Apolipoprotein C-III	0.32	0.001	10.8
C9JF17	APOD	Apolipoprotein D (Fragment)	0.03	0.000	24.1
P02649	APOE	Apolipoprotein E	0.25	0.000	36.1
D9IWP9	APOH	Apolipoprotein H (Fragment)	0.37	0.001	36.2
A0A1B2FIX0	APOL1	Apolipoprotein L1 (Fragment)	0.13	0.000	27.1
O75882	ATRN	Attractin	0.49	0.001	158.4
P98160	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	0.58	0.027	468.5
Q96KN2	CNDP1	Beta-Ala-His dipeptidase	0.29	0.001	56.7
P43251	BTD	Biotinidase	0.55	0.000	61.1
B4E1Z4	n/a	C3/C5 convertase	0.57	0.000	140.9
P15169	CPN1	Carboxypeptidase N catalytic chain	0.25	0.000	52.3
B0AZL7	IGFALS	cDNA, FLJ79457, highly similar to Insulin-like growth factor-binding	0.28	0.000	66
B2R815	SERPINA4	cDNA, FLJ93695, member 4 (SERPINA4), mRNA	0.48	0.000	48.5
B2R9F2	SERPINA6	cDNA, FLJ94361 member 6 (SERPINA6), mRNA	0.46	0.000	45.1
Q19UG4	F9	Christmas factor (Fragment)	0.36	0.001	20.7
P00742	F10	Coagulation factor X	0.04	0.000	54.7
P03951	F11	Coagulation factor XI	0.03	0.000	70.1
X6RLJ0	C1QA	Complement C1q subcomponent subunit A (Fragment)	0.05	0.000	23.3
A0A0A0MSV6	C1QB	Complement C1q subcomponent subunit B (Fragment)	0.23	0.000	24
Q9NZP8	C1RL	Complement C1r subcomponent-like protein	0.11	0.000	53.5
P01024	C3	Complement C3	0.64	0.000	187
P13671	C6	Complement component C6	0.21	0.000	104.7
Q02985	CFHR3	Complement factor H-related protein 3	0.15	0.000	37.3
P05156	CFI	Complement factor I	0.32	0.000	65.7
A5PL27	CP	CP protein	0.54	0.000	122.1
H6VRF8	KRT1	Cytokeratin-1	0.06	0.000	66

V9HWI6	HEL-S-51	Gc-globulin	0.18	0.000	52.9
A0A0C4DGZ8	GP1BA	Glycoprotein Ib (Platelet), alpha polypeptide	0.53	0.005	68.9
A0A024R962	hCG_40889	HCG40889, isoform CRA_b	0.65	0.000	139
P02790	HPX	Hemopexin	0.58	0.000	51.6
P05546	SERPIND1	Heparin cofactor 2	0.32	0.000	57
Q04756	HGFAC	Hepatocyte growth factor activator	0.32	0.000	70.6
P26927	MST1	Hepatocyte growth factor-like protein	0.43	0.000	80.3
B3KX75	CHL1	highly similar to Neural cell adhesion molecule L1-like	0.06	0.000	124.7
A6XND1	IGFBP3	Insulin-like growth factor-binding protein 3	0.21	0.000	29
P19823	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	0.27	0.000	106.4
A8K9A9	KLKB1	Kallikrein B	0.29	0.000	71.3
A0A1B0GVI3	KRT10	Keratin, type I cytoskeletal 10	0.02	0.000	63.3
P35527	KRT9	Keratin, type I cytoskeletal 9	0.02	0.000	62
P35908	KRT2	Keratin, type II cytoskeletal 2 epidermal	0.04	0.000	65.4
D3DNU8	KNG1	Kininogen 1, isoform CRA_a	0.15	0.000	47.8
H7C2X7	LSG1	Large subunit GTPase 1 homolog (Fragment)	0.36	0.000	32
B4DJI1	n/a	L-lactate dehydrogenase	0.09	0.000	33.6
P14151	SELL	L-selectin	0.62	0.004	42.2
P51884	LUM	Lumican	0.56	0.008	38.4
P13473	LAMP2	Lysosome-associated membrane glycoprotein 2	0.51	0.000	44.9
Q96PD5	PGLYRP2	N-acetylmuramoyl-L-alanine amidase	0.58	0.034	62.2
Q6UXB8	PI16	Peptidase inhibitor 16	0.39	0.001	49.4
P80108	GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	0.19	0.000	92.3
P55058	PLTP	Phospholipid transfer protein	0.13	0.000	54.7
B2RMS9	ITIH4	Plasma Kallikrein-sensitive glycoprotein	0.09	0.000	103.3
A0A7I2V2D2	SERPING1	Plasma protease C1 inhibitor	0.21	0.000	53.2
P05154	SERPINA5	Plasma serine protease inhibitor	0.04	0.000	45.6
P00747	PLG	Plasminogen	0.59	0.000	90.5
E9PAQ1	CFP	Properdin	0.04	0.000	45.1
P02760	AMBP	Protein AMBP	0.17	0.000	39
A0A1C7CYW9	TTLL8	Protein monoglycylase TTLL8 (Fragment)	0.23	0.000	94.5
A0A024R6P0	SERPINA3	Serpin peptidase inhibitor, member 3, isoform CRA_c	0.54	0.039	47.6
P02743	APCS	Serum amyloid P-component	0.48	0.000	25.4
P27169	PON1	Serum paraoxonase/arylesterase 1	0.27	0.000	39.7
P09486	SPARC	SPARC	0.03	0.000	34.6
A0A140VK00	n/a	Testicular tissue protein Li 227	0.12	0.000	34.2
A0A052Z4K6	TGFBI	Transforming growth factor-beta-induced protein ig-h3	0.38	0.000	57.3

B-Upregulated proteins					
Accession ID	Gene name	Description	FC	adj. p-value	MW [kDa]
P02741	CRP	C-reactive protein	36.5	0.000	25
P14780	MMP9	Matrix metalloproteinase-9	17.9	0.000	78.4
P02042	HBD	Hemoglobin subunit delta	14.1	0.002	16
V9HWF6	HEL-S-153w	Alpha-1-acid glycoprotein	13.3	6.1E-12	23.5
P18065	IGFBP2	Insulin-like growth factor-binding protein 2	13.2	0.011	34.8
P04275	VWF	von Willebrand factor	9.4	0.000	309.1
P68871	HBB	Hemoglobin subunit beta	9.1	0.001	16
B4DUV1	FBLN1	Fibulin-1	9.0	0.000	70.1
H0YLC7	FAH	Fumarylacetoacetase (Fragment)	8.6	0.000	18
P19652	ORM2	Alpha-1-acid glycoprotein 2	7.3	1.2E-10	23.6
A8KAJ3	EFEMP1	cDNA FLJ77823, highly similar to Homo sapiens EGF-containing fibulin-like extracellular matrix protein 1, transcript variant 3, mRNA	7.2	0.000	54.6
P18428	LBP	Lipopolysaccharide-binding protein	6.7	0.000	53.4
H0Y512	APMAP	Adipocyte plasma membrane-associated protein (Fragment)	5.8	1.3E-06	45.4
O43866	CD5L	CD5 antigen-like	5.6	0.008	38.1
P01033	TIMP1	Metalloproteinase inhibitor 1	5.2	0.000	23.2
P33908	MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	5.0	0.000	72.9
Q9UP60	SNC73	SNC73 protein	4.9	0.021	40.9
P23142	FBLN1	Fibulin-1	4.8	0.000	77.2
P04040	CAT	Catalase	4.5	0.006	59.7
P41222	PTGDS	Prostaglandin-H2 D-isomerase	4.4	0.026	21
Q92954	PRG4	Proteoglycan 4	4.0	0.000	151
E9PHK0	CLEC3B	Tetranectin	3.8	0.000	17.8
Q0ZCI9	n/a	Immunoglobulin heavy chain variable region (Fragment)	3.7	0.000	14.1
Q6FHW3	DF	Adipsin	3.5	1.6E-02	24.4
V9GYM3	APOA2	Apolipoprotein A-II	3.2	0.006	14.9
Q16853	AOC3	Membrane primary amine oxidase	3.1	0.000	84.6
Ferritin	FERMT3	Fermitin family homolog 3	3.1	0.010	75.9
P12259	F5	Coagulation factor V	3.1	0.000	251.5
Q8N9G5	n/a	cDNA FLJ37429 fis, clone BRAWH2001666	3.1	0.000	42.2
Q15063	POSTN	Periostin	3.0	0.037	93.3
P02765	AHSG	Alpha-2-HS-glycoprotein	2.9	0.000	39.3

B2R888	n/a	Monocyte differentiation antigen CD14	2.8	0.000	40
A0A087WTF6	NCAM1	Neural cell adhesion molecule 1	2.9	0.001	93.3
H7C0V9	APP	Amyloid-beta A4 protein (Fragment)	2.8	0.018	55.1
A0A024R462	FN1	Fibronectin	2.4	0.004	259
B7Z3Y2	PCYOX1	cDNA FLJ51879, highly similar to Prenylcysteine oxidase	2.3	0.001	48.3
B4DPM2	F7	cDNA FLJ55738, highly similar to Coagulation factor VII	2.3	0.000	43.6
A0A140VJ17	n/a	Testicular tissue protein Li 61	2.2	0.000	60.6
B2R5G8	n/a	Serum amyloid A protein	2.2	0.000	14.8
G3V2W1	SERPINA10	Protein Z-dependent protease inhibitor	2.2	0.000	55.1
B2R6W1	C7	cDNA, FLJ93143, highly similar to Homo sapiens complement component 7 (C7), mRNA	2.1	0.004	93.5
P40197	GP5	Platelet glycoprotein V	2.0	0.000	60.9
P08697	SERPINF2	Alpha-2-antiplasmin	2.0	2.4E-10	54.5
Q13790	APOF	Apolipoprotein F	1.9	0.029	35.4
E9PEK4	CSF1R	Macrophage colony-stimulating factor 1 receptor	1.9	0.003	74.2
Q53XB4	RAB1	Epididymis secretory sperm binding protein	1.8	0.015	16.8
Q86U17	SERPINA11	Serpin A11	1.8	0.001	47
A0A096LPE2	SAA2-SAA4	SAA2-SAA4 readthrough	1.7	0.026	23.3
Q13885	TUBB2A	Tubulin beta-2A chain	1.6	0.005	49.9
O00187	MASP2	Mannan-binding lectin serine protease 2	1.6	0.017	75.7

Proteins were listed along with their accession numbers (UniprotKB AC/ID), gene name, description, fold change, adj. p-values and molecular weight (Mw) in kilodaltons. The p-value adjusted using Benjamini-Hochberg correction for the false-discovery rate.

Finally, we showed the top 10 pathways in which DEPs play a role, with the protein interaction network generated in the STRING database based on the Reactome Pathways. Accordingly, platelet degranulation, hemostasis, platelet activation, signaling and aggregation, regulation of Insulin-like growth factor (IGF) transport and uptake by IGFs, post-translational protein phosphorylation, and complement cascade were among the remarkable pathways.

DISCUSSION

Type 2 diabetes is a metabolic disease that is initially silent and then causes micro- and macrovascular complications (9). Serum and plasma samples contain abundant proteins as well as numerous proteins of tissue origin. Therefore, proteomic analyses performed on serum samples are important to obtain information about proteins that play a role in the course of diabetes. To date, several biomarker candidates have been proposed for the early detection of diabetes using MS-based proteomic methods (1,2). Despite the results obtained, the molecular and cellular pathways associated with the disease and its complications are still not clear. In

this study, we carried out label-free quantification and LC-MS/MS to identify the proteomic alterations in serum samples of atherosclerotic T2D patients. Differentially expressed proteins were generally associated with inflammation, oxidative stress, lipid transportation, and coagulation, and these changes were consistent with the literature. As is known, patients with T2D show a strong predisposition to atherosclerotic vascular diseases due to various factors. Increased oxidative stress and inflammation are the processes most blamed in this relationship (9,10). High levels of CRP, a well-known inflammatory marker, are also seen in the therapeutic monitoring of T2D and cardiovascular diseases (10,11). In addition, some studies have found an inverse correlation between blood CRP levels and PON1 and adiponectin levels (12-14). According to our MS analysis results, serum CRP levels increased 36.5 times in patients with atherosclerotic T2D compared to the control. Moreover, adiponectin and PON1 were found to be significantly lower, showing a negative correlation with CRP. Accordingly, the adiponectin level in the patients was significantly reduced (0.20-fold) compared to the control. It has been reported that adiponectin, which has anti-inflammatory, anti-atherogenic, and insulin-sensitizing effects, is decreased in T2D (12,15,16).

Table 2. Gene ontology (GO) Analysis of Differentially Expressed Proteins.

Term ID	Description	Gene Count	p-Value
Cellular Component			
GO:0005615	Extracellular space	103	4.09e-63
GO:0005576	Extracellular region	106	1.21e-56
GO:1903561	Extracellular vesicle	82	2.56e-49
GO:0070062	Extracellular exosome	81	1.34e-48
GO:0072562	Blood microparticle	32	1.17e-39
GO:0062023	Collagen-containing extracellular matrix	43	5.47e-39
GO:0031012	Extracellular matrix	46	3.86e-38
GO:0031982	Vesicle	87	2.69e-35
GO:0034774	Secretory granule lumen	26	2.23e-19
GO:0031093	Platelet alpha granule lumen	16	3.76e-18
Biological Process			
GO:0006950	Response to stress	69	8.44e-20
GO:0016192	Vesicle-mediated transport	45	1.04e-14
GO:0006952	Defense response	41	4.01e-16
GO:0045055	Regulated exocytosis	32	4.01e-16
GO:0051346	Negative regulation of hydrolase activity	26	3.69e-15
GO:0002576	Platelet degranulation	23	3.01e-22
GO:0010466	Negative regulation of peptidase activity	22	1.29e-15
GO:0006956	Complement activation	17	1.08e-19
GO:0006958	Complement activation, classical pathway	13	3.34e-15
GO:0072378	Blood coagulation, fibrin clot formation	12	3.36e-15
Molecular Function			
GO:0061134	Peptidase regulator activity	22	1.63e-16
GO:0030414	Peptidase inhibitor activity	20	7.72e-16
GO:0004866	Endopeptidase inhibitor activity	19	6.37e-15
GO:0004857	Enzyme inhibitor activity	23	6.89e-14
GO:0004867	Serine-type endopeptidase inhibitor activity	15	7.48e-14
GO:0030234	Enzyme regulator activity	29	1.09e-09
GO:0004252	Serine-type endopeptidase activity	13	6.90e-09
GO:0008201	Heparin-binding	13	2.23e-08
GO:0005539	Glycosaminoglycan binding	14	7.49e-08
GO:0070325	Lipoprotein particle receptor binding	7	2.27e-07

According to the order of p-adjust value, only the top 10 terms were displayed.

There is even growing evidence that high adiponectin levels in the blood are associated with a lower risk of type 2 diabetes and complications (14,17,18). PON1 also has antioxidant and anti-atherogenic activity like adiponectin. PON1, which is bound to serum HDL shows its antioxidant enzyme property by reducing the accumulation of lipid peroxide, which is responsible for the onset and progression of atherosclerosis (19). In our study, the level of PON1 was also found to be 0.27-fold lower in patients. It has been reported in a recent study that the decrease in PON1 activity and the lack of correlation with HDL and APOA1 may increase the potential risk for atherosclerosis-related diseases by causing HDL dysfunction (20). Some studies have suggested that PON1 activity may be dependent on HDL particle content (19,21). Fourteen different apolipoproteins that can be found in the structure of HDL and regulate cholesterol transport and metabolism have been identified (22). In our study, significant differences were found in serum levels of APOA1, APOA2, APOA4, APOC3, APOE, APOH, and APOL1 compared to the control. Of these, APOA1, APOA2, APOA4, and APOE are known as structural and functional apolipoproteins. The major structural proteins of the HDL-cholesterol complex are APOA1 and APOA2. It has been reported that APOA1, which is of interest due to its role in reverse cholesterol transport, shows a positive correlation with PON1 activity in serum samples of coronary artery patients (23).

Increasing evidence suggests that the micro and macro complications of diabetes may be related to the accumulation of free oxygen radicals and lipid peroxidation products (24). Malondialdehyde (MDA) is an important indicator of lipid peroxidation, and its increase has been reported in diabetic patients in many studies (25-30). However, the antioxidant status in patients with T2D is not as clear as the finding of increased oxidative products. Superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase are the primary antioxidant enzymes involved in the oxidant defense mechanism, and controversial reports have been reported regarding SOD and catalase enzyme levels. In some studies, no changes were detected in these enzymes, while a decrease or increase was reported in some studies (25-32). It has been reported in recent studies that the decrease in SOD activity indicates the presence of glycation and excessive lipid peroxidation in T2D (29,30). Other studies have reported that SOD activity is higher in serum and plasma samples of patients with T2D compared to controls. The authors interpreted this increase as an adaptive response to increased oxidative stress (27,31). According to the results of LC-MS/MS-based proteomic analysis performed by Andújar-Vera et al., a decrease in SOD1, SOD2, APOE, and APOM proteins, an increase in CAT and APOA4 proteins were detected in the calcified femoral arteries from 7 patients (32). In another study, catalase was significantly increased in the plasma of T2D patients with the absence and existence of nephropathy (29). These results suggest that a CAT increase in diabetic patients may be a compensatory mechanism against oxidative damage (25,29,32). In our study, the 4.5-fold increase in serum levels of catalase compared to the control group highlights the

supportive role of antioxidant enzymes. However, whether this alteration in catalase levels is related to the decrease in APOA1 and PON1 needs to be investigated further.

On the other hand, hyperglycemia in diabetic patients can increase the expression of MMP in macrophages and endothelial cells due to oxidative damage. MMPs are known as endopeptidases that target extracellular matrix proteins, some of which play an important role in remodeling venous tissue (33,34). Due to its role in diabetes, MMP9 is one of the most interesting MMP family members. In our study, the serum MMP9 level was found to be 17.9 times higher than the control. Various findings have shown that MMP9 level is significantly increased in macrophages and endothelial cells during hyperglycemia (35,36). The results obtained in these studies reveal that overexpression of MMP9 in particular is strongly associated with atherosclerotic plaque instability, which is an important risk factor for acute coronary syndrome (35-38). The activity of MMP9 is mostly controlled by TIMP1. Taken together, the effects of high glucose on MMP9 and TIMP1 expression may disrupt the MMP/TIMP balance. In addition, higher TIMP1 expression levels in endothelial cells and plasma are considered as an indicator of endothelial dysfunction (39,40). Inokubo et al. detected elevated MMP-9 and TIMP-1 in plasma samples of patients affected by acute coronary syndrome (41). Derosa et al. also reported that plasma levels of both MMP9 and TIMP1 were increased in a diabetic group (37). In agreement with their findings, our analysis revealed a significant increase in TIMP1 and MMP9 levels. Interestingly, the increase we observed in MMP9 expression was almost 4 times that of TIMP1. These findings may be an important sign in MMP/TIMP imbalance. In this context, both our study and the existing studies in the literature shed light on future studies by showing that MMP9 and TIMP1 may be new targets for the prevention of vascular complications in diabetic patients.

In addition, our LC-MS/MS analyses allowed the identification of several proteins, such as fibronectin (FBLN1), tetranectin (CLEC3B), periostin (POSTN), adipisin (DF), kininogen 1 (KNG1), lumican (LUM), SERPIND1, SERPINA6, SERPINF2, actin-depolymerizing factor proteoglycan (GSN), biotinidase (BTD) and fibulin (FBLN1), in agreement with the literature (39,42-46).

CONCLUSION

In summary, it has been emphasized in the literature that inflammation and oxidative stress may play an important role in T2D and its complications. The changes in CRP, adiponectin, PON1, MMP9, CAT, TIMP1, and apolipoproteins that we observed in our study support this view. Our study also showed that many proteins involved in interrelated mechanisms are differentially expressed in atherosclerotic patients with T2D. However, there is a need to confirm the expression changes detected in these proteins by different methods.

As a result, LC-MS/MS analyses highlighted the proteins that will provide predictions in the treatment and course of the disease. Quantification of these proteins with targeted

proteomic approaches in a larger sample in further studies will contribute to the creation of clinically usable panels.

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