Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy

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ORIGINAL RESEARCH

Proteomic analysis to identify candidate biomarkers associated with type 1 diabetes

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Purpose: Type 1 diabetes mellitus (DM1) is one of the most common chronic diseases observed during childhood. The incidence of DM1 is increasing worldwide, and there is currently no way to prevent or delay the onset or to cure the disease. Most diseases, including diabetes, stem from abnormalities in the functioning of proteins, and some studies have reported the expression of protein variation to be involved in the development of DM1. Thus, the aim of this study was to investigate the differential expression of serum proteins in patients with DM1.

Materials and methods: Serum of patients with DM1 (n=30) and healthy controls (n=30) was collected. A proteomic approach was used with depletion of albumin and immunoglobulin G chromatography on serum samples followed by data-independent, label-free mass spectrometric analysis.

Results: A total of eight serum proteins were identified as being differentially expressed and involved in the immune system, lipid metabolism, and pathways of coagulation. DM1 was associated with the upregulation of six proteins: alpha-2-macroglobulin, apolipoprotein A-II, β2 glycoprotein I, Ig alpha-2 chain C region, alpha-1-microglobulin, and prothrombin. A total of two proteins were downregulated, including pregnancy zone protein and complement C4.

Conclusion: To the best of our knowledge, these findings show differential expression of proteins revealing new proteins that may be involved in the development and progression of diabetes.

Keywords: proteome, mass spectrometry, precision medicine, diagnosis

Introduction

Type 1 diabetes mellitus (DM1) is characterized by the destruction of insulin-producing β-cells in the pancreas, with consequent insulin deficiency.[1] It is a metabolic and endocrine condition more common in childhood development and is preceded by the dysregulation of many biological pathways.[2] At present, no therapy exists to halt the immune-mediated destruction of β-cells,[3] and DM1 has a substantial long-term impact on the quality of life, particularly in children and adolescents.

According to the International Diabetes Federation,[4] the total population with DM was equivalent to 415 million and is estimated to increase to 471 million by 2035. A recent report of World Health Organization estimated that, globally, 422 million adults aged >18 years were living with diabetes in 2014, compared with 108 million in 1980.[5] Following the same trend, the incidence of DM1 has increased by ~3% worldwide and represents 5%–10% of the entire population with diabetes.[6]

There has been a lot of research conducted on diabetes over the decades. Several proteomic approaches have been undertaken to identify biomarkers for monitoring the

prediction[7,8] and progression[9] of the disease. However, there are some limitations due to low specificity or late application to the disease stage.[10]

DM1 is a complex disease with altered expression of many genes and their products.[11] Therefore, different approaches were developed using current proteomic technologies to explore its clinical potential and enable the discovery of new biomarkers for DM1.

Recent advances in proteomics have triggered rapid progress in mass spectrometry (MS)-based proteomics. It enables more than simple protein identification and can accurately and reliably quantitate differences in protein abundance in an organism, a cell, or a tissue at a given time or under a particular condition.[12] The label-free technique has been shown to be more accurate in estimating absolute abundance of proteins in complex samples.[13]

In the current study, we performed a quantitative and qualitative analysis using a sample pretreatment immunodepletion of serum albumin and immunogloblin G (IgG) combined with data-independent label-free mass spectrometry (MS^E) analysis with the aim of identifying differentially expressed levels of serum proteins in DM1 patients and healthy controls (HCs).

Materials and methods

Study participants

The present study enrolled DM1 patients selected among those in a regular follow-up in the outpatient of Endocrinology and Diabetes at the Federal University Hospital of Ceara, Fortaleza, Brazil. DM1 patients aged ≥18 years, both male and female, who had attended the outpatient department of Endocrinology and Diabetes for a minimum of 6 months, with clinical and cognitive conditions to answer an interview and the capacity to stand up normally were eligible for the DM1 group. Patients using immunosuppressive medications (renal, heart, or hepatic dysfunction; type 2 diabetes; etc.); those with alcoholism and mental disorders (including dementia and Alzheimer's disease); those diagnosed with leprosy or HIV infection; and those with autoimmune disease, amputation or ulcers on the lower limbs, acute infectious frames, or diabetic ketoacidosis in the 3 months preceding the evaluation, as well as pregnant or lactating females, were excluded. HCs were selected from the Federal University and Ceará State University, considering the same criteria used for DM1 patients, besides not having a familiar history of diabetes and with fasting blood glucose ≤99 mg/dL.

The study initially enrolled all patients attending the outpatient department as potential participants. After applying rigorous inclusion and exclusion criteria to achieve a group with highly homogeneous clinical features and accounting for loss during the selection process, a total of 60 subjects were included as study participants: 30 DM1 patients and 30 HCs. The DM1 and HC groups were matched by gender, age, body mass index (BMI), total cholesterol, high-density lipoprotein cholesterol (HDL-C), serum low-density lipoprotein cholesterol (LDL-C), total protein, urea, and creatinine. General health features, demographics, and clinical data were obtained through medical records and direct interviews. The study protocol (number 1357544) was approved by the Federal University Hospital of Ceará Ethics Committee, and the study was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all the participants.

Anthropometry and health-related behaviors

Weight and height were measured according to the SISVAN (Food and Nutritional Surveillance System; 2004).[14] BMI was calculated as weight (in kg) divided by height (in m^2).[15] The considered standard doses for alcohol consumption and categories were in accordance with World Health Organization.[16] Cigarette smoking status was consistent with that shown in Vital and Health Statistics.[17]

Serum samples

Blood samples were collected from all subjects after an overnight fast (12 hours) for biochemical analysis. All the samples were centrifuged at 3000 rpm for 15 minutes at room temperature, and the serum obtained was kept frozen at −80°C until analyzed. Fasting blood sugar, total cholesterol, HDL-C, triglyceride, urea, and creatinine were measured by colorimetric enzymatic method using commercially available kits (Bioclin, Quibasa Quimica Basica Ltda, Belo Horizonte, Minas Gerais, Brazil) and an automated biochemical analyzer (Mindray BC-2800; Shenzhen Mindray Bio-Medical Electronics Co. Ltd., Shenzhen, China). LDL-C was calculated using the Frederickson–Friedewald equation.[18] C-peptide analysis was performed by immunoassays of microparticles by quimioluminescence (Kit ARCHITECT C-Peptide) using Architec Asystema no. i2000 (Chicago, IL, USA). Samples were analyzed in triplicate.

The serum protein concentration was determined by the Bradford method.[19] The serum samples were pooled in the same group for proteomic analysis. Using 5.33 μg of protein from each individual sample, 15 samples were pooled to form each group of patients with diabetes and the HC groups. Therefore,

two pools of samples from patients with DM1 (DM1a and DM1b) and two pools of HCs (HCa and HCb) were generated.

Immunodepletion of high-abundance proteins

In order to improve the chance of detecting a wide range of serum proteins of medium to low abundance, proteins of high abundance, albumins, and immunoglobulins were depleted from the serum. Aliquots of serum were filtered through 0.22-μM membrane (KASVI, São José do Pinhais, Brazil). Then, 150 μL of filtered serum was applied to a HiTrap® Albumin & IgG Depletion column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) attached to an AKTA Purifier 10 fast protein liquid chromatography system (GE Healthcare). The column was preequilibrated with a solution of 20 mM Tris–HCl (pH 7.4) and 0.15 M NaCl. The elution of fraction rich in albumin and immunoglobulin was realized with 0.1 M Glycine–HCL buffer (pH 2.7) delivered at 1 mL/min, and absorbance was monitored at 216 and 280 nm.

Nano-ultra performance liquid chromatografy MS analysis

After immunodepletion, the fractions of the pools were dialyzed, concentrated, and quantified using a Vivaspin™ (GE Healthcare) and a NanoVue™ Spectrometer (GE Healthcare), respectively, at an absorbance at 280 nm. The denatured samples (100 μg) were diluted in 50 mM ammonium bicarbonate, denatured with a solution of 0.2% RapiGest™ SF (Waters Corporation, Milford, MA, USA) at 80°C for 15 minutes in a dry bath, and reduced with 100 mM dithiothreitol at 60°C for 60 minutes, and alkylation with 300 mM iodoacetamide for 30 minutes was performed in the dark at room temperature. The digestion occurred with the addition of 1 μg of modified trypsin (Promega, Madison, WI, USA) to each sample at 37°C, and the samples were incubated overnight. The reaction was stopped by adding 10 μL of 5% of trifluoroacetic acid, the samples were incubated for 90 minutes at 37°C, centrifuged, and the supernatant was transferred to vial (Waters, Manchester, UK). Tryptic peptides of yeast alcohol dehydrogenase (ADH) were added to vials containing digested proteins to a final concentration of 50 fmol/μL as internal standards. The final protein concentration was ~1 μg/μL.

The tryptic peptides were separated using a nanoACQUITY UPLC® system (Waters) equipped with an HSS T3 C18 reverse-phase column (1.8 μm, 75 μm×20 mm; Waters) for 110 minutes using 0%–40% gradient for 90 minutes and 40%–85% for 5 minutes. The column was reequilibrated for 15 minutes at 35°C. The flow rate was 0.35 μL/min, and mobile Phases A and B contained 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. All the samples were measured in triplicate. MS^E was performed using a Synapt HDMS (nano-electrospray ionization quadrupole /orthogonal acceleration time-of-flight mass spectrometer; Waters, Manchester, UK). The equipment was operated in "V" mode with a precursor double-charge resolution ≥10,000 full width at half maximum and in electrospray positive-ion mode nano-electrospray ionization (+). Mass spectrometer data were acquired using a NanoLockSpray probe channel infusion of Glu-fibrinogen peptide (Glu-Fib) derived from fibrinopeptide B human $(M + 2H)^2$ and final calibration of the equipment used MS/MS Glu-Fib fragments.

Data processing and protein identification and quantification

Liquid chromatography (LC)/MS^E data were processed, and the proteins were identified using the ProteinLynx Global Server (PLGS) version 2 software with the UniProt reverse Homo sapiens annotated database. For searching spectra and the database, the default parameters of PLGS were used, followed by a maximum of one missed trypsin cleavage. The absolute quantification of each run was calculated according to the three most intense peptides (label-free Hi3 method) using ADH peptides as internal standards.[13] Relative quantification of identified proteins from each expression group was performed using alpha-1-antitrypsin (UniProt) as an internal common housekeeping protein to normalize expression levels, using PLGS Expression^E software (Waters).[20] The average quantitative values of all the samples were calculated, and the *p*-values (<0.05) were calculated using Expression^E software to refer to the differences between biological replicates.

Gene ontology analysis

The identified proteins were classified into different categories, as biological processes, cellular location, and molecular function in accordance with the information found in the Gene Ontology database, UniProtKB, and EBI GOA.

Statistical analysis

The data obtained concerning demographics and biochemical and anthropometric parameters were analyzed using R software Version 3.2.2 (2017). For the comparison of two means, Student's *t*-test was used for independent samples; otherwise, Mann–Whitney *U* test was used. Correlations between variables were tested using Pearson and Spearman correlation tests. Data were considered significant with *p*-values <0.05. Figure 1 presents the experimental strategy used in this study.

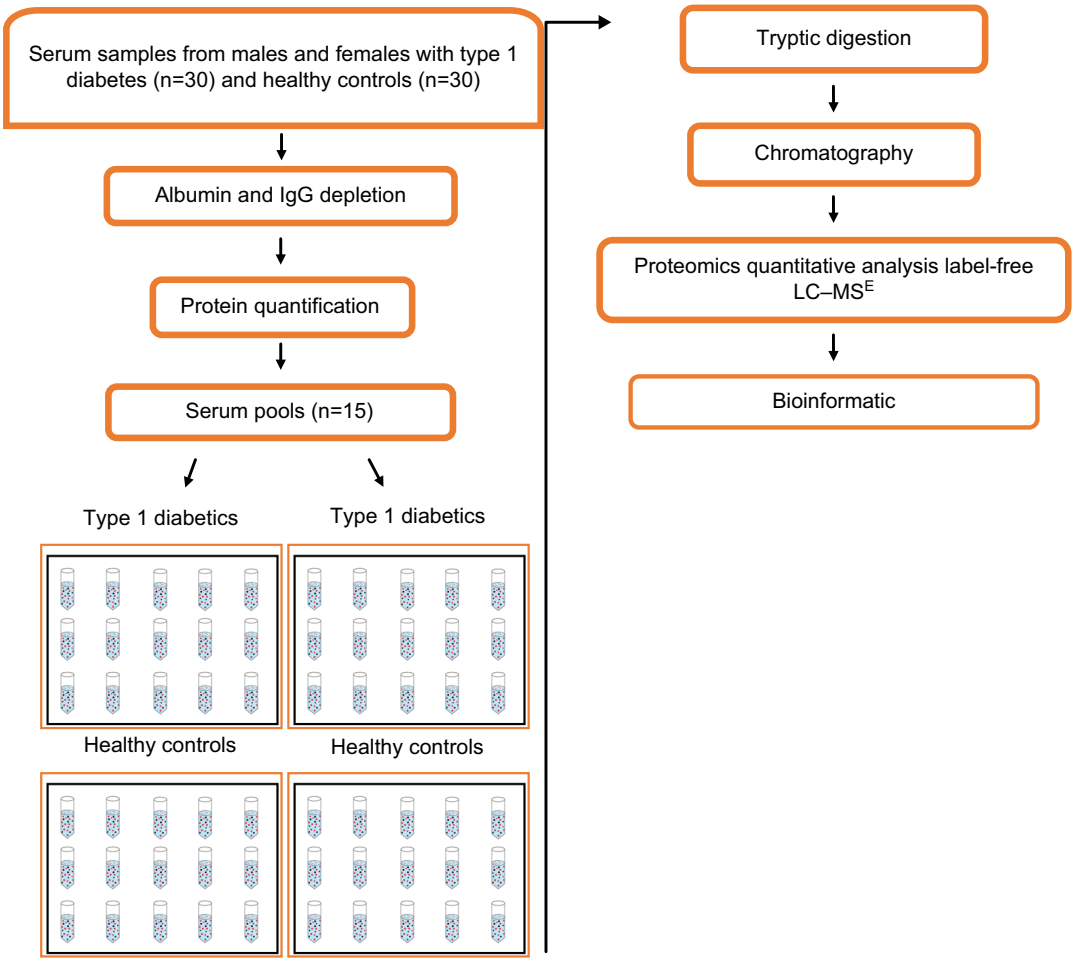

Figure 1 Diagram of the study design used for research of proteomic biomarker in the serum of individuals.
Abbreviations: LC–MSE, liquid chromatography—data-independent label-free mass spectrometry; IgG, immunoglobulin G.

Results

Clinical features

A total of 60 individuals with an average age of 33.30±9.80 years (ranging from 18 to 59 years) were studied and grouped into 30 DM1 patients and 30 HC individuals. Table 1 shows the biochemical and anthropometric characteristics of the patients enrolled for the study. DM1 and HC groups were matched, and there were no significant differences between the groups regarding age, gender, and BMI or in the serum concentrations of total cholesterol, HDL-C, LDL-C, total proteins, and creatinine. As expected, fasting blood glucose and C-peptide were significantly different between DM1 patients and HCs ($p<0.001$). The DM1 group also exhibited a negative correlation between C-peptide and duration of disease ($r=-532$; $p<0.001$). Moreover, no significant differences were observed between the groups relative to alcohol consumption and cigarette smoking status.

Expression of proteins

A comparative proteomic analysis was performed between DM1 and HC groups to investigate alterations in serum proteins in DM1 patients. In the current study, a total of 130 proteins were identified in the serum of the DM1 and HC groups in the total data set (Table S1). Eight proteins were differentially expressed in the serum of the DM1 group compared with the HC group (six proteins were upregulated and two were downregulated). Of the list of proteins identified, the criteria stated for the differential expression in each of the two biological replicates only considered proteins with differential expression (DM1/HC) with absolute ratios >1.5 (upregulated) and <0.66 (downregulated) and the levels of those proteins with ratios between 1.5 and 0.66 (unchanged). Based on gene ontology analysis, most of the proteins that were identified as related to molecular function were involved in binding (48%), catalytic (12.35%) and antioxidant (4.16%)

Table 1 Demographic, anthropometric, and clinical characteristics of participants in study

Parameters	Patients with DM1	HC	*p*-value
Subjects (n)	30	30	–
Age (years)	35.03 (8.60)	31.50 (10.67)	0.163[a]
BMI (kg/m^2)	24.76 (3.10)	24.74 (4.03)	0.982[a]
DM1 duration (years)	15.63 (10.47)	–	–
HbA1c (%)	7.5 (1.2)	–	–
Blood glucose (mg/dL)	145.44 (56.96)	89.93 (6.02)	<0.001[b]
Peptide C (ng/mL)	0.14 (0.17)	3.92 (0.56)	<0.001[b]
Total cholesterol (mg/dL)	179.68 (39.12)	192.56 (36.59)	0.193[a]
LDL (mg/dL)	87.88 (36.34)	88.90 (31.73)	0.907[a]
HDL (mg/dL)	73.70 (25.65)	76.83 (22.86)	0.619[a]
TG (mg/dL)	141.80 (34.80)	95.11 (26.01)	<0.001[b]
Total proteins (mg/dL)	7.24 (1.01)	7.24 (1.26)	0.564[b]
Creatinine (mg/dL)	0.91 (0.21)	0.92 (0.23)	0.863[a]
Urea (mg/dL)	33.03 (11.47)	25.97 (7.72)	0.007[a]
Gender			
Male n (%)	12 (63.16)	7 (36.84)	0.165[a]
Female n (%)	18 (43.90)	23 (56.10)	
Smoking n (%)			
Yes	2 (66.67)	1 (33.33)	1. 000[b]
No	28 (49.12)	29 (50.88)	
Drinking n (%)			
Yes	0 (0.00)	1 (100.00)	1. 000[b]
No	30 (50.85)	29 (49.15)	

Notes: Values are expressed as mean (SD) and number (percentage). [a]Student's *t*-test; [b]Mann–Whitney test; *p*-values <0.05 are considered significant.
Abbreviations: BMI, body mass index; DM1, type 1 diabetes mellitus; HbA1c, glycated hemoglobin; HC, healthy controls; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides.

activities, and other functions. They also participated in biological processes related to regulation (19.81%), cellular process (19.81%), localization (14.12%), stimulus response (9.58%), and immune system (9.75%), among others. In addition, the majority of proteins were located in the extracellular region (Figure 2). Table 2 shows the proteins identified as differentially expressed in the serum from DM1 group compared with HC group.

Discussion

Diabetes occurs as a result of a range of minor changes in protein expression that alters the stability of the health β-cell to a prediabetic phenotype of instability and possibly β-cell destruction.[21] Thus, the proteomic analysis of protein expression is an important tool for understanding the molecular modifications associated with disease progression and may contribute to the therapeutic monitoring of this autoimmune disease.

In the present study, a proteomic approach using a label-free quantitative MS analysis identified eight differentially expressed proteins in the serum of DM1 patients compared with HCs. All the proteins that were either upregulated (alpha-2-macroglobulin [α2M], apolipoprotein A-II [ApoA-II], β2 glycoprotein I, Ig alpha-2 chain C region [IGHA2], alpha-1-microglobulin [AMBP], and prothrombin [THRB]) or downregulated (pregnancy zone protein [PZP] and complement C4 [C4]) are discussed below.

α2M belongs to the α-M family of proteins and is the largest major antiproteinase present in the plasma of vertebrates.[22] In the present study, α2M expression levels were upregulated in the DM1 group compared with HCs. In support of our data, Takada et al also showed that serum levels of α2M were clearly upregulated in many individuals with DM1 and type 2 diabetes mellitus (DM2).[23] Interestingly, upregulation of α2M has been suggested as a potential biomarker for diabetic retinopathy and nephropathy,[23–25] and studies have indicated that α2M can also act as a binding and carrier protein for cytokines, including IL-Iβ and IL-6, among others,[26] leading to the induction of several defense reactions, such as immune response, acute phase reaction, and hematopoiesis.[22] Therefore, acting as a carrier and binding protein could be the mechanism by which α2M performs its immune functions, acting in inflammatory processes, and could explain its enhanced expression in the serum of people with DM1.

Proteomic analysis has shown upregulated expression levels of IGHA2 in DM1 patients. IGHA2 is an immunoglobulin that is a membrane-bound or secreted glycoprotein produced by B lymphocytes.[27] This immunoglobulin has an important

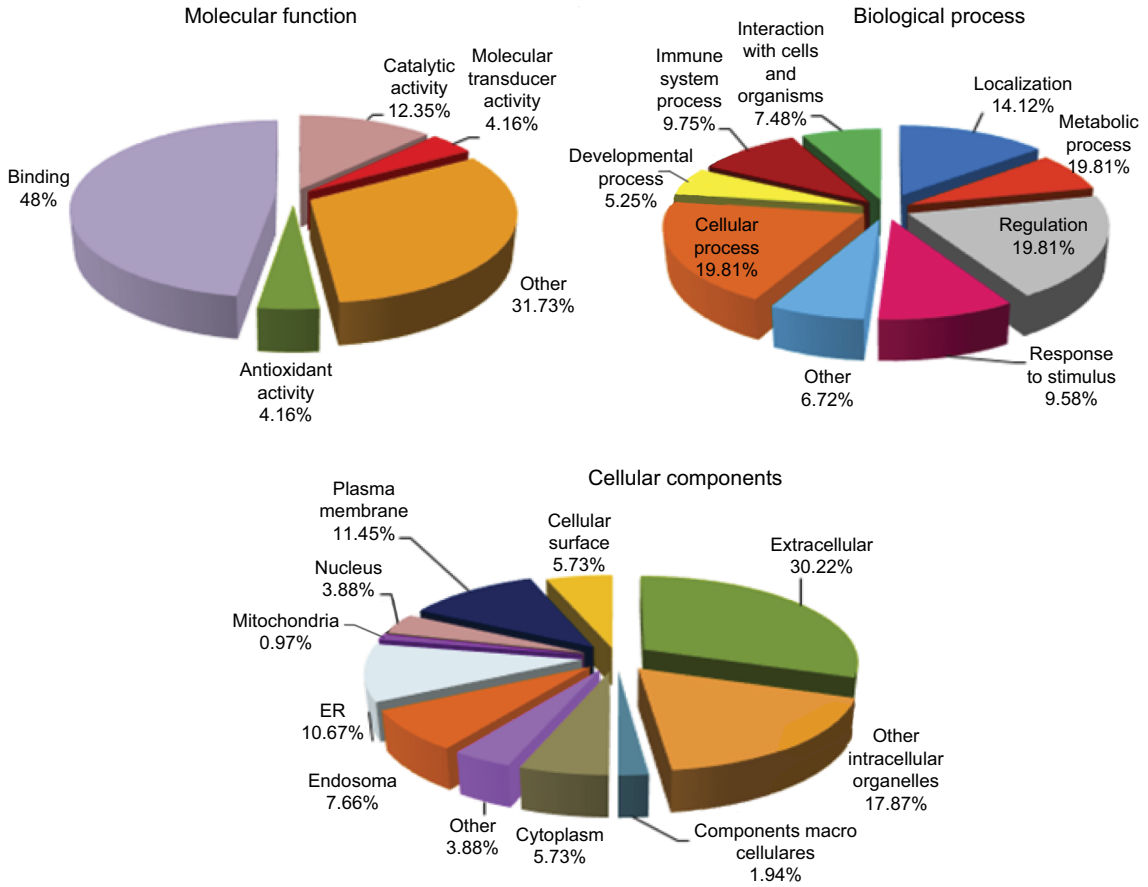

Figure 2 Percentage of serum proteins identified from albumin and immunoglobulin G depleted serum by LC–MS^E for patients with type 1 diabetes and healthy controls based on their molecular function, biological process, and cellular components.
Abbreviations: ER, endoplasmic reticulum; LC–MS^E, liquid chromatography—data-independent label-free mass spectrometry.

role in immune defense, especially at the mucosal surfaces.[28] In our study, the high levels of IGHA2 identified are in keeping with previous reports that have shown increased concentrations of circulating IgHA2 in DM1 and DM2 patients.[29–31] In DM1 patients, the increased expression in the serum of IGHA2 could be triggered in response to antigenic stimuli, forming continuous stimulation of antibody production.[29] Moreover, the literature shows that the relationship between diabetes and IGHA2 has been described as involving advanced glycation products. Some studies reported a significant increase in the glycation of IGHA2 in the plasma of patients with DM1 and with complications of diabetes, especially in retinopathy, nephropathy, and neuropathy.[32,33] In addition, a cross-sectional study reported that increased levels of IgA tended to be associated with hyperglycemia.[34] In this context, it was suggested that high levels of IGHA2 result from an immune response to advanced glycosylation end products that increase and accumulate with persistent high blood glucose in patients with diabetes.[31]

ApoA-II expression levels were upregulated in the DM1 group compared with the HC group. APOA-II is the second most abundant apolipoprotein constituent of HDL, and the increased expression of APOA-II has been associated with several aspects of HDL, triglycerides, obesity, and insulin resistance.[35,36] DM1 patients often show lipid disorders, such as abnormalities in the regulation of plasma lipid metabolism,[37] in different amounts of classes of lipoproteins, and in the transfer of lipids.[38] Lipoprotein abnormalities might contribute to the development of atherogenesis and diabetic nephropathy.[39,40] In fact, Soedamah-Muthu et al reported that a lower APOA-I to APO-II ratio in patients with microalbuminuria or macroalbuminuria could contribute to an increased risk of cardiovascular disease.[41] Increased expression of APOA-II in transgenic mice showed enhanced HDL levels and increased size of HDL particles. Larger HDL observed is caused, in part, by the inhibition of the ability of hepatic lipase (HL) to hydrolyze phospholipids and triglycerides by APOA-II as well as an increased ratio of APOA-I to APOA-II. Therefore, interactions of APOA-II and APOA-I and HL influence the HDL structure and antiatherogenic properties.[42,43] Furthermore, an excess of APOA-II in HDL may contribute to hypertriglyceridemia by inhibiting the

Table 2 Characterization of differentially expressed proteins in serum of DM1 patients compared with HCs as identified by LC–MS^E

Accession number	Protein name	Molecular weight	Matched peptides	Coverage (%)	Score	DM1/HC[a]	Expression	Function
P01023	Alpha-2-macroglobulin	163,291	66/95	69.54	11,454.35	1.64	Up	Cellular process/regulation
P02760	Protein AMBP	38,999	10/26	37.78	2,146.99	1.58	Up	Cellular process/process metabolic/immune system
P02652	Apolipoprotein A II	11,175	6/11	72.00	9,656.85	1.84	Up	Cellular process/developmental process/response to stimulus/ metabolic process
P02749	Beta-2-glycoprotein 1	38,298	14/22	62.03	4,930.08	1.72	Up	Cellular process/localization/ metabolic process/regulation
P01877	Ig alpha-2 chain C region	36,526	15/22	37.58	11,700.44	2.14	Up	Cellular process/regulation/ immune system
P00734	Prothrombin	70,037	14/47	35.69	1,449.93	1.60	Up	Cellular process/developmental and metabolic process/regulation/ response to stimulus
P0C0L4	Complement C4 A	192,785	52/118	54.59	5,352.24	0.54	Down	Immune system/regulation/ response to stimulus
P20742	Pregnancy zone protein	163,863	16/87	21.52	1,774.43	0.41	Down	Interaction with cells and organisms

Notes: [a]Average differences of replicate samples run to type 1 diabetes patients compared with healthy control. Proteins are shown as 1.5-fold differences either upregulated or downregulated ($p<0.05$). Functions were assigned according to the String databases and literature search.
Abbreviations: AMBP, alpha-1-microglobulin; DM1, type 1 diabetes mellitus; HC, healthy control; LC–MS^E, liquid chromatography–data-independent label-free mass spectrometry.

lipolysis of triglyceride-rich lipoproteins and by modifying the lipoprotein lipase activity.[44] In diabetes, deficient function of insulin-dependent lipoprotein lipase may lead to hypertriglyceridemia as well as decreased HDL cholesterol levels due to increased lipid exchanges between triglyceride-rich lipoproteins and HDL.[37] Nevertheless, studies using various approaches have described APOA-II as both proatherogenic and antiatherogenic; however, the role of this protein in lipid metabolism and atherogenesis is not well understood. Nevertheless, evidence supports the hypothesis that APOA-II is antiatherogenic, and further studies are required.[45] Taken together, these data indicate that APOA-II plays an important role in regulating lipid and β-cell metabolism. Thus, in the current study, our observation supports previous reports that show an association between apolipoproteins and DM1.

β2 glycoprotein I, also known as apolipoprotein H (APOH), is involved in the inhibition of the intrinsic pathway of the activation of blood coagulation. There is evidence to support the association between APOH and lipid metabolism, inflammation, thrombosis, autoimmune disease, and atherosclerosis.[46,47] Here, expression levels of APOH were upregulated in DM1 patients. The analysis of plasma levels of APOH from people with DM2 showed that patients with hypertriglyceridemia had significantly higher APOH values.[46] Others studies have shown an association between APOH and diabetic retinopathy and found increased levels of APOH in the vitreous fluid of DM1 patients with proliferative diabetic retinopathy.[48,49] However, to our knowledge, no study has yet shown increased levels of APOH in the serum of DM1 patients. Interestingly, APOH binds apoptotic cells in vivo, and this might trigger or enhance complement activation, thus facilitating the recognition and clearance of apoptotic cells.[50] The role of APOH in the pathogenesis of diabetic retinopathy remains to be elucidated, but it could be related to complement activation or its potential role in the recognition of dying cells.[49] Although the relationship between cell apoptosis and autoimmunity remains to be fully established, there is evidence that T-cell-induced apoptosis is a mechanism in DM1.[51] Here, we show for the first time the upregulation of APOH in the serum of patients with DM1.

C4 was downregulated in the serum from patients with DM1. The complement system plays an important role in innate and acquired immunity, and evidence suggests that relevant target regulation of the complement system might be efficient in controlling autoimmune diseases.[52] Abnormalities in complement proteins in DM1 patients have been reported, and reduced levels of C4 were attributed to hypercatabolism or reduction in protein synthesis.[53] Moreover, decreased levels of C4 in children and adolescents with DM1 were related to impaired metabolic control.[54] High glucose interferes with the innate function of the complement system and impairs the ability of the host to combat infection.[55] Deficiencies in C4

are strongly associated with autoimmunity humoral and lead to impaired humoral responses.[52] Thus, the upregulation of C4 observed in this study is suggestive of loss of some functions of the complement system and thus increased risk of infectious processes and impaired metabolic control in DM1.

AMBP is a serum protein filtered by the glomerular membrane in the proximal tubule.[56,57] Increased urinary excretion of this protein was observed in DM1 patients in the presence of microalbuminuria and normoalbuminuria.[57] In this sense, new research has indicated some biomarkers that precede albuminuria and have been detected in both DM1 and DM2 patients with early renal dysfunction.[58] AMBP has been proposed as a biomarker of tubular dysfunction in the early stages of diabetic nephropathy and in the late stages of chronic kidney disease.[59–62] Increased excretion urinary of AMBP has been frequently identified in patients with diabetes, compared with the control group.[57] Increased levels of AMBP suggest that serum AMBP may play a role in the regulation of inflammatory processes,[62] such as that occurs in diabetes. Interestingly, our study also showed that increased levels of AMBP occur in the serum proteome of DM1 patients, and to the best of our knowledge, this is the first study to observe this protein in serum from DM1 patients.

THRB is the protein precursor of thrombin, which plays a key role in thrombosis by converting fibrinogen to fibrin.[63]

Diabetes is associated with a number of changes in thrombotic and fibrinolytic coagulation factor level/activity, which collectively increase the risk of thrombus formation.[63] Thrombin generation is enhanced in both DM1 and DM2 individuals, secondary to low-grade activation of the coagulation system.[64] It has been suggested that elevated THRB levels can contribute both to thrombotic risk and to shortened clotting times in patients with diabetes.[65] In this sense, hyperglycemia has been associated with enhanced thrombin production, and controlling glucose levels results in reduced thrombin generation, suggesting that hyperglycemia is prothrombotic.[64] Conversely, hypoglycemia is also associated with enhanced clot formation.[63] Here, we identified that THRB was upregulated in the serum of DM1 patients, which is an important component in the pathway of coagulation. This is in accordance with previous studies showing that this protein is suggestive of poorly controlled diabetes.

PZP was shown to be downregulated in the serum of DM1 patients compared with HCs. PZP is one of the most abundant pregnancy glycoprotein-associated plasma proteins with described immune inhibitory activities and is remarkably homologous to α2M.[66,67] Several studies have suggested that PZP has immunosuppressive properties in pregnancy, indicating a role for this protein in maintaining immune tolerance to the fetus and equilibrating the maternal immune response.[66,68] In fact, serum levels increase during gestation (up to 1,000 mg/L).[69] Furthermore, high serum levels of PZP have been observed in relation to some diseases, including inflammatory conditions, such as rheumatoid arthritis and also in people who later developed Alzheimer's disease.[70,71] In the current study, we observed downregulation of PZP expression in the DM1 group compared with HCs. This finding is very relevant because, to our knowledge, this is the first report of low levels of PZP in the serum of DM1 patients. The reduction of this protein in DM1 patients suggests that there could be a suppression mechanism of it, thereby avoiding inhibition of the activation of T lymphocytes against the pancreatic β-cells. It has been suggested that PZP contributes to immune regulation by noncovalently sequestering a variety of other ligands such tumor necrosis factor-α, IL-2, and IL-6.[72] In addition, Skornicka et al demonstrated that both native and activated PZP isoforms inhibited the activation of T-cell growth and IL-2 production.[66] DM1 is thought to involve chronic inflammation, leading to the release and increased activity of various immune system cells with a central role for T cells and macrophages.[73] Studies have shown that some inflammatory markers are enhanced in individuals with DM1 compared with those without.[74,75] Taken together, our results suggest that PZP could be a target for a potential DM1 therapy.

Conclusion

In conclusion, we used a proteomic approach with label-free MS^E analysis as a strategy to discover differential protein profiles between the serum of patients with DM1 and HCs.

We identified proteins that were expressed in differential levels in the serum of patients with DM1 compared with HCs. In addition, to our knowledge, we described the first evidence for changes in expression levels of PZP and APOH in the serum of DM1 patients.

Our results showed differential expression of proteins related to immune system, lipid metabolism, and coagulation pathways, which may be correlated to the development and progression of DM1. Accordingly, these findings need to be fully investigated and validated in future studies.

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Author contributions

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

Table S1 List of serum proteins between type 1 diabetes patients and healthy controls as identified by LC–MS^E analysis

Accession number	Protein name	Molecular weight	Matched peptides	Coverage (%)	Score
P43652	Afamin	69,069	4/61	11.52	100.66
P02763	Alpha-1-acid glycoprotein 1	23,512	9/17	53.73	11,680.58
P19652	Alpha-1-acid glycoprotein 2	23,603	8/19	39.80	8,975.25
P01011	Alpha-1-antichymotrypsin	47,651	16/31	45.63	4,340.51
P01009	Alpha-1-antitrypsin	46,737	34/35	62.44	13,832.54
P04217	Alpha-1B-glycoprotein	54,254	8/26	51.31	6,138.02
P08697	Alpha-2-antiplasmin	54,566	7/34	25.46	694.29
P02765	Alpha-2-HS glycoprotein	39,325	4/18	50.68	2,842.23
P01023	Alpha-2-macroglobulin	163,291	66/95	69.54	11,454.35
P01019	Angiotensinogen	53,154	12/25	35.26	4,547.05
Q9UJ72	Annexin A10	37,278	4/35	10.19	134.28
P02647	Apolipoprotein A I	30,778	27/32	68.54	29,954.64
P02652	Apolipoprotein A II	11,175	6/11	72.00	9,656.85
P06727	Apolipoprotein A IV	45,399	12/42	54.55	2,142.37
P02656	Apolipoprotein C III	10,852	3/10	36.36	12,643.42
P05090	Apolipoprotein D	21,276	7/12	30.69	1,265.36
P02649	Apolipoprotein E	36,154	6/30	20.19	350.88
P02749	Beta 2 glycoprotein 1	38,298	14/22	62.03	4,930.08
Q9Y5Z0	Beta secretase 2	56,181	3/23	9.46	140.23
P22792	Carboxy peptidase N subunit 2	60,557	5/30	13.03	334.61
O43866	CD5 antigen like	38,088	8/31	43.80	614.25
Q7L2Z9	Centromere protein Q	30,595	4/28	17.16	121.48
O43303	Centriolar coiled coil protein of 110 kDa	113,424	9/80	10.67	226.74
P00450	Ceruloplasmin	122,205	27/80	57.28	3,369.44
P10909	Clusterin	52,495	12/36	32.96	3,176.77
P00748	Coagulation factor XII	67,792	4/40	5.37	96.90
P13671	Complement component C6	104,787	10/65	9.31	159.79
P01024	Complement C3	187,149	61/136	51.41	8,628.71
P0C0L4	Complement C4 A	192,785	52/118	54.59	5,352.24
P0C0L5	Complement C4 B	192,752	44/117	48.57	5,333.65
P00751	Complement factor B	85,533	23/64	38.09	1,924.60
P08603	Complement factor H	139,097	33/96	40.70	1,155.49
P05156	Complement factor I	65,751	2/42	5.49	117.71
P08185	Corticosteroid binding globulin	45,141	6/23	27.90	371.10
P04003	C4b binding protein alpha chain	67,033	13/45	30.82	1,198.61
A6NLW8	Double homeobox protein A	23,817	1/21	13.24	142.05
O43543	DNA repair protein XRCC2	31,957	2/19	10.36	182.94
Q9NPG1	Frizzled 3	76,263	2/54	6.16	148.64
P06396	Gelsolin	85,968	17/51	32.86	661.02
Q96I13	Glycine N-acyltransferase like protein1	35,101	3/17	11.26	149.87
Q13588	GRB2 related adapter protein	25,337	2/20	26.27	389.12
O96020	G1 S specific cyclin E2	46,757	4/29	11.88	105.55
P00738	Haptoglobin	45,205	27/28	70.94	17,966.31
P00739	Haptoglobin related protein	3,903	20/25	46.26	7,405.70
P69905	Hemoglobin subunit alpha	15,256	6/9	76.76	3,268.27
P68871	Hemoglobin subunit beta	15,998	7/13	57.82	4,909.04
P02042	Hemoglobin subunit delta	16,056	5/13	29.25	4,275.18
P02100	Hemoglobin subunit epsilon	16,203	2/14	21.77	4,275.18
P69891	Hemoglobin subunit gamma 1	16,140	5/13	40.82	4,306.96
P69892	Hemoglobin subunit gamma 2	16,126	4/13	38.10	4,295.91
P02790	Hemopexin	51,677	21/34	50.87	16,740.46

(*Continued*)

Table S1 (*Continued*)

Accession number	Protein name	Molecular weight	Matched peptides	Coverage (%)	Score
P05546	Heparin cofactor 2	57,071	7/36	11.62	295.97
P04196	Histidine rich glycoprotein	59,578	12/33	24.95	1,886.61
P17482	Homeobox protein Hox B9	28,059	1/23	15.20	155.80
P01876	Ig alpha-1-chain C region	37,655	20/23	67.42	19,361.04
P01877	Ig alpha-2-chain C region	36,526	15/22	69.41	11,700.44
P01857	Ig gamma 1 chain C region	36,106	9/21	37.58	1,570.00
P01859	Ig gamma 2 chain C region	35,901	4/20	17.18	300.21
P01860	Ig gamma 3 chain C region	41,287	4/24	12.20	702.83
P01861	Ig gamma 4 chain C region	35,941	7/20	33.94	1,236.51
P01766	Ig heavy chain VIII region BRO	13,227	4/6	40.83	4,324.36
P01767	Ig heavy chain VIII region BUT	11,906	1/8	9.57	976.53
P01774	Ig heavy chain VIII region POM	12,710	1/8	15.97	2,043.78
P01777	Ig heavy chain VIII region TEI	12,559	2/6	36.13	4,324.36
P01765	Ig heavy chain VIII region TIL	11,612	2/6	33.04	2,043.78
P01779	Ig heavy chain VIII region TUR	12,188	1/7	16.38	2,043.78
P01776	Ig heavy chain VIII region WAS	12,847	2/7	29.91	2,043.78
P01763	Ig heavy chain VIII region WEA	12,142	1/6	9.65	976.53
P01764	Ig heavy chain VIII region 23	12,582	2/8	18.80	965.64
P01871	Ig mu chain C region	49,307	25/38	63.94	8,892.79
P04220	Ig mu heavy chain disease protein	43,057	22/35	52.94	12,444.26
P01834	Ig kappa chain C region	11,609	7/7	80.19	26,627.65
P01614	Ig kappa chain VII region Cum	12,676	1/7	11.30	787.52
P06309	Ig kappa chain VII region GM607 Fragment	12,664	2/5	31.62	567.77
P06310	Ig kappa chain VII region RPMI 6410	14,707	2/7	29.32	622.24
P06617	Ig kappa chain V II region TEW	2,059	2/5	32.74	571.31
P04206	Ig kappa chain VIII region GOL	11,830	2/8	31.19	1,520.77
P18135	Ig kappa chain VIII region HAH	14,073	2/8	26.36	1,260.29
P18136	Ig kappa chain VIII region HIC	14,089	2/7	26.36	1,260.29
P01621	Ig kappa chain VIII region NG9 Fragment	10,729	2/6	56.00	590.59
P01620	Ig kappa chain VIII region SIE	11,775	4/6	66.06	1,600.43
P01623	Ig kappa chain VIII region WOL	11,746	3/7	51.38	1,622.15
P01622	Ig kappa chain VIII region TI	11,778	3/6	51.38	1,520.77
P04206	Ig kappa chain VIII region GOL	11,830	2/8	31.19	1,520.77
P0CG04	Ig lambda 1 chain C region	11,348	5/7	65.09	38,924.68
P0CG05	Ig lambda 2 chain C region	11,294	6/7	77.36	39,000.63
P0CG06	Ig lambda 3 chain C region	11,238	5/7	76.42	39,000.63
P0CF74	Ig lambda 6 chain C region	11,277	5/6	77.36	24,818.13
A0M8Q6	Ig lambda 7 chain C region	11,303	4/7	46.23	24,610.28
P01591	Immunoglobulin J chain	18,099	4/11	34.59	1,818.20
P35858	Insulin like growth factor binding protein complex acid labile subunit	66,035	6/35	12.56	119.96
B9A064	Immunoglobulin lambda like polypeptide 5	23,063	6/12	42.99	38,934.43
P19827	Inter-alpha trypsin inhibitor heavy chain H1	101,389	19/56	31.61	1,735.56
P19823	Inter-alpha trypsin inhibitor heavy chain H2	106,464	21/70	32.77	1,415.98
Q14624	Inter-alpha trypsin inhibitor heavy chain H4	103,358	22/64	40.00	1,410.90
Q5T749	Keratinocyte proline rich protein	64,136	1/29	9.33	133.79
P01042	Kininogen 1	71,958	13/61	39.13	1,470.83
P02750	Leucine-rich alpha-2-glycoprotein	38,178	6/24	26.80	925.55
O15165	Low-density lipoprotein receptor class A domain containing protein	33,900	2/19	12.42	142.67
Q96PD5	N acetylmuramoyl L alanine amidase	62,217	7/36	23.61	381.10
Q13516	Oligodendrocytetranscriptionfactor2	32,385	2/16	20.74	356.38
P05155	Plasma protease C1 inhibitor	55,154	7/29	21.60	1,325.77
P00747	Plasminogen	90,569	15/63	26.91	318.40
P02775	Platelet basic protein	13,894	3/9	46.09	801.19

(*Continued*)

Table S1 (*Continued*)

Accession number	Protein name	Molecular weight	Matched peptides	Coverage (%)	Score
P20742	Pregnancy zone protein	163,863	16/87	21.52	1,774.43
P02760	Protein AMBP	38,999	10/26	37.78	2,146.99
Q9UJC3	Protein Hook homolog 1	84,648	8/69	13.87	65.59
P00734	Prothrombin	70,037	14/47	35.69	1,449.93
Q9H5L9	Putative uncharacterized protein C5orf66	1,557	1/8	24.83	115.20
O00472	RNA polymerase II elongation factor ELL2	7,232	5/74	9.69	163.86
Q9NP77	RNA polymerase II subunit A C terminal domain phosphatase SSU72	22,575	7/17	43.30	179.28
Q96HS1	Serine threonine protein phosphatase PGAM5 mitochondrial	32,005	3/23	11.76	229.10
P02787	Serotransferrin	77,064	42/76	72.78	42,299.91
P02768	Serum albumin	69,367	54/55	38.92	4,317.47
P27169	Serum paraoxonase arylesterase 1	39,731	7/18	50.99	2,611.12
P04278	Sex hormone binding globulin	43,779	4/29	25.62	210.35
O00337	Sodium nucleoside cotransporter	71,584	3/35	3.39	102.95
Q13596	Sortingnexin1	5,907	3/40	11.88	178.19
O60235	Transmembrane protease serine 11D	46,264	2/20	11.48	122.25
P02766	Transthyretin	15,887	4/9	50.34	302.37
Q9NZQ9	Tropomodulin 4	39,336	3/32	26.09	192.01
Q5SQH8	Uncharacterized protein C6orf 136	35,794	2/16	29.84	122.10
Q8N865	Uncharacterized protein C7orf 31	68,465	4/49	12.03	47.34
Q6UXV3	Uncharacterized protein UNQ6126	16,883	1/8	36.31	161.41
P02774	Vitamin D binding protein	52,964	24/45	67.93	3,508.70
P04004	Vitronectin	54,306	10/28	38.49	2,808.10
P25311	Zinc alpha-2-glycoprotein	34,259	9/27	48.66	2,246.30
P21506	Zinc finger protein 10	66,455	4/45	9.60	220.02
Q86UK7	Zinc finger protein 598	98,637	6/71	10.18	161.99

Abbreviations: AMBP, alpha-1-microglobulin; LC–MS^E, liquid chromatography–data-independent label-free mass spectrometry.