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Identification of differentially expressed plasma proteins in atherosclerotic patients with type 2 diabetes

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ABSTRACT

Besides hyperglycaemia and insulin resistance, several factors are associated with a higher cardiovascular risk in type 2 diabetes mellitus (T2DM), many of them being closely related to each other owing to common origins or pathways. The pathophysiological mechanisms underlying vascular dysfunctions in diabetes include reduced bioavailability of nitric oxide, increased ROS and prothrombotic factors production, as well as activation of receptors for advanced glycation end-products. These alterations contribute to create a pro-inflammatory/thrombotic state that ultimately leads to plaque formation and complication.

This study aimed at identifying differentially expressed plasma proteins between T2DM and non-diabetic patients undergoing carotid endarterectomy, by means of two-dimensional electrophoresis coupled with LC-MS/MS. Before analysis, plasma samples were enriched in low-expression proteins through combinatorial hexapeptide ligand libraries. Both mono- and two-dimensional western blotting were performed for data validation. Differentially expressed proteins were mapped onto STRING v10 to build a protein–protein interaction network.

Sixteen differentially expressed spots were identified with a high score. Among them, there were fibrinogen beta and gamma chains, complement C1r, C3 and C4-B subcomponents, alpha-1-antitrypsin (AAT), vitronectin and CD5 antigen-like. Protein–Protein interaction analysis evidenced a network among differentially expressed proteins in which vitronectin seems to represent a potentially pivotal node among fibrinolysis, complement dependent immune responses and inflammation in accordance with a number of *in vitro* and *in vivo* evidences for a contributory role of these proteins to the development of diabetic atherosclerosis.

1. Introduction

Diabetes mellitus is a huge global health problem affecting more than 380 million people worldwide (International Diabetes Federation, 2013). It represents the fifth-leading cause of mortality and a major risk factor for cardiovascular diseases, such as coronary artery disease, stroke and peripheral vascular disease (hazard ratio of 2.5 for women and 2.4 for men). Overall, people with diabetes mellitus older than 50 years show a significant reduction of life expectancy (8.2 years for women and 7.5 for men) (Franco, Steyerberg, Hu, Mackenbach, & Nusselder, 2007).

The pathophysiological mechanisms underlying vascular dysfunctions in diabetes include reduced bioavailability of nitric oxide, increased ROS and prothrombotic factors production, as well as activation of receptors for advanced glycation end-products. Both endothelial cells and smooth muscle cells as well as platelets are involved. These alterations in vascular homeostasis foster a pro-inflammatory/thrombotic state which ultimately leads to athero-thrombosis (Beckman, Paneni, Cosentino, & Creager, 2013; Paneni, Beckman, Creager, & Cosentino, 2013). Indeed, atherosclerosis is a chronic inflammatory condition, characterized by the accumulation of lipids, collagen and calcium in medium and large arteries, which could turn into an acute clinical event due to plaque rupture and thrombosis (Libby, 2002; Lutgens et al., 2003).

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

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Type 2 diabetes mellitus (T2DM), or adult-onset diabetes, represents over 90% of cases of diabetes mellitus and is characterized by hyperglycaemia caused by insulin resistance. A major issue of T2DM is that it may remain undetected for several years and its diagnosis is often made incidentally, through an abnormal blood or urine glucose test, when vascular complications are already present in most of patients (Paneni et al., 2013). Furthermore, hyperglycaemia and insulin resistance alone could not justify the high cardiovascular risk associated with T2DM suggesting that other factors significantly contribute to increase the residual cardiovascular risk in diabetic patients. Although apparently independent, many of these risk factors are correlated with each other owing to common origins or pathways (Sattar, Wannamethee, & Forouhi, 2008).

In this scenario, a better understanding of the mechanisms underlying diabetic vascular disease is mandatory, as it may provide novel approaches to prevent or delay the development of its complications.

In the last years our research team have been applying proteomics to identify circulating (Lepedda, Nieddu, Rocchiccioli, et al., 2013) and tissue filtered markers (Lepedda et al., 2009; Lepedda, Zinellu, Nieddu, Zinellu, et al., 2013) of the presence and stability of advanced carotid atherosclerotic plaque. Contextually, we have focused on type 1 and 2 diabetes identifying urinary trypsin inhibitor as a useful marker of these chronic inflammatory conditions (Lepedda, Nieddu, Zinellu, et al., 2013).

The aim of this study was to identify differentially expressed plasma proteins between T2DM and non-diabetic patients undergoing carotid endarterectomy, by means of two-dimensional electrophoresis (2-DE) coupled with LC-MS/MS analysis. As plasma is a very complex biological fluid in terms of both dynamic range of protein concentration, exceeding 10 orders of magnitude, and the very high number of protein species, up to 5,000 different isoforms (Anderson & Anderson, 2002; Issaq, Conrads, Janini, & Veenstra, 2002), we performed a preliminary enrichment step using combinatorial hexapeptide ligand libraries that allowed the analysis of the so called "deep proteome" (Righetti, Candiano, Citterio, & Boschetti, 2015).

2. Material and methods

2.1. Patient Population

Proteomic analyses were conducted on plasma samples from 29 patients undergoing carotid endarterectomy for severe artery disease at Centro Cardiologico "F. Monzino", IRCCS (Milan). Carotid atherosclerosis was assessed by ultrasonography using a Mylab 70 X vision echocolor Doppler equipped with a LA332 AppleProbe 11–3 MHz (Esaote). All patients underwent surgery according to NASCET guidelines for carotid stenosis (Brott et al., 2011). Plaques were classified according to the Gray-Weale classification (Gray-Weale, Graham, Burnett, Byrne, & Lusby, 1988) in soft (S), with hypoechoic features (types 1 and 2), and hard (H), with hyperechoic features (types 3, 4, and 5). Before surgery, fasting blood samples were collected into Vacutainer tubes containing EDTA, immediately centrifuged at 2000 × g for 10 min at 4 °C and stored at −80 °C until analysis. Informed consent was obtained before enrolment. The study was conducted in accordance with the ethical principles of the current Declaration of Helsinki.

All patients were under pharmacological treatment for both hypertension and dyslipidaemia. Among them, 15 subjects were affected by T2DM whereas the other 14 atherosclerotic patients represented the control group. Among T2DM patients, ten were under glucose-lowering therapy, two were under dietary recommendations and three were not under medical control. Main demographic and clinical data are reported in Table 1.

Table 1
Main demographic and clinical data.

Parameters	T2DM patients (15)	Controls (14)	p^*
Age (years)	72.7 ± 7.7	70.4 ± 7.9	0.414
Sex ratio (m/f)	14/1	12/2	
Plaque typology (S/H)	11/4	8/4	
BMI (kg/m^2)	27.5 ± 3.5	24.9 ± 2.6	0.028
Triglycerides (mg/dL)	122.2 ± 51.3	110.4 ± 51.1	0.523
Total cholesterol (mg/dL)	158.3 ± 41.3	164.9 ± 49.9	0.685
HDL cholesterol (mg/dL)	43.4 ± 18.7	40.7 ± 10.5	0.629
LDL cholesterol (mg/dL)	94.2 ± 40.2	103.6 ± 42.4	0.529
Homocysteine (μmol/L)	11.0 ± 3.0	11.9 ± 6.4	0.689
C-reactive protein (mg/dL)	0.95 ± 1.4	1.9 ± 4.7	0.532
Glycaemia (mg/dL)	152.7 ± 34.0	93.6 ± 12.8	<0.001
HbA1c (%)	7.3 ± 0.9	6.2 ± 0.5	0.002
Diastolic pressure (mmHg)	75.2 ± 9.1	76.7 ± 10.0	0.65
Systolic pressure (mmHg)	133.2 ± 16.3	133.7 ± 12.3	0.926

* Significance was set at $p < 0.05$.

2.2. Plasma low-abundance proteins enrichment

In order to reduce the very high dynamic range of protein concentration in plasma, we performed a low-abundance proteins enrichment step by using combinatorial peptide ligand libraries commercially known as the ProteoMiner™ enrichment kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions with slight modifications. Briefly, 1 mL plasma sample was incubated with the functionalized beads for 2 h at room temperature followed by two consecutive elution steps with 150 μL of an elution buffer containing 2% CHAPS, 8 mol/L urea and 5% acetic acid. Then, eluate was concentrated and dialyzed against a buffer containing 8 mol/L Urea and 4% CHAPS by using Amicon Ultra-0.5 mL Centrifugal Filter Units (Millipore, Billerica, MA). Protein concentration was assessed using DC Protein Assay Kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions, using bovine serum albumin as standard. Proteins were stored at −20 °C before analysis.

2.3. Differential protein expression analysis

2-DE was performed as previously described (Lepedda, Zinellu, Nieddu, Zinellu, et al., 2013; Lepedda et al., 2009). Briefly, 300 μg of proteins added with 1% (w/v) DTT and 0.4% (v/v) carrier ampholytes (pH 3–10, Bio-Rad, Hercules, CA) were applied to 70 mm IPG strips (pH 4–8, Bio-Rad, Hercules, CA) for 6 h by rehydration loading at 20 °C and subsequently focused at 50 μA/IPG strip for 22 kVh at 20 °C in a Protean IEF Cell (Bio-Rad, Hercules, CA). Once isoelectric focusing (IEF) was completed, proteins were in gel reduced by incubating IPG strips with a 50 mmol/L Tris buffer containing 6 mol/L urea, 30% glycerol (v/v), 3% SDS (w/v) and 1% DTT (w/v), followed by in gel alkylation, using the same solution containing 2.5% iodoacetamide (w/v) in place of DTT. Each step was performed keeping strips under continuous shaking for 15 min. Then IPG strips were sealed with 0.5% low melting point agarose (w/v) in SDS running buffer at the top of second dimension gels (8 cm × 7 cm × 0.1 cm). SDS PAGE was carried out on 10–15%T/3%C polyacrylamide gels for 15 min at 50 V and then at 150 V until the Bromophenol dye front reached the lower limit of the gel, in a Mini-Protean Tetra Cell (Bio-Rad, Hercules, CA). Gels were then fixed in 30% ethanol (v/v), 2% phosphoric acid (v/v) solution for 1 h, washed twice in 2% phosphoric acid (v/v) solution for 10 min, equilibrated in 18% ethanol (v/v), 2% phosphoric acid (v/v), and 15% ammonium sulphate (w/v) solution for 30 min, and stained in the same solution added with Coomassie Brilliant Blue G250 (0.02% w/v final concentration) for 48 h. Gel images were acquired by using GS-800 calibrated densitometer (Bio-Rad, Hercules, CA) at 36.3 μm resolution. Image analyses were performed using PD-Quest 2-D analysis software V8.0.1 (Bio-Rad, Hercules, CA). Total Quantity in valid spots was the adopted method for data normalization. By this method, the raw quantity of each spot in a member gel was divided by the total quantity of all the spots in that gel that were included in the master.