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Journal of Experimental and Clinical Neurosciences (JECNS)

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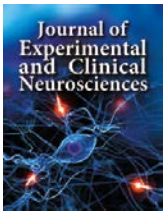

Research Article

Proteomic Screening of Cerebrovascular Diseases Markers in Activated Platelets

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Keywords:

Cerebrovascular diseases
Oxidized LDL
Platelet
Proteomics

Received: 2014-05-25
Accepted: 2014-10-19

DOI: 10.13183/jecns.v1i1.3

Abstract

Purpose: The circulating anucleated platelets are involved in pathophysiology of several cerebrovascular diseases (CVD) such as atherosclerosis, Alzheimer and stroke. Platelets should be activated before involving in the inflammatory or hemostatic events. Oxidized low density lipoprotein (oxLDL) as oxidative stress inducer and one of the major risk factors in atherosclerosis leads to vast changes in platelets proteome. The objective here is to study proteins involved in such phenomena.

Methods: In the current study, quiescent human platelets activated by oxLDL.2D SDS-PAGE (pH 3-10) were used to separate platelet proteins. Progenesis SameSpots statistical software was used to compare the gel images of resting and activated platelets with each other. Finally, comparatively expressed proteins were identified using Matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectroscopy technique (MALDI-TOF MS).

Results: This study showed altered levels of actin binding proteins such as myosin-9, Cofilin-1 and transgelin2. It has also pointed to overexpression of Thrombospondin (TSP)-1, Fibrinogen, Proto-oncogene tyrosine-protein kinase Src, and underexpression of Enoyl-CoA hydratase, mitochondrial, Ubiquitin-like modifier-activating enzyme 7.

Conclusion: Alteration in actin associated protein hints towards the cytoskeletal changes necessary to oxidative stress effect on platelets. Additionally, altered expression levels of Thrombospondin (TSP)-1and Proto-oncogene tyrosine-protein kinase following oxidative stress signifies that cerebrovascular events can partly modulate via Src kinase pathway or/and TSP- TNFα- TLR4/NF-Kb pathway. These findings can raise our basic knowledge about platelet function and novel drug targets, leading to the discovery of the mechanism(s) of action of new antiplatelet drugs.

Introduction

Stroke is one of the most common causes of death [1] and the main reason for permanent disability in adults all over the world [2]. So there is a vital need for novel developments in stroke management including prevention and treatment. The importance of blood biomarkers is becoming increasingly felt in cerebrovascular diseases [3] since biomarkers might help physicians in some steps of stroke assessment. Several events such as inflammation [4], reperfusion damage [4, 5], and oxidative stress could be the main goals to look for probable biomarkers. In recent years many scientists have focused on platelet biomarkers because of their involvement in hemostasis, inflammation innate in the immunity system [6] and production of oxidative agents. Platelets should be activated before playing their roles [3, 7]. Throughout activation, they undergo some changes in size, shape, expression of proteins and post translational modification [8]. Many studies have reported high average levels of mean platelet volume (MPV) after acute ischemic stroke and chronic cerebrovascular diseases. Also, it is proved that high levels of MPV have an association with a weak survival rate after ischemic stroke and myocardial infarction and with an elevated risk of restenosis after coronary angioplasty [9]. This association is in accord with what is known to be more reactivity of bigger platelets, including aggregation and release of thromboglobulin, thromboxane A2, and platelet

factor 4 [10]. In another study spontaneous platelet aggregation was shown in transient ischemic attacks or cerebral infarction [11]. On the other hand, anticoagulants suppressing platelets are established in the management of patients at risk for ischemic stroke in patients with well-known symptomatic cerebrovascular disease or atherothrombosis [12]. Among platelet activators, oxidized low density lipoprotein (oxLDL) as a oxidative agent has been widely studied particularly in a wide range of inflammatory underlying diseases [13, 14] such as atherosclerosis [15], cancers [16] and diabetes [17]. The insertion of lipoproteins in intima is the first event in atherosclerotic plaque formation [18]. There is a trace amount oxLDL in healthy individuals [19]. The normal intima has 1.96±0.99 ng/µg Apo lipoprotein B100 [20] but concentrations increase 7-fold in atherosclerotic plaque (14.9±1.7 ng/µg apoB100) [19, 20]. The Blood samples obtained from atherosclerotic individuals have anti-ox LDL that binds oxidized specific epitopes in oxLDL demonstrating that oxLDL exists in the vessels as well [21]. Therefore, in the circulation, platelets can come into contact with oxLDL and become activated [19, 21]. Recently, several platelet receptors for ox LDL counting integrin αIIbβ, the class B scavenger receptor CD36, lectin-type oxidized LDL receptor 1and scavenger receptor A (SR-A) have been identified. A new report has shown that binding of oxLDL to CD36 leads to platelet activation via Src kinase and p38 mitogen-activated protein kinase–dependent pathways. Prominently, CD36 involvement in platelet hyperactivity and accelerated thrombosis has been demonstrated in murine models of hyperlipidemia. As far as we know, the mechanisms of these activatory effects are still not very clear [22].

Nowadays the most powerful technology for comprehensive analysis of proteins is proteomics [23]. The proteome may be described as the whole expressed proteins in distinctive area such as a cell, organelle or fluid at one time, containing all intact and modified proteins [24]. Many proteomics studies on platelets have been performed so far [25]. The latest Proteomic and Phospho-Proteomic Profile of Human Platelets in Basal and Resting State were published by Qureshi et al in 2009 [26]. They succeed in noticeably increasing the previous platelet proteome which had been presented by Zahedi et al [27, 28]. They cataloged a total of 1507 unique proteins in human platelets from ten Individual proteomic analyses and a total of 140 1D SDS PAGE gel slices. Additionally several comparative proteomics studies have been conducted on platelets [26, 27, 28, 29] one of which was Majek et al's study [30]. They targeted changes within proteins, in platelets activated by three potent agonists Arachidonic Acid (AA), collagen and thrombin. They chose two proteins including WD repeat-containing protein 1 and mitochondrial glycerol-3-phosphate dehydrogenase as thrombin pathway specific proteins [30].

We performed a different proteomics study on platelets which underwent oxidative stress. In our recent publication about protein changes in cu^{2+}-oxLDL induced activated platelets, we focused on plasma membrane proteins such as gamma-aminobutyric acid A receptor gamma 2, WD repeat domain 52, isoform CRA a and relevant entities [31]. We found some candidate signaling proteins such as kinesin-like protein KIF21A ([swiss-prot; Q7Z4S6]) and leucine-rich PPR-motif, containing protein and c-mer proto-oncogene tyrosine kinase ([swiss-prot; Q08828]) involved in innate immune and hemostasis. Moving on, we decided to present some other proteins from the same experiment related to injury-related pathways in cerebrovascular diseases and stroke.

Methods

Materials

Prostaglandin E1, urea, CHAPS, TEMED were purchased from Sigma-Aldrich, (Gillingham, Dorset, UK). Immobilized pH gradient IPG strips (pH 3-10) and the electrophoresis instruments were acquired from Bio-Rad (California, USA). Formic acid, ammonium bicarbonate, and ammonium Sulfate, DTT and ammonium persulfate were obtained from Fluka Chemie (Buchs, Switzerland). All other chemicals were purchased from Promega (Southampton, UK).

Methods

Platelets Preparation and treatment with oxLDL

Human drug -free whole blood was obtained from six healthy donors (aged 45±50) who were referred to Tabriz central blood bank (in Iran). The main exclusion criterion was plasma P-

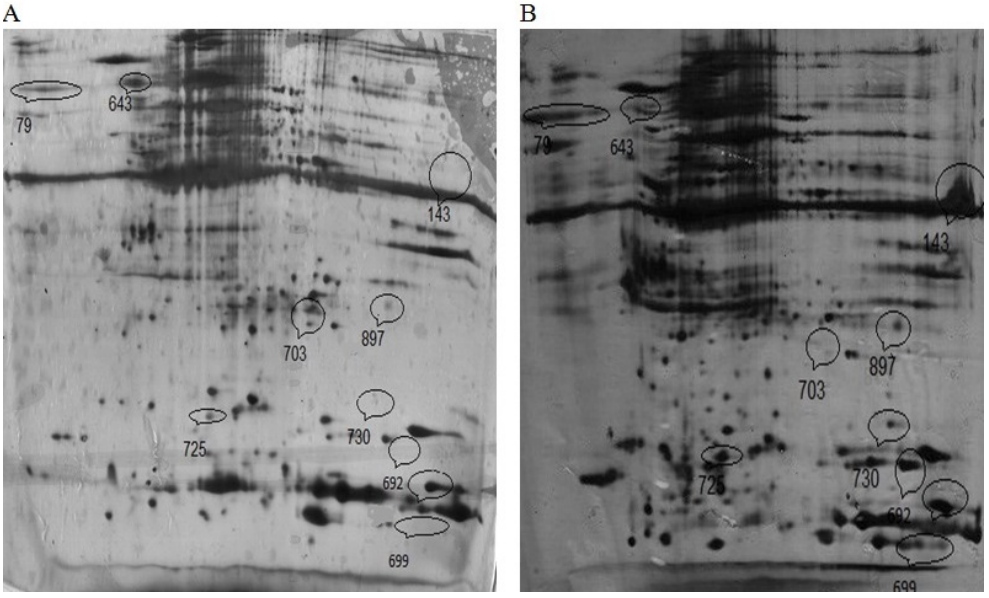

Figure 1. 2D gel analysis of proteins extracted from platelets. The numbers assigned to the proteins spots correspond to those listed in Table 1. (A) Representative 2-DE gel scanned image of resting platelets proteins is exposed to 1g/l in polypropylene tubes at 37°C for 1 hour. (B) Representative 2-DE gel scanned image of activated platelet proteins is exposed to 75µg/ml cu2+ OxLDL in polypropylene tubes at 37°C for 1 hour. Protein concentration was 1500µg/ml.

selectin < 40ng/ml [32]. The whole blood was collected into a ACD (65mM citric acid, 85 mM citrate, 111 mM glucose) solution (8.1:1.9**)** v/v. Plasma rich platelet (PRP) was obtained by the centrifugation of blood at 200 × g at 37°C for 20 min (Centrifuge Hetich universal) before incubation with1 µM prostaglandin E1 (Sigma-Aldrich, Gillingham, Dorset, UK) in 37°C for 15 min and subsequent centrifugation at 1000 × g at 37°C for 15 min. Ca^{2+} free Tyrode's buffer (140 mM NaCl, 2 mM MgCl2, 5.6 mM glucose, 3 mM KCl**,** 0.4 mM NaH2PO4, 12 mM NaHCO3; pH 6.2) was added to platelet pellet before being centrifuged at 700 × g at 37°C for 10 min. The Tyrode's buffer (140 mM NaCl, 5.6 mM glucose; 3 mM KCl**,** 0.4 mM NaH2PO4, 12 mM NaHCO3, 1 mM MgCl2, 2 mM $CaCl_2$; pH 7.4) was then used to make a standard platelet suspension (SPS) (10^8/mL) [33, 34]. Onyx Coulter Counter blood counter (Beckman Coulter, Brea, CA, USA) was used for counting the platelet in PRP. The dialyzed commercial LDLs were Oxidized (1 mg/ml) by 10 µM CuSO4 in PBS for 24 h at 37 °C. Platelets were dialyzed for desalting of PRP. We confirmed the lipid peroxidation by Thiobarbituric acid assay [35]. An aggregometer (Bio/Data) was used to adjust required amount of ox-LDL and optimum aggregation. Finally, the platelets were divided in two groups resting platelets (RP) treated with 1g/l LDL and activated platelets (AP) with 75µg/ml cu^{2+}oxidized LDL /SPS/ml in polypropylene tubes at 37°C for 1 hour.

Platelets were sonicated (Bandelin Sonoplus, Berlin, Germany) in ice-cold sonication buffer (0.34 M sorbitol, 10 mM Hepes), one tablet Complete Mini (Sigma-Aldrich,Gillingham, Dorset, UK) in 50 ml of buffer, 0.3 unit/ml aprotinin (Sigma-Aldrich,Gillingham, Dorset, UK), 1 mM DTT, 200 µM PMSF, for 30 s at 70% maximum power. Subsequently, precipitation of proteins was performed at four times of the sample volume of cold acetone (-20° C), incubation at -20°C for at least 2 hr, and centrifugation at 15,000 × g at 4°C for 15 min pellets stored at -70° C.

Protein separation

Pellets were resuspended in a sample buffer (7 M urea, 65 mM DTT)**,** 0.5% v/v ampholytes (pI 3-10), 4% w/v CHAPS (Bio-Rad California, USA) and a trace bromophenol blue. Rehydration of IPG strips (pI 3-10, 18 cm) (Bio-Rad California, USA) was performed in 350 µL of the sample for 10 hr at RT. The first dimension of protein separation was done at: 100 V for 40 min, 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, reaching 2000 V in 10 min, and 4000 Vh (max 0.125 mA and 0.125 W per strip) using Ettan™ IPGphor™II Isoelectric Focusing System. The strips were Equilibrated 15 min in a DTT buffer (50 mM Tris pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT, and a little bromophenol blue). Subsequent equilibration was performed in the same buffer with 2.5% w/v iodoacetamide, a replacement to DTT, for another 15 min. Proteins were separated in the second dimensions by SDS Polyacrylamide Gels 15% (SDS-PAGE), using a vertical electrophoresis separation system (Ettan DALT II System), step1/ 2.5W/gel/ 0:30min-step2/17 (max 180) W/gel/ 4:30 h. Silver staining was used for visualizing the spots that is briefly described in the following; we used 50% ethanol, 5% acetic acid for fixation and 0.02% Na2S2O3.5H2O for sensitization. Then the gels were incubated in cold 0.1% AgNO3 for 20 minutes at 4 °C 0.05%. Formalin and 3% Na2CO3 were used as developer. The developer was changed when it turned yellow. The staining was stopped with 5% acetic acid.

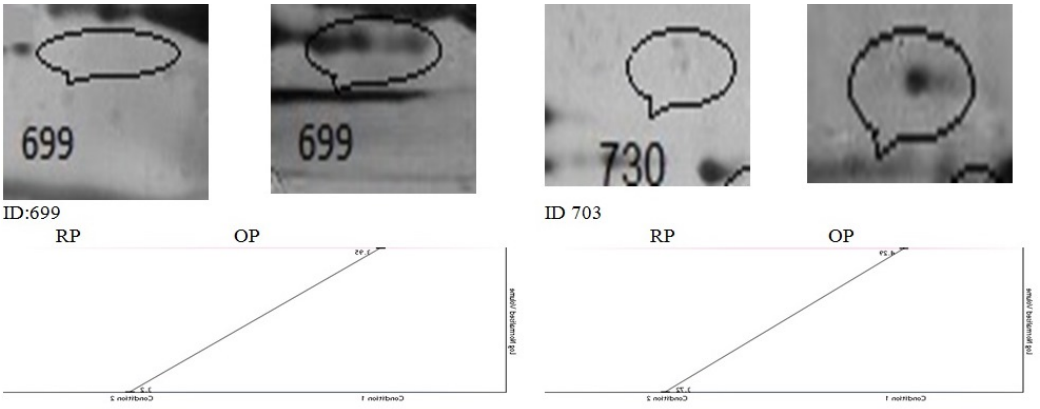

Figure 2. Two overexpressed spots profile with the logarythm of expression volume for each groups.
RP: resting platelets. OP:oxLDLactivated platelets.

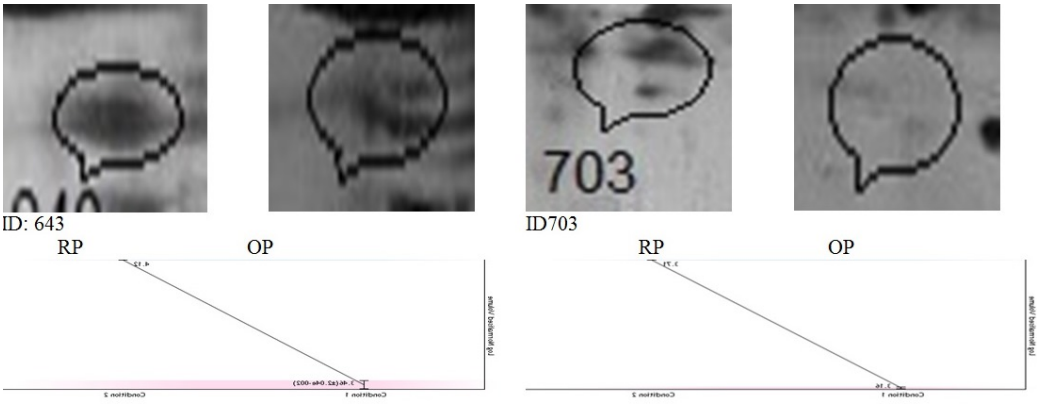

Figure 3. Two underexpressed spots profile with the logarythm of expression volume for each groups.
RP: resting platelets. OP: oxLDLactivated platelets.

Image analysis

Scanned images of gels in (tiff format, Resolution300 dpi and Bit Depth 16) were analyzed using Progenesis SameSpots software (Nonlinear Dynamics, Newcastle upon Tyne, UK).This software was used for computing fold values and p-values of all spots by one way Anova analysis. The major applied filtering was pvalue ≤ 0.05 and fold ≥2.

In-gel Digestion and Peptide Extraction

Excised spots from the gel were destained in a 1:1 ratio of 100 mM NH4HCO3 and acetonitrile**.** Afterward, completely dried gel pieces were subjected to tryptic (modified sequence-grade porcine; Promega, Madison, WI, USA) (14 ng/μL of trypsin in 30 mM NH4HCO3) digestion at 37°C overnight. Then, the peptide extraction was done with 50% Acetonitrile/5% TFA for 60 minutes each time at room temperature. Then Peptides were eluted using Zip Tips (Millipore Billerica, MA, USA) and applied onto the metal target between two layers of matrix a-cyano-4-hydroxycinnamic acid and analyzed by Matrix Assisted laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF-MS) on a BrukerBiflex (BrukerDaltonics). Calibration was performed using Trypsin autolytic peptides as internal calibrator.

Results

More than 700 spots were detected by the software on average in each gel. Statistical analysis of comparatively expressed spots identified 241 protein spots (Figure 1). Locations of 241 identified spots are showed in 2D gel image of reference. Figure 2 shows a gel image without annotation for better clarity. Among them 60 spots were selected for identification and 45 proteins were successfully identified by MALDI-TOF MS. The majority of them was platelets protein, defined as using the PlateletWeb (a platform for all platelet- related findings consisting of proteome, interactome, transcriptome, UniProt, NCBI database). Two human but non-platelet protein were Afadin Swiss-Prot: J3KN01; spot 72), Growth hormone secretagogue receptor type 1(Swiss-Prot: Q92847; spot 112). The criteria for identification were values of significance, Z score, and number of detected peptides. More than three unique peptides were found for Identification of each protein. Profound Z score higher than 1.65 calculated by the software was used as correction index.

In Table 1 the properties of 10 proteins including, protein identification, Protein swiss-prot accession number, both theoretical and examined, amount of pI and MW, Anova p-value, fold and sequence coverage have been cataloged. We manually categorized these proteins based on up/down regulation. Overexpressed proteins were Thrombospondin (TSP)-1([swiss-prot: P07996]), Cofilin-1([swiss-prot; P23528]), Fibrinogen beta chain ([swiss-prot; P02675]), Multimerin-1 ([swiss-prot; Q13201]), Proto-oncogene tyrosine-protein kinase Src ([swiss-prot; P12931]), Cofilin-1 ([swiss-prot; P23528]) and Transgelin-2 ([swiss-prot; P37802). Underexpressed proteins were Adenylyl cyclase-associated protein 1([swiss-prot; Q01518), ([swiss-prot; P37802]), Enoyl-CoA hydratase, mitochondria ([swiss-prot; P30084]), Ubiquitin-like modifier-activating enzyme 7 ([swiss-prot; P41226]). For instance, TSP-1(spot 699) and Transgelin-2 (spot 730) are two presented proteins in Figure 2. We presented two downregulated proteins, Adenylyl cyclase-associated protein 1 (spot 703) and Ubiquitin-like modifier-activating enzyme 7 (spot 643) in Figure 3. Their Expression profile has been presented in Table 1. By using Cytoscape 5 as a strong network analysis tool, we found many proteins which may be connected with identified proteins and several pathways that are involved in cerebrovascular diseases particularly in atherosclerosis (Figure 4). For TPS-1 we found 26 interacting proteins in platelets (part A-Figure 4). TSP-1 has no phosphorylation site. Multimerin-1 interacts with only one platelet protein (Figure 4-B) and, twelve platelet proteins interacting with Fibrinogen have been identified among which two proteins with phosphorylation site are integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) and ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin) (Figure 4-C). We found one kinases, LIM domain kinase 1(LIMK1) which interact with cofiln-1; however no phosphorylation site was detected on cofilin-1 in platelets (Figure 4-D). Other analyzed pathways aren't presented in the paper.

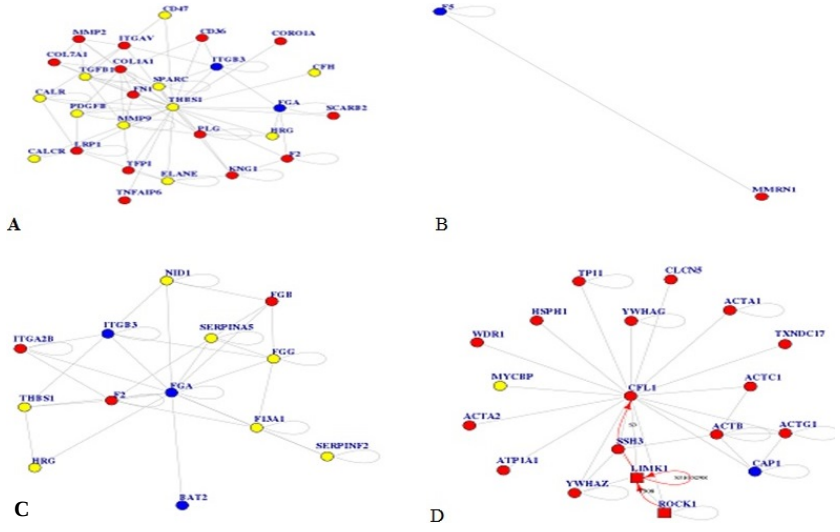

Figure 4. Networks are showing interactions between selected proteins with other platelet proteins. A-TSP-1 network , B-Mutimrin-1network ,c-fibrinogen network, D- Cofilin-1.(all network adapted from platelet web).

Denotes platelet proteins with phosphorylation sites detected in platelets

Denotes platelet proteins with phosphorylation sites detected in human cells

Denotes platelet proteins with no detected phosphorylation sites

Denotes platelet kinases with phosphorylation sites detected in human cells

Discussion

We carried out a simple differential proteomics study that focused on human platelets at two situations in activated and quiescent platelets to extend our information on platelets and its involvement in CVD. In the current paper, we presented ten oxLDL-induced platelet proteins already proved to contribute to CVD and stroke. We discussed some of these proteins and their established involvement in CVD.

1. Thrombospondin (TSP)-1([swiss-prot: P07996]) which has gained increasing centrality in recent studies. Thrombospondin-1, stored in platelet α-granules is a 450-kD multimeric extracellular matrix glycoprotein [36] released by a wide range of cells, including epithelial cells (ECs) [37], polymorphonuclears (PMNs) [38] and smooth-muscle cells (SMCs) [39]. It is known as a potent inhibitor of angiogenesis and nitric oxide/cGMP signaling [40, 41]. This protein was previously presented in Májek et al's proteomic study too [30]. They conducted a differential proteomics experiment in the presence of three potent agonists of platelets, Thrombin, Arashidonic acid (AA) and Collagen. Like us, they also found increased amount of TSP-1 in Thrombin activated platelets. No significant difference was observed in TPS-1 expression in AA or collagen activated platelets. Based on network in platelet web (Figure 4-A), TPS-1 has two known receptors, so called SCARB3 (antigen CD36) and integrin beta 3 (antigen CD61) [42]. This protein links to many aggregation and hemostasis pathways too [43]. Through these pathways, TSP-1 can be involved in numerous contexts. For instance, TSP-1 can suppress endothelial cell proliferation and induce apoptosis [44]. TSP-1can be distinctively regulated by Rac pathway leading to increased ROS production. TSP-1 has an important role in platelet adhesion to damaged endothelium inhibiting from nitric oxide (NO)-induced vasodilation [45] and tissue perfusion in ischemic rat model [45, 46].

The data has shown that the alteration of TSP-1 and TSP-2 may have a close relationship with intracerebral hemorrhage (IHC) -induced angiogenesis in rat brains following hemorrhagic stroke [44]. Following ICH the considerable rise of TSP-1 mRNA at the early phase may prove an initial challenge of antiangiogenic efforts to increase EC migration and cell cycle development [46]. However, their studies have demonstrated that ICH can lead to upregulation of proangiogenic factors including VEGF, Ang-1, and their receptors too. In another study, Navarro et al showed that a pro-angiogenic shift in plasma was associated with mild short-term neurological deficit while an acute anti-angiogenic status [47] that was mainly determined by a high level endostatin and TSP-1 was associated with worse long-term functional outcomes. [45, 47] Liauw et al in a study on knockout (KO) mice by unilateral occlusion of the common carotid artery (CCA) and the distal middle cerebral artery showed that loss of Thrombospondin-1/2 impairs behavioral recovery, synaptic recovery and axonal sprouting after stroke [48]. Based on evidence, TSP-1 released from oxLDL-activated platelets can lead to some cerebrovascular events. It seems that the complete set of the functions for this protein is elusive.

2. Fibrinogen ([swiss-prot P02675]) is another overexpressed protein in stress oxidative induced by oxLDL. This result is in conflict with Májek et al's study [30]. They showed an increase of fibrinogen in Thrombin activated platelets while it was noticeably decreased in AA group. Fibrinogen is able to form fibrin polymer and to act as a cofactor in platelet aggregation. Fibrin Polymerization following the exposition of the fibrinogen N-terminal is triggered by thrombin and is obviously considered an atherogenic marker, involved at almost all phases of lesion development [49]. Fibrin, alongside micro thrombus, is deposited on normal intima in early proliferative lesions followed by recruiting smooth muscle cells (SMCs) into intima and participating for plaque development [24, 48]. Moreover Fibrin provides a lot of fibrin degradation products (FDP) proliferating SMCs [49]. Collected fibrin in fibrous plaques may also bind the lipoprotein Lpa with high affinity, thus fix its lipid core within the lesions [50].

3. Multimerin 1 swissprot: Q13201,the third identified protein in our study, is a transporter protein and a stabilizing one for platelet factor 5 (F5) [51]. It is also Ligand for integrin alpha-IIb/beta-3 and integrin alpha-V/beta 3 on activated platelets [48]. This gene is involved in pathophysiology of two hereditary illnesses, autosomal recessive hemorrhagic diathesis and thrombophilia [52], which are known as activated protein C resistance. Multimerin-1 has strong association with myocardial infarction (MI) and stroke [53]. It also significantly improves its subsequent outcomes compared with other risk factors alone [52]. VacA, an exotoxin released by H. pylori, can activate platelets via binding to Multimerin 1 in peptide sequence AA 321-340 [53].

Transgelin-2 swiss-prot: P37802 unexpectedly was increased in our experiment. In Májek et al's report, both thrombin and AA group have higher levels of Transgelin-2 but in collagen group no difference was observed between collagen and control groups [54]. Nonetheless, it is taken to be an inflammatory marker [55]. Van Laanen et al in their proteomics study on debris collected from distal protection filter after carotid angioplasty showed that Transgelin-2 in patients with acute inflammation is higher than in patients without acute inflammation [56].

Actin binding proteins such as myosin-9, Cofilin-1 expression were altered in this experiment. Changes in actin associated proteins suggest that the cytoskeletal changes necessary for oxidative stress have effects on platelets.

4. Cofilin-1 as another identified protein binds to F-actin and acts as inhibitor of F-actin reorganization. It controls mitosis via actin cytoskeleton dynamics [57] and promotes cytokinesis so it can play a vital role in the platelet remodeling [58]. This change is essential for the overexpression of atypical chemokine receptor (ACKR2) from reticulum endoplasmic vesicles to cell surface, raising its ability in degradation and particle engulfment [58]. Platelets can uptake the LDL particles [27] and present an oxidized form of them to PMNs [23, 26, 42]. This is so important in early stages of atherosclerotic plaque formation [25, 28]. Kim et al in their recent paper showed that by preventing the inactivation of cofilin, oxidative stress–induced inhibition of 14-

Table 1. The list of identified proteins RP – resting platelets. OP–oxLDL activated platelets. AN – accession number (SWISS-PROT), SC -sequence coverage of protein.

					Experimental		Calculated		SC%
Protein identification	AN	spots	fold	Anova (p)	pI	MW	pI	MW	12
Fibrinogen beta chain	P02675	699	5.6	1.293e-011	9.2	20	8.54	27	23
Adenylyl cyclase-associated protein 1	Q01518	703	4.1	6.804e-011	7.1	49	8.2	52	21
Cofilin-1	P23528	725	11	1.455e-010	6.4	37	7	29	15
Thrombospondin-1 N-Terminal Domain	P07996	692	3.7	1.148e-010	8.9	33	8.3	28	31
Transgelin-2	P37802	730	4.2	2.203e-009	7.9	39	8.4	29	13
Myosin-9	P35579	79	7.4	3.747e-008	3	126	5.5	128	22
Multimerin-1	Q13201	143	2.5	4.518e-008	9.4	98	8.1	108	9
Enoyl-CoA hydratase, mitochondrial	P30084	893	12	5.332e-008	8.7	28	8.2	32	16
Ubiquitin-like modifier-activating enzyme 7	P41226	643	2.9	6.476e-008	4.8	148	4.58	133	24
Proto-oncogene tyrosine-protein kinase Src	P12931	897	3.4	8.560e-008	8.6	53	7.1	60	11

3-3zeta leads to the change of blood monocytes into proatherogenic macrophages [57]. Phosphorylated cofilin can modulate reorganization of dendritic spines in hippocampal neurons. Regulation of cofilin activity in turn is mediated by beta;-Arrestins in dendritic spines [56, 59].

The mentioned evidence proves that platelets have a strong relationship with CVD and are involved in many processes; therefore, identification of platelets biomarker can improve our knowledge in pathophysiology of these diseases. In this way, we will gradually be able to plan novel drugs for new biomarkers as well.

Acknowledgements: We are grateful to Mr Vatankhah; staff in Drug applied Research Center, Tabriz University of Medical Sciences in Tabriz (Iran) for their kind help in sample preparation steps.

Ethical issues: The protocol for the research project has been approved by the ethic committee at TUMS (Tabriz University of Medical Sciences) which is in compliance with the Helsinki Declaration**.**

Conflict of interests: The authors had no competing interests to declare in relation to this article.

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