

Proteomics investigation of human platelets by shotgun nUPLC-MSE and 2DE experimental strategies: a comparative study

Francesco Finamore^{1,2}, Luisa Pieroni^{1,2#}, Maurizio Ronci^{1,2}, Valeria Marzano^{1,2}, Stefano Levi Mortera^{1,2}, Mario Romano^{3,4}, Claudio Cortese², Giorgio Federici^{2,5}, Andrea Urbani^{1,2}

¹IRCCS-Santa Lucia Foundation, Rome; ²Department of Internal Medicine, University "Tor Vergata", Rome; ³Centro Studi sull'Invecchiamento (CeSI), Fondazione università "G. d'Annunzio", Chieti; ⁴Department of Biomedical Sciences, University "G. d'Annunzio", Chieti; ⁵Ospedale Pediatrico Bambino Gesù, Roma; Italy.

Background. Platelets, the smallest human blood cells component, have a key role in the control of haemostasis and thrombosis but they have also been shown to be implicated in a number of different pathological states because of their involvement also in the process of inflammation and its resolution. Their peculiar anucleated morphology renders the proteomics an intriguing approach to understand their biology. Given the high impact of platelets in different diseases we have started a systematic investigation of protein repertoire in controlled platelet preparation.

Material and methods. Platelets have been extracted from blood of healthy donors (n=6) collected by venipuncture in Vacutainer. The quality of the preparation was assessed by observation and enumeration in a Bürker chamber with a conventional tissue culture microscope. To characterize human platelets proteome we analysed the pool of purified platelets combining two proteomic approaches: 2-DE separation combined with Mass Spectrometry and nanoscale ultra performance LC-MS^E shotgun proteomics experiments.

Results. The 2D gel analysis leads to an average of 1900 protein spots, after the filtering of "noise" and "false positive" spots, over 500 were selected to be eligible for further analysis given their optimal spot quality value. To perform the analysis by ion counting shotgun proteomic approach, based on nano ultra performance liquid chromatography (nUPLC) coupled to MS^E processing of continuum LC-MS data, the same pool of samples was subject to liquid phase tryptic digestion and the peptide obtained used for the experiments. All the data obtained were analysed using ProteinLynx GlobalServer v2.3 (PLGS, Waters). Three analytical replicates were acquired in high/low energy modes and associated to a human protein database returning the identification of 100 distinct genes. Comparative analysis of the Gene Ontology has been performed to evaluate the differential functional representation of the molecular repertoire investigated with these two orthogonal approaches.

Discussion. The overall molecular function classification revealed differences between the two proteomic approaches. In particular, we found significant differences in cytoskeletal proteins (19.65% 2-DE versus 45.60% Shotgun) and receptors (0.92% 2-DE versus 6.90% Shotgun).

Keywords: platelets, proteome, mass spectrometry (MS), gene ontology.

Introduction

Platelets are the smallest blood circulating cells involved in several biological processes such as haemostasis, wound repair, inflammation and pathological events causing cardiovascular diseases,

including stroke and myocardial infarction¹⁻⁵. Platelets arise from fragmentation of membrane-delimited cytoplasmic area of terminally differentiated megakaryocytes, located in the bone marrow¹. Although translation events and protein synthesis have

been recognized in platelets², their anucleate nature renders genomic and transcriptomic techniques not very suitable for genotypic characterization, nevertheless significant analysis can be carried out on platelet protein level. In this respect, available proteomics technologies offers huge sensitivity, in terms of characterization, quantitation and identification of the proteins content of biological samples, thus representing very promising tools for the comprehensive research of platelet biology. At the beginning of the proteomics era the platelet proteome has principally been investigated by high-resolution 2-D IEF/SDS-PAGE maps as primary separation technique⁶⁻¹⁰. However, this technique is not very efficient for the separation of several kind of proteins such as, for instance, hydrophobic proteins. Hydrophobic proteins, in fact, tend to aggregate and precipitate when they reach their pI, which represents the point of their lowest solubility. As a consequence, these proteins will be underrepresented in the second dimension gel. Moreover an analysis only based on protein separation studies can be affected by protein changes or degradation due to long term storage of the analyte, that's why alternative approaches, such as peptide analysis based strategies, mainly used for quantitation studies, has been more recently shown to be a necessary complement to have a whole image of the entire expression profile of a sample^{11,12}.

Therefore, in this study, we have employed two different but complementary approaches, namely the classic 2-DE, in association with mass analysis and nanoscale ultra-performance LC-MS^E. We compared these techniques for sensibility, accuracy and data reproducibility and we consistently observed that combining these techniques resulted in a more detailed characterization of the platelet proteome.

Improving advances in proteomics technologies achieved in the last decade, leads to the possibility to find platelets proteomes available in several integrated database and the combination with transcriptomes data allowed the generation of comprehensive in silico model of platelets specific interactomes and platelets phosphorylations and kinases functional map^{13,14}.

In our study we carried out a meta-analysis by bioinformatic investigation employing The **PANTHER** (Protein ANalysis THrough Evolutionary Relationships) Classification System¹⁵ combining both our experimental data with bibliographic

references⁹ to estimate the functional distribution of the identified proteins.

Materials and methods

Platelet preparation and protein isolation

Blood samples, collected in sodium citrate-containing tubes were obtained from six healthy volunteers. Each blood sample was processed individually by adding acid citrate dextrose (ACD) (45 mM sodium citrate, 25 mM citric acid, 80 mM D-glucose) and centrifuged for 15 min at $150 \times g$ at room temperature to obtain the platelet rich plasma (PRP). The upper third of the PRP was centrifuged at the same conditions to remove any contaminating leukocytes. Platelets were pelleted at $1000 \times g$ and washed in ACD twice. Purity of isolated platelets was confirmed by microscopic inspection and a percentage of leukocyte contamination $< 0.02\%$ and of RBC $< 1\%$ was calculated. Pellets were suspended in 200 μ l sample buffer (7 M urea, 2 M thiourea, 40 mM Tris pH 7,5, CHAPS 4%, DTT 50 mM). The extracted proteins were subjected to precipitation with a solution of ice-cold acetone, ethanol and methanol at -20°C overnight and then air-dried. Proteins were suspended in 6 M urea dissolved in 100 mM Tris pH 7,5 and protein concentration was determined by the Bradford method. Individual samples were pooled and subjected to 2-DE and nUPLC-MS^E analysis.

2-DE

2-DE was performed using the IPGphor II (Amersham Biosciences) as previously described⁹ with some modifications. Proteins (triplicates of 100 μ g for each condition) were loaded on a pH 3-10 NL IPG strips by in-gel rehydration for 8 h at the voltage of 30V. Proteins were electrofocused at 80000 V/h at a maximum voltage of 8000V. After focusing, IPG strips were subjected to protein reduction and alkylation by two sequential immersion in the equilibration buffer containing 1% DTT for 10 min, and in the equilibration buffer containing 4% iodoacetamide for 10 min. Then, IPG strips were loaded at the top of 8-16% polyacrylamide linear gradient gels for the separation in second dimension. SDS-PAGE was carried out at a constant current of 40 mA per gel. Gels were stained by a silver staining protocol compatible with MS¹⁸. Image analysis was carried out with the DELTA2D (DECODON-Germany)

software. Statistical analysis of differential protein expression was performed by the Student's T-test. Changes were considered significant at $p = 0,05$.

Protein excision and tryptic digestion

Protein spots were excised manually and transferred to eppendorf tubes (0,2 mL). Protein-containing gel pieces were destained with 200 μ L of 30 mM $K_3Fe(CN)_6$ and 100 mM $Na_2S_2O_3$ and then washed with sequential incubation in 100 μ L of 0,1 M ammonium bicarbonate and dried with 100 μ L of 100% ACN. Gel immobilised proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and subsequently reswollen with 10 ng/ μ L trypsin in 50 mM ammonium bicarbonate and digested overnight at 37 °C. Peptides were purified and concentrated by solid phase extraction (SPE) in ZipTip C18 pipette tips (Millipore) and spotted directly onto a MALDI target upon elution with 2 μ L of CHCA matrix (5 mg/mL in 50% ACN, 0,1% TFA).

Protein identification by MS

MALDI-MS and MALDI-MS/MS were performed on an UltraFlex III MALDI-TOF/TOF mass spectrometer (Bruker-Daltonik, Bremen, Germany). Data were acquired in positive reflectron mode. Two hundred shots per spectrum were accumulated. All acquisition were performed in a mass range of 700-3500 Thomson (m/z) with voltages of 25 and 21.7 kV for the first and second ion extraction stages, 9 kV for the lens, 26.3 and 13.8 kV for reflector 1 and 2 respectively. Quadratic external calibration of TOF was performed on monoisotopic mass of bradykinin (clip 1-7) [M+H]⁺, angiotensin II [M+H]⁺, angiotensin I [M+H]⁺, substance P [M+H]⁺, bombesin [M+H]⁺, ACTH (clip 1-17) [M+H]⁺, ACTH (clip 18-39) [M+H]⁺, somatostatin [M+H]⁺. MS and MS/MS data were analyzed by the Bruker FlexAnalysis 3.0 software. Peptide mass fingerprint obtained from MS analysis were used for protein identification in the Swiss-Prot database using the peptide search routine MASCOT (<http://www.matrixscience.com>). All peptide mass values were considered mono-isotopic and mass tolerance was set at 50 ppm. MASCOT scores greater than 56 were considered significant ($p = 0,05$). For MS/MS analysis, peaks were searched against the Swiss-Prot database, using the same setting of MS analysis, with a fragment tolerance of 0,3 Da.

Protein identification by nUPLC-MS^E

Samples (25 μ g of total protein for each pool) were diluted in 25 μ L 6 M urea in 100 mM Tris pH 7,5. Proteins were reduced in the presence of 10 mM DTT for 1 h at 37 °C and subsequently alkylated with 20 mM iodoacetamide for 1 h at RT in the dark. Modified proteins were digested with 1 μ L of 0,5 μ g/ μ L trypsin solution at 37 °C overnight. The reaction was stopped by adding 1 μ L of TFA 10% (v/v). A 2 μ L aliquot of the digested peptides was loaded, three times for each pool, on the nano-ACQUITY UPLCTM chromatographic system. Peptides were trapped on a 5 μ m Symmetry C18 column (180 μ m \times 620 mm) and washed for 10 min at 5 μ L/min with mobile phase A (0.1% FA). Peptides were then eluted and separated using a 200 min RP gradient at 300 nL/min (3-40% ACN over 120 min) on a 1.7 mm BEH 130 C18 NanoEaseTM (75 μ m \times 625 cm) nanoscale LC column. The column temperature was set at 50 °C. Lock mass ([Glu1]-fibrinopeptide B, 250 fmol/mL) was constantly infused by the NanoAcquity auxiliary pump at a constant flow rate of 250 nL/min. The Q-ToF PremierTM mass spectrometer was programmed to switch between low (4 eV) and high (15-40 eV) energies in the collision cell, with a scan time of 1.5 s per function over a mass range of 50-1990 Th. LC-MS^E data were processed with ProteinLynx GlobalServer v2.3 (Waters) and searched in the associated human protein database (UniProtKB/SwissProt Protein Knowledge Base, Release 56.0, July 2008.)

Bioinformatic pathway analysis

Proteins identified by 2DE and LC-MS^E were combined with bibliographic references⁹ in a unique dataset and uploaded into PANTHER (ProteinANalysis THrough Evolutionary Relationships) Classification System available online on the free web site <http://www.pantherdb.org/>, PANTHER Pathway version 2.5 released on line on January 06, 2009. This software allows to predict function using published scientific experimental evidence and evolutionary relationships. Proteins are classified by expert biologists into families and subfamilies of shared function, which are then categorized by molecular function and biological process ontology terms and detailed biochemical interactions are included in canonical pathways and can be viewed interactively¹⁵⁻¹⁷.

Results and discussion

Mapping platelets proteome

The initial analysis of platelet proteome was carried out on silver stained 2-DE maps of platelets proteins pools, solubilised under reducing condition and in the presence of urea, thiourea and CHAPS. These experimental conditions allowed effective separation and representation of platelet proteins by 2-DE over a broad range of pH gradient for the IEF steps. The 2D gels of platelet proteins were characterized also by the presence of contaminant plasma proteins, such as albumin, present in large amount, haemoglobin and some members of the apolipoprotein family. From imaging analysis by DECODON Delta 2D software, we obtained more than 1,900 distinct spots, which were compared with each other. We search for spots with a significantly high spot volume (>0.5 %Vol) and we obtained more than 500 spots matching these criteria (Figure 1A).

Regardless biological and gel-to-gel variation, the majority of these protein spots were found to be reproducible across the three gels. Binary comparison of the log ratios of the relative spot volumes gave the degree of variation among the three replicates. Deviation from the main 45-degree diagonal line reflects fold changes of spot volumes (Figure 1B).

The protein spot separation pattern of the three replicates is consistent with previously published work^{8,9} done under the same conditions of pI and MW range. In order to confirm these correspondence and the related purity of our preparation, some protein spots were selected as reference points, excised from the gel, trypsin-digested and subjected to MS analysis. Comparative analysis of experimental identification

versus reference proteins identified by O'Neill et al.⁹ was successful, even though some of these proteins, in particular the big ones (e.g. talin), appeared in different gel position respect to the theoretical molecular weight and pI, probably because of post translational modification or sample degradation.

In order to obtain a better characterization of platelet proteome, thus overcoming the limitations of 2-DE, we decided to complement the analysis with the recently-developed nano LC-MS^E.

Platelet shotgun proteomics investigation

Total protein extracts were digested and analyzed in triplicate run injections. All extracted peptides were subjected to an alternate scanning acquisition method, designed to obtain high resolution and accurate mass information for each detected precursor and any associated fragments. The acquisition mode was configured to alternate between two collision energy conditions. Low energy allows detection of eluting precursor ion peptides, while high energy allows detection of associated product ions with no precursor ion selection prior to CID. Data were collected during the entire LC-MS^E experiment as pairs of chromatographic profiles and their associated ion mass measurements for all the detected peptides¹⁹.

We identified a total of 114 proteins (Table I) and 102543 EMRT (Exact Mass Retention Time clusters). Quality control measures were performed on the replicates to determine the analytical reproducibility of the analysis (Figure 2). The final results from the clustering algorithm contain all mass spectrometric and chromatographic characteristics for each peptide component.

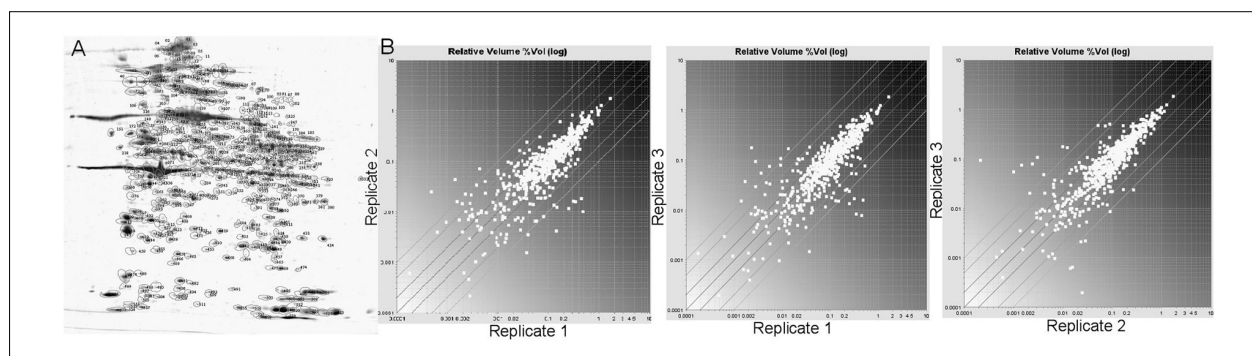


Figure 1 - 2D_E analysis of platelets proteome. (A) Representative 2-DE map of human platelets. Spots with a volume higher than 0.5 % Vol. are enclosed in boundaries. (B) Binary comparison among the log ratios of relative spot volumes detected in the three replicate gels.

Table I - Proteins identified by LC-MS^c

SwissProt Acc.N°	Protein name	MW (Da)	pI	PLGS score	Matching peptides	Coverage (%)
A5A3E0	ANKRD26 like family C member 1B	121366	5.8	1303.003	37	41.3
A6NKZ8	Putative tubulin beta chain-like protein	41748	4.6	300.3834	6	26.1
A6NNZ2	Tubulin beta-8 chain B	49540	4.6	306.393	5	17.8
O00151	PDZ and LIM domain protein 1	36049	6.6	443.2623	17	76.9
O00299	Chloride intracellular channel protein 1	26905	4.9	336.7437	12	68.9
O14950	Myosin regulatory light chain 12B	19766	4.5	469.5623	6	51.7
O43707	Alpha-actinin-4	104788	5.1	1246.604	32	48.1
O94888	UBX domain-containing protein 7	54828	4.9	270.1815	11	38.9
O95810	Serum deprivation-response protein	47144	5.0	494.3606	14	49.9
P00338	L-lactate dehydrogenase A chain	36665	8.4	309.2319	12	39.8
P00488	Coagulation factor XIII A chain	83214	5.7	794.0464	31	54.5
P00918	Carbonic anhydrase 2	29227	7.0	182.8219	5	34.6
P02042	Hemoglobin subunit delta	16045	8.2	526.4562	8	82.3
P02100	Hemoglobin subunit epsilon	16192	9.2	224.1096	3	12.9
P02671	Fibrinogen alpha chain	94914	5.6	975.3052	28	39.8
P02675	Fibrinogen beta chain	55892	8.3	814.1464	33	78.4
P02679	Fibrinogen gamma chain	51478	5.2	935.0375	24	59.4
P02768	Serum albumin	69321	5.9	1230.0095	32	60.4
P02775	Platelet basic protein	13885	9.1	640.334	7	38.3
P02776	Platelet factor 4	10837	8.8	264.0247	4	35.6
P04075	Fructose-bisphosphate aldolase A	39395	8.1	506.852	14	58.2
P04350	Tubulin beta-4 chain	49553	4.6	471.5167	11	48.4
P04406	Glyceraldehyde-3-phosphate dehydrogenase	36030	8.7	929.9182	18	67.8
P06396	Gelsolin	85644	5.8	912.8763	26	47.1
P06733	Alpha-enolase	47139	7.2	539.9177	19	60.8
P06753	Tropomyosin alpha-3 chain	32798	4.5	326.7619	19	53.5
P07195	L-lactate dehydrogenase B chain	36615	5.6	271.8883	5	18.3
P07437	Tubulin beta chain	49638	4.6	633.4846	11	47.1
P07737	Profilin-1	15044	8.5	637.4518	10	80.0
P07951	Tropomyosin beta chain	32830	4.5	328.07	7	27.5
P07996	Thrombospondin-1	129299	4.5	2156.4783	46	49.1
P08107	Heat shock 70 kDa protein 1A/1B	70009	5.3	459.2476	14	41.2
P08514	Integrin alpha-IIb	113319	5.0	535.3934	25	29.9
P08567	Pleckstrin	40071	8.3	934.5118	21	58.6
P09417	Dihydropteridine reductase	25773	7.2	142.1144	9	50.8
P09486	SPARC	34609	4.5	190.9292	9	51.8
P09493	Tropomyosin alpha-1 chain	32688	4.5	297.0772	6	21.8
P09972	Fructose-bisphosphate aldolase C	39431	6.4	367.2415	10	31.9
P10720	Platelet factor 4 variant	11545	9.5	309.8934	3	27.9
P11021	78 kDa glucose-regulated protein	72288	4.9	360.8539	18	34.4
P11142	Heat shock cognate 71 kDa protein	70854	5.2	520.1844	19	37.3
P12814	Alpha-actinin-1	102992	5.1	1519.6946	41	62.9
P13929	Beta-enolase	46957	7.7	124.1352	6	20.7
P14618	Pyruvate kinase isozymes M1/M2	57900	7.8	611.7149	21	62.9
P14649	Myosin light chain 6B	22749	5.4	264.5406	7	47.1
P18206	Vinculin	123721	5.3	2245.8167	62	64.0
P19105	Myosin regulatory light chain 12A	19781	4.5	469.5623	10	80.1
P23528	Cofilin-1	18490	8.2	582.6157	9	56.0
P24071	Immunoglobulin alpha Fc receptor	32244	6.5	158.8546	5	35.2
P24844	Myosin regulatory light polypeptide 9	19814	4.6	372.3448	9	70.3
P28065	Proteasome subunit beta type-9	23249	4.7	216.371	9	60.3
P30041	Peroxiredoxin-6	25019	6.0	164.9216	4	31.7
P31146	Coronin-1A	50993	6.2	307.7916	10	35.1
P34931	Heat shock 70 kDa protein 1-like	70331	5.6	378.9034	22	41.0
P35579	Myosin-9	226390	5.3	4097.9404	114	57.3
P35580	Myosin-10	228856	5.3	1592.002	67	43.5
P35749	Myosin-11	227197	5.2	1598.0902	73	39.4
P37802	Transgelin-2	22377	8.4	1125.636	15	86.4
P50552	Vasodilator-stimulated phosphoprotein	39805	9.3	397.6586	13	34.2

follows next page

Table I - Proteins identified by LC-MS[®]

SwissProt Acc.N°	Protein name	MW (Da)	pI	PLGS score	Matching peptides	Coverage (%)
P54652	Heat shock-related 70 kDa protein 2	69977	5.4	377.9034	21	39.1
P60174	Triosephosphate isomerase	26652	6.5	294.145	7	47.4
P60660	Myosin light polypeptide 6	16919	4.4	588.86	11	61.6
P60709	Actin, cytoplasmic 1	41709	5.1	2612.1707	27	76.8
P62736	Actin, aortic smooth muscle	41981	5.1	1535.4171	15	48.3
P62937	Peptidyl-prolyl cis-trans isomerase A	18000	7.9	245.5191	8	57.6
P63104	14-3-3 protein zeta/delta	27727	4.5	574.0399	14	51.8
P63261	Actin, cytoplasmic 2	41765	5.2	2617.9204	22	73.3
P63267	Actin, gamma-enteric smooth muscle	41849	5.2	1502.7444	15	45.2
P67936	Tropomyosin alpha-4 chain	28504	4.5	581.3867	18	57.7
P68032	Actin, alpha cardiac muscle 1	41991	5.1	1570.227	15	48.3
P68133	Actin, alpha skeletal muscle	42023	5.1	1523.284	18	54.9
P68363	Tubulin alpha-1B chain	50119	4.8	845.3544	12	40.1
P68366	Tubulin alpha-4A chain	49892	4.8	964.94	16	55.1
P68371	Tubulin beta-2C chain	49799	4.6	536.0964	10	44.9
P68871	Hemoglobin subunit beta	15988	6.9	1363.5991	13	93.9
P69891	Hemoglobin subunit gamma-1	16130	6.8	211.2016	8	58.5
P69892	Hemoglobin subunit gamma-2	16116	6.8	207.7864	4	32.7
P69905	Hemoglobin subunit alpha	15247	9.2	625.6528	8	89.4
Q01518	Adenylyl cyclase-associated protein 1	51822	8.1	426.7084	21	52.8
Q05209	Tyrosine-protein phosphatase non-receptor type 12	88065	5.3	504.4341	20	37.7
Q13418	Integrin-linked protein kinase	51385	8.0	406.3417	13	44.9
Q13509	Tubulin beta-3 chain	50400	4.6	427.0488	9	32.9
Q13748	Tubulin alpha-3C/D chain	49927	4.8	566.2676	9	36.0
Q13885	Tubulin beta-2A chain	49874	4.6	521.9	15	47.9
Q14112	Nidogen-2	151299	4.9	757.1977	28	31.8
Q14185	Dedicator of cytokinesis protein 1	215207	7.3	991.6309	39	26.1
Q15404	Ras suppressor protein 1	31520	9.1	262.5601	10	46.9
Q15942	Zyxin	61238	6.2	517.1403	16	51.2
Q3ZCM7	Tubulin beta-8 chain	49743	4.6	331.9643	9	32.7
Q562R1	Beta-actin-like protein 2	41975	5.3	793.0007	20	63.8
Q58FF3	Putative endoplasmic-like protein	45829	5.0	295.9952	16	53.4
Q6NUK1	Calcium-binding mitochondrial carrier protein SCaMC-1	53320	5.9	291.726	16	40.3
Q6PEY2	Tubulin alpha-3E chain	49884	4.8	388.5545	7	31.1
Q6Q0C0	E3 ubiquitin-protein ligase TRAF7	74560	6.7	432.0255	21	44.3
Q6S8J3	ANKRD26 like family C member 1A	121285	5.8	1470.8132	18	23.4
Q71U36	Tubulin alpha-1A chain	50103	4.8	576.6373	9	31.3
Q7Z406	Myosin-14	227861	5.6	1663.2179	62	39.0
Q80930	Regulatory protein E2	45528	9.2	336.3838	17	52.8
Q86UX7	Fermitin family homolog 3	75905	6.5	717.6672	35	67.2
Q8IZ40	REST corepressor 2	57976	9.3	243.9844	12	35.4
Q8NGU1	Putative olfactory receptor 9A1	29526	7.4	168.0808	2	9.1
Q99867	Putative tubulin beta-4q chain	48403	4.9	366.3576	11	35.5
Q9BQE3	Tubulin alpha-1C chain	49863	4.8	629.9761	16	51.2
Q9BUF5	Tubulin beta-6 chain	49825	4.6	337.3611	8	34.1
Q9BV86	Methyltransferase-like protein 11A	25370	5.2	152.797	7	37.2
Q9BVA1	Tubulin beta-2B chain	49920	4.6	538.4824	9	36.6
Q9BYX7	Beta-actin-like protein 3	41988	5.9	587.8795	7	34.4
Q9GZV4	Eukaryotic translation initiation factor 5A-2	16782	5.2	101.176	5	73.9
Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	10431	4.6	159.5535	5	69.9
Q9H4B7	Tubulin beta-1 chain	50294	4.9	1078.2789	23	79.8
Q9HBI1	Beta-parvin	41688	6.3	227.9087	9	39.8
Q9NY65	Tubulin alpha-8 chain	50061	4.8	451.669	11	41.4
Q9UBW5	Bridging integrator 2	61836	4.9	463.2477	18	56.3
Q9UI15	Transgelin-3	22458	7.2	165.844	6	43.7
Q9Y281	Cofilin-2	18724	8.2	362.7851	6	47.6

These were subjected to statistical calculations after the clustering process. The clustering algorithm utilized the analytical reproducibility of the mass measurement and the reproducibility of the chromatographic retention time of each peptide.

The mass precision of the extracted peptide components was within ± 5 ppm (approximately 2 ppm) of the mean mass measurement (Figure 2A). The variability of intensity among the replicate injections for these EMRT components showed an average coefficient within 1.6 and 2.3% (Figure 2B). The reproducibility of retention times for most of these clusters showed a RSD centered at 0.4% (Figure 2C). Variations in intensity were evaluated by conducting binary comparisons of the intensities of all matched peptide components from two replicate injections (Figure 2D). Under ideal conditions, the binary comparison would rely on a perfect 45-degree diagonal ($\ln(\text{ratio})=0$) intersecting through 0. The scatter plot showed a minimal degree of deviation of the peptide components intensity values throughout the detected range, confirming that this methods is highly reproducible. We compared datasets of identified proteins to those obtained with 2-DE experiments, in order to cluster them according to the Gene Ontology hierarchy, based on molecular functions and biological processes categories.

Gene ontology

Following 2-DE and nanoscale reversed phase LC-MS^E, all the identified proteins were analyzed for their involvement in known biological processes and their molecular function, respectively. Protein identification datasets derived both from our

experimental work and from bibliographic reference⁹ have been merged and loaded on PANTHER software.

Proteins were grouped by category and compared for their ontology class. We observed differences in the functional distribution of the identified proteins between the two methods used. Using Shotgun approach, we found a major representation of proteins involved in cell structure and motility (44.40%) respect to 2-DE (19.70%). On the contrary, using 2-DE we observed a predominance of proteins involved in cell metabolism (26.10%), respect to Shotgun analysis (8.80%). Notably, when we combined these two different but complementary proteomic tools, we were able to detect additional classes of proteins with different biological functions (Figure 3A).

Moreover, the molecular function classification revealed differences between the two proteomic approaches. In particular, we found significant differences in cytoskeletal proteins (19.65% 2-DE versus 45.60% Shotgun) and receptors (0,92% 2-DE versus 6.90% Shotgun), as expected due to the limited resolution of membrane hydrophobic proteins in the IEF first dimension. We found also differences in protein of immunity response (0.42% 2-DE versus 6.30 Shotgun) as well as in other classes of proteins (Figure 3B).

In conclusion, present results indicate that molecular profiling performed with two orthogonal proteomics analysis methodologies, enhances the possibility to obtain deeper information on the biology and pathophysiology of the systems under investigation. This becomes particularly relevant with platelets, in light of their multiple physiological functions and their involvement in a variety of human diseases.

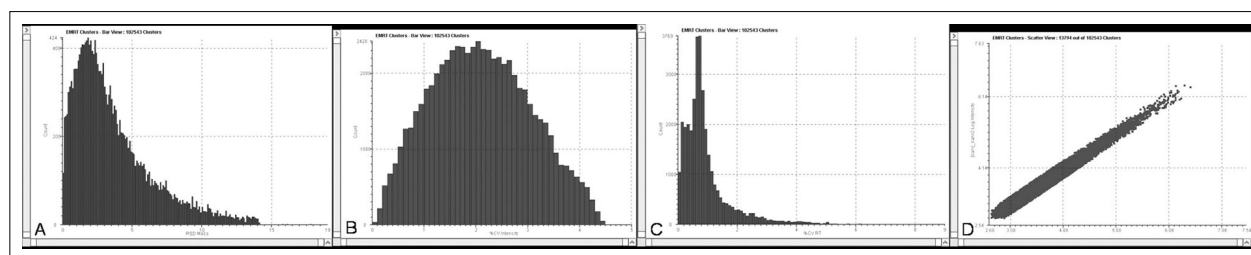


Figure 2 - Analytical reproducibility of replicate LC-MS^E experiments. **(A)** Relative standard deviation from all the EMRT components within ± 5 ppm of the mean mass measurement. **(B)** Average coefficient of variation of the measured signal intensity of the clusters. **(C)** Average retention time coefficient of variation was centered at 0.4%. **(D)** Binary comparison of the log intensity measurement obtained from the matched EMRT clusters for two replicate injections.

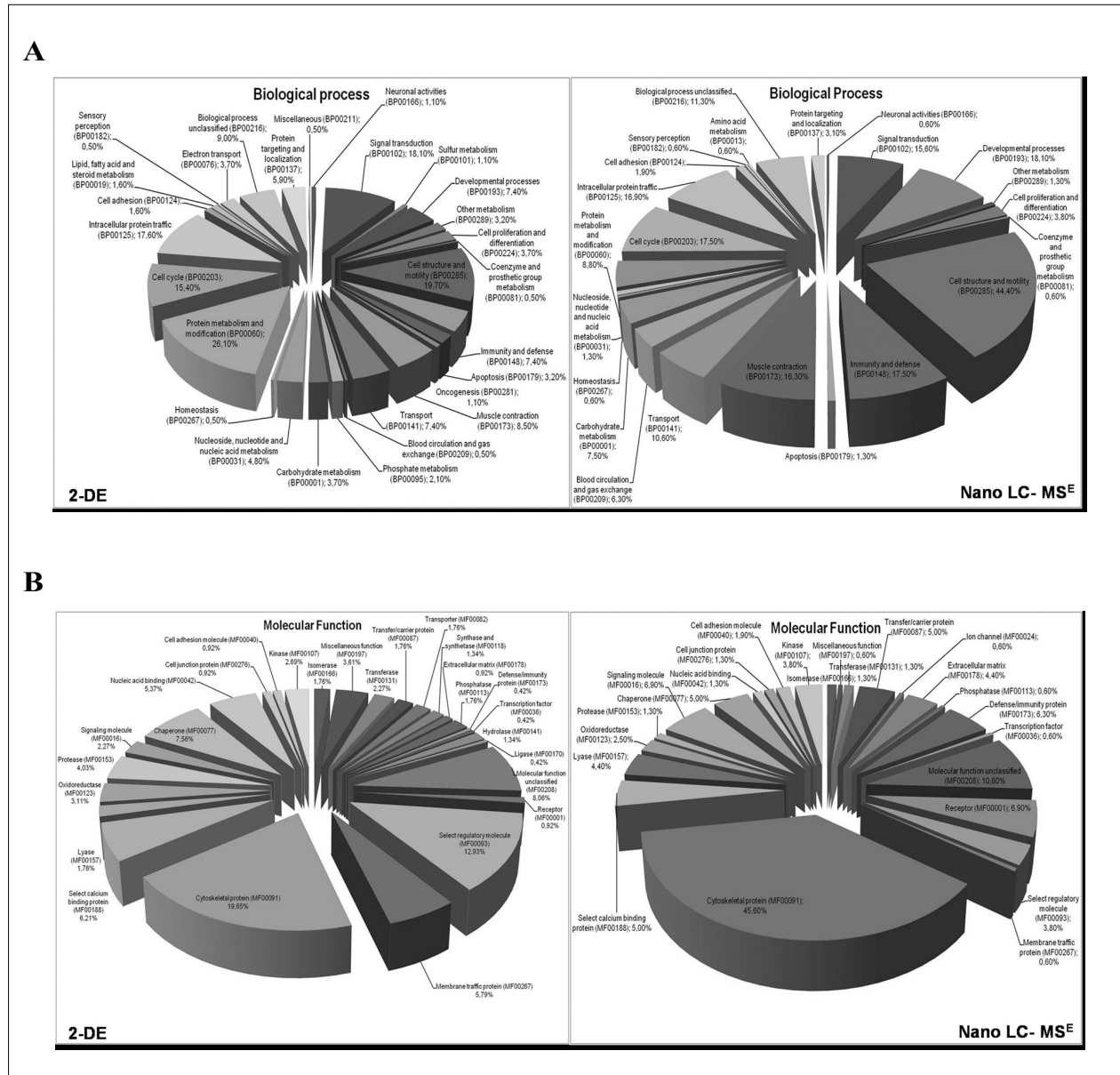


Figure 3 - Gene ontology distribution terms obtained from platelets proteome analysis. The proteins identified by 2-DE and LC-MS^E analysis were clustered based on their **(A)** biological process and **(B)** molecular functions

References

- Brown AS, Erusalimsky JD, Martin JF, in: von Bruchhausen F, Walter U (Eds), Platelets and their Factors, Springer-Verlag, Berlin, Germany 1997, pp. 3-19.
- Denis MM, Tolley ND, Bunting M, et al. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. *Cell* 2005; **122**(3): 379-91.
- Macaulay IC, Carr P, Gusnanto A, et al. Platelet genomics and proteomics in human health and disease. *J Clin Invest* 2005; **115**(12): 3370-7.
- Ruggeri ZM. Platelets in atherothrombosis. *Nat Med* 2002; **8**: 1223-1234.
- Savage B, Cattaneo M, Ruggeri ZM. Mechanisms of platelet aggregation. *Curr Opin Hematol* 2001; **8**: 270-276.
- Gravel P, Sanchez JC, Walzer C, et al. Human blood platelet protein map established by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* 1995; **16**: 1152-1159.
- Marcus K, Immler D, Sternberger J, Meyer HE. Identification of platelet proteins separated by two-dimensional gel electrophoresis and analyzed by matrix

- assisted laser desorption/ionization-time of flight-mass spectrometry and detection of tyrosine-phosphorylated proteins. *Electrophoresis* 2000; **21**: 2622-2636.
- 8) Marcus K, Moebius J, Meyer HE. Differential analysis of phosphorylated proteins in resting and thrombin-stimulated human platelets. *Anal Bioanal Chem* 2003; **376**: 973-993.
 - 9) O'Neill EE, Brock CJ, von Kriegsheim AF, et al. Towards complete analysis of the platelet proteome. *Proteomics* 2002; **2**: 288-305.
 - 10) Garcia A, Prabhakar S, Brock CJ, et al. Extensive analysis of the human platelet proteome by two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* 2004; **4**: 656-668.
 - 11) Kubota K, Kosaka T, Ichikawa K. Combination of two-dimensional electrophoresis and shotgun peptide sequencing in comparative proteomics. *Journal of Chromatography B* (2005); **815**: 3-9.
 - 12) Thon JN, Schubert P, Duguay M, et al. Comprehensive proteomic analysis of protein changes during platelet storage requires complementary proteomic approaches. *Transfusion* 2008; **48**(3): 425-35.
 - 13) Dittrich M, Birschmann I, Mietner S, et al. Platelet protein interactions: map, signaling components, and phosphorylation groundstate. *Arterioscler Thromb Vasc Biol* 2008; **28**(7): 1326-31.
 - 14) Senzel L, Gnatenko DV, Bahou WF. The platelet proteome. *Curr Opin Hematol* 2009; **16**(5): 329-33.
 - 15) Thomas PD, Campbell MJ, Kejariwal A, et al. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* 2003; **13**: 2129-2141.
 - 16) Mi H, Guo N, Kejariwal A, Thomas PD. PANTHER version 6: protein sequence and function evolution data with expanded representation of biological pathways. *Nucl Acids Res* 2007; **35**: D247-D252.
 - 17) Thomas PD, Kejariwal A, Guo N et al. Applications for protein sequence-function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. *Nucl Acids Res* 2006; **34**: W645-W650.
 - 18) Shevchenko A, Wilm M, Vorm O, et al. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996; **68**: 850-858.
 - 19) Silva JC, Denny R, Dorschel CA, et al. Quantitative proteomic analysis by accurate mass retention time pairs. *Anal Chem* 2005; **77**: 2187-2200.

Correspondence: Luisa Pieroni
 IRCCS - S. Lucia Foundation
 Via del Fosso di Fiorano, 64/65
 00143 Rome, Italy
 E-mail: luisa.pieroni@uniroma2.it
