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

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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 end its resolution .Their peculiar anucleated morphology render the proteomics an intriguing approach to understand their biology. Given the high impact of platelet 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 performances LC-MS^E shotgun proteomics experiments.

Results. The 2D gel analysis leads 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 accounting 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 run were acquire 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 onthology.

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 infarction[1–5]. Platelets arise from fragmentation of membrane-delimited cytoplasmic area of terminally differentiated megakaryocytes, located in the bone marrow[1]. Although translation events and protein synthesis have

been recognized in platelets[2], 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 technique[6-10]. 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** (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships) Classification System[15] combining both our experimental data with bibliographic references[9] 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 × 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 × g and washed in ACD twice. Purity of isolated platelets was confirmed by microscopic inspection and a percentage of leukocite contamination < 0.02% and of RBC < 1% was calculated. Pellets were suspended in 200 µl 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 described[9] with some modifications. Proteins (triplicates of 100 µg for each condition) were loaded on a pH 3-10 NL IPG strips by in-gel rehydratation 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 MS[18]. Image analysis was carried out with the DELTA 2D (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 µL of 30 mM K3Fe(CN)6 and 100 mM $Na_2S_2O_3$ and then washed with sequential incubation in 100 µL of 0,1 M ammonium bicarbonate and dried with 100 µL of 100% ACN. Gel immobilised proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and subsequently reswollen with 10 ng/µL 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 µL 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 µg of total protein for each pool) were diluted in 25 µL 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 µL of 0,5 µg/µL trypsin solution at 37 °C overnight. The reaction was stopped by adding 1 µL of TFA 10% (v/v). A 2 µL aliquot of the digested peptides was loaded, three times for each pool, on the nano-ACQUITY UPLC™ chromatographic system. Peptides were trapped on a 5 µm Symmetry C18 column (180 µm × 620 mm) and washed for 10 min at 5 µL/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 NanoEase™ (75 µm × 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 Premier™ 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 references[9] in a unique dataset and uploaded into PANTHER (Protein ANalysis 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 interactively[15-17].

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 peptides[19].

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.

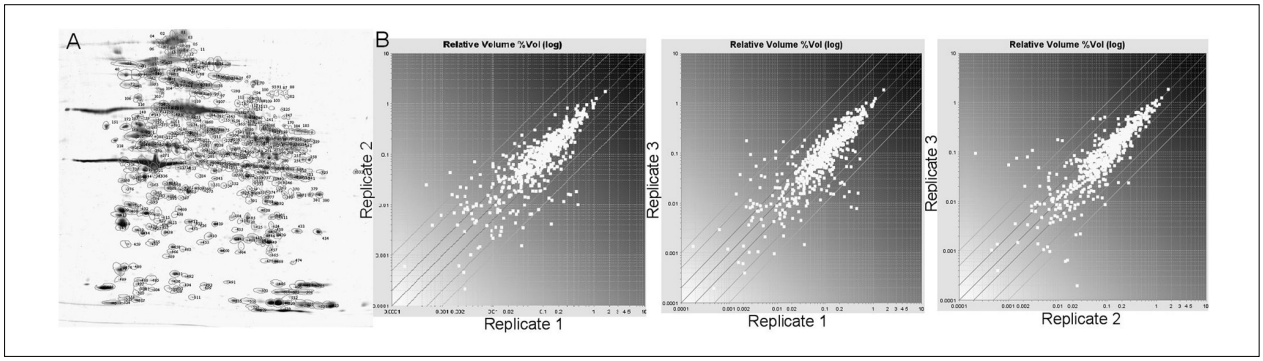

Figure 1 - 2D_E analysis of platelets proteome. **(A)** Representative 2-DE map of human platelets. Spots with a volume higher than 0.5 % Vol. are enclosed in boundaries. **(B)** Binary comparison among the log ratios of relative spots volumes detected in the three replicate gels.

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

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

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 endoplasmin-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(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 reference[9] 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.

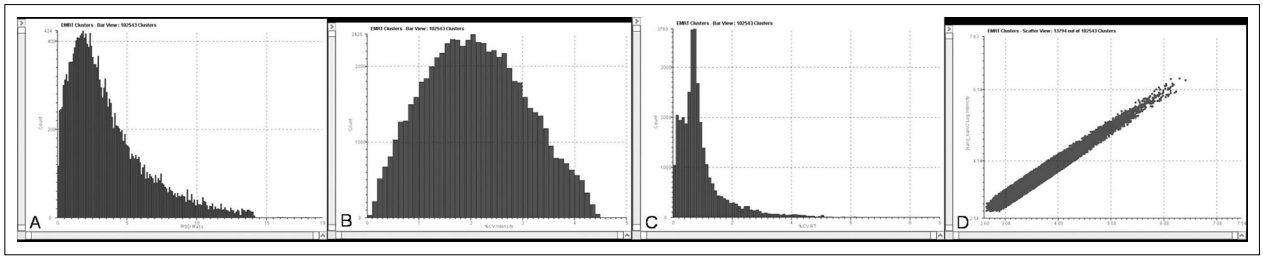

Figure 2 - Analytical reproducibility of replicate LC-MS^E experiments. **(A)** Relative standard deviation from all the EMRT components within ± 5 ppm of the mean mass measurement. **(B)** Average coefficient of variation of the measured signal intensity of the clusters. **(C)** Average retention time 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.

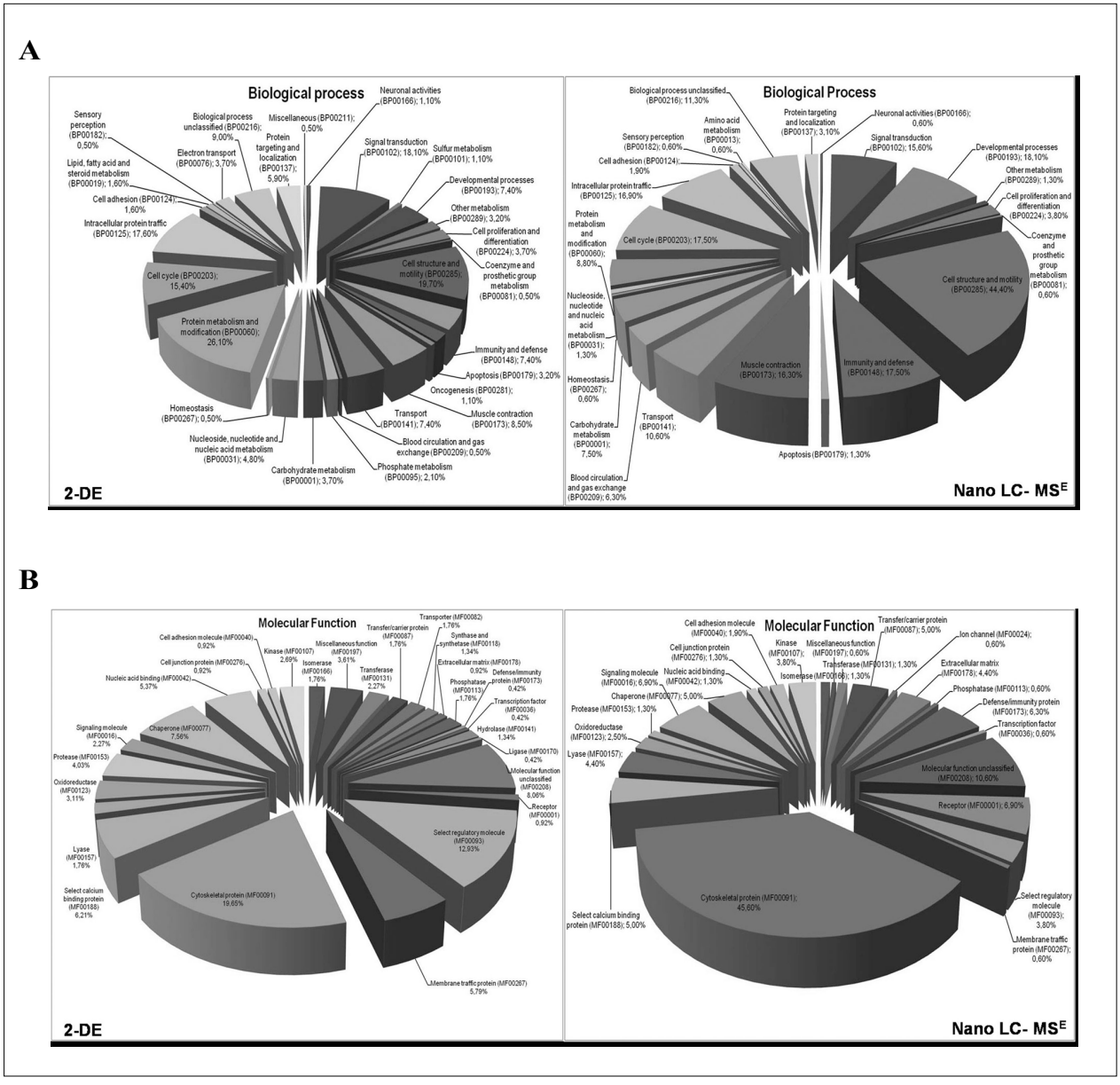

Figure 3 - Gene ontology distribution terms obtained from platelets proteome analysis. The proteins identified by 2-DE and LC-MS^E analysis were clustered based on their **(A)** biological process and **(B)** molecular functions

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