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Proteomics Study of Human Platelets in Additive Solution

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ABSTRACT

Proteomics is the study of platelet proteins and the molecules that regulate unknown platelet functions and diseases related to platelets. Application of proteomics includes to the study of (a) whole platelet proteome in resting or activated condition (b) platelet subproteomes granules, micro-particles, plasma membrane and (c) platelet PTMs (phosphosproteome, glycoproteome, palmitoylome). LCMS analysis was performed in Thermo Orbitrap fusion tribird mass spectrometer coupled with an EASY-NLC 1200 series system after the protein pellets were dissolved in urea buffer (8M urea/2M thiourea) and then sample concentration were estimated using Nanodrop. 34 *de-novo* proteins were identified and analyzed. Fold change >1.5 consider as down-regulation of protein expression as compared to control group while fold change <0.98 consider as down-regulation of protein expression. Platelet proteomics provides astounding information about underline platelet-related diseases, though several changes still remain to be studied. However, we are confident that the in-cooperation of the most recent advances in MS-based techniques in platelets proteomics studies will allow further description of the mechanisms implicated in diseases and find out new biomarkers or drug targets.

KEYWORDS

Platelet; Proteomics; LC-MS; Protein expression; Molecular level

INTRODUCTION

Proteomics is a powerful tool of identify and monitor changes during platelet storage and in combination with biochemical and physiologic studies, facilitates the development of a sophisticated mechanistic view [1].

Proteomics can provide detailed insight into the (quantitative) protein composition of platelets, of their sub compartments (e.g., the plasma membrane) or platelet-derived micro particles, examined at resting or activated conditions (Figure 1). In addition, proteomics helps in providing quantitative information about changes in protein and PTM levels, for instant on perturbation of the resting sate (e.g. agonist treatment) or when comparing platelets derived from different donors (genomic background or healthy versus disease) [2].

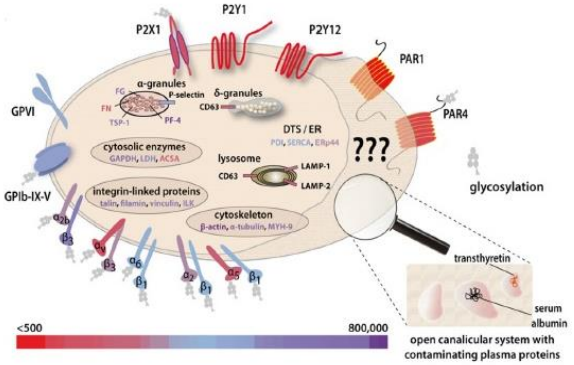

Figure 1: Diagrammatic representation of platelet proteins and their glycosylation sites.

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Purpose of the study

Proteomics of platelets can unveil (1) quantitative changes in the abundance of thousands of proteins, (2) post-translation modifications, (3) protein –protein interactions, and (4) protein localization, while requiring only small blood volume (Figure 2).

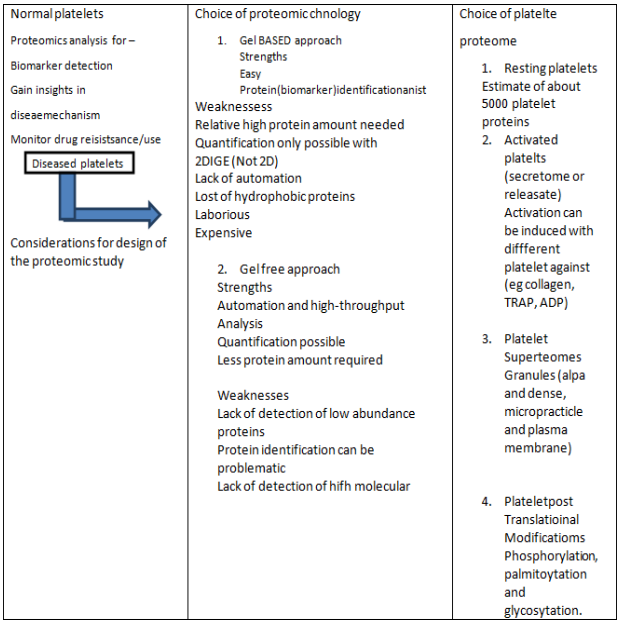

Figure 2: Overview of platelet proteomic used in platelet research

Objectives of the study

1. To study the platelet proteome determine the platelet protein with or without platelet additive solution.
2. To study the platelet proteome determine the platelet protein with or without platelet additive solution during the extended storage period.
3. To study and ascertain the role of various platelets proteome towards the pathogeneses towards platelet storage lesions.

MATERIALS AND METHODS

Reagents and materials

The chemicals for protein digestion, dithiothreitol, iodoacetamide, urea, thiourea and ammonium bicarbonate were purchased from Sigma-Aldrich. The chemicals required for PAS, trisodium citrate dihydrate, sodium acetate trihydrate, sodium hydrogen phosphate, disodium hydrogen phosphate, sodium chloride, potassium and magnesium chloride hexahydrate were also purchased from Sigma-Aldrich. Sodium dodecyl sulphate, Tris-HCl and EDTA for lysis buffer were collected from Bio-Rad. Human α-Thrombin (Factor II a) **(**catalogue#: HT 1002a, Size: 1000units) was bought from Enzyme Research Laboratories (South Bend, IN, USA).

Institutional ethical committee approval was obtained. Blood was collected from 10 healthy voluntary donors (between age group of 18-60 years after taking the written consent from all the blood donors prior to the collection. Donors were selected between age groups of (18-60 years), Weight >45 kg for RDP & 60 kg for SDP, Haemoglobin >12.5 g/dl and Platelet count between $150x10^3/\mu L$ - $250x10^3/\mu L$. Mandatory physical examination of donors and TTI screening were done.

Buffy coat platelet concentrates preparation method

BC-PC were prepared as per the routine procedure as mentioned in the SOP.

Platelets purification and ex–vivo activation with thrombin for proteomic analysis

BC-PC samples were rinsed twice with the Tyrode's Buffer containing 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO4, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.4) and centrifuged at 750xG for 10minutes. Platelet count was estimated with a Beckman Coulter LH780 system (Beckman Coulter, Inc., Brea, CA). The FC assessment showed that the platelet samples contained 99.15-99.35% CD61 positive cells (platelets), the rest being represented by leukocyte population (CD45 positive), a purity was within the same range as published research studies on whole platelets proteome [3]. To obtain thrombin activated platelets, wash platelets were re-suspended in 0.5mL of washing buffer with 1.8 mM $CaCl_2$ (to have 2x108 platelets/ ml) and

treated overnight with 1 IU of human alpha-thrombin for each sample (derived from three healthy volunteers).

Platelets treatment with pas and proteins extraction

Thrombin activated PC-BC was prepared and was separated into two equal parts. One half of the PC-BC was re-suspended in 65% of (PAS; SSP) and the other portion was kept without PAS. Both sets of PCs were stored at 20^0C - 24^0C for 7 days [4]. Protein extraction was done by employing the platelets cryogenic lysis using 5 cycles of freezing in liquid nitrogen and thawing at 37^oC followed by centrifugation at 18,000 rpm for 30 min at 4^oC. Pellet was re-suspended in 50 mM ammonium bicarbonate buffer (pH 8.5) containing 8M urea and sample was stored at -20^oC until further LC-MS/MS analysis.

Protein tryptic digestion

The protein pellets were dissolved in urea buffer (8M urea/2M thiourea) and the concentration of the samples were noted using Nanodrop (NanoDrop 2000/2000c).

Reduction of protein samples was done with 10 mM DTT and incubated at 37°C for duration of 30 minutes followed by addition of 30 mM IAA kept and incubated in the dark (room temperature, 30 minutes) to undergo alkalinisation of the free cysteine residues. The samples were diluted to 1M urea prior to the addition of trypsin (1:50 (w/w)). Sample was kept at 37°C for overnight digestion. Final concentration of 0.1% formic acid was used toquench the reaction. The digested peptides underwent vacuum drying and were desalted using C-18 ziptip (Pierce C18 Tips, 10 µL bed). The eluted peptides were dried and re-dissolved in 2% acetonitrile/0.1% formic acid.

LC-MS/MS analysis

The analysis was done in Thermo Orbitrap fusion tribird mass spectrometer coupled with an EASY- nLC 1200 series system. The peptides were administered onto revere-phase C18 pre column (Acclaim PepMap 100 C18 3 µm, 75 µm × 2 cm NanoViper) and then separated on C18 analytical column (Easy spray Pep map RSLC C18 2µm, 15 cm × 75 µm) for a resolved separation. Peptides were rinsed using a linear gradient of 168 minutes from 5% to 45% solvent B (80% acetonitrile in 0.1% formic acid) at a flow rate of 300 nL/min, 45% B to 98% B for 12 min and solvent A composition was 0.1% formic acid in LC grade water.

The mass spectrometer was operated with a positive ionization voltage of 1900 V and 273°C temperatures was used for ion transfer tube. MS spectra were acquired in the orbitrap with a resolution of 120000 over a mass range of 375-1700 m/z, automatic gain control (AGC) value was set to 4.0 e5 and a maximum injection time was kept as 50 ms. 20 highly intense ions for fragmentation was selected by Top-Speed acquisition mode which were isolated by quadruple with an isolation width of 1.2 Da. Charged states of these ions ranged from 2+ to 7+, were fragmented by HCD with an optimized collision energy of 30% with step energy +5 (Figure 3). The fragmented ion spectra were obtained by Ion trap in centroid mode, AGC value was set to 1e4 and a maximum injection time of 35 s was used. The data acquisition was done with Excalibur software (version-4.1.31.9).

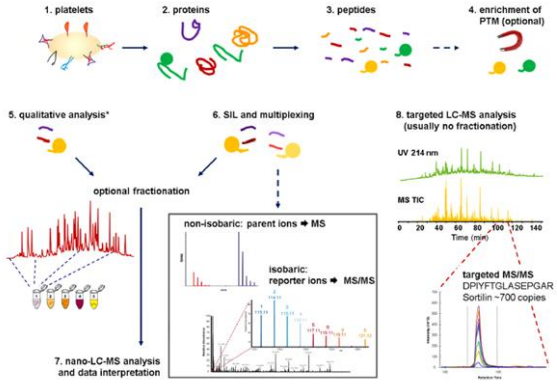

Figure 3: Diagrammatic representation of LC-MS workflow.

Data analysis

Raw data was processed into MGF peak list by using Proteome Discoverer (version 2.1). MGF peak list was

further analysed against human proteome database (Downloaded from Uniprot on 2018) by Mascot Daemon (version 2.6). (Refer Figure 4-5)A mass tolerance of 10 ppm was used for the precursor ions and 0.6 Da for the fragmented ions. Maximum missed cleavages were allowed up to two; "Trypsin" was set as enzyme specificity and charge states were set to 2+, 3+ and 4+. The fixed modification was set as Carbamidomethylation at cysteine whereas the dynamic modification was set as Oxidation at methionine. A FDR of 1% was used at the protein as well as the peptide level. emPAI based Label Free method was used to quantitatively compare the effect of PAS treatment on blood platelets.

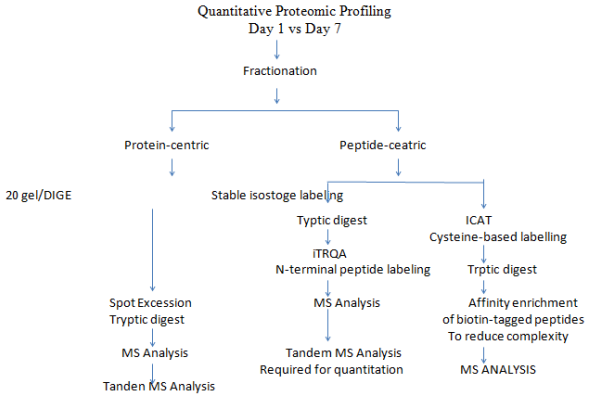

Figure 4: A schematic of the experimental setup and workflow for a complementary proteomic assessment of changes occurring in a platelet unit during storage.

RESULTS

Differential expression profile

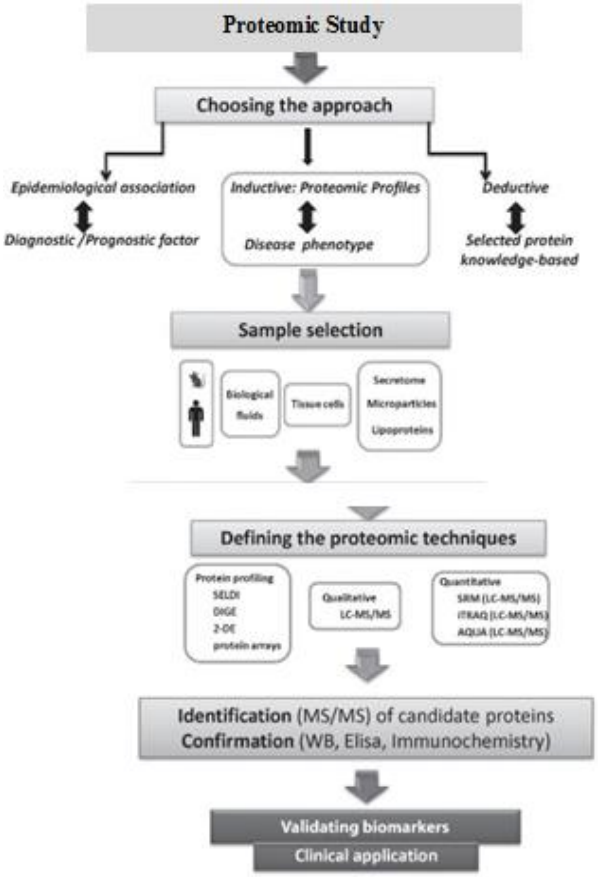

Figure 5: In a proteomic approach, an inductive strategy (unbiased) is selected and applied to using several techniques.

Protein ID	Protein Description	Fold Change	Standard Deviation
P02747_C1QC	Complement C1q subcomponent subunit C	2.08	0.63
P01031_CO5	Complement C5	1.71	1.06
F5H1C6	Fermitin family homolog 3 (Fragment)	1.76	0.67
A0A087X232	Complement C1s subcomponent	1.65	0.85
P08185_CBG	Corticosteroid-binding globulin	1.77	0.76
A0A286YEY1	Immunoglobulin heavy constant alpha 1 (Fragment)	1.39	0.33
P04217_A1BG	Alpha-1B-glycoprotein	-0.98	0.33

Table 1: Showing differential expression of proteins as compared to the control group along with fold change. Fold change >1.5 consider as up-regulation of protein expression as compared to control group while fold change < -0.98 consider as down-regulation of protein expression.

Note: Student T test was carried out to consider significantly differential protein expression.

Protein ID	Protein Description
P06702_S10A9	Protein S100-A9
P08514-2_ITA2B	Isoform 2 of Integrin alpha-IIb
P35908_K22E	Keratin, type II cytoskeletal 2 epidermal
Q7Z2Y8_GVIN1	Interferon-induced very large GTPase 1
A0A0G2JPR0	Complement C4-A
K7EQQ3	Keratin, type I cytoskeletal 9

Table 2: Protein expressed only in Platelets with additive solution.

Protein ID	Protein Description
A0A075B6S6 KVD30	Immunoglobulin kappa variable 2D-30
P00488 F13A	Coagulation factor XIII A chain
P00739 HPTR	Haptoglobin- related protein
P01780 HV307	Immunoglobulin heavy variable 3-7
P29622 KAIN	Kallistatin
P35858-2 ALS	Isoform 2 of Insulin –like growth factor- binding protein Complex acid labile subunit
A0A024R617	Alpha-1-antitrypsin
A0A2R8Y7R2	Hemoglobin subunit beta
F5H2D0	Complement Clr subcomponent
Q60FE5	Filamin –A
U3KPZ0	Triosephosphate isomerase (Fragement)
A0A0B4J2D9 VKD13	Immunoglobin kappa variable ID -13
A0A0C4DH33 HVI24	Immunoglobulin heavy variable 1-24
A0A0C4DH41 HV61	Immunoglobulin heavy variable 4-61
O14791-2 APOLI	Isoform 2 of Apolipoprotein L1
P05160 F13B	Coagulation factor XII B chain
P19438-5_TNRIA	Isoform 5 of tumor necrosis receptor
P19438-2_STOM	Isoform 2 of Erthycrocyte band 7 integral membrane protein
Q9H497-3_TOR3A	Isoform 3 of Torsin -3A
A0A1W2PQM2	Tubulin alpha-1C chain
A0A2R8Y5V9	Tropomyosin alpha-4 chain
B0AZ56	cDNA, FLJ79516, highly similar to 14-3-3 protein zeta/delta
D6RAR4	Hepatocyte growth factor activator
E7EUTS	Glyceraldehyde-3-phosphate dehydrogenase
G3V264	Plasma serine protease inhibitor (Fragment)
J3QSES	Phosphatidylcholine -sterol acytransferase (Fragment)
Q5T0R1	Adenlyl cyclase-associated protein 1 (Fragment)

Table 3: Showing newly identified platelet proteins along with their functions.

DISCUSSION

Proteomics of platelets can unveil (1) Quantitative changes of proteins. (2) Post translational protein modifications (3) protein- Protein interactions (4) Localization of protein with only few millilitres of blood [1].

Proteomics thus provides an excellent tool to decode complex processes by identifying novel platelet-expressed proteins-expressed proteins (refer Fig. 6) and analysing functional changes of the platelet proteome [5]. Proteomics thus provides an excellent tool to decode complex processes by identifying novel platelet-expressed proteins and analysing functional changes of the platelet proteome [6].

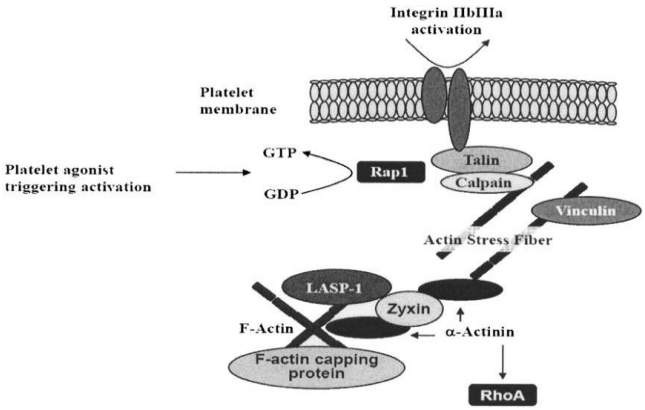

Figure 6: Integrin signaling pathway mediated by the GPIIb/IIIa. All proteins displayed are identified to be changing during platelet storage by proteomic approaches, leading to the potential involvement of this pathway to the storage lesion.

Proteomics is the wide ranging study of proteins demonstrated by a genome, cell, issue or organisms, which are in general termed the proteome (Figure 7). The protein profile can alter with time, or specific treatment as part of the normal response of living organism [8].

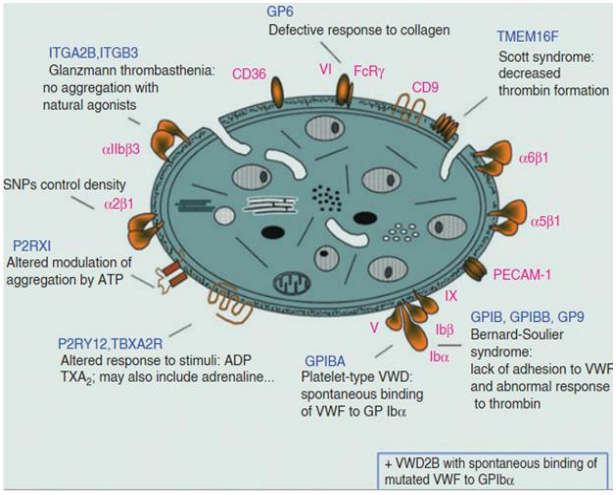

Figure 7: Identifying genes and proteins whose mutations gives rise to disorders of surface components of Platelets.

Proteome is a blend of protein and genomics which was used by Marc Wilkins in 1994 [9]. Proteomics was first introduced in 1997 in analogy to genomics [10].

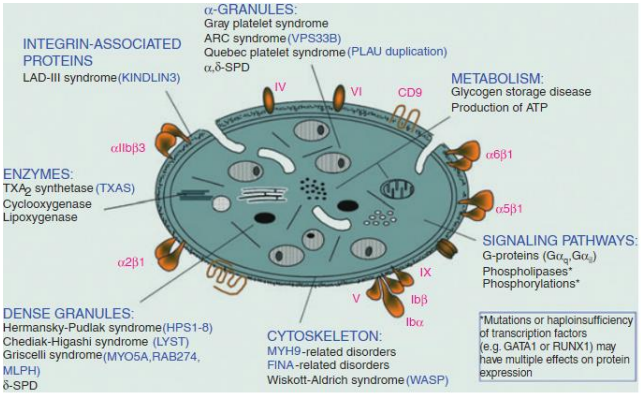

Figure 8: Identifying genes and proteins whose mutations gives rise to disorders of surface components of Platelets.

From a transfusion point of view, studying the process that lead to development of the storage lesion has been of longstanding interest (Figure 8). Storage-related changes in the pattern of cytosolic and membrane proteins were first noted in 1987 by Snyder and colleagues using 2-dimesnional gel electrophoresis (2D) [11]. Only two actin fragments were able to be identified as significantly accumulating in platelets during the first 7 days of storage in genome sequencing and bioinformatics at that time.

In order to decrease the disadvantages of the 2-DE technology as mentioned above, a complementary proteomic study was employed that addressed the relative differences using DIGE, ICAT, and iTRAQ in the analysis of the PSL (Figure 9). This strategy combines the potential of the power of the protein-centric approach specified by high resolution achieved through the protein pre-separation, hints for changes in PTM as well as the detection of potential degradation [12].

The study of protein alternations during storage period comprised the differences in the pattern between day 1 and day 7 and further studies are necessary to determine more detailed information using a day by day assessment because the actual levels of protein may be more dynamic and reflect the summation of de novo synthesis and degradation [13].

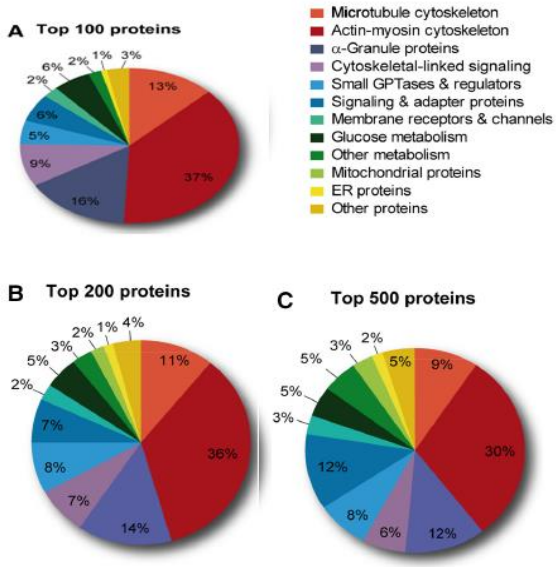

Figure 9: Distribution among functional categories of the 500 most abundantly expressed proteins.

Greenings et al. [14] have performed a comparison of human platelet membrane-cytoskeleton proteins with the plasma proteome. This correlation permits the

identification and classification of proteins that are selectively obtained from plasma by the PLTs such as L-lactate dehydrogenase, serum albumin, fibrinogen, carbonic anhydrase, endoplasmin, and multimerin 1 from those that are endogenous to platelets such as a actin, actinin, filamin, tropomyosin, thrombospondin-1, platelet basic protein, platelet factor 4, and stomatin which are ultimately released into the circulation or made available for concentrated (Figure 10) and focal release at vascular sites of injury [15].

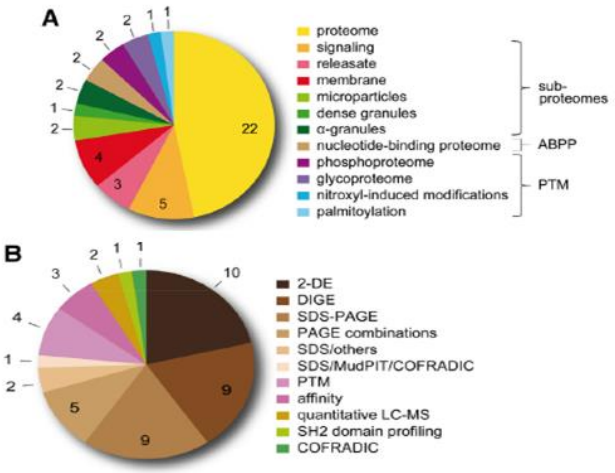

Figure 10: A) Diagram: Platelet proteomics use to characterize global proteome, sub proteome, activity based protein profiling. B) Diagram: Based on two dimensional electrophoresis.

Because of recent advances, proteomics now enables the estimation of properties of protein like abundance, modifications. Sub cellular localization and protein interaction etc. in thousands of proteins simultaneously (Figure 11). These studies have generated extensive protein database of >5000 proteins of platelets that are also useful to study diseases under normal physiological conditions [16].

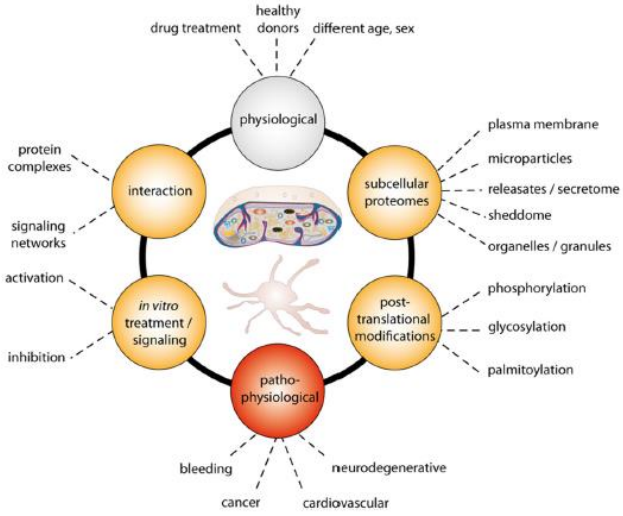

Figure 11: Diagrammatic representation of potential use of platelet proteomics.

According to the study done by Thone et al (Table 4) the following are the examples of results from Platelet Quality in Vitro Measures Correlating with Results from Proteomic Approaches [17].

Activation	Increased in CD62P Expression due to α-granule release.	Some proteins found to be changing during storage agree with changes observed.
Morphology	Shape change from discoid to spheroid monitored by ESC.	Appearances of actin isoforms as well as changed in actin binding proteins etc.
Metabolic activity	Increased in PO_2 and lactate; Decreased in pCO2 and glucose.	Changes in metabolic path way protein such as pyruvate, kinases and acyl-protein.
Signalling	Slight decrease in with respect to binding.	Amount of subunits of the GPIBIX/V with complexes remain.
Adhesion	Decreased in fibrinogen binding.	Reduced protein amount of different fibrinogen chains.
Coagulation	Increases in phosphatidyl- serine, phosphatidyl-serine by annexin V binding.	Change in coagulation factors V and XII

Table 4: Showing results from Platelet Quality in Vitro Measures Correlating with Results from Proteomic Approaches.

We have identified 34 proteins where proteins like tubulin Alpha -1C Chain, Alpha-1-B glycoprotein, Filamin A and Kallistatin A have been well studied in the past (Table 5). Although many proteins along with their physiological activities have been identified, further studies are required to acknowledge PTM and also identify their exact role towards the development of PSL. These newly recognized proteins can act as "target proteins" which can be further modified and manipulated to improve our understanding of PSL.

Some of the newly identified platelet proteins are mentioned below in the table:

No.	Name of the Protein	Protein description
1.	C1S complements [18]	This gene encodes a serine protease, which is a major constituent of the human complement subcomponent C1
2.	Alpha-1-B glycoprotein [19]	It is a 54.3 kDa protein in humans that is encoded by the A1BG gene.
3.	Ferritin family homolog [20]	It is also known as kindlin-3 (KIND3), MIG2-like protein (MIG2B), or unc-112-related protein 2 (URP2) is a protein that in humans is encoded by the *FERMT3*gene.
4.	S100-A9 [21]	It belongs to the S100 family of proteins containing 2 EF hand calcium-binding motifs.
5.	Integrin alpha-IIb [22]	It is a protein that in humans is encoded by the *ITGA2B*gene. ITGA2B, also known as CD41, encodes integrin alpha chain 22b.
6.	Keratin, type II cytoskeleton 2 epidermal [23]	It belongs to the keratin gene family. The type II cytokeratins consist of basic or neutral proteins which are arranged in pairs of heterotypic keratin chains co expressed during differentiation of simple and stratified epithelial tissues.
7.	Interferon induced very large GTPase [24]	The complex, partially overlapping, cellular responses to IFN type I (IFN-α and -β) and IFN type II (IFN-γ) involve several hundred genes that can be largely classified in terms of specific cellular programs functional in innate and adaptive immunity
8.	Interferon induced very large GTP ase [25]	The complex, partially overlapping, cellular responses to IFN type I (IFN-α and -β) and IFN type II (IFN-γ) involve several hundred genes that can be largely classified in terms of specific cellular programs functional in innate and adaptive immunity
9.	Complement C4- A [26]	It is a part of the classical activation pathway. The trimer provides a surface for interaction between the antigen-antibody complex and other complement components.
10.	Keratin 9 [27]	It is a human protein encoded by the KRT9 gene. It is a type I cytokeratin which is found only in the terminally differentiated epidermis of palms and soles.
11.	Immunoglobulin kappa Variable 2D[28]	It is 30 V region of the variable domain of immunoglobulin light chains that participates in the antigen recognition.
12.	Factor XIII [29]	It is activated by thrombin and calcium ion to a transglutaminase that catalyzes the formation of gamma-glutamyl-epsilon-lysine cross-links between fibrin chains.
13.	Haptoglobin – related protein [30]	It encodes a haptoglobin-related protein that binds haemoglobin as efficiently as haptoglobin.
14.	Immunoglobulin heavy chain [31]	It is a region of the variable domain of immunoglobulin heavy chains that participates in the antigen recognition.
15.	Kallistatin A [32]	It is Kallistatin is a serine proteinase inhibitor which binds to tissue kallikrein and inhibits its activity.
16.	Insulin like growth factor binding protein (IGFBP) [33]	It consists of six structurally similar proteins (IGFBP-1 to -6), which bind insulin-like growth factors (IGFs) with high affinity.
17.	Alpha-1 antitrypsin [34]	It is a protein belonging to the serpin super family. It is encoded in humans by the SERPINA1 gene
18.	Hemoglobulin subunit beta [35]	It is globin protein which along with alpha globin which makes HbA.
19.	Complement C1r subcomponent [36]	It is a protein involved in the complement system of the innate immune system. In humans,
20.	Filamin A′[37]	It provides instructions for producing the protein filamin A.
21.	Triosephosphate isomerase, TIM(1-28) [38]	It competes with VIP for binding to the VIP receptor on rat liver plasma.
22.	Immunoglobulin kappa variable [39]	It is also known as antibodies, are membrane-bound or secreted glycoproteins produced by B lymphocytes.
23.	ISOFORM ApolipoproteinL1 [40]	It is a minor apoprotein component of HDL (High-density lipoprotein) or 'good cholesterol'
24.	Coagulation factor XIIIB Chain [41]	This gene encodes coagulation factor XIII B subunit.
25.	Isoform 5 of tumor Necrosis factor receptor super family member 1A [42]	It encodes a member of the TNF receptor super family of proteins.
26.	Isoform 2 of erthrocyte band 7 integral membrane protein [43]	It encodes gene encodes a member of a highly conserved family of integral membrane proteins.
27.	Isoform 3 of Torsin -3A [44]	One of the two protein isoforms encoded by this gene is a type II integral membrane protein.
28	Tubulin Alpha -1C Chain [45]	It is a protein and plays a central role in association with the troponin complex.
29.	cDNA, FLJ79516[46]	It is small, highly conserved, acidic proteins with molecular found in all eukaryotic species.
30.	Hepatocyte growth factor activator[47]	It is protein encoded by this gene belongs to peptidase family.
31.	Glyceraldehyde-3phosphate dehydrogenase [48]	It is protein involved in glycolysis.
32.	Plasma seine protease inhibitor (Fragment) [49]	It is a serpin type protease inhibitor found in most tissues and fluids.
33.	Phosphatidylcloline sterol acyltransferase [50]	It is produces cholesterol esters and is responsible from removal of excess cholesterol from tissues.
34.	Adenylyl cyclase-associated protein [51]	It helps in actin cytoskeleton reorganization.

Table 5: Limited level of protein synthesis due to absence of nucleus.

CONCLUSIONS

Due to the absence of a nucleus (and consequently limited levels of protein synthesis) and the regulation of platelet activity at the level platelets in health and disease. Proteomics provides an excellent tool to decode complex by identifying novel platelet-expressed proteins, dissecting mechanisms of signaling or metabolic pathways, and analyzing functional changes of the platelet proteome [52].

Despite significant strides in proteomics, alteration in individual protein concentration (donor-donor variability) continues to represent an important limiting factor in the study of the PSL. This observation is complicated by clear differences in the types and number of proteins identified by the different proteomics tools that are currently available. As a result, careful observation must be done to determining which technology yields the most appropriate information [53].

Another big challenge in platelets proteomics is pre-analytical interference for instance because of impurity of pre-activation as the levels of known platelet biomarkers are known to be influenced by platelet isolation protocols. To prevent erroneous results, there is a need for robust and standardized protocols of platelet preparation and activation, along with protein isolation [54].

The experimental design of platelet proteomic studies should take into consideration the appropriate controls, technical /biological replicates, and sample preparation protocols. However, more proteomics studies are required to fully understand and appreciate the various proteins involved towards the pathogenesis of PSL.

As the known platelet biomarkers level are known to be determined by platelet isolation protocols. To avoid artificial results, there is serious requirement need for vigorous and standardized protocol of protein isolation, activation and preparation. The exploratory design of platelet proteomic studies should take into a consideration the appropriate controls.

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