# Comparison of Human Platelet Membrane-Cytoskeletal Proteins with the Plasma Proteome: Towards Understanding the Platelet-Plasma Nexus

David W. Greening[1,2], Kristen M. Glenister[3], Eugene A. Kapp[1], Robert L. Moritz[1], Rosemary L. Sparrow[3], Garry W. Lynch[4], Richard J. Simpson[1]

[1] Joint ProteomicS Laboratory, Ludwig Institute for Cancer Research & The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Parkville, VIC, Australia.
[2] Department of Biochemistry and Molecular Biology, University of Melbourne, VIC, Australia.
[3] Research Unit, Australian Red Cross Blood Service (ARCBS), Melbourne, VIC, and
[4] ARCBS, NSW, Australia.

**To whom correspondence should be addressed:**
Prof. Richard J. Simpson
Ludwig Institute for Cancer Research
PO Box 2008, Royal Melbourne Hospital
Royal Parade, Parkville, Victoria 3050, Australia

Tel: +61 03 9341 3155
Fax: +61 03 9341 3104
Email: Richard.Simpson@ludwig.edu.au

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**ABBREVIATIONS**

| | |
|---|---|
| 1-DE | one-dimensional electrophoresis |
| Ac | protein accession number |
| BC | buffy-coat |
| emPAI | exponentially modified protein abundance index |
| FDR | false-discovery rate |
| HUPO | Human Proteome Organisation |
| PE | phycoerythrin |
| PPP | Plasma Proteome Project |
| PRP | platelet rich plasma |
| PS | Protein Score |
| RBC | red blood cell |
| WB | whole blood |
| WBC | white blood cell |

**SUMMARY**

Platelets are essential for maintaining vascular integrity. Given the anucleate nature of platelets, definition of their proteome is essential for understanding platelet pathophysiology. We describe here a detailed MS-based proteomic analysis of the platelet membrane/cytoskeletal sub-proteome from purified, normal, non-activated human platelets. In contrast to previous platelet proteomic purification strategies, the buffy-coat method was utilized in this study to isolate and purify non-activated platelets, yielding significantly-reduced contaminants for leukocytes ($0.02 \pm 0.007 \times 10^6$/L) and erythrocytes ($0.21 \pm 0.02\%$). Using a false discovery rate of 1%, 203 proteins were identified and characterized with respect to their sub-cellular localization, biological function, and cellular processes. Of these, 16 have not been identified in previous human platelet proteome studies. As a first approach towards understanding the dynamic platelet-plasma protein composition nexus, we re-analysed the entire HUPO plasma proteome project dataset (647 plasma proteins identified) and compared these data with our platelet proteome dataset. Co-identified proteins (41) were further analysed with respect to their relative abundances (emPAI) and functional enrichment in these two proteomes, as well as their correlation with the platelet transcriptome. Both platelet membrane/cytoskeletal and plasma proteome reference datasets, comprising both processed and unprocessed MS/MS spectra, are publicly accessible (http://www.ludwig.edu.au/archive/).

## INTRODUCTION

Platelets play a pivotal role in maintaining vascular integrity by detecting, and responding to, endothelial damage [1, 2]. Central to platelet function is the diverse interplay of adhesive, signalling and secretory responses, amplifying the activation process and initiating thrombus production. Such responses are mediated via a complex membrane system that releases proteins, cytokines, exosomes, and microparticles into the circulation [3, 4]. The interaction of platelets with blood vessel walls and the subsequent contribution to atheroma formation and thrombosis is important in the aetiology and pathogenesis of peripheral, coronary, cerebrovascular and other vascular diseases [5].

Platelets are membrane bound entities that bud from megakaryocytes in the bone marrow [1]. While platelets are anucleate and void of genomic DNA [6], they do contain proteins synthesized by megakaryocytes, in addition to proteins synthesised from platelet mRNA during platelet activation [7]. For these reasons, only proteomic technologies can provide the necessary tools for characterizing, at the protein level, the vicissitudes of platelet function. Several reports on the mapping of select platelet proteome compartments have been published, ranging from global profiling of proteins present in non-stimulated platelets, analyses of signalling complexes [8], to characterization of "sub-proteomes" of membrane extracts [9] or microparticle extracts from activated platelets *in vitro* [10] (reviewed in [11]).

It has recently been shown that platelet-derived proteins and peptides released upon platelet activation directly influence the composition of the plasma proteome [12-14]. This issue is gaining greater attention, as one of several important pre-analytical variables that must be controlled in order to realise effective blood-based biomarker discovery programs. Circulating platelets are of particular importance because of the dynamic, two-way relationship they have with plasma, with which they play alternate roles to incorporate and store plasma proteins under some conditions, or to release soluble or granule proteins under others [15, 16]. Despite the fundamental importance for haemostasis and

thrombogenesis, the nature of this inter-relationship between blood plasma and platelets is not clearly understood.

In this study, we examined a sub-proteome of resting human platelets from platelet concentrates produced for transfusion purposes using the buffy-coat method. In an attempt to enhance low-abundance platelet proteins, we enriched for the membrane/cytoskeletal sub-proteome. Two independent platelet preparations from pooled blood-type matched donors were analysed separately using mass spectrometry-based proteomics, in order to assess experimental reproducibility. The combined data were analysed using independent forward- and reversed-sequence database searching [17-19]. Stringent manual validation was defined at the peptide level, in addition to characterization and analysis of the ensuing protein dataset. We have compared our data with several previously published platelet proteomic studies, including whole platelet lysate [20, 21], purified platelet membrane [9, 22] and platelet microparticle [10] proteomes. Several novel proteins not previously observed in these studies have been noted and discussed. In order to examine the contribution of platelet proteins in normal human plasma, we independently re-analysed the Human Proteome Organization (HUPO) Plasma Proteome Project (PPP) dataset [23]. Proteins co-identified in the two proteomes are described in terms of their relative abundance, using the exponentially modified protein abundance index (emPAI) [24], as well as their molecular functionality. This correlation provides for the first time a baseline of platelet and plasma proteomes of normal individuals upon which future protein profiling of thromboactive and diseased states can be compared.

## EXPERIMENTAL PROCEDURES

**Platelet Preparation.** Human platelets were purified under non-activating conditions from whole blood (WB) using two different platelet preparation techniques, one employed for clinical transfusion purposes (buffy-coat, BC), the other for standard laboratory practice (i.e., platelet-rich plasma, PRP) [25].

***Buffy-Coat Platelet Preparation.*** Whole blood was collected with informed consent from healthy volunteer donors at the Australian Red Cross Blood Service, Melbourne, according to routine- and strictly-regulated procedures. Briefly, approximately 450 mL of WB was collected into a standard PL146 Optipac blood pack containing CPD anticoagulant (63 mL anticoagulant containing 1.66 g sodium citrate, 1.61 g dextrose, 206 mg citric acid, 140 mg sodium phosphate; Baxter, La Châtre, France) and then centrifuged at 4200*g* for 10 min at 22$^{o}$C. Blood components were then separated automatically using an automated blood component separator (Optipress II, Baxter, Maurepas, France) to obtain the platelet rich BC fraction. BCs from five ABO matched donors were pooled using platelet additive solution (T-sol™; 6.75 g/L sodium chloride, 4.08 g/L sodium acetate trihydrate, 2.94 g/L sodium citrate dihydrate, Baxter, Maurepas, France). Pooled BCs were centrifuged at 445*g* for 7.5 min at 22$^{o}$C and the platelet-rich supernatant was leukofiltered (OptiPure PC PLX-5, Baxter) to deplete intact leukocytes and leukocyte-derived proteins that might be secreted upon storage [26]. Platelet concentrates were stored in standard platelet concentrate storage bags (PL2410) on a platform agitator at 22$^{o}$C under constant agitation (50-70 oscillations per min, Linear Platelet Reciprocator, Melco, Glendale, CA) until processing.

***Platelet Rich Plasma Preparation.*** Whole blood (8.5 mL from 8 normal donors) was collected using 21 gauge needles into standard vacutainer tubes containing acid citrate dextrose anticoagulant (1.5 mL anticoagulant containing 33 mg sodium citrate, 36.75 mg dextrose, 10.95 mg citric acid; BD

Biosciences, San Jose, CA). The PRP was obtained by centrifugation (110*g*, 15 min, 22ºC). The upper 25% was carefully removed for analysis to limit contamination by leukocytes and erythrocytes.

***Estimation of Platelet Numbers.*** Platelet counts were determined using a haematology analyser (CellDyn 3200, Abbott, Santa Clara, CA).

***Assessment of Platelet Purity.*** Levels of contaminating erythrocytes in BC and PRP platelet preparations were estimated by flow cytometry (FACScan, with Cellquest software, Becton Dickinson, Palo Alto, CA). Briefly, the platelet and erythrocyte populations were identified by their forward and side light scatter characteristics and a gate placed around the erythrocyte population. Correct positioning of the gate was verified by staining with fluorescein isothiocyanate conjugated anti-CD41 and phycoerythrin (PE) conjugated anti-glycophorin A to label platelets and erythrocytes, respectively. Residual leukocytes were determined using an absolute counting assay using TRUCount tubes (BD Biosciences). The level of platelet activation was monitored by the surface expression of P-selectin (CD62) on released secretory storage granules [4], using flow cytometry and PE-conjugated anti-CD62P. All antibodies were from BD Biosciences, San Diego, CA.

**Enrichment of the Platelet Membrane / Cytoskeleton.** The platelet membrane/cytoskeleton sub-proteome was prepared as previously described for the extraction of G-proteins from the cortex brain [27]. Briefly, pooled BC-derived platelet concentrates (n=5) were processed on the day following blood collection. Platelet concentrates (10 mL; mean 9.95 ± 0.74x$10^9$ platelets for each preparation) were centrifuged at 500*g* for 1 min at 22ºC to sediment any agglutinates. The suspensions were transferred to fresh tubes and platelets pelleted at 1100*g* for 15 min at 22ºC. Sedimented platelets were washed five times with platelet wash buffer (2.49 g/L $Na_2HPO_4$, 3.35 g/L $Na_2EDTA$, 8.2 g/L NaCl, pH 7). Washed platelet pellets were re-suspended in lysis buffer (1 mL; 10 mM Tris pH 8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) and incubated on ice for 10 min. Lysates were centrifuged at 3000*g* for 10 min at 22ºC to separate soluble and insoluble fractions. The insoluble fraction

(representing a crude membrane/cytoskeletal preparation) was re-suspended in SDS reducing sample buffer (200 μL) and samples stored at -20ºC. The total protein content of the unsuspended lysate was estimated using the 2-D Quant kit (GE Healthcare), using bovine serum albumin of known concentration to obtain a standard curve. Equivalent amounts of total protein (50 μg) were separated using a SDS polyacrylamide gel (4-20% Tris-Glycine precast gel, Gradipore) in Tris-Glycine-SDS electrophoresis buffer at 150 V (constant voltage) until tracking dye had reached the bottom of the separation gel. Proteins were visualised with colloidal Coomassie [28]. Mass spectrometric (MS) analysis was performed on two independently prepared BC platelet concentrates.

**Nano-Liquid Chromatography-Tandem Mass Spectrometric Analysis.** For each BC platelet preparation (track length approximately 8 cm), 34 gel sections were excised (1.0-1.5 mm sections) and extensively washed with deionized water. Excised gel sections were individually digested with trypsin (0.05 μg) and generated peptides concentrated to ~10 μL by centrifugal lyophilization (Savant, U.S.A.) for electrospray-Ion Trap (ESI-IT) tandem mass spectrometry (MS/MS) (LCQ-Deca, Finnigan, San Jose U.S.A.). Protein digests (~10 uL in 1% (v/v) formic acid) were transferred into 100 μL glass autosampler vials for injection and fractionation by capillary reversed-phase-HPLC (Model 1100, Agilent, Germany) using a butyl-silica (C4) 150 x 0.15 mm I.D. RP-capillary column (Vydac™-C4MS, 5 μm, 300 Å Vydac, U.S.A.) developed with a linear 60-min gradient from 0-100% B with a flow rate of 0.8 μL/min at 45°C. Solvent A was 0.1% (v/v) aqueous formic acid and Solvent B was 0.1% aqueous formic acid/60% (v/v) acetonitrile. The capillary HPLC was coupled on-line to the ESI-IT mass spectrometer for automated MS/MS analysis of individually isolated peptide ions [29, 30].

Peak lists were extracted using *extract-msn* version 3 as part of Bioworks 3.2 (Finnigan, San Jose, U.S.A.). The parameters used to create the peak lists were as follows: minimum mass 700; maximum mass 5000; grouping tolerance 1.4; intermediate scans 1; minimum group count 1; automated calculation of charge state - peptide charge states of either 1+, or 2+ or higher; 30 peaks minimum per

spectrum; ±2 Da peptide mass tolerance; ±0.5 Da MS/MS fragment mass tolerance. The mass spectrometer was operated in data-dependent mode (triple-play) to automatically switch between MS, Zoom MS (automated charge state recognition), and MS/MS acquisition, selecting the most intense precursor ion for fragmentation using CID. Where four consecutive precursor ions of the same mass were observed, dynamic exclusion was invoked for a period of 240 s.

**Protein Sequence Database.** The protein subset database (LudwigNR_subset), comprising 205,780 entries (derived from human, mouse, rat, bovine, and mycoplasma, October 2005 (110 Mb)) was generated from the non-redundant LudwigNR protein database (comprising approximately 2,000,000 entries (1.20 Gb)) sourced from UniProt and EnsEMBL [18, 31, 32] Re-arrangement of the order of protein entries in the LudwigNR_subset was performed to ensure that all human proteins were at the beginning of the database file.

**Database Searching.** Acquired MS/MS spectra were searched against the LudwigNR_subset database using the MASCOT search algorithm (v2.1.04, Matrix Science, U.K.) [33]. Searches were conducted with carbamidomethylation of cysteine as a fixed modification (+57 Da) as well as variable modifications consisting of $NH_2$-terminal acetylation (+42 Da) and oxidation of methionine (+16 Da), and the allowance for up to two missed tryptic cleavages. In total, 67,714 MS/MS spectra, including duplicate 2+ / 3+ spectra, were submitted to MASCOT, where 33,956 spectra were assigned peptides with Ions score (IS) ≥15. Peptides with IS ≥15 were retained and classified as either discrete (i.e., matching a unique protein record) or degenerate (i.e., matching multiple protein records) using an in-house developed program (*MSPro*). Of these assigned peptides, 4,165 were classified as significant matches where the IS ≥ the reported Homology score (HS) (if available), otherwise Identity score was used. Overall, ~10% of the acquired MS/MS spectra yielded significant peptide identification and inferred proteins.

To estimate levels of false positive protein identifications, MS/MS spectra were also searched against the corresponding reversed-sequence (decoy) database. The utility of reversed-sequence searches to assess the false protein assignment distribution has been explored by various groups [18, 34, 35]. Guidelines for publishing peptide and protein identification data have recently been proposed for proteomics, in order to ensure a strict requirement for data validation [36]. Specifically, the issue in determining protein false-positive rates was raised (e.g., the results of randomized database searches or other computational approaches). In our studies data from independent[1] forward and reverse database searches was clustered and analysed by *MSPro* (Kapp *et al*, manuscript in preparation).

**Protein Score and False-Discovery Rate Calculations.** For each MS/MS spectrum, MASCOT reports a peptide probability-based IS and HS. Peptides that have IS ≥ HS are deemed significant and contribute to the Protein Score (PS). MASCOT reports a "standard" or "MudPIT" protein score as a non-probabilistic way of ranking hits. The "MudPIT" protein score ($PS_M$) is calculated by taking into account all matched significant peptides (i.e., including duplicates) (*Equation 1*). In this study, the Protein Score was modified to exclude duplicate peptide matches and to place more emphasis on the highest scoring peptide match (*Equation 2*). Importantly, this modification does not affect the Protein Score if there is only one significant peptide match.

[1] The independent database search strategy is preferred for a probabilistic-based scoring algorithm such as MASCOT, since a) it attempts to assign low scores to incorrect (random) matches, regardless of spectrum quality, and b) score thresholds (e.g., identity threshold) are calculated based on the number of precursor ion matches (http://www.matrixscience.com/help/decoy_help.html).

$$PS_M = \sum (IS - HS) + \left( \frac{\sum HS}{n} \right) \qquad \text{(Equation 1)}$$

$$PS = {}^{h}IS + \sum_{i=2}^{n} (IS - HS) \qquad \text{(Equation 2)}$$

Where *n* is the number of significant peptides, and ${}^{h}IS$ refers to the highest Ions Score (also $i = 1$).

Inferred proteins are sorted on Protein Score (*Equation 2*) and grouped based on parsimonious analysis (similar to DBParser [37]) as distinct, differentiable, superset and subset. Proteins grouped as distinct, differentiable and superset are classed as primary proteins.

For a defined protein score, the false discovery rate (FDR) is calculated by dividing the number of primary grouped protein hits for the decoy search by the number of primary grouped protein hits for the normal search (Figure 1). A 1% FDR threshold at the protein level (corresponding to a protein score of 50 in this analysis) was utilised in the platelet proteome analyses.

**Validation of MS Identified Peptides and Proteins.** To ascertain the validity of peptide identifications and hence inferred proteins, a combination of automated (using *MSPro*) as well as manual validation of spectra (Java™ spectrum applet) was performed in accordance with previously established guidelines [38]. For inferred proteins scoring >50 only the top two scoring peptides were manually verified. Proteins were then submitted to *BLAST* (http://www.ncbi.nlm.nih.gov/BLAST/) with a cut-off of 95% to remove redundancy. The platelet reference dataset comprising of both processed and unprocessed MS/MS spectra is publicly available (http://www.ludwig.edu.au/archive/).

**Protein Annotation.** Swiss-Prot/TrEMBL (http://www.expasy.org), Ensembl (http://www.ensembl.org) and the Gene Ontology databases (http://geneontology.org) were used for

annotation. *MSPro* was used to extract the GOSlim ontology [39] for molecular function(s), sub-cellular localization, and cellular process(es) for each individual protein. Duplicate GO terms for different categories were automatically removed. Data obtained from annotation databases requires critical evaluation to ascribe the most likely function(s) when several are available. Annotated data, presented in *Supplementary Material Tables 1* and *2*, should be viewed as an effort to assemble all available information via Uniprot (comprising Swiss-Prot and Trembl) and Ensembl databases using primary protein accession numbers.

Known or predicted transmembrane-spanning helices were determined through the literature, or by using the web-based prediction program TMHMM version 2.0, based on a hidden Markov model (provided by the Center for Biological Sequence Analysis of the Technical University of Denmark, http://www.cbs.dtu.dk/services/TMHMM-2.0) [40].

**Statistical Analysis of Proteins Co-identified in Platelets and Plasma.** Statistical analyses were performed in order to monitor the enrichment of functional categories of platelet proteins co-identified in human plasma. This comparative study is based on the validated dataset of platelet proteins, and an independent re-analysis of the human plasma proteome dataset (HUPO Plasma Initiative [23]). The pilot phase of the HUPO PPP was a combined effort from 47 different laboratories for the comprehensive proteomic analysis of human plasma and serum, of which 18 laboratories submitted peptide and protein identification datasets [18, 23]. The entire submitted MS/MS spectra were searched independently against the non-redundant International Protein Index (IPI) human sequence database (IPI, v2.21, July 2003, 56,530 entries, European Bioinformatics Institute, www.ebi.ac.uk/Databases/) [41] using MASCOT software [33]. Data was subsequently integrated and validated similarly to the platelet sub-proteome dataset, as described. To determine levels of false positive protein identifications, spectra were also searched against the corresponding reversed-sequence database. The estimated protein false positive rate was <1%. The final dataset consisted of 647 proteins (*HUPO 647*). For comparison against

our platelet proteome dataset, all PPP entries were transcribed to corresponding UniProt protein accession number entries (Swiss-Prot, TrEMBL and PIR protein database) [42].

The functional classes (derived from GOSlim) of proteins co-identified from *HUPO 647* and our platelet membrane/cytoskeletal sub-proteome datasets were compared statistically, based on hypergeometric distribution [43]. This enabled the degree of functional enrichment for a given protein functional class or cluster to be quantitatively assessed. The hypergeometric distribution assumes independent tests (i.e., each protein has a single classification), enabling the summation of data resulting in clusters of proteins having differing functions, or properties. The probability (*p*-value) can therefore be calculated, representative of whether a given protein category is enriched in comparison to a randomized sample (in this case IPI human protein entries). Platelet proteins co-identified in *HUPO 647* were assessed on the basis of enrichment or depletion with respect to each functional GOSlim class.

**Relative Abundance of Proteins Co-identified in Platelets and Plasma.** Relative protein abundances of our platelet and plasma proteomes were determined using the exponentially modified protein abundance index (emPAI) procedure [24, 44]. Experimentally observed peptides from each protein (*observed*) can be compared with the theoretical number of peptides generated from an *in silico* digest (*observable*) in order to calculate relative protein abundance [24]. The numbers of *observed* peptides per protein from both datasets were determined counting unique (non-duplicate) peptide sequences from co-identified proteins. *Observable* peptides were generated on the basis of tryptic digestion of co-identified proteins, with two missed cleavage sites and a mass range from 700-5000 Da. The emPAI data were ranked independently for *HUPO 647* dataset and platelet sub-proteome dataset according to derived abundances.

## RESULTS & DISCUSSION

**Platelet Preparation.** Any detailed platelet protein profiling effort requires strict attention to blood collection and handling procedures that may influence platelet activation, as well as strategies aimed at minimizing erythrocyte and leukocyte contamination [15]. In preliminary studies, we prepared and evaluated platelets from normal donor whole blood using the BC and PRP methods (see *Experimental Procedures*). The extent of platelet activation in these preparations was monitored using P-selectin (CD62), a membrane protein localised in secretory granules of platelets, and released upon activation [4]. For platelets prepared using the BC method, the extent of platelet activation was found to be significantly reduced (9%) when compared with platelets prepared by the PRP method (47%) (data not shown).

The levels of erythrocyte and leukocyte contamination were assessed using flow cytometry. In Figure 2, pooled BC platelet concentrates (n=8) containing 295 ± $5x10^9$ platelets per litre showed minimal leukocyte (0.02 ± $0.007x10^6$/L) and erythrocyte contamination (0.21 ± 0.02%). For platelets prepared using the PRP method, the yield of platelets varies with the percentage of the PRP fraction analysed. In Figure 2, PRP-derived platelets (n=6) containing 140 ± 22 $x10^6$ platelets were obtained from the upper 25% of the PRP fraction. Importantly, this PRP preparation generated significant numbers of leukocytes (15.71 ± $8.31x10^6$/L) and erythrocytes (0.72 ± 0.22%). This represents an approximate 800- and 4-fold greater leukocyte and erythrocyte contamination, respectively. Efforts to increase platelet yield by analysing a larger percentage of the PRP (i.e., >25%), while increasing the number of platelets, resulted in a concomitant increase in the levels of erythrocyte and leukocyte contaminants. For these reasons, we decided to utilise the BC method for preparing platelets for our detailed proteomic analysis. In order to address the potential issues of biological variation and variation associated with MS experimentation, two independent platelet preparations, each derived from 5 pooled blood-type matched donors, were studied.

**Mass Spectrometric Analysis and Annotation of the Platelet Membrane/Cytoskeletal Sub-proteome.** Both BC platelet preparations were solubilised in SDS containing DTT and PMSF and individually separated electrophoretically (SDS-PAGE, 4-20% gradient gel). Each 1D-gel lane was excised into 34 portions and individual gel slices were trypsinised. Peptides were extracted from each gel slice and individually analysed by capillary-LC-ESI LCQ MS/MS. Acquired MS/MS spectra were searched against the protein subset database (LudwigNR_subset) using MASCOT software, and proteins identified from matched peptide sequences. For these studies we utilised a forward- and reversed-protein database to estimate the rate of false identifications (false-discovery rate; FDR) (*Experimental Procedures*). For our data, inferred proteins were grouped and tabulated using *MSPro* and summarised based on a FDR threshold of 1%.

Stringent manual validation of the top two scoring peptides for each inferred protein above the protein FDR of 1% was also performed. By these means, all automated protein identifications were manually verified. Additionally, extending the manual validation approach below the FDR threshold of 1%, an additional 36 proteins (43 peptides) were identified. This highlights that when using an automated approach based on the FDR, there is the potential for overlooking several *bone-fide* proteins/peptides of low-abundance identified based on a single peptide (*Supplementary Material Table 1*). This combined automated and manual approach resulted in the identification of 203 proteins from 921 unique peptides; 73 proteins correlated with a single peptide (35.9%, *Supplementary Material*), 34 from two peptides (16.7%), 33 from three peptides (16.3 %), 17 from four peptides (8.3%), and 46 from greater than four peptides (22.6%). These datasets are listed in *Supplementary Material Tables 1 and 2*. Interestingly, an overlap of ~65% was observed in the proteins identified in our duplicate platelet preparations; this correlation in sample replicates is in agreement with the study by Durr and colleagues [45].

We subsequently investigated the 203 protein identifications with respect to their cellular localization and functionality using GOSlim ontology (Figure 3). In cases where there was no localization data or where localization data was incomplete, proteins were assigned to the group “no information”. Using this approach 38 plasma integral membrane proteins (18%) were identified (Figure 3A). Additionally, we found 36 membrane and membrane-associated proteins that localize to other cellular compartments, such as mitochondria, endoplasmic reticulum, and intracellular vesicles. We also analyzed potential transmembrane proteins using TMHMM™ (prediction algorithm based on a hidden Markov model) for the calculation of putative transmembrane domains, identifying an additional 15 proteins (8%). The cytoskeletal group (29%) comprises proteins responsible for cellular morphology and includes many known structural proteins, such as actin, myosin, and filamin, which are present at high abundance and in multiple isoforms. The presence of a significant number of transcription factors and elongation factors may indicate that such proteins are incorporated into platelets during the budding process from the megakaryocyte, or obtained from other blood cells, tissues, or plasma via endocytosis [46, 47].

Platelet proteins were also categorized according to their molecular function and biological process(es) (Figure 3B,C). The majority of proteins were assigned into the physiological processes category (115), with lower numbers falling into the cellular (37) and regulatory (14) process categories. Regulatory mechanisms in platelets can be subdivided into regulation of cellular processes, physiological processes or enzyme activities. The most common cellular regulatory activities identified were implicated with regulating transport, signal transduction and programmed cell death. Of the signalling proteins, nine are reported to be involved in general signalling events, ten linked to surface receptor signal transducers, and two have specific roles in intracellular regulatory cascades

(*Supplementary Table 2*). Similarly, numerous components of protein processing pathways (proteasome and ubiquitins) were also identified.

**Correlation with Previous Platelet Proteomic Studies.** We performed a detailed comparison of our enriched membrane/cytoskeletal platelet proteome dataset (203 proteins) with previously published data from whole platelets using 2D-PAGE [20, 21], platelet membranes [9, 22], and platelet microparticles [10]. The overlap of protein identifications in these studies is shown (Figure 4).

Using whole platelets prepared from PRP, O'Neill [21] and Garcia [20] reported a total of 416 protein identifications. A significant number of these proteins are involved in intracellular signalling and cytoskeleton regulation. Membrane proteins were significantly under-represented, with only abundant membrane proteins such as platelet glycoproteins alpha-IIB (Ac:P08514), IB (Ac:P13224), and IX (Ac:P14770) identified. When compared with our platelet membrane/cytoskeleton preparation, we identified an additional 78 proteins, most of which are associated with platelet membrane compartments.

More recently, Moebius and colleagues presented a detailed proteome study on human platelet membranes [9]. Proteins from membrane fractions were separated by 1D-SDS-PAGE and benzyldimethyl-n-hexadecylammonium chloride (16-BAC)/SDS prior to MS analysis by nano-LC-ESI-MS/MS. When compared with the 298 platelet membrane proteins reported by Moebius *et al*., we co-observe 99 proteins, including numerous platelet membrane receptor glycoproteins. In contrast, we observe an additional 105 proteins, including 24 integral membrane proteins and 8 cytoskeletal proteins, including Myosin and Actin-related proteins.

A recent proteomic study provided the first overview of platelet-derived microparticles (~0.1µm membrane vesicles), identifying 578 proteins that comprise this sub-cellular proteome [10]. In comparison, we observe 163 proteins in common and an additional 41 proteins, including 11 plasma

membrane proteins, such as Actin-related protein 2/3 complex subunit 3, Lipopolysaccharide-responsive and beige-like anchor protein, and Ubiquitin-protein l.

A recent proteomic and genomic study, focusing on the purification and enrichment of transmembrane proteins in human platelets and mouse megakaryocytes, was conducted by Senis and colleagues [22]. This study used three separate methods (lectin affinity chromatography; biotin/NeutrAvidin affinity chromatography; and free flow electrophoresis) to enrich platelet surface proteins prior to identification by LC-MS/MS. In comparison with our proteomic study, we co-observe 17 out of 164 proteins, including numerous type 1 and 2 membrane proteins, in addition to several integral membrane proteins.

Compared to previous platelet proteomic analyses, the buffy-coat method utilized in our present study to isolate and purify non-activated platelets, minimized the presence of contaminating proteins from erythrocytes and leukocytes.

Overall, we have identified 16 platelet-associated proteins not seen in previous platelet proteome studies (Table 1). These include cytoskeletal related proteins (e.g., Alpha-cardiac actin (Ac:P68032), Macrophage migration inhibitory factor (Ac:P14174), Gamma-2 tubulin (Ac:Q9NRH3), and transport proteins (e.g., ATP synthase f chain (Ac:P56134), Phosphate carrier protein (Ac:Q00325), in addition to proteins associated with the plasma or integral membranes. A salient finding was the identification of trafficking protein particle complex subunit 6B (isoform 1 or 2) (Ac:Q86SZ2), which in yeast, forms part of the multi-subunit TRAPP (transport protein particle) complex [48, 49]. This identification of the TRAPP subunit was based upon a single peptide, the N-terminal tryptic peptide (Ac-ADEALFLLLHNEmVSGVYK, residues 2-20, Figure 5). Given that the N-terminal amino acid of TRAPP precursor protein is methionine, presumably the acetylation of Ala-2 arose after the co-translational processing of TRAPP involving N-terminal methionine excision and subsequent acetylation

via an N-terminal acetyltransferase [50]. The tissue distribution of this protein in humans is yet to be determined ([51], September 2007, http://www.proteinatlas.org).

**Annotating the Human Plasma Proteome.** It is well recognized that platelet lysis frequently occurs upon blood collection and storage [12, 14], and that the variability of this phenomenon is a major obstacle to any blood-based biomarker initiative. For these reasons we decided to examine whether there was any correlation between platelet proteins that we identified with the human plasma proteome. This information will further provide a basis for future holistic studies aimed at determining the contribution of various organs and tissues to the overall protein composition of blood.

In 2005, 18 individual laboratories analyzed human plasma as part of the HUPO PPP initiative. The collective findings were compiled to form the core PPP dataset [23, 52]. As a first step, it was essential to subject the existing and most comprehensive plasma proteome dataset to the same rigorous validation metrics that we employed for our platelet study. Using the MASCOT search algorithm and a 1% FDR threshold resulted in the identification of *HUPO 647* (http://www.ludwig.edu.au/archive/). We assigned a controlled vocabulary for describing proteins according to the Gene Ontology convention for their molecular functions, biological processes, and sub-cellular localization (Figure 6). In general proteins were found to be distributed evenly across the extracellular compartment, plasma membrane, cytoplasm and nucleus. Extensive interactions were observed with extracellular proteins, suggesting that many extracellular proteins may exhibit unrecognized intracellular localization or that such interactions may occur when interacting with plasma membrane-bound proteins.

**Integration of Platelet and Plasma Proteomes.**

*Functional Enrichment.*

We evaluated platelet proteins co-identified with the human plasma proteome with respect to the enrichment of specific functional categories (e.g., protein binding, signal transduction, etc). A

comparison of the platelet and plasma proteins revealed the co-identification of 41 proteins (Table 2). In Table 3, each *p*-value reflects, for each Gene Ontology term, the relative expression of each protein at significantly higher or lower levels in platelets. Intensities for each category were subsequently ranked. These data show that proteins associated with *protein binding* and *adhesion* ($p=6.38 \times 10^{-25}$), *enzymatic* and *catalytic activity* ($p=5.98 \times 10^{-9}$), *structural activity* ($p=4.31 \times 10^{-6}$), and *transporter activity* ($p=1.02 \times 10^{-5}$) were significantly enriched in platelet compared with the plasma pool. These findings are in accordance with the functional annotation data (Figure 3). Given the diverse functional activities of platelets in normal physiology it is not surprising that co-identified proteins in blood plasma have extracellular functions predominantly associated with *enzymatic function*, as well as *transport* and *adhesion*. Functional categories such as *electron transport* ($p=4.42 \times 10^{-1}$), *translation regulation* ($p=5.27 \times 10^{-1}$), and *nucleic acid binding* ($p=9.99 \times 10^{-1}$) functions were expressed at reduced levels in platelets, which is consistent with their anuclear state. Together, these ontologic comparisons reveal a prominence of membrane receptor, cytoskeletal, and signal transduction (catalytic) proteins in our platelet membrane/cytoskeletal study, and a lack of biosynthetic and metabolic proteins.

*Relative Protein Abundance.*

As a first step towards understanding the contribution of platelet proteins to the overall blood composition, i.e., determining whether platelet proteins originate solely from platelets or from other tissues as well, thereby contributing to the plasma pool, we correlated the relative abundances of co-identified proteins in both proteomes. Using the relative protein abundance index (emPAI), reported by Mann and colleagues [24], the relative abundances of co-identified proteins were calculated and listed in Table 2. A high proportion of cytoskeletal proteins, such as actin, actinin, filamin, and tropomyosin were found to be more abundant in platelets. Likewise, cell surface proteins (such as the integral membrane and membrane associated proteins, such as platelet glycoproteins, integrins, thrombospondin-1

(Ac:P07996), platelet basic protein (Ac:P02775, PBP), platelet factor 4 (Ac:P02776, PF4), and stomatin (Ac:P27105)) are also more relatively abundant in platelets. In contrast, soluble chaperone and extracellular proteins, such as L-lactate dehydrogenase (Ac:P00338), serum albumin (Ac:P02768), fibrinogen (Ac:P02671, P02675, P02679), carbonic anhydrase (Ac:P00918), endoplasmin (Ac:P14625), and multimerin 1 (Ac:Q13201) found in our platelet preparation are examples of proteins which are endogenous to the plasma pool, derived from other tissue sources, and selectively acquired and stored by platelets. This study identifies proteins derived from human platelets contributing directly to the human plasma pool. Such a correlation sets the basis for proteins selectively acquired from plasma by platelets, with those that are endogenous to platelets and potentially released into the circulation or available for concentrated and focal release at vascular sites of injury.

*Comparison with Platelet Transcriptome.*

We further compared our findings for the 41 proteins co-identified in platelets and plasma with a recent transcriptional profile for platelets [53]. The 2,928 distinct messages reported in the platelet transcript analysis were compared with co-identified platelet/plasma proteins (Table 2). Of the 41 proteins co-identified in platelets and plasma, only 25 correlated with the platelet transcriptome. Interestingly, RNA messages for 16 co-identified proteins were not detected in the platelet transcript study. Of these, 8 proteins were shown to possess functions ranging from endocytotic/granule storage to vesicle release activities, including endoplasmin (Ac:P14625), multimerin 1 (Ac:Q13201), glucose-regulated protein 78 (Ac:P11021), heat shock protein Hsp70 (Ac:P11142), and glyceraldehyde-3-phosphate dehydrogenase (Ac:P04406) [54-56].

The challenges of analysing plasma and other biofluid samples by MS have become apparent since the focus of HUPO on the human plasma proteome. Specifically, the HUPO study highlighted

significant complications, including aspects inherent to the sample itself, in addition to sample handling, transport, storage, and processing [12]. These variables are all pre-analytical processes, and influence the quality and/or reproducibility of the data, in addition to the myriad of analytical process variables themselves. The contribution of platelet-derived peptides or enzymes to plasma has been suggested to induce further, albeit *ex vivo*, proteolysis [57]. In this initial study we aimed to further the understanding of the platelet contribution towards the human plasma proteome.

The elevated abundance of PPBP (Ac:P02775) and PF4 (Ac:P02776)in our platelet sub-proteome, as well as the HUPO human plasma specimen, ranked #12 and #21, and #201 and #236, respectively (Table 2) in part, can be explained by the *ex vivo* release of these proteins during blood collection and/or plasma preparation [12-14]. The presence of PPBP and PF4 in plasma has been shown to be associated with *in vitro* activation of platelets [58], and can result from tissue factor and other activators from venous trauma as a result of narrow gauge needles used in venipuncture [59].

In conclusion, our study has examined for the first time the direct crossover between human plasma and platelets. This information provides insights into the direct influence that platelets have on the plasma proteome via activation, granule release, lysis and selective uptake and release of plasma proteins. It is noteworthy that normal healthy human plasma contains microparticles from many different cell types and that 70-90 % of such microparticles are thought to be platelet-derived [60]. Our initial study has enabled the relative global mapping of similarities and differences in the abundance and function of proteins co-identified in each of the platelet and plasma proteomes, thereby providing the first global assessment of the platelet-plasma protein nexus.

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