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Differences between Platelets Derived from Neonatal Cord Blood and Adult Peripheral Blood Assessed by Mass Spectrometry

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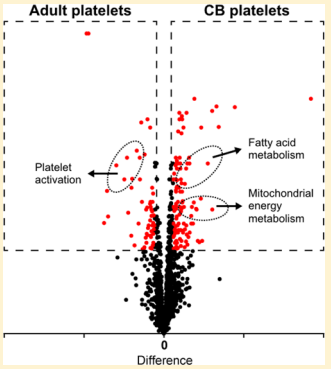
Supporting Information

ABSTRACT: It has been proposed that differences may exist between umbilical cord blood (CB) platelets and adult peripheral blood (APB) platelets, including altered protein levels of the main platelet integrins. We have now compared the protein expression profiles of CB and APB platelets employing a label-free comparative proteomics approach. Aggregation studies showed that CB platelets effectively aggregate in the presence of thromboxane A2 analogue, collagen, and peptide agonists of the proteinase-activated receptors 1 and 4. In agreement with previous studies, higher concentrations of the agonists were required to initiate aggregation in the CB platelets. Mass spectrometry analysis revealed no significant difference in the expression levels of critical platelet receptors like glycoprotein (GP)Ib, GPV, GPIX, and integrin αIIbβ3. This was confirmed using flow cytometry-based approaches. Gene ontology enrichment analysis revealed that elevated proteins in CB platelets were in particular enriched in proteins contributing to mitochondrial energy metabolism processes. The reduced proteins were enriched in proteins involved in, among others, platelet degranulation and activation. In conclusion, this study reveals that the CB and APB platelets are distinct. In particular, changes were observed for proteins that belong to metabolic and energy generation processes and not for the critical adhesive platelet integrins and glycoproteins.



KEYWORDS: *platelets, cord blood, proteome, mass spectrometry*

INTRODUCTION

It has been described that the protein composition of plasma and circulating cells in cord blood (CB) are distinct from those in adult peripheral blood (APB). The levels of the hemostatic proteins factor II, factor VII, factor IX, and factor X are, for instance, decreased in CB.[1] Reduced plasma levels have also been reported for proteins that belong to the cholesterol−lipid transporting system, the iron transporting system, and the complement system.[2−4] The presence of hemoglobin γ chains in CB-derived erythrocytes rather than the adult hemoglobin β chains represents another well-known example.[5] It has also been proposed that CB platelets are distinct from APB platelets.[6−9] Detailed insight is, however, still lacking.

Platelets are small enucleated cellular fragments of megakaryocytes and are essential for effective bleeding arrest and blood vessel repair at sites of injury.[10] To this end, platelets express a unique set of adhesive glycoproteins and integrins including the complex of the glycoproteins Ib, IX, and V (GPIb-IX-V) and the integrin αIIbβ3. Platelets also contain specialized secretory dense and α granules, storing pro-hemostatic small molecules and a multitude of functionally distinct proteins.[11] The platelet surface is irregularly shaped and contains many invaginations, which form open channels that remain in contact with the extracellular space. This open canalicular system provides access for plasma proteins and other substances to the platelet interior. It also facilitates secretion of the secretory granules when platelets are activated.[12]

Compared to APB platelets, it has been shown that CB platelets have a decreased response to agonists, including impaired aggregation in response to thrombin, thromboxane A2 analogue U46619, and collagen.[13,14] This illustrates that there are differences between the platelets derived from CB and APB. Some studies have reported that this hyporeactivity is caused by decreased levels of integrin αIIbβ3,[15,16] while others described

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that it was not the basal expression level of αIIbβ3 but its activation that is impaired on CB platelets.[9] This shows that the differences in the expression level and activation potential of this critical platelet protein in CB and APB platelets is still unclear. Moreover, there is very little information available about the differences in expression levels of other platelet proteins. In this study, we now used a label-free quantitative mass spectrometry approach to assess the differences between CB and APB platelets in detail.

EXPERIMENTAL SECTION

Blood Sampling

Umbilical cord blood (CB) was obtained from five healthy full-term infants immediately following normal vaginal delivery after uncomplicated pregnancies. Then 40−80 mL of CB was collected into precitrated cord blood collection MSC 1201DU bags (Macopharma, Tourcoing, France) via a 12-gauge needle. From five healthy adults, approximately 50 mL of blood was drawn from the antecubital vein via a 21 gauge needle and collected into citrated collection tubes (Greiner Bio One Vacuette sodium citrate blood collection coagulation tube, 3.8%). Details of the CB (newborns) and adult donors are provided in Table I. Both CB and APB platelets were isolated within 48 h after the blood was drawn. All human materials were obtained in accordance with the Declaration of Helsinki and the Dutch rules and regulations with respect to the use of human materials from volunteer donors. CB was obtained from the cord blood bank of Sanquin according to the guidelines of Eurocord. Mothers gave written informed consent to participate in the study. Blood from healthy anonymized donors was also obtained after their written informed consent. The used system was approved by Sanquin's internal ethical board.

Table I. Demographic Features of the Study Population[a]

newborns (CB)	
n	5
gender (% M)	20
gestational age (w)	40.3 ± 1.3
birth weight (g)	3641 ± 461
Apgar score (1 min)	9.2 ± 0.7
adults (APB)	
n	5
gender (% M)	60
age (y)	48.5 ± 10.6

[a]Results are reported as mean ± SD. None of the participating newborns and adults had a known disorder or sickness. Only infants with Apgar scores[41] ≥8, indicating a healthy condition,[42] were included.

Platelet Isolation

Platelet-rich plasma (PRP) was collected via centrifugation of whole blood for 20 min at 120*g*. Then 10% (v/v) ACD-A (BD-Plymouth) and 1 nM prostaglandin E1 (PGE1) were added to the PRP. Platelets were spun down by centrifugation for 10 min at 2000*g* and resuspended in a buffer comprising 36 mM citric acid, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 5.6 mM D-glucose, 10% (v/v) ACD-A, and 1 nM PGE1 at pH 6.5. Platelets were again washed and resuspended in the same buffer but with 0.1 nM PGE1. Next, platelets were collected by centrifugation for 10 min at 2000*g* at 20 °C. Platelets were processed immediately for aggregation tests and flow cytometry analyses. For proteomics, platelets were stored at −30 °C until sample preparation for mass spectrometry analysis.

Extra Purification of Platelets Using Magnetic Beads

Extra purification of CB platelets was performed using an EasySep positive selection kit (StemCell) containing anti-CD41 tetrameric antibody complexes (TAC) and magnetic beads, following the manufacturer's instructions. In brief, 500 μL of PRP was diluted 1:3 in a buffer containing 36 mM citric acid, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 5.6 mM D-glucose, 10% (v/v) ACD-A and 1 nM PGE1, pH 6.5. After the addition of anti-CD41 TAC (1:50 ratio) and magnetic beads (1:25 ratio), the platelets were washed three times with the same buffer using an EasySep magnet. Finally, platelets were collected by centrifugation for 10 min at 1000*g* and stored at −30 °C until preparation for mass spectrometry analysis.

Platelet Aggregation

Platelets were washed and resuspended in Tyrode's salt solution (Sigma, St. Louis, USA) to a concentration of 200 × 10^6 platelets/mL. Platelet aggregation was initiated by employing increasing concentrations of U46619 (Tocris, Bristol, UK), collagen (Chrono-log, Havertown, USA), and PAR1- or PAR4-activating peptides (SFLLRN-amide and AYPGKF-amide, respectively; Peptides International, Louisville, USA). Reactions were performed at 37 °C in a final volume of 500 μL of Tyrode's buffer under continuous stirring (1000 rpm). The change in light transmission in a Chrono-log 700 aggregometer was used to follow platelet aggregation. Aggregation profiles were recorded for 10 min and maximum aggregation responses were determined using AGGRO/LINK software (Chrono-log).

Flow Cytometry Analysis

Washed platelets were resuspended to a final concentration of 50 × 10^6 platelets/mL in a buffer containing 20 mM Hepes, 100 mM NaCl, 5 mM D-glucose, 2.5 mM EDTA, pH 6.5, containing 0.5% (w/v) human serum albumin. Platelets were stained with anti-CD41 (integrin subunit αIIb)-PE-Cy7 or anti-CD42b (GPIb)-APC antibodies (BioLegend) for 20 min, followed by fixation in 0.5% (w/v) *para*-formaldehyde. For the assessment of integrin αIIbβ3 (CD41/CD61) activation, platelets were first activated with 10 μM PAR1 activating peptide for 5 min at 37 °C (negative control: buffer incubation for 5 min at 37 °C). The platelets were subsequently labeled using PAC1-Alexa Fluor 647 antibody (BioLegend) for 20 min, followed by fixation in 0.5% (w/v) *para*-formaldehyde. Antibody binding was always performed without permeabilizing the platelets. Flow cytometry was performed on a BD Canto II cytometer (BD Biosciences), and the results were analyzed using FlowJo version 10 (FlowJo LLC).

Sample Preparation for Mass Spectrometry

All fine chemicals were from Thermo Scientific (Breda, The Netherlands) unless indicated otherwise; 100 × 10^6 washed platelets per donor were lysed in 100 μL of 8 M urea, 100 mM Tris-HCl (Life Technologies, Carlsbad, USA) at pH 8. Platelet samples were sonicated for 10 min at room temperature and centrifuged to remove insoluble material. The protein concentrations of the lysates were assessed using a Bradford assay. Platelet lysates comprising 5 μg of protein were diluted at least 7-fold (v/v) in 50 mM ammonium bicarbonate (Fluka). Proteins were reduced for 60 min at 25 °C in 10 mM dithiothreithol (DTT) and alkylated using 55 mM iodoacetamide (IAM) for 45 min at room temperature in the dark.

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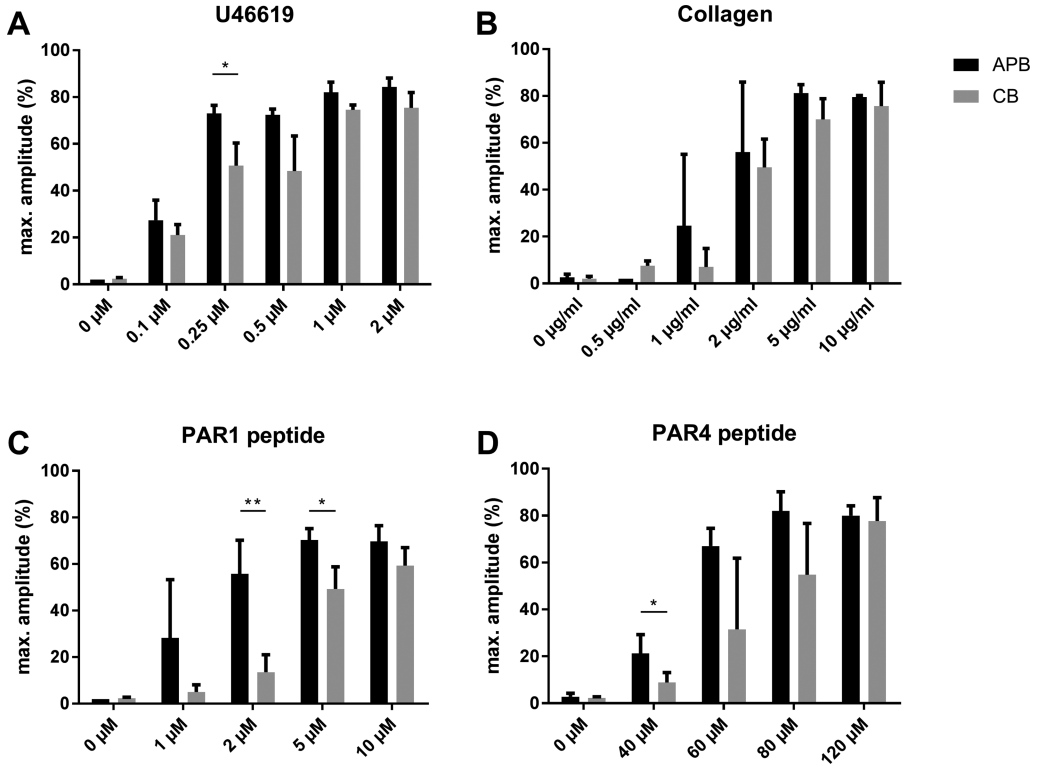

Figure 1. CB and APB platelets show effective aggregation. Platelet aggregation was assessed in the presence of increasing concentrations of (A) the thromboxane A2 analogue U46619, (B) collagen, (C) PAR1 activating peptide, and (D) PAR4 activating peptide. Platelet aggregation is expressed as percentage of maximal aggregation. Error bars represent the mean ± standard deviation. Significant differences (unpaired *t* test with Welch's correction) are indicated with * $p < 0.05$, ** $p < 0.01$.

Proteins were proteolyzed by trypsin (Promega, Madison, USA) overnight at 25 °C using a protein:trypsin ratio of 1:20 (mg/mg). Protein samples were acidified with 5 µL 99% formic acid and prepared for MS analysis using Empore-C18 StageTips.[17]

Mass Spectrometry Data Acquisition

Mass spectrometry analysis was performed essentially as described by Gazendam et al.[18] Tryptic peptides were separated by nanoscale C18 reverse phase chromatography coupled online to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on a 20 cm 75−360 µm inner−outer diameter fused silica emitter (New Objective) packed in-house with ReproSil-Pur C18-AQ, 1.9 µm resin (Dr Maisch GmbH). The column was installed on a Dionex Ultimate3000 RSLC nanoSystem (Thermo Scientific) using a MicroTee union formatted for 360 µm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15 kV. Buffer A was composed of 0.5% acetic acid and buffer B of 0.5% acetic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nL/min at 5% buffer B, equilibrated for 5 min at 5% buffer B (17−22 min) and eluted by increasing buffer B from 5 to 15% (22−87 min) and 15−38% (87−147 min), followed by a 10 min wash to 90% and a 5 min regeneration to 5%. Survey scans of peptide precursors from 400 to 1500 m/z were performed at 120 K resolution (at 200 m/z) with a 1.5×10^5 ion count target. Tandem mass spectrometry was performed by isolation with the quadrupole with isolation window 1.6, HCD fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS2 ion count target was set to 10^4 and the max injection time was 35 ms. Only those precursors with charge state 2−7 were sampled for MS2. The dynamic exclusion duration was set to 60 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3 s cycles. All MS data were acquired with Xcalibur software (Thermo Scientific).

Data Analysis

The RAW mass spectrometry files were processed with the MaxQuant computational platform 1.5.2.8.[19] Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot-Swiss-Prot database (release 2015-02; 89796 entries, downloaded February 2015).[20] Standard settings with the additional options match between runs, Label free quantification (LFQ), and unique peptides for quantification were selected. The RAW files as well as details about the settings can be found in the parameter files deposited in the PRIDE repository database[21] with the data set identifier PXD004578. The generated "proteingroups.txt" table was filtered for reverse hits and "only identified by site" using Perseus 1.5.1.6. The LFQ values were transformed in $\log_2$ scale. Five analyzed samples from CB and five from APB were divided into two groups and filtered for five valid values in at least one of the experimental groups. Missing values were imputed by normal distribution (width = 0.3, shift = 1.8), assuming these proteins were close to the detection limit. Normalized LFQ (z-score) values were imported in the R (v3.2.2) environment. Potential contaminant proteins were removed, except for ALB and THBS1. The removed contaminants can be found in the online available "proteingroups.txt" file in the "potential contaminants" column (PRIDE repository database[21]). Empiri-

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cal Bayes moderated t-statistic in limma[22] (v3.24.15) was used to find differentially expressed proteins; proteins with FDR adjusted p-values lower than 0.05 and more than 1.5-fold change difference in expression were considered significant. Enrichment of biological processes, molecular functions, and cellular components was performed and visualized using the Cytoscape[23] (version 3.3.0) plug-in BiNGO[24] (version 3.0.3). Ontology and annotation data sets were downloaded on 23-3-2016 from the Gene Ontology Consortium Web site (www.geneontology.org). For the comparison of significantly different expressed proteins to all identified proteins, a p-value cutoff of 0.05 was used. Subsequently, the BiNGO-output was loaded into Enrichment Map[25] (version 2.0.1) with a Jaccard similarity coefficient cutoff value of 0.5.

RESULTS

CB Platelets Are Hyporeactive Compared to APB Platelets

Prior to assessing the differences in protein expression between CB and APB platelets, we evaluated the aggregation potential of the platelets. To this end, platelet aggregation was induced by employing increasing concentrations of the platelet agonists thromboxane A2 analogue (U46619), collagen, and peptide agonists for the thrombin receptors proteinase-activated receptor (PAR)-1 and PAR4. The response of the platelets was measured by aggregometry (Figure 1). Both CB and APB platelets showed effective aggregation in the presence of each of the agonists. However, higher concentrations of the agonists were required to initiate aggregation in the CB platelets. In particular, this was observed for platelet aggregation in response to the PAR1 activating peptide.

It has been suggested that the activation potential of the αIIbβ3 integrin on CB platelets is decreased compared to APB platelets.[9,16] To verify this finding, we made use of a fluorescently labeled PAC-1 antibody in a flow cytometry-based approach. This antibody has been shown to mainly bind to the activated αIIbβ3 integrin on the platelet surface.[26] While both CB and APB platelets showed increased PAC-1 binding upon platelet activation, this increase was significantly higher for the APB than the CB platelets (Figure 2). This finding implies decreased levels of the activated αIIbβ3 integrin on activated CB platelets compared to activated APB platelets. This may be explained either by a reduced activation potential of the αIIbβ3 integrin or by a decreased presence of this integrin at the CB platelet surface. Compatible with previous studies,[9,27] our findings show that CB platelets are less responsive to platelet agonists compared to APB platelets. However, the CB platelets do have the mechanism to fully support aggregation.

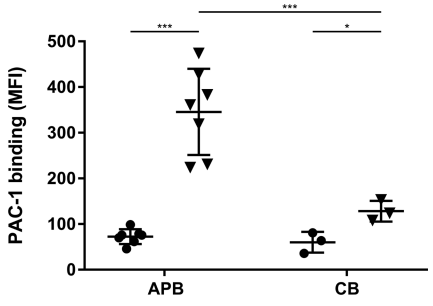

Figure 2. CB platelets show reduced activation of integrin αIIbβ3. Antibody PAC-1, which binds the activated integrin αIIbβ3, was incubated with the CB and APB platelets after activation with buffer alone (circles) or 10 μM PAR1 activating peptide (triangles). Mean fluorescence intensity (MFI) values as assessed by flow cytometry analysis are shown. Significant differences (unpaired t test with Welch's correction) are indicated with * $p < 0.05$, *** $p < 0.005$.

CB and APB Platelets Can Be Distinguished Based on Their Protein Expression Profiles

Label-free comparative proteomics was employed to assess the differences in protein levels, including the αIIbβ3 integrin and platelet glycoproteins such as GPIb, between CB and APB platelets. Proteomic analysis of five platelet samples from APB and five from CB resulted in the identification of 3036 proteins. To assess the variation between and within the platelet groups, Pearson correlation testing was performed on the protein intensities omitting all missing values. This showed a high correlation between all platelet samples ($R^2 > 0.9$; Figure 3). Using the protein selection criteria as described in the Experimental Section, quantitative information was obtained for 1999 proteins. An overview of these proteins is given in Supporting Information, Table S-1. Irrespective of the high correlation between all platelet samples, multidimensional scaling analysis revealed that CB platelets and APB platelets are distinct (Figure 4). An empirical Bayes moderated t test was performed to assess the differential protein levels between APB and CB samples. This analysis revealed increased levels for 113 proteins and decreased levels for 57 proteins in CB platelets compared to APB platelets (Figure 5). These findings together show that CB and APB platelets are distinct but that the differences are relatively small.

Evaluation of the Nonplatelet Proteins

Co-purified proteins that are not from platelet origin but derived from other blood components form a special point of attention in platelet proteomics studies. This is difficult to avoid as platelets take up proteins and microvesicles from their surroundings, like "sponges".[28] In an elegant study by Zeiler et al., the copurified nonplatelet proteins were identified by comparing the proteomes of platelets with different levels of purity.[29] Although these studies were performed on murine platelets, the identified proteins may represent a good model for proteins of nonplatelet origin also in the human context. For the identified proteins in our study, we indicated in Supporting Information, Table S-1, whether they match with the copurified proteins identified in the study by Zeiler et al.[29] In addition, we show whether a protein is among the top 150 most abundant plasma proteins.[30] Results show that especially the significantly downregulated proteins in CB platelets (26 out of 57) are rich in proteins that originate from plasma, including apolipoproteins, complement proteins, and proteins of the iron transport system. The reduced proteins also comprise the hemoglobin δ and β chains, which are normally expressed in adult erythrocytes.[5] The fetal hemoglobin γ-1 chain was, in turn, among the elevated proteins. The elevated proteins in CB platelets are further remarkably rich in several mitochondrial proteins, including members of the mitochondrial membrane respiratory chain. These proteins have not been identified in the study by Zeiler et al.[29] as putative copurifying proteins. To confirm that the elevated proteins originate from CB platelets and not from other cells, we included an extra purification step in the procedure to obtain CB platelets. Comparative proteomics between the "standard" and "extra" purified CB platelets revealed a marked reduction in typical erythrocyte proteins, including hemoglobin β and α, peroxiredoxin-2, and

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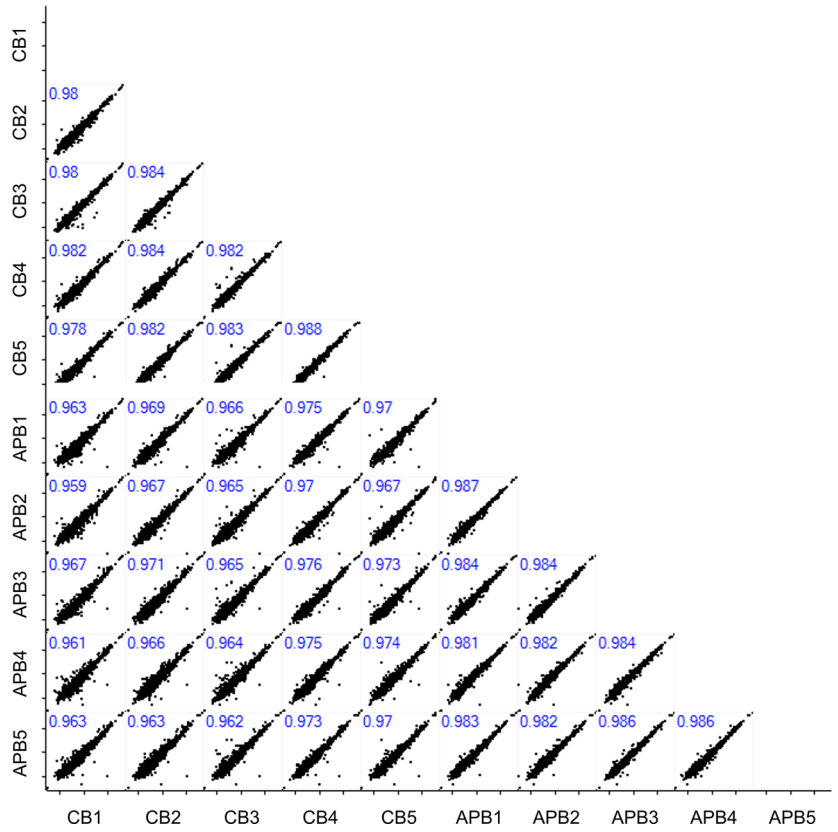

Figure 3. LFQ protein intensities are highly correlated between and within the platelet groups. Shown is a multiscatter plot of the $\log_2$ transformed LFQ values of the proteins identified in five CB platelet samples (CB1−CB5) and five APB platelet samples (APB1−APB5). All invalid values were omitted from the analysis. The values indicated in blue represent the squared Pearson correlation coefficient.

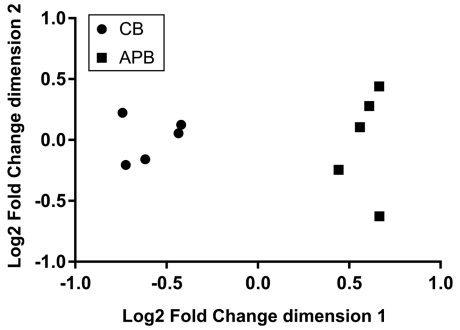

Figure 4. Multidimensional scaling (MDS) plot of CB and APB platelets. MDS plot visualizes, in two dimensions, the distance between the samples based on the pairwise Euclidean distance calculated with the protein expression values of the 500 most variable proteins.

band 3 anion transport protein (HBB, HBA, PRDX2, and SLC4A1, respectively) (Supporting Information, Figure S-1). Further, 107 out of the 113 proteins that were found to be elevated in CB platelets, were again identified and quantified in this new analysis. Only four of these proteins (EPB41, SPTA1, TGM2, TIMP3) were >1.5-fold reduced in the extra purified CB platelets, while the levels of the other 103 proteins remained similar (Supporting Information, Figure S-1, Table S-2). These results imply that the significantly elevated proteins in the CB samples are most likely of platelet origin.

There Is No Difference in Protein Levels of the Critical Platelet Surface Proteins GPIb and Integrin αIIbβ3

It has been suggested that CB platelets exhibit a decreased level of integrin αIIbβ3.[15,16] Our proteomics analysis, however, showed no difference between APB and CB platelets for this protein (Figure 5A; Supporting Information, Table S-1). Also for other platelet integrin subunits, such as α2, β1, and α6, as well as the glycoproteins GPIb, GPIX, and GPV, no significant differences were found (Figure 5A; Supporting Information, Table S-1). We also used flow cytometry analysis to assess the surface expression of integrin αIIbβ3 and GPIb on the platelets. The result showed comparable fluorescence values for both these proteins on CB and APB platelets (Figure 6). Compatible with the proteomics analysis, this result suggests that the levels of the αIIbβ3 integrin and GPIb on CB and APB platelets are similar. On the other hand, the activation potential of integrin αIIbβ3 (see Figure 2) indeed appears to be decreased on the CB platelets.

Gene Ontology Enrichment Analysis of the Elevated and Decreased CB Platelet Proteins

To gain further insight into the nature of the 170 differentially expressed proteins, they were annotated for their molecular functions, cellular components, and biological processes.[31] In particular, proteins related to mitochondrial energy metabolism (oxidative phosphorylation) were enriched among the elevated proteins in CB platelets. This is shown by the upregulation of proteins that contribute to NADH dehydrogenase activity, oxidoreductase activity, and electron transport chain processes. Other significantly enriched proteins contribute to (long-chain) fatty acid metabolism and iron ion binding (Figure 7A;

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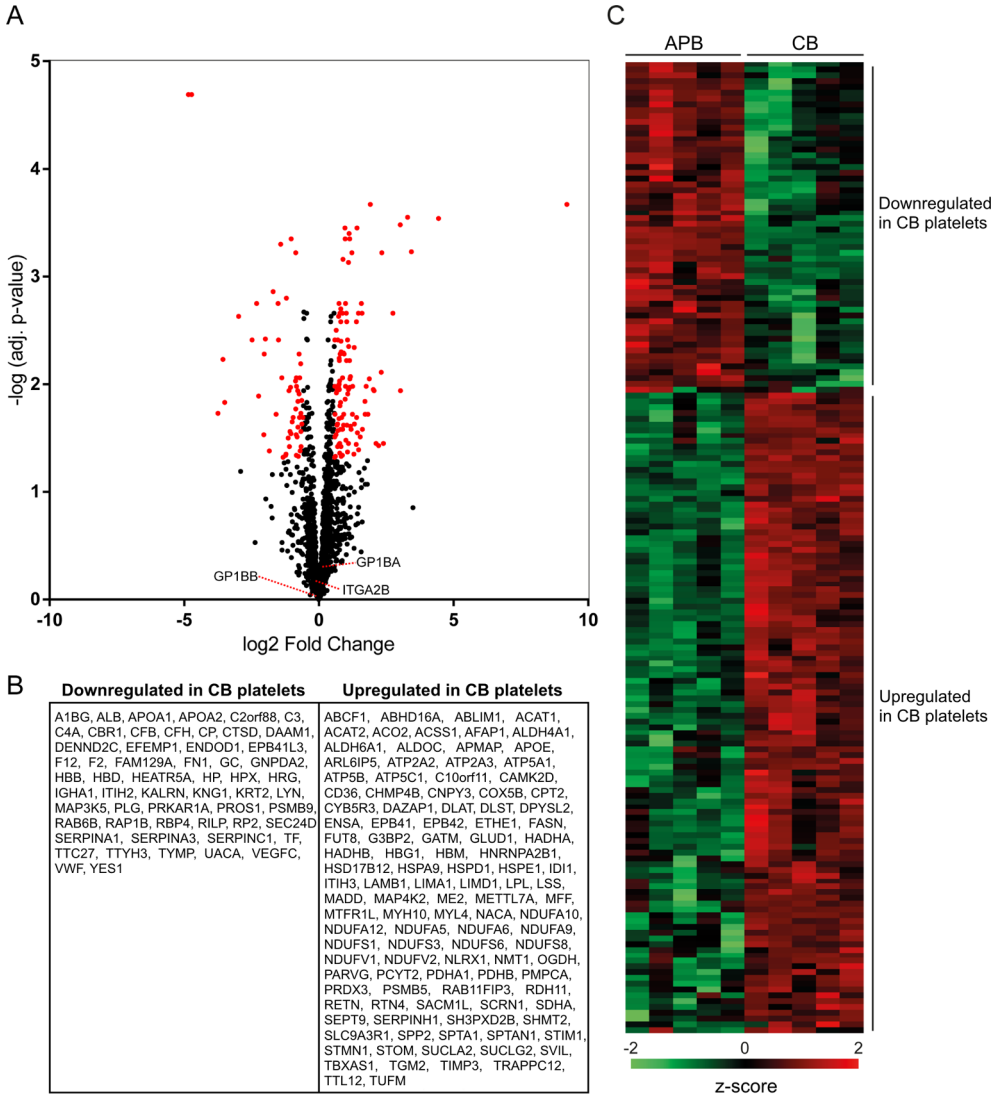

Figure 5. Significantly differentially expressed proteins between CB and APB platelets (170 out of 1999). (A) Volcano plot showing the negative logarithm of the adjusted *p*-value against the fold change in relative protein levels between CB and APB platelets, with each protein represented by a single point in the graph. Points indicated in red correspond to significant proteins ($p < 0.05$) showing more than 1.5-fold change (FC) in expression level between CB and APB platelets. A positive FC corresponds to upregulated proteins and a negative FC to downregulated proteins in CB platelets. Data points representing the critical adhesive platelet proteins GPIBA, GPIBB, and integrin αIIbβ3 (ITGA2B) are indicated in the figure. (B) List of the gene names of the differentially expressed proteins in CB and APB (red points in A). (C) Heat-map and hierarchical clustering of the 170 significantly different proteins between CB and APB platelets. Heat-map colors are based on the *z*-scored ($\log_2$) LFQ values. Green shades correspond to decreased expression levels and red shades to increased expression levels.

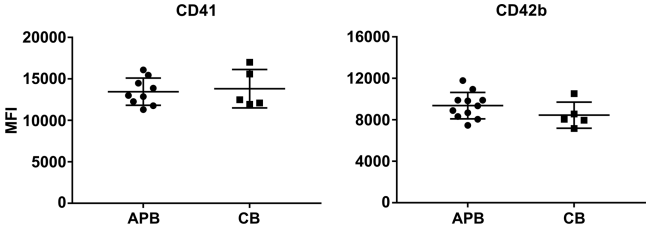

Figure 6. Flow cytometry analysis reveals no difference in surface expression of αIIbβ3 and GPIb between CB and APB platelets. Integrin subunit αIIb (CD41, left panel) and GPIb (CD42b, right panel) were stained with fluorescently labeled antibodies as described in the Experimental Section. The mean fluorescence intensity (MFI) of the platelets was assessed using flow cytometry.

Supporting Information, Table S-3A). Proteins related to the inflammatory response, fibrinolysis, platelet activation, blood coagulation, and complement activation were significantly enriched among the reduced proteins in CB platelets (Figure 7B; Supporting Information, Table S-3B). Together, these data together show that mainly proteins involved in (mitochondrial) energy processes and metabolism are elevated in CB platelets compared to APB platelets.

DISCUSSION

It has previously been proposed that there are differences between CB and APB platelets.[7−9,14] Studies directed to identify these differences are, however, inconclusive. Studies have, for instance, not only reported reduced levels of integrin αIIbβ3 but also normal levels of this protein on CB platelets.[9,15,16] In the present study, we employed a label-free comparative proteomics approach combined with functional

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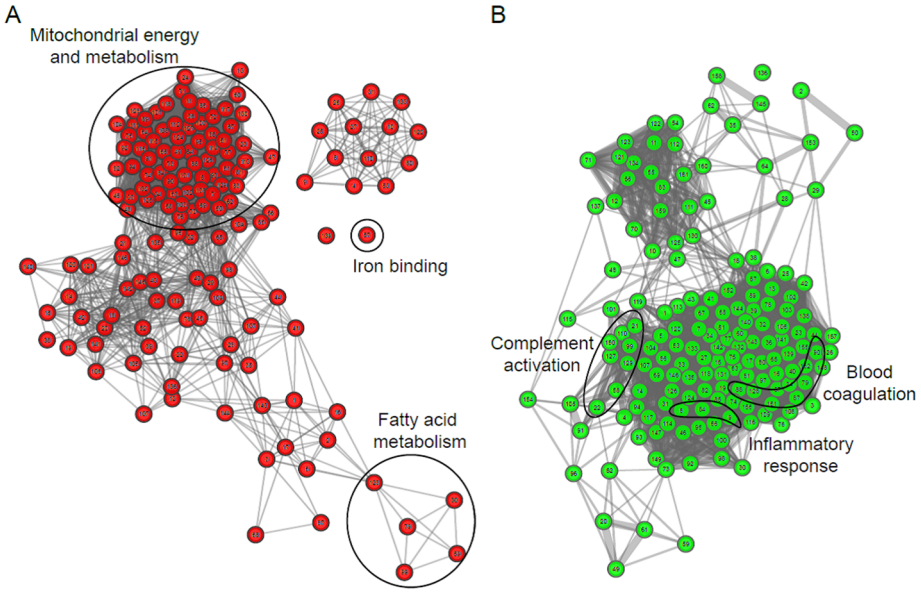

Figure 7. Gene ontology (GO) category enrichment analysis reveals increased expression in CB platelets of proteins that contribute to respiratory mitochondrial processes. Enrichment of biological processes, molecular functions, and cellular components was assessed as described in the Experimental Section. Significantly enriched GO terms of the 170 differentially expressed proteins are presented, showing GO terms associated with (A) upregulated proteins and (B) downregulated proteins in CB platelets. Each node indicates an enriched process and lines between two nodes indicate that these processes share overlapping genes. Numbers in the nodes correspond to the processes listed in Supporting Information, Table S-3. Big circles around several nodes in this figure indicate that most of the nodes included in the circle contribute to the process that is written close to the circle; the names of these overarching terms are derived from the Word Cloud tool in Cytoscape.

studies to assess the differences between CB and APB platelets in more detail.

In agreement with previous studies,[9,13,14] we found a reduced aggregation response of the CB platelets to several agonists and a reduced activation potential of the integrin αIIbβ3 (Figures 1 and 2). Yet, the proteomics data did not reveal a significant difference in the expression levels of the critical adhesive platelet integrins and glycoproteins (Figure 5; Supporting Information, Table S-1). One could argue that the proteomics approach does not distinguish between cell surface and intracellular proteins and that there may be a specific reduction of integrin αIIbβ3 and GPIb on the CB platelet surface only. However, the employed flow cytometry approach using nonpermeabilized platelets resulted in the detection of similar levels of αIIbβ3 and GPIb on the surface of CB and APB platelets as well (Figure 6). On the basis of these combined findings, we propose that there are no differences in the levels of integrin αIIbβ3 and GPIb between CB and APB platelets.

The cause of the reduced activity of CB platelets remains a topic for further investigation. Our results show a reduction of proteins in CB platelets that may contribute to cellular signaling, i.e., LYN, MAP3K5, and FAM129A (Figure 5B; Supporting Information, Table S-1). Notably, LYN has been proposed to be directly involved in platelet activation by mediating the release of intracellular Ca^{2+} in platelets.[32] Although our study does not provide direct evidence for this, these observations may together indicate that the intracellular signal transduction efficiency is altered in CB platelets. This is in agreement with a study by Israels et al., who studied the response of CB-derived platelets to thromboxane analogue U46619 and observed a reduced intracellular Ca^{2+} mobilization during activation of these platelets.[13] As the levels of critical adhesive integrins and glycoproteins are not altered in CB platelets, we propose that future studies should be directed to unravel the putative altered signal transduction pathways in CB platelets.

Several studies have been published in which the human platelet proteome is characterized.[33−36] To increase protein identifications, Burkhart et al. also isolated and analyzed phosphopeptides in addition to the proteins identified in their more standard proteomics approach (4191 identified proteins).[33] Lee et al. used a peptide prefractionation strategy to increase the number of protein identifications (5423 identified proteins).[35] Using the platelet processing protocol without any prefractionation of the peptides in this study resulted in the identification of 3036 proteins in the combined CB and APB platelet samples. When we performed an offline pH-based prefractionation of the peptides of the APB platelets, we increased the protein identifications to 4954 proteins. Because of the increased depth in protein identifications, we now also identified low abundant proteins including PAR1, PAR4, P2Y, and P2X. The Supporting Information, Table S-4 and Figure S-2, show an overview of the identified proteins as well as overlap with the proteins that were identified in the other two studies.

The assessment of the platelet proteome is challenged by the inevitable copurification of proteins from plasma and other blood components. This problem is difficult to completely overcome as plasma-derived proteins and proteins from other cells or microvesicles can get trapped in the open canalicular space of a platelet.[12,28] This phenomenon, however, also provides information about the differential levels of these proteins in CB and APB samples. Our analysis reveals, for instance, reduced expression levels of plasma proteins APOA1 and APOA2 and elevated levels of APOE in CB samples, confirming that newborns may have a distinct lipid cholesterol transport system compared to adults.[37] The proteomic data further confirms the decreased levels of plasma proteins involved in the iron transport system (e.g., haptoglobin and

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hemopexin) and several proteins of the complement system (e.g., C3, complement factor B (CFB) and CFH (Supporting Information, Table S-1).[2,3]

The CB platelet samples also reveal elevated levels of the hemoglobin γ chain and reduced levels of the hemoglobin β chain compared to the APB platelet samples (Supporting Information, Table S-1). This is fully compatible with the notion that CB erythrocytes mainly express the hemoglobin γ-1 chain.[5] Remarkably, the embryonic hemoglobin μ (HBM) was among the proteins identified exclusively in the CB platelets. Although the HBM transcript has been identified and described in reticulocytes previously,[38] no detectable protein has been identified so far. Apparently, the embryonic hemoglobin μ chain is still present in the newborns.

The most pronounced elevated proteins in the CB platelets contribute to energy metabolic processes including the Krebs cycle and hepatic β-oxidation. An increased energy generation potential of CB platelets has also been observed by Sjövall et al.[39] They showed that mitochondrial respiration levels from CB platelets were generally higher compared to those from adult cohorts. The reason for this elevation is at present unclear and is a topic for further investigation.

CONCLUSIONS

Our findings together provide several novel targets for future investigations directed to gain insight into the differences between CB and APB platelets. Further, it remains to be established whether platelets obtained from the peripheral blood of newborns show the same differences with APB platelets as CB platelets. Results of this study may also provide the basis for the development of novel diagnostic tools to assess congenital platelet disorders in newborns. We show that many proteins that are critical for platelet function are equally expressed in CB and APB platelets. Using APB platelets as a reference, a deficiency of a critical platelet protein in newborns caused by a mutation in the involved gene can be assessed with proteomics on CB platelets. A major advantage of using CB-derived platelets is that they can be easily obtained without causing any burden for the newborn. Examples of congenital platelet disorders[40] that may be readily identified using proteomics include Glanzmann's thrombasthenia (reduction or absence of integrin αIIbβ3), Bernard Soulier syndrome (reduction or absence of GPIb), Gray platelet syndrome (absence of Neurobeachin-like protein 2 (NBEAL2)), and Hemansky–Pudlak syndrome type II (absence of AP3B1).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00298.

Mass spectrometry analysis of standard and extra purified CB platelets; proteins identified employing a pH-based subfractionation approach, compared with published proteomics studies (PDF)

Quantified proteins in CB and APB platelets; list of all 1999 identified and quantified proteins in five CB and five APB samples (XLSX)

Relative levels of proteins identified in CB platelets isolated via the standard purification protocol or a protocol involving an extra purification step (XLSX)

List of all significant gene ontology terms associated with enriched and down-regulated proteins in CB platelets; numbers correspond to the numbers in the nodes in Figure 5A,B (XLSX)

List of proteins identified in APB platelets employing a pH-based subfractionation approach of the peptides (XLSX)

Accession Codes

RAW files and the MaxQuant search results have been deposited in the PRIDE repository database[21] with the data set identifier PXD004578.

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Notes

The authors declare no competing financial interest.

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