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ORIGINAL ARTICLE

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A method based on plateletpheresis to obtain functional platelet, $CD3^+$ and $CD14^+$ matched populations for research immunological studies

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Abstract

Background: In previous studies with peripheral blood cells, platelet factors were found to be associated with severe allergic phenotypes. A reliable method yielding highly concentrated and pure platelet samples is usually not available for immunological studies. Plateletpheresis is widely used in the clinics for donation purposes. In this study, we designed a protocol based on plateletpheresis to obtain Platelet-Rich Plasma (PRP), Platelet-Poor Plasma (PPP) as well as $CD3^+$ and $CD14^+$ cells matched samples from a waste plateletpheresis product for immunological studies.

Methods: Twenty-seven subjects were voluntarily subjected to plateletpheresis. PRP, PPP and blood cell concentrate contained in a leukocyte reduction system chamber (LRSC) were obtained in this process. $CD3^+$ and $CD14^+$ cells were isolated from the LRSC by density-gradient centrifugation and positive magnetic bead isolation. RNA was isolated from PRP, $CD3^+$ and $CD14^+$ cell samples and used for transcriptomic studies by Affymetrix. PRP and PPP samples were used for platelet protein quantification by multiplex assays.

Results: A reliable high yield method to obtain matched samples of PRP, PPP, $CD3^+$ and $CD14^+$ from a single donor for RNA and protein analyses has been designed. The RNA quality indicators (RQI) routinely used for other cell types were not suitable for platelet RNA characterization. Despite this, the platelet RNA was valid for

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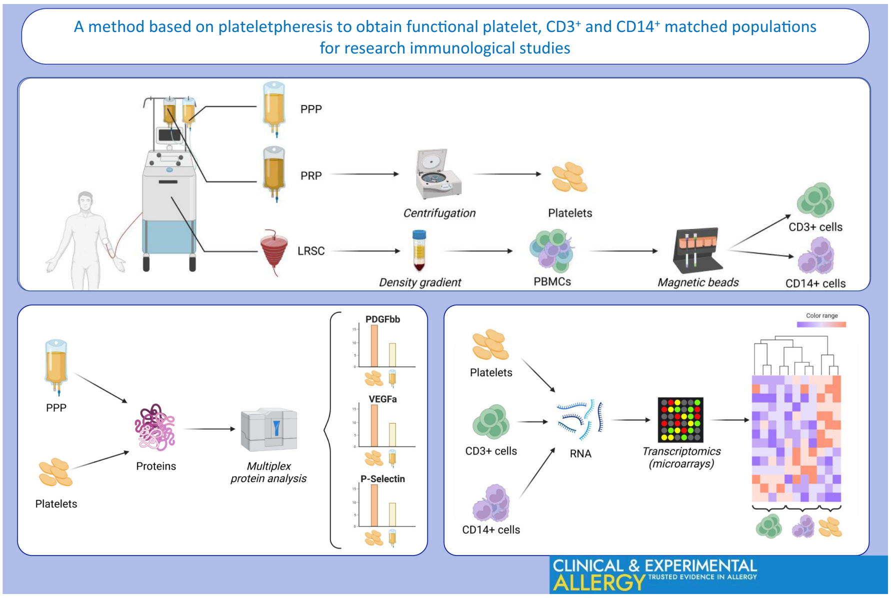
transcriptomic studies by Affymetrix, as platelet transcripts obtained in our previous studies were confirmed in PRP samples. Platelet samples were enriched in platelet factors as determined in protein multiplex analysis.

Conclusions: We have developed a method that yields not only high content and pure platelet samples from a single donor but also CD3$^+$ and CD14$^+$ matched samples that can be used for RNA and protein analyses in immunological studies.

KEYWORDS

leukocyte reduction system chamber (LRSC), multiplex, plateletpheresis, platelet-rich plasma (PRP), platelets, transcriptomics



GRAPHICAL ABSTRACT

We describe a platelet isolation method based on plateletpheresis that allows to obtain pure, highly concentrated and functional platelet samples from a single donor. Additionally, matched CD3$^+$ and CD14$^+$ populations can be obtained from a waste product of plateletpheresis procedure. All the samples are suitable for *omics* studies.

1 | INTRODUCTION

Platelets are anucleate blood cells generated from megakaryocytes. They are loaded with a wide range of mediators contained in secretory granules, which not only have coagulation-related functions, but also play a key role in inflammation.[1,2] There is increasing evidence supporting the potential of platelets as a source of biomarkers in inflammatory diseases.[3] This is particularly interesting in the field of allergy, in which severe allergy management often represents a difficult challenge.

Platelet activation markers, such as β-thromboglobulin (β-TG) and platelet factor 4 (PF4), have been found increased in allergic asthmatic patients after allergen challenge.[4] This is also the case for patients with atopic dermatitis, which also presented increased plasma levels of such markers.[5,6] In our previous studies, alteration of the platelet function was found to be associated with severe allergic phenotypes.[7] Severe allergic patients do not respond to treatment, suffer exacerbations and present a reduced quality of life.[8]

Key Messages

- A high yield method to obtain platelets from a single donor based on plateletpheresis.
- Matched samples of other peripheral blood mononuclear cell populations are also obtained with this procedure.
- All the cell samples are suitable for RNA and protein analyses in research studies.

The mechanisms that explain the acquisition of a severe phenotype are still poorly understood.

The study of platelets and their role in inflammation requires pure, non-activated platelet isolates. A single leukocyte possesses 12.500-fold higher mRNA content and 65-fold higher protein content than one platelet.[9,10] Therefore, even very low numbers of contaminating leukocytes could shed misleading results in transcriptomic and proteomic platelet studies. Currently, a reliable method yielding highly concentrated and pure platelet samples for immunological studies is not commonly available.

The most used techniques for isolating pure platelets are based on multiple centrifugation steps, which cause physical stress leading to platelet activation and granule content release.[11] This mechanical activation could lead to a misinterpretation in the quantification of protein and mRNA. In addition, protocols based on successive centrifugation steps require large blood volumes and pooling samples from multiple subjects to obtain sufficient mRNA concentration for performing platelet gene expression studies.[12] This requirement makes these techniques impractical for use in clinical research and underlies why many studies use pools of platelets from different donors.[13]

Plateletpheresis is a technique mainly used for platelet donation purposes that allows the generation of a platelet-rich plasma (PRP) product from a single donor with no leukocyte or red blood cell contamination. Whole blood is processed by a cell separator, which separates the blood components based on centrifugation parameters. Platelets are retained and collected as PRP, while the remaining blood components are returned to the donor through automated circulation.[11] Although the process is highly efficient, a remaining of highly concentrated blood sample is collected in a leukocyte reduction chamber system (LRCS), which is usually discarded as a waste product. This technique is not commonly used in basic research since specialized equipment and training are required. However, its advantages over platelet isolation methods based on centrifugation make plateletpheresis an attractive alternative for performing multi-omics assays, in which concentration and purity of samples are essential.

In this study, we describe a protocol based on plateletpheresis to obtain PRP as well as $CD3^+$ and $CD14^+$ matched cell samples. The PRP and cell samples obtained by plateletpheresis are suitable for transcriptomic and protein analyses. Therefore, this methodology could be used for phenotyping platelets as well as other cell populations in immune diseases in order to elucidate their role in inflammation.

2 | MATERIAL AND METHODS

2.1 | Study subjects

Twenty-seven individuals were recruited between October 2018 and February 2021 at the blood bank and the Allergy Service of the Puerta de Hierro-Majadahonda University Hospital. The protocol was approved by the Ethics Research Committee from the hospital, and written informed consent was obtained from all study subjects. Individuals younger than 18 years old, with cancer or haematological diseases were excluded from the study.

2.2 | Plateletpheresis

Plateletpheresis donor's standard exclusion criteria were used to select participants. Plateletpheresis was performed in the Apheresis Unit of the Haematology department of the hospital. Trima Accel machine (Terumo BCT) was set to obtain PRP (85 ml) and platelet-poor plasma (PPP) (50 ml) samples using Adenine Citrate Dextrose-A (ACD-A) as anticoagulant. PRP and PPP samples were collected sterile and transferred to two different storage bags (Paediatric Transfer Bags, Grifols). After plateletpheresis, samples were allowed to rest at room temperature (RT) for 2 h. After resting, a hemogram of both samples was performed to assess cell counts. A minimum concentration of 500×10^9 platelets/L was considered a quality requirement for PRP. Moreover, the content of other cell types was negligible. We also obtained the LRSC and used it for peripheral blood mononuclear cell (PBMC) isolation.

2.3 | Isolation of PBMCs from the LRSC

Blood contained in the LRSC (7–9 ml) was diluted 1:1 in RPMI medium (Thermo Fisher Scientific). The total volume of diluted blood was carefully dispensed onto 1 V of Ficoll (Thermo Fisher Scientific) and centrifuged for 20 min, 500 *g* with no brake at RT. The PBMC fraction was collected from the buffy layer. Two washing steps with PBS (Thermo Fisher Scientific) and centrifugation for 5 min, 300 *g* at 4°C were performed. Supernatant was discarded, and cells were resuspended in 5 ml PBS to quantify cell numbers with a N-20 Sysmex (Roche).

2.4 | CD3+ and CD14+ isolation with magnetic beads

$CD14^+$ and $CD3^+$ cells were sequentially isolated from the PBMC fraction with magnetic MicroBeads (Miltenyi Biotec) following manufacturer instructions. Once isolated, cell populations were stored in Rneasy Lysis (RLT) buffer containing 1% β-mercaptoethanol at −20°C until transcriptomic analysis.

2.5 | $CD3^+$ and $CD14^+$ RNA extraction

RNA was extracted from $CD3^+$ and $CD14^+$ cells using Rneasy® Mini Kit (Qiagen) with Dnase treatment following manufacturer procedure. RNA concentration was determined using a NanoDrop™ 2000/2000c Spectrophotometer, and its integrity was assessed with Experion RNA StdSens analysis kit (Bio-Rad Laboratories Inc.), establishing an RNA quality indicator (RQI) ≥ 7 as a requisite for transcriptomic analysis.

2.6 | PPP and PRP processing for transcriptomic analysis and protein quantification

Two different methods to process PRP samples for transcriptomic analyses were used. PRP and PPP samples PL-1 to PL-14 were aliquoted in 50 ml tubes and directly frozen at −80°C. For subsequent samples, the protocol described by Amisten et al[12] with modifications was followed. From samples PL-15 to PL-27, 45 ml PRP were distributed into three previously weighed tubes (each tube containing 15 ml PRP). The process was repeated for PPP, but tubes were not weighed in this case. Weighed tubes containing PRP were centrifuged for 10 min, 300 *g* at RT, with full acceleration and no brake. Supernatant was discarded and tubes were again weighed to assess the pellet mass. One ml of TRIzol (Sigma) was added to every 100 mg of platelet pellet. Resuspended pellet was divided into 1 ml aliquots and stored at −80°C until RNA extraction.

Seventy-five µl of the remaining PPP and PRP volumes were collected for multiplex protein assays and stored at −80°C until assay performance.

2.7 | Platelet RNA isolation, quantification and quality control

Directly frozen PRP and PPP samples (PL-1 to PL-14) were thawed and RNA was isolated using different methods (TRIzol [Sigma] and Rneasy kits [Qiagen]). The amount of RNA obtained was in every case insufficient for further experiments (<10 ng/µl). Therefore, the protocol was changed for subsequent samples.

Samples PL-15 to PL-27 (frozen in TRIzol) were thawed and maintained at RT for 5 min. Then, 200 µl of cold chloroform (Panreac) were added to each tube containing 1 ml TRIzol. After mixing by inverting the tubes 5 times, they were left to rest for 3 min at RT and later centrifuged for 15 min, 14,000 *g* at 4°C. Approximately 400 µl of supernatant were transferred to new tubes containing 10 µg of ultra-pure glycogen (Thermo Fisher). 500 µl of cold isopropanol (Panreac) were added to each tube and mixed by inversion. Samples were stored overnight at −20°C. Next, samples were centrifuged for 15 min, 14,000 *g* at 4°C. Supernatant was discarded and pellets of the same study subject were pooled and resuspended in 1 ml of ethanol 70% (Panreac). After 10 min of 5500 *g* centrifugation at 4°C, supernatant was discarded. Pellets were dried out at 35–37°C in a thermoblock for 2 min with open tube lids. RNA pellets were resuspended in 30 µl of water, mixed by vortex and frozen at −80°C. Finally, RNA isolation was performed following MinElute Cleanup (Qiagen) kit instructions. RNA contamination with proteins and salts was assessed and RNA was quantified with a NanoDrop™ 2000/2000c Spectrophotometer. RNA integrity was evaluated for two samples (PL-15 and PL-16) by Experion RNA StdSens Starter Kit (Bio-Rad Laboratories Inc.), following manufacturer instructions.

2.8 | Affymetrix

Transcriptomic analysis of $CD3^+$, $CD14^+$ cell and PRP samples (n = 13) was performed using GeneChip Human Gene 2.1 ST strips (Affymetrix, Thermo Fisher Scientific). Following manufacturer instructions, 100 ng RNA from each sample were hybridized using GeneChip™ WT PLUS Reagent Kit. Hybridization details can be found elsewhere.[7]

2.9 | Multiplex protein analysis

Three platelet-associated proteins, P-Selectin, platelet-derived growth factor ββ (PDGFββ) and vascular endothelial growth factor a (VEGF-a), were measured using Luminex technology in undiluted PPP and PRP samples. Proteins were quantified in duplicate in 96-well plates following manufacturer instructions (Thermo Fisher). The plate was analysed on a LUMINEX 200 (Luminex Corp) equipment and xPONENT software (Luminex corp). Samples with a low number of counts (<70) were discarded.

2.10 | Statistical analysis

Mean and standard deviation (SD) were calculated for continuous demographic variables. Affymetrix results were analysed with TAC software (Thermo Fisher). Ebayes ANOVA method was used for comparing gene expression between the three different cell types. Genes were filtered by selecting those with a gene-level fold change <−2 or >2. Statistical significance was set at p-value <.05, and multiple correction was performed using FDR <0.1. Luminex results were analysed with GraphPad Prism 9 software. Protein concentrations in PPP and PRP were compared by paired-Wilcoxon Test, setting statistical significance at p-value <.05.

3 | RESULTS

3.1 | Characteristics of study subjects

Twenty-seven individuals were recruited and subjected to plateletpheresis donation. Of those, three individuals were excluded of the study for different reasons: PL-1 was excluded because $CD3^+$ and $CD14^+$ were not isolated; PL-6 did not complete the plateletpheresis process due to vein rupture during the procedure; and PL-12 PRP did not reach the minimum platelet concentration.

Some of the subjects experienced sickness or dizziness along the process due to the hypocalcaemia caused by the action of the ACD-A anticoagulant. In these cases, a calcium supplement was administered without further incidences.

Of the 24 included study subjects, only 4 were male. The study population was aged 29.5 years (median), ranging between 21 and 53 years (minimum and maximum). Demographics and blood

TABLE 1 Demographics and blood parameters of the study subjects before plateletpheresis

Subject	Sex	Age	Weight (kg)	Height (cm)	WBC (10^9/L)	RBC (10^9/L)	HGB g/dL	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (10^9/L)	NEUT (10^9/L)	LYMPH (10^9/L)	MONO (10^9/L)	EO (10^9/L)	BASO (10^9/L)
PL-02	F	36	74	159	6.23	4.89	13.6	40.2	82.2	27.8	33.8	240	3.18	2.5	0.47	0.05	0.02
PL-03	M	43	87	170	7.63	5.31	16.10	45.90	86.40	30.30	35.10	255.00	4.94	1.86	0.61	0.15	0.05
PL-04	F	35	64	161	6.38	4.59	13.60	39.40	85.80	29.60	34.50	262.00	3.19	2.69	0.40	0.07	0.02
PL-05	F	29	58	164	6.54	4.38	12.70	38.80	88.60	29.00	32.70	311.00	3.79	2.13	0.44	0.10	0.07
PL-07	F	23	61	158	5.38	4.48	13.10	38.50	85.90	29.20	34.00	304.00	3.27	1.59	0.27	0.19	0.02
PL-08	F	26	54	162	7.98	4.81	13.90	40.90	85.00	28.90	34.00	316.00	4.41	2.50	0.67	0.32	0.06
PL-09	F	35	54	167	5.09	5.04	14.80	43.60	86.50	29.40	33.90	161.00	2.79	1.91	0.32	0.05	0.02
PL-10	M	38	76	175	5.85	4.48	14.90	42.50	94.90	33.30	35.10	199.00	3.71	1.34	0.69	0.06	0.03
PL-11	F	26	55	167	5.77	4.29	12.90	39.20	91.40	30.10	32.90	254.00	2.43	2.56	0.57	0.13	0.05
PL-13	F	30	67	160	5.14	4.37	13.00	38.80	88.80	29.70	33.50	206.00	2.77	1.83	0.37	0.12	0.04
PL-14	F	53	73	158	6.35	4.81	13.40	42.10	87.50	27.90	31.80	210.00	3.51	2.12	0.34	0.27	0.09
PL-15	F	46	63	168	4.27	4.54	13.90	40.90	90.10	30.60	34.00	244.00	2.27	1.45	0.30	0.16	0.08
PL-16	F	28	75	168	5.06	4.23	11.80	35.70	84.40	27.90	33.10	203.00	2.57	1.90	0.43	0.11	0.04
PL-17	F	28	68	165	7.06	4.49	13.60	41.10	91.50	30.30	33.10	313.00	3.76	1.88	0.90	0.45	0.06
PL-18	F	40	68	166	11.01	3.91	11.40	34.80	89.00	29.20	32.80	288.00	8.35	1.88	0.55	0.15	0.04
PL-19	M	24	77	178	6.85	5.95	17.70	53.20	89.40	29.70	33.30	194.00	3.25	2.76	0.64	0.14	0.05
PL-20	F	21	56	156	5.70	5.12	14.20	42.90	83.80	27.70	33.10	326.00	2.68	1.86	0.47	0.61	0.07
PL-21	F	27	114	172	7.38	4.13	12.40	36.90	89.30	30.00	33.60	299.00	3.75	2.89	0.49	0.20	0.03
PL-22	F	22	75	163	8.18	4.68	13.20	38.80	82.90	28.20	34.00	263.00	4.58	2.78	0.55	0.16	0.03
PL-23	M	50	85	170	10.79	4.47	13.20	38.80	86.80	29.50	34.00	334.00	6.92	2.79	0.60	0.28	0.05
PL-24	F	41	60	171	7.01	4.50	13.60	41.40	92.00	30.20	32.90	355.00	4.27	2.25	0.31	0.13	0.04
PL-25	F	26	70	168	7.86	4.69	12.60	39.40	84.00	26.90	32.00	225.00	5.32	1.77	0.45	0.22	0.06
PL-26	F	36	52	160	7.63	4.01	12.10	36.80	91.80	30.20	32.90	222.00	4.76	1.94	0.61	0.21	0.07
PL-27	F	26	58	160	7.39	4.41	13.50	40.10	90.90	30.60	33.70	200.00	5.55	1.13	0.50	0.17	0.03
Mean + SD or frequency	84% F/16%M	32.64 ± 8.91	68.48 ± 13.51	165.44 ± 5.7	6.76 ± 1.66	4.58 ± 0.45	13.49 ± 1.34	40.24 ± 3.76	96.79 ± 45.15	31.92 ± 12.48	33.4 ± 1.03	243.68 ± 68.43	3.92 ± 1.47	2.08 ± 0.48	0.49 ± 0.14	0.18 ± 0.12	0.04 ± 0.01

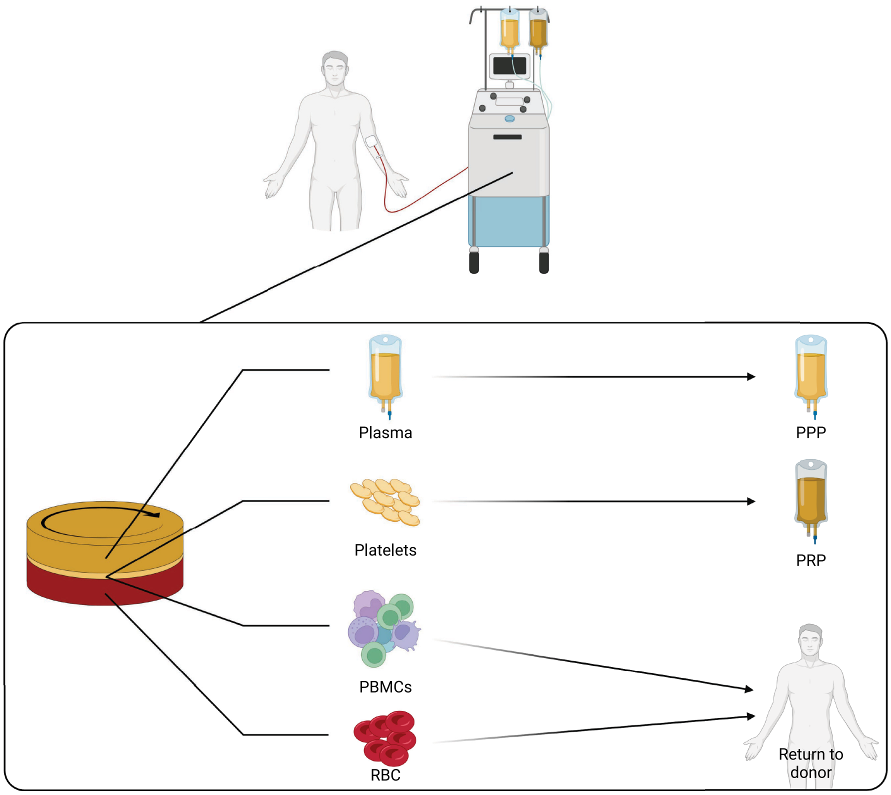


FIGURE 1 Simplified diagram of plateletpheresis. Whole blood is collected from the subject and separated by centrifugation into plasma, red blood cells (RBC) and platelets + white blood cells (WBC). Plasma (or PPP: platelet-poor plasma) is collected in donation bags. Platelets are separated from WBC using Leukoreduction system chambers (LRSC). Purified platelets are then collected in donation bags as PRP (platelet-rich plasma). Finally, RBC and WBC are transferred back to the donor via the same puncture point. Some RBC and WBC remain in the LRSC, usually discarded as a waste plateletpheresis product. PBMCs can be then isolated from it

parameters of the study subjects before the plateletpheresis process are shown in Table 1.

3.2 | Plateletpheresis yielded pure, highly concentrated platelet samples from single donors

Once completed the plateletpheresis process (Figure 1) and after 2 h resting at RT without shaking, PRP samples were analysed on a haemocytometer to assess platelet concentration (Table 2). All the individual samples yielded at least 500×10^9 platelets/L. However, the duration of the process and the processed volume of blood were different for each study subject. The duration of the plateletpheresis procedure mainly depends on the blood platelet counts of the donor before the procedure (Table 1). Other minor affecting factors are the donor's haematocrit levels and the vein access flow. In our study, the average time to complete the donation was 32.14 ± 6.57 min (mean ± SD), and the processed volume of blood was 1127 ± 166.6 ml (mean ± SD) (Table 2).

3.3 | $CD3^+$ and $CD14^+$ cell populations were isolated from a waste product of plateletpheresis process

The cell content of the PBMC fraction obtained from the LRSC was analysed on a haemocytometer. The numbers of total white blood cells (WBC), lymphocytes, monocytes and platelets are collected in Table 3. From those, 10 million monocytes and the corresponding number of lymphocytes were sequentially isolated by positive selection with magnetic beads from each subject. $CD3^+$ and $CD14^+$ fractions were subsequently used for RNA isolation. The purity of the isolated ranged 97.6–98.9% for $CD3^+$ and 96.9–98.8% for $CD14^+$ cells (data not shown). The remaining PBMCs that were not used for $CD3^+$ and $CD14^+$ isolation were frozen and stored for future experiments.

3.4 | Platelet RNA obtained with TRIzol is suitable for Affymetrix transcriptomic analysis and enriched in platelet transcripts compared to $CD3^+$ and $CD14^+$ cell populations

Once all the cell populations (PRP, $CD3^+$ and $CD14^+$) were collected, RNA was isolated by established procedures. We could not obtain valid platelet RNA samples for further transcriptomic analyses from PL-2 to PL-14, as PRP samples were directly frozen after plateletpheresis. Freezing/thawing cycles lyse platelets releasing all their components to the medium. For PRP samples PL-15 to PL-27, the protocol developed by Amisten et al[12] based on TRIzol extraction was used. When assessing the quality of the platelet RNA, the ratios obtained on the Nanodrop were low (Figure 2A). Moreover, the RNA integrity of two platelet samples was analysed by Experion. Neither of them complied with the minimum RQI = 7, established as a quality requirement for transcriptomic studies (Figure 2B–E). This is because the composition of 18s/28s is different in platelets, resulting in a lower RQI than that from nucleated cells[13]; therefore, we did not evaluate more samples with this technique. Nevertheless, we decided to continue further processing platelet RNA samples and analysed their

TABLE 2 Characteristics of plateletpheresis and obtained PRP samples after 2 h resting

Subject	Process duration (min)	Processed blood volume (ml)	PRP volume (ml)	PLT (10^9/L)	PDW (fL)	MPV (fL)	P-LCR (%)	Platelet mass (µl)
PL-02	27	1074	83	1419	10.80	10.00	24.20	15,240.06
PL-03	32	1080	83	1100	9.80	9.60	20.20	11,404.80
PL-04	29	1056	83	1687	9.80	9.70	21.50	17,280.28
PL-05	26	953	83	1310	10.70	10.10	24.70	12,609.14
PL-07	27	964	83	1346	8.80	8.90	15.20	11,548.14
PL-08	28	945	84	1213	10.70	10.00	25.10	11,462.85
PL-09	50	1519	83	561	9.20	9.20	17.30	7839.86
PL-10	38	1406	84	903	7.50	8.40	9.70	10,664.79
PL-11	42	1096	83	700	7.80	8.40	10.40	6444.48
PL-13	35	1286	84	1164	9.30	9.30	18.50	13,921.21
PL-14	32	1228	84	1085	10.40	10.00	23.80	13,323.80
PL-15	35	1151	83	1113	7.40	8.10	8.60	10,376.61
PL-16	-	1275	84	670	8.90	9.00	14.80	7688.25
PL-17	28	955	83	1390	9.00	9.20	17.40	12,212.54
PL-18	29	1035	80	1053	10.80	10.00	24.20	10,898.55
PL-19	40	1358	83	869	8.20	8.80	13.40	10,384.90
PL-20	28	943	83	1189	8.40	8.90	14.30	9978.92
PL-21	-	1072	118	1366	9.10	9.30	18.20	13,618.47
PL-22	26	1035	84	1124	8.40	8.90	15.00	10,353.73
PL-23	22	985	110	1157	7.20	8.10	8.20	9231.12
PL-24	26	903	85	1500	8.50	9.00	15.50	12,190.50
PL-25	35	1208	83	1098	8.40	8.90	14.50	11,804.82
PL-26	36	1236	84	706	8.90	9.00	15.40	7853.54
PL-27	36	1276	83	1256	10.90	10.00	25.00	16,026.56
Mean ± SD	32.13 ± 6.57	1126.62 ± 166.57	85.83 ± 8.80	1124.12 ± 278.98	9.12 ± 1.15	9.20 ± 0.61	17.29 ± 5.35	11,431.58 ± 2661.27

TABLE 3 Concentration and total counts of white blood cells (WBC), lymphocytes, monocytes and platelets collected in the PBMC fraction isolated from the LRSC

Subject	WBC count	LYMPH (10^9/L)	MONO (10^9/L)	PLT (10^9/L)	Total WBC	Total lymph	Total Mono	Total PLT
PL-02	40.60	31.54	8.84	64	2.03E+08	1.58E+08	4.42E+07	3.20E+08
PL-03	20.50	15.68	4.68	137	1.03E+08	7.84E+07	2.34E+07	6.85E+08
PL-04	58.54	50.06	8.15	121	2.93E+08	2.50E+08	4.08E+07	6.05E+08
PL-05	45.18	36.79	8.18	206	2.26E+08	1.84E+08	4.09E+07	1.03E+09
PL-07	14.11	11.03	2.86	5	7.06E+07	5.52E+07	1.43E+07	2.50E+07
PL-08	10.84	7.80	2.82	192	5.42E+07	3.90E+07	1.41E+07	9.60E+08
PL-09	47.05	37.71	8.90	242	2.35E+08	1.89E+08	4.45E+07	1.21E+09
PL-10	96.97	57.08	37.22	333	4.85E+08	2.85E+08	1.86E+08	1.67E+09
PL-11	59.68	46.12	13.27	184	2.98E+08	2.31E+08	6.64E+07	9.20E+08
PL-13	23.19	16.11	6.74	39	1.16E+08	8.06E+07	3.37E+07	1.95E+08
PL-14	55.98	42.34	13.15	411	2.80E+08	2.12E+08	6.58E+07	2.06E+09
PL-15	85.20	66.49	17.99	353	4.26E+08	3.32E+08	9.00E+07	1.77E+09
PL-16	71.08	58.65	11.39	267	3.55E+08	2.93E+08	5.70E+07	1.34E+09
PL-17	24.38	16.10	7.38	259	1.22E+08	8.05E+07	3.69E+07	1.30E+09
PL-18	16.03	12.31	3.39	246	8.02E+07	6.16E+07	1.70E+07	1.23E+09
PL-19	76.43	64.64	11.38	526	3.82E+08	3.23E+08	5.69E+07	2.63E+09
PL-20	37.73	28.71	8.69	670	1.89E+08	1.44E+08	4.35E+07	3.35E+09
PL-21	71.60	71.60	13.27	685	3.58E+08	3.58E+08	6.64E+07	3.43E+09
PL-22	64.18	47.68	16.02	641	3.21E+08	2.38E+08	8.01E+07	3.21E+09
PL-23	88.38	62.00	24.14	1071	4.42E+08	3.10E+08	1.21E+08	5.36E+09
PL-24	7.07	4.91	1.92	52	3.54E+07	2.46E+07	9.60E+06	2.60E+08
PL-25	32.81	19.43	12.82	101	1.64E+08	9.72E+07	6.41E+07	5.05E+08
PL-26	26.33	14.57	14.57	135	1.32E+08	7.29E+07	7.29E+07	6.75E+08
PI-27	23.38	10.35	11.04	100	1.17E+08	5.18E+07	5.52E+07	5.00E+08
Mean ± SD	26.33 ± 23.38	14.57 ± 10.35	14.57 ± 11.04	135 ± 100	1.31E+8 ± 1.16E+8	7.28E+7 ± 5.17E+7	7.28E+7 ± 5.52E+7	6.75E+8 ± 5E+8

transcriptome on the Affymetrix platform, together with CD3+ and CD14+ samples, whose RNA was optimal for further transcriptomic analysis, as determined by the purity ratios and the RQI (Figure 2F,G).

When RNA isolated from PRP, CD3+ and CD14+ cell samples was analysed on the Affymetrix platform, the different cell types were clustered apart from the others in a non-supervised principal component analysis (PCA) (Figure 3A). Moreover, it was confirmed that platelet-related transcripts obtained in previous studies with PBMC samples (presumably containing platelets)[7] were enriched in the PRP (Figure 3B) when compared to the CD3+ and CD14+ cell populations. Among these transcripts were those involved in different platelet functions, such as formation of adhesion complexes (*GP1BA* and *SELP*), aggregation of complexes (*ITGA2B*) or granule secretion and trafficking (*RAB27B*).

CD3+ and CD14+ populations also depicted classical transcriptomic profiles for these populations (Figure S1).

3.5 | PDGFββ, VEGF-a and P-Selectin proteins are increased in the PRP fraction

Finally, we assessed whether PRP and PPP samples could be used for protein measurements. We quantified the content of proteins described to be related to platelets, such as PDGF*ββ*, VEGF-a and P-Selectin by multiplexing assays. We could confirm that PRP samples were enriched in platelet-associated proteins when compared to their matched PPP samples (Figure 4).

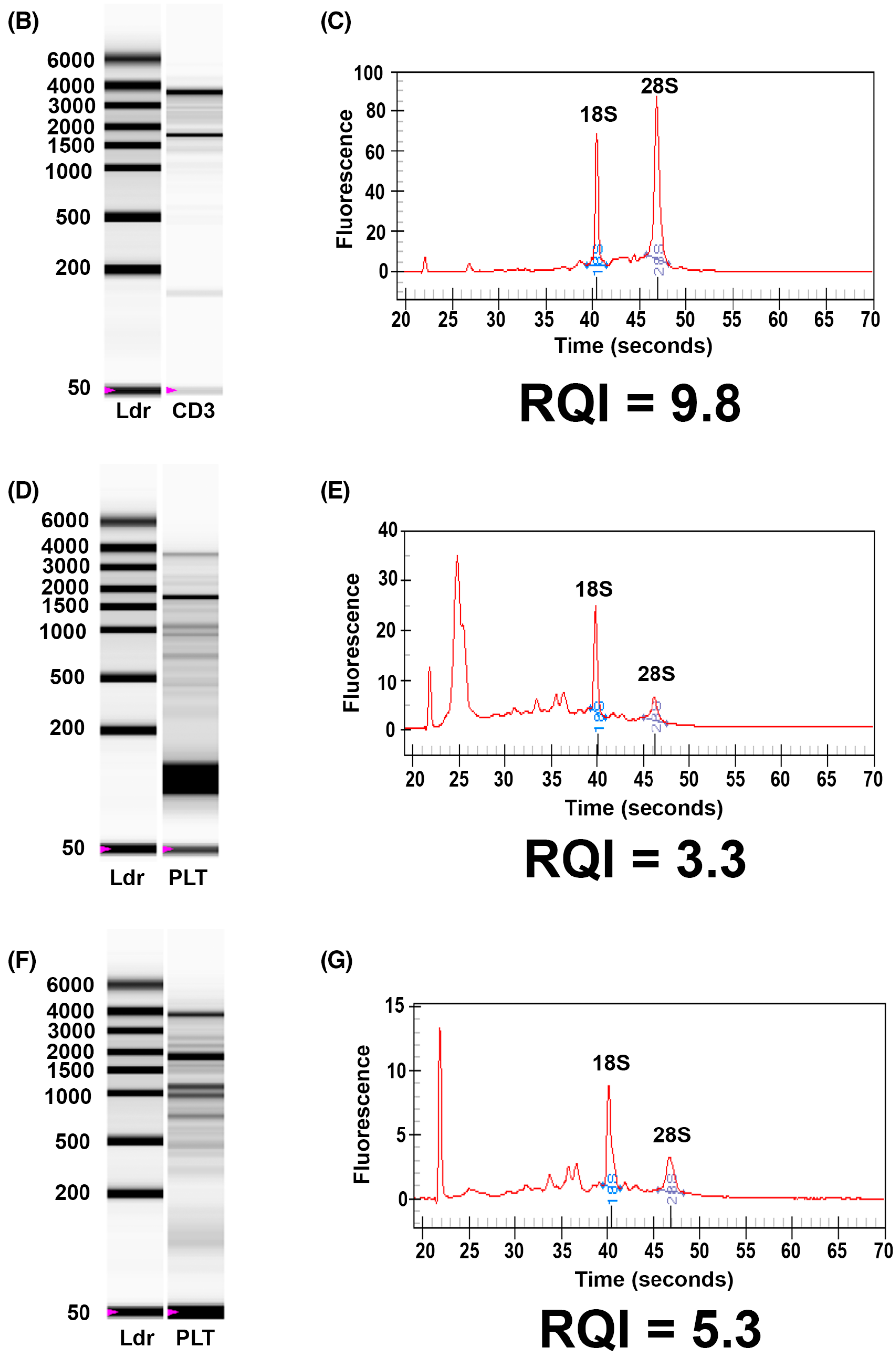
4 | DISCUSSION

There is increasing evidence that platelets can no longer be considered as mere mediators of coagulation, but as important players in

FIGURE 2 RNA quality assessment. Concentration and contamination of platelet RNA was analysed by NanoDrop® before and after amplification for Affymetrix (A). Quality and integrity of platelet RNA was analysed by Experion® before (B, C) and after (D, E) treatment with a CleanUp column (Qiagen). RNA from CD3+ cells was analysed as a control (F, G). RQI: RNA Quality Indicator; Ldr: ladder; PLT: platelet sample

(A)

Subject	PLATELETS					
	[RNA] (ng/ml)	260/280	260/230	[ss-cDNA] (ng/ml)	260/280	260/230
PL-17	115.3	1.96	0.75	492	2.04	1.99
PL-18	81.3	1.52	0.51	354.8	2.07	1.89
PL-19	41.1	1.74	0.75	313.7	2.09	1.88
PL-20	39.8	1.77	0.36	312.7	2.05	1.84
PL-21	167.1	2.01	0.79	303.5	2.1	1.96
PL-22	54.9	1.82	1.07	321.1	2.09	1.9
PL-23	102.2	1.89	1.18	333.4	2.07	1.91
PL-24	25.1	1.3	0.14	115.9	1.88	1.44
PL-25	18.4	1.44	0.05	130.6	1.98	2,32
PL-26	35.9	1.59	0.07	91.1	1.92	2.35
PL-27	42.4	1.7	0.31	65.8	1.9	2.45



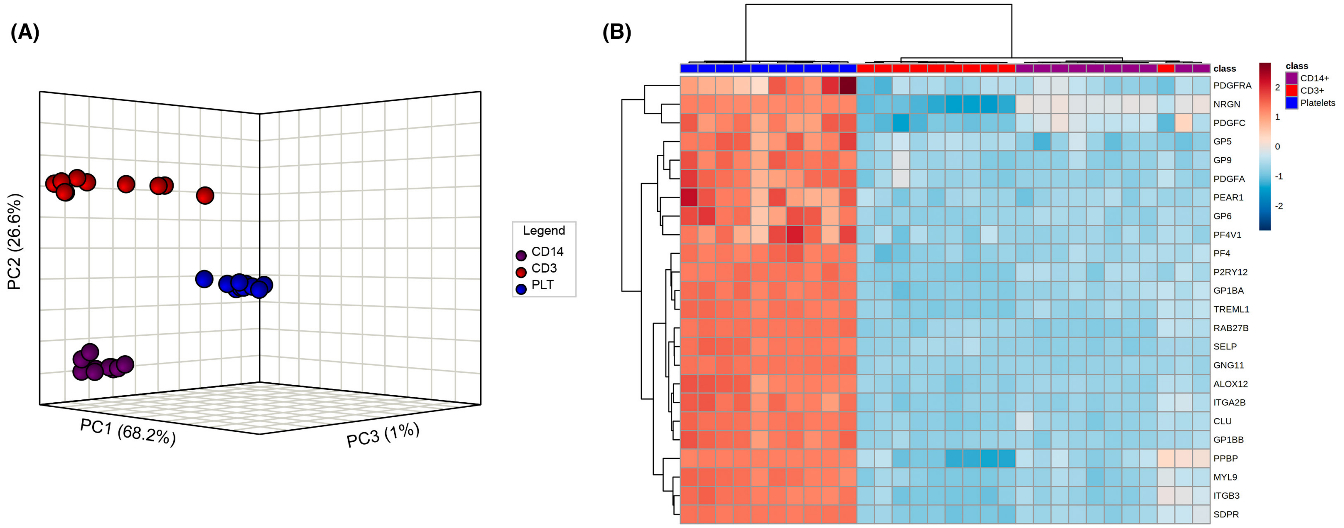


FIGURE 3 Transcriptomic differences between platelets, $CD3^+$ and $CD14^+$ samples. (A). Unsupervised multivariate analysis of transcriptomic data was used to analyse the clustering of platelet (blue), $CD3^+$ (purple) and $CD14^+$ (red) samples. (B) Heatmap of platelet-related genes expressed in platelet (blue), $CD3^+$ (purple) and $CD14^+$ (red) samples. Upregulated genes are represented in red and downregulated genes are shown in blue. ANOVA *p-value* <.05. FDR *p-value* <.05

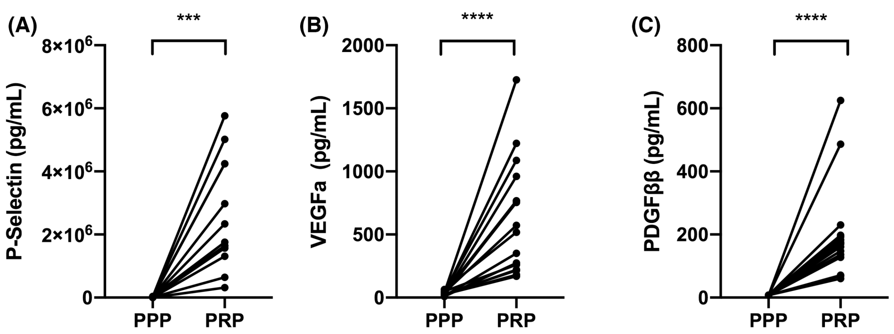


FIGURE 4 Platelet-associated molecules are increased in PRP samples. P-Selectin (A), VEGF-a (B) and PDGFββ (C) quantification by Luminex in PPP and PRP samples. ***Paired-Wilcoxon Test *p-value* <.005, **** Paired-Wilcoxon Test *p-value* <.001

inflammatory and immune-modulatory processes.[2] The transcriptional landscape of human platelets has been reported altered during sepsis.[14] Several studies have pointed out modifications on platelet functions in inflammatory diseases such as Chronic Obstructive Pulmonary Disease (COPD)[15] or COVID-19 severity and mortality.[16] Moreover, increased levels of platelet-derived mediators have been noted in peripheral blood and bronchoalveolar lavage fluid (BALF) of asthmatic patients, suggesting increased levels of platelet activation.[17] We previously found that severe food-associated respiratory allergy could be associated with the alteration of platelet functions.[7] Since then, our aim had been to study platelet phenotype and role in severe allergic patients. However, the lack of an optimized method to obtain pure and functional platelet samples from single donors has made their characterization so far difficult or inaccurate. Here, we describe a high-yielding method for the collection of a pure platelet-rich product from single donors based on plateletpheresis for multi-omic studies.

Plateletpheresis is not commonly used for research purposes. Efficient platelet collection is nevertheless routinely performed by plateletpheresis in blood donation centres for donation purposes.[18,19] Since specialized equipment and staff training are required, few research studies have so far used this technique.[20] The most commonly used technique for isolating pure platelets is based on successive centrifugation steps. As previously discussed, this technique requires large blood volumes[12]; thus, many studies report using pools of samples from different donors, instead of individual samples.[13] Moreover, platelets suffer mechanical stress during the multiple centrifugation steps, which leads to platelet activation and release of molecules contained in platelet granules.[11] Therefore, the interpretation of results could be altered. Nevertheless, this activation can be minimized by allowing the isolated platelets to rest, as it is described in our protocol for PRP samples obtained by plateletpheresis.

General research has traditionally focused on the study of blood leukocyte populations. For that, density-gradient centrifugation has been widely applied for the isolation of these cell types. However, due to their density, platelets are also retained in the cell layer after blood centrifugation together with leukocytes. This was the case in our previous study,[7] where we identified a set of transcripts involved in platelet functions contained in PBMC samples. This highlights the fact that the extraction of pure platelet samples is difficult using classical laboratory techniques.

In contrast, plateletpheresis allows the collection of pure rich platelet samples from a single donor.[18] Hence, plateletpheresis not only provides a higher purity of the collected platelet samples, but also a higher yield when compared to traditional blood centrifugation. This opens an avenue to perform platelet research studies without contamination with other blood cell populations and without the need of pooling samples.

Moreover, plateletpheresis allows collecting acellular plasma (PPP) and functional leukocytes from the LRSC, usually discarded as a waste product of the plateletpheresis procedure.[21] Therefore, from a single donor we can obtain matched samples of PRP, PPP and leukocytes, simplifying sampling and minimizing sample contamination.

In the present study, we have validated the suitability of platelet samples obtained by plateletpheresis for omic studies. With the method described here, PRP samples can be used for protein determination by multiplex and RNA isolation for transcriptomic studies.

We described that the protein levels of platelet-related factors such as VEGF-a, PDGFββ and P-Selectin are higher in PRP samples than in their PPP counterparts. Previous studies have reported platelet-rich concentrates as a source of platelets and growth factors, including transforming growth factor-beta (TGF-beta) and PDGF.[22] VEGF-a is a member of the PDGF/VEGF family.[23] Current evidence implies that platelets not only contribute structurally but instructively to vascular remodelling. In this sense, platelets are major storage and delivery vehicles for pro- and anti-angiogenic growth factors including VEGF-a.[24] PDGFββ is involved in the recruitment and differentiation of pericytes that are smooth muscle-like cells found in close contact with the endothelium in capillaries, where they regulate the morphology and function of the vessels during vessel formation.[25] Recent research has also implicated PDGFββ as a potential contributing factor to cell signalling pathway of mesenchymal stem cells, human dermal fibroblasts, pericytes and smooth muscle cells, among others, in various interrelated diseases.[26] In turn, P-selectin is one of the most bioactive molecules contained in α-granules and involved in inflammation. It promotes platelet aggregation and platelet interactions with both leukocytes and endothelial cells.[27]

At the transcriptional level, we have observed an enrichment in platelet-related transcripts in PRP samples compared to their matched $CD3^+$ and $CD14^+$ counterparts. The transcriptomic results presented here, therefore, validate plateletpheresis as a suitable method for obtaining pure platelet samples and suggest that the findings involving platelet transcripts previously obtained from PBMCs were most presumably due to platelet presence in the cell layer fraction obtained by density-gradient centrifugation.[7,28] This set of platelet transcripts includes those involved in the following platelet functions: formation of adhesion complexes (*GP1BA* and *SELP*)[29,30]; receptors (*GP6*, *P2RY12*)[31]; aggregation of complexes (*ITGA2B*)[31]; granule secretion and trafficking (*RAB27B*)[32–34] and activation and response to elevated platelet cytosolic Ca^{2+} (*PF4*).[35]

It is worth mentioning that RNA of PRP samples was valid and used for transcriptomic analysis, regardless of the integrity and quality determinations. In this regard, it can be concluded that the methods usually employed to assess RNA quality are not suitable for platelet RNA. Platelets are known to have a complex transcriptome including mRNA and miRNA, among others.[36] RNA was detected in platelets over 30 years ago; however, we are currently only beginning to unravel the complexity of the platelet transcriptome.[37] The use of RNA sequencing or genome-wide studies with platelet RNA has enabled us to expand our knowledge on platelet biology and functions since a rising number of studies have been dedicated to this topic.[13,38–40] Therefore, it is essential to use standardized and appropriate tools for isolating, quantifying and characterizing the complex and rich platelet RNA repertoire.

In this study, we have developed a protocol based on plateletpheresis to obtain pure PRP samples, as well as PPP, $CD3^+$ and $CD14^+$ matched cell samples from single donors. These samples are suitable for protein and transcriptomic studies. Therefore, this methodology could be used for phenotyping platelets as well as other cell populations in immune diseases and contribute to unravel their role in inflammatory diseases such as allergy.

AUTHOR CONTRIBUTION

CG-C and JLB designed the study and together with DB and MME supervised the research. JLB supervised sample collection and together with LNMB and MRM recruited the study subjects. JS-S, LM-B, MID-D and CP-T processed the samples and performed the experiments. CP-T, MID-D and CG-C drafted the manuscript. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest in relation to this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GEO (Gene Expression Omnibus) at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE203196, reference number GSE203196.

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REFERENCES

1. Luo L, Zhang J, Lee J, Tao A. Platelets, not an insignificant player in development of allergic asthma. *Cell*. 2021;10(8):2038.
2. Gomez-Casado C, Villaseñor A, Rodriguez-Nogales A, Bueno JL, Barber D, Escribese MM. Understanding platelets in infectious and allergic lung diseases. *Int J Mol Sci*. 2019;20(7):1730.
3. Chen Y, Zhong H, Zhao Y, Luo X, Gao W. Role of platelet biomarkers in inflammatory response. *Biomark Res*. 2020;8(1):28. doi:10.1186/s40364-020-00207-2
4. Kowal K, Pampuch A, Kowal-Bielecka O, DuBuske LM, Bodzenta-Łukaszyk A. Platelet activation in allergic asthma patients during allergen challenge with Dermatophagoides pteronyssinus. *Clin Exp Allergy*. 2006;36(4):426-432.
5. Nastałek M, Potaczek DP, Wojas-Pelc A, Undas A. Plasma platelet activation markers in patients with atopic dermatitis and concomitant allergic diseases. *J Dermatol Sci*. 2011;64(1):79-82. doi:10.1016/j.jdermsci.2011.07.001

6. Tamagawa-Mineoka R, Katoh N, Ueda E, Masuda K, Kishimoto S. Elevated platelet activation in patients with atopic dermatitis and psoriasis: increased plasma levels of β-thromboglobulin and platelet factor 4. *Allergol Int*. 2008;57(4):391-396.
7. Obeso D, Mera-Berriatua L, Rodríguez-Coira J, et al. Multi-omics analysis points to altered platelet functions in severe food-associated respiratory allergy. *Allergy Eur J Allergy Clin Immunol*. 2018;73(11):2137-2149.
8. Barber D, Villaseñor A, Escribese MM. Metabolomics strategies to discover new biomarkers associated to severe allergic phenotypes. *Asia Pac Allergy*. 2019;9(4):e37. doi:10.5415/apallergy.2019.9.e37
9. Amisten S, Braun OÖ, Bengtsson A, Erlinge D. Gene expression profiling for the identification of G-protein coupled receptors in human platelets. *Thromb Res*. 2008;122(1):47-57. doi:10.1016/j.thromres.2007.08.014
10. Bugert P, Dugrillon A, Günaydin A, Eichler H, Klüter H. Messenger RNA profiling of human platelets by microarray hybridization. *Thromb Haemost*. 2003;90(4):738-748.
11. Jang C-S, Kim S-I, Kim H-K, et al. Plateletpheresis: the process, devices, and indicators of product quality. *J Life Sci*. 2014;30(24):1030-1038.
12. Amisten S. A rapid and efficient platelet purification protocol for platelet gene expression studies. In: Gibbins JM, Mahaut-Smith MP, eds. *Platelets and Megakaryocytes: Volume 3, Additional Protocols and Perspectives*. Springer New York; 2012:155-172. doi:10.1007/978-1-61779-307-3_12
13. Rowley JW, Oler AJ, Tolley ND, et al. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood*. 2011;118(14):e101-e111. doi:10.1182/blood-2011-03-339705
14. Middleton EA, Rowley JW, Campbell RA, et al. Sepsis alters the transcriptional and translational landscape of human and murine platelets. *Blood*. 2019;134(12):911-923. doi:10.1182/blood.2019000067
15. Maclay JD, McAllister DA, Johnston S, et al. Increased platelet activation in patients with stable and acute exacerbation of COPD. *Thorax*. 2011;66(9):769-774. http://thorax.bmj.com/content/66/9/769.abstract
16. Hottz ED, Azevedo-Quintanilha IG, Palhinha L, et al. Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. *Blood*. 2020;136(11):1330-1341. doi:10.1182/blood.2020007252
17. Turkalj M. The role of platelets in allergic inflammation and asthma. In: IBE-C P, ed. *Asthma*. IntechOpen; 2019:Ch. 5. doi:10.5772/intechopen.85114
18. Bueno JL, García F, Castro E, Barea L, González R. A randomized crossover trial comparing three plateletpheresis machines. *Transfusion*. 2005;45(8):1373-1381.
19. Bueno JL, Barea L, García F, Castro E. A comparison of PLT collections from two apheresis devices. *Transfusion*. 2004;44(1):119-124.
20. Bueno JL, Ynigo M, de Miguel C, et al. Growth differentiation factor 11 (GDF11) - a promising anti-ageing factor - is highly concentrated in platelets. *Vox Sang*. 2016;111(4):434-436.
21. Pfeiffer IA, Zinser E, Strasser E, et al. Leukoreduction system chambers are an efficient, valid, and economic source of functional monocyte-derived dendritic cells and lymphocytes. *Immunobiology*. 2013;218(11):1392-1401.
22. Mehta S, Watson JT. Platelet rich concentrate: basic science and current clinical applications. *J Orthop Trauma*. 2008;22(6):432-438. https://journals.lww.com/jorthotrauma/Fulltext/2008/07000/Platelet_Rich_Concentrate__Basic_Science_and.12.aspx
23. Shibuya M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J Biochem*. 2013;153(1):13-19. doi:10.1093/jb/mvs136
24. Rafii DC, Psaila B, Butler J, Jin DK, Lyden D. Regulation of vasculogenesis by platelet-mediated recruitment of bone marrow-derived cells. *Arterioscler Thromb Vasc Biol*. 2008;28(2):217-222.
25. Hellberg C, Ostman A, Heldin C-H. PDGF and vessel maturation. *Recent Results Cancer Res*. 2010;180:103-114.
26. Wang C, Liu Y, He D. Diverse effects of platelet-derived growth factor-BB on cell signaling pathways. *Cytokine*. 2019;113:13-20. https://www.sciencedirect.com/science/article/pii/S1043466618304071
27. Nurden AT. Platelets, inflammation and tissue regeneration. *Thromb Haemost*. 2011;105(S 06):S13-S33.
28. Uerpmann-Wittzack R. European Directorate for the Quality of Medicines and Healthcare (EDQM). *The Council of Europe: Its Law and Policies*. European Directorate for the Quality of Medicines & HealthCare; 2017:394-406.
29. Othman M, Emsley J. Gene of the issue: GP1BA gene mutations associated with bleeding. *Platelets*. 2017;28(8):832-836.
30. Rondina MT, Voora D, Simon LM, et al. Longitudinal RNA-seq analysis of the repeatability of gene expression and splicing in human platelets identifies a platelet SELP splice QTL. *Circ Res*. 2020;126(4):501-516. doi:10.1161/CIRCRESAHA.119.315215
31. Rumbaut RE, Thiagarajan P. No Title. San Rafael (CA); 2010.
32. Tanya T, Magnus Å, Futter CE, Authi KS, Seabra MC. Rab27b regulates number and secretion of platelet dense granules. *Proc Natl Acad Sci*. 2007;104(14):5872-5877. doi:10.1073/pnas.0609879104
33. Tiwari S, Italiano JEJ, Barral DC, et al. A role for Rab27b in NF-E2-dependent pathways of platelet formation. *Blood*. 2003;102(12):3970-3979.
34. Chen D, Guo J, Miki T, Tachibana M, Gahl WA. Molecular cloning and characterization of rab27a and rab27b, novel human Rab proteins shared by melanocytes and platelets. *Biochem Mol Med*. 1997;60(1):27-37.
35. Sikara MP, Vlachoyiannopoulos PG. FRI0273 plasma levels of PF-4var/cxcl4l1, a nonallelic variant of platelet factor-4 (PF-4/CXCL4), are elevated in patients with antiphospholipid syndrome (APS). *Ann Rheum Dis*. 2013;72(Suppl 3):A466 LP-A466. http://ard.bmj.com/content/72/Suppl_3/A466.3.abstract
36. Davizon-Castillo P, Rowley JW, Rondina MT. Megakaryocyte and platelet transcriptomics for discoveries in human health and disease. *Arterioscler Thromb Vasc Biol*. 2020;40(6):1432-1440. doi:10.1161/ATVBAHA.119.313280
37. Rowley JW, Schwertz H, Weyrich AS. Platelet mRNA: the meaning behind the message. *Curr Opin Hematol*. 2012;19(5):385-391.
38. Ranucci M, Ballotta A, Di Dedda U, et al. The procoagulant pattern of patients with COVID-19 acute respiratory distress syndrome. *J Thromb Haemost*. 2020;18(7):1747-1751. doi:10.1111/jth.14854
39. Sol N, Wurdinger T. Platelet RNA signatures for the detection of cancer. *Cancer Metastasis Rev*. 2017;36(2):263-272.
40. Supernat A, Popęda M, Pastuszak K, Best MG, Grešner P, et al. Transcriptomic landscape of blood platelets in healthy donors. *Sci Rep*. 2021;11(1):15679. doi:10.1038/s41598-021-94003-z

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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