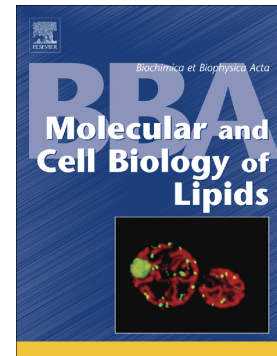# Accepted Manuscript

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Lipid mediators in platelet concentrate and extracellular vesicles: molecular mechanisms from membrane glycerophospholipids to bioactive molecules

Sami Valkonen[a,b§], Minna Holopainen[b,c§], Romain A. Colas[d], Ulla Impola[b], Jesmond Dalli[d], Reijo Käkelä[c,e], Pia R.-M. Siljander[a], Saara Laitinen[b*]

[a]*EV group, Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Biocenter 1, Viikinkaari 9, 00790, Helsinki, Finland*

[b]*Finnish Red Cross Blood Service, Kivihaantie 7, 00310, Helsinki, Finland*

[c]*Helsinki University Lipidomics Unit, Helsinki Institute for Life Science (HiLIFE), Biocenter 3, Viikinkaari 1,00790, Helsinki, Finland*

[d]*Lipid Mediator Unit, William Harvey Research Institute, Barts and the London School of Medicine, Queen Mary University of London, John Vane Science Centre, Charterhouse Square, London, EC1M 6BQ, United Kingdom*

[e]*Molecular and Integrative Biosciences Research Programme, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland*

[§]*Equal contribution*

[*]*Corresponding author*

Abstract

Platelets are collected for transfusion to patients with different hematological disorders, and for logistical reasons, platelets are stored as concentrates. Despite the carefully controlled conditions, platelets become activated during storage, and platelet concentrates (PLCs) may cause adverse inflammatory reactions in the recipients. We studied by mass spectrometry the lipidomic changes during storage of the clinical PLCs, the platelets isolated from PLCs, and the extracellular vesicles (EVs) thereof. The release of EVs from platelets increased with the prolonged storage time. The molar percentages of arachidonic acid -containing species were increased during storage especially in the phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine classes of glycerophopholipids. The increase of these species in the membrane glycerophopholipid composition paralleled the production of both proinflammatory and proresolving lipid mediators (LMs) as the amount of the arachidonic acid-derived LMs such as thromboxane B_2 and prostaglandin E_2 also increased in time. Moreover, several monohydroxy pathway markers and functionally relevant proinflammatory and proresolving LMs were detected in the PLC and the EVs, and some of these clearly accumulated during storage. By Western blot, the key enzymes of these pathways were shown to be present in the platelets and in many cases also in the EVs. Since the EVs were enriched in the fatty acid precursors of LMs, harbored LM-producing enzymes, contained the related monohydroxy pathway markers, and also secreted the final LM products, the

PLC-derived EVs appear to have the potential to regulate inflammation and healing, and may thereby aid the platelets in exerting their essential physiological functions.

Keywords

Platelet, Extracellular vesicle, Glycerophospholipid, Lipid mediator, Specialized proresolving mediator, Immunology

Uncommon abbreviations

EV, Extracellular vesicles

LM, Lipid mediator

NTA, Nanoparticle tracking analysis

PLC, Platelet concentrate

SPM, Specialized proresolving mediator

1 Introduction

Fatty acids and membrane lipids, besides being structural components and the means for storing energy, play significant roles in cell signaling pathways (1–3). For platelets, membrane lipids are a prerequisite for their optimal functioning and therefore, membrane lipidome is under constant enzymatic remodeling and regulation (4). The glycerophopholipid (GPL)-derived polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), n-3 docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), and arachidonic acid (AA), are used to produce LMs, which play vital roles in the different phases of inflammation, including the resolution and healing process (5–8). In contrast to the well-characterized time-dependent platelet GPL alterations and the related mechanisms (9–12), the role of LMs in platelets is incompletely understood. Specific LMs, like thromboxane (Tx)A_2 and prostaglandin (PG)E_2 are well-known for their functions in platelets (13,14), but the biological actions of the recently discovered proresolving LMs such as resolvins (Rvs), protectins and maresins remains of interest (15–18) in both platelets and extracellular vesicles (EVs) thereof.

Platelet concentrates (PLC) are widely used in clinical settings to treat patients with thrombocytopenia (low platelet count), to replace platelets in the case of massive bleeding, and to prevent bleeding of patients with cancer, malignant blood diseases, or other malfunctions of platelets. The lifespan of a platelet in the circulation is typically 8 to 10 days (19), and currently the storage time of clinical PLCs is 5 to 7 days (20). In addition to replenishing the patient's platelet count to a haemostatically functional level, clinical PLCs may contribute to the overall immunological status of a patient, as platelets communicate with and modulate other cells involved in immunological reactions (21–26).

One way of cell communication is mediated through the secretion of EVs which are protein-, lipid-, metabolite-, and nucleic acid -containing lipid bilayered particles produced by most cells (27,28). Platelets are sensitive cells that are activated through multiple ways including mechanical stress

(29), chemical agonists (30), as well as aging (31). When activated, platelets produce EVs with an activation-dependent composition (32,33). During storage, the platelet activation state as well as the concentration of EVs increase in the PLC (34–36), which may have an impact on the recipient's response. The molecular mechanisms underlying the adverse transfusion responses, such as allergic and febrile nonhemolytic reactions, are however, still poorly understood (37). Analogously, the increasing numbers of EVs of an aging erythrocyte concentrate have been linked to a proinflammatory host response (38).

During aging, at least two types of phospholipid-related alterations occur in platelets within the PLC: following the depletion of ATP, the membrane phospholipid asymmetry cannot be maintained by the lipid translocating enzymes and is eventually lost (39), an event tied to the EV formation (40). In addition to the defective control of transbilayer lipid distribution, lipid peroxidation and EV secretion cause losses of phospholipid content, especially the polyunsaturated lipid species (12,41). As the acyl chains of the membrane GPLs may be cleaved and converted to LMs, it is important to investigate the abundance of the molecular species within the different GPL classes along with the enzymes required for the LM biosynthesis (42). Depending on the presence of precursor PUFAs and the enzymes modifying them, the resulting downstream LMs exhibit proinflammatory properties, proresolving properties, or have dual functions (7,43,44), and depending on the function, they are generally categorized as proinflammatory mediators or specialized proresolving mediators (SPMs). SPMs modulate the resolution, i.e. the active dampening phase of inflammation, in several disease models (5,45,46). It has been demonstrated that only pico- to nanogram quantities of SPMs are required to exert their effects on cellular functions (6), and the recent technological development has now enabled discovery of novel SPM structures. Therefore, comprehension of the time-dependent LM alterations in the clinical PLC and within the EV lipidomes is crucial to uncover the impact of lipids on the immunological cells of a patient receiving the clinical PLC. Thus, in PLCs, it is of paramount importance to study i) how the shifts in the relative amount of PUFA-containing GPL-precursors translate to the amount and profiles of LMs, ii) whether certain LMs or their pathway markers are transported in EVs or platelets, and iii) to what extent the LMs originated from platelets or EVs thereof influence the surrounding cells. By solving these questions, we can begin to elucidate the effects of transfusion from an immunological perspective.

In the current study, the time-dependent alterations in the GPL composition during storage of clinical PLCs were investigated from the PLCs, platelets, and the PLC EVs. Secondly, the presence of specific enzymes required for LM production was verified. Furthermore, the LMs of the PLC and EVs were profiled in order to unravel, whether and where certain LMs become more prevalent in the PLC as a function of time.

2 Materials and Methods

2.1. Sample preparation

Standard leukocyte-reduced clinical grade PLCs derived from buffy coats of four ABO RhD -matched whole blood donations were obtained from the Finnish Red Cross Blood Service (Helsinki, Finland) and were handled anonymously, as accepted by Finnish Supervisory Authority for Welfare and Health (Valvira, Finland).

Sterile sampling was done using 50 mL syringes (Henke-Sass, Wolf GmbH, Tuttlingen, Germany) and 18 gauge needles (Terumo, Tokyo, Japan). Before sampling, the contents of the storage bag's tube were emptied into the storage bag and the PLC was mixed by gently turning it from side to side 5 times. This procedure was repeated 3 times to obtain a representative sample. After extracting 20 mL of sample via the storage bag's tube, the tube was resealed. The sampling days (d) were d1, d2, d5, or d8 counting from the blood donation (d0), where d1 was the production day of a PLC. The d1 sampling was done within 2 hours after the PLCs were available from the production line, at around 3 p.m., but the d2 - d8 samplings were done 9 a.m. PLCs were stored at 22 °C under constant horizontal agitation and subjected to standard quality control of Finnish Red Cross Blood Service (visual inspection of PLC, and determination glucose concentration, lactate concentration, potassium concentration, pCO_2, pO_2, pH, residual leukocyte count). Additionally microbiological cultures were prepared from the PLCs after final sampling day to exclude possible microbial contamination (data not shown).

Aliquots of 250 µL of untreated PLC were snap-frozen and put under argon gas to store the lipids unaffected. The PLC samples were stored in -70 °C until the analysis. A total of 17 mL of PLC was used for pEV isolation. To prevent platelet activation, Anticoagulant Citrate Dextrose Solution pH Eur Solution A (Terumo BCT, Lakewood, CO, USA) and Apyrase (Sigma-Aldrich, St. Louis, MO, USA) were added to the final concentrations of 4.25% v/v and 2 U/mL, respectively, and the PLCs were diluted 1:4 with phosphate buffered saline (PBS (Thermo Fisher Scientific, Waltham, MA, USA)). The diluted PLCs were centrifuged at $650 \times g$ at RT for 7 minutes (Eppendorf centrifuge 5810R, (Eppendorf, Hamburg, Germany)) without brake, and the pelleted platelets were placed under argon and snap frozen. The supernatant was centrifuged $1560 \times g$ in RT for 20 minutes (Eppendorf centrifuge 5810R) and platelet content of the supernatant was reduced to 1×10^6 platelets/mL, as confirmed with Coulter Cell counter T-540 (Beckman Coulter). To extract the whole pEV population from the PLC without differentiating exosomes and microvesicles, the supernatant was ultracentrifuged at $100000 \times g$ at 4 °C for 1 hour (MLA-50 rotor, k-factor 92 (Beckman Coulter)). The supernatant was carefully decanted and remaining supernatant was removed with a pipette, after which the EV pellet was resuspended into 200 µL of PBS and divided to two aliquots of 90 µL for lipid analysis and 20 µL aliquot for Nanoparticle Tracking Analysis (NTA) in Protein LoBind tubes (Eppendorf). Immediately after preparing the aliquots, samples were snap-frozen, stored under argon and placed in -70 °C until lipid analysis.

2.2. Nanoparticle Tracking Analysis

Particle number and size distribution of the particles in EV samples were analyzed with NTA instrument LM14C equipped with violet (405 nm, 70 mW) laser (Malvern Instruments Ltd., Malvern, UK) and sCMOS camera (Hamamatsu photonics K.K., Hamamatsu, Japan) in controlled temperature of 22.0 °C and camera level 14 using Nanosight software 3.0 (Malvern Instruments Ltd.). EV samples were diluted 1:1000, 1:2000, 1:5000 and 1:10000 with filtered (0.2 µm) PBS on d1, d2, d5 and d8 samples, respectively, and three videos of 90 seconds were recorded from samples, mixing the sample manually between measurements. Data were analyzed with NanoSight NTA 3.0 software using detection threshold 5 and gain 10.

2.3. Glycerophospholipid analysis

GPLs of the PLC, platelet, and EV samples were analyzed as reported previously (47). Briefly, total lipids were extracted using Folch extraction (48), and the samples, spiked with internal standards and supplemented with 1% NH4OH, were infused into electrospray ionization source of triple quadrupole mass spectrometer (MS) Agilent 6490 Triple Quad LC/MS with iFunnel technology (Agilent Technologies, Santa Clara, CA, USA) using a flow rate of 10 μL/min, ion source temperature of 250 °C, instrument collision energies of 5 – 45 eV depending on the lipid class, and nitrogen as the nebulizing (20 psi) and the drying gas (11 μL/min at 250 °C).

After the MS+ or MS− scan survey, the GPL species were detected using MS/MS scans specific to a given GPL class (49). The resulting spectra were processed using MassHunter Workstation Qualitative Analysis Software (Agilent Technologies, Inc.) and the quantification of individual GPL species was conducted using free software called Lipid Mass Spectrum Analysis (50) through comparison of the sample peak intensities to the internal standards with known concentrations. Results were determined and expressed as molar percentages (mol%) calculated separately to each GPL class. In order to keep technical methodological variation at minimum level, the average relative concentration of a GPL species had to exceed 0.5 mol% across all sample types and time points within given GPL class in order to be included in the quantification. For bar graph visualization, GPL species abundance threshold was set to > 1 mol% (after normalizing data to 100 mol%, when species contributing < 0.5 mol% were deducted) in every sample type studied.

2.4. Western blotting

To ensure sufficient sample material for Western blot analysis, a separate sampling was conducted, where the sampling volume was increased to 50 mL. Samples were collected from two PLCs on d1 and d8, and sample collection and processing was conducted similarly as above. One tablet of cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland) was dissolved to 10 mL of PBS and a volume equaling to 1/6 of sample volume was added to samples before determining the protein content using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The samples containing 25 μg of protein for each well were prepared together with 4x Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 10% 2-mercaptoethanol (Sigma-Aldrich) and boiled for 5 minutes. Then samples were loaded to Mini-PROTEAN TGX Stain-Free protein gels with 4-20% gradient (Bio-Rad) in biological duplicates together with Precision Protein Plus WesternC Blotting Standard (Bio-Rad). Gels were run for 50 minutes with 170 V in 1× Tris/Glycine/SDS Buffer (Bio-Rad), and the proteins were blotted for 20 minutes with 1.3 A up to 25 V using semi-dry blotting machine Trans-Blot Turbo (Bio-Rad), 1× Transfer Buffer (Bio-Rad) including 20% methanol (Merck, Darmstadt, Germany), and Trans-Blot Turbo Mini Nitrocellulose Transfer Packs (Bio-Rad), where original 0.2 μm nitrocellulose membrane was replaced with 0.45 μm nitrocellulose membrane (Bio-Rad). Transfer of the proteins was confirmed by imaging gels and membranes using ChemiDoc Touch Imaging System (Bio-Rad), followed by 1 hour blocking of the membrane in room temperature with 6% milk solution (Valio, Helsinki, Finland), prepared in 1 × Tris buffer (Sigma-Aldrich) containing 0.05% Tween20 (Sigma-Aldrich).

Antibodies against 12-Lipoxygenase ((12-LOX) Novus Biologicals, Littleton, CO, USA, clone 1C3), 15-Lipoxygenase 1 ((15-LOX1) Novus Biologicals, clone 3G8), 15-Lipoxygenase 2 ((15-LOX2) Novus Biologicals, clone 4A7), Apolipoprotein (Apo)A1 (Medix Biochemica, Espoo,

Finland, product 100264), ApoB (Medix Biochemica, product 100261), CD9 (Becton Dickinson, Franklin Lakes, NJ, USA, clone M-L13), CD41 (Beckman Coulter, clone sz22), CD63 (Becton Dickinson, clone H5C6), Cyclooxygenase (COX)1 (Thermo Fisher Scientific, Waltham, MA, USA, clone AS70), COX2 (Thermo Fisher Scientific, clone AS66) Cytochrome p450 (CYP) 1A1 (Novus Biologicals, clone 6G5), CYP2J2 (Novus Biologicals, clone 2D10), CYP5A1 (Novus Biologicals, clone OTI2C1), cytosolic Phospholipase A_2 ((cPLA$_2$) Santa Cruz Biotechnology, Inc., Dallas, TX, USA, clone sc-454), and sectetory Phospholipase A_2 ((sPLA$_2$), Abcam plc, Cambridge, UK, ab23705) were diluted 1:200 (15-LOX1, 15-LOX2 and CYP2J2), 1:250 (CD9 and CD63), 1: 1:500 (COX1, COX2, cPLA$_2$), 1:1000 (ApoA1, ApoB, CYP1A1, CYP5A1, sPLA$_2$), 1:2000 (12-LOX), or 1:10000 (CD41) in $1 \times$ Tris buffer containing 2% milk and 0.05% Tween20 and incubated overnight. The membranes were first rinsed and then washed with Tris buffer containing 0.05% Tween20 3×10 minutes followed by incubation with Goat Anti-Mouse or Anti-Rabbit IgG (H + L)-HRP Conjugate secondary antibodies (Bio-Rad), containing Precision Protein StrepTactin-HRP Conjugate (Bio-Rad) diluted 1:3000 and 1:10000, respectively, to Tris buffer containing 2% milk and 0.05% Tween20. After incubation, the membranes were first rinsed and then washed 2×10 minutes in Tris buffer containing 0.05% Tween20 and 10 minutes in Tris buffer, followed by addition of 1 mL of Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Chicago, IL, USA) per membrane, mixed 1:1 as instructed. After 1 minute incubation in room temperature, the chemiluminescence of membranes was captured using ChemiDoc Touch Imaging System.

2.5. Analyses on the ImageStreamX MkII

PLC EVs were labelled with CD41 AF647 (BioLegend, San Diego, CA, USA, clone HIP8) and CD63 FITC (Becton Dickinson, clone H5C6) for 30 min at RT. At least 10000 events for each sample were acquired using a 12 channel Amnis® ImageStream®X Mark II (Merck KGaA, Darmstadt, Germany) imaging flow cytometer. Samples were acquired at $60 \times$ magnification with low flow rate/high sensitivity. The integrated software INSPIRE® (Merck) was used for data collection. The instrument and INSPIRE software were set up as follows: Excitation lasers 488, 642 and 785 and channels 01 and Ch1 (bright field, BF), Ch06 (scattering channel), plus fluorescence channels Ch02, Ch011 were activated for signal detection. Single color controls were used for compensation and unlabelled EVs were used to determine auto fluorescence. Buffer with and without antibody molecules were used to determine the background noise. Compensated data files were analyzed using image-based algorithms available in the IDEAS® statistical analysis software package (version 6.2.188.0). Positive events for were gated based on the intensity values on each channel and images.

2.6. Lipid mediator profiling

PLC and EV samples were thawed on ice and 4 volumes of ice cold methanol (Thermo Fisher Scientific) containing internal standards (d$_8$-5S-hydroxyeicosatetraenoic acid (HETE), d$_5$-RvD2, d$_5$-lipoxin (LX)A$_4$, d$_4$-PGE$_2$, d$_4$-leukotriene (LT)B$_4$, d$_5$-LTC$_4$, d$_5$-LTD$_4$, and d$_5$-LTE$_4$; 500 pg each (Cayman Chemical, Ann Arbor, MI, USA) were added to the sample. LMs were extracted and identified as described previously (8,51,52). Briefly, the samples in methanol were incubated for 45 minutes at -20 °C for protein precipitation, and centrifuged at $1900 \times$ g at 4 °C for 10 minutes. The

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methanol content of the supernatant was evaporated to less than 1 mL using nitrogen gas stream, and the LMs were extracted with automated Extra-Hera system (Biotage, Uppsala, Sweden) employing solid-phase extraction as described previously (8). LMs were eluted with methyl formate (Thermo Fisher Scientific) and methanol (for sulfido-conjugates). The samples were concentrated and injected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (LC-20AD HPLC (Shimadzu, Kyoto, Japan) and SIL-20AC autoinjector (Shimadzu) paired with QTrap 6500+ (ABSciex, Framingham, MA, USA)). LMs were identified with multiple reaction monitoring by identifying the parent (Q1) and daughter (Q3) ions in both negative and positive (for sulfido-conjugates) ionization modes (8,51,52). Each LM was identified using previously published criteria by matching the retention time of authentic and synthetic standards (from Cayman Chemical, prepared in house or provided by Charles N. Serhan, Harvard Medical School, Boston, MA, USA) and identifying at least 6 diagnostic ions from the MS/MS spectra (8,52). For chiral analysis of monohydroxy pathway markers, the methyl formate fraction was extracted as above. Chiral LC-MS/MS was conducted for platelet PLC samples at day 5 and day 8 as previously described (53).

2.7. Statistical analysis

Results are expressed as median with interquartile range. Statistical significance was determined using Dunn's multiple comparison test, and p-values ≤ 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism v7.02 (GraphPad Software Inc., La Jolla, CA, USA).

Principal component analysis (PCA) was conducted and visualized using free online tool Metaboanalyst (54). For analysis, GPL class data including all quantifiable GPL classes, sample types, and sample days were imported as mol% excluding species with mol% < 0.5. In data input for Metaboanalyst features with > 25%, missing values were removed and missing values were estimated using Bayesian PCA. The imported data were log transformed and auto scaled (mean centered and divided by the square root of each variable) for the analysis.

3 Results

3.1. Extracellular vesicles accumulate to the platelet concentrate during storage

During storage, the particle concentration in the EV samples isolated from the PLC significantly increased ($p = 0.0087$) from a median of 7.5×10^9 particles/mL in d1 sample to 2.7×10^{10} particles/mL in d8 sample (Fig. 1a). Despite the increased particle concentration, the particle size of EV samples remained unchanged throughout the storage, 60% of the particles being 100 – 200 nm (Fig. 1b).

3.2. Storage alters the glycerophospholipid composition of platelet concentrates

Mass spectrometry of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) species compositions in PLCs, platelets, and EVs revealed a set of storage time -dependent changes, which were common for all the sample types. In the PC species of PLC, platelets, and EVs, consistent and statistically significant increases were detected for the species 32:0 and 38:4, while the species 34:2 decreased with the increasing storage time (Fig. 2). In the EVs, the 38:4 was the only polyunsaturated PC species the percentage of which did not decrease during the storage, whereas all the other detected species were statistically significantly declined by

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d8. The time-dependent decrease in polyunsaturated PC species was reflected as n respective relative increase in monounsaturated PC species. In general, the PC species profiles of the d8-EVs were very similar to the corresponding profiles of the PLCs, whereas the PC species profile of platelets was notably different compared to these two.

Regarding PE, the levels of a minor species 34:1 consistently declined in all sample types. Quantitatively, the most important PE species 38:4 showed a remarkable and statistically significant accumulation in the d5- and d8-EVs, and it was also significantly increased in the d8 platelets. Moreover, in the EVs the relative abundance of PE 38:5 was significantly increased, although it decreased in the PLC and platelets (Fig. 3). The shorter polyunsaturated species of PE (e.g. 34:5 and 36:5) were present in relatively high percentages in the d1- EVs, but they decreased in d5- and d8 samples.

In PS species, the only statistically significant change common to all sample types was the increase in the most prominent PS species 38:4 (Fig. 4). In the EVs, the percentages of the relatively short polyunsaturated species (e.g. 36:4 and 36:5) decreased in time, similarly to the trend observed in PE.

While the GPL contents of 38:4, which is the major source of AA, notably increased during the storage in the PLC, platelets and EVs, only modest changes were found in the GPL species serving as the source of the n-3 PUFAs, EPA, DPA and DHA.

In the PCA scores plot, the PLC and platelet samples were clustered forming clearly defined populations with uniform GPL species composition (Fig. 5), whereas the EV samples were dispersed on a larger area, showing a concentrate age-dependent, large variation in the GPL species composition, when all quantified GPL classes (Fig. 2-4, Supplementary Fig. 1-3) were included in the analysis. Notably, the three different populations did not overlap with each other when 95% confidence intervals were visualized.

3.3. Enzymes linked to lipid mediator biosynthesis are expressed in platelet concentrates

Next, the Western blot analysis was performed to prove the presence of LOX, COX, CYP and PLA_2 species in the samples isolated from PLCs. From lipoxygenases 12-LOX, and 15-LOX2 were detected in all the sample types in both d1 and d8 samples (Fig. 6), whereas 15-LOX1 was only slightly present in different sample types. Additionally cyclooxygenases COX1 and COX2 were detected in PLC and EVs, but only COX1 was detected in platelets. From the analyzed CYP species only CYP5A1 and CYP2J2 were detected in all sample types, as CYP1A1 was detected only in PLC and EVs. From PLA_2 species $cPLA_2$ was detected in all sample types, but $sPLA_2$ was present only in platelets and EVs.

As controls, ApoA1, ApoB, CD9, CD41, and CD63 were also detected from the samples. ApoA1 and ApoB levels were detected to show the presence of HDL and LDL, normal components in plasma containing samples such as PLCs. As expected, no change in the levels of the apolipoprotein intensities was seen between the d1 and d8 samples. CD41, a platelet membrane marker prominently present also in platelet-derived EVs was equally present in the d1 and d8 samples from the PLC and platelets, but in the EVs the level of CD41 markedly increased from the d1 samples to

d8 samples similarly to EV marker CD9. The increase in the intensities of the CD41 and CD9 bands was paralleled with the enhanced intensities in the d8 bands of the enzymes 12-LOX, COX1, CYP1A1, CYP5A1, and cPLA$_2$. The expression of CD63 in EV samples was stable in both time points.

3.4. Two distinct platelet EV populations are present in platelet concentrates

PLC EV samples were further characterized by analyzing the CD41 and CD63 expression of sample particles with Amnis® ImageStream®X Mark II imaging flow cytometer. Two platelet-derived particle populations were detected: Major population of EVs (71% of Carboxyfluorescein succinimidyl ester positive particles of EV sample, data not shown) expressed only CD41, whereas both CD41 and CD63 were expressed only on the surface of 1,2% from CD41 positive events (Fig. 7a and b).

3.5. Lipid mediators accumulate in platelet concentrates during storage

Several LMs and their pathway markers were identified from the PLCs and EVs (Supplementary Table 1 and 2) in accordance with the previously published criteria for lipid species recognition: the retention times (Fig. 8a) and a minimum of six diagnostics ions including at least one backbone break in the MS/MS spectrum (Fig. 8b) of the analyzed molecules were matched with the corresponding authentic or synthetic standards (8).

When investigating the chirality of the monohydroxy pathway markers resulting from the enzymatic processing of GPLs, a clear tendency towards either *R* or *S* configuration was observed, as the relative abundance of the dominant configuration was over > 80% (Fig. 9). The majority of the analyzed 13 monohydroxy pathway markers were in the *S* configuration, and only in the case of 4 monohydroxy pathway markers (13-hydroxydocosahexaenoic acid (HDHA), 13-hydroxydocosapentaenoic acid (HDPA), 11-hydroxyeicosapentaenoic acid (HEPE), and 11-HETE) *R* was the dominant configuration.

In the PLCs, several bioactive metabolites of DHA, n-3 DPA, EPA and AA, SPMs and proinflammatory mediators, were detected (Fig. 10, Supplementary Table 1). The content of some mediators (e.g. sulfido-conjugate LTE$_4$) remained unchanged, whereas certain LMs and pathway markers showed a statistically significant, time-dependent accumulation during storage, such as DHA-derived RvD4; RvD5; 17*R*-RvD1; 17*R*-RvD3; 10*S*,17*S*-diHDHA (also known as PDX); 7*R*,Δ12-*trans*,14*S*-diHDHA; 4*S*,14*S*-diHDHA, EPA-derived RvE2, and AA-derived 5*S*,15*S*-diHETE; 15-epi-LXA$_4$; TxB$_2$; PGE$_2$ (Fig. 10, Supplementary Table 1). The most notable accumulation in SPMs was in 17*R*-RvD1, where an almost 100-fold increase was detected (median from 2.7 to 209.5 pg / 2 ×10^8 platelets, p = 0.0001). In the case of TxB$_2$, which is the hydrolyzed form of the most prevalent and extremely short-lived proinflammatory mediator from platelets, TxA$_2$, over 10-fold increase in the accumulation was observed during storage (median from of 34.4 to 407.1 pg / 2 ×10^8 platelets, p = 0.0003), but the absolute amount of TxB$_2$ was higher than that of 17*R*-RvD1. Additionally, the levels of monohydroxy pathway markers derived from DHA, n-3 DPA, EPA and AA metabolomes accumulated drastically in the PLC samples (Supplementary Table 1).

The EV samples contained to a large extent the same LMs as the PLCs, but they were present in lower amounts due to the small amount of the EV material (Supplementary Table 2). However, some LMs were only detected in the EV samples (RvD3, MaR1, n-3-DPA, and RvE3). Proresolving 17*R*-RvD1 and a dual function LM, PGE_2, were the only LMs detected that showed a statistically significant (*p* = 0.0066 and 0.0044, respectively), time-dependent accumulation during storage along with several monohydroxy pathway markers, such as DHA-derived 17-HDHA; 14-HDHA, n-3 DPA-derived 17-HDPA; 14-HDPA; 13-HDPA, EPA-derived 18-HEPE, and AA-derived 15-HETE (Fig. 11, Supplementary Table 2).

4 Discussion

Currently, different molecular indicators are employed in the determination of platelet activation (34). Since EVs are produced as a result of cell activation (55), they have also been suggested to serve as a measure of platelet activation (35,36,56). However, it is presently demanding to create an EV particle number-based quality criteria for platelets, because EV quantification methods are still not accurate nor reproducible, and they lack standardization (57–59). Further, a low number of EVs is thought to be constitutively formed by platelets during storage (31,32). Therefore, in order to develop improved EV-based platelet activation indicators, the composition rather than the quantity of EVs deserves more investigation. As demonstrated by our group and others, the platelet EV protein composition is activator/condition-dependent (32,60). Therefore, acquisition of compositional data may also help to elucidate the role of platelet EVs in intercellular communication i.e. immunomodulation and the underlying molecular mechanisms. In this study, we observed a compositional change in both the membrane GPL profile in addition to the LM profile of PLCs and PLC-derived EVs during storage. The identification of enzymatic machinery and proresolving SPMs from EVs provides insights on the role of EVs in the immunology of platelets.

From the perspective of lipids, the fundamental element of EVs is their surrounding membrane bilayer derived from the membranes of the parent cell. The time-dependent alterations in the GPL composition of clinical grade PLCs, its platelets and also EVs have been studied previously (10,11), and our lipidome data of platelets and EVs is comparable to these results. The changes in the GPL composition of platelets and EVs thereof have been suggested to arise from the loss of lipid bilayer asymmetry, lipid peroxidation or effects due to the altered rate of EV budding (12,39,41). In this work, a clear difference was seen in the GPL profiles between the platelets and the EVs during the first 2 days of storage. Later at d5 and d8, however, the platelet and EV lipidomes resembled each other in many respects.

An important and consistent finding in our study was the increase of the AA-containing species 38:4 found in PC, PE and PS of the PLC, platelets and EVs. The elevated relative abundance of this AA source was more pronounced in the PE and PS species than in the PC species. AA is the main PUFA precursor for production of proinflammatory LMs by the COX and LOX enzymes (61), and it is important for the platelet function. Since the isolated EV yield was small during the first day sampling, we cannot rule out the possibility that a part of the profile changes in the PC species of the EVs could be due to lipoprotein presence in the samples, which was proven with Western blot. Lipoproteins are known to be rich in PC (62), especially in PC 34:2, the amount of which decreased with the storage time in contrast to the increased EV number. However, since lipoproteins carry

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very little PE and PS, the time-dependent elevation of PE 38:4 and PS 38:4 species in the EVs are firm findings and suggest that also the observed PC 38:4 shift was based on changes in the EVs. Importantly, the d1-2 EVs contained elevated levels of relatively short and highly unsaturated PE and PS species, the kind of GPL species known to have a high efflux propensity (63), which likely promotes their exit from plasma membranes with the early budding EVs. Production of EVs from platelets provides an abundant and AA-rich precursor pool for the LM production. In platelets and EVs, the relative concentrations of the highly unsaturated GPL species (such as 38:6, 38:7, 40:6, 40:7, which have DHA as their main PUFA component) were in general constant during the PLC storage period. Since the EV particle number released by platelets increased roughly 3-fold from d1 to d8, and the PUFA composition of GPL species remained virtually unchanged, enhanced enzymatic activity is required to produce the observed, even 30-fold increase in the monohydroxy pathway markers.

Although there are previous reports on the GPL composition of platelets during storage, no systematic studies exist regarding the molecular mechanisms of LM production or LMs themselves. In this study, we investigated the LM profiles of PLCs and EVs. We observed a clear increase in the monohydroxy pathway markers, proinflammatory and, more importantly, proresolving LMs during storage. Previously, only the monohydroxy pathway markers, but not their bioactive downstream products SPMs, have been identified from EVs derived from inflammatory exudates (64). Here, for the first time, we report the SPM profile of PLC EVs. Moreover, we identified a large repertoire of enzymes involved in the biosynthesis of LMs from the cleavage of acyl chains from GPLs (e.g. sPLA$_2$, cPLA$_2$) into the oxygenation of the free fatty acids (12-LOX, 15-LOX) in all the samples (PLC, platelet, EV). Previously, the presence of several key enzymes has been reported in platelets (65–71), and the enzyme activity of 5-LOX has been demonstrated with neutrophil EVs (72). It is important to highlight the presence of the enzymes related to LM biosynthesis in the PLC EVs, since these results indicate that the EVs contain the enzymatic machinery required for LM production.

Next, we investigated the chirality of monohydroxy pathway markers, which revealed that these pathway markers were rather specific products of enzymatic modification than a result of auto-oxidation because enzymes have a clear tendency to produce molecules with a specific configuration. For instance, the observed production of various LMs with *R* configuration, such as 17*R*-RvD1 and 15-epi-LXA$_4$, was consistent with the expression of CYP enzymes in the EVs (isoforms CYP1A1 and CYP2J2) which insert a hydroxyl group mainly into the *R* position (73). Overall, the literature on the roles of the different CYP isoforms in the biosynthesis of SPMs is lacking, hence, the relationships of the CYP isoforms and produced SPMs are unclear (69). Furthermore, COX1 and COX2 enzymes were found to be present in both PLC and EV samples. A recent study demonstrated that there is an endogenous acetylation mechanisms for COX2 that increases 15-epi-LXA$_4$ formation (74) that might have played a role in the biosynthesis of this SPM with *R* configuration also in our study. Additionally, sPLA$_2$ and cPLA$_2$, which are required in the remodeling of the GPL membrane, were expressed in both PLC and EV samples. The PLA$_2$ enzymes may also free esterified monohydroxy fatty acids from the GPL membrane enabling quick downstream LM synthesis (75). The results indicate that the detected enzymes can be active and contribute to the composition of the lipid cargo of PLC EVs. It is important to acknowledge, that to

some extent the LMs might be also produced in cells via active enzymatic hydroxylation and delivered in EVs. Although we did not measure the enzyme activity directly, the chirality analysis of the monohydroxy pathway markers indicates that there is active enzyme machinery in PLCs. Whether the enzymes are active in platelet EVs and how the residual leukocytes and EVs thereof in PLCs contribute to LM production requires further studies.

During the storage of the PLCs, we observed an accumulation of multiple LMs and monohydroxy pathway markers, and the effect was also apparent in the EV fraction. EVs mainly contained monohydroxy pathway markers and enzymes required in the biosynthesis of LMs, rather than LMs itself, as cargo. However, the LM analysis was limited by the amount of material available and limits of sensitivity of the equipment, therefore the results should be interpreted cautiously. Nonetheless, our results imply that at different phases of inflammation, the monohydroxy compounds of EVs may reinforce the impact of platelet LM deposits (13,17,18). Our data suggest that the EVs in circulation contain the monohydroxy compounds and the enzymatic machinery required to convert them to bioactive LMs. In addition, the monohydroxy chains could be esterified into the membrane GPLs to promote fast LM biosynthesis (75,76). Taking into consideration that SPMs are bioactive only at pico- to nanogram quantities (6), EVs could be regarded as first aid kits (77); when required, the enzymes present in the EVs could convert the monohydroxy fatty acids into functional SPMs to rapidly influence the surrounding cells *e.g.* platelets (16), macrophages (78), neutrophils (79), or endothelial cells (80). LM have been shown previously to be in pivotal role in the he interaction of platelets with the conventional immune cells, particularly neutrophils, or their EVs (70,81–86), underlining the importance of studying the LMs and SPMs particularly in the inflammation and the immune cell interactions.

The activation of platelets has been reported to induce the production of TxA_2 (87) and we also observed a drastic accumulation of TxB_2, the inactive metabolite of bioactive TxA_2 with a half-life of 30 seconds. Although from a clinical perspective the accumulation of inactive TxB_2 in the PLCs may bear no immunological relevance upon transfusion, it remains a measurable indicator of TxA_2 and platelet activation (88). One interesting finding related to the heterogenic cell interactions and the contribution of an ample AA source for TxA_2, active COX1 or activation of inducible COX2 is in cancer, where aspirin inhibition of platelet COX1 is beneficial for patients (89). Overall, the effects of proinflammatory mediators in platelet function have been investigated to a greater extent than the role of SPMs. We found that the levels of several SPMs increased during the storage, especially 17*R*-RvD1 which accumulated in both PLCs and EVs. Only few interactions between proresolving and proinflammatory mediators have previously been described in platelets, one of them involving 10*S*,17*S*-diHDHA (also known as PDX) secreted by platelets, which is shown to inhibit TxA_2–mediated prothrombotic aggregation (18). We observed an increase in the levels of 10*S*,17*S*-diHDHA in the PLCs during storage, however, the consequent interactions influencing platelet functions and ultimately the effects on PLC recipients require further studies.

Circulating platelets in the human body are constantly replenished, meaning that at any given time only a fraction of the uneven-aged platelet population is comprised of ageing platelets, which may also contribute to the total EV population in plasma. Thus, the EVs in stored PLCs, more coeval, cannot be compared directly to the platelets and EVs in plasma. As the PLCs are sealed products, nothing is added or removed from them after preparation. Platelets, whether alive or dysfunctional,

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stay in the PLCs, whereas the dysfunctional and aging platelets are removed from the circulation (90). The detection of enzymes involved in the LM production was conducted using highly specific antibodies. In addition to known platelet enzymes, we detected enzymes that typically are not associated to platelets (COX2 and 15-LOX1). To what extent other residual cells, e.g. leukocytes and EVs thereof, contribute to the detected enzymes and furthermore, enzymatic activity, remains unknown. Additionally, the instructions for the blood donors dictate that aspirin should not be consumed but we cannot exclude the possibility of aspirin intake. Thus, it is possible that COX acetylated by aspirin together with residual leukocytes and EVs thereof may have contributed to the biosynthesis of SPMs with the configuration *R* (6). All these factors contribute to the lipidome, so the results are not directly comparable to lipidome of pure platelet population or platelet EVs, but rather represent the lipidome of clinical grade PLC transfused to patients.

To conclude, this study examined the biosynthesis pathway of LMs covering the membrane GPL precursor, enzymes, and bioactive end products in clinical grade PLCs, and more interestingly, in the PLC EVs. We demonstrated storage time-dependent variation in the relative abundance of GPL species, the sources for precursor PUFAs utilized in LM synthesis. Furthermore, we confirmed the presence and activity of the enzymatic machinery required for biosynthesis of the LMs, and finally, we identified and quantified the bioactive metabolomes derived of DHA, n-3 DPA, EPA, and AA in the whole PLC and EV samples that had not been described previously. In addition to shedding light on the mechanisms of the intercellular signaling, the current study adds another level of complexity to the platelet-mediated interactions, as we have demonstrated that the platelet age contributes to the composition of EVs. The detection of monohydroxy pathway markers and proresolving LMs in the platelets and EVs is of great importance and suggests that the EVs may strengthen platelet-mediated cell communication with immune cells. The data given here proves that the PLC EVs possess a powerful machinery to mediate lipid-dependent functions in inflammation. Platelet function in different phases of inflammation and healing requires further studies, including how platelets employ the lipid machinery via EVs, and to which cells / LM receptors platelet-derived EV-borne LMs are ultimately targeted.

Considering the time-dependent compositional changes from the perspective of clinical transfusions, the d5 PLCs were fairly similar to the d1 in terms of their EV content, GPL composition, and LM profile. Significant compositional changes were observed only after 8 days of storage with significantly increased EV content, a large variation in the GPL content, and the accumulation of LMs to both PLCs and the EVs. These observations indicate that the clinical PLCs remained stable for the duration of the current storage time of up to d5 from the perspective of the metabolipidome.

5 Acknowledgements

6 Figure legends

Figure 1: The particle concentration (A) and the size distribution (B) of particles in the extracellular vesicle samples. In Figure A each dot represents an averaged result of three replicate measurements of a single extracellular vesicle sample. Bars represent median with interquartile range. In Figure B columns represent average (n = 5) and bars standard deviation. $**p \leq 0.01$ using Dunn's multiple comparisons test.

Figure 2: Variation in the relative abundance of phosphatidylcholine (PC) species in the platelet concentrates, platelets, and extracellular vesicles. Columns represent median and bars interquartile range. n = 5; $**p \leq 0.01$, $*p \leq 0.05$ using Dunn's multiple comparisons test.

Figure 3: Variation in the relative abundance of phosphatidylethanolamine (PE) species in the platelet concentrates, platelets, and extracellular vesicles. Columns represent median and bars interquartile range. n = 5; $**p \leq 0.01$, $*p \leq 0.05$ using Dunn's multiple comparisons test.

Figure 4: Variation in the relative abundance of phosphatidylserine (PS) species in the platelet concentrates, platelets, and extracellular vesicles. Columns represent median and bars interquartile range. n = 5; $**p \leq 0.01$, $*p \leq 0.05$ using Dunn's multiple comparisons test.

Figure 5: Visualization of principal component analysis of GPL composition in the platelet concentrates, platelets, and extracellular vesicles. All quantified glycerophospholipid species were used as loadings. Samples were assigned codes consisting of the sample type (EV/Platelets/PLC), platelet concentrate number (1-5), and the sampling day (d1, d2, d5, or d8); shaded areas represent 95% confidence intervals.

Figure 6: Enzyme detection from the platelet concentrates, platelets and extracellular vesicles by Western blot. 12-lipoxygenase (LOX), 15-LOX1, 15-LOX2, cyclooxygenase (COX)1, COX2, cytochrome p450 (CYP)1A1, CYP2J2, CYP5A1, cytosolic phospholipase 2 (cPLA$_2$), secretory phospholipase 2 (sPLA$_2$), CD41, apolipoprotein (Apo)A1, and ApoB were detected from the platelet concentrates, platelets and extracellular vesicles. Samples were isolated from two different platelet concentrates on day (d)1 and d8. The size of detected proteins is indicated on the right side of the figure, STD = molecular weight standard.

Figure 7: Extracellular vesicle sample characterization using ImageStreamX MkII. A representative image of the CD41 and CD63 localization (A) and statistics of CD41 and CD63 expression (B) in the extracellular vesicle sample isolated from platelet concentrate.

Figure 8: Identified lipid mediators from platelet concentrates and extracellular vesicles. A) A representative multiple reaction monitoring chromatograms for each of the identified lipid mediator and pathway marker. B) Tandem mass spectrometry fragmentation spectra employed in identification for resolvin (Rv) D4, 17R-RvD1, and thromboxane (Tx)B$_2$. Peaks assigned with bolded m/z values indicate backbone breaks of the molecule.

Figure 9: Chirality of monohydroxy pathway markers in platelet concentrates. Multiple reaction monitoring chromatograms (MRM) for monohydroxy pathway markers derived from docosahexaenoic acid, n-3 docosapentaenoic acid, eicosapentaenoic acid, and arachidonic acid in platelet whole products (day 5 and 8) were identified with chiral liquid chromatography-tandem mass spectrometry. MRM transitions used in the detection of each monohydroxy pathway marker were as follows: 17-HDHA m/z 343>245, 14-HDHA m/z 343>205, 14-HDPA m/z 345>207, 13-HDPA m/z 345>195, 18-HEPE m/z 317>259, 15-HETE m/z 319>219, 12-HETE m/z 319>179. For each enantiomer pair, the R isomer was eluted before the S isomers. Results are a representative of three similar experiments. HDHA, hydroxydocosahexaenoic acid; HDPA, hydroxydocosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid.

Figure 10: Lipid mediators and pathway markers accumulate in the platelet concentrates during storage. Samples were collected on day (d)1, d2, d5, and d8 post the preparation of the concentrates. Results are expressed as pg / 2.0 x 10^8 platelets; n = 5 per group. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ vs. day 1 using Dunn's multiple comparisons test. HDHA, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; LX, lipoxin; PG, prostaglandin; Rv, resolvin; Tx, thromboxane.

Figure 11: Lipid mediators and monohydroxy pathway markers accumulate in the extracellular vesicles of platelet concentrate during storage. Samples were collected on day (d)1, d2, d5, and d8 post the preparation of platelet products. Results are expressed as pg / 2.0 x 10^8 platelets; n = 5 per group. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$ vs. day 1 using Dunn's multiple comparisons test. HDHA, hydroxydocosahexaenoic acid; HDPA, hydroxydocosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; Rv, resolvin.

Supplementary Figure 1: Variation in the relative abundance of phosphatidylcholine alkyl-acyl (PCa) species in the platelet concentrates, platelets, and extracellular vesicles. Columns represent median and bars interquartile range. n = 5; **$p \leq 0.01$, *$p \leq 0.05$ using Dunn's multiple comparisons test.

Supplementary Figure 2: Variation in relative abundance of lysophosphatidylcholine (LysoPC) species in the platelet concentrates, platelets, and extracellular vesicles. Columns represent median and bars interquartile range. n = 5; **$p \leq 0.01$, *$p \leq 0.05$ using Dunn's multiple comparisons test.

Supplementary Figure 3: Variation in relative abundance of sphingomyelin (SM) species in the platelet concentrates, platelets, and extracellular vesicles. Columns represent median and bars interquartile range. n = 5; **$p \leq 0.01$, *$p \leq 0.05$ using Dunn's multiple comparisons test.

Supplementary Figure 4: Original Western blot membranes showing the detection of 12-lipoxygenase (12-LOX) and cyclooxygenase 1 (COX1) in platelet concentrates, platelets, and extracellular vesicles on day (d)1 and d8 samples. STD = molecular weight standard.

Supplementary Figure 5: Original Western blot membranes showing the detection of cytochrome p450 5A1 (CYP5A1) and cytosolic phospholipase A$_2$ (cPLA$_2$) in platelet concentrates, platelets, and extracellular vesicles on day (d)1 and d8 samples. STD = molecular weight standard.

Supplementary Figure 6: Original Western blot membranes showing the detection of platelet glycoprotein IIb (CD41), 15-lipoxygenase 2 (15-LOX2), cytochrome p450 1A1 (CYP1A1), and secretory phospholipase A$_2$ (sPLA$_2$) in platelet concentrates, platelets, and extracellular vesicles on day (d)1 and d8 samples. STD = molecular weight standard.

Supplementary Figure 7: Original Western blot membranes showing the detection of apolipoprotein (Apo)A1, ApoB, cyclooxygenase 2 (COX2), cytochrome p450 2J2 (CYP2J2), and secretory phospholipase A$_2$ (sPLA$_2$) in platelet concentrates, platelets, and extracellular vesicles on day (d)1 and d8 samples. STD = molecular weight standard.

Supplementary Figure 8: Original Western blot membranes showing the detection of CD9 and CD63 in extracellular vesicles on day (d)1 and d8 samples. STD = molecular weight standard.

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Highlights

- AA-containing FA species increase in major GPL classes of platelet concentrates.

- Enzymes processing FAs to lipid mediators were detected in all sample types.

- Lipid mediator pathway markers were found to be a result of enzymatic processing.

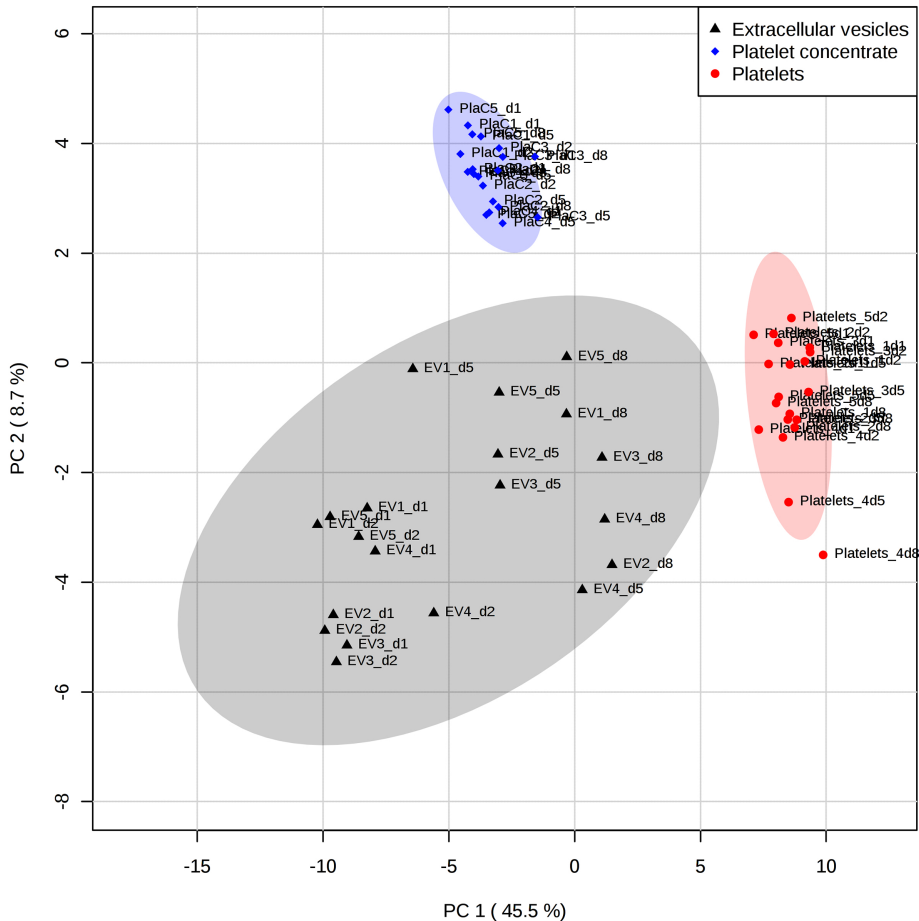- Lipid mediators were detected in platelet concentrates and extracellular vesicles.
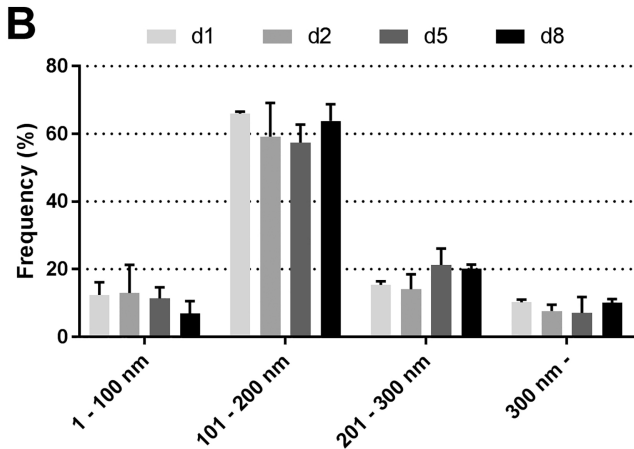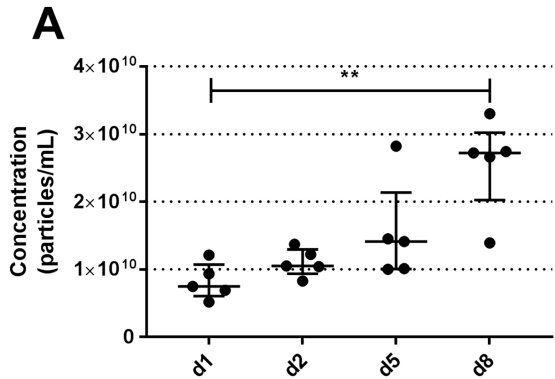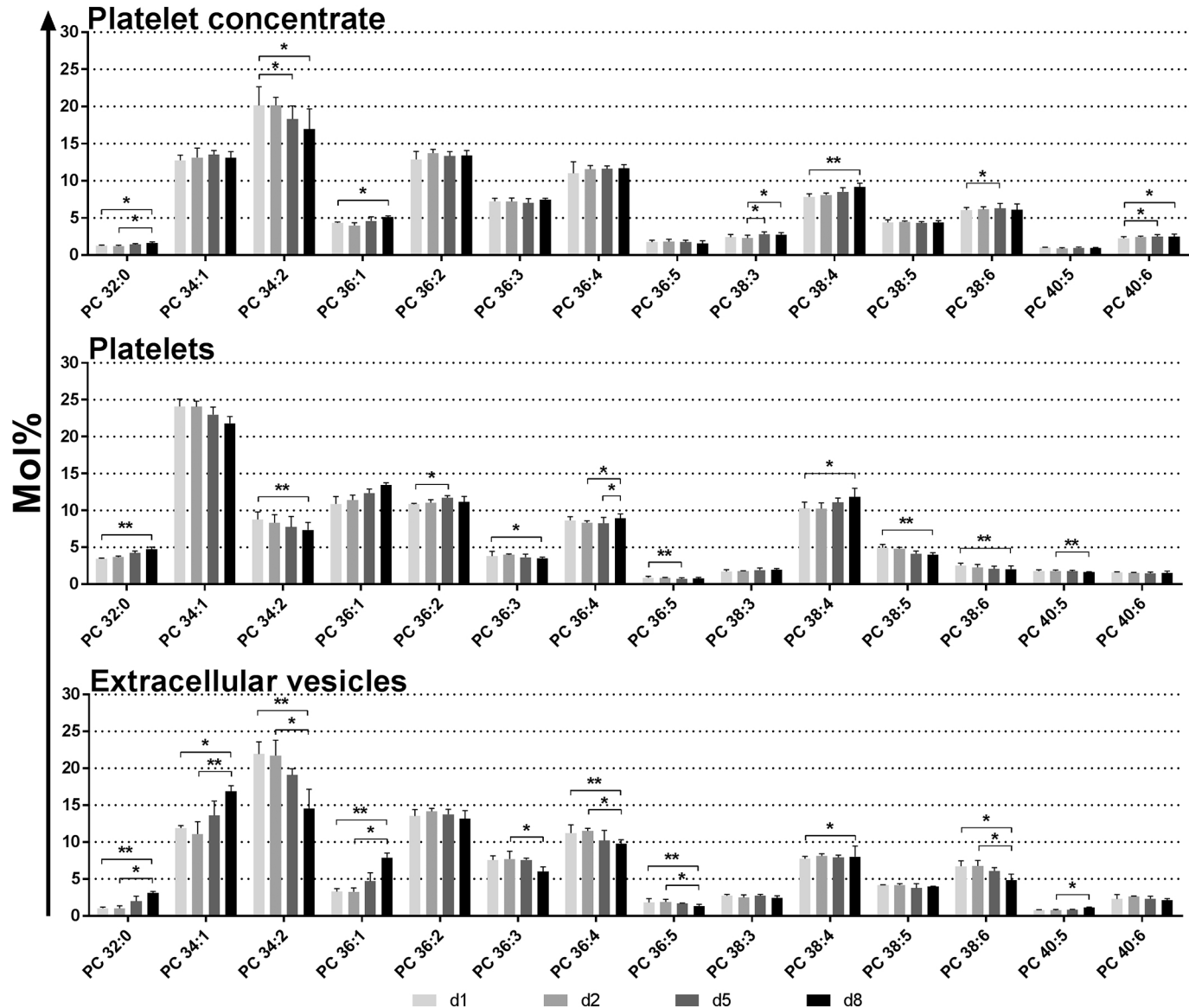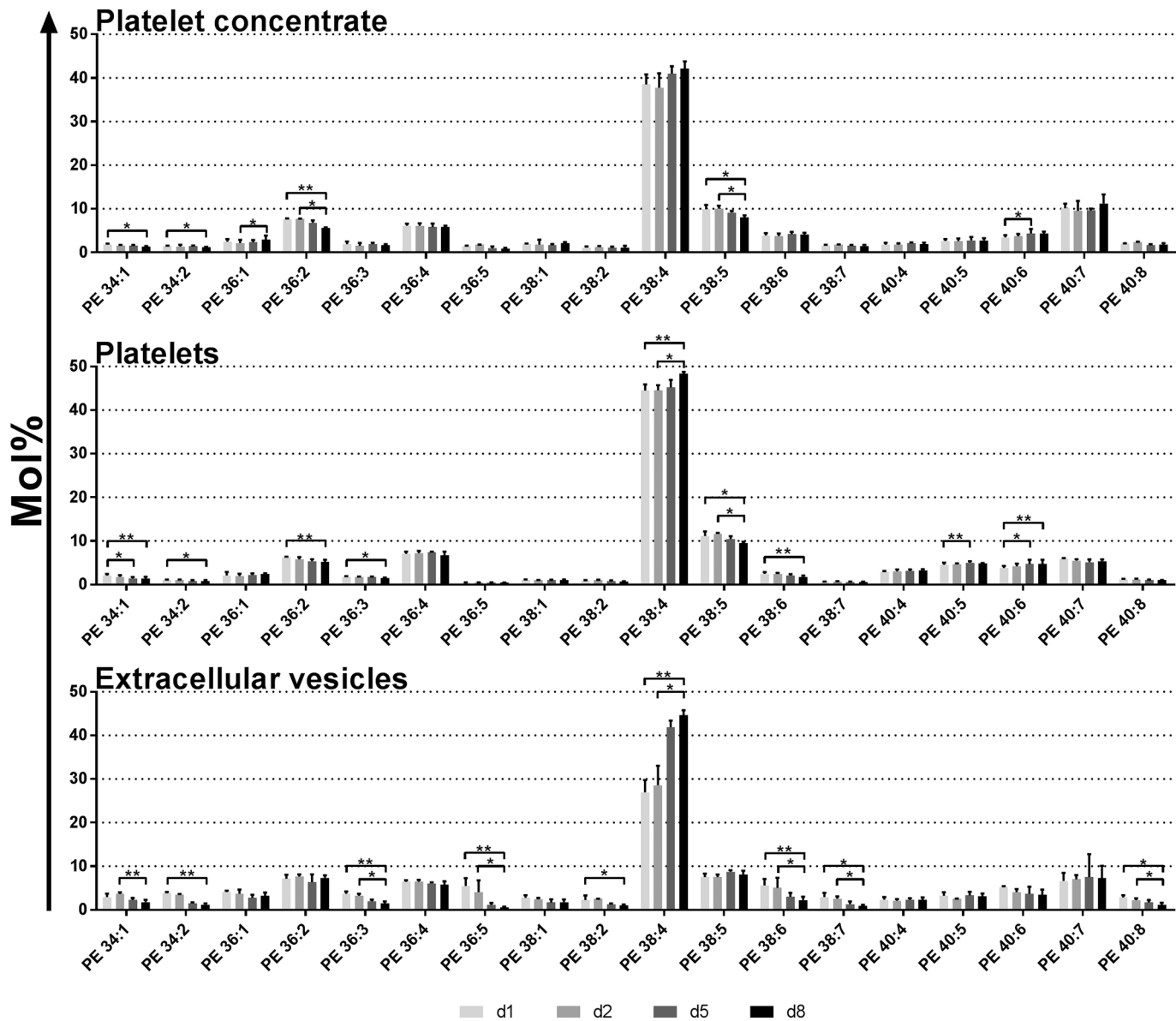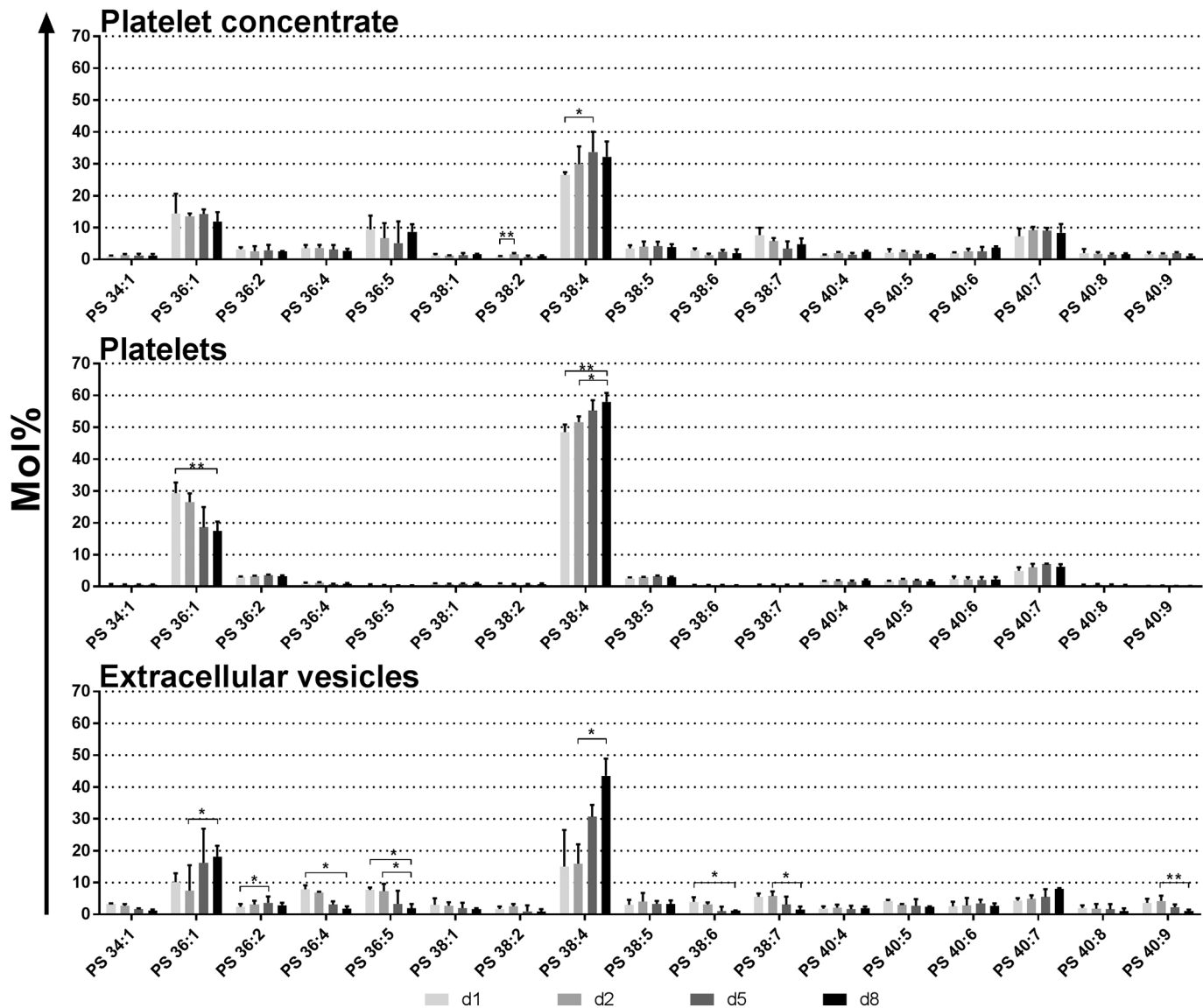
Figure 1

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