



Lysophosphatidylcholine is a Major Component of Platelet Microvesicles Promoting Platelet Activation and Reporting Atherosclerotic Plaque Instability

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Abstract

Background Microvesicles (MVs) are small cell-derived vesicles, which are mainly released by activated cells. They are part of a communication network delivering biomolecules, for example, inflammatory molecules, via the blood circulation to remote cells in the body. Platelet-derived MVs are known to induce vascular inflammation. Research on the mediators and mechanisms of their inflammatory effects has attracted major interest. We hypothesize that specific lipids are the mediators of vascular inflammation caused by platelet-derived MVs.

Methods and Results Liquid chromatography electrospray ionization–tandem mass spectrometry was used for lipid profiling of platelet-derived MVs. Lysophosphatidylcholine (LPC) was found to be a major component of platelet-derived MVs. Investigating the direct effects of LPC, we found that it induces platelet activation, spreading, migration and aggregation as well as formation of inflammatory platelet–monocyte aggregates. We show for the first time that platelets express the LPC receptor G2AR, which mediates LPC-induced platelet activation. In a mouse model of atherosclerotic plaque instability/rupture, circulating LPC was detected as a surrogate marker of plaque instability. These findings were confirmed by matrix-assisted laser desorption ionization imaging, which showed that the LPC concentration of human plaques was highest in vulnerable plaque regions.

Keywords

- lysophosphatidylcholine
- microvesicles
- platelet activation
- unstable atherosclerotic plaques

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Conclusion LPC is a major component of platelet-derived MVs and via its interaction with G2AR on platelets contributes to platelet activation, spreading, migration and aggregation and ultimately to vascular inflammation. Circulating LPC reports on atherosclerotic plaque instability in mice and is significantly increased in unstable areas of atherosclerotic plaques in both mice and humans, linking LPC to plaque instability.

Introduction

Microvesicles (MVs), also called microparticles, are small cell blebs typically released during the activation and apoptosis of multiple cell types.^{1–3} They possess the surface characteristics of their parental cells; hence, their cells of origin can be identified.⁴ Even though it was initially believed that MVs are only cellular debris, their shedding into the blood is a highly regulated process.^{1,2} Once released from their parental cells into circulation, MVs bind in a receptor-mediated way to distinct target cells, transferring their cell membrane as well as their vesicle components to their destination cell.^{5,6} The biology of MVs is highly complex and only partly understood, but there is increasing evidence that they contain inflammatory ribonucleic acids (RNAs) and cytokines, which they deliver to their destination cells.^{7,8} Furthermore, MVs are 'loaded' by their parental cells with distinct RNA molecules, underlining the highly complex biology of MV release.⁶ The MV-dependent transfer of bioactive molecules often results in altered pro-inflammatory function and phenotype of the target cells, remote from the original source of these MVs, as demonstrated for endothelial cells, smooth muscle cells, monocytes, neutrophils and T cells.^{9–13} Hence, MVs are part of a unique communication network involved in a multitude of diseases.^{3,14,15} In particular, platelet-derived MVs (PMVs) have been associated with cardiovascular diseases.^{2,10}

Lysophosphatidylcholine (LPC; 2-acyl-sn-glycero-3-phosphocholines) is a phospholipid generated from phosphatidylcholine (PC) after removal of a fatty acid group. LPC is increased in cardiovascular diseases driven by acute and chronic inflammation.¹⁶ As circulating, unbound LPC is immediately degraded by plasma lysophospholipases, it remains only temporarily in circulation if unprotected from degradation. However, MVs have been proposed to be transport vehicles for LPC.^{17,18}

This study describes LPC as a major component of circulating PMVs and investigates its effects on platelet function and its potential link to atherosclerotic plaque instability.

Methods

Platelet-Derived MV Preparation

Blood was taken from the antecubital veins of healthy individuals and anticoagulated by citrate (Fc 0.01 M). Samples were centrifuged ($150 \times g$, 15 minutes) to obtain platelet-rich plasma (PRP). To obtain highly pure PMV samples, platelets were washed using the following protocol. PRP was centri-

fuged ($720 \times g$, 10 minutes, room temperature [RT]) and the platelet-containing pellets were re-suspended in acid citrate dextrose (ACD) Tyrode's buffer containing prostaglandin (1 μ M). After a second centrifugation step ($500 \times g$, 10 minutes, RT), platelets were re-suspended in the initial volume of PRP with Tyrode's buffer containing 1 mM of $MgCl_2$ and $CaCl_2$. MV release was induced by adding agonists (adenosine diphosphate [ADP] 20 μ M, thrombin 0.1 U/mL and collagen 10 μ g/mL) for 15 minutes at RT. Platelets were pelleted by centrifugation ($16,000 \times g$, 2 minutes) and MVs were purified by ultracentrifugation ($100,000 \times g$, 60 minutes, 4°C), washed in phosphate-buffered saline (PBS) and subjected to lipid mass spectrometry. Significant contamination of PMV preparations by lipoproteins, which could carry LPC, has been excluded by lipoprotein analysis of the PMV samples.

Lipid Analysis with Liquid Chromatography Electrospray Ionization–Tandem Mass Spectrometry

Lipid extraction of MVs was performed as follows: Samples underwent total lipid extraction using a single-phase chloroform/methanol (2:1) technique, as described previously.¹⁹ A 10- μ L aliquot of cell lysate or MVs was combined with 200 μ L $CHCl_3/MeOH$ (2:1) and 15 μ L internal standard mix, then briefly vortexed. Samples were mixed (rotary mixer, 10 minutes), sonicated (water bath, 30 minutes) and allowed to stand (20 minutes) at RT. Samples were centrifuged ($16,000 \times g$, 10 minutes) and the supernatant dried under a stream of nitrogen (N_2) at 40°C. The extracted lipids were re-suspended in 50 μ L water-saturated BuOH with sonication (10 minutes), followed by 50 μ L of 10 mM NH_4COOH in MeOH. Extracts were centrifuged ($3,350 \times g$, 5 minutes) and the supernatant transferred into 0.2 mL glass vials with Teflon insert caps. Mass spectrometric analysis was performed using 5 μ L injections of the lipid extracts. Lipid analysis was performed by liquid chromatography electrospray ionization–tandem mass spectrometry (LC ESI-MS/MS) using an Agilent 1200 liquid chromatography system with an Applied Biosystems API 4000 Q/TRAP mass spectrometer with a turbo ion-spray source (350°C) and the Analyst 1.5 data system. Quantification of lipids was based on signal intensity relative to the corresponding internal standard, as described previously.¹⁹ For lyophilized samples, background values of buffer and media were subtracted. Values displayed for each lipid class were calculated as the sum of each individual species within the class.

For lipid analysis of vessel tissue, tissue was first homogenized in PBS. Note that 100 μ L of homogenized sample were incubated with $CHCl_3/MeOH$ (2:1, 20 volumes, 10 minutes,

RT), with shaking (90 revolutions per minute [rpm]), followed by a second incubation step (50 minutes, RT). Note that 0.4 mL of MQ H₂O was added to each sample followed by incubation on a platform shaker for 10 minutes at 90 rpm. Then, samples were centrifuged at 3,500 rpm for 5 minutes before the upper aqueous layer was discarded. The lower hydrophobic layer, which contains phospholipids, was transferred into a clean glass tube. The hydrophobic layer was dried under N₂ at 40°C. For mass spectrometry analysis, dried samples were re-suspended in water-saturated butanol (50 µL) and 10 mM ammonium formate in MeOH (50 µL). Lipidomic analysis was then performed by LC ESI-MS/MS as described above.

Preparation of LPC and PC Liposomes

Organic solvents (e.g. chloroform and methanol), in which polar lipids such as LPC and PC need to be re-suspended, can affect cell integrity. To avoid any artificial effect of the lipid solvent, experiments were performed with lipid micelles that can be re-suspended in PBS.²⁰ Therefore, as recommended by the manufacturer, LPC and PC powder (both AVANTI, Alabama, United States) were diluted in chloroform:methanol (1:2) and dried under N₂ before the solvent was eliminated under vacuum. PBS (3 mL) was added to the dried lipid powder and lipids were re-suspended under ultrasound exposure (30 seconds, RT), incubated at 80°C (20 minutes) and samples again sonicated (30 seconds). Lipid micelles were stored at 4°C and sonicated for 30 seconds before use. The molarity of the lipids in the micelle preparations was determined by mass spectrometry.

Cell Culture Assay

To investigate whether the LPC receptor, G2A receptor (G2AR), is expressed on platelets and to avoid any contamination-related artificial effect on G2AR expression analysis, DAMI (ATCC, United States) cells were used. DAMI cells are a megakaryoblastic cell line showing several platelet characteristics and often used as a contamination-free model to study platelets.²¹ The human cell line HeLa (ATCC) was used as a negative control. G2AR expression was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as described below.

To obtain highly pure platelet samples for functional assays, washed platelets were used. PRP was centrifuged (720 × g, 10 minutes, RT) and the platelet-containing pellets were re-suspended in ACD Tyrode's buffer containing prostaglandin (1 µM). After a second centrifugation step (500 × g, 10 minutes, RT), platelets were re-suspended in the initial volume of PRP with Tyrode's buffer containing MgCl₂ and CaCl₂. Washed platelets were counted (Sysmex KX-21N, Germany), normalized and used within 60 minutes for further assays.

Flow Cytometry

The effects of LPCs on the cell surface expression of different receptors were assessed with flow cytometry. All flow cytometric measurements were performed with a FACSCanto (BD Bioscience, United States) and data were processed with a FACSDiva (BD Bioscience).

Platelet surface receptor expression was assessed in washed, highly pure platelets incubated with LPC (10 and 25 µM), PC (10 and 25 µM) or ADP (20 µM) (10 minutes, 37°C), followed by anti-CD62P or procaspase-activating compound 1 incubation (both BD Biosciences, 10 minutes, RT) and fixation (cell FIX, BD Bioscience). For G2AR blockage, a commercial anti-G2AR antibody (Santa Cruz, United States) was used.

As an additional indicator of vascular inflammation, the effects of LPC and PC on the number of platelet-monocyte aggregates (PMAs) were investigated. Whole blood was haemolyzed (BD FACS Lysing Solution, United States) and the remaining leukocytes and platelets were incubated with LPC, PC or ADP (10 minutes, 37°C). Samples were stained with anti-CD14 and anti-CD41 antibodies (both Beckman Coulter, United States) and PMAs were defined as CD41-positive events located within the flow cytometric gate for monocytes.

For the assessment of PMV surface characteristics, annexin V binding buffer (1× final solution; BD Bioscience) was added to PMVs. The samples were incubated for 15 minutes in the dark with AF647-conjugated Annexin V (Thermo Fisher, United States), anti-CD41, anti-CD61 and anti-CD42b antibodies. After incubation, 450 µL of annexin V binding buffer (1× final solution) was added to the samples.

Immunofluorescence Microscopy of Permeabilized Platelets

Purified platelets were re-suspended in PBS with 0.1% bovine serum albumin (BSA) plus 0.1% glucose and adjusted to 10 × 10³/µL. Platelets were incubated with LPC, PC, PBS or ADP (20 µM) for 30 minutes at 37°C and fixed with 2% paraformaldehyde. Platelets were placed into 6-well plates, each well containing a coverslip (MENZEL-Glaeser, Germany) coated with 0.01% poly-L-lysine (Sigma Aldrich, Germany). To fix platelets on the coverslips, the 6-well plates were centrifuged (250 × g, 5 minutes) and the platelets subsequently permeabilized (0.5% Triton X 100, Sigma Aldrich, 5 minutes) before the slides were blocked overnight. Specimens were then incubated with a phycoerythrin-labelled anti-CD62P antibody (BD Bioscience) for 2 hours followed by several washing steps. Microscopy was performed on a Zeiss 510 Meta Confocal Microscope with a 40× water lens and images were processed with ImageJ 1.46n (United States).

Platelet Static Adhesion Assay

To investigate LPC effects on platelet adhesion and spreading, platelet static adhesion assays on immobilized fibrinogen were performed.²² Twelve-well plates, each well containing glass coverslips, were coated with fibrinogen (100 µg/mL overnight, 4°C) and blocked (0.1% BSA, 60 minutes, RT), and approximately 50 × 10⁹/L platelets were placed into each well before LPC (25 µM), PC (25 µM), PBS (negative control) or ADP (positive control, 20 µM) were added (20 minutes, 37°C). For the immunostaining, platelets were labelled with anti-CD41 antibody and phalloidin for 30 minutes. After several washing steps, platelet attachment and spreading of 10 platelets per treatment were visualized microscopically by measuring the surfaces of the attached platelets (Olympus FSX100, Germany).

Platelet Aggregometry

Washed platelets re-substituted with fibrinogen (150 µg/mL) were incubated with LPC or PC micelles, and light-transmission aggregometry (AggRAM, Helena Laboratories, United States) performed. Platelet samples were also pre-incubated with a P2Y₁₂ blocker (2MeSamp, 100 µM) before LPCs were added. Platelet aggregation was measured over 600 seconds and the area under the curve used for statistical analysis.

Platelet Migration Assay

Collagen-coated µ-slide chemotaxis assays (ibidi, Germany) were used to investigate whether washed platelets migrate towards LPCs (50 µM). Stromal cell-derived factor 1 (SDF-1) (3 µg/mL, R&D Systems, United States) was used as a positive control, and cell culture medium as a negative control. Platelet migration was video-recorded for up to 7 hours and migration direction and speed were analysed using ImageJ. Statistical analysis was performed with software from ibidi and the Rayleigh test was used to verify the level of significance of the directed movement.

RNA Isolation and qRT-PCR

RNA of DAMI cells and platelets was isolated using the TRIzol isolation method. Briefly, cells were incubated with trizol (5 minutes, RT) before chloroform was added. Cell lysates were centrifuged (12,000 × g, 15 minutes, 4°C) and the aqueous phase transferred into a new tube containing glycogen. RNA precipitation was induced by isopropanol (5 minutes, 4°C) and precipitated RNA harvested by centrifugation (12,000 × g, 10 minutes, 4°C). RNA-containing pellets were washed twice in 75% EtOH and re-suspended in 10 µL diethylpyrocarbonate-treated water. qRT-PCR was done with standard protocols and gene expression levels were calculated using the Δ CT method with 18s as the house-keeping gene.²³

To investigate whether platelets express the LPC receptor G2AR, qRT-PCR of platelet complementary deoxyribonucleic acid was performed using commercial (Qiagen, Germany) as well as custom-made primers (Geneworks). qRT-PCR products were run on a 1.5% agarose gel and bands were visualized on a BIORAD (Gel Doc System, United States). As a negative control for G2AR expression HeLa cells, which are human fibroblastic cells, were used.

To investigate LPC-induced endothelial microRNA expression, human umbilical vein endothelial cells (HUVECs) were cultured under standard conditions and used at passage 4 before cells were stimulated with 50 µM LPC micelles or PBS for 6 hours. RNA isolation, qRT-PCR kits and primers were from Qiagen (Germany) and standard protocols were used.

Mouse Model of Atherosclerotic Plaque Instability

To assess LPC concentrations in stable and unstable atherosclerotic plaques, a recently developed tandem stenosis (TS) mouse model was used that reflects the characteristics of unstable, vulnerable atherosclerotic plaques as seen in humans.^{24,25} Briefly, 6-week-old apolipoprotein E-deficient (ApoE^{-/-}) mice were placed on a high-fat diet for 6 weeks before a TS with a 150-µm outer diameter was introduced at

the right carotid artery. The left carotid artery remained healthy and did not develop atherosclerosis. The aortic arch developed stable atherosclerosis. Mice were euthanized 8 weeks after surgery and vessel segments (right carotid artery and aortic arch), healthy arterial tissue (left carotid artery) and blood were collected for lipid analysis. For blood lipid analysis, a control group of ApoE^{-/-} mice were subjected to a sham operation (TS with 450 µm TSs of the carotid artery) and their values compared with the lipid levels of mice with 150 µm diameter stenoses in the TS model.

Immunohistochemistry and Histochemical Staining of Mouse Tissue Slides

Cryosections were obtained 500 µm proximal to the proximal suture in the right common carotid artery of the TS mouse model representing unstable atherosclerotic plaques, the corresponding location in the left common carotid artery representing healthy arteries and from the aortic arch representing stable plaques. Haematoxylin and eosin (H&E) staining was used for morphology. For Oil red O staining, frozen sections were fixed in 10% formalin, washed, stained in Oil red O solution (0.3% in isopropanol, Sigma) for 1 hour, differentiated in 60% isopropanol, dehydrated and mounted by Aquatex (Merck, United States). For CD68 staining, frozen section were incubated with primary antibody (Bio-Rad, United States) 1:200 at 4°C overnight. Following wash steps and incubation in avidin-biotin complex reagent, 3,3'-diaminobenzidine reagent was applied to each section. As soon as sections turned brown, slides were immersed in dH₂O to stop the reaction. Slides were dehydrated and mounted on coverslips with dibutylphthalate polystyrene xylene mounting media. Images were obtained using a light microscope (Leica Brightfield microscope).

Matrix-Assisted Laser Desorption Ionization and Histology

After increased levels of different LPC species were found in mouse plaques, LPC distributions were investigated in human plaque using matrix-assisted laser desorption ionization (MALDI). Human atherosclerotic plaque samples were obtained from patients with symptomatic carotid artery stenosis who underwent carotid endarterectomy. All patients gave written informed consent and the study was approved by the ethics committee of the Alfred Hospital, Melbourne, Australia.

Plaque tissue samples were cryosectioned and fixed on slides before histology staining or MALDI imaging. To detect plaque areas that met the criteria of vulnerable, unstable atherosclerotic plaques, tissue slides were stained for markers known to be increased in unstable plaques. In particular, staining for matrix metalloproteinase 9 (MMP-9) (R&D Systems) and collagen (Masson's trichrome staining) was performed using standard staining protocols. Corresponding sections of slides in which plaque instability was shown, were used for LPC detection with MALDI. Note that 30 mL of matrix solution (25 mg/mL in 700:300:1 v/v/v ethanol, Merck, Kilsyth, VA, Australia; Milli-Q water: trifluoroacetic acid, Sigma-Aldrich, St Louis, Missouri, United States) was used

for each slide. Microscope slides were mounted on a cardboard support (35 cm × 20 cm) using sticky tape and placed against the back wall of a fume cupboard. Matrix solution was sprayed from a distance of 50 cm using an Eclipse HP-CS airbrush with a 0.3-mm nozzle (Anest Iwata, Yokohama, Japan) equipped with a Smart Jet Pro air compressor (Anest Iwata) operating at 0.3 mPa. The matrix was applied in coats by passing the spray over the sample in a sweeping motion from left to right, then right to left, then back again over 3 seconds. Each coat was allowed to dry before another coat was applied. This process was repeated until 30 mL of the matrix solution had been sprayed onto the target.

All mass spectrometric analyses were performed on a SYNAPT HDMS (Manchester, United Kingdom) orthogonal geometry time-of-flight (TOF) mass spectrometer fitted with an intermediate pressure MALDI source. This instrument was equipped with an imaging mass spectrometry device located between the quadrupole and TOF mass analyser. Prior to all analyses, the mass spectrometer was mass calibrated over an m/z range from 100 to 1,000 using a mixture of two poly(ethylene glycol) (PEG) standards (Sigma-Aldrich) with average molecular weights of 600 and 1,000 Da. PEG standards were prepared at 1 mg/mL in a 1:1 v/v mixture of acetonitrile (Honeywell, Morristown, New Jersey, United States) and water, combined with α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (CHCA at 3 mg/mL in 1:1 v/v acetonitrile–water) and aqueous NaCl at 1 mg/mL (Sigma-Aldrich) at 5:5:1 v/v/v, then spotted onto a stainless steel MALDI target plate in 1 μ L aliquots and allowed to dry. PEG standards were mass analysed on the mass spectrometer in the positive ion V-mode of operation and resultant data were used to mass calibrate.

Statistics

Comparison of two variables was done with the Student's *t*-test. Multiple comparisons were done with a one- or two-way analysis of variance and Tukey's or Sidak's, respectively, post hoc analysis (version 8, GraphPad Software, United States). If not stated otherwise, data are shown as the mean \pm standard error of the mean. All tests were two-tailed and *p*-values of ≤ 0.05 are described as significant.

Results

Platelet-Derived MV Characterization and Lipid Distribution in Comparison to Platelets and MVs of Different Origins

PMVs were characterized using flow cytometry. After gating using forward and sideward light scatter, PMVs were characterized by binding of annexin V, measuring the surface expression of phosphatidylserine, and the expression of CD41, CD61 and CD42b, the latter representing glycoprotein (GP) Ib known to be partially shed with platelet activation, which explains the lower expression level of GPIb compared with the other surface markers (►Supplementary Fig. S1A–S1I, available in the online version).²⁶ In our lipidomic profiling, mass spectrometry revealed that MVs released from ADP-stimulated platelets contained up to 3.4% LPC (►Fig. 1A), while

the LPC content of ADP-activated platelets was only 2.1%, indicating an active packaging mechanism of LPC into platelet MVs (►Fig. 1B). MVs from THP-1 cells contained less LPC, 2.2% (►Fig. 1C), as did MVs of the megakaryocytic cell line DAMI with 1.5% LPC (►Fig. 1D). We also investigated whether the type of agonist used to activate platelets influences the LPC content of PMVs. In general, the LPC content in PMVs was higher than in activated platelets. Thrombin as the strongest platelet agonist increased the LPC content of platelet MVs more than ADP or collagen (►Fig. 1E). Overall, MVs represent a potential source of LPC in the circulation and potentially as deposits in the tissue.

LPC Induces Inflammatory Changes in Endothelial Cells

It has been recently described that LPC induces intercellular adhesion molecule-1 expression on human endothelial cells.²⁷ As an additional measure of pro-inflammatory changes, we investigated potential changes induced by LPC focusing on a spectrum of microRNAs known to be regulated during endothelial dysfunction.^{28–30} MicroRNAs 19a, 34a, 132, 155 and 200c were all strongly increased after incubation of HUVECs with LPC (►Supplementary Fig. S2, available in the online version).

LPC Activates Platelets and Induces Platelet–Monocyte Aggregates

It is known that PMVs are involved in inflammation and coagulation.³¹ To investigate whether this is mediated by the high LPC content of PMVs, platelets were incubated with LPC or PC micelles, then platelet activation, as well as PMA formation, were assessed by flow cytometry. LPC induced significant activation of the platelet integrin GPIIb/IIIa in comparison to PC (10 μ M LPC vs. 10 μ M PC: 8.5 ± 1.765 vs. 16.33 ± 1.085 , $p = 0.025$; 25 μ M LPC vs. 25 μ M PC: 114.7 ± 28.29 vs. 15.17 ± 1.138 , $n = 6$, $p = 0.0173$, ►Fig. 2A). Additionally, the platelet-activation marker CD62P was up-regulated in LPC-treated platelets (10 μ M LPC vs. 10 μ M PC: 245.5 ± 48.92 vs. 76.17 ± 21.22 , $p = 0.048$; 25 μ M LPC vs. 25 μ M PC: $1,522 \pm 388$ vs. 79.00 ± 17.77 , $n = 6$, $p = 0.0147$) (►Fig. 2B). Flow cytometry data showing LPC-induced platelet activation were confirmed in a proof-of-concept experiment demonstrating that LPC induces translocation of intracellularly stored CD62P to an extent comparable to ADP (►Figs. 2C–F). CD62P (P-selectin) is the ligand of the monocyte receptor P-selectin glycoprotein ligand (PSGL)-1 and essential for the formation of highly inflammatory PMAs. In agreement, LPC significantly induced PMA formation (CD41/CD14 positive events in percentage of all CD14-positive events: PBS vs. LPC: $11 \pm 1.628\%$ vs. $23.66 \pm 2.138\%$, $p = 0.022$; PBS vs. PC: $11 \pm 1.628\%$ vs. $13.44 \pm 1.847\%$, $p = \text{n.s.}$, $n = 5$).

LPC Induces Platelet Adhesion and Spreading

To investigate whether LPC affects platelet adhesion, platelets were allowed to adhere to a fibrinogen matrix under static conditions. As shown in ►Fig. 3A, LPC-activated platelets spread on fibrinogen-coated slides to an extent similar to that of ADP-stimulated platelets (►Fig. 3B). Significantly less platelet spreading was observed for PC-

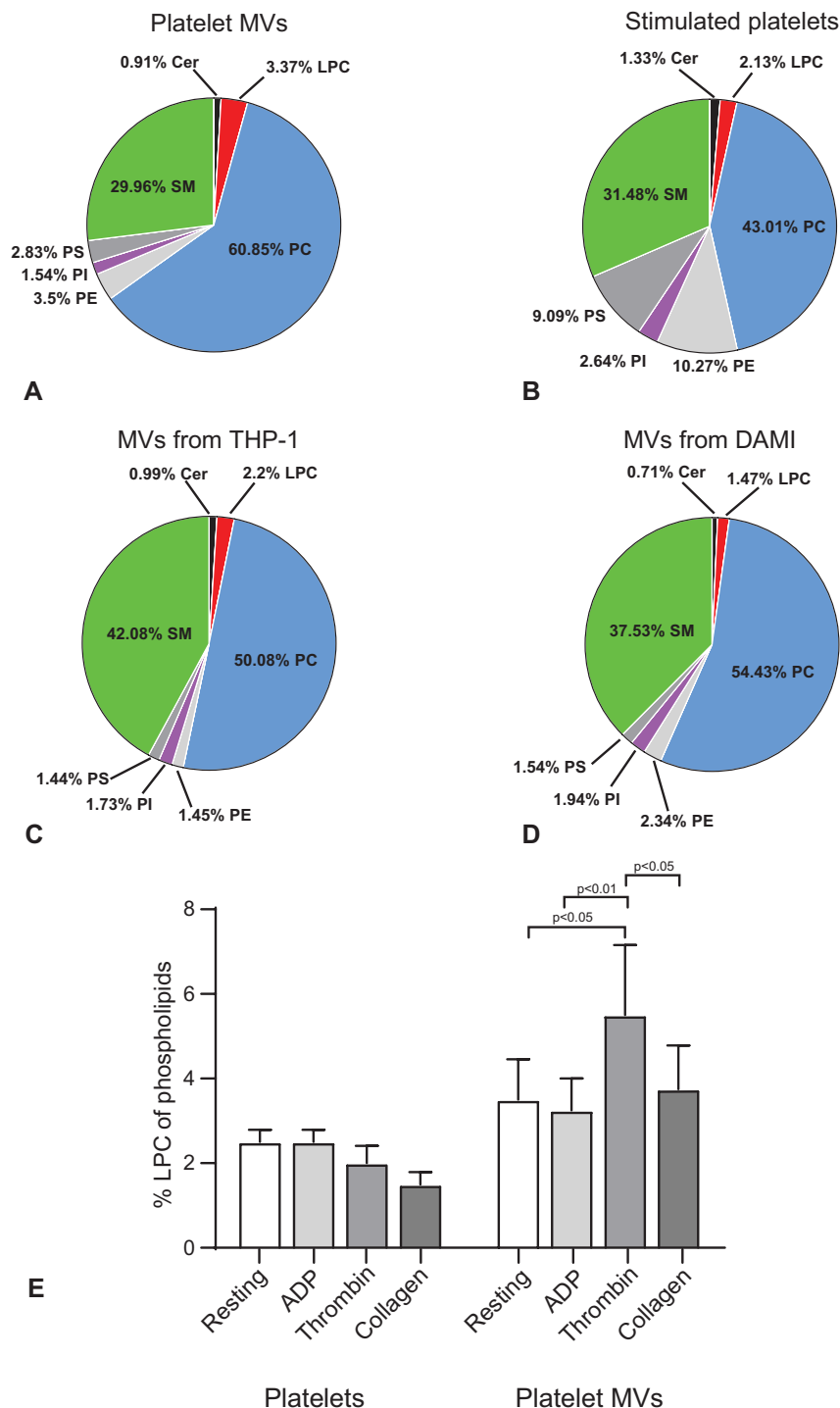


Fig. 1 Lipid distribution in activated platelets and microvesicles (MVs) of different origins. (A) Platelet-derived MVs (PMVs) contain more LPC than activated platelets (B). In comparison to PMVs, MVs from THP-1 cells and DAMI cells contain less LPC (C and D). The mean of $n = 3$ samples are given. The platelet agonists adenosine diphosphate (ADP), thrombin and collagen used to activate platelets and to generate PMVs all showed an overall statistically significant increased LPC content in PMVs compared with platelets. However, the individual agonist used did not result in a statistically significant difference in LPC content of PMVs (E). The overall comparison of platelet versus platelet MVs LPC content revealed that platelet MVs contain more LPC than the platelets from which they were released. $n = 4$. Cer, ceramides; LPC, lysophosphatidylcholines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; PS, phosphatidylserines; SM, sphingomyelins.

treated (**Fig. 3C**) and PBS-incubated (**Fig. 3D**) platelets. In agreement, surface areas of LPC-incubated platelets were significantly larger than those of PC-incubated platelets (PBS vs. ADP vs. LPC vs. PC: $1,315 \pm 114$ vs. $3,509 \pm 333$ vs. $2,829 \pm 453$ vs. 866 ± 73 , $n = 10$, $p < 0.0001$, **Fig. 3E**).

Platelets attached to fibrinogen and those pre-treated with LPC or ADP showed strong formation of lamellipodia and filopodia in comparison to those incubated with PBS or PC (**Fig. 3F**) as shown by anti-CD41 and phalloidin staining on platelets.

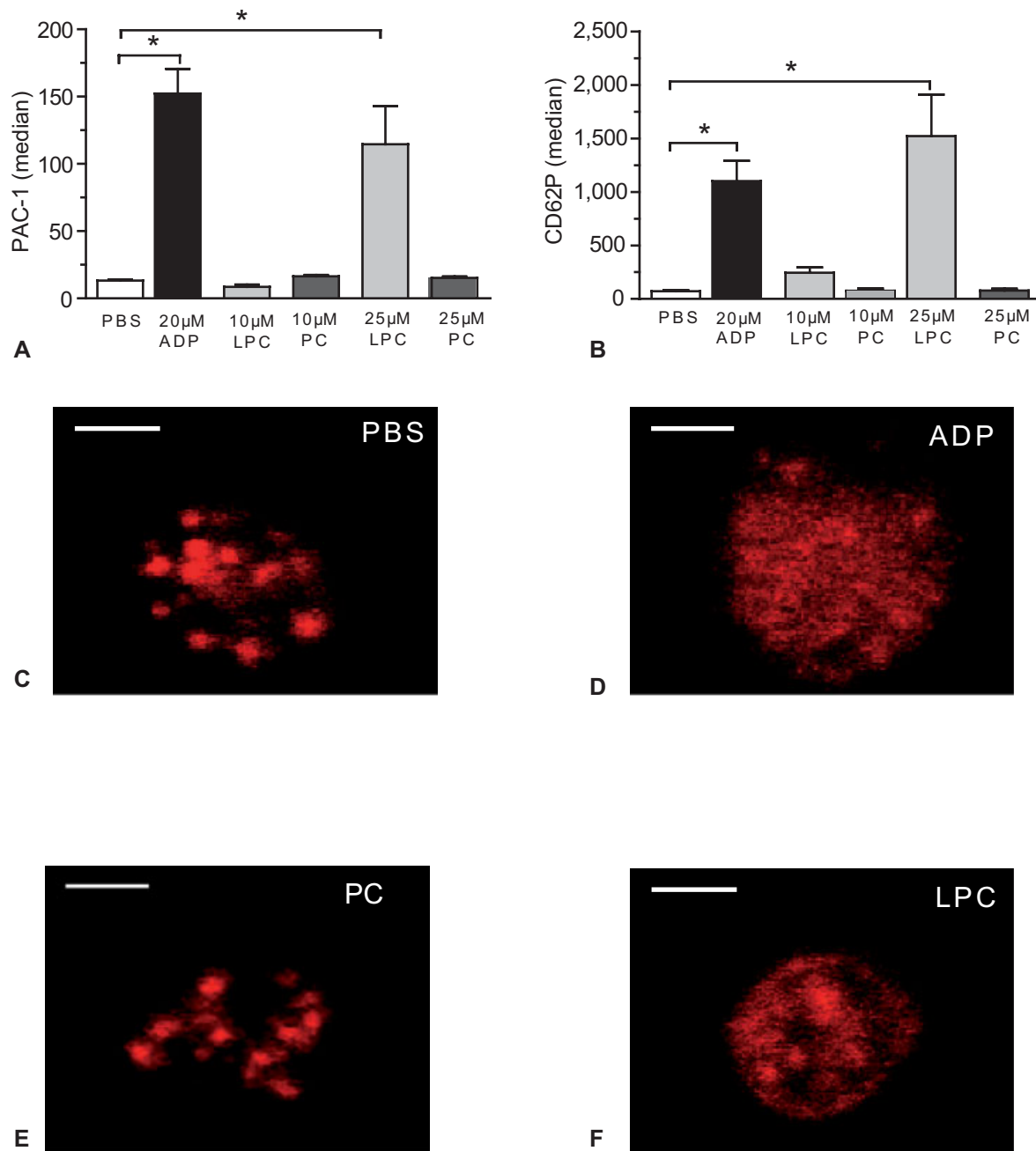


Fig. 2 Lysophosphatidylcholine (LPC) is a potent platelet activator. LPC but not phosphatidylcholine (PC) significantly induces activation of the platelet integrin glycoprotein (GP) IIb/IIIa, as detected by procaspase-activating compound 1 (PAC-1) binding in flow cytometry (A). Additionally, the platelet-activation marker CD62P is up-regulated after LPC treatment in flow cytometry (B). The effect of LPC on CD62P was confirmed in fluorescence microscopy with anti-CD62P staining, revealing that adenosine diphosphate (ADP) (D) and LPC (F), but not phosphate-buffered saline (PBS) (C) or PC (E), induce degranulation and translocation of CD62P. Bar indicates 2 μ m. * p < 0.05, n = 3.

LPC Induces Platelet Aggregation

Platelet aggregation is a critical step in atherothrombosis. To assess whether LPC affects platelet aggregation, light-transmission aggregometry was performed. LPC induced platelet aggregation to an extent similar to that of 20 μ M ADP (PBS vs. 50 μ M LPC: 3.752 ± 1.874 vs. 61.39 ± 12.57 , $p = 0.0011$; PBS vs. 50 μ M PC: 3.752 ± 1.874 vs. 3.193 ± 1.869 , $p = 0.6007$, $n = 6$, ►Figs. 4A and B). LPC-induced aggregation was also investigated in platelets pre-treated with a P2Y₁₂ receptor blocker (2MeSamp). 2MeSamp did not affect LPC-induced

platelet aggregation (50 μ M LPC + 2MeSAMP vs. 50 μ M LPC: 63.33 ± 9.748 vs. 61.39 ± 12.57 , $n = 6$, $p = 0.7561$), indicating that LPC acts independently of the P2Y₁₂ receptor.

LPC Acts as a Chemoattractant for Platelet Migration

Platelet migration is a more recently recognized component of platelet function. We adapted a migration assay and assessed the hypothesis that LPC acts as a chemoattractant. ►Figs. 4C–E shows three migration assays demonstrating platelet migration towards SDF-1, which is a well-known platelet chemoattractant.

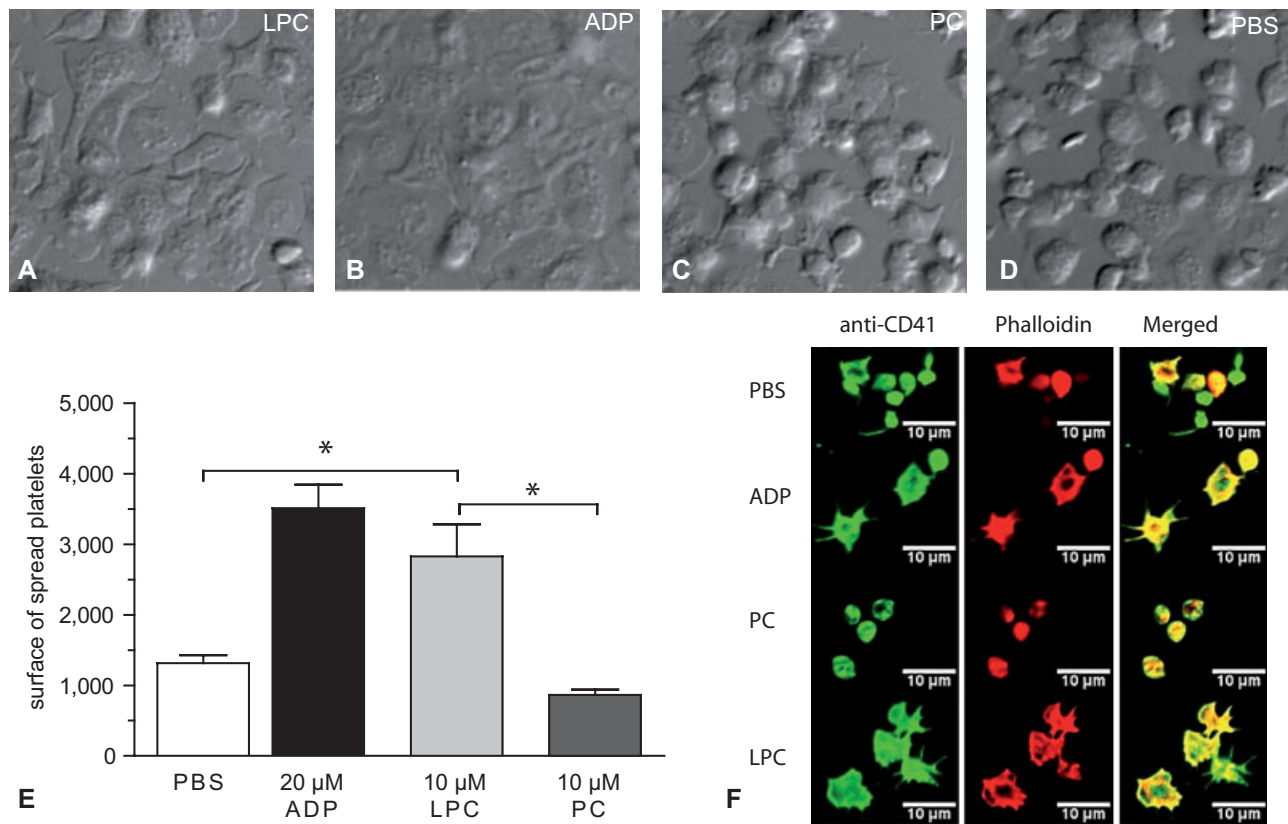


Fig. 3 Lysophosphatidylcholine (LPC) induces platelet adhesion and spreading to immobilized fibrinogen. (A) LPC-treated platelets show strong platelet adhesion and spreading on fibrinogen, similar to adenosine diphosphate (ADP) (B). In comparison to LPC and ADP, phosphatidylcholine (PC) (C) and phosphate-buffered saline (PBS) (D) cause significantly less platelet spreading. In agreement, LPC-treated platelets spread over a significantly larger surface area than platelets treated with PC (E, * $p < 0.05$, $n = 10$). Furthermore, platelets were treated with PBS (negative control), ADP (positive control), PC or LPC and platelet adhesion to fibrinogen as well as formation of platelet lamellipodia and filopodia was investigated. Stainings were performed with anti-CD41 and phalloidin. It was found that platelets after ADP and LPC treatment showed strong formation of platelet lamellipodia and filopodia, which was not found in platelets treated with PBS and PC (exemplary pictures out of 3 experiments are shown) (F).

Platelets stimulated with SDF-1 migrated with an average velocity of 178 $\mu\text{m}/\text{h}$ and a mean distance of 198 μm . Platelets moved with an average velocity of 144 $\mu\text{m}/\text{h}$ and a mean distance of 128 μm from the intersection of the x-axis and y-axis towards high LPC concentrations (\rightarrow Figs. 4I–K). The Rayleigh test showed a significant ($p < 0.05$) migration preference along the x-axis for the side on which the LPC or SDF-1 was located. Platelets not influenced by any chemoattractant moved with an average speed of 184.5 $\mu\text{m}/\text{h}$ and a mean distance of 124 μm but in different directions with no side preferred. No directional movement was observed when both chambers were filled with medium only (\rightarrow Figs. 4F–H). The Rayleigh test showed no significant preference ($p > 0.05$).

G2A Receptor Expression on Platelets

After finding that LPC specifically acts on platelets, we assessed whether platelets express the LPC receptor G2AR. Using qRT-PCR, we demonstrated that platelets and the megakaryocyte cell line DAMI, used as a cell contamination-free control, expressed G2AR (\rightarrow Fig. 5A). Human fibroblasts (HeLa cells) did not have relevant G2AR expression levels and were used as a negative control (\rightarrow Fig. 5B). Additionally, we showed G2AR expression in histological assessments of platelet thrombi (\rightarrow Figs. 5C–G). Supporting these

data, a commercially available anti-G2AR antibody inhibited the effect of LPC on platelet activation (CD62P expression) in a dose-dependent manner ($p < 0.0001$, $n = 6$, \rightarrow Fig. 5H).

LPC is a Marker of Unstable Atherosclerosis in Mice

To investigate whether LPC is associated with atherosclerosis, plasma from ApoE^{-/-} mice with TS surgery, a mouse model of plaque instability, was measured in comparison to mice with sham surgery. Typical histological characteristics of unstable plaques were found with Oil Red O, H&E and anti-CD68 staining. As shown in \rightarrow Fig. 6A, the unstable plaque demonstrates accumulation of macrophages in the plaque with a significant reduction of the vessel lumen.

We found the total LPC content in plasma was significantly increased in mice with TS representing plaque instability (mice with sham surgery vs. TS mice: $488 \pm 5 \mu\text{M}$ vs. $970 \pm 27 \mu\text{M}$, $p < 0.0001$, $n = 3$, \rightarrow Fig. 6B). Furthermore, we investigated the LPC content in various vessel segments and found it was significantly increased in unstable plaque segments compared with stable plaque and healthy vessel segments (healthy arteries vs. stable plaques vs. unstable plaques: 0.111 ± 0.014 vs. 0.297 ± 0.026 vs. 0.392 ± 0.054 , $n = 5$, $p = 0.0039$, \rightarrow Fig. 6C), indicating LPC represents a potential surrogate marker for unstable atherosclerosis.

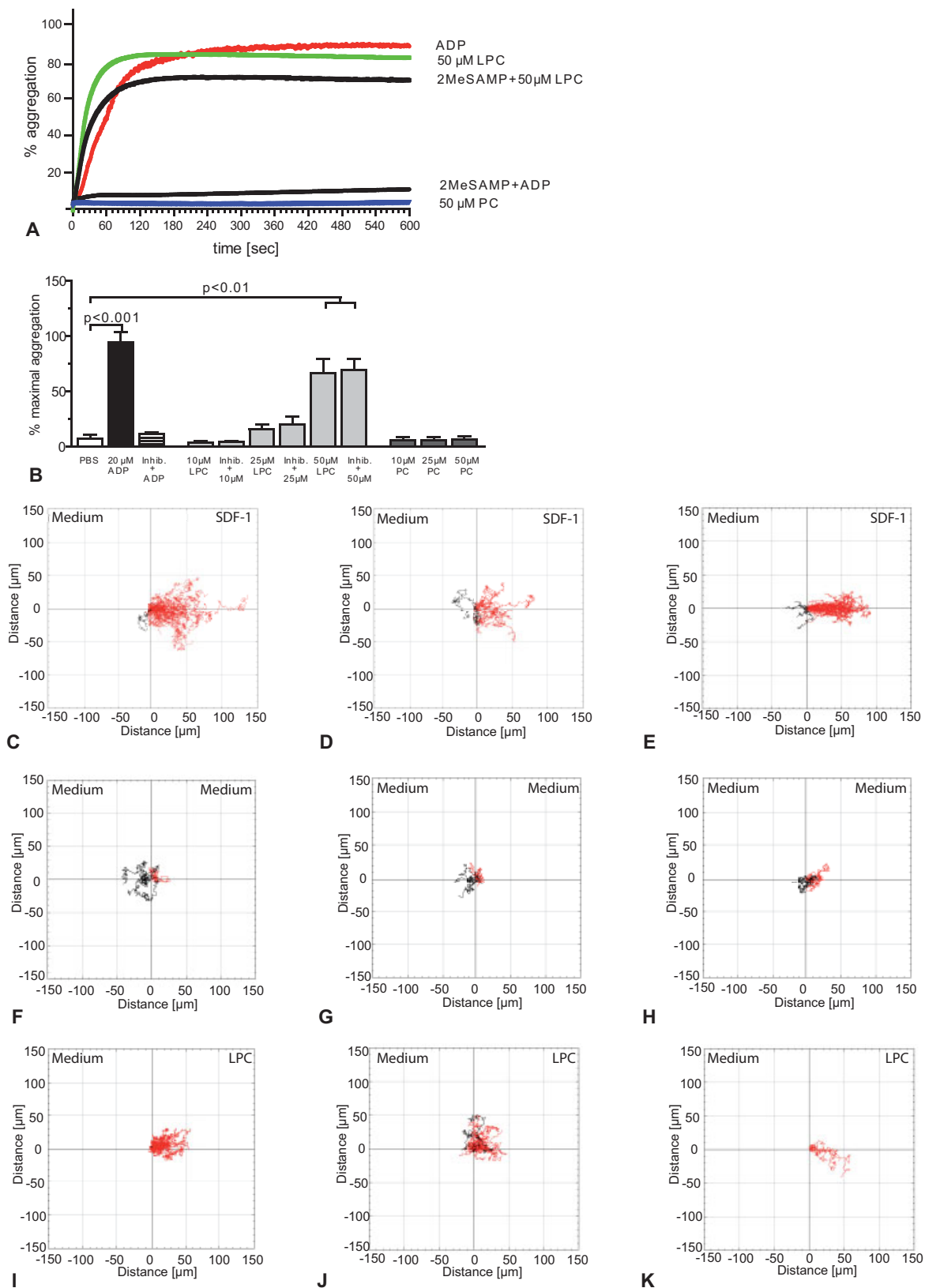


Fig. 4 Lysophosphatidylcholine (LPC) induces platelet aggregation and migration. As quantified by light-transmission aggregometry, LPC (green line) induces platelet aggregation to a similar extent as that of adenosine diphosphate (ADP) (red line), whereas phosphatidylcholine (PC) (blue line) has no effect on platelet aggregation (A). P2Y₁₂ inhibition by 2MeSAMP (black line) does not affect LPC-induced platelet aggregation (B), indicating that LPC activates platelets independently of P2Y₁₂. PC as a control does not induce platelet aggregation. * $p < 0.05$, $n = 6$. Using a μ -slide migration assay, we found washed platelets migrate towards higher stromal cell-derived factor 1 (SDF-1) concentrations (positive control) (C–E). No directional migration of platelets towards medium without any chemoattractant (negative control) was observed (F–H). Platelets migrate directional towards LPC (I–K).

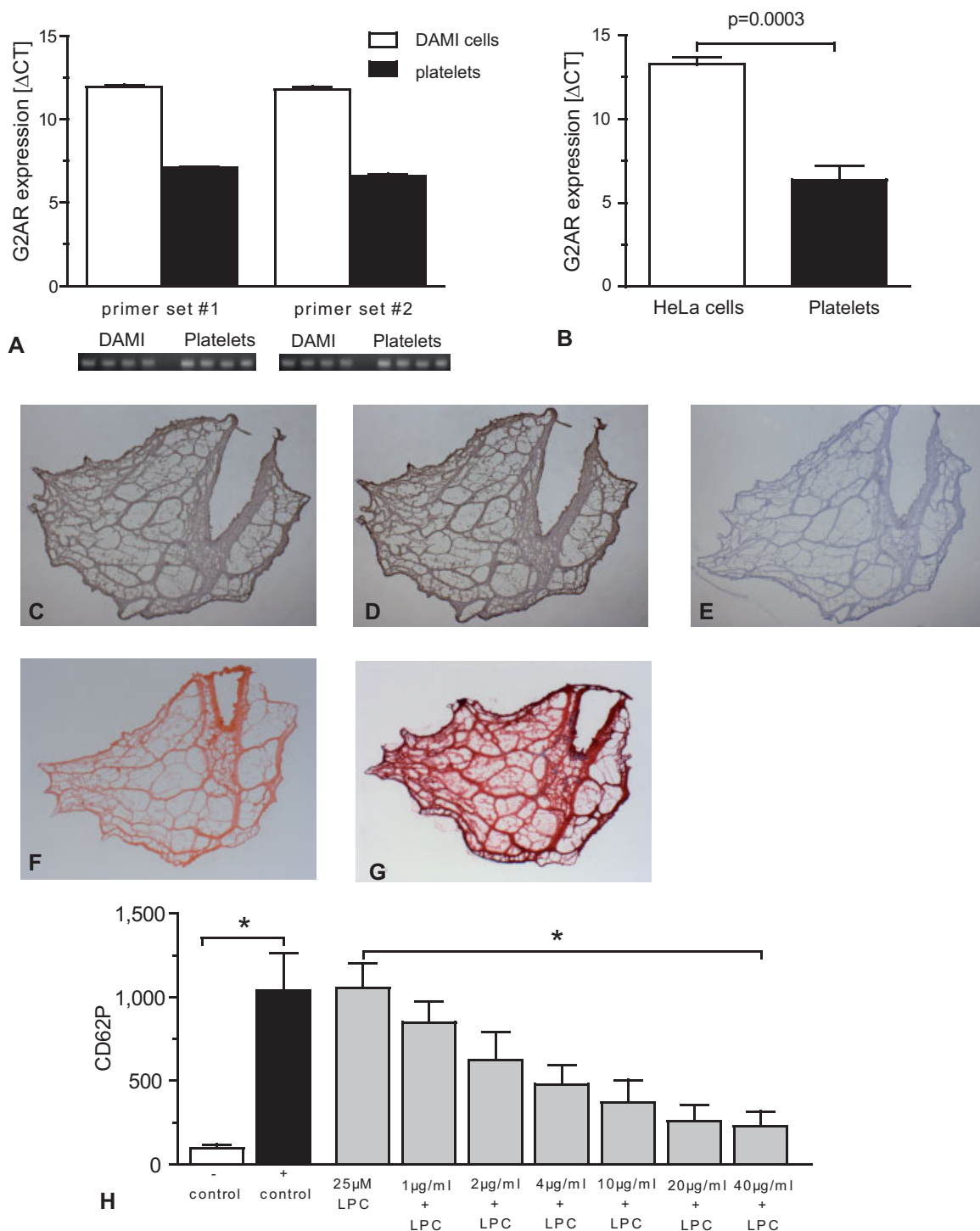


Fig. 5 Platelets express the lysophosphatidylcholine (LPC) receptor G2A. Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with two different G2AR primer sets, we found that washed platelets, as well as the megakaryocytic cell line DAMI, express significant levels of G2AR (A, $n = 4$). Human HeLa cells were used as a negative control and did not show significant G2A receptor expression ($n = 4$) (B). As shown via immunohistochemistry, thrombi express G2AR (C) and CD41 (D). (E) Phosphate-buffered saline (PBS) control (F) haematoxylin and eosin staining. (G) Masson's trichrome staining. Each of these stainings is representative of three independent stainings. Use of a specific G2AR-blocking antibody significantly reduces LPC-induced CD62P expression on platelets in flow cytometry in a dose-dependent manner (doses from 1 to 40 μ g/mL shown); indicating LPC acts on G2AR on platelets (H). * $p < 0.05$, $n = 6$.

LPC Distributions in Human Unstable Plaques

To confirm the data from the mice experiments and to indicate translational relevance, LPC content and local distribution were for the first time assessed in human plaques using MALDI.

To understand the localization of LPC in the human plaques, additional sections were stained with Masson's trichrome (**Fig. 7A**) as well as immunohistochemistry staining with MMP-9 (**Fig. 7B**), demonstrating extracellular protease activities, which is also considered a marker of

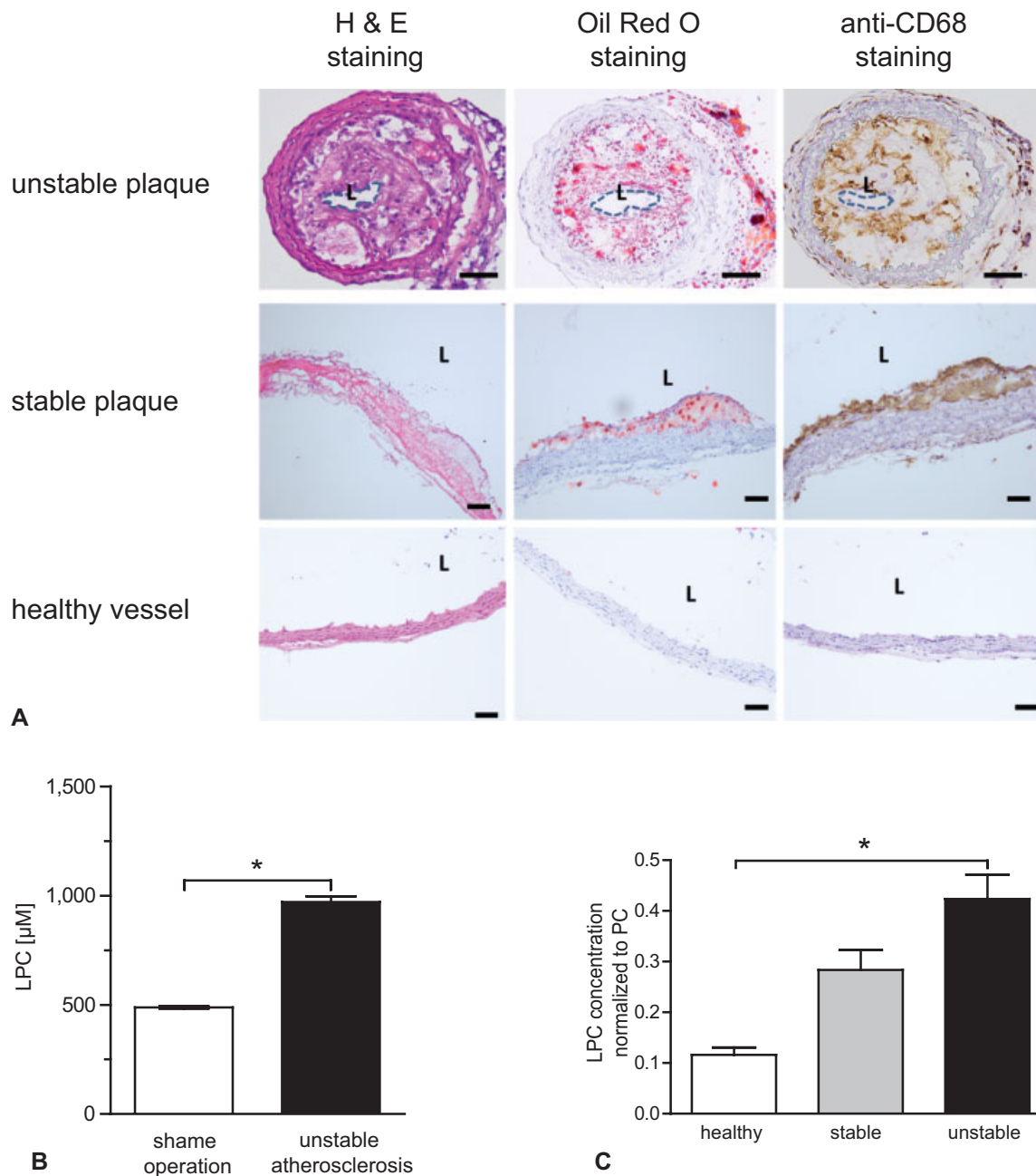


Fig. 6 Lysophosphatidylcholine (LPC) in blood of atherosclerotic mice and in unstable and stable atherosclerotic plaques of mice. Blood and tissue of healthy vessels, stable atherosclerotic plaques and unstable atherosclerotic plaques were characterized and investigated for their LPC contents. Oil Red O, haematoxylin and eosin (H&E) and anti-CD68 staining of tissue slides from the tandem stenosis mouse model of plaque instability/rupture showed the typical accumulation of macrophages in the unstable plaque with a significant reduction of the vessel lumen (A). Blood of mice with unstable atherosclerosis contains significantly higher amounts of LPC than those with stable atherosclerosis (B). Furthermore, LPC concentrations in murine vessels with stable atherosclerotic lesions are higher than those of healthy arterial tissue, while they are the highest in arteries with unstable atherosclerotic plaques (C). * $p < 0.05$, $n = 3$ for blood analysis and $n = 5$ for vessel tissue analysis. L indicates the vessel lumen, bar indicates 100 μ m.

plaque instability. MALDI imaging revealed that the most abundant LPC species, LPC 16:0 (\rightarrow Fig. 7C), LPC 18:0 (\rightarrow Fig. 7D) and LPC 18:1 (\rightarrow Fig. 7E), were enriched in the necrotic cores of atherosclerotic plaques.

Discussion

The major findings of the study are: (1) LPC is a major lipid of PMVs. (2) LPC induces platelet activation, aggregation, adhe-

sion, spreading and migration, as well as the formation of PMAs. (3) G2AR is expressed on platelets and mediates the effects of LPC on platelets. (4) LPC is increased in plasma of mice with unstable atherosclerosis. (5) LPC is highly abundant in unstable atherosclerotic plaques in both mice and humans.

Increasing evidence has emerged that MVs are important biovectors that transfer cellular components (e.g. proteins, cell organelles, RNA) to their target cells, thereby mediating

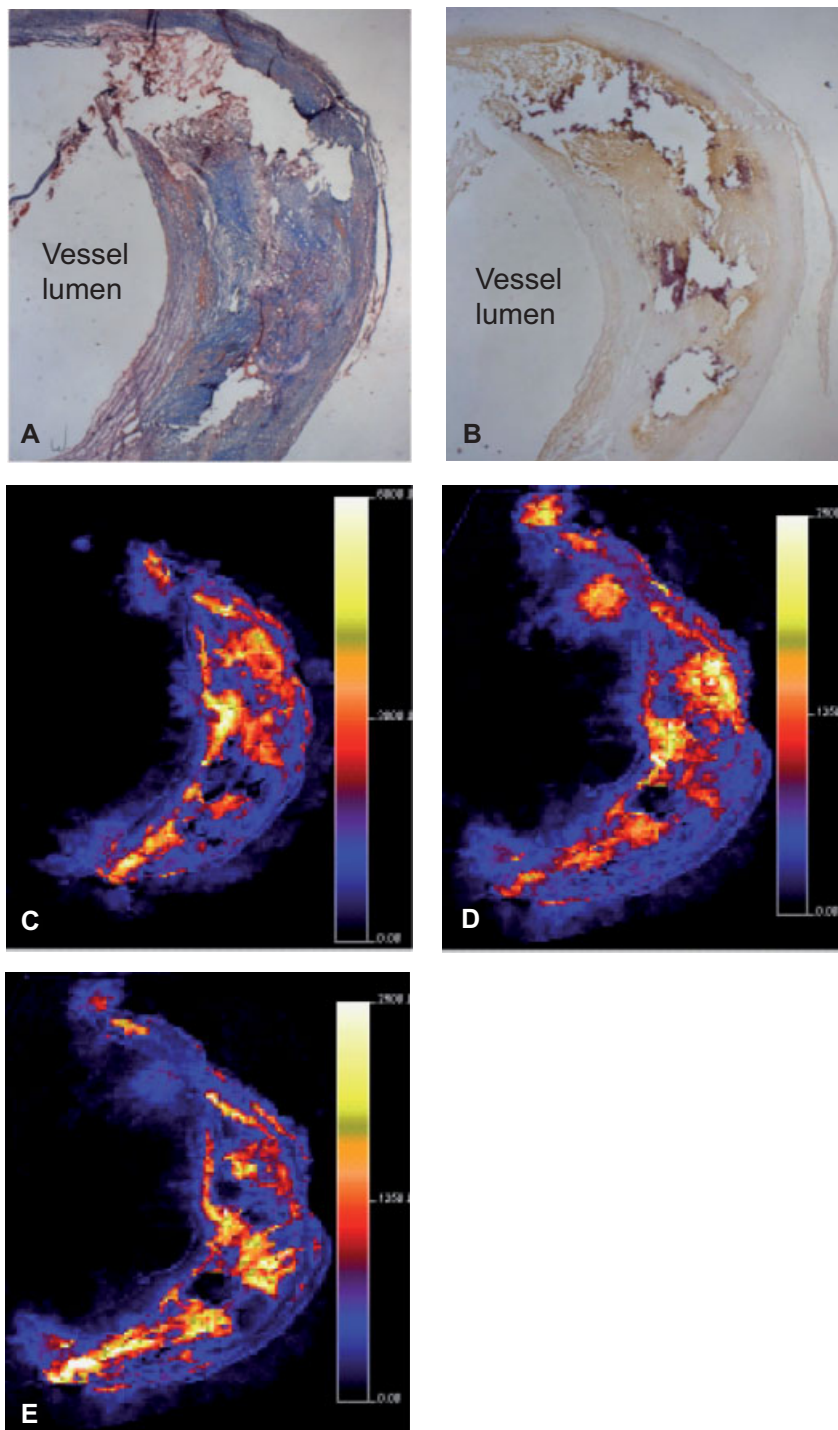


Fig. 7 Lysophosphatidylcholine (LPC) mapping of human vulnerable plaques. To investigate the morphology of atherosclerotic human plaque, tissue slides were stained for collagen (A) and matrix metalloproteinase 9 (MMP-9) (B). LPC 16:0 (C), 18:0 (D) and 18:1 (E) content was determined in these tissue sections. LPC mapping of human vulnerable plaques shows LPC concentrations are highest in areas of plaque vulnerability.

strong inflammation and coagulation in acute and chronic cardiovascular diseases.^{2,4,32,33} Because circulating lipids are involved in atherogenesis, we assessed whether the inflammatory effect of MVs can be attributed to the transport of inflammatory lipids such as LPC.³⁴ LPC is one of the main bioactive molecules in oxidized low-density lipoprotein and is involved in several conditions associated with vascular oxidative stress and inflammation.^{35,36} However, as LPC is

promptly metabolized in circulation by serum lysophospholipases, LPC is typically found to be associated with carrier proteins.³⁷ In this study, we demonstrate that PMVs, the most abundant type of MVs circulating in blood, contain large amounts of LPC and represent an important vehicle for LPC. Interestingly, these MVs contain relatively more LPC than do activated platelets, suggesting an active packaging mechanism of LPC into MVs.

PMVs are postulated to have strong pro-coagulant effects.³⁸ Hypothesizing these effects might be related to their LPC content, we found LPC causes activation of the platelet integrin GPIIb/IIIa, as well as increased surface expression of CD62P (P-selectin). Additionally, as shown by confocal microscopy, LPC induces the translocation of CD62P within the platelet to an extent similar to ADP. Several studies have proven that platelet CD62P interacts with the monocyte surface receptor PSGL-1, facilitating the formation of highly inflammatory PMAs.^{39,40} In agreement, we found LPC additionally increases PMA formation. These data indicate that LPC is a central component of PMV-induced platelet activation and platelet-monocyte crosstalk in cardiovascular disease.

Atherothrombotic complications are feared in patients with coronary artery disease and platelet activation plays a major role in these events as well as throughout atherogenesis.^{41,42} We hypothesized that LPC is a mediator of platelet activation in atherothrombotic events. Interestingly, it was reported that *Trypanosoma cruzi*, the cause of life-threatening Chagas disease, induces platelet aggregation in an LPC-dependent manner.⁴³ We found that LPC induces platelet activation, aggregation and spreading, confirming its central role. We then aimed to identify the platelet receptor mediating these LPC effects on platelets. It is well known that LPC binds to the G protein-coupled membrane receptor G2AR, which is expressed on a wide range of immune cells (e.g. lymphocytes and macrophages). This interaction mediates neutrophil and macrophage activation, as well as macrophage migration, and so is essential for both innate and adaptive immunity. However, to the best of our knowledge, the expression of G2AR in platelets has never before been reported. Using PCR, we found significant levels of G2AR in platelets, as well as the megakaryocytic cell line DAMI, suggesting the LPC effect on platelets is mediated by G2AR. Supporting this, histological staining of platelet thrombi confirmed significant G2AR expression and G2AR inhibition significantly reduced platelet activation by LPC in a dose-dependent manner.

It is evident that circulating PMVs bind to leukocytes and activate them.⁴⁴ Gómez-Muñoz et al found that LPC induced phospholipase D in mouse macrophages, which promoted the synthesis of inflammatory second messengers such as phosphatidic acid, lysophosphatidic acid and diacylglycerol, and thereby contributed to the cellular response involved in atherosclerosis and inflammation.⁴⁵ These data were confirmed by Huang et al, who described how LPC enhanced interferon γ secretion of peripheral blood monocytes, as well as antibody production.⁴⁶ Thus, once bound to circulating leukocytes or macrophages, platelet MVs might deliver their LPC to these cells, contributing to their activation and ultimately to vascular inflammation.

MVs of different cellular origin such as PMVs and T cell-derived MVs have been found to be involved in atherogenesis. However, their impact on the progression of atherosclerotic lesions still remains controversial.⁴⁷ Additionally, recent data indicate that also endothelial cells and smooth muscle cells of atherosclerotic plaques can release MV.⁴⁸

Whether atherogenesis and plaque instability is mainly driven by circulating MVs attaching to the plaque or by MVs released from the plaque itself also needs to be elucidated in future studies.

It has been suggested that oxidized lipids as components of MVs are involved in monocyte-endothelial cell interactions and thus in vascular inflammation at the early stages of atherosclerosis.⁴⁹ Otherwise, the impact of PMVs and their lipids on the progression of atherosclerosis is not well understood. In this study, enhanced levels of LPC were found in the plasma of mice exposed to unstable atherosclerotic plaques. These data imply that LPC in circulating MVs can be used as a marker for plaque instability.

A plasma risk marker of atherosclerotic plaque instability is highly sought-after and several advanced 'omics' approaches have been applied towards the discovery of such a marker.⁵⁰ Lipidomics as applied in our characterization of MVs and platelets has only become available based on the technical advances in liquid chromatography and mass spectrometry systems over the recent years. Recently, Chatterjee et al reported lipidomics of platelets from patients with coronary artery disease and acute coronary syndromes allowing the identification of specific patterns of lipids associated with cardiovascular risk.⁵¹ Our work extends this approach using lipidomics on MVs, which has not been described before. Increasingly high-throughput capabilities, increased sensitivities and the extension of detectable lipid species make lipidomics of platelets and/or MVs an attractive option for cardiovascular biomarker discovery.

Hypothesizing that PMVs are a potential source of LPC in circulation, our finding is in line with reports that PMVs are more abundant in atherosclerosis than controls.⁵² Our data are supported by those of Lavi et al, who found enhanced levels of Lp-PLA2, the enzyme that metabolizes PC to LPC, and increased amounts of LPC, in patients with coronary artery disease.⁵³ Wilensky et al found therapeutic inhibition of Lp-PLA2 with darapladib significantly reduced coronary atherosclerosis.⁵⁴

PMVs are not only markers of atherosclerosis in the circulation but were also found to accumulate in atherosclerotic lesions.^{52,55} Using fluorescent angiography, LPC has been detected ex vivo in coronary artery plaques.⁵⁶ These data were supported by Ménégaut et al, who reported ex vivo increased levels of 2-arachidonoyl-lysophosphatidylcholine in carotid atheroma plaques of diabetic patients.⁵⁷ In agreement with these report, we found LPC to be more abundant in atherosclerotic plaques compared with normal arterial tissues.

Our unique LPC mapping of vulnerable plaques using MALDI imaging is consistent with a role of LPC in plaque instability. Our finding is also consistent with a previous lipidomic comparison of unstable and stable plaque tissue describing an accumulation of LPC in unstable atherosclerotic plaques.⁵⁸ Similarly, it was shown that plaques from patients with symptomatic carotid artery stenosis contained more LPC than those of asymptomatic patients.^{59,60} These findings are also in line with a study by Herrmann et al, who

showed Lp-PLA2 was highly expressed in carotid artery plaques and furthermore predicted the risk for cardiovascular complications.⁶¹ Despite this ample evidence of LPC accumulation in unstable plaques, it has to be acknowledged that the source of LPC in plaques is not yet defined. MVs are shown to be abundant in plaques,³³ as such they are a potential source of plaque-associated LPC. However, further lipid tracing studies are warranted to ultimately clarify the source of unstable plaque-associated LPC.

Using MALDI to map LPC distribution in human unstable carotid plaque sections, we further found LPC was particularly enriched in necrotic plaque areas, those areas that were highly inflamed and prone to rupture. Considering that LPC in atherosclerotic plaques attracts further monocytes, which contain high levels of phospholipase A2 and thus increase LPC levels inside the plaque, LPC might in fact be a key contributor to plaque instability, promoting a vicious cycle of monocyte recruitment with consequently increased PLA2 and LPC levels.⁶² These data are supported by a finding that LPC itself induces macrophage polarization towards inflammatory M1 macrophages.⁶³

Peter et al showed that LPC is a strong chemoattractant for macrophages, leading to macrophage accumulation at atherosclerotic sites of high LPC content and thus promoting further plaque instability.⁶⁴ Within the last decade, it has become evident that platelets also have the ability to migrate towards chemokines, opening a whole new view into platelet (patho)physiology.⁶⁵ As a follow-up of our findings of increased LPC concentration in unstable atherosclerotic plaques, and identification of G2AR as an LPC receptor on platelets, we assessed whether LPC acts as a chemoattractant for platelets. We found that platelets migrate towards increasing LPC concentrations. These data suggest that platelets migrate into areas of unstable, vulnerable plaques and thereby promote plaque inflammation.

At present, the intracellular signalling cascade on which LPC acts can only be hypothesized. Stafford et al found the protein kinase D2, an enzyme essential for intracellular LPC signalling in leukocytes, is also expressed in platelets. Therefore, the intracellular pathway by which LPC act on platelets could be similar to that of leukocytes.⁶⁶ Moreover, recent data indicate exogenous LPC increases intracellular Ca^{2+} levels by opening transmembrane Ca^{2+} channels and by mobilizing Ca^{2+} from intracellular stores.⁶⁷ Both mechanisms are vital for platelet activation and aggregation. Schmidt et al showed platelets migrated towards increased SDF-1 concentration, and Ca^{2+} ion channels were essential for this platelet migration.⁶⁸ As LPC is known to increase intracellular Ca^{2+} concentrations, this may in fact be the underlying mechanism to explain why LPC acts as a chemoattractant for platelets.

In conclusion, PMVs are a major source of LPC in circulation. Binding of LPC to G2AR on platelets results in platelet activation, adhesion, spreading, migration and aggregation, as well as in the formation of PMAs. Additionally, LPC in circulating MVs in mice represents a marker for atherosclerotic plaque instability. Two-dimensional MALDI imaging/mapping in human atherosclerotic plaques shows LPC is

particularly enriched in areas of plaque instability, where it may contribute to plaque inflammation.

What is known about this topic?

- Platelet-derived microvesicles are involved in vascular inflammation.
- LPC is a pro-inflammatory lipid that promotes activation of white blood cells.
- Activated platelets contribute to atherothrombosis with consecutive thromboembolic complications.

What does this paper add?

- Lysophosphatidylcholine (LPC) is a major component of platelet-derived microvesicles.
- LPC binds to the G2A receptor, which was newly identified to be expressed on platelets, and thereby causes platelet activation, spreading, migration and aggregation.
- Circulating LPC is a surrogate marker for plaque instability in mice and was significantly increased in vulnerable areas of atherosclerotic plaques, in both mice and humans.

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Conflict of Interest

None declared.

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