Kinetics of Circulating Extracellular Vesicles Over the 24-Hour Dosing Interval After Low-Dose Aspirin Administration in Patients at Cardiovascular Risk

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Extracellular vesicles (EVs) are small vesicles deriving from all cell types during cell activation, involved in transcellular communication, and regarded as predictors of vascular damage and of cardiovascular events. We tested the hypothesis that, in patients on chronic low-dose aspirin treatment for cardiovascular prevention, aspirin may affect the release of EVs within the 24-hour interval. We enrolled 84 patients, mostly at high or very high cardiovascular risk, on chronic low-dose aspirin treatment. The numbers of circulating EVs (cEVs) and annexinV+ cEVs (*total*, *platelet-derived*, *endothelial-derived*, and *leucocyte-derived*) were assessed immediately before, and after 10 and 24 hours of a witnessed aspirin administration. Platelet cyclooxygenase 1 (COX-1) recovery was characterized by measuring serum thromboxane B_2 ($sTXB_2$) at the same timepoints. Nine healthy participants were also enrolled. In patients, daily aspirin administration acutely inhibited after 10 hours following aspirin administrations the release of cEVs (*total* and *leukocyte-derived*) and annexinV+ cEVs (*total*, *platelet-derived*, *endothelial-derived*, and *leukocyte-derived*), with a rapid recovery at 24 hours. The inhibition after 10 hours suggests a COX-1–dependent mechanism. Interestingly, the slope of platelet-derived and of annexinV+ platelet-derived cEVs were both directly related to $sTXB_2$ slope and COX-1 messenger RNA, raising the hypothesis that *vice versa*, cEVs may affect the rate of COX-1 recovery and the subsequent duration of aspirin effect. In healthy participants, no circadian difference was observed, except for leukocyte-derived cEVs. Our findings suggest a previously unappreciated effect of aspirin on the kinetics of a subset of cEVs possibly contributing to the cardioprotective effects of this drug.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ The extracellular vesicles (EVs) are (i) fragments of several cell types (i.e., endothelium, platelets, leukocytes); (ii) released during apoptosis or cell activation; (iii) play a role in microvascular and macrovascular complications in diabetic patients, and (iv) can be used as markers of vascular damage and as predictors of cardiovascular events. Studies evaluating the effects of pharmacological therapies on circulating EVs and the impact of aspirin on the release of EVs have produced divergent results.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Evaluate in patients treated with low-dose aspirin for cardiovascular prevention (i) the potential effects of chronic treatment with aspirin on cEV levels and (ii) the role of cEVs as a potential determinant of interindividual variability in aspirin response over the 24-hour interval.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Our findings suggest a bidirectional interplay between aspirin and cEV. On the one side, a previously unappreciated effect of aspirin on the kinetics of a subset of circulating EVs possibly contributing to the cardioprotective effects of this drug. On the other side, a contribution of cEV release to the interindividual variability in COX-1 recovery over the 24-hour dosing interval during chronic low-dose aspirin administration.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ The discovery of previously uncharacterized effects of low-dose aspirin on platelets and their interaction with extraplatelet, endothelial, and immune cells may help improve our understanding of the mechanisms of action of an old drug on cardioprotection and possibly chemoprevention.

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Aspirin is the cornerstone of cardiovascular prevention in high-risk patients.[1] The best characterized target of aspirin at low-doses is platelet cyclooxygenase (COX)-1 or prostaglandin endoperoxidase synthase, whose selective and irreversible acetylation at serine 529 residue inhibits the access of arachidonic acid to the site of active bond.[2] Acetylation of platelet COX-1 translates into a reduction in the release of thromboxane A_2 (TXA_2), a metabolite downstream COX-1 pathway.[3] The development of serum TXB_2 ($sTXB_2$), as a mechanism-based biomarker of platelet COX-1 inhibition and as the most reliable tool for assessing aspirin response,[3] has played a fundamental role in defining the human pharmacology of aspirin as an antiplatelet agent.[4] Pharmacodynamic studies evaluating time-dependent acetylation levels of platelet COX-1 after low-dose aspirin show a maximum inhibition of COX-1 acetylation and $sTXB_2$ formation 8 hours after administration.[5] Due to the lack of nucleus, and to the irreversible nature of COX-1 acetylation, this effect is long-lasting during the 24-hour dosing interval. Indeed, in healthy participants, $sTXB_2$ biosynthesis is persistently and constantly inhibited between two administrations.[6] Platelet COX–1 acetylation and inhibition of $sTXB_2$ are cumulative and saturable mechanisms for repeated daily doses.[7] Despite the irreversibility of action of aspirin, a percentage of patients on chronic therapy still experience atherothrombotic events due to an accelerated platelet turnover, with newly released platelets escaping aspirin inhibition, yielding a rapid recovery of COX-1 and incomplete suppression of TXA_2 production over the 10-to-24-hour dosing interval.[8]

Extracellular vesicles (EVs) are fragments of virtually all cell types (i.e., *platelets*, *endothelium*, *leukocytes*) released during cell apoptosis or activation. They are characterized by an integral plasma membrane expressing the phenotype of the cell from which they originated[9,10] and can be identified and enumerated by flow cytometry using specific markers.[9] EVs are thought to play a role in microvascular and macrovascular complications in diabetic patients and can be used as markers of vascular damage and as predictors of cardiovascular events.[11] The cargo of EVs includes proteins, lipids, and genetic material such as messenger RNAs (mRNAs) and noncoding RNAs, suggesting a role in intercellular communication.[12] Interestingly, the content of EVs may be significantly different from their parental cells, indicating an active mechanism of selective "packaging" from cells into EVs, thus suggesting an interesting mechanism for transferring gene-regulatory function from EV-releasing cells to target cells, thus influencing cell phenotype and biological behavior.[13] Circulating EVs (cEVs) can exhibit phosphatidylserine (PS) on their surface, as an index of their procoagulant activity,[14] apoptosis,[15] and prothrombotic status, and are elevated in many cardiovascular and metabolic disorders, such as atherosclerosis, acute coronary syndromes, hypertension, heart failure, type 2 diabetes mellitus, and obesity.[16] Studies evaluating the effects of pharmacological therapies on cEVs and the impact of aspirin on the release of EVs have produced divergent results,[17] possibly due to the heterogeneity of clinical settings and methods used to assess EV numbers. Bulut *et al*. showed that treatment with aspirin (100 mg) for 8 weeks reduced endothelial-derived and platelet-derived EV levels by 62.7% ($P < 0.05$) and 28.4% ($P < 0.05$), respectively, in patients with coronary artery disease.[18] At variance, a significant reduction in EVs derived from monocyte and smooth muscle cells was observed after 10 days of aspirin treatment (100 mg) in previously aspirin-naïve patients with type 2 diabetes mellitus, while EVs derived from leukocyte, platelet, and endothelial cells did not show significant changes.[19] Consistently, a 3-day aspirin treatment (150 mg) did not affect platelet EV numbers in previously aspirin-naïve healthy participants despite its effective inhibition of platelet aggregation.[20]

Aims of the study were to evaluate in patients on low-dose aspirin treatment for cardiovascular prevention (i) the potential time-dependent effects of chronic treatment with aspirin on levels of cEVs over the 24-hour dosing interval and (ii) the role of cEVs as a potential determinant of the interindividual variability in aspirin response.

METHODS

Study participants

A total of 84 patients on chronic treatment with low-dose aspirin (enteric coated, Cardio aspirin 100 mg, Bayer, Milan, Italy) once daily for at least 1 month were enrolled at the Diabetes and Cardiovascular Prevention Clinics, Dept. of Clinica Medica, Chieti SS. Annunziata Hospital, Italy. We also enrolled nine healthy participants, not on aspirin treatment, as controls.

Exclusion criteria included clinically significant hepatic, renal, cardiac, and/or pulmonary insufficiency; history of malignant neoplasms (diagnosed and treated within the past 5 years); pregnancy or lactation; history of malabsorption; regular (daily) alcohol consumption; regular (i.e., more than 3 days per week) nonsteroidal anti-inflammatory drug intake; type 1 diabetes excluded by islet autoantibodies evaluation (antiglutamic acid decarboxylase, islet cell cytoplasmic, and IA-2 antibodies), in the presence of any of the following: family history of type 1 diabetes, age less than 40 years, lean phenotype, or early requirement for insulin therapy. Patients with arterial hypertension or hypercholesterolemia were included if it was well controlled with stable drug therapy.

This study was performed according to Good Clinical Practice regulations (Good Clinical Practice for Trial on Medicinal Product for Human Use (CHMP)/European Commission—July 1990; Decreto Ministeriale 27.4.1992—Ministero della Sanità and the Declaration of Helsinki, Hong Kong 1989; Clinical Trial Registry number NCT05584943).

The protocol was approved by the institutional Ethics Committee of the University of Chieti (GR-2011-02350450). Participants provided written informed consent.

Study design

The study was preceded by a 7-day run-in during which patients were instructed to take aspirin at 8 a.m. The protocol was divided into 2 days: Day 1, 8:00 a.m. (T0), first sampling for general blood tests, $sTXB_2$, EVs, followed by a witnessed aspirin intake; 6:00 p.m. (T10), second sampling for $sTXB_2$ and EVs; Day 2, 8:00 a.m. (T24), third sampling for $sTXB_2$, COX-1 mRNA and cEVs (**Figure 1**).

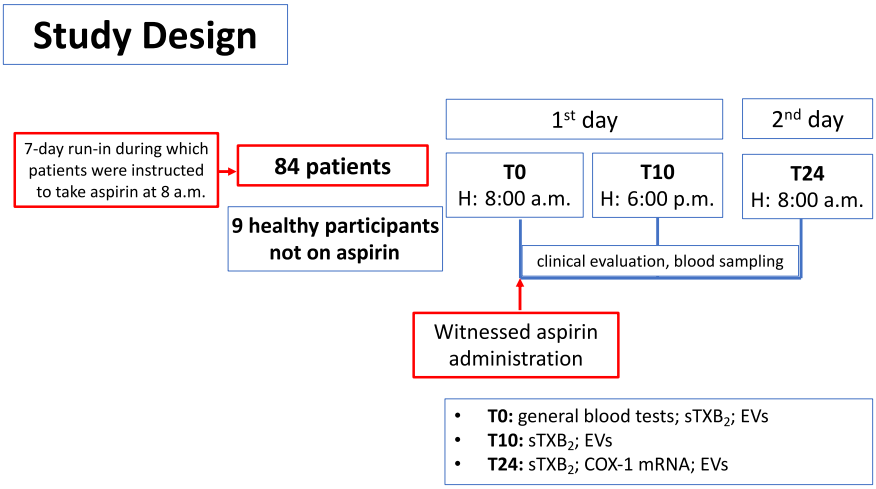

Figure 1 Study design. COX-1, cyclooxygenase 1; EVs, extracellular vesicles; mRNA, messenger RNA; $sTXB_2$, serum thromboxane B2; T0, 0 hours postaspirin intake; T10, 10 hours postaspirin intake; T24, 24 hours postaspirin intake.

Analytical measurements

All participants underwent three subsequent blood samplings (Day 1: 8:00 a.m. and 6:00 p.m.; Day 2: 8:00 a.m.). Blood samples for routine hematochemistry were obtained according to the protocols of the centralized laboratory of Chieti SS. Annunziata Hospital, Italy.

Biochemical measurements

Serum TXB_2 produced *ex vivo* during whole blood clotting[21] and index of platelet COX-1 activity[22] was measured with a commercial enzyme-immunoassay kit (Cayman Chemical, Ann Arbor, MI).[23] Serum TXB_2 slope (($sTXB_2$ T24—$sTXB_2$ T10)/14) is the linear fitting of $sTXB_2$ measured at T10 and T24 postaspirin witnessed intake that reflects the extent of platelet COX-1 recovery and of low-dose aspirin response.

Quantitative real-time polymerase chain reaction

Platelet COX-1 mRNA was analyzed in a small subset of 22 patients to verify whether platelet COX-1 content may be related to cEV number. Platelet-rich plasma was separated (100 g, 15 min) from ACD-A (Acid Citrate Dextrose Solution, Solution A) anticoagulated blood, mixed with prostaglandin E_1 (PGE_1, 4 μM) and EDTA (10 mM), and filtered on Pall Purecell PL (Pall Medical, New York) to remove leukocyte contaminant.

Filtered platelet-rich plasma was analyzed by flow cytometry to exclude red blood cell (CD235+) contamination and platelet activation (CD62P+). Platelet-rich plasma was centrifuged (1,900 *g*, 8 minutes) and pellet lysed with RL-Buffer Kit (Norgen, Thorold, ON, Canada).

Platelet RNA was extracted by using Total RNA Purification Kit (Norgen), and the reverse transcription of 500 ng was performed by Omniscript RT Kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reactions were performed using miSCRIPT Syber Green PCR Kit (Qiagen). Primers (5′ to 3′) used were:

- COX-1 forward TCATCAGGGAGTCTCGGGAG, reverse ATTCCTCCAACTCTGCTGCC;
- β2-microglobulin forward GCTCGCGCTACTCTCTCTTT, reverse TGTCGGATGGATGAAACCCA.

Extracellular vesicles: flow cytometry analysis

Peripheral blood (PB) samples (3.5 mL of sodium citrate tubes; Becton Dickinson Biosciences-BD, Franklin Lakes, NJ) were drawn, using 19G needles, and processed within 4 hours from venipuncture. PB samples for EV analyses were stained as already described.[24] Briefly, to stain PB samples, a reagent mix was prepared by adding to 195 μL of binding buffer 1× (BD Biosciences) the dyes and antibodies (0.5 μL CD31 Pe-Cy7 563651 BD Biosciences; 1 μL Lypophilic Cationic Dye (LCD) APC 626267 BD Biosciences; 1 μL CD45 BB515 564585 BD Biosciences; 5 μL CD41a BV510 563250 BD Biosciences; 1 μL annexinV V450 560506 BD Biosciences). Then 5 μL of PB samples were added to the reagent mix and incubated for 45 minutes (room temperature, in the dark). Finally, samples were adequately diluted with binding buffer 1× (1:143), and no swarm effects occurred[24] when they were acquired (1×10^6 events/samples) by flow cytometry (FACSVerse, BD Biosciences). All requirements imposed for polychromatic flow cytometry EV analysis were considered.[25] The trigger threshold was placed on the channel in which the dye used to stain EVs emits (lipophilic cationic dye, allophycocyanin—APC—channel, threshold value = 200/262.144). For all used parameters the height signals and biexponential or logarithmic modes were selected. Instrument performances, data reproducibility, and fluorescence calibrations were sustained by the Cytometer Setup & Tracking Module (BD Biosciences). The evaluation of nonspecific fluorescence was obtained by acquiring fluorescence minus one controls combined with the respective isotype control.[9] Compensation was automatically calculated. Data were analyzed using FACSuite v 1.0.6.5230 (BD Biosciences) and Flow Jo X v 10.0.7 (BD Biosciences) software. EV concentrations were obtained by the volumetric count function.[26] EVs were identified and subtyped as already reported (including "small EVs" (range sizes 50–100 nm), medium/large EVs (>100 nm)).[24,26] Briefly, within the gate of intact EVs (LCD+/Phalloidin−), platelet-derived EVs were identified as CD41a+ events, while leukocyte-derived and endothelial-derived EVs were detected as CD45+ and CD45−/CD41a−/CD31+ EVs, respectively, as also suggested by the position paper of the International Society of Extracellular Vesicles.[25] Each of the above-mentioned EV subsets was also analyzed for the surface expression of annexinV+, reflecting their procoagulant activity[14] and/or apoptosis.[15]

Nonalcoholic fatty liver disease

The grading of nonalcoholic fatty liver disease has been obtained using a high-resolution ultrasound system (General Electric GE Heathcare). Participants were examined in the supine position. Steatosis was graded as score 0 when the echotexture of the liver is normal; score 1when there is a slight and diffuse increase of liver echogenicity with normal visualization of the diaphragm and of the portal vein wall; score 2 when a moderate increase of liver echogenicity with slightly impaired appearance of the portal vein wall and the diaphragm; and score 3 when marked increase of liver echogenicity with poor or no visualization of the portal vein wall, diaphragm, and posterior part of the right liver lobe.[27]

Statistical analysis

The primary end point was the kinetics of total cEV numbers across the 24-hour dosing interval after a witnessed aspirin administration, in participants already on chronic low-dose aspirin treatment. Secondary preplanned end points were the kinetics of platelet-derived, leukocyte-derived, and endothelial-derived EV and total, platelet-derived, leukocyte-derived, and endothelial-derived annexinV+ subsets of cEV. Comparisons of variables between timepoints (overall comparison of three timepoints: T0 vs. T10 vs. T24; and pairwise comparisons: T0 vs. T10; T10 vs. T24; T0 vs. T24) over the 24-hour aspirin dosing interval, were performed by Friedman *U*-Test followed by Sidak correction for $N = 3$ multiple comparisons. Spearman rank correlation test was used to assess relationships among continuous variables. Comparisons of cEV number between patients and healthy participants was performed by Mann–Whitney *U*-Test. Only two-tailed probabilities were used for testing statistical significance, and $P < 0.05$ was considered statistically significant. The data analysis was generated using SAS/STAT software, Version 9.1.3 of the SAS Statistical System for Windows 2009.

RESULTS

Baseline characteristics

Baseline characteristics of all patients are detailed in **Table 1**. Approximately three quarters of patients had hypertension (73.0%) and nonalcoholic fatty liver disease (74.7%, 20 patients score 1, 30 score 2, and 9 score 3) and half had obesity and/or type 2 diabetes mellitus. Overall, 22 participants out of 84 were on aspirin treatment for secondary prevention (13 previous myocardial infarction or revascularization, 8 previous stroke, 3 peripheral artery disease, of whom 2 participants with concurrent peripheral artery disease and previous stroke) and an additional 37 had other manifestations of atherosclerotic cardiovascular disease (stable coronary artery disease or carotid stenosis >50%). Forty-four percent were on statin treatment.

Kinetics of COX-1 recovery

Serum TXB_2 was reduced after 10 hours following a witnessed aspirin intake (T10 vs. T0 $P = 0.0003$ and vs. T24, $P = 0.0003$), and returned to baseline after 24 hours (T0 vs. T24, $P = 0.8377$; **Figure 2**). Although with interindividual variability, this pattern reflects the maximum inhibition of COX-1 after 8 hours.[5]

The number of cEVs and annexinV+ cEVs (*total, platelet-derived, endothelial-derived, and leukocyte-derived*) were lower after 10 hours following aspirin administration

Observation of the kinetics of cEV number in the 24-hour dosing interval between two consecutive aspirin administrations unraveled a pattern of inhibition and subsequent recovery. Overall, the numbers of cEVs (*total* and *leukocyte-derived*) and of annexinV+ cEVs (*total* and *platelet-derived*) displayed time-dependent changes across the 24 hours after a witnessed aspirin administration (T0 vs. T10 vs. T24; **Table 2**).

The number of Total cEVs ($P = 0.003$, $P = 0.003$; **Figure 3** and **Table 2**) and of Total annexinV+ cEVs ($P = 0.003$, $P = 0.003$; **Figure 3** and **Table 2**) were lower at T10 vs. T0 and vs. T24, respectively. Platelet-derived cEV numbers did not change over time while platelet-derived annexinV+ cEV numbers ($P = 0.003$, $P = 0.003$; **Figure 3** and **Table 2**) were lower at T10 vs. T0 and vs. T24, respectively. Endothelial-derived cEV numbers were not different

Table 1 Baseline characteristics of all patients

Variable	*n* (%) or median (25th–75th percentile)
N	84
Age, years	68.0 (61.7–73.2)
Male gender, *n* (%)	48 (57.0)
Smokers, *n* (%)	10 (11.6)
Weight, kg	80.0 (72.0–90.5)
BMI, kg/m^2	29.9 (25.9–33.0)
Waist circumference, m	104.0 (96.0–112.2)
WHR	1.0 (0.9–1.0)
Obesity, *n* (%)	42 (50.0)
Systolic arterial pressure, mmHg	140.5 (133.8–152.0)
Diastolic arterial pressure, mmHg	78.0 (70.0–84.2)
Hypertension, *n* (%)	73 (87.2)
Type 2 diabetes mellitus, *n* (%)	45 (53.6)
Glycated hemoglobin, %	6.1 (5.7–6.9)
Fasting plasma glucose, mg/dL	103.5 (93.0–125.2)
Serum creatinine, mg/dL	0.8 (0.7–0.9)
eGFR, mL/min	88.3 (76.2–96.2)
Uric Acid, mg/dL	5.7 (4.6–7.0)
Total cholesterol, mg/dL	177.5 (152.7–208.0)
HDL cholesterol, mg/dL	50.0 (42.0–57.2)
Triglycerides, mg/dL	121.0 (87.7–155.5)
Dyslipidemia, *n* (%)	46 (55.8)
AST, U/L	24.5 (20.0–30.0)
ALT, U/L	29.0 (22.7–36.0)
Total Bilirubin, mg/dL	0.7 (0.5–0.9)
NAFLD, *n* (%)	59 (74.7)
hs-C-reactive protein, mg/dL	0.3 (0.1–0.5)
Red blood cell count, $\times 10^6/\mu L$	4.7 (4.4–5.1)
Hemoglobin, g/dL	13.9 (13.0–14.8)
Hematocrit, %	41.1 (38.4–43.4)
Platelet count, $\times 10^3/\mu L$	233.0 (197.5–283.5)
Mean platelet volume, fL	11.1 (10.3–11.6)
White blood cell count, $\times 10^3/\mu L$	6.8 (5.9–7.7)
Cardiovascular disease, *n* (%)	
Stable CAD	7 (8.1)
Carotid stenosis (>50%)	30 (37.2)
MI or revascularization	13 (15.3)
Stroke, TIA or revascularization	8 (9.3)
Peripheral artery disease	3 (3.5)
Therapy, *n* (%)	
Metformin	27 (34.1)
Glinide	4 (4.7)
PPAR-γ agonists	6 (8.1)
Sulfonylurea	3 (3.5)
DPP-IV	1 (1.2)

(Continued)

Table 1 (Continued)

Variable	*n* (%) or median (25th–75th percentile)
Acarbose	1 (1.2)
ACE-I	28 (32.6)
ARBs	20 (25.6)
Diuretics	25 (29.1)
β-blockers	32 (37.2)
CCA	30 (34.9)
Other antihypertensives	1 (1.2)
Statins	38 (44.2)
Ezetimibe	3 (3.5)
Omega 3	2 (2.4)
Proton pump inhibitors	35 (40.7)
ASA	84 (100)

ACE-I, angiotensin-converting enzyme inhibitor; ALT, alanine amino transferase; ARBs, angiotensin receptor blockers; ASA, acetylsalicylic acid (aspirin); AST, aspartate aminotransferase; BMI, body mass index; CAD, coronary artery disease; CCA, calcium channel antagonist; CCA, common carotid artery; DPP-IV, dipeptidyl peptidase IV; eGFR, estimated glomerular filtration rate; GLP1 RA, glucagon-like peptide 1 receptor agonist; HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; MI, myocardial infarction; NAFLD, nonalcoholic fatty liver disease; PPAR-γ, peroxisome proliferator-activated receptor gamma; TIA, transient ischemic attack; WHR, waist to hip ratio.

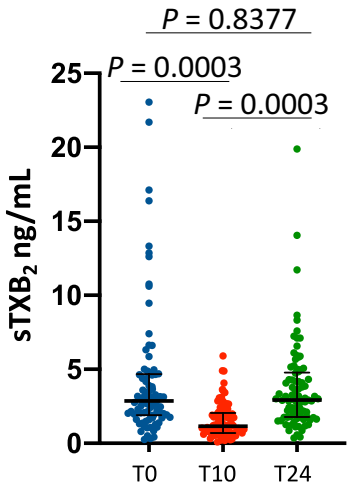

Figure 2 Levels of total serum thromboxane B_2 in all patients. Serum thromboxane B_2 ($sTXB_2$), *ex vivo* index of COX-1-dependent TXA_2 production, measured at 0 (T0), 10 (T10), and 24 hours (T24) postaspirin intake in all patients ($n = 84$). Significance was calculated by Friedman *U*-Test, followed by Sidak correction for multiple comparisons. COX-1, cyclooxygenase 1.

over the three timepoints, while endothelial-derived annexinV+ cEV numbers ($P = 0.036$; Figure 3 and Table 2) were lower at T10 vs. T24. Leukocyte-derived cEV numbers ($P = 0.003$, $P = 0.003$, $P = 0.047$; Figure 3 and Table 2) and leukocyte-derived annexinV+ cEV numbers ($P = 0.012$, $P = 0.003$, $P = 0.999$; Figure 3 and Table 2) were lower at T10 vs. T0 and vs. T24, and at T0 vs. T24.

The pattern of cEVs (*total* and *leukocyte-derived*) and of annexinV+ cEVs (*total, platelet-derived endothelial-derived*, and *leukocyte-derived*) reduction and subsequent increase after aspirin administration, mirroring the kinetics of $sTXB_2$ inhibition and recovery, suggests a COX-1 dependent mechanism underlying its release, at least for a few EV subphenotypes.

The number of cEVs (*total, platelet-derived, endothelial-derived*) and annexinV+ cEVs (*total, platelet-derived, endothelial-derived,* and *leukocyte-derived*) in healthy participants not on aspirin therapy did not change across the 24-hour dosing interval

To exclude that the observed changes in the number of cEVs over 24 hours were dependent on a circadian rhythm and not on the effect of aspirin, we recruited nine sex-matched healthy volunteers not on aspirin treatment and evaluated the levels of the cEVs at the same timepoints. We did not observe any difference in the number of cEVs in healthy volunteers among T0 vs. T10 and vs. T24 (Figure 4), reinforcing the hypothesis that the reduction of the cEVs (*total* and *leukocyte-derived*) and annexinV+ cEVs (*total, platelet-derived, endothelial-derived*, and *leukocyte-derived*) observed in patients after 10-hour aspirin administration was an effect of the drug and not due to a circadian rhythm.

Correlations between slope of platelet EVs and slope of $sTXB_2$ and COX-1 mRNA

The apparent similarity between the kinetics of cEVs (*total* and *leukocyte-derived*) and annexinV+ cEVs (*total, platelet-derived, endothelial-derived*, and *leukocyte-derived*) after chronic aspirin intake and the pattern of inhibition and recovery of $sTXB_2$ in settings of enhanced platelet turnover prompted us to challenge the relationship between the two. We observed a direct correlation between the slope of $sTXB_2$, mirroring the recovery rate of platelet COX-1 activity during the 10–24 hour dosing interval, and the slope of cEVs derived from platelets ($\rho = 0.206$, $P = 0.068$) and slope of annexinV+ cEVs derived from platelets ($\rho = 0.250$, $P = 0.029$), thus reinforcing the hypothesis of a potentially bidirectional link between thromboxane (TX)–dependent platelet inhibition and EV release (Figure 5). We also observed, in a subset of patients, a direct correlation between COX-1 mRNA and the slope of cEVs derived from platelets ($\rho = 0.499$, $P = 0.018$) or the slope of annexinV+ cEVs derived from platelets ($\rho = 0.605$, $P = 0.003$) (Figure 5).

Levels of cEVs and annexinV+ cEVs (*total, platelet-derived, endothelial-derived*, and *leukocyte-derived*) were higher in patients than in healthy participants

The number of cEVs (*total*: T0 $P < 0.001$; T10 $P = 0.001$; T24 $P < 0.001$) and of annexinV+ cEVs (*total*: T0 $P < 0.001$; T10 $P < 0.001$; T24 $P < 0.001$; *platelet*: T0 $P < 0.001$; T10 $P = 0.019$; T24 $P = 0.001$; *endothelial*: T24 $P = 0.031$; and *leukocyte*: T0 $P = 0.001$; T10 $P = 0.021$; T24 $P = 0.006$) were higher in patients vs. in healthy participants not on aspirin therapy, except for platelet, endothelial, and leukocyte cEVs (Figure S1).

Table 2 Extracellular vesicle kinetics at T0 vs. T10 vs. T24, T0 vs. T10, T10 vs. T24 and T0 vs. T24 after aspirin administration

EVs	T0	T10	T24	*P* (overall)	*P* (T0 vs. T10)	*P* (T10 vs. T24)	*P* (T0 vs. T24)
Total EVs, *n*/μL (range)	29,323 (15,160–84,661)	20,481 (11,816–37,046)	27,861 (16,495–70,474)	<0.000	0.003	0.003	0.999
CD41a+PLT-derived EV, *n*/μL (range)	3,944 (2,000–7,649)	3,815 (1,671–7,128)	4,352 (2,061–7,118)	0.089	0.974	0.842	0.999
CD31+ END-derived EV, *n*/μL (range)	451 (124–1,132)	467 (245–897)	483 (152–938)	0.986	0.978	0.995	0.986
CD45+ LEUKO-derived EV, *n*/μL (range)	385 (192–707)	207 (75–332)	255 (136–500)	<0.000	0.003	0.003	0.047
AnnexV+ Total, *n*/μL (range)	11,259 (3,509–33,001)	5,171 (1,192–17,440)	13,490 (3,983–43,069)	<0.000	0.003	0.003	0.476
CD41a+/AnnexV+ PLT-derived EV, *n*/μL (range)	1,260 (740–1,960)	576 (232–1,314)	1,169 (722–2,457)	<0.000	0.003	0.003	0.999
CD31+/AnnexV+ END-derived EV, *n*/μL (range)	150 (29–454)	152 (44–375)	154 (69–501)	0.203	0.584	0.036	0.937
CD45+/AnnexV+ LEUKO-derived EV, *n*/μL (range)	82 (31–242)	41 (16–82)	82 (41–182)	0.006	0.012	0.003	0.999

Data are median (25th–75th percentile) unless otherwise specified. Significance was calculated by Friedman *U*-Test followed by Sidak correction for $N = 3$ multiple comparisons.
AnnexV+, annexin V+; EVs, extracellular vesicles; END, endothelial; LEUKO, leukocyte; *n*/μL, number/microliter; PLT, platelet.

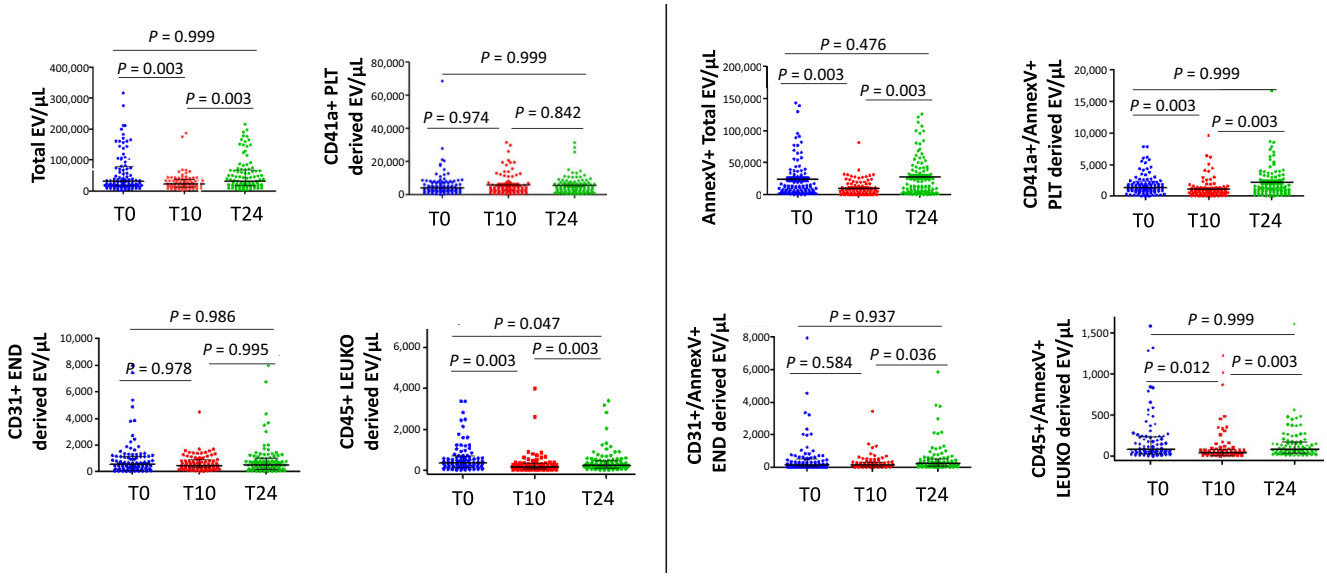

Figure 3 Kinetics of cEVs (circulating extracellular vesicles) and annexinV+ cEV numbers (total, platelet, endothelial, and leukocyte) in all patients during the 24-hour aspirin dosing interval. The number of cEVs (total, CD41a+ platelet, CD31+ endothelial, CD45+ leukocyte) and the number of annexinV+ cEVs (total, CD41a+ platelet, CD31+ endothelial, CD45+ leukocyte) measured at 0 (T0), 10 (T10), and 24 hours (T24) postaspirin intake in all patients (n = 84). Significance was calculated by Friedman U-Test, followed by Sidak correction for multiple comparisons. AnnexV+, annexinV+; END, endothelial; LEUKO, leukocyte; PLT, platelet.

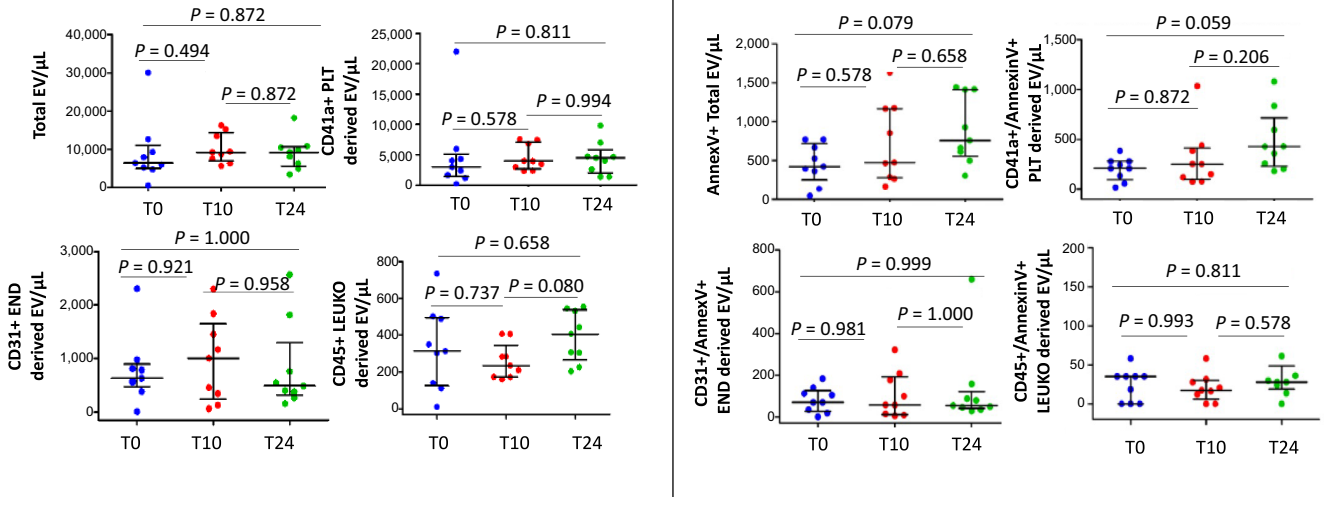

Figure 4 Kinetics of cEVs (circulating extracellular vesicles) and annexinV+ cEV numbers (total, platelet, endothelial, and leukocyte) in healthy participants not on aspirin therapy. The number of cEVs (total, CD41a+ platelet, CD31+ endothelial, CD45+ leukocyte) and the number of annexinV+ cEVs (total, CD41a+ platelet, CD31+ endothelial, CD45+ leukocyte) measured at 0 (T0), 10 (T10), and 24 hours (T24) in healthy participants (n = 9) not on aspirin therapy. Significance was calculated by Friedman U-Test, followed by Sidak correction for multiple comparisons. AnnexV+, annexinV+; END, endothelial; LEUKO, leukocyte; PLT, platelet.

DISCUSSION

The main findings of our study are (i) a subset of cEVs (*total, leukocyte-derived, annexinV+ total and annexinV platelet-derived, endothelial-derived*, and *leukocyte-derived*) are transiently reduced after 10 hours following low-dose aspirin administration, with a pattern of inhibition mirroring the effect of aspirin on COX-1 dependent $sTXB_2$ release; (ii) the slope of recovery of platelet-derived EV is related to the rate of COX-1 recovery and may be implicated in the duration of aspirin effect over 24 hours.

It was well-established that cEVs are mediators of cell-to-cell communication. During their formation, they inherit from their parental cell a specific cargo which can include RNA, free fatty acids, surface receptors and proteins. The biological function of EVs is to maintain cellular and tissue homeostasis by transferring critical biological cargos to distal or neighboring recipient cells. They have been regarded as markers of cardiovascular diseases, including thrombosis.[28] In addition, cEVs may be considered biomarkers to monitor therapeutic efficacy in various cardiometabolic

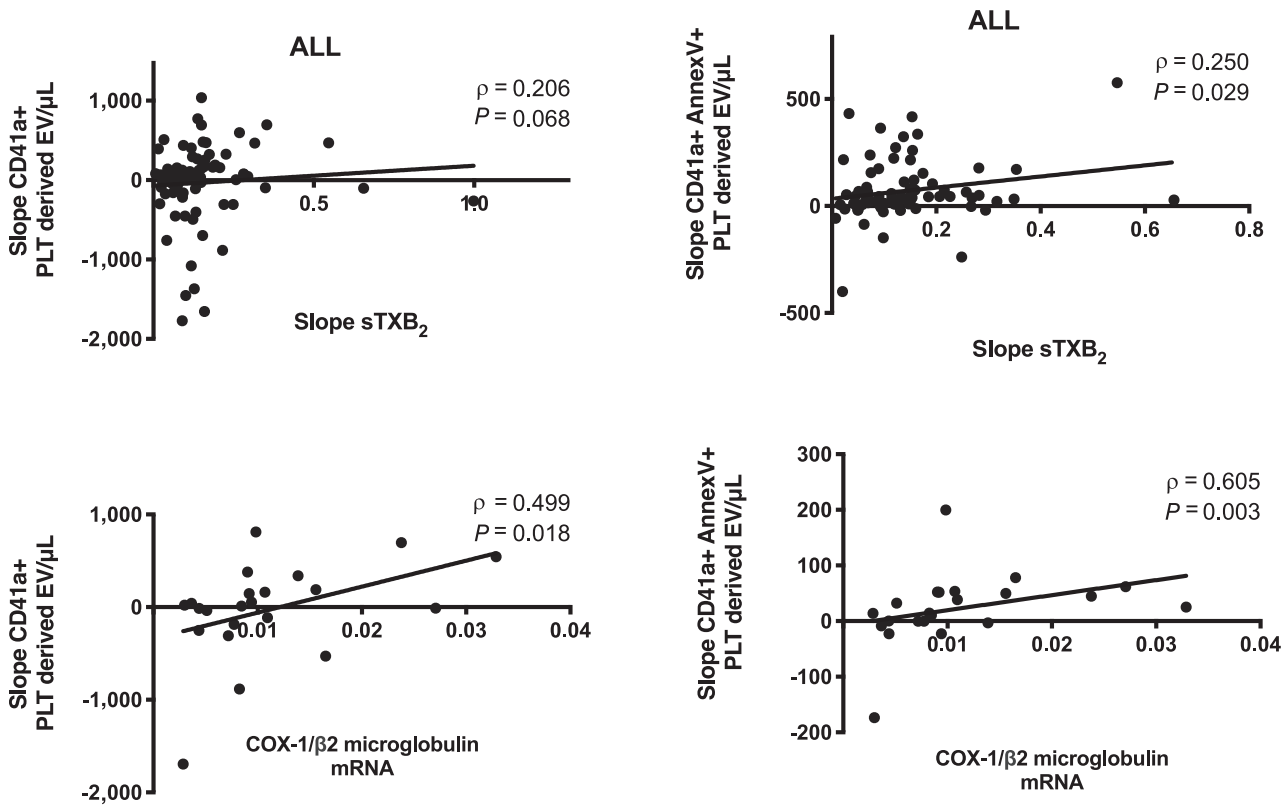

Figure 5 Correlations between slope of cEVS (circulating extracellular vesicles) derived from platelet and slope of $sTXB_2$ (serum thromboxane B_2) and COX-1 mRNA. Correlation between cEVs and cEVs annexinV+ derived from platelets (CD41a+) all patients (n = 84) and slope of $sTXB_2$. Correlation between cEVs and cEVs annexinV+ derived from platelets (CD41a+) and COX1/β2 microglobulin mRNA in a small subset (n = 22). Spearman correlation coefficient and P value are reported. AnnexV+, annexinV+; COX, cyclooxygenase; mRNA, messenger RNA; PLT, platelet.

diseases.[29] Cardioprotective drugs, such as statins,[30] angiotensin II receptor blockers,[31] calcium blockers,[32] clopidogrel,[33] and aspirin,[18] have been shown to reduce blood levels of cEVs in patients. The effects of these drugs may result from their direct effects or be the consequences of the decreased cholesterol, inflammation, or overall cardiovascular risk.

Antiplatelet agents, such as aspirin, are widely used for the prevention and treatment of vascular disease, but their impact on EV release remains poorly understood and contradictory mainly due to the multiple, nonuniform, and standardized protocols used.[20] Monocytes, platelets, and their aggregates, accepted as a predictive marker of several cardiovascular pathologies, may have longer lasting pathogenic effects through generation of vesicles which may propagate proinflammatory actions. Modulation of platelet reactivity, by antiplatelet therapies, could help attenuate the detrimental properties of these vesicles.[34]

Here we showed that the numbers of cEVs (*total* and *leukocyte-derived*) and annexinV+ cEVs (*total, platelet-derived, endothelial-derived*, and *leukocyte-derived*), are reduced after 10 hours following low-dose aspirin administration in patients on chronic aspirin treatment.

Circulating EVs play a role in coagulation and hemostasis, as well as in various diseases associated with thromboembolic events, such as cancer, atherosclerosis, sepsis, and preeclampsia.[35] Expression of annexinV on platelet-derived cEVs, index of PS expression, may mirror their procoagulant activity or platelet activation, since the binding of annexinV dramatically increases in platelet-derived EVs after exposure to different platelet agonists facilitating the assembly of protease complexes of the clotting cascade.[36–39] Platelet-derived PS-exposing EVs are prothrombotic, proinflammatory, and associated with cardiovascular and metabolic disease.[40] Exposure of PS contributes to shedding of EVs[41] since PS-exposing EV release is reduced in Scott syndrome, a rare bleeding disorder caused by mutations in TMEM16F.[42] The mechanisms by which PS-exposing EVs are cleared by activated platelets are not well characterized. Cholesterol-rich lipid rafts provide a platform for coordinating signaling through calcium ion receptors and channels in platelets and are required for the release of PS-exposing EVs from platelets.[16] It has been reported that $P2Y_{12}$ and thromboxane prostanoid α, receptors for adenosine diphosphate (ADP) and TXA_2, respectively, are detectab in platelet lipid rafts. However, the P2Y12 antagonist, AR-C69931MX, or aspirin, had no effect on A23187-induced release of PS-exposing EVs.[16] The effect of aspirin on regulating vesicle levels *in vitro* confirms how its effect is strictly dependent on the type of agonist used.[36] Similarly annexinV on endothelial-derived EV may reflect endothelial apoptosis and is associated with a worse clinical outcome, including an increased incidence of adverse cardiovascular and cerebral events in coronary artery disease.[15]

Other studies have addressed the issue of the effect of aspirin on cEVs, by studying healthy participants or patients of different

clinical settings before and after several days of aspirin treatment, yielding inconsistent results. To the best of our knowledge, this is the first study analyzing the kinetics of cEVs within the dosing interval between two subsequent aspirin administrations in participants already on chronic low-dose aspirin treatment. Notably, we showed that the pattern of cEV reduction and subsequent increase after aspirin reflects the kinetics of $sTXB_2$ inhibition and recovery, suggesting a TX-dependent mechanism underlying EV release (both platelet-derived and not). Indeed, the best characterized mechanism of action of aspirin at low doses consists in the irreversible acetylation of platelet COX-1 and subsequent inhibition of TXA_2 release. This effect is quite stable for the whole 24-hour dosing interval, although with the maximum effect appreciated after 8 hours, as previously described,[5] due to the irreversible nature of the inhibition and the lack of nucleus in platelets, which are not able to resynthesize unacetylated COX-1.

Platelets generate large amounts of EVs when stimulated with strong (thrombin, collagen, and calcium ionophores) and weak agonists (TXA_2, ADP, and epinephrine).[43] Both exogenous TXA_2 and ADP directly promote *in vitro* platelet EV shedding.[36]

Antiplatelet agents modulate EV generation by acting on intracellular signaling pathways. Pretreatment of platelets with aspirin or SQ-29548, a highly selective TP receptor antagonist, results in a significant reduction of microvesiculation induced by the tested agonists, except for ADP and epinephrine, suggesting a role of endogenous TXA_2 on agonist-triggered EV shedding.[36] For all the above-mentioned reasons, the COX-1–dependent aspirin-induced modulation of platelet-derived EV release may be the underlying mechanism for the platelet subset of cEVs.

Surprisingly, we also observed a similar aspirin-induced modulation on other EV subtypes that we measured (*total, endothelial-derived*, and *leukocyte-derived*).

Interaction among immune cells through EVs may explain, at least in part, the common pattern of different EV subtypes in response to aspirin exposure. Indeed, cooperation and bidirectional exchange of lipid metabolites between neutrophils and platelets through EVs maximize the synthesis of prostaglandins, such as TXA_2.[44]

Previous studies showed reverse transcellular communication by EV transport from activated platelets to neutrophils, implying a possible COX-1 shuttling from platelets to neutrophils.[45] *Vice versa*, the interaction of platelets with neutrophils is required for enhancing TXA_2 production from activated platelets. COX-1 in platelets uses predominantly neutrophil-derived arachidonic acid as a substrate for TXA_2 production, and the physical proximity between platelets and neutrophils leads to enhanced COX-1 activity. Neutrophil-derived arachidonic acid (AA) is shuttled from neutrophils to platelets by EVs.[44] In addition, platelet-derived EVs transfer AA between platelets and endothelial cells, and EVs also metabolize AA to TXA_2.[38,46]

Leukocyte-derived EV concentrations may reflect the fact that aspirin at low doses is able to inhibit innate immune-mediated responses, by reducing the frequency of activated CD4+ T cells.[47] It is also well known that low doses of aspirin ameliorate endothelial function, improving vasodilation, reducing thrombosis, and inhibiting atherosclerosis.[48,49] Since the concentrations of endothelial-derived EVs exposing PS were significantly lower at T10 vs. T24, suggesting lower endothelial cell apoptosis, it is intriguing to speculate that low doses of aspirin shift such a balance toward the antiapoptotic side for the endothelial compartment, also influencing the release of EVs.

Consistent with our findings, a 10-day aspirin therapy has been shown to reduce EV shedding from smooth muscle cells and erythrocytes, but not from platelets, in patients with types 1 and 2 diabetes mellitus.[19] In healthy participants, aspirin did not affect the release of platelet-derived EVs and their phenotypes, defined using the surface expression of proinflammatory (CD40L, CD62P, and CD31) and procoagulant (PS and PAC-1) markers, despite inhibition of platelet aggregation.[20] However, EV levels were evaluated only after 3 days of taking 150 mg of aspirin. In contrast, patients with stable angina showed higher platelet-derived EVs vs. healthy participants, which decreased after administration of low-dose aspirin.[50]

To exclude that the observed reduction may depend on a circadian rhythm instead of an aspirin-dependent effect, we evaluated the kinetics of EV number in healthy participants not on aspirin therapy. Daily rhythmic changes have been previously observed in the content of the synaptic vesicle of nerve endings.[51] The relatively stable values observed in nonaspirin-treated healthy participants reinforced our hypothesis of a modulation by aspirin.

Finally, we tested the hypothesis that EV release may contribute to excess unacetylated COX-1 and/or TXB_2, thus explaining the accelerated recovery of COX-1 and shorter duration of aspirin effect over the habitual dosing interval.

The variable turnover rate of aspirin target, platelet COX-1, is the most convincing determinant of the interindividual variability in aspirin response.[52] The significant direct correlations between the slope of platelet-derived EVs between 10 and 24 hours after aspirin administration and $sTXB_2$ slope on the one hand, and COX-1 mRNA on the other hand, suggest a possible involvement of platelet EVs in accelerated recovery of COX-1, yielding shorter duration of aspirin response in a fraction of patients. Of note, platelet EVs metabolize AA to TXA_2[38,46] and may contain COX-1 and possibly release TXA_2. Thus, EV rapid recovery in a fraction of patients may influence $sTXB_2$ values in the 10–12-hour dosing interval. In addition, EV cargo in terms of microRNAs and proteins may include signals promoting platelet activation and TXA_2 release.[12] An alternative explanation for the observed correlations is that platelet-derived EV recovery may mirror residual platelet activation due to newly released platelets, with uninhibited COX-1. Indeed, EVs are released from platelets during activation.[43] *Vice versa*, incubation of normal platelets with aspirin significantly inhibited AA-induced platelet reactivity, EV formation, and procoagulant activity.[14] Thus, the correlation that we observed could also be due to an opposite effect, with the recovery of COX-1 resulting in increased EV release.

Limitations of the study include its cross-sectional nature, which prevented ascertainment of the cause–effect relationship between EV number and COX-1 recovery, lack of analysis of EV cargo, lack of baseline values in patients before aspirin initiation, and healthy participants younger than patients. However, aspirin withdrawal

would not be ethical, and the aim of our study was to analyze the kinetics of EV number during daily aspirin administration.

Strengths include accurate clinical characterization of patients, and repeated measurements of both $sTXB_2$ and number of EV over the 24-hour aspirin dosing interval, EV detection protocol, which requires minimal sample handling, thus avoiding possible artifacts generated by the enrichment procedures that can activate or damage cells/platelets and artificially generate or disrupt EVs or induce their fusion.[24]

In conclusion, with the limits highlighted, our findings suggest a previously unappreciated effect of aspirin on the kinetics of a subset of circulating EVs. Further studies are needed to assess whether EVs release and their inhibition may interfere with the efficacy of aspirin or affect aspirin response.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

F.S., R.L., P.G.S., and P.L. wrote the manuscript. F.S. designed the research. R.L., P.G.S., R.T., G.B., D.D., V.C., and R.P. performed the research. R.L., P.G.S., and R.T. analyzed the data. M.M., G.L., and F.C. contributed new reagents/analytical tools.

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