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Full Length Article

Effects of clopidogrel with or without aspirin on the generation of extracellular vesicles in the microcirculation and in venous blood: A randomized placebo controlled trial☆

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ABSTRACT

Background: Dual-antiplatelet therapy (DAPT) is a standard strategy in acute coronary heart disease; however, it confers a considerable bleeding risk. Single-antiplatelet therapy (SAPT) inhibits haemostatic system activation ex vivo to a similar extent as DAPT. Extracellular vesicles (EV) are procoagulant and contribute to haemostatic system activation. We aimed to investigate the effect of DAPT compared with SAPT on EV.

Methods: In a randomized, double-blind, placebo-controlled trial, 44 healthy volunteers received DAPT (clopidogrel + aspirin) or SAPT (clopidogrel + placebo) for 7 days. Blood was obtained from a standardized microvascular injury and through venipuncture at baseline (BL) and at 2 h, 24 h, and 8 days after treatment initiation. The number, origin, and surface expression of EV were assessed using flow cytometry. Data are given as median (quartiles). Non-parametric tests were used to evaluate the short-term (BL vs 2 h) and long-term differences (2 h to 8 days), as well as the differences between treatment groups.

Results: There was no difference either in the short-term effects on the number ($\times 10^3\,mL^{-1}$) of EV in microvascular blood between DAPT [BL: 1433 (653; 3184) vs 2 h: 862 (545; 2026), p = 0.39] and SAPT [(BL: 614 (552; 1402) vs 2 h: 1079 (781; 1538), p = 0.75)] or in the long-term effects. DAPT and SAPT did not exhibit differential short-term effects on the number and proportion (36% and 27% vs 55% and 36%) of platelet-derived EV. DAPT and SAPT resulted in a significant short-term increase in phosphatidylserine expression in microvascular blood. The effects of DAPT and SAPT on EV in venous blood were similar to those in microvascular blood.

Conclusion: DAPT and SAPT have comparable effects on the amount, origin, and surface characteristics of EV.

1. Introduction

Coronary heart disease (CHD) is the most frequent cause of death in North America and Europe [1,2], and is associated with considerable morbidity and high hospitalization rates [3–5]. Revascularization therapy with percutaneous coronary intervention plus stent placement combined with coagulation suppression and platelet inhibition is the preferred treatment strategy in acute CHD. The backbone of antithrombotic CHD regimens is the inhibition of platelet function. Aspirin together with a P2Y12 inhibitor represents the standard antiplatelet therapy in acute CHD [6–8]. However, the combination of platelet function inhibitors increases the bleeding risk, which may set off the benefits in terms of their antithrombotic efficacy [9–12].

Recently, we studied the effects of monotherapy with a P2Y12 inhibitor versus dual-antiplatelet therapy (DAPT) consisting of a P2Y12 inhibitor plus aspirin on markers of haemostatic system activation. The analyses were performed using blood obtained directly at the site of plug formation after a microvascular injury rather than using venous blood. This model is more representative of conditions inside a coronary artery because of a greater homology in shear rates, and captures

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the procoagulant effects resulting from the interplay of blood cells, endothelium, and coagulation factors. As a result, we did not find a difference in the inhibition of platelet activation or coagulation activation between single-antiplatelet therapy (SAPT) with a P2Y12 inhibitor and DAPT [13].

On the basis of these findings, we sought to investigate the effect of clopidogrel monotherapy versus DAPT consisting of clopidogrel plus aspirin on extracellular vesicles (EV). EV are submicron-sized vesicles shed from membranes of various cells. They contribute to haemostatic system activation by exposing negatively charged phospholipids and tissue factor (TF), and are increased in patients with hypercoagulable states, including acute CHD [14]. We previously showed that EV are abundant in blood obtained from a microvascular injury and that the majority of these EV originate from platelets [15]. We studied the amount, origin, and surface expression of EV in blood obtained from the microcirculation because of the advantage of investigating the effects of platelet inhibition on haemostatic system activation under conditions comparable to in vivo circumstances.

2. Methods

2.1. Study population

The study was carried out in healthy, male, non-smoking volunteers. The exclusion criteria were history of bleeding or disorders associated with an increased bleeding risk, history of cardiovascular or systemic inflammatory diseases, obesity, allergy or contraindication to any study drug, history or symptoms suggestive of gastrointestinal disease, any other significant finding in physical or laboratory examination, excessive alcohol consumption, or use of any medication within 2 weeks before blood sampling.

The study protocol was approved by the Ethics Committee of the Medical University of Vienna, Austria (EK 184/2010). The study was conducted according to the Declaration of Helsinki including current revisions and the ICH Good Clinical Practice guidelines. The trial is registered at clinicaltrials.gov (NCT02120092) and at the European clinical trials database (EudraCT 2010-019643-19). Written informed consent was obtained from all subjects before any study-related procedures were conducted.

2.2. Study design

The study was conducted as a randomized, parallel-group, double-blind, placebo-controlled trial at the Departments of Medicine I and Clinical Pharmacology, Medical University of Vienna, Austria. Randomization was performed using the method of permuted blocks with a block size of four. A person not directly involved in study-related procedures performed concealment of the respective drugs. Investigators involved in the study were not aware of the randomization code, which was broken after finalizing the study and all laboratory analyses were completed.

The trial was scheduled for 8 consecutive days. The study treatment was administered in the fasting state once daily from day 1 to day 7. Blood sampling was performed on day 1 before treatment [baseline (BL)] and after 2 h, 6 h, 24 h, and 8 days. On day 1, volunteers received 600 mg clopidogrel (Plavix®; Sanofi Pharma Bristol-Myers Squibb, Paris, France) together with 100 mg aspirin (DAPT group) or 600 mg clopidogrel together with placebo (SAPT group), followed by 150 mg clopidogrel together with 100 mg aspirin or 150 mg clopidogrel together with placebo from day 2 until day 7.

2.3. Blood sampling

2.3.1. Microvascular blood

As previously described [13,15,16], standardized bleeding-time incisions were performed using a commercially available disposable device (Surgicutt®; ITC, Edison, NJ, USA). A sphygmomanometer cuff was placed on the upper arm and inflated to 40 mmHg. Subsequently, 1-mm-deep and 5-mm-long incisions were made parallel to the antecubital crease on the lateral volar aspect of the forearm. Throughout the study, the same person performed all incisions and blood samplings.

To avoid platelet activation and activation of coagulation factors because of skin contact, microvascular blood was harvested directly from the edge of the incision by using disposable plastic pipettes (TipOne®; STARLAB Corporation, Hamburg, Germany). The blood was collected over a period of 4 min and immediately transferred to ice-cooled plastic tubes (Microcentrifuge tubes, VWR collection, VWR, Radnor, PA, USA) containing 100 μL of a buffer suspension consisting of 3.8% sodium citrate, 10% aprotinin, and 0.5% indomethacin. Each buffer-filled tube was weighed before and after addition of microvascular blood to calculate the amount of blood in each tube [15]. The tubes were centrifuged at 1500*g* for 10 min at 4 °C using a microcentrifuge (Centrifuge 5424 R, Eppendorf, Hamburg, Germany). The clear supernatant was separated and removed for immediate flow cytometric (FCM) analysis.

2.3.2. Venous blood

Venous blood was collected from an antecubital vein with a loosened tourniquet by using a 21-gauge needle or an 18-gauge venous catheter. The first tube was used for determining blood counts (EDTA, Vacuette®; Greiner Bio-One, Kremsmünster, Austria) and served at the same time as a discard tube. Venous blood samples used for FCM were collected in tubes containing a 3.8% (0.129 mol L^{-1}) solution of sodium citrate (Vacuette®; Greiner Bio-One, Kremsmünster, Austria). The samples were centrifuged at 2600*g* for 15 min at 4 °C. The clear supernatant was separated and removed for immediate FCM analysis.

2.4. Laboratory analyses

2.4.1. Flow cytometry

Flow cytometry was performed using a FACSCalibur™ cytometer with CellQuest 4.0.2 software (Becton Dickinson, Franklin Lakes, NJ, USA) [15,17]. EV were defined according to size (< 1 μm, forward scatter) and binding to annexin-V. In order to set the gate for size and annexin-V binding, 1 μm beads (microparticles based on melanin resin, FITC-marked, 1 μm, Fluka, Buchs, Switzerland) suspended in a buffer suspension were used (Supplemental Fig. 1). Size was measured on forward scatter and annexin-V binding on side scatter. In order to evaluate annexin-V binding, samples were measured with and without added calcium to the suspension (Supplemental Figs. 2 and 3). By comparing measurements with and without calcium the size gate for annexin-V binding was set. Antibodies directed against specific cell surface molecules were used to further classify EV: CD142 to identify TF-positive EV (TF^+ EV), CD41a to identify EV originating from platelets (PLT^+ EV), and CD105 to identify EV originating from endothelial cells (EC^+ EV). The acquisition time of FCM analysis was 180 s. During this time, the cytometer analysed 180 μL of sample suspension. The quantity of EV in 1 mL of plasma was calculated using the predetermined volume of plasma in each sample suspension and the volume of the analysed sample suspension.

Microvascular blood supernatants and venous blood supernatants were added to filtered annexin-V binding buffer [10 mmol L^{-1} Hepes/NaOH (pH 7.4), 140 mmol L^{-1} NaCl, 2.5 mmol L^{-1} $CaCl_2$; Bender MedSystems, Vienna, Austria; filtered by Millex GP, Millipore Corporation, Bedford, MA, USA] in a 1:1 (microvascular blood) and 1:1.5 (venous blood) ratio, respectively. To assess the cellular origin and surface marker expression, specific antibodies (ABs) were added to a 60-μL (microvascular blood) or 100-μL (venous blood) aliquot of this mixture, respectively: 10 μL of an anti-CD142-phycoerythrin (PE) AB (BD Biosciences, San Jose, CA, USA; clone HTF-1, mouse IgG1, kappa), 2.5 μL (microvascular blood) or 5 μL (venous blood) of a cell-type-specific monoclonal AB against CD105 PE (Beckman Coulter, Brea, CA,

USA; clone 1G2, mouse IgG3), and a peridinin-chlorophyll-protein complex (PerCP) labelled AB against CD41a (BD Biosciences, San Jose, CA, USA; clone HIP8, mouse IgG1, kappa). A 2.5 μL (microvascular blood) or 10 μL (venous blood) volume of annexin V-fluorescein isothiocyanate (FITC) (Annexin V FITC; Bender MedSystems, Vienna, Austria) was added to the sample after incubation in the dark at room temperature for 20 min, followed by another incubation in the dark at 4 °C for 15 min. After resuspension in 500 μL (microvascular blood) or 1000 μL (venous blood) annexin-V binding buffer, FCM analysis was performed within 2 h. The EV counts for microvascular blood were corrected using a factor calculated from the ratio of the stop solution to the individual microvascular blood volume in the collection tubes. Buffer controls were run on a daily basis by measuring the respective annexin-V/AB combinations in tubes without samples. The blank values were subtracted from the results of the respective samples. Controls to assess day-to-day variations were not used as pre-study experiments showed stable results.

A total of 36 (2%) out of 1.760 samples could not be assessed because of technical issues or haemolytic blood samples.

2.5. Statistical methods

We analysed number, origin and surface characteristics of EV as the per protocol predefined secondary endpoint of a recently published study [13]. The number of subjects was powered to detect a difference between ß-thromboglobulin levels (the primary endpoint) in microvascular blood at baseline and 2 h after the first study drug intake. Data are given as median and quartiles (25th and 75th percentile), if not stated otherwise. Owing to the skewed distribution of all outcome parameters, non-parametric analyses were performed. To evaluate the short-term treatment effect, the non-parametric Wilcoxon signed rank test was used, comparing BL measurements with values after 2 h. The differences due to aspirin were tested by comparing the changes after 2 h between the two treatment groups (with aspirin or placebo), by using the non-parametric Wilcoxon rank sum test. To test for differences in effects over the total observation time, the area under the curve was calculated and compared using the non-parametric Wilcoxon signed rank test. All p-values were results of two-sided tests, and p-values of < 0.05 were considered statistically significant.

3. Results

3.1. Subjects

The study population consisted of 44 healthy male volunteers [median age 25 (25th and 75th percentile: 23; 27) years, median body mass index 23.3 (25th and 75th percentile: 21.7; 25.2) kg/m^2]. Of them, 23 subjects were randomized to receive clopidogrel and aspirin (DAPT group) and 21 subjects were randomized to receive clopidogrel and placebo (SAPT group). The two groups did not differ with regard to platelet count, activated partial thromboplastin time, prothrombin time, fibrinogen, or haemoglobin at BL. None of the volunteers had a severe adverse event.

3.2. Effects of DAPT vs SAPT on the amount and origin of EV in blood obtained from the microcirculation

Compared with BL, the total amount of EV after 2 h did not significantly differ between subjects receiving clopidogrel monotherapy and those receiving clopidogrel combined with aspirin (Table 1; Fig. 1, panel A). The total EV counts non-significantly declined from BL to 2 h when clopidogrel was combined with aspirin, whereas a non-significant increase was seen in the clopidogrel monotherapy group. Similarly, the amount of EV originating from platelets (PLT$^+$ EV) decreased non-significantly from BL to 2 h in the DAPT group. When clopidogrel was given alone, the amount of PLT$^+$ EV increased non-significantly from BL to 2 h (Table 1; Fig. 1, panel B). PLT$^+$ EV accounted for about 30% of the total amount of EV in the DAPT group and for about 50% in the clopidogrel monotherapy group at BL.

Compared with BL, endothelial cell-derived EV (EC$^+$ EV) were lower after 2 h in the DAPT group ($p = 0.02$). No such effect was observed in the SAPT group (Table 1; Fig. 1, panel C). EC$^+$ EV accounted for about 2% of the total amount of EV in the DAPT group and for about 6% in the clopidogrel monotherapy group at BL.

The DAPT and SAPT groups did not significantly differ in terms of the overall amount of EV as well as PLT$^+$ EV and EC$^+$ EV throughout the whole study period (from 2 h to 8 days) (Fig. 1, panels A–C; Supplemental Table 1).

3.3. Effects of DAPT vs SAPT on TF and phosphatidylserine expression of EV in blood obtained from the microcirculation

At BL, only a small proportion of EV expressed TF (5% in the DAPT group, 11% in the SAPT group). There was no significant difference between BL and 2 h in the number of TF$^+$ EV in volunteers receiving DAPT or SAPT (Table 2).

Moreover, no difference in the number of TF$^+$ EV was seen between the two groups over a period of 8 days (Supplemental Table 2).

Surface phosphatidylserine expression of EV significantly increased from BL to 2 h in the DAPT group ($p = 0.0188$) and in the SAPT group ($p = 0.008$); however, there was no difference in effect between treatment groups ($p = 0.74$) (Table 2). Neither DAPT nor SAPT had a significant effect on phosphatidylserine expression of EV throughout the study (Supplemental Table 2).

3.4. Effects of DAPT and SAPT on the amount and origin of EV in venous blood

Treatment with either DAPT or SAPT had an impact on the total amount of EV or EC$^+$ EV (Table 3; Fig. 2, panels A and C). The number of EV derived from platelets significantly declined after 2 h in the DAPT group, whereas no effect of SAPT was seen. This difference between treatment groups at 2 h was not significant ($p = 0.08$) (Table 3; Fig. 2, panel B). PLT$^+$ EV accounted for about 50% of the total amount of EV in both treatment groups at baseline.

Over the whole study period of 8 days, DAPT or SAPT did not differ in terms of effects on the amount and origin of EV (Fig. 2, panels A–C; Supplemental Table 3).

3.5. Effects of DAPT and SAPT therapy on TF and phosphatidylserine expression of EV in venous blood

The TF expression of EV did not change from BL to 2 h in either of the two treatment groups. Phosphatidylserine expression of EV significantly increased in both groups ($p < 0.001$ for DAPT and $p = 0.001$ for clopidogrel alone) but without a significant difference between groups ($p = 0.27$) (Table 4).

Furthermore, no difference in TF or phosphatidylserine expression of EV was detectable from BL to day 8 (Supplemental Table 4).

4. Discussion

We studied the antithrombotic potency of DAPT and SAPT in male volunteers and did not find a differential effect between clopidogrel monotherapy and clopidogrel in combination with aspirin on the amount, origin, and surface characteristics of EV.

Our study has several distinct features and specific findings. We have previously described a method for quantifying EV in blood emerging from the microvasculature [15]. We applied this technique to evaluate the effects of antiplatelet therapy on EV, as they have prothrombotic properties and are released from various cells including platelets. Platelets are the major source of EV in human blood [18]. In

Table 1
Effects of dual- and single-antiplatelet therapy on the amount and cellular origin of EV in microvascular blood from baseline to 2 h in healthy subjects.

	Total EV ($\times 10^3$ mL^{-1})		PLT$^+$ EV ($\times 10^3$ mL^{-1})		EC$^+$ EV ($\times 10^3$ mL^{-1})	
	Clopidogrel + aspirin	Clopidogrel + placebo	Clopidogrel + aspirin	Clopidogrel + placebo	Clopidogrel + aspirin	Clopidogrel + placebo
Baseline	1433 (653; 3184)	614 (552; 1402)	521 (214; 869)	341 (246; 622)	36 (22; 101)	39 (20; 71)
% total EV	–	–	36	55	2	6
2 h	862 (545; 2026)	1079 (781; 1538)	232 (75; 548)	391 (254; 567)	18 (9; 73)	37 (12; 58)
% total EV	–	–	27	36	2	3
p-Value[a]	0.39	0.75	0.26	0.67	0.02	0.45
p-Value[b]	0.36		0.54		0.19	

EV, extracellular vesicles; PLT$^+$ EV, platelet-derived extracellular vesicles; EC$^+$ EV, endothelial cell-derived extracellular vesicles.
[a] From baseline to 2 h.
[b] Differences between treatment groups; differences were compared using non-parametric tests; values are given as median (quartiles).

the present study, at baseline, 36% of EV in the DAPT group and 55% in the SAPT group originated from platelets (no significant difference). Two hours after drug intake, the number of EV slightly decreased in the DAPT group but increased in the SAPT group. The number of PLT$^+$ EV followed a similar course. These fluctuations were either non-significant (microvascular blood) or very short-lived (venous blood). Importantly, the amount of EV over the whole study period of 8 days was not affected by either treatment regimen. Thus, we do not consider these findings as indicative of a relevant difference between DAPT and SAPT.

In line with our previous findings [15], the number of EV was significantly lower in venous blood than in blood obtained from the microvasculature (Tables 1 and 3).

The decrease of platelet-derived EV in venous blood in subjects receiving clopidogrel combined with aspirin was only mild, short-lived, and most likely a chance finding. However, we cannot entirely exclude an additive effect on platelet inhibition by aspirin on top of clopidogrel within the first 2 h after drug intake.

A small proportion (between 2% and 6% in microvascular and venous blood) of EV originated from endothelial cells. In blood obtained from the microvasculature, the number of EC$^+$ EV significantly declined 2 h after starting DAPT, whereas it remained stable after SAPT. Given the absence of a similar effect in venous blood and over a longer treatment period in both groups, this observation could represent a chance finding; however, a short-lived effect of DAPT on endothelial cell activation cannot be completely ruled out.

The results of this analysis complement our previous findings [13]. In the previous study, monotherapy with clopidogrel or ticagrelor was compared with either agent combined with aspirin in healthy male volunteers. We did not detect any difference in terms of platelet and coagulation activation when clopidogrel or ticagrelor monotherapy was compared with DAPT. These results are also concordant with recent results from clinical trials investigating different combinations of antiplatelet and anticoagulant agents and omitting aspirin in selected treatment arms [19–23].

EV have procoagulant properties by expressing TF on their surface and/or by providing a phospholipid-rich surface. Thus, we also assessed the surface characteristics of EV before and after antiplatelet treatment. Overall, the proportion of EV expressing TF and the amount of phosphatidylserine expression of EV was small both in microvascular blood and venous blood. We did not observe changes in the amount of either TF positive EV or phosphatidylserine expression of EV over time or between treatment groups, except for an increase in phosphatidylserine expression after 2 h in microvascular blood and venous blood in both groups. This is unlikely to be explained by an antiplatelet effect but may be considered as injury related.

The role of EV is a matter of intensive research. EV generation is markedly increased in conditions known to be associated with activation of the haemostatic system, such as acute vascular events [24]. Mallat and colleagues demonstrated the presence of high levels of EV in patients with acute coronary syndrome [14]. Suades et al. studied EV in patients with ST-segment elevation myocardial infarction (STEMI), by analysing blood collected during percutaneous coronary intervention from the culprit lesion and venous blood from the periphery. They investigated the origin of EV, and found a significant increase in EV in venous blood originating from white blood cells and endothelial cells in patients with STEMI when compared with healthy controls. Compared with venous blood, blood collected during percutaneous coronary

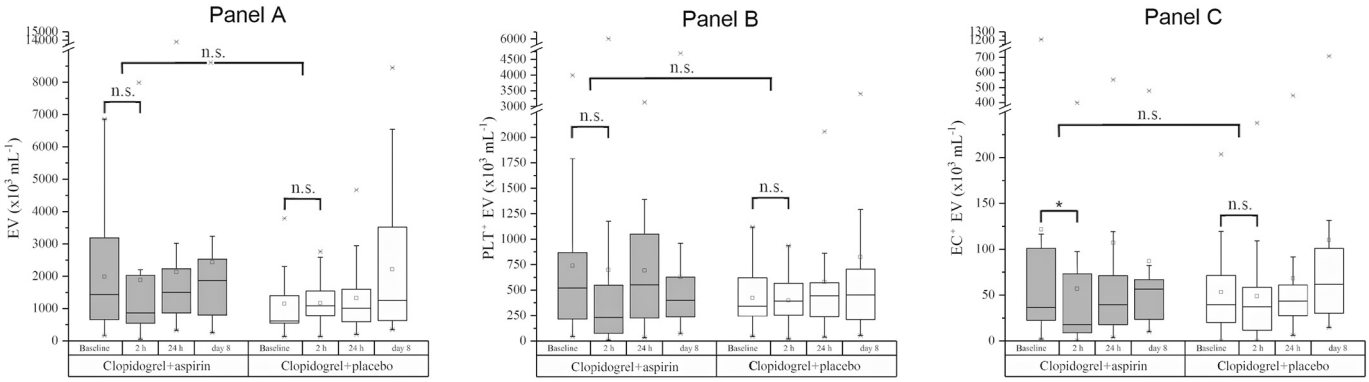

Fig. 1. Boxplots demonstrating the effects of dual- and single-antiplatelet therapy on the amount of overall EV generation (panel A), generation of PLT$^+$ EV (panel B), and generation of EC$^+$ EV (panel C) in microvascular blood at different time points over the observational period compared with baseline levels. The boxes depict the data range from the first to the third quartile. The horizontal bar denotes the median. The whiskers represent the outermost data point within 1.5 × interquartile range from the first and third quartiles. The crosses represent outliers. * = $p < 0.05$. n.s. = not significant. EV, extracellular vesicles; PLT$^+$ EV, platelet-derived extracellular vesicles; EC$^+$ EV, endothelial cell-derived extracellular vesicles.

Table 2
Effects of dual- and single-antiplatelet therapy on TF and phosphatidylserine expression of EV in microvascular blood from baseline to 2 h in healthy subjects.

	TF^+ EV ($\times 10^3$ mL^{-1})		Phosphatidylserine expression (AU)	
	Clopidogrel + aspirin	Clopidogrel + placebo	Clopidogrel + aspirin	Clopidogrel + placebo
Baseline	69 (37; 179)	69 (29; 114)	266 (159; 326)	261 (170; 340)
% Total EV	5	11	–	–
2 h	81 (26; 180)	61 (33; 151)	310 (197; 443)	332 (260; 378)
% Total EV	9	6	–	–
p-Value[a]	0.89	0.58	0.0188	0.008
p-Value[b]	0.91		0.74	

TF, tissue factor; EV, extracellular vesicles; TF^+ EV, tissue factor-positive extracellular vesicles; AU, arbitrary units.
[a] From baseline to 2 h.
[b] Differences between treatment groups; differences were compared using non-parametric tests; values are given as median (quartiles).

Table 3
Effects of dual- and single-antiplatelet therapy on the amount and cellular origin of EV in venous blood from baseline to 2 h in healthy subjects.

	Total EV ($\times 10^3$ mL^{-1})		PLT^+ EV ($\times 10^3$ mL^{-1})		EC^+ EV ($\times 10^3$ mL^{-1})	
	Clopidogrel + aspirin	Clopidogrel + placebo	Clopidogrel + aspirin	Clopidogrel + placebo	Clopidogrel + aspirin	Clopidogrel + placebo
Baseline	533 (369; 755)	486 (322; 598)	276 (206; 407)	242 (153; 347)	10 (7; 17)	12 (11; 20)
% total EV	–	–	52	50	2	2
2 h	534 (375; 782)	514 (400; 897)	222 (167; 289)	237 (159; 410)	10 (9; 19)	13 (9; 17)
% total EV	–	–	41	46	2	2
p-Value[a]	0.91	0.18	0.02	0.78	0.38	0.56
p-Value[b]	0.3		0.08		0.47	

EV, extracellular vesicles; PLT^+ EV, platelet-derived extracellular vesicles; EC^+ EV, endothelial cell-derived extracellular vesicles.
[a] From baseline to 2 h.
[b] Differences between treatment groups; differences were compared using non-parametric tests; values are given as median (quartiles).

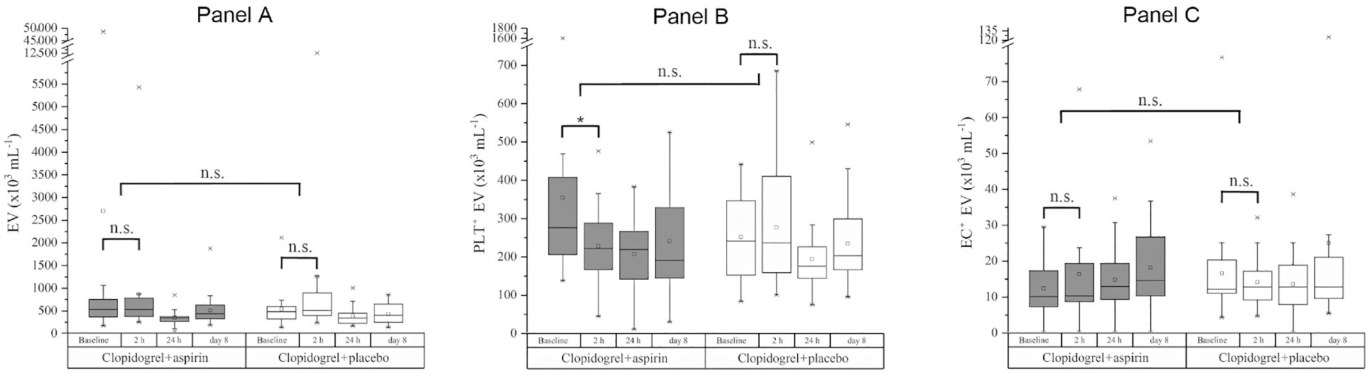

Fig. 2. Boxplots demonstrating the effects of dual- and single-antiplatelet therapy on the amount of overall EV generation (panel A), generation of PLT^+ EV (panel B), and generation of EC^+ EV (panel C) in venous blood at different time points over the observational period compared with baseline levels. The boxes depict the data range from the first to the third quartile. The horizontal bar denotes the median. The whiskers represent the outermost data point within 1.5 × interquartile range from the first and third quartiles. The crosses represent outliers. * = $p < 0.05$. n.s. = not significant. EV, extracellular vesicles; PLT^+ EV, platelet-derived extracellular vesicles; EC^+ EV, endothelial cell-derived extracellular vesicles.

intervention showed a significantly increased proportion of EV originating from white blood cells, endothelial cells, and platelets as well as TF-positive EV [25]. Recently, Connor and colleagues studied the effects of aspirin and P2Y12 inhibition on platelet EV release in healthy volunteers and CHD patients. In vitro platelet stimulation in healthy volunteers resulted in increased platelet EV counts. This effect was partly abolished by aspirin and entirely blocked by P2Y12 inhibition [26].

Our study has strengths and limitations. We applied a sound methodology by performing a randomized, parallel-group, double-blind, placebo-controlled study. To avoid pre-analytic irregularities due to freeze/thawing, we performed FCM analysis in fresh, unfrozen, and unthawed samples [15]. The number of EV at baseline in microvascular blood was higher in patients receiving DAPT compared to SAPT. This did not affect our analysis since we did not perform a direct comparison between the number of EV but rather compared changes within and between the two treatment groups. The number of subjects was powered to detect a difference between β-thromboglobulin levels (the primary endpoint) in microvascular blood at baseline and 2 h after the first study drug intake. Therefore, we may have missed differences between treatment groups owing to lack of statistical power. Although we determined the effects of antiplatelet therapy under conditions close to the in vivo circumstances, microvascular blood experiments may not reflect all the mechanisms leading to coagulation and platelet activation

Table 4
Effects of dual- and single-antiplatelet therapy on TF and phosphatidylserine expression of EV in venous blood from baseline to 2 h in healthy subjects.

	TF^+ EV ($\times 10^3$ mL^{-1})		Phosphatidylserine expression (AU)	
	Clopidogrel + aspirin	Clopidogrel + placebo	Clopidogrel + aspirin	Clopidogrel + placebo
Baseline	19 (15; 75)	23 (13; 50)	335 (285; 397)	364 (319; 399)
% total EV	3	5	–	–
2 h	24 (14; 73)	17 (13; 47)	571 (394; 715)	488 (384; 613)
% total EV	5	3	–	–
p-Value[a]	0.88	0.29	< 0.0001	0.0011
p-Value[b]	0.31		0.27	

TF, tissue factor; EV, extracellular vesicles; TF^+ EV, tissue factor-positive extracellular vesicles; AU, arbitrary units.
[a] From baseline to 2 h.
[b] Differences between treatment groups; differences were compared using non-parametric tests; values are given as median (quartiles).

in patients with acute CHD. In this respect, studying healthy volunteers rather than patients and the lack of a clinical end point must also be considered limitations. We kept the treatment period as short as possible (7 days) to avoid prolonged exposure of healthy volunteers to a potential bleeding risk. Therefore, we could have missed the long-term effects of antiplatelet treatment on the generation and characteristics of EV. Another limitation concerns the methodology of EV analysis. We used forward scatter rather than side scatter for size definition to allow a comparison with our previous works [15,17].

5. Conclusion

We studied haemostatic system activation in the microvasculature under conditions resembling those in a coronary artery, and did not observe marked differential effects between DAPT and SAPT on the amount, origin, and surface characteristics of EV.

These findings add to the notion that SAPT with a P2Y12 inhibitor alone inhibits coagulation and platelet activation to a similar extent as DAPT. Thus, the results of our study provide further support to emerging concepts of omitting aspirin from DAPT or triple therapy in the treatment of cardiovascular disease. Further research in patients with CHD is needed to translate the findings of this hypothesis-generating study into clinical practice.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Appendix A. Supplementary data

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