Randomized controlled trial protocol to investigate the antiplatelet therapy effect on extracellular vesicles (AFFECT EV) in acute myocardial infarction

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Abstract
Activated platelets contribute to thrombosis and inflammation by the release of extracellular vesicles (EVs) exposing P-selectin, phosphatidylserine (PS) and fibrinogen. P2Y12 receptor antagonists are routinely administered to inhibit platelet activation in patients after acute myocardial infarction (AMI), being a combined antithrombotic and anti-inflammatory therapy. The more potent P2Y12 antagonist ticagrelor improves cardiovascular outcome in patients after AMI compared to the less potent clopidogrel, suggesting that greater inhibition of platelet aggregation is associated with better prognosis. The effect of ticagrelor and clopidogrel on the release of EVs from platelets and other P2Y12-exposing cells is unknown. This study compares the effects of ticagrelor and clopidogrel on (1) the concentrations of EVs from activated platelets (primary end point), (2) the concentrations of EVs exposing fibrinogen, exposing PS, from leukocytes and from endothelial cells (secondary end points) and (3) the procoagulant activity of plasma EVs (tertiary end points) in 60 consecutive AMI patients. After the percutaneous coronary intervention, patients will be randomized to antiplatelet therapy with ticagrelor (study group) or clopidogrel (control group). Blood will be collected from patients at randomization, 48 hours after randomization and 6 months following the index hospitalization. In addition, 30 age- and gender-matched healthy volunteers will be enrolled in the study to investigate the physiological concentrations and procoagulant activity of EVs using recently standardized protocols and EV-dedicated flow cytometry. Concentrations of EVs will be determined by flow cytometry. Procoagulant activity of EVs will be determined by fibrin generation test. The compliance and response to antiplatelet therapy will be assessed by impedance aggregometry.

Keywords
ADP receptors, extracellular vesicles, P2Y12 antagonists, platelets, ticagrelor

Introduction
Activation and aggregation of platelets on a ruptured or eroded atherosclerotic plaque leads to acute myocardial infarction (AMI) [1]. During AMI, activated platelets release proinflammatory mediators and bind to leukocytes, leading to leukocyte activation [1]. In addition, activated platelets release fragments of their outer cell membrane, called platelet-derived extracellular vesicles (EVs) [2]. A transmission electron microscope image of EVs from human plasma is shown in Figure 1.

Platelet EVs are nanoparticles surrounded by a phospholipid membrane which contains platelet-derived proteins [3]. The presence of glycoprotein IIb/IIIa (CD41/CD61) enables to establish the cellular origin of EVs released from platelets or megakaryocytes among other EVs present in blood [4]. If EVs expose the glycoprotein IIb/IIIa along with P-selectin (CD62P) and/or phosphatidylserine (PS), or if EVs
Platelet EVs exposing P-selectin, PS and/or fibrinogen are likely involved in inflammation and thrombosis [7–10], and potentially contribute to the development and progression of atherosclerosis [10–12]. P-selectin mediates binding of platelets and platelet EVs to monocytes via P-selectin glycoprotein ligand-1 on the monocyte, which leads to monocyte activation, cytokine release and exposure of tissue factor (TF) [7]. PS along with other negatively charged phospholipids binds clotting factors in the presence of calcium ions, thereby propagating thrombin generation [8]. Fibrinogen binds both to the CD11b/CD18 receptor (Mac-1) on monocytes, thereby activating monocytes, and to activated glycoprotein IIb/IIIa, thereby enabling platelet cross-linking and aggregation [9].

Results of the Canakinumab Anti-inflammatory Thrombosis Outcomes (CANTOS) study showed that inhibition of the interleukin-1β pathway decreases the rate of recurrent cardiovascular events in patients after AMI, compared to placebo [13]. CANTOS proved that atherosclerosis is an ongoing inflammatory disease, indicating the need for treatment strategies that reduce inflammation and improve cardiovascular outcome. At present, patients after AMI receive a multitude of drugs, of which the anti-inflammatory effects are not or incompletely known. Drugs routinely administered after AMI include antagonists of the P2Y12 receptor for adenosine diphosphate (ADP), such as ticagrelor and clopidogrel, which prevent recurrent cardiovascular events in patients after AMI [14]. Ticagrelor inhibits platelet activation more than clopidogrel, being a more potent antiplatelet drug [15]. Since activated platelets contribute both to inflammation and to thrombosis, P2Y12 receptor antagonists offer an opportunity for a combined anti-inflammatory and antithrombotic strategy after AMI.

The P2Y12 receptor is exposed on platelets [16], leukocytes [17,18] and vascular endothelial cells [19,20]. Binding of ticagrelor or clopidogrel to platelet P2Y12 receptors increases the intracellular concentration of cyclic adenosine monophosphate, thereby making platelets less sensitive to activation by other agonists [16]. If platelets are less sensitive to activation, they are expected to expose and release less proinflammatory mediators, such as P-selectin and CD40 ligand (CD40L), and to release less EVs. Because the more potent antiplatelet drug ticagrelor improves the cardiovascular outcome of patients after AMI compared to the less potent clopidogrel, greater inhibition of platelet aggregation (IPA) seems associated with better prognosis [21]. However, both ticagrelor and clopidogrel decrease the exposure of P-selectin, and concentrations of soluble P-selectin and CD40L to a similar extent, both in patients with AMI and in healthy individuals administered endotoxin [22–25]. Reliable analysis of platelet P-selectin can only be performed in freshly collected whole blood, i.e. within 15 minutes following blood collection [26]. Soluble P-selectin and CD40L, in turn, can be released also from cells other than platelets [26,27]. Therefore, measurement of P-selectin exposure on platelets and soluble platelet-derived molecules do not reflect the extent of platelet activation in vivo.

Because platelet EVs are platelet-specific and can be analyzed in biorepositories, platelet EVs are advantageous over other available biomarkers of platelet activation [28,29]. Both ticagrelor and clopidogrel inhibit the release of platelet EVs [30,31], but the effects of ticagrelor and clopidogrel on EVs have never been compared in a randomized clinical study. We hypothesize that plasma from patients treated with ticagrelor (1) contains lower concentrations of EVs from activated platelets, exposing fibrinogen, exposing PS, from leukocytes and endothelial cells and (2) has lower procoagulant activity, when compared to patients treated with clopidogrel. We aim to compare the effects of ticagrelor and clopidogrel on plasma concentrations of different EV subtypes and plasma EV procoagulant activity in patients with AMI.

**Methods**

**Study Design**

This is a prospective, single-center, randomized, investigator-blinded, parallel group design study conducted at the 1st Chair and Department of Cardiology, Medical University of Warsaw, Poland in collaboration with the Vesicle Observation Center and Laboratory of Experimental Clinical Chemistry, Amsterdam University Medical Centers, The Netherlands. The duration of this study is expected to be 12 months.

**Selection of Participants**

The study inclusion and exclusion criteria are listed in Table I. Patients will be enrolled by the principal investigator among those who were admitted to 1st Chair and Department of Cardiology, Medical University of Warsaw due to the first ST-segment elevation acute myocardial infarction (STEMI) or non-STEMI, and then underwent percutaneous coronary intervention (PCI) with stent implantation. Since the majority of patients with STEMI is pretreated with clopidogrel prior to hospital admission, initially all patients will be administered a loading dose of clopidogrel (600 mg) to obtain a homogenous patient group. Since the reported concentrations of EVs in plasma of healthy volunteers differ between studies [28], 30 age- and gender-matched healthy volunteers will be enrolled in the study to investigate the physiological concentrations and procoagulant activity of EVs using recently standardized protocols and EV-dedicated flow cytometry [29,32,33], confirming the results of previous studies [34–36]. The principal investigator will describe all study procedures and all patients and healthy volunteers will provide written informed consent.
Table I. Eligibility criteria for the study.

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<tr>
<th>Inclusion criteria</th>
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<tr>
<td>Age &gt;18 years</td>
<td>Known coagulopathy</td>
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<td>Informed consent to participate in the study</td>
<td>Active pathological bleeding</td>
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<td>First ST-elevation myocardial infarction (STEMI) or non-STEMI</td>
<td>Known history of bleeding disorder</td>
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<td>Percutaneous coronary intervention (PCI) with stent implantation</td>
<td>Susception of intracranial hemorrhage</td>
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<td>Administration of the loading dose of clopidogrel (600 mg) prior to PCI</td>
<td>Need for oral anticoagulation therapy</td>
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<td>Administration of GPIIb-IIIa antagonists</td>
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<td>Cardiogenic shock</td>
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<td>Severe chronic renal failure (eGFR &lt;30 mL/min)</td>
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<td>Severe liver insufficiency</td>
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<td>Infectious disease</td>
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<td>Chronic dyspepsia</td>
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<td>Increased risk of bradycardia</td>
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<td>Known pregnancy, breast-feeding, or intention to become pregnant during the study period</td>
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<td>Study drug intolerance</td>
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<td>Coadministration of ticagrelor or clopidogrel with strong CYP3A4 inhibitors</td>
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<td>Participation in any previous study with ticagrelor or clopidogrel</td>
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eGFR: estimated glomerular filtration rate.

Randomization and Blinding

Following written informed consent, patients who meet all inclusion criteria and do not meet any of the exclusion criteria will be included and randomized. Block randomization with fixed block size of size 8 without stratification will be carried out using sealed envelope system in a 1:1 ratio either to replace clopidogrel with ticagrelor (study group) or to continue the treatment with clopidogrel (control group). Study drugs will be administered based on the randomization list. Both randomization and administration of the drugs will be conducted by an independent operator (K.P.), not involved in sample collection and analysis. During the clinical trial, participants will be identified solely by an individual randomization number. All collected samples will be coded with a unique number and analyzed in one block by an operator blinded to patient- and treatment-related data (A.G).

Treatment Arms

All drugs will be administered orally. Patients randomized to switch to ticagrelor will receive a loading dose of ticagrelor (180 mg), followed by a maintenance dose (90 mg twice daily) [14]. Patients randomized to clopidogrel will continue the treatment with a maintenance dose of clopidogrel (75 mg once daily) [14]. At hospital discharge, patients (1) will receive either ticagrelor or clopidogrel for 6 months of treatment and (2) will be advised to contact the principal investigator in case of recurrent thrombotic event or bleeding. Patients who experience a recurrent thrombotic or bleeding event will be admitted to 1st Chair and Department of Cardiology for thorough examination, assessment and monitoring. In case of suspected or confirmed nonresponsiveness to the initial P2Y12 antagonist, patients will switch to another P2Y12 antagonist. At 6 months, all patients will be invited for the follow-up visit. At the follow-up visit, compliance will be checked by counting of tablets, and ticagrelor will be recommended for the remaining 6 months of double antiplatelet therapy (altogether 12 months of treatment, as recommended by the guidelines of European Society of Cardiology) [14]. Patients who choose to switch to clopidogrel at the follow-up visit due to financial constraints or other reasons will be prescribed clopidogrel.

Trial Schedule

The trial schedule is presented in Figure 2. Venous blood will be collected from fasting patients (1) 24 hours after administration of clopidogrel (randomization), (2) 48 hours following randomization to ticagrelor or clopidogrel group (matching the length of the hospital stay of patients with AMI) and (3) 6 months following the index hospitalization (follow-up visit). Venous blood will also be collected from 30 gender- and age-matched fasting healthy volunteers.

Blood will be collected and processed according to the recently standardized protocol for collection, handling and storage of human plasma for analysis of EVs [29]. Briefly, blood will be collected using a 19-gauge needle to 10-mL plastic tube containing citrate (final concentration 0.109 mol/L). The tourniquet will be removed promptly after the venipuncture. The first 2.5 mL of collected blood will be discarded to avoid pre-activation of platelets. To remove cells, blood will be centrifuged twice using a Rotina 380 R centrifuge equipped with a swing-out rotor and a radius of 155 mm (Hettich Zentrifugen, Tutlingen, Germany). The centrifugation parameters will be 2500 g for 15 minutes at 20°C, acceleration speed 1, no brake. After the first centrifugation, plasma will be transferred to a new 5-mL plastic tube, leaving ~1 cm plasma above the buffy layer. After the second centrifugation, cell- and platelet-free plasma (PFP) will be collected and transferred carefully to a new 5-mL plastic tube, leaving ~100 µL at the bottom of the old tube. PFP will be mixed with a pipet and 250 µL aliquots will be made in 1.5-mL tubes. PFP will be stored in −80°C freezer until analyzed. Prior to the analysis, frozen samples will be transported on dry ice to the Vesicle Observation Center, Amsterdam University Medical Centers, The Netherlands. Since EV concentrations are affected by numerous pre-analytical variables, such as diameter of a needle, type of tube (plastic/glass), type of system (free flow/vacuum), use of tourniquet (released promptly/kept during the collection), blood will be collected and processed only by operators who underwent an appropriate training with subsequent quality check of the samples in LEKC, Amsterdam UMC (C.E., M.B.).

Analyses will compare (1) the concentrations of EVs from activated platelets, exposing fibrinogen, exposing PS, from leukocytes and endothelial cells and (2) the procoagulant activity of plasma EVs between patients treated with ticagrelor and clopidogrel at 48 hours and at 6 months. In addition, IPA will be assessed in all patients at randomization, 48 hours and 6 months to check...
the compliance and the response to acetylsalicylic acid (ASA) and P2Y12 antagonists. Data regarding demographic characteristics, comorbidities and concomitant pharmacotherapy will be collected, and thorough clinical examination will be conducted during the index hospitalization and at the follow-up visit.

Concomitant Medications

If not administered prior to hospital admission, patients will receive a loading dose of aspirin (300 mg) prior to PCI. Unfractionated heparin will be administered during PCI in the dosage left at the discretion of the interventional cardiologist. At hospital discharge, all patients will receive treatment with ASA 75 mg once daily (as part of the prescribed double antiplatelet therapy) and atorvastatin at least 10 mg once daily.

Analytical Methods

Concentration and Composition of EVs

Concentrations of different EV subtypes, defined as a number of EVs (exceeding the detection limit) per mL plasma, will be determined by EV-dedicated flow cytometry (A60 Micro, Apogee Flow Systems, Hertfordshire, UK) according to the recent guidelines [32,33]. EVs released from different cells will be defined as follows: activated platelets: CD61+/P-selectin+; fibrinogen-exposing EVs: CD61+/fibrinogen+; PS-exposing EVs: PS+; leukocytes: CD45+; endothelial cells: CD31+/CD146+. Scatter detectors will be calibrated with Rosetta Calibration (Exometry, Amsterdam, The Netherlands), fluorescence detectors will be calibrated with SPHERO PE Calibration kit (ECFP-F2-5K, Spherotech, Lake Forest, IL, USA), Quantum FITC-5 MESF beads (555A, Bangs Laboratories, Fishers, IN, USA) and Quantum APC MESF beads (823A, Bangs), and the flow rate will be calibrated with TruCount beads (BD Biosciences, Franklin Lakes, NJ, USA). To enable data comparison, detection limits of scatter and fluorescence detectors will be quantified in units of nm to mean equivalent soluble fluorophore (MESF), respectively. Flow-SR will be applied to determine the size and refractive index of particles and improve specificity by enabling label-free differentiation between EVs and lipoprotein particles [37].

Procoagulant Activity of EVs

The procoagulant activity of EVs will be determined as the ability of EVs in PFP to generate fibrin, as described previously [30]. Briefly, after pre-incubation for 5 min at 37°C, clotting will be initiated by addition of CaCl2. Fibrin formation over 1 hour will be determined by measuring the optical density (δ = 405 nm) in duplicate on a spectrophotometer at 37°C. Because TF is the key initiator of the coagulation [38], and because plasma EVs in patients with AMI expose TF [39], the procoagulant activity of plasma EVs will be evaluated in the absence and presence of antibody against human TF (coagulation factor VII). Recombinant human TF will be used as a positive control, and saline will be used as a negative control.

Inhibition of Platelet Aggregation

IPA in response to double antiplatelet therapy (ASA and P2Y12 antagonist) will be assessed by impedance aggregometry (Multiplate Analyzer, Roche Diagnostics, Risch-Rotkreuz, Switzerland) using the ASPI test (arachidonic acid, 0.5 mM) and the ADP high sensitivity test (ADP, 6.5 μM with addition of prostaglandin E1, 9.4 nM), respectively [40,41].

End points

Study end points refer to two patient groups (ticagrelor vs. clopidogrel). The primary end point is the concentrations of EVs from activated platelets at 6 months. The secondary end points are (1) the concentration of EVs from activated platelets at 48 hours and (2) the concentrations of EVs exposing fibrinogen, exposing PS, from leukocytes and endothelial cells at 48 hours and 6 months. The tertiary outcome is the procoagulant activity of the total plasma EVs at 48 hours and 6 months. The study is not powered for mortality and other adverse events.

Sample Size

Because insufficient data are available to assess the impact of ticagrelor and clopidogrel on the concentrations of platelet EVs and EVs from other types of cells in patients with AMI, the standard deviation (SD) and mean difference between the two treatment arms were estimated based on the preliminary in vitro experiments, which we conducted during preparation for this study [30]. In our experiments, ticagrelor decreased ADP-induced platelet EVs release twofold, with the SD ± 1.0 value of the mean [30]. It was not feasible to investigate the effect of clopidogrel on EV release in vitro due to the instability of the clopidogrel active metabolite. However, data from the literature show that clopidogrel decreases the release of platelet EVs ex vivo by twofold, with SD ± 1.0 [31]. The required sample size was calculated by a two-sided t-test at a significance level of 0.05 with the following assumptions: (1) SD in each group ± 1.0, (2) mean difference between the groups = 1 and (3) nominal test power = 0.9. Based on this sample size estimation, a total of 46 patients (23 per group) should be enrolled in the trial. Since 5–30% of patients in clopidogrel group may have inadequate platelet inhibition [41], requiring to switch to a more potent P2Y12 receptor antagonists in case of recurrent thrombotic event, and since the overall rate of switching between P2Y12 antagonists reported in registries ranges from 5% to 50% [32,42,43], we assumed that up to 30% of patients may be potentially lost to follow-up. Based on this assumption, we estimated that 60 patients (30 per group) should be enrolled in the trial.

Statistical Plan

A single statistical analysis will be performed at the end of the study using IBM SPSS Statistics 20. Since the differences between healthy volunteers and controls will be reported descriptively, no statistical matching of healthy volunteers and patients will be performed. Intention to treat (ITT) and per protocol populations will be defined. The ITT population will consist of all patients who were randomized and received at least one dose of the study drug, regardless of protocol violations. The per protocol population will consist of all patients who were randomized and treated completely in accordance with the study protocol. Categorical variables will be presented as number and percent; continuous variables will be presented as mean and SD or median with interquartile range. Data will be displayed graphically for visual examination. Shapiro–Wilk test will be used to test for non-Gaussian distribution of continuous variables. No formal statistical testing will be performed on the differences between the two treatment arms among patients with AMI at randomization. Linear regression with EV concentration at randomization as an addition covariate will be used to compare the concentrations between the two treatment arms at 48 hours and 6 months. Student’s two-sided t-test or Mann–Whitney U test will be used to compare the EV procoagulant activity between the two treatment arms at 48 hours and 6 months. No corrections for multiple testing
will be performed. Pearson or Spearman correlation coefficient will be used to analyze a correlation between IPA and EV concentrations in both study groups, separately in patients responding and not-responding to clopidogrel, according to the impedance aggregometry results. The non-responsiveness to clopidogrel will be defined as platelet aggregation >46 aggregation units in response to 6.5 µM ADP [44]. A p-value below 0.05 will be considered significant. Mortality and other adverse events as well as comparison of EV concentrations and EV procoagulant activity between healthy volunteers and all AMI patients will be reported descriptively.

Legal Considerations

The study protocol was approved by the Bioethical Committee Approval of Medical University of Warsaw (approval number: KB/112/2016), and registered in the ClinicalTrials database (NCT02931045). Recording of adverse events will be conducted according to good clinical practice, the ethical principles described in the Declaration of Helsinki, the requirements of the European Medicines Agency and local legal and regulatory requirements. Data storage will be performed in accordance with local data protection laws. Competent authorities and sponsor authorized persons may request access to trial documentation in case of an inspection or audit. Direct access to these documents must be guaranteed by the principal investigator. Documentation can be copied during inspection or audit in case the identity of the participant has been made unrecognizable.

Expected Results

We expect lower concentrations of EVs from activated platelets, exposing fibrinogen, exposing PS, from leukocytes and endothelial cells, as well as lower procoagulant activity of plasma EVs in patients treated with ticagrelor, compared to clopidogrel. Consequently, we expect to identify a new mechanism of action of ticagrelor. If present, this mechanism would create a basis for future studies to investigate whether lower EV concentrations are associated with improved clinical outcomes in patients treated with P2Y12 antagonists.

We expect higher concentrations of all EV subtypes in patients with AMI at randomization, compared to healthy volunteers, thereby confirming the results of preceding studies [31–33] using recently standardized protocols and EV-dedicated flow cytometry [28–30].

Discussion

Antiplatelet therapy effect on extracellular vesicles (AFFECT EV) is the first study which directly compares the effects of ticagrelor and clopidogrel on the concentrations and procoagulant activity of EVs in patients with AMI in a randomized and investigator-blinded way. The state-of-the art methods to collect and handle samples and to analyze platelet extracellular vesicles (PEVs) will account for the reliability of results. The translational collaboration between the 1st Chair and Department of Cardiology, Medical University of Warsaw, Poland and the Vesicle Observation Center, Amsterdam University Medical Centers, The Netherlands will further ensure scientifically sound interpretation of data and optimize dissemination of results.

AFFECT EV is expected to establish whether EVs can be affected by drugs routinely used in patients after AMI, thereby identifying one of the potential novel mechanisms of action contributing to a combined antithrombotic/anti-inflammatory benefit of the P2Y12 receptor antagonists. Regarding the exponentially growing interest in EVs during the last decade [39], determining the effect of the P2Y12 receptor antagonists on EVs may be the first step to explain the clinical benefits of long-term treatment with P2Y12 antagonists, and may provide a basis for future studies aimed to associate this effect with clinical outcomes.

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Declaration of interest

E. van der Pol is a cofounder and shareholder of Exometry BV. All other authors report no declarations of interest.

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