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FLOW ANALYSIS OF INDIVIDUAL BLOOD EXTRACELLULAR VESICLES IN ACUTE CORONARY SYNDROME

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Abstract

A diverse population of small extracellular vesicles (EVs) that are released by various cells has been characterized predominantly in bulk, a procedure whereby the individual characteristics of EVs are lost. Here, we used a new nanotechnology-based flow cytometric analysis to characterize the antigenic composition of individual EVs in patients with acute coronary syndrome (ACS). Plasma EVs were captured with 15-nm magnetic nanoparticles coupled to antibodies against CD31 (predominantly an endothelial marker), CD41a (a marker for platelets), and CD63 or MHC class I (common EV markers). The total amounts of EVs were higher in the ACS patients than in the controls, predominantly due to the contribution of patients with acute myocardial infarction. For all captured fractions, the differences in the EV amounts were restricted to CD41a⁺ EVs. The increase in the numbers of EVs in the ACS patients, predominantly of platelet origin, probably reflects platelet activation and may indicate disease progression.

Keywords

extracellular vesicles; platelets; acute coronary syndrome; acute myocardial infarction; flow cytometry

Introduction

Over recent years it has become clear that small (<300 nm) extracellular vesicles (EVs), which were initially considered to be “platelet dust” [1], constitute an important physiological system of cell–cell communication. Together with two other systems of intercellular communication, namely cell–cell contact interactions and the release of soluble

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Declaration of Interest statement

The authors declare having no conflict of interest.

molecules (cytokines), EVs coordinate normal body physiology and are altered in various pathologies. In particular, it has been shown that EVs released from platelets and endothelial cells regulate normal blood coagulation [2–7].

Many cell types release EVs into the blood stream, and these EVs may carry proteins characteristic of the cells that released them. Bulk analysis has shown that blood EVs carry tetraspanin CD63, which is expressed by cells of many types and is involved in the EV formation process [8]. Also, it has been reported that two molecules are common to blood EVs: CD41a, a glycoprotein IIb/IIIa that is characteristic of platelets [9,10], and CD31, a cell adhesion molecule expressed by endothelia [11–14] and less abundantly by other cell types [4,15,16]. However, a combination of at least two of the following antigens, CD31, CD62E or CD146 is required to attribute EVs to endothelia rather than to platelets [17][18]. By incorporating cellular proteins into their membranes, EVs may reflect not only the types of the cells from which they were released but also the physiological state of these cells [19,20]. Accordingly, EV composition may change in different pathologies, in particular in acute forms of coronary artery disease. The composition of EVs in the blood of patients with acute coronary syndromes (ACS) differs significantly from that found in healthy controls [21,22].

Although the pool of EVs in blood is highly diverse, up to now EVs, especially the small ones (<300 nm) that constitute the majority of the released vesicles [23], have been characterized predominantly in bulk by various biochemical methods. With these strategies, individual EV features, in particular their antigenic composition, are lost. To characterize individual EVs in a high-throughput manner, it is necessary to apply to them a technique similar to flow cytometry [24]. However, EVs cannot be analyzed by means of standard flow cytometry because they are too small to generate the light-scattering signal on which most cytometers rely for triggering their analysis. Moreover, if EVs are stained with fluorescence-labeled antibodies and fluorescent triggering is applied, it is difficult to distinguish EVs from free antibodies and their aggregates because of their small size.

Recently, we overcame these obstacles and developed a nanotechnology-based system that permits the analysis of the antigenic composition of individual EVs [25]. Here, we applied this method to identify the antigenic composition of individual EVs in the blood of healthy volunteers and patients with acute forms of coronary artery disease.

Methods

Characteristics of patients

In this study, we enrolled 12 ACS patients and 15 healthy volunteers without cardiovascular diseases. Inclusion criteria for ACS patients were determined according to the existing guidelines [26]. Exclusion criteria for patients were symptoms of infectious diseases, any record of neoplasms, cardiogenic shock, and thrombolytic therapy. The ACS cohort was then separated into two groups: a group with acute myocardial infarction (AMI), both non-ST-elevation and ST-elevation, and a group with unstable angina (UA). The diagnosis for subgroups was determined according to the standard criteria [27,28]. All patients were hospitalized in Davidovsky Moscow City Hospital. During transportation to the hospital and

prior to blood sampling all patients were treated with dual antiplatelet therapy (aspirin 250-300 mg and clopidogrel 300-600 mg) and the majority of them (83%) also received parenteral anticoagulants. Blood samples were collected within the first 24 h of patient admission (before coronary angiography). In hospital, all patients were subjected to standard pharmacological therapy according to the current guidelines [27,28], including dual antiplatelet therapy, anticoagulants, statins, beta-blockers, and ACE inhibitors. The study protocol and participants' consent was approved by the Interuniversity Committee of Ethics, protocol number 12-12 date 20.12.2012; all participants signed a written informed consent to participate in this study.

Blood collection

Peripheral blood was collected by intravenous withdrawal in vacuum tubes with sodium citrate as anticoagulant (Sarstedt, Nuembrecht, Germany, cat#05.1071.001). The first tube with blood was discarded because it contained platelets activated during venipuncture and skin fibroblasts. Platelet poor plasma (PPP) was prepared within less than 1 h after blood collection by centrifugation at 3,000 g for 15 min. Then, plasma was aliquoted in 600 µl fractions and frozen at -80°C . EV isolation with magnetic nanoparticles, in contrast to gradient centrifugation, allowed us to use one centrifugation instead of two [29] to avoid additional disturbance of the preparation. Indeed, the second centrifugation removes only a small fraction of particles between 300 and 500 nm (Fig.S2), and thus was purposefully avoided in our protocol.

Coupling of monoclonal antibodies to magnetic nanoparticles

The procedure of coupling was as described previously [30]. Briefly, prior to coupling, monoclonal antibodies (Abs) against CD31 (Biolegend, San Diego, CA, cat# 303108), CD41a (Biolegend, cat# 303712), CD63 (Biolegend, cat# 353014), and MHC (HLA-A,B,C Biolegend, cat #311423) were dissolved in coupling buffer at a concentration of 2 mg/ml. For coupling, 1 mg of 15-nm iron oxide magnetic nanoparticles with carboxylic acid (MNPs) (OceanNanoTech, San Diego, CA, cat# ICK-15-005) was incubated in 400 µl of activation buffer. Afterwards, 400 µl of coupling buffer was added to activated MNPs followed by the addition of 1 mg of antibody and the mix was incubated for 2 h at room temperature. The reaction was stopped by addition of 10 µl of quenching solution and followed by two washings on SuperMag SeparatorTM. In order to visualize nanoparticles, prior to capture of the EVs we mixed the Abs–MNPs with fluorescent anti-mouse IgG1 Fab fragment (Zenon® Alexa Fluor® 488 Mouse IgG1 Labeling Kit, Life Technologies, Grand Island, NY). We washed away the unbound Fab fragments using a 100-kDa Nanosep® centrifugal device (Pall Corporation, Timonium, MD) for two 5-min centrifugations at 1,100 g. Labeled Abs–MNPs were diluted in their initial volume with PBS (Gibco, Life Technologies).

Capture and detection of EVs with nanoparticles

PPP (100 µl, freshly thawed) was incubated with 60 µl of labeled Abs–MNPs for 1h at $+4^{\circ}\text{C}$ followed by addition of 2.5% (v/v) Zenon® blocking reagent (Life Technologies). The suspension of EVs conjugated to Abs–MNPs was labeled with fluorescent antibodies or isotype controls for 20 min at room temperature with continuous mixing. For CD31-coupled

MNPs we used the following specific monoclonal antibodies: anti-CD41a-APC (BD Bioscience, San Jose, CA, cat# 559777) and anti-CD63-PE (Biolegend, cat# 353004). For CD41a-coupled MNPs we used anti-CD31-AlexaFluor® 647 (Biolegend, cat# 303112) and anti-CD63-PE (Biolegend, cat# 353004). For CD63-coupled MNPs we used anti-CD31-PE (Biolegend, cat# 303106) and anti-CD41a-APC (BD Bioscience, cat# 559777). Afterwards, suspensions were transferred into MACS® magnetic columns (Miltenyi Biotec, San Diego, CA) mounted on an Octomag® magnet separator (Miltenyi Biotec). Unbound antibodies were washed away by gravity-mediated flowing of the input sample followed by the flowing of 500 µl of washing buffer for two times (PBS with 2 mM EDTA and 0.5% normal mouse serum). Then, magnetic columns were unmounted and EVs conjugated to Abs–MNPs were eluted with 2 × 400 µl of PBS and fixed with 200 µl of 4% formaldehyde solution. Directly prior to flow cytometry analysis, 50 µl of well-mixed AccuCheck® beads (Invitrogen, Life Technologies) were added.

The procedure is summarized in Fig.S1.

For distinguishing endothelia-derived vesicles we are used monoclonal antibodies anti-CD146-PE (Beckman Coulter cat#A07483), anti-CD62E-PE (Biolegend cat#336008), and anti-CD42a-eFluor450® (eBioscience, cat#48-0428-42).

Flow cytometry analysis

Suspensions of EVs conjugated to Abs–MNPs were analyzed with a FACS Aria II SORP (BD Bioscience) flow cytometer equipped with 355-, 407-, 488-, and 633-nm lasers. Fluorescence activated by the 488-nm laser set at a power of 180.3 mW and measured between 505 and 550 nm (Blue-525-channel) was set as the threshold. The flow cytometer sheath fluid was filtered through a series of in-line filters with nominal filtration rating of 100 nm and 40 nm (Meissner Filtration Products, Inc, Camarillo, CA). First, we acquired filtered PBS and set up the value of the threshold. We set fluorescence parameters in the highest voltage that maintained the positive peak of labeled CompBeads (BD Bioscience) in the range of linearity of the trigger channel (Fig.S3). We calculated compensation in FlowJo Software (TreeStar, LLC, Ashland, OR, USA) using single-stained CompBeads. EV events were counted in a single-event gate according to height and weight parameters of MNP fluorescence (Fig.S4).

This gating strategy allowed us to include in our analysis EVs of predominantly less than 300 nm in diameter. According to the sizing with the calibration beads beads (Megamix – Plus SSC, Biocytex, Marseille, France), less than 4% of the events corresponded to particles between 300 and 500 nm and all larger particles were excluded by the gating strategy. This was confirmed with an NTA assay using a NanoSight equipped with 405nm laser and analyzed by NTA 3.0 software (NanoSight NS300, Malvern Instruments, UK) (Fig.S4). The measurements were performed with constant sample flow using syringe pump for 60 sec at camera level 13. The average size of the EVs captured with anti-CD31–MNPs was 128.1 ± 3.2 nm.

Earlier we demonstrated that the efficiency of our technique for capture of viruses and EVs of interest is about 99% [25,30]. In the current work we confirmed this result: we captured

EVs via MNP-coupled antibodies against CD41a, stained them with antibodies against CD31 and CD63 and isolated on magnetic columns. The flow-through fraction was again subjected to the same procedure. We found that on the first run, we captured 97% of the EVs recognized by anti-CD41a antibodies (data not shown).

Statistical analysis

We assessed the normality of our data distribution using the Shapiro-Wilk normality test. For the normally distributed data we used the t-test to compare different fractions of EVs in patients with ACS and in healthy volunteers. For comparison of the data across multiple groups, i.e., subgroups of unstable angina, acute myocardial infarction, and the control group, we used a one-way ANOVA. We examined pair-wise comparisons using Tukey's multiple comparisons test. When the distribution was not normal, we used the Mann-Whitney test for two-group comparison and the Kruskal-Wallis test for several groups. We used the nonparametric Dunn's multiple comparisons test to examine pair-wise comparisons. Data with normal distributions are presented as means $\pm$ SD; non-parametric data are presented as median [25th percentile; 75th percentile]. We performed statistical analysis using Prism 6.0 (GraphPad Software, San Diego, CA). Statistical significance was defined as a p-level < 0.05 .

Results

ACS patients

We analyzed EVs in platelet poor plasma from 12 ACS patients and from 15 control individuals without coronary artery disease. The ACS cohort included 9 males and 3 females, average age 61.3 ± 8.8 . ACS patients were separated into two groups: acute myocardial infarction, consisting of non-ST-elevation and ST-elevation myocardial infarction (5 males and 1 female, median age 60.5 [55.3; 67.5]), and unstable angina (4 males and 2 females, median age 59.0 [53.3; 67.3]). A control group of healthy volunteers contained 7 males and 8 females, average age 55.5 ± 9.5 . There were no significant differences ($p < 0.05$) in age and gender between the ACS patients and the control volunteers; neither were there statistical differences in these parameters between the UA and AMI subgroups or between either of these groups and control volunteers.

Blood was collected according to a protocol approved by the Institutional Review Board and separated from cells, in particular from platelets, as described in Materials and Methods. Briefly, EVs in PPP were captured with MNPs coupled to specific antibodies, stained for various antigens separated on a magnetic column and analyzed in a flow cytometer. Isotype control antibodies were used to check for nonspecific staining (Fig. 1, S5).

Quantification of captured EVs in patients with ACS

First, EVs from PPP were captured by fluorescent MNP-coupled antibodies against CD31 or against CD41a, a typical platelet antigen. Also, we used MNPs coupled to antibodies against CD63, a common marker of EVs. Captured EVs were stained with detection fluorescent antibodies against CD31 and CD63 for CD41a-captured EVs, against CD41a and CD63 for CD31-captured EVs, and against CD31 and CD41a for CD63-captured EVs. EVs of interest

were defined as flow events positive for the MNP label and for at least one of the detection antibodies. For each staining, we performed an isotype control experiment using non-specific isotype-matched immunoglobulins with the same fluorophores as in specific antibodies (Fig. S5). The numbers of events positive for isotype control staining typically constituted less than 1% of events positive for staining for specific antibodies. The volume of the acquired samples was controlled by the AccuCheck beads added to the test tube at a known concentration prior to flow analysis. With this approach, we were able to enumerate EVs in our samples and calculate their concentrations in PPP.

We found that in blood of patients from both groups as well as in blood of healthy volunteers the most abundant fraction of vesicles was CD41a-positive. With all the capture strategies used, the total amounts of EVs positive for one or two of the detection antibodies in ACS patients were significantly higher than in healthy volunteers. The amount of EVs captured by CD31–MNPs and positive for one or two of the detection antibodies in ACS patients was 3,359 [2,328; 5,472] EVs/μl in comparison with 1,272 [714; 2,157] EVs/μl in healthy volunteers ($p=0.001$). The total amount of EVs captured by CD63 in ACS patients in comparison with healthy volunteers was 3,541 [1,318; 5,173] EVs/μl vs. 806 [488; 2,112] EVs/μl ($p=0.007$). There were 4,752 [3,238; 7,173] EVs/μl in plasma of patients with ACS captured by CD41a–MNPs, whereas in plasma of healthy volunteers this number was significantly lower, 2,623 [1,927; 4,188] EVs/μl ($p=0.015$) (Fig. 2). For normally distributed data, we also compared the differences in means and found that in patients there was approximately a 2.4-fold higher amount of CD63-captured EVs ($3,207 \pm 1,827$ vs. $1,321 \pm 1,052$; $p=0.002$) and a 1.7-fold higher amount of CD41a-captured EVs ($5,296 \pm 2,590$ vs. $3,069 \pm 1,555$; $p=0.018$) than in healthy volunteers (Table 1).

Furthermore, we investigated whether EV distribution in patient samples was associated with their clinical diagnosis. Towards this goal, we compared samples obtained from control volunteers with samples from the patients in the UA and AMI subgroups. We found that the amount of EVs captured with CD31–MNPs in samples from AMI patients was significantly higher than in the control group (4,261 [2,994; 6,256] EVs/μl vs. 1,272 [714; 2,157] EVs/μl; $p=0.003$) (Fig. 3). Also, there was significantly higher amount of EVs captured with CD63–MNPs in samples from the AMI group than in controls (4,559 [3,489; 5,238] EVs/μl vs. 806 [488; 2,112] EVs/μl; $p=0.002$) (Fig. 4) (Table 1). We also observed a higher amount of EVs captured by CD41a–MNPs in the AMI group, but in this case the difference did not reach statistical significance. For EVs in UA patient samples, we did not find significant changes compared with controls.

Thus, in ACS patients the amounts of all captured EVs were higher than in healthy volunteers, and these differences were most pronounced for patients with AMI.

The spectra of EVs of different phenotype in ACS patients

We investigated whether the increase in EV concentrations in ACS patient plasma was restricted to a particular fraction of the EVs, or whether the numbers of EVs were proportionally increased in all antigenically different fractions of EVs that we tested. To answer this question, we enumerated EVs in three different subsets for all the captured populations: EVs captured by CD31–MNPs stained for CD41a and CD63, EVs captured by

CD63-MNPs stained for CD31 and CD41a, and EVs captured by CD41a-MNPs stained for CD31 and CD63.

The amounts of CD31-captured EVs of CD41a⁺CD63⁺ or CD41a⁺CD63⁻ phenotypes were significantly higher in ACS patients than in healthy volunteers (940 [456; 1,415] EVs/ μ l vs. 342 [246; 622] EVs/ μ l; $p=0.009$; and 2,133 [1,764; 4,211] EVs/ μ l vs. 761 [385; 1,807] EVs/ μ l; $p=0.003$, respectively). There were virtually no vesicles (presumably of endothelial origin), CD31-captured and negative for the platelet marker CD41 (Fig. 5) (Table 1). To confirm this, we stained CD31-MNPs captured EVs with antibodies against CD62E, CD146 and CD42a. The number of EVs positive for these markers in ACS patient was negligible (approximately three orders of magnitude lower than CD31+CD41+ EVs) (Fig.1). Also, there were almost no EVs of CD31⁺CD41a⁻ phenotype within EVs captured by CD63-MNPs. Quantitative analysis revealed that CD41a⁺ fractions in ACS patients were significantly larger than in healthy volunteers (2,559 [931; 3,885] EVs/ μ l vs. 659 [463; 1,708] EVs/ μ l for CD31⁺; $p=0.009$; and 758 [337; 1,282] EVs/ μ l vs. 147 [36; 568] EVs/ μ l for CD31⁻; $p=0.012$, respectively) (Fig. 6).

In all the fractions of EVs captured by CD41a-MNPs (CD31⁺CD63⁻, CD31⁺CD63⁺, and CD31⁻CD63⁺), we observed increased numbers of EVs in PPP of ACS patients compared with controls. However, only in the CD31⁺CD63⁻ fraction did this increase reach statistical significance ($4,356 \pm 2,391$ EVs/ μ l vs. $2,199 \pm 1,112$ EVs/ μ l; $p=0.010$) (Fig.7) (Table 1).

Furthermore, in several experiments we investigated the presence of the above-mentioned antigens on a larger pool of vesicles. For these experiments, we captured EVs with MNPs that carry antibodies against MHC class I, since this antigen is expressed on a wide range of different cells in the human body [31] and can be considered to be a “ubiquitous” EV marker. EVs from the blood of 5 patients (3 males and 2 females, median age 63.0 [57.0; 71.0]) and of 5 control volunteers (1 male and 4 females, median age 54.0 [44.5; 59.0]) were captured with MHC class I-MNPs and stained with the detection antibodies against CD41a and CD31. The two groups did not differ significantly in age/gender characteristics. The amounts of EVs captures through MHC class I and positive for CD41a or CD31 were not different from the amounts of EVs captured through CD41a or CD31. In samples obtained both from control individuals and from patients, there were more CD41a⁺ than CD31⁺ EVs (1,465 [794; 2,729] EVs/ μ l vs. 1,143 [586; 1,284] EVs/ μ l; $p=0.011$). Also, the numbers of CD31⁺ EVs captured with the MHC-I-MNPs from PPP of the control individuals were not significantly different from those captured from the PPP of patients (586 [346; 1,219] EVs/ μ l vs. 1,197 [1,020; 2,279] EVs/ μ l; $p=0.1775$). In contrast, the amounts of CD41a⁺ EVs captured with these MNPs from the ACS patients were significantly higher than those in controls (2,156 [1,453; 3,074] EVs/ μ l vs. 794 [522; 1,415] EVs/ μ l; $p=0.017$) (Fig. 8).

Thus, although the amounts of EVs were generally increased in ACS patients, the magnitude of the increase was different in different sub-populations of EVs.

Discussion

The release of extracellular vesicles is part of normal cellular physiology [32,33]. When cells undergo pathological changes, EVs released by these cells reflect the pathologies developed. Therefore, EVs are widely studied as candidates for diagnostic markers, and it is conceivable that altered EVs may become therapeutically significant targets. Moreover, altered EVs may contribute to the pathogenesis of disease itself.

Multiple reports have demonstrated that EVs are associated with cardiovascular diseases. It has been reported on the basis of bulk analysis that the total amounts of EVs of different sizes and origins are elevated in patients with higher cardiovascular risk scores [11], endothelial dysfunction [34–36], ischemic stroke [12,37], stable atherosclerotic plaques [13,38], and acute coronary syndromes [15,21,22,39–43]. Moreover, it has been shown that EV levels correlate with clinical outcomes in patients with coronary artery disease [44,45] and are reduced with statin therapy [46,47].

Various cells in different activation statuses can potentially release EVs into blood, where they are pooled. Although EVs are released by cells of different types and activation status, the individual characteristics of these EVs are lost in bulk analysis (e.g., by Western blot, ELISA or proteomics), by which EVs are routinely studied. Meanwhile, analysis of individual EVs can potentially report about the pathological states of the cells of their origin, provided that they carry cellular antigens by which they can be traced to these particular cells. Here, we attempted to trace the origin of EVs by determining the antigenic composition of individual EVs isolated from blood of healthy volunteers and from blood of patients with acute forms of coronary artery disease, in particular myocardial infarction. This task was accomplished with the new technology “flow virometry”, which was originally developed for the analysis of individual virions [30] and later adapted to analyze individual small EVs [25].

With this technology, we captured EVs with magnetic nanoparticles conjugated with antibodies against EV antigens and stained for other antigens with fluorescent antibodies. Using fluorescent triggering and high-power lasers, we visualized predominantly small (<300 nm) particles that are below the resolution of conventional cytometers that use light-scattering trigger. Earlier, we showed that with our protocol of EV capture with antibody-coupled MNPs and flow cytometer settings the events correspond predominantly to single EVs. Indeed, when we used a mixture of vesicles of different colors to evaluate the fractions of aggregates or swarms (both of them would represent predominantly two-color events), 95% of events constituted single vesicles. This is in agreement with the result of NanoSight analysis performed here, which showed a narrow peak of EVs of approximate 120 nm in size.

We chose a set of antibodies specific for antigens that in different combinations characterize platelet- and endothelium-derived EVs, namely CD31, CD41a, CD63, CD62E, and CD146 and evaluated their presence on EVs isolated from blood. We used some of these antibodies either to capture EVs by coupling them to MNPs or to detect their respective antigens on EVs. Specifically, in each experiment, we used CD31, CD41a, CD63, one for capture and

the other two for detection, and we enumerated EVs in plasma from control individuals and from patients with acute coronary artery disease. In other experiments, we captured EVs through MHC-class I, which is present on the majority of EVs [48,49], since it is expressed on most cell types.

Moreover, the amount of EVs captured with CD41a–MNPs that were positive for CD31 was not different from the amount of EVs captured through MHC class I that also carry CD41a and CD31 indicating that with CD41a–MNPs we captured virtually all EVs that express this antigen in combination with CD31 or CD63.

We compared the total numbers of EVs captured by all three antibodies. In all cases, we observed a significant increase of EVs in blood plasma of ACS patients compared with healthy controls. That was confirmed for EVs captured through MHC class I. Next, we investigated whether this increase correlated with a more specific diagnosis: we split ACS patients into two groups, UA and AMI, and compared EVs isolated from the blood of these patients with EVs from control donors. We found that the largest difference in EV count was between the group of controls and the subgroup of AMI patients. Although this conclusion is based on a relatively small cohort of patients, these data are in general agreement with earlier published results of analyses of microparticles in patients with stable angina and MI [19, 20, 37]. Our technology allowed us to switch to a fine analysis of small EVs that constitute the majority of blood vesicles [23] and to compare EVs of particular antigenic composition in control individuals and ACS patients. We found that the majority of EVs in all the subpopulations captured with various MNPs carry CD41a, indicating their platelet origin. The lack of endothelial-derived EVs was confirmed by the lack of positivity in staining for CD62E or CD146. This result is in agreement with previous studies of EV bulk analysis in ACS patients [41,43,50]. However, it differs from the results of experiments reported in [39] where the investigators found significantly higher numbers of CD31⁺CD41a[−] EVs. This difference may stem from the fact that we are focused on small EVs, which constitute more than 90% of all EVs, while the work cited above may have reported on the population of EVs of larger size (microparticles and apoptotic bodies) that are revealed with regular flow cytometry, which cannot detect particles with a size below 300 nm. The increase of platelet-originated EVs in blood of ACS patients that we report here may reflect platelet activation in such patients [51,52]. In summary, we found that the predominant population of EVs captured through various antigens including MHC class I carry CD41a, indicating their platelet origin. The amounts of EVs were higher in the ACS patients than in controls. Predominantly, this difference is due to the inclusion in the studied group of patients with AMI. This group of patients demonstrated the largest numbers of EVs in blood compared with controls. The increase in the numbers of EVs in the ACS patients does not occur across the board in all the EVs with different antigenic compositions, but rather is restricted to CD41a-positive vesicles, probably reflecting the activated status of platelets associated with the disease. The difference between patients and healthy volunteers does not seem to be caused by the therapy applied to the patients upon admission. Indeed, both ACS and UA patients received similar therapy, but the numbers of EVs in plasma of UA patients, but not of AMI patients, did not differ significantly from those in plasma of healthy volunteers. Furthermore, we did not find any difference in the number of EVs between a few ACS patients whose blood was collected two hours after the start of therapy start and the rest

of the cohort, however, this conclusion was based on a relatively small number of cases. Moreover, the anti-platelet therapy would rather decrease the number of the released EVs in patients compared to healthy controls, while we and others [41,53,54] observed the opposite effect, that is the increase of the number of various EVs in patients' blood compared to healthy control. Thus, the described difference in EVs seems to be a part of the pathological process rather than the result of treatment. However more systematic studies are required to analyze the effect of anti-platelet therapy on kinetics of EVs release.

The lack of such an analysis is one of the significant limitations of the current study. Another limitation is a small cohort of patients. Although in this cohort we found significant differences between different groups in the concentrations of plasma EVs, this finding should be confirmed on a larger scale to prove that CD41a⁺ EVs may be a marker of ACS in general and of AMI in particular, as our results indicate. Finally, in our study we focused on EVs that carry one of the three antigens CD31, CD41a and CD63. A larger set of antibodies against different antigens may reveal other differences in EVs' spectra between ACS patients and healthy volunteers, indicating changes in physiology of various cells in the course of the disease.

Future studies should reveal whether the increase in the number of platelet-derived EVs precedes some other symptoms of ACS; if so, this increase may be one of the early indicators of the disease. In general, analysis of antigenic composition of individual EVs that traces them to their cells of origin may report on the status of these cells and indicate disease progression. In general, analysis of the antigenic composition of individual EVs may lead to the development of new diagnostic tools.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMI	acute myocardial infarction
MNP	magnetic nanoparticle
PPP	platelet poor plasma
UA	unstable angina

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Fig. 1. Visualization of EVs captured with CD31-MNPs and stained for CD41a, CD62E and CD146

EVs were captured with MNPs coupled to anti-CD31 antibodies. MNPs-antibody complexes were stained with Zenon Alexa Fluor 488. Captured EVs were stained with APC-labeled anti-CD41a antibodies, CD62E-PE and CD146-PE; to avoid aggregation MNPs were added in excess [25]. **A, C and E** Specific staining. **B, D and F** Isotype control staining.

Fig. 2. Total amounts of EVs in blood of patients with ACS in comparison with healthy volunteers

EVs from patients with ACS (brown symbols) and from healthy volunteers (green symbols) were captured with MNPs coupled to one of three Abs against CD31, CD41a, or CD63 and stained with the other two (fluorescent) Abs. Stained EV–MNP complexes were isolated on magnetic columns and subjected to flow analysis. EVs of different phenotypes have been enumerated. Data presented as dot plot with median and IQR.

Fig. 3. CD31-captured EVs in blood of patients with UA and AMI

EVs from patients with UA (brown symbols) and AMI (yellow symbols), and from healthy volunteers (green symbols) were captured with CD31-MNPs and stained with fluorescent Abs against CD41a and CD63. Stained EV-MNP complexes were isolated on magnetic columns, subjected to flow analysis and enumerated. Data presented as dot plot with median and IQR.

Fig. 4. CD63-captured EVs in blood of patients with UA and AMI

EVs from patients with UA (brown symbols) and AMI (yellow symbols), and from healthy volunteers (green symbols) were captured with CD63-MNPs and stained with fluorescent Abs against CD41a and CD31. Stained EV-MNP complexes were isolated on magnetic columns, subjected to flow analysis and enumerated. Data presented as dot plot with median and IQR.

Fig. 5. CD31-captured EVs in blood of patients with ACS and healthy volunteers

EVs from patients with ACS (brown symbols) and from healthy volunteers (green symbols) were captured with MNPs coupled to Abs against CD31 and stained with fluorescent Abs against CD41a and CD63. Stained EV-MNP complexes were isolated on magnetic columns and subjected to flow analysis. EVs of different phenotypes have been enumerated. Data presented as dot plot with median and IQR.

Fig. 6. CD63-captured EVs in blood of patients with ACS and healthy volunteers

EVs from patients with ACS (brown symbols) and from healthy volunteers (green symbols) were captured with MNPs coupled to Abs against CD63 and stained with fluorescent Abs against CD41a and CD31. Stained EV-MNP complexes were isolated on magnetic columns and subjected to flow analysis. EVs of different phenotypes have been enumerated. Data presented as dot plot with median and IQR.

Fig. 7. CD41a-captured CD31⁺CD63⁻ EVs in blood of patients with ACS and healthy volunteers EVs from patients with ACS (brown symbols) and from healthy volunteers (green symbols) were captured with MNPs coupled to Abs against CD41a and incubated with fluorescent Abs against CD31 and CD63. Stained EV–MNP complexes were isolated on magnetic columns and subjected to flow analysis. EVs of different phenotypes have been enumerated. Data presented as dot plot with mean $\pm$ SD.

Fig. 8. MHC-captured CD41a⁺ EVs in blood of patients with ACS and healthy volunteers
 EVs from patients with ACS (brown symbols) and from healthy volunteers (green symbols) were captured with MNPs coupled to Abs against MHC class I and incubated with fluorescent Abs against CD31 and CD41a. Stained EV–MNP complexes were isolated on magnetic columns and subjected to flow analysis. EVs of different phenotypes have been enumerated. Data presented as dot plot with median and IQR.

Table 1
Comparative concentration of various populations of EVs captured with three different MNPs

Concentrations shown as median of EVs per 1 µl PPP with 25th percentile-75th percentile.

MNPs	anti-CD41		anti-CD63		anti-CD31	
	Control	ACS	Control	ACS	Control	ACS
anti-CD41			802 [481; 2102]	3535 [1305; 5167]	1269 [701; 2143]	3349 [2311; 5467]
anti-CD63	786 [415; 1359]	984 [570; 1146]			354 [253; 634]	954 [466; 1427]
anti-CD31	2595 [1840; 4154]	4720 [3215; 7140]	672 [471; 1710]	2565 [943; 3890]		