Monocyte-derived circulating microparticles (CD14+, CD14+/CD11b+ and CD14+/CD142+) are related to long-term prognosis for cardiovascular mortality in STEMI patients

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All authors take responsibility for all aspects of the reliability and declare that they have no financial and personal relationships that might bias the data presented and their discussed interpretation.

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Running head: Monocyte microparticles and long-term CV mortality
Abstract

Background: Circulating microparticles (cMPs) have been proposed as novel biomarkers of cardiovascular disease (CVD). We aimed to investigate the prognostic relevance of cMPs for future major adverse cardiovascular events (MACE) in STEMI patients.

Methods: We included 200 STEMI patients treated with percutaneous coronary intervention (PCI). One hundred patients with a primary composite end point (recurrent nonfatal acute MI, rehospitalization for heart failure, unscheduled PCI or death because of CV causes) were case-matched for sex, age, and CVD risk factors to 100 patients without a primary endpoint at the end of study follow-up (4.4 (1.4) years). cMPs from vascular cells were measured by flow cytometry at a mean of 28 hours after onset of symptoms.

Results: No differences were observed in MP shedding between patients with or without a MACE at the end of the study follow-up. However, compared to patients who survived during follow-up, patients who died because of CV causes (n=24) presented with increased total cMPs (Annexin V -AV-), cMPs carrying tissue factor, and increased MP shedding from platelets, lymphocytes, monocytes, and activated leukocytes, and ~10% lower left ventricular ejection fraction (LVEF). ROC-curve analyses showed that monocyte-derived cMPs (CD14+/AV+, CD11b+/CD14+/AV+ and CD142+/CD14+/AV+) considered together with LVEF best predicted cardiovascular mortality.

Conclusions: Monocyte-derived cMPs assessed in the acute phase relates to the prognosis of CV death at the long term. These findings may be of clinical interest in the risk assessment of STEMI patients.
**Keywords:** Circulating Microparticles, Cardiovascular mortality, STEMI, Monocytes, Tissue factor.

**Abbreviations:** Acute myocardial infarction (AMI), annexin V (AV), cardiovascular disease (CVD), circulating microparticles (cMPs), coronary artery disease (CAD), cardiovascular (CV), cluster of differentiation (CD), cardiovascular event (CVE), heart failure (HF), major adverse cardiovascular event (MACE), percutaneous coronary intervention (PCI), phosphatidylserine (PS), smooth muscle cells (SMC), ST-elevation myocardial infarction (STEMI), tissue factor (TF, CD142).
1. Introduction

Circulating microparticles (cMPs) are small extracellular vesicles ranged 0.1-1µm diameter that are released when cells undergo necrosis, apoptosis or are injured. These MPs play a role in cell-to-cell communication because they contain cytoplasmatic material and cell surface proteins from their parental cells of origin (1), and therefore they can be defined in the flow cytometer by size and expression of cell-specific antigens such as clusters of differentiation (CD) and phosphatidylserine (PS, which binds Annexin V, AV), on their surface.

Hence, cMPs participate in cardiovascular disease (CVD) progression, and have been proposed as sensitive biomarkers of CVD (2). In fact, increased levels of cMPs have been found in acute coronary syndrome patients (3), including STEMI (4-6), and in patients suffering a primary major adverse cardiovascular event (MACE) (7).

However, although it is known that low left ventricular ejection fraction (LVEF), older age and C-reactive protein (CRP) impact long-term prognosis of acute coronary heart disease (8, 9), there is a paucity of data on the role of cMPs or their use as biomarkers in the development of future MACE on STEMI patients.

Therefore, the aim of our study was to investigate prognostic relevance of cMPs from different cell origins for future clinical endpoints in a STEMI population.

2. Materials and methods

2.1. Study design

Patients from this retrospective observational study belong to a cohort of STEMI patients admitted to Oslo University Hospital Ullevål, Norway between June 2007 and August 2011, all treated with primary percutaneous coronary intervention (PCI).
Patients below age 18 years and patients unable (clinically unstable or sedated patients) or unwilling to give written informed consent were not included.

STEMI was defined as ST segment elevation of >2 mm in two or more contiguous chest leads or ≥1 mm in two or more limb leads or left bundle branch block, with typical chest pain and elevated troponin levels above the recommended diagnostic threshold. Patients were followed for a period of 4.4 years for recordings of clinical endpoints, done by telephone contact or mail. Hospital records were collected for each endpoint and then further classified by an Endpoint Committee. Mortality endpoints were obtained from the Cause of Death Registry, provided by the Norwegian Institute of Public Health.

A primary endpoint of MACE in this cohort was defined as a composite of recurrent nonfatal AMI, rehospitalization for heart failure (HF), unscheduled PCI or death because of cardiovascular causes (cardiovascular –CV- death).

2.2. Patients

We randomly selected 100 patients with a primary endpoint of MACE. Each case was matched for sex, age, diabetes, hypertension, smoking habits and family history of CVD to 100 patients without a clinical endpoint at the end of study follow-up. Patients on warfarin treatment at inclusion, patients with previous malignant diseases, peripheral artery disease, heart failure (HF), previous AMI, previous cerebrovascular disease, and patients who died from non-CV causes were excluded.

Clinical information was collected from hospital records and questionnaires acquired at the time of inclusion. Left ventricular ejection fraction (LVEF) was measured by echocardiography before hospital discharge or at a clinical follow-up within 3 months after the AMI. In case of repeated examinations, an average value was used.
The study protocol was approved by the Regional Committee for Medical Research Ethics and following the principles outlined in the Declaration of Helsinki, and all participants signed an informed consent.

2.3. Blood sampling

Blood samples were collected at median time of 28 h after symptoms and 21 h after the PCI procedure after overnight fasting. Sodium citrate (3.8%) tubes were used. Blood was centrifuged within 30 minutes at 2500 x g for 20 min at 4°C and plasma was frozen at -80°C until analyzed. Additionally, biochemical and hematological parameters were quantified with standardized routine methods.

2.4. Circulating microparticles isolation and quantification

The cMP fraction was isolated from plasma by a two-step high-speed centrifugation. Five hundred µL of frozen plasma aliquots were thawed on melting ice and centrifuged at 2500xg 15 min at room temperature (RT) (10). Then, 250 µL of plasma collected from the upper part of the vial was transferred to another vial and centrifuged at 20,000×g for 30 min at RT to pellet cMPs. The supernatants were discarded and the cMP enriched pellet was washed once with citrate-phosphate buffered saline (PBS) solution (citrate-PBS; 1.4 mM phosphate, 154 mM NaCl, 10.9 mM trisodium citrate, pH 7.4) before a second equal centrifugation step was made. Finally, the remaining cMP pellets were resuspended in 100 µL citrate-PBS.

Triple-label flow cytometric analysis was performed as follows: 5 µL of washed cMP suspensions were diluted in 30 µL PBS buffer containing 2.5 mM CaCl₂ (Annexin Binding Buffer, ABB). Thereafter, combinations of 5 µL of allophycocyanin (APC)-
conjugated AV (BD-horizon) with two specific monoclonal antibodies for MP characterization (mAb, 5 µL each, Supplemental Table 1) labeled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE), or the isotype-matched control antibodies were added. Samples were incubated 20 min at RT in the dark and diluted with ABB before being immediately analyzed with the Auto Collect mode in 96-well plates on an AccuriC6 flow cytometer (BD, Accuri® Cytometers, Inc., San Diego, CA), except for MPs from smooth muscle cells (SMC). For the detection and quantification of cMPs from SMC origin, 5 µL of the cMPs suspension were incubated 20 min at RT in the dark with 5 µL AV-APC and 5 µL CD142-FITC (TF) in a final volume of 50 µL ABB. cMPs were fixed with 250 µL ABB/paraformaldehyde 2% for 30 min and centrifuged at 20,000×g for 30 min to pellet cMPs. After eliminating the supernatant, cMPs were permeabilized with 45 µL of ABB/saponin 0.1% 20 min at RT in the dark. After permeabilizing, 5 µL of smooth muscle actin (SMA)-α-PE were added to the cMPs suspension and incubated 20 min at RT in the dark and finally diluted with ABB prior to flow cytometer analyses.

For all cMP characterization and quantification, acquisition was performed at 2 minutes per sample. Flow rate was set at 14 µL/min. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with the settings in the logarithmic scale. The upper threshold for FSC and SSC to ≤1 µm was set with the Megamix-Plus FSC beads (BioCytex, Marseille, France, Supplemental Figure 1). cMPs within the established gate limits (≥0.1 to ≤1 µm) were identified and quantified based on their binding to Annexin V and reactivity to cell-specific mAb (Supplemental Figure 2). To identify positive marked events, thresholds of fluorescence were set based on samples incubated with the same final concentration of isotype-matched control antibodies after titration experiments. AV binding level was corrected for
autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer (PBS). To reduce background noise, buffers were prepared on the same day and filtered through 0.2 μm pore size filters under vacuum.
cMPs derived from leukocytes other than lymphocytes and monocytes were inferred by subtracting agranulocyte MPs (CD3+, lymphocyte-derived plus CD14+, monocyte-derived) from total leukocyte-derived cMPs (CD45+) instead of labeling with specific mAbs.
Data were analyzed with the BD CSampler software (version 1.0.264.21, Accuri® Cytometers, Inc.). The cMP concentration (number of cMPs per μL of plasma) was determined according to Nieuwland’s formula (11).

2.5. **Statistical analysis**

Statistical analyses were performed using the SPSS Statistical Analysis System (version 23.0). Results are expressed as mean (SD) or n (%), when indicated.
Skewed variables (assessed with the Shapiro-Wilk test) were transformed to their natural logarithms for parametric analyses, and are shown as antilogarithmic values to facilitate the interpretation of the results. One-way ANOVA was performed to examine the differences in MP concentrations as related to cardiovascular risk factors, potential confounding factors and primary outcomes. To analyze the main associations between cMPs and relevant clinical variables, we performed multivariate linear regression adjusting for age, sex, acute administration of GPIIb/IIIa inhibitors, hours from symptoms to PCI and hours from symptoms to blood sampling and time of follow-up, according to the ANOVA results. ROC-Curve analyses were performed
to identify the cMPs and other clinical biomarkers able to predict a primary endpoint, and their corresponding C-statistics [areas under the curve (AUC) with their 95% confidence interval (CI)] were calculated. A cut-off level of cMPs was determined with the shortest distance from upper left corner of the ROC curve (where sensitivity=1 and specificity=1), and therefore, minimizing [(1-sensitivity)^2 + (1-specificity)^2]. Multivariable models for the prediction of a primary endpoint were performed with a logistic regression model with cMP levels from different cell origins, as well as for LVEF by creating predicted probabilities, which then were transferred to the ROC curve algorithm to estimate the likelihood of a primary outcome by calculating the corresponding AUC along with their 95% CI. A two-tail P-value of <0.05 was considered statistically significant.

3. Results

3.1. Characteristics of patients

Characteristics of the included patients are shown in Supplemental Table 2. There were no differences in the time frame from AMI symptoms to PCI between patients with and without a primary endpoint, or in the time between symptoms and blood sampling. All patients received aspirin and clopidogrel, and in the acute phase all patients received heparin, 25 patients received thrombolytics and 71 patients received GPIIb/IIIa inhibitors.

Within patients with a primary endpoint (n=100), 24 patients died of CV causes, 34 patients suffered an AMI, 41 patients underwent an unscheduled PCI, and 1 patient suffered a rehospitalization for HF.
3.2. Associations between circulating microparticles and biomarkers of CVD and AMI severity

No differences were observed in cMP concentrations from any cell origin in relation to sex, family history of CVD, previous use of aspirin or statins or acute administration of thrombolytics. Acute administration of GPIIa/IIIb inhibitors significantly reduced total, platelet-, endothelial-, erythrocyte-, leukocyte- and SMC-derived MP shedding, and cMPs carrying TF (Supplemental Table 3).

LVEF correlated inversely to peak TnT (r=-0.274, P=0.001) and NT-proBNP (r=-0.326, P<0.0001). As shown in Supplemental Table 4, LVEF also correlated inversely to monocyte-derived cMPs (CD14+/AV+, CD11b+/CD14+/AV+ and CD142+/CD14+/AV+). The results were consistent after multivariate adjustments (all p<0.05). No significant correlations were observed between cMPs from any cell origin and levels of TnT or NT-proBNP (data not shown).

3.3. Associations between circulating microparticles and MACE

No differences were observed in total number of MPs between patients with or without a MACE at the end of study follow-up. However, there were significant differences in the phenotype of shed MPs between patients who died because of CV causes and patients who survived during follow-up. As shown in Figure 1 and compared to patients who survived during follow-up, patients who died because of CV causes presented with significantly higher levels of total AV+ cMPs, cMPs carrying TF (CD142+/AV+), and increased MP shedding from platelets (CD61+/AV+, CD142+/CD61+/AV+, CD42b+/AV+, CD31+/CD42b+/AV+ and CD62P+/AV+), lymphocytes (CD3+/CD45+/AV+), monocytes (CD14+/AV+, CD11b+/CD14+/AV+ and CD142+/CD14+/AV+) and activated leukocytes (CD62L+/AV+). Multivariate adjustment did not modify the observed results (not shown), except for CD31+/CD42b+/AV+ and CD3+/CD45+/AV+ that
loosed the statistical significance. Patients who died because of CV causes were older and women in a higher proportion, and had higher CV risk burden and higher NT-proBNP (Table 1) in comparison to patients who survived during follow-up. In addition, patients who died because of CV causes had ~10% lower LVEF (mean of 40.1 (12.3)%) compared to patients who survived during follow-up (49.5 (8.4)%), P<0.001, one-way ANOVA.

3.4. Circulating microparticles as prognostic biomarkers of CV mortality

In order to evaluate any predictive power of cMPs for CV mortality, ROC-curve analyses were performed for MPs increased in patients who died because of CV causes.

As displayed in discontinuous lines on Figure 2, CD14+/AV+ cMPs at 640 cMPs/ 100 µl (P=0.011) were able to discriminate between patients who died because of CV causes and patients who did not, with a 43.5% sensitivity and 85.6% specificity [area under de curve (AUC)=0.695 (95% CI 0.544 to 0.847)] followed by CD142+/CD61+/AV+ cMPs at a cut-off point of 190 cMPs/ 100 µl, P=0.006, 52.2% sensitivity and 82.5% specificity, AUC=0.712 (0.556 to 0.868), and CD11b+/CD14+/AV+ cMPs at a cut-off point of 100 cMPs/ 100 µl, P=0.001, 69.6% sensitivity and 56% specificity, [AUC= 0.759 (0.629 to 0.889)].

On the other hand, LVEF at a cut-off point of 45.5% discriminated mortality with 70.6% sensitivity and 67.6% specificity [P=0.001, AUC= 0.763 (0.616 to 0.910)]. The best AUC was reached when the three types of cMPs were included in the clustered model along with LVEF [P< 0.0001, AUC= 0.807 (0.671 to 0.942)] (Figure 2, black continuous line). Thus, CD14+/AV+, CD11b+/CD14+/AV+ and
CD142⁺/CD14⁺/AV⁺ cMPs considered together with LVEF best predicted future cardiovascular mortality.

4. Discussion

The main finding of this study was that cMPs derived from monocytes measured during the acute phase of STEMI in patients undergoing a primary PCI were not associated with non-fatal MACE, but were shown to independently predict CV mortality. We also observed that adding LVEF to the model improved the predictive power for CV death, as evidenced by the increased C statistics. This clustered model of prediction could be of potential clinical utility in the risk assessment of cardiovascular mortality from STEMI patients, and potentially in the evaluation of the efficacy of new treatment modalities.

Several clinical and biochemical risk markers as well as functional tests, have been proposed to classify AMI severity and prognosis. Elevated levels of CRP and serum amiloid A are predictors of CV events in patients with severe unstable and stable angina, patients with severe coronary artery disease and patients with AMI (9), and elevated levels of interleukin (IL)-6 and IL-6 receptor have also been found to predict long-term CV mortality in patients with AMI (12, 13). Nevertheless, new proposed biomarkers may increase the power of CV prognosis in clustered models combined with “classical” biomarkers.

LVEF measured at the time of hospital discharge after the AMI has long been recognized as a strong predictor of short- and long-term mortality (8, 14), as also found in our study. LVEF correlated with monocyte-derived cMPs, suggesting that these MPs may reflect ongoing processes related to long-term CV injury during
myocardial infarction in patients who died because of CV causes. LVEF also correlated with peak TnT and NT-proBNP, in line with previous reports (15, 16). No correlations were, however, observed between cMPs and TnT or NT-proBNP, indicating that the pathological source of these three read outs is unrelated. Indeed, monocyte MP shedding reflects a generalized systemic innate immunity activation process in addition to the myocardial injury in itself. However, whether monocyte MP shedding is the consequence of an active process that contributes to CVD worsening or it is a residual activity of the AMI inflammatory reaction needs to be elucidated in further studies.
cMPs are key players at the crossroads between inflammation, coagulation, and vascular repair, depending on their cellular origin and molecular cargo (2), and are being proposed as new prognosis biomarkers of CVD. In patients with stable CAD, CD31+/AV+ cMPs were shown to be associated with major adverse cardiovascular and cerebral event presentation after 6 years follow-up (17). In high CV risk patients CD144+/AV+ cMPs were reported to be independent predictors of future cardiovascular events (18) and also to predict CV events, but not CV death in HF patients (19). In our study of STEMI patients, only monocyte-derived cMPs were predictive for CV mortality, but patients who died because of CV causes, showed increased cMPs from several cell origins, indicating that different cells are activated as to release cMPs during CVD progression.
Our results also suggests that monocyte activation, as reflected by their release of cMPs bearing CD11b and TF, also plays a key role in the worsening of CVD. It is know that monocyte-derived cMPs loaded with TF elicit procoagulant activity (20), constituting the second largest pool of thrombogenic cMPs after platelet-derived MPs (21) and therefore promoting a prothrombotic state. Besides TF-loaded monocyte-
derived MP, CD11b+ cMPs are captured by activated platelets within thrombi by a P-selectin/P-selectin glycoligand-1 dependent mechanism leading to the accumulation of TF and contributing to thrombus propagation (22). In addition, increased levels of CD14+ MP have been associated with increased common carotid intima-media thickness in children (23), and in adults with previous CVD. CD14+ cMPs were also positively associated with the number of atherosclerosis affected vascular territories (24), and with an increased risk for CV morbidity and mortality (25). Thus, monocyte MPs shown in our study to be predictive of CV death in STEMI patients (namely CD14+/AV+, CD14+/CD11b+/AV+ and CD14+/CD142+/AV+ cMPs) may be principal actors in the crossroad between inflammation and thrombosis.

Patients who received an acute administration of GPIIb/IIIa inhibitors showed reduced MP shedding from almost all vascular cell origins. Interestingly, treated patients showed decreased MP shedding from monocytes (CD14+/AV+), but not from activated monocytes (CD14+/CD11b+/AV+ and CD14+/CD142+/AV+) or activated endothelial cells (CD146+/CD62E+/AV+). Patients treated with GPIIb/IIIa inhibitors also showed 2-3 fold lower levels of platelet-derived cMPs, which is supported by previous studies (26, 27). GPIIb/IIIa complex plays a crucial role in MP release from platelets (28), and about three quarters of platelet-derived cMPs carry the glycoprotein IIa/IIIb receptor (5, 29).

It might be discussed, as a limitation, that samples were collected in the acute phase of STEMI. However, this means that blood samples have information derived from cellular activation during the first few days following the STEMI. The fact that monocyte-derived cMPs in the acute phase of the STEMI can predict CV death to a certain degree up to 4.4 years later, may rather be considered as a major strength of our study. Assessing the risk of CV death at the acute phase may have potential
clinical applications in the long-term management of these patients. Another strength of our study relies on its sample size, which is relatively high for cMPs studies, although the number of patients who died was modest, and the cohort was somewhat selected in a certain degree. Nevertheless, this study is observational and as such is subjected to confounding factors. Additionally, LVEF data was collected between 2 days and 3 months after the STEMI. Prior use of aspirin and statins, known to decrease MP shedding (30, 31), differs a little between cases and controls, although in a non-significant manner. Notwithstanding, after STEMI all patients started aspirin and statin therapy, therefore equalizing the associated long-term effects on MACE attributable to the use of these medications.

5. Conclusions
In our population of STEMI patients, monocyte-derived cMPs (CD14⁺) and monocyte-derived cMPs carrying markers of cell activation (CD11b and TF) measured in the acute phase, are adding statistical power to LVEF for the prognosis of long term CV mortality. These findings may be useful in the clinical assessment of patients and improving prognosis at the long-term, but have to be confirmed in other populations.

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Conflicts of interest
The authors report no relationships that could be construed as a conflict of interest.
References


Table 1. Characteristics of the total population and according to cardiovascular mortality.

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<th>CV DEATH</th>
<th>NO CV DEATH</th>
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<tr>
<td></td>
<td>(n=24)</td>
<td>(n=176)</td>
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<td>Males, n (%)</td>
<td>16 (66.7)</td>
<td>144 (81.8)</td>
<td>&lt;0.0001</td>
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<td>Age (years)</td>
<td>71.5 (8.6)</td>
<td>59.5 (11.8)</td>
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<td>BMI (Kg/m^2)</td>
<td>27.3 (5.1)</td>
<td>27.0 (3.8)</td>
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<td>Systolic blood pressure (mm Hg)</td>
<td>144 (36)</td>
<td>137 (27)</td>
<td>0.307</td>
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<td>Diastolic blood pressure (mm Hg)</td>
<td>83 (19)</td>
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<td>5.2 (1.2)</td>
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<td>Triglycerides (mmol/L)</td>
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<td>HbA1C (%)</td>
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<td>NT-proBNP (pg/L)</td>
<td>271 (289)</td>
<td>79 (137)</td>
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<td>Left ventricular ejection fraction (%)</td>
<td>40.1 (12.3)</td>
<td>49.5 (8.4)</td>
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<td>Hypertension, n (%)</td>
<td>12 (50)</td>
<td>52 (29.5)</td>
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<td>11 (45.8)</td>
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<td>Family history of CVD, n (%)</td>
<td>5 (20.8)</td>
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<td>Medication, n (%)</td>
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<td>Acetylsalicylic acid</td>
<td>6 (25)</td>
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<td>Statins</td>
<td>1 (4.2)</td>
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<td>2 (8.3)</td>
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<td>Glycoprotein IIb/IIIa inhibitors, n (%)</td>
<td>6 (25)</td>
<td>65 (36.9)</td>
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<td>Time from symptoms to PCI (hours)</td>
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<td>6.5 (8.2)</td>
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<td>Time from symptoms to blood sampling (hours)</td>
<td>30.3 (16.0)</td>
<td>27.8 (22.6)</td>
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Results are expressed as mean (SD) or n (%) when indicated. *P from the comparison between patient who will die because of CV causes (CV death) and patients who survived during follow-up (no CV death), assessed with one-way ANOVA for continuous variables and the Chi-squared test for categorical variables. BMI indicates body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; TnT, troponin T; NT-proBNP, N-terminal of the prohormone brain natriuretic peptide; CVD, cardiovascular disease; and AMI, acute myocardial infarction.
FIGURE CAPTIONS

Figure 1. Circulating microparticles in patients who died because of cardiovascular causes and patients who survived during the study follow-up.

Results are expressed as mean ± SEM. P values from the comparison between patients who died from cardiovascular causes (in black, n= 24) and patients who survived (in white, n= 176) at the end of study follow-up (one-way ANOVA). A) Total and platelet-derived cMPs; B) Tissue factor (CD142), von Willebrand factor receptor- and PECAM-carrying cMPs; C) P-Selectin (CD62P)- and L-Selectin (CD62L)-carrying cMPs and lymphocyte-derived cMPs (CD3/CD45); and D) monocyte (CD14)-derived cMPs carrying Macrophage-1 Antigen (Mac-1, CD11b) and tissue factor. cMPs denotes circulating microparticles and AV, annexin V.

Figure 2. ROC curve analyses to evaluate the predictive power of monocyte-derived cMPs and ejection fraction for cardiovascular death.

ROC curve analyses used to evaluate the prognostic power of monocyte-derived cMPs, LVEF and the combination of LEVF and monocyte-derived cMPs probabilities as predictors of cardiovascular death with their corresponding areas under the curve (AUC) and their 95% confidence intervals (CI). CD14 is a biomarker of monocytes, CD11b is Macrophage-1 Antigen (Mac-1, a marker of leukocyte activation) and CD142 is tissue factor. cMPs indicates circulating microparticles; AV, annexin V and LVEF, left ventricular ejection fraction.
Figure 1
Figure 2