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Supervised and unsupervised learning to define the cardiovascular risk of patients according to an extracellular vesicle molecular signature

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ABBREVIATIONS

Area under the curve, AUC; Coronary Artery Disease, CAD; Cardiovascular, CV; Chronic Heart Failure, CHF; Chronic Kidney Disease, CKD; Diastolic Blood Pressure, DBP; Ejection Fraction, EF; Endothelial Vesicle(s), EV(s), EEV(s); Estimated Glomerular Filtration Rate, eGFR; Extracellular Vesicle(s), EV(s); European Society of Cardiology, ESC; Hypertension, HTN; Flow Cytometry, FC; Low-Density Lipoprotein, LDL; Left Ventricular Hypertrophy, LVH; Leukocyte-derived EV(s), LEV(s); Linear Discriminant Analysis, LDA; Microalbuminuria, MA; Median Fluorescence Intensity, MFI; Nanoparticle Tracking Analysis, NTA; Normalized MFI, nMFI; Organ Damage, OD; Platelet-derived EV(s), PEV(s); Systolic Blood Pressure, SBP.

KEYWORDS

Extracellular Vesicles; Cardiovascular Risk; Machine Learning; Biomarker.

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ABSTRACT

Cardiovascular (CV) disease represents the most common cause of death in developed countries. Risk assessment is highly relevant to intervene at individual level and implement prevention strategies. Circulating extracellular vesicles (EVs) are involved in the development and progression of CV diseases and are considered promising biomarkers. We aimed at identifying an EV signature to improve the stratification of patients according to CV risk and likelihood to develop fatal CV events.

EVs were characterized by nanoparticle tracking analysis and flow cytometry for a standardized panel of 37 surface antigens in a cross-sectional multicenter cohort (n=486). CV profile was defined by presence of different indicators (age, sex, body mass index, hypertension, hyperlipidemia, diabetes, coronary artery disease, cardiac heart failure, chronic kidney disease, smoking habit, organ damage) and according to the 10-year risk of fatal CV events estimated using SCORE charts of European Society of Cardiology.

By combining expression levels of EV antigens using unsupervised learning, patients were classified into three clusters: Cluster-I (n=288), Cluster-II (n=83), Cluster-III (n=30). A separate analysis was conducted on patients displaying acute CV events (n=82). Prevalence of hypertension, diabetes, chronic heart failure, and organ damage (defined as left ventricular hypertrophy and/or microalbuminuria) increased progressively from Cluster-I to Cluster-III. Several EV antigens, including markers for platelets (CD41b-CD42a-CD62P), leukocytes (CD1c-CD2-CD3-CD4-CD8-CD14-CD19-CD20-CD25-CD40-CD45-CD69-CD86), and endothelium (CD31-CD105) were independently associated with CV risk indicators and correlated to age, blood pressure, glucometabolic profile, renal function, and SCORE risk.

EV profiling, obtained from minimally invasive blood sampling, allows accurate patient stratification according to CV risk profile.

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INTRODUCTION

Cardiovascular (CV) diseases are the leading cause of death in Europe, accounting for 39% and 47% of deaths in females and males, respectively[1]. Of the major contributing factors, the respective prevalence of uncontrolled hypertension, hypercholesterolemia, diabetes, obesity, and smoking habit, is 24.8%, 15.6%, 6.8%, 22.8%, and 21%, in European mid-to-high income countries[1]. In apparently healthy subjects, the interaction between environmental factors, genetics, and the aforementioned CV risk factors, results in the development of organ damage (OD) and potentially fatal CV events[2]. Identifying the unrecognized risk of CV disease in asymptomatic subjects is highly relevant[2]. In this context, the use of unconventional biomarkers as risk modifiers could improve CV risk prediction compared to conventional risk indicators.

Extracellular vesicles (EVs) are nanosized membrane particles generated by all cells following cellular activation/injury or actively released in response to different stimuli[3]. EVs have been involved in the development and progression of CV disease, where they can play either a protective or detrimental role[4]. The effects of circulating EVs largely depend on the cell type and its functional state, which influences its cargo and the expression of surface membrane proteins[4,5]. Based on their surface antigens, circulating EVs may be classified according to their cellular origin: platelet-derived EVs (PEVs) are the most ubiquitous subpopulation, followed by immune system/leukocyte-derived EVs (LEVs) and endothelial EVs (EEVs)[6,7]. PEVs, LEVs, and EEVs have been associated with CV burden, patient outcome, and even the development of CV events[8-11]. Being easily accessible and mirroring the complex intravascular environment, circulating EVs are considered ideal biomarkers, able to identify the multifactorial contribution of each CV risk indicator to the overall CV risk[4,12].

Unfortunately, pre-analytical work-up and application of different protocols to isolate and characterize EVs, as well as the lack of standardized methods with appreciable sensitivity and reproducibility, hinder inter-study comparisons[13] and rapid conversion of current knowledge into clinical practice[12].

The aims of the present study are: (i) to investigate the performance of a standardized panel of EV surface antigens for the stratification of patients according to their CV risk and (ii) to identify and quantify the specific and independent associations between the evaluated EV surface antigens and several CV risk indicators. To achieve these goals, we conducted an analysis on a large multicenter cross-sectional cohort of 486 patients employing both unsupervised and supervised machine learning approaches to characterize circulating EVs using a validated flow cytometry (FC) platform[14,15]. This allowed the simultaneous profiling of several surface antigens, including markers from platelets as well as immune system and endothelial cells. EV antigens were evaluated either individually or in combination with a specific signature determined by supervised and unsupervised machine learning strategies, allowing in-depth phenotyping of our patients according to their CV profile.

MATERIAL AND METHODS

This is a cross-sectional observational cohort study, and all relevant data are available from the corresponding author upon reasonable request. An extended description of the methods is provided in the Online Supplement.

Patients

Samples were obtained from a serum bank created during previous studies involving patients recruited between March 2017 and August 2020[16-18] by different medical centers based in Italy and Switzerland. The following institutions were involved in patient recruitment: Cardiocentro Ticino Institute, Lugano; Neurocenter of Southern Switzerland, Lugano; Hypertension Unit of the University of Torino. All patients gave informed consent according to the Helsinki declaration. We recruited a first cohort of 404 ambulatory patients referred to the aforementioned institutions, who gave informed consent and being over 18 years of age. Patients were excluded in case of: (1) concomitant acute/chronic inflammatory disease (infections, autoimmune disease); (2) cancer; (3)

acute or recent (less than one year) cardiovascular event; (4) pregnancy; (5) missing data. A second cohort of 82 patients admitted to the emergency department for an acute CV event (cerebrovascular event or myocardial infarction with ST-segment elevation, STEMI) was included and analyzed separately. All study participants underwent an in-depth assessment of cardiovascular risk factors (extended methods); 10-year risk of fatal CV events was estimated using the SCORE risk charts of the European Society of Cardiology (ESC)[19]. As the cohort was composed of Italian and Swiss subjects, we used the charts for low-risk countries[2].

Sample handling and EV characterization

Peripheral venous samples were collected in serum separator tubes (BD Vacutainer SST II); after clot formation, samples underwent serial low-speed centrifugations: 1600g for 15 min at 4°C, 3000g for 20 min, 10000g for 15 min to separate serum from cellular components and remove cellular debris and larger EV (Figure S1A). Serum was then stored at -80°C and thawed immediately before analysis. EV concentration (number of particles per mL) and diameter were determined in pre-cleared serum samples by nanoparticle tracking analysis (NTA); 1 µL of serum was diluted 1:1000 in phosphate-buffered saline (PBS); three videos of 60 s were recorded and analyzed for each sample. EV surface antigens were quantified by FC, as previously described[16]. EVs were isolated by immuno-capture using 37 different fluorescent-labeled capture bead populations (MACSPlex human Exosome Kit, Miltenyi Biotec), each coated with a specific antibody against common membrane antigens expressed by circulating EVs (Table S1); 15 uL of capture bead mix was added to 50 uL of serum and diluted to a final volume of 120 uL with MACSPlex buffer. After overnight bead-capture, EVs were incubated with 15 uL of a detection reagent (fluorescent-labeled antibodies against CD9-CD63-CD81) and finally analyzed by MACSQuant-Analyzer-10 flow cytometer (Miltenyi Biotec). Median fluorescence intensity (MFI; arbitrary unit, a.u.) was expressed for each EV surface antigen after subtraction of MFI for blank control and normalization with mean MFI for CD9-CD63-CD81 (normalized MFI, nMFI-%),

providing both a qualitative and semi-quantitative analysis[14,15]. Gating strategy and approach to discriminate different beads subsets are reported in Extended Methods (see Online Supplement and Figure S1A). Data on assay reproducibility are reported as Extended Results (see Online Supplement and Figure S1B-C-D). Pre-analytical sample management complied with minimal information for studies of extracellular vesicles (MISEV) indications[13].

Statistics and machine learning analysis

Normally and non-normally distributed variables are expressed as mean ± standard deviation and median and interquartile range, respectively. They were analyzed by one-way ANOVA with post-hoc Bonferroni test or non-parametric tests, as appropriate. Ordinal variables were expressed as absolute number (percentage) and analyzed by *Chi*-squared or Fisher's test. Correlations were assessed by Pearson's r test. Multivariate regression models were used to confirm the association of single CV risk indicators with clusterization according to EV surface profile. Associations of single EV antigens with each CV risk indicator were evaluated by linear regression models and calculation of β estimates (β>1 means an increased likelihood of the explored CV risk indicator). Values of $P<0.05$ were considered statistically significant.

Unsupervised learning was applied to classify patients into 3 clusters (Cluster-I, -II, and -III) according to the expression levels of EV surface antigens. Principal component analysis was used to visualize patient clustering. Supervised learning (linear discriminant analysis) was used to obtain a specific EV signature by linear weighted combination of nMFI for the 37 EV surface antigens and to discriminate patients according to the presence/absence of CV risk indicators. Discriminant performance of the specific EV signature was explored using ROC curves.

RESULTS

Patient characteristics and correlation with EV surface antigens

Overall, we recruited 486 patients; a first cohort of 404 referred ambulatory patients and a second cohort of 82 patients admitted to the emergency department for an acute CV event, which was analyzed separately (see methods).

Regarding subjects from the initial study cohort, 38.6% were females, with a mean overall age of 58 years. Hyperlipidemia (64.4%) and hypertension (53.2%) were highly prevalent in study participants, followed by a history of coronary artery disease (CAD), smoking habit, chronic kidney disease (CKD), diabetes, and chronic heart failure (CHF). OD was also relatively frequent, with 10.4% of patients displaying microalbuminuria (MA) and 16.6% left ventricular hypertrophy (LVH). The median 10-year likelihood of fatal CV events was 2%, which represents a low-to-moderate CV risk, with 105 patients (26% of the cohort) displaying a risk lower than 1%, and 8 patients (2%) a risk equal to or higher than 10%, according to the ESC SCORE risk charts. The clinical and biochemical parameters are shown in Table 1.

EV parameters were evaluated after stratification of the overall cohort for age, sex, and body mass index (Figure S2). Mean MFI for CD9-CD63-CD81, which represents a reliable parameter to quantify circulating EVs[16-18], displayed a gradual, significant increase with age (3.4-fold increase from patients <40 years to patients ≥80 years; P=0.030). This increase in the amount of circulating EV in older patients mirrored a rise in the smaller sub-fraction of vesicles (Table S2). Compared to the youngest patients, EV surface antigens from leucocytes, platelets, and endothelium were higher in older patients (Figure S2A-B), while no differences were found between females and males, or patients stratified based on their BMI (Supplemental Results; Tables S3-S4). Significant correlations were observed between several EV markers and age, systolic blood pressure (SBP), glucose, total cholesterol, estimated glomerular filtration rate (eGFR), and SCORE risk (Supplemental Results and Table S5).

Unsupervised patient clustering according to an EV specific signature

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Unsupervised learning was applied to divide the initial study cohort into 3 clusters based exclusively on the antigens expressed on the EV surface, i.e., regardless of the clinical and biochemical characteristics of the patients. Expression levels for all evaluated EV surface antigens (except for EV-specific tetraspanins CD9-CD63-CD81 that were used as references to normalize antigen expression to the concentration of vesicles) gradually increased from Cluster-I to -III, with Cluster-III displaying a 0.8- to 108.3-fold increase compared to Cluster-I (P<0.01; Table S6). This increase is visible in the heat map showing MFI of the 37 EV antigens in the overall cohort after stratification for clusters (Figure 1A). Patient clustering according to EV marker expression was carried out by principal component analysis; a specific EV signature allowed the discrimination between clusters (Figure 1B).

Inter-cluster comparisons of patient characteristics and CV risk indicators revealed significant differences between clusters (Table 1).

Cluster-I (n=288) – Cluster-I was the largest cluster with the lowest proportion of men (60.4%) and a mean age of 57 years. Patients in this cluster had the lowest mean systolic and diastolic blood pressure (SBP/DBP; 132/83 mmHg), total cholesterol, LDL, and creatinine (P<0.01; Table 1). Moreover, they displayed the lowest prevalence of hypertension (49.3%), CHF (1.7%), and diabetes (8%) (Figure 1C). Furthermore, Cluster-I displayed the lowest proportion of patients with MA (6.6%), LVH (10.8%), as well as 78 patients (27.1%) in the lowest CV risk category of SCORE<1% (10-year likelihood of fatal CV event), and only 1 patient (0.3%) with SCORE ≥ 10% (Figure 1D-E).

Cluster-II (n=86) – In this cluster (62.8% males), the mean age was 58 years and the mean BP 140/85 mmHg; both represent intermediate values compared to Clusters I and III. The average levels of total cholesterol and LDL were similar to Cluster-III (Table 1). Prevalence of hypertension, CHF, and diabetes was 60.5%, 4.7%, and 8.1%, respectively, while MA and LVH were observed in 12.8% and 18.6% of patients, respectively. The 10-years risk of fatal CV events

was again intermediate compared to Clusters I and III: 22 patients (25.6%) with SCORE<1% and 4 patients (4.7%) with SCORE≥10% (Figure 1C-E).

Cluster-III (n=30) – Cluster-III contained patients with the highest CV risk and the highest proportion of men (66.7%). The mean age was 57 years. Patients in this cluster had the highest values of SBP and DBP (143/90 mmHg), as well as glucose, total cholesterol, LDL, and creatinine (P<0.01; Table 1). In addition, they displayed the highest prevalence of hypertension (70.0%), CHF (30.0%), diabetes (43.3%) and OD (MA 40.0%; LVH 66.7%) (Figure 1C-D). Patients in Cluster-III had the highest CV risk: only 5 patients (16.7%) had a SCORE <1%, while 3 out of 30 patients (10%) had SCORE≥10% (Figure 1E). Multivariate regression analysis confirmed a significant association of SCORE risk, CHF, diabetes, and OD with Cluster-III, independently from all the other considered factors (age, BMI, sex, and each single CV risk indicator; Table S7), with an OR ranging between 1.27 and 8.06.

Supervised learning to define the cardiovascular risk of patients by EV profiling

To assess the reliability of the above results, we compared all EV parameters in patients from the initial study cohort with and without the explored CV risk indicators (Tables S8-S17). Differentially expressed EV surface antigens after stratification according to single CV risk indicators are described in the Supplemental Results. Normalized medians of all evaluated EV parameters are shown in Figure 2A; the heat map shows how CHF, diabetes, MA, and LVH are associated with the highest median levels of all the evaluated EV surface antigens. Supervised learning algorithms were used to combine the levels of the 37 surface EV markers in a specific molecular signature that discriminated patients according to their CV risk indicators. The performance of each specific EV signature as a discriminant was determined by analyzing the ROC curves for each CV risk indicator (Table 2), after bootstrap cross-validation (see Extended Methods). AUC ranged between 0.818 and 0.973 (Figure 2B-C), with the highest accuracy observed in the discrimination of CHF (90.1%), followed by CAD (89.1%), MA (88.1%), OD (86.3%), and LVH (86.1%).

Considering that CV diseases are multifactorial disorders and that each patient may display multiple CV risk indicators at the same time, we built linear regression models to identify a potential association between each individual EV parameter and each individual CV risk indicator (age, sex, BMI, hypertension, hyperlipidemia, CAD, CHF, diabetes CKD, smoking habit, SCORE risk, and OD) (Table S18). Associations between EV parameters and CV risk indicators are described in detail in the Supplemental Results and summarized in Figure 3. Interestingly, several EV markers were associated with hypertension, CAD, CHF, diabetes, CKD, SCORE risk, and OD, independently from the remaining CV risk indicators. In particular, CD62P, CD42a, and CD31 were directly associated to SCORE risk (Figure 3F), with a β estimate ranging from 1.082 to 1.122, which corresponds to an 8.2 to 12.2% increase in nMFI for each 1% increase in the 10-year risk of a fatal CV event, irrespective of whether other CV risk indicators were present. Single patient data were reported for three representative subjects, selected from Cluster-I, -II, and -III (Table S19).

Sub-analysis on patients with established CV disease

To assess the performance of EV profiling on risk assessment in patients with established CV disease, we performed two sub-analyses, one in patients from the initial study cohort with a 10-year risk of fatal CV events higher than 4% according to SCORE charts, and one in a second cohort of patients, which was analyzed during an acute CV event.

Tables S20 and S21 reports EV profiling and characteristics of patients at high/very high CV risk according to SCORE charts, after clusterization for the respective EV surface profiling. Prevalence of hypertension, hyperlipidemia, CAD, CHF, diabetes, and OD still increased from cluster-I to cluster-III. Accordingly, levels of 31 out of the 37 evaluated EV surface antigens were higher in cluster-III compared to cluster-I or -II (P<0.05 for all comparisons).

Clinical and biochemical characteristics of patients with an acute CV event are reported in Table S22 and compared with the initial study cohort. Patients with an acute event displayed a higher number of particles at NTA, and, consistently, a higher expression of CD9-CD63-CD81, compared

to those from the initial cohort; moreover, they differed for 33 of the 37 evaluated EV surface antigens (see Table 23). In particular, expression levels of CD31, CD42a, and CD62P displayed a 2.3- to 2.9-fold increase (Figure S3A-B-C) in patients with an acute CV event and considering the merged cohorts (initial study cohort plus patients with acute events), they were directly correlated to SCORE risk (R ranging between 0.202 and 0.237 – $P<0.001$; Figure S3D-E-F). Of note, 64.6%, 76.6%, and 73.9% of patients with expression levels of CD31, CD42a, and CD62P, respectively above the 95th percentile and with a SCORE risk equal to or lower than 10%, displayed an acute CV event (Figure S3D-E-F, left upper quadrant of the plots), while 55.6%, 44.4%, and 28.6% of patients with acute CV events had a SCORE risk > 10% and levels of CD31, CD42a and CD62P equal to or lower than 95th percentile (Figure S3D-E-F, right lower quadrant of the plots). Table S19 reports single patient data; as expected, nMFI for CD62P-CD42a-CD31 progressively increased from cluster-I to cluster-III, reaching the maximum levels in the subject selected from the cohort with an acute CV event.

DISCUSSION

Here, we report on immuno-profiling of intravascular EVs in a large and well-characterized multi-center cross-sectional cohort. We found a significant correlation between levels of expression of specific EV surface antigens and patient characteristics, including major CV risk factors. Unsupervised learning was applied to divide our cohort into 3 clusters on the exclusive basis of EV antigens, and when we unveiled cluster patients' characteristics, we observed an increased prevalence of hypertension, CHF, diabetes, OD, and risk of fatal CV events from cluster I to III. Using supervised learning and linear regression modelling approach, we confirmed the independent association between EV antigens and their combination in specific EV signatures with single CV risk indicators. Notably, several EV antigens, including endothelial and platelet markers, were associated with 10-year risk of suffering a fatal CV event, as assessed by SCORE risk charts. A further increase in the expression of EV antigens associated with CV risk indicators was observed

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in a separate cohort of patients with an acute CV event. Of note, some patients experiencing an acute CV event despite a low-moderate risk, displayed high levels of the SCORE-associated surface proteins CD31, CD42a, CD62P. If from one side this analysis suggests that a combined approach using EV profiling and SCORE charts may improve CV risk stratification, from the other side raises the question of whether EV antigen profiling might outperform the classical CV risk estimators. At this regard, future prospective studies are warranted to establish if EV surface antigen profiling might overcome some of the limitations of the current risk estimation scores. We must also underline that sampling of the cohort with a CV event was performed during acute phase; therefore, levels of expression of EV surface antigens could be biased by inflammatory response and platelet activation which occur during an acute event. Even with this important limitation, these data may reinforce our hypothesis on potential role of EV profiling as unconventional biomarker to stratify patients according to CV risk.

The role of EVs as mediators of intercellular communication through the transfer of their contents (proteins, lipids, and nucleic acids) or by interacting with target cells through surface epitopes has been known for many years[20,21]. Recent evidence highlighted their prominent role in modulating target cells toward pro-fibrotic and pro-inflammatory phenotypes, leading to aging-related impairment of organ homeostasis[22-24]. In our cohort, EV markers from activated platelets and immune cells as well as injured and/or activated endothelium correlated to age; their levels were remarkably increased in the eldest subjects and those with an increased CV risk. This was particularly evident in patients that clustered in the group with the highest CV risk (Cluster-III). Markers of activated platelets (CD41b-CD42a-CD62P), endothelial cells (CD31-CD105), and leukocytes (CD1c-CD2-CD3-CD4-CD8-CD14-CD19-CD20-CD25-CD40-CD45-CD69-CD86) were highly expressed in these subjects and correlated to the likelihood of hypertension, hyperlipidemia, diabetes, CKD, previous CAD, CHF, and OD.

EEVs are released from endothelial cells in response to activation or injury and, consistently, an increase of circulating EEVs has been demonstrated for different CV diseases. CD31-positive

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vesicles increased in patients with severe hypertension and were directly correlated to blood pressure[9,25]. A rise in $CD31^+$ EEVs was also observed in patients with diabetes mellitus, atherosclerosis, smoking habit, CHF, and CAD[16,25-27] and was associated with the incidence of major cardio-cerebrovascular events[8,16,18,28]. PEVs mainly derive from membrane shedding, possess pro-coagulant activity, and may exert stimulatory/inhibiting effects on a large variety of cells, including leukocytes, endothelium, and other platelets[29]. PEVs are considered a marker of platelet activation[29] and increase in different CV pro-thrombotic diseases, such as acute coronary syndrome and stroke, potentially resulting from vessel occlusion[7,16,18,30]. They have also been associated with atherosclerosis, severe hypertension, diabetes mellitus, and a 10-year risk of CAD[25,31,32]. Finally, circulating LEVs may indicate immune cell activation and have been involved in vascular inflammation, atherosclerosis, and endothelial dysfunction[6,7]. Their increase was demonstrated in patients with stable/unstable atherosclerotic plaques and familial hypercholesterolemia[33-35]. Vesicles carrying CD14+ increased in patients with CHF[27], while the combinations CD11+/CD14+, CD3+/CD45+, or CD45+ LEVs predicted CV events and were associated to CV mortality[10,11,28,36].

While these studies focused on a few individual EV surface proteins, recent technological advances have allowed us to quantify 37 different antigens simultaneously, including almost all the previously evaluated markers. Furthermore, this made it possible to assess the discrimination performance of an EV signature obtained by linear combination of single antigens. In our study, we were able to identify a specific signature for each CV risk indicator by applying supervised and unsupervised learning algorithms. Unsupervised clustering establishes non-linear boundaries to discover intrinsic patterns of association of multi-dimensional data obtained by the simultaneous profiling of multiple EV subpopulations. This strategy allowed in-depth phenotyping and accurate stratification of patients according to their CV risk, with superior results compared to previous studies using EVs as biomarkers.

Given the complex interplay of several risk factors in the determination of the overall CV risk, we established, for the first time, a specific association between each individual EV marker and each

individual CV risk indicator and to precisely quantify each association through linear regression. Both EEV and PEV levels were associated with the Framingham risk score[9,32,37]. Here, we described the relationship between increased levels of EV antigens and the 10-year risk of fatal events according to SCORE charts[19]: CD31 (endothelial marker), CD42a (platelets marker), and CD62P (activated platelets and endothelium marker) expressed on EV surface were directly and independently associated to the risk of fatal CV events and further increased during an acute CV event, thus representing promising biomarkers to improve patient stratification according to CV risk.

The present study has some limitations. First, EV surface antigens included in our assay mainly mirror inflammatory response and platelet activation and are influenced by several acute or chronic conditions. Therefore, the resulting EV signature is most likely reflective of a dynamic "state". In line with this, we have published previous work showing that in patients with myocardial infarction, EV concentrations as well as the expression of specific markers, peaked at hospital admission and rapidly declined over the next 2 days[16]. However, we do not have data to precisely dissect the hour-to-hour, day-to-day, week-to-week inherent variability, which remains a crucial aspect that needs to be addressed in the future. Second, we only recruited Caucasian patients at low-to-moderate CV risk referred to specialized centers; thus, a selection bias cannot be excluded. Nevertheless, prevalence of common contributors to CV risk and demographic characteristics roughly reflect those described in a recent report on the ESC Atlas[1]. Moreover, at present, it is still unclear whether specific subpopulations of circulating EVs increased because of endothelial injury, immune cell, and platelet activation, or in response to active regulation, directly contributing to OD progression and CV events. A further technical limit of our experimental approach is the impossibility to detect more than two antigens of interest on the same particle, thus not allowing to count double/triple - positive EVs. Finally, the study design did not allow a direct demonstration of improvement in CV risk stratification by an EV profiling approach compared to SCORE classification; to this goal, a prospective longitudinal study would be required.

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In conclusion, to the best of our knowledge, this is the largest study characterizing circulating EV subpopulations by simultaneous evaluation of several surface antigens using a standardized flow cytometry platform while employing advanced computational algorithms.

Presence of different CV risk factors may influence the biogenesis, characteristics, and release of EVs. We demonstrated that an EV-specific molecular signature is associated with each CV risk indicator and reflects patients' overall CV risk profile. Furthermore, we demonstrated that individual EV surface antigens might serve as independent predictors of CV risk and 10-year likelihood of fatal events, and precisely quantified their association with major risk factors.

If validated in future prospective and longitudinal studies, in the era of personalized medicine, the immuno-profiling of circulating EVs, obtainable from minimally invasive blood sampling, could be a promising tool for the stratification of CV risk and the tailored management of CV risk patients in addition to the management of traditional risk factors.

BRIEF COMMENTARY

Background. Extracellular vesicles (EVs) are nano-sized bilayer membrane particles involved in the development of cardiovascular (CV) diseases. EV cargo reflects cells of origin and their activation state, and it can be used as source of potential biomarkers.

Translational Significance. We characterized circulating EVs using a flow-cytometry platform, potentially scalable for clinical application, and employing machine learning approaches, to assess whether EV surface antigens may be used to stratify patients according to their CV risk and likelihood to develop CV events. We demonstrated that single EV surface antigens and their combination in a specific biomolecular signature are independent predictors of CV risk and 10-years likelihood of fatal events. Besides traditional risk factors, EV profiling represents a promising tool for CV risk stratification.

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Disclosures

All authors have read the journal's policy on disclosure of potential conflicts of interest, and they have nothing to disclose

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Figure Legends

Figure 1. Unsupervised patient clustering according to EV signature

Unsupervised learning (k-means algorithm; see Methods) was used to classify patients into three clusters according to normalized median fluorescence intensity (nMFI) for the 37 extracellular vesicles (EV) surface antigens evaluated by flow cytometry. (**A**) Heat map showing the expression of EV surface antigens in patients stratified using cluster analysis (Table S6). Blue and red indicate low and high nMFI after normalization between 1 and 100, respectively. (**B**) Principal component analysis to visualize patient clustering according to k-means algorithm; the ellipses include patients within mean +/- 95% confidence interval (principal component 1, and 2 +/- 1.96*SD). (**C-E**) Prevalence (%) of cardiovascular risk indicators in the 3 patient clusters (Cluster-I, blue, n=288; Cluster-II, grey, n=86; Cluster-III, red, n=30). Statistic is shown in Table 1. *P*-value was obtained by chi-square test, (or Fisher test when appropriated) and considered significant when $P<0.05$ (*$P<0.05$; ***$P<0.001$).

Figure 2. Supervised learning to define CV risk according to EV specific signature

Supervised learning (linear discriminant analysis; see Methods) was used to discriminate patients according to a predefined cardiovascular (CV) risk indicator and a specific Extracellular Vesicle (EV) signature. (**A**) Heat map showing EV parameters (expressed as median levels) for patients stratified according to CV indicators: hypertension, hyperlipidemia, coronary artery disease (CAD), chronic heart failure (CHF), diabetes, chronic kidney disease (CKD), smoking habit, microalbuminuria (MA), left ventricular hypertrophy (LVH), or organ damage (OD). We considered EV concentration (EVs per mL), EV diameter (nm), median fluorescence intensity (MFI) for CD9-CD63-CD81 (arbitrary unit; a.u.) and normalized MFI for the 37 EV surface antigens (nMFI; %). Blue and red indicate low and high values for the considered EV parameters after normalization between 1 and 100. Detailed statistic is reported in Tables S8-S17. (**B**) Violin plot showing the accuracy (median and interquartile range) for models discriminating patients with and without a predefined CV risk indicator (75% of the initial cohort used to train the model, and 25% for validation, randomly permuting train and validation samples by bootstrapping for 100 iterations; see also Extended Methods and Table 2). (**C**) ROC curves (median and interquartile range were obtained by bootstrap cross-validation) for models discriminating patients with and without a predefined CV risk indicator; median AUC is reported for each CV risk indicator (Table 2).

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Figure 3. Association between individual EV parameters and CV risk indicators

Forest plots showing significant associations between individual EV parameters and predefined cardiovascular (CV) risk indicators (P<0.05; see Table S18). Linear regression models were used to correct the measurement of each EV parameter (concentration, diameter, and median fluorescence intensity for the 37 evaluated surface antigens) for age, sex, BMI, presence of hypertension (HTN), hyperlipidemia, coronary artery disease (CAD), chronic heart failure (CHF), diabetes, chronic kidney disease (CKD), smoking habit, SCORE risk (%), and organ damage (OD). β estimate is reported with its 95% confidence interval (95% CI). A β estimate greater than 1 indicates an increased likelihood of the explored CV risk indicator, independently from all other indicators, according to the following equation: Y = e^β * e^ (β *X). i.e., β=1.122 (for EVs/mL and hypertension) means a significant increase of the average number of circulating EVs by 12.2% in the presence of hypertension and increase that is independent from age, sex, BMI, hyperlipidemia, CAD, CHF, diabetes, CKD, smoking habit, and OD.

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Table 1. Patient characteristics. Clinical and biochemical characteristics of patients included in the study (n=404). Unsupervised learning (k-means algorithm; see Methods) was used to classify patients into 3 clusters. Data are reported as mean ± one standard deviation (age; SBP/DBP, systolic/diastolic blood pressure; weight; BMI; glucose; total cholesterol; HDL; LDL; triglycerides; creatinine; eGFR, estimated glomerular filtration rate; WBC, white blood cells; neutrophils; lymphocytes; monocytes; basophils; eosinophils; hemoglobin; platelets), median [interquartile range] (SCORE risk), or number (percentage - sex; BMI; hypertension; hyperlipidemia; CAD, Coronary Artery Disease; CHF, Chronic Heart Failure; diabetes; CKD, Chronic Kidney Disease; smoking habit; microalbuminuria; LVH, Left Ventricular Hypertrophy; organ damage), as appropriate. P<0.05 was considered significant and reported in bold (Figure 1C-E). EF, Ejection Fraction; CV, Cardiovascular.

Variable	Study Cohort (n=404)	Cluster-I (n=288)	Cluster-II (n=86)	Cluster-III (n=30)	*P*-value
Age (years)	58 ± 15.3	57 ± 15.2	58 ± 16.6	57 ± 12.2	0.890
Sex (Male; %)	248 (61.4)	174 (60.4)	54 (62.8)	20 (66.7)	0.764
SBP (mmHg)	135 ± 20.3	132 ± 21.0	140 ± 17.2	143 ± 17.0	**<0.001**
DBP (mmHg)	84 ± 11.1	83 ± 11.3	85 ± 9.6	90 ± 9.8	**0.002**
Weight (Kg)	74 ± 12.9	75 ± 13.1	72 ± 12.4	77 ± 12.1	0.116
BMI (Kg/sqm)	25.7 ± 3.55	25.9 ± 3.67	24.8 ± 3.23	25.9 ± 3.00	0.052
Overweight (25-29.9 Kg/sqm)	181 (44.8)	128 (44.4)	41 (47.7)	12 (40.0)	0.382
Obese (≥ 30 Kg/sqm)	49 (12.1)	39 (13.5)	6 (7.0)	4 (13.3)	
Hypertension (%)	215 (53.2)	142 (49.3)	52 (60.5)	21 (70.0)	**0.031**
Hyperlipidemia (%)	260 (64.4)	180 (62.5)	59 (68.6)	21 (70.0)	0.466
CAD (%)	86 (21.3)	58 (20.1)	19 (22.1)	9 (30.0)	0.445

CHF (EF<35%; %)	18 (4.5)	5 (1.7)	4 (4.7)	9 (30.0)	**<0.001**
Diabetes (%)	43 (10.6)	23 (8.0)	7 (8.1)	13 (43.3)	**<0.001**
CKD (eGFR<60mL/min; %)	50 (12.4)	29 (10.1)	14 (16.3)	7 (23.3)	0.051
Smoking habit (%)	59 (14.6)	37 (12.8)	15 (17.4)	7 (23.3)	0.212
Glucose (mmol/L)	5.6 ± 1.86	5.5 ± 1.80	5.3 ± 1.48	6.7 ± 2.80	**0.002**
Total Cholesterol (mmol/L)	5.2 ± 1.10	5.0 ± 1.00	5.5 ± 1.24	5.5 ± 1.33	**<0.001**
HDL (mmol/L)	1.4 ± 0.48	1.3 ± 0.44	1.4 ± 0.59	1.4 ± 0.48	0.774
LDL (mmol/L)	3.0 ± 0.91	2.9 ± 0.87	3.3 ± 0.96	3.3 ± 1.07	**0.003**
Triglycerides (mmol/L)	1.7 ± 1.06	1.6 ± 1.03	1.8 ± 1.14	1.8 ± 1.16	0.357
Creatinine (mg/dL)	1.07 ± 0.552	1.03 ± 0.439	1.11 ± 0.620	1.38 ± 1.027	**0.003**
eGFR (mL/min)	83 ± 26.3	85 ± 25.8	79 ± 26.2	78 ± 30.0	0.080
WBC (n/L per 10e9)	7.1 ± 2.28	7.1 ± 2.07	7.0 ± 2.80	7.1 ± 2.72	0.976
Neutrophils (n/L per 10e9)	4.6 ± 2.09	4.7 ± 1.93	4.3 ± 2.47	4.8 ± 2.55	0.640
Lymphocytes (n/L per 10e9)	1.9 ± 0.72	1.8 ± 0.65	2.2 ± 0.86	1.8 ± 0.82	0.054
Monocytes (n/L per 10e9)	0.40 ± 0.149	0.43 ± 0.156	0.36 ± 0.122	0.30 ± 0.057	0.051
Basophils (n/L per 10e9)	0.05 ± 0.031	0.05 ± 0.033	0.05 ± 0.026	0.04 ± 0.054	0.616
Eosinophils (n/L per 10e9)	0.15 ± 0.105	0.15 ± 0.111	0.15 ± 0.097	0.15 ± 0.084	0.990
Hemoglobin (g/L)	142 ± 13.9	143 ± 15.2	140 ± 9.1	142 ± 14.5	0.703
Platelets (n/L per 10e9)	249 ± 64.3	254 ± 69.1	236 ± 45.4	238 ± 65.3	0.358
Microalbuminuria (%)	42 (10.4)	19 (6.6)	11 (12.8)	12 (40.0)	**<0.001**
LVH et echocardiography (%)	67 (16.6)	31 (10.8)	16 (18.6)	20 (66.7)	**<0.001**
Organ Damage (%)	82 (20.3)	42 (14.6)	20 (23.3)	20 (66.7)	**<0.001**
SCORE Risk (%)	2.0 [0.0; 3.0]	2.0 [0.0; 3.0]	2.0 [0.0; 5.0]	2.5 [1.0; 5.0]	**0.029**
CV Risk <1%	105 (26.0)	78 (27.1)	22 (25.6)	5 (16.7)	**0.004**
CV Risk =1%	84 (20.7)	64 (22.2)	15 (17.4)	5 (16.7)	
CV Risk =2%	75 (18.6)	56 (19.4)	14 (16.3)	5 (16.7)	
CV Risk =3-4%	67 (16.6)	51 (17.7)	12 (14.0)	4 (13.3)	
CV Risk =5-9%	65 (16.1)	38 (13.2)	19 (22.1)	8 (26.7)	
CV Risk ≥10%	8 (2.0)	1 (0.3)	4 (4.7)	3 (10.0)	

Table 2. EV signature predicts CV risk indicators. Supervised learning (linear discriminant analysis; see methods) was used to discriminate patients according to predefined cardiovascular (CV) risk indicators and a specific EV signature. EV signature was obtained by weighted linear combination of expression levels of the 37 EV surface antigens evaluated by flow cytometry. Discriminant performance was evaluated by ROC curve analysis. The area under the curve (AUC) and related accuracy are reported together with their interquartile range for each model (75% of the dataset used to train the model and 25% for validation, randomly permuting train and validation samples by bootstrapping for 100 iterations: Figure 2B-C; see also Extended Methods). CAD, Coronary Artery Disease; CHF, Chronic Heart Failure; CKD, Chronic Kidney Disease; LVH, Left Ventricular Hypertrophy.

Variable	AUC (95% CI)	Accuracy (%)
Hypertension	0.898 (0.868-0.928)	84.2 (81.2-87.1)

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Hyperlipidemia	0.818 (0.773-0.847)	75.7 (73.3-79.0)
CAD	0.944 (0.912-0.961)	89.1 (82.5-92.3)
CHF (EF<35%)	0.945 (0.838-0.981)	90.1 (89.1-93.1)
Diabetes	0.915 (0.864-0.954)	85.1 (84.2-88.1)
CKD (eGFR<60mL/min)	0.873 (0.815-0.922)	82.2 (76.2-85.1)
Smoking habit	0.861 (0.825-0.906)	80.2 (78.2-83.2)
Microalbuminuria	0.931 (0.880-0.996)	88.1 (86.1-91.1)
LVH et echocardiography	0.959 (0.941-0.996)	86.1 (83.2-89.9)
Organ Damage	0.973 (0.947-0.995)	86.3 (84.2-89.0)

Fig. 1.

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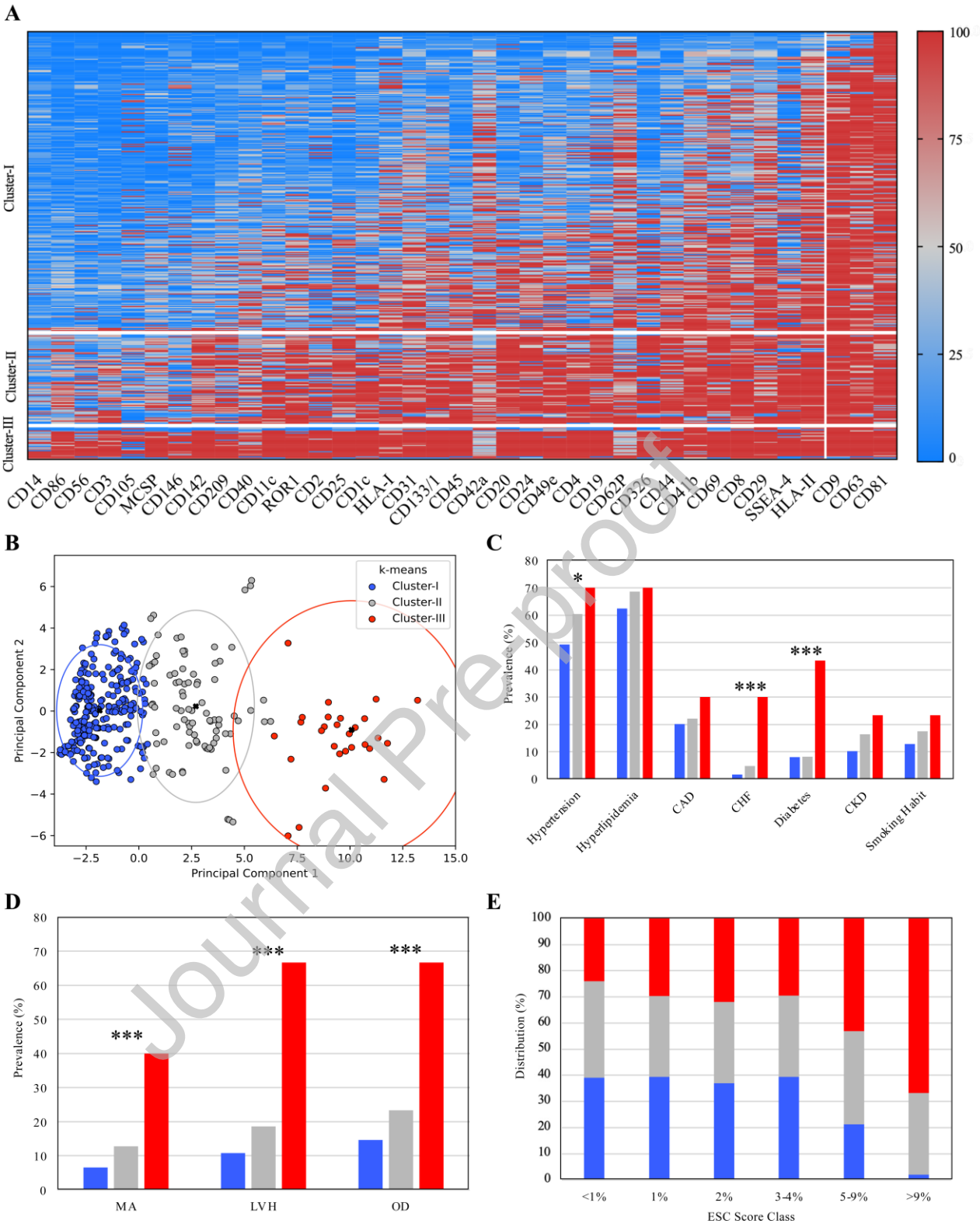

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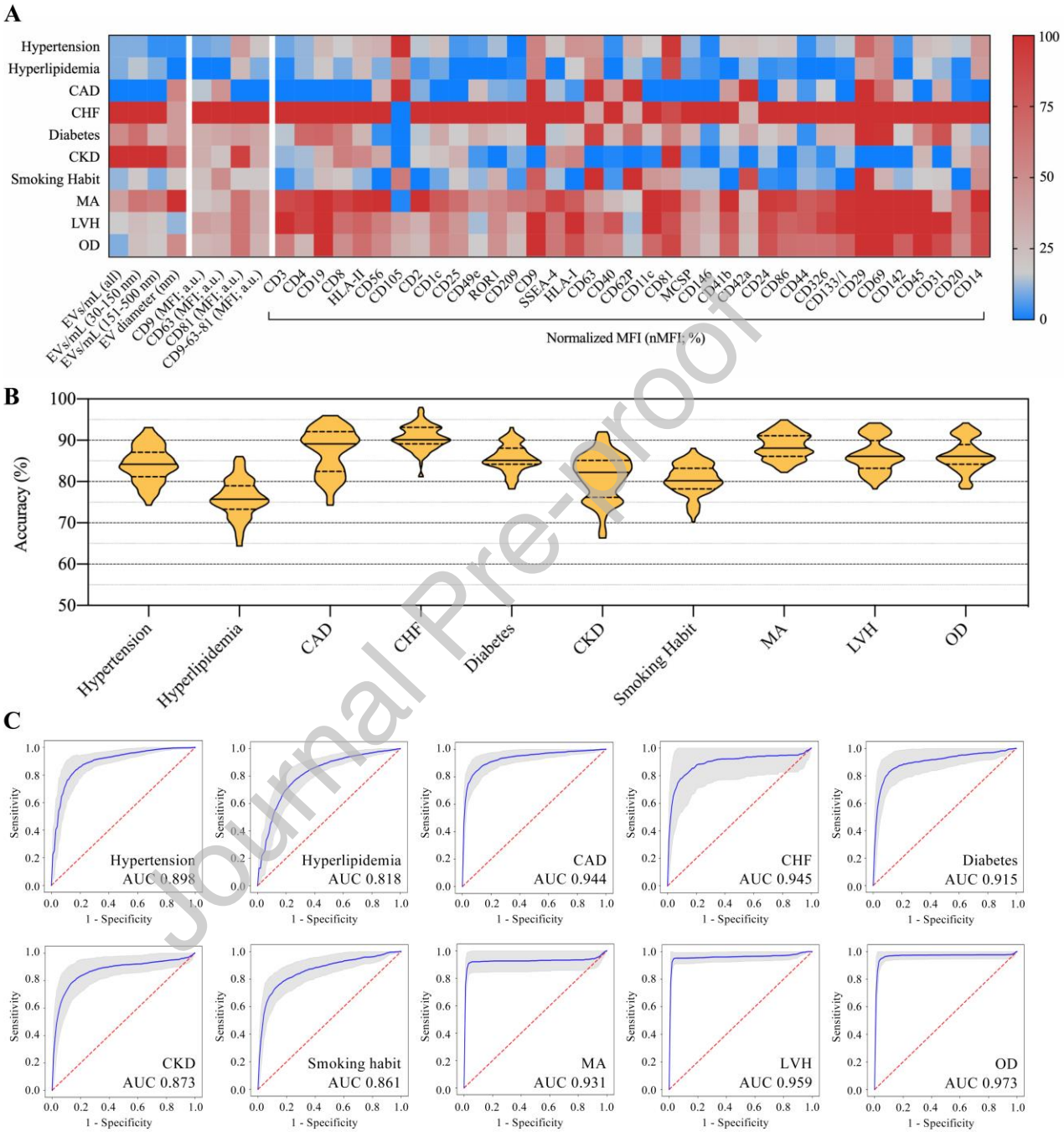
Fig. 2.


A
Hypertension
Hyperlipidemia
CAD
CHF
Diabetes
CKD
Smoking Habit
MA
LVH
OD
EVs/mL (all)
EVs/mL (30-150 nm)
EVs/mL (151-500 nm)
EV diameter (nm)
CD9 (MFI; a.u.)
CD63 (MFI; a.u.)
CD81 (MFI; a.u.)
CD9-63-81 (MFI; a.u.)
CD3 CD4 CD19 CD8 HLA-II CD56 CD105 CD2 CD1c CD25 CD49e ROR1 CD209 CD9 SSEA-4 HLA-I CD63 CD40 CD62P CD11c CD81 MCSP CD146 CD41b CD42a CD24 CD86 CD44 CD326 CD133/1 CD29 CD69 CD142 CD45 CD31 CD20 CD14
Normalized MFI (nMFI; %)
100
75
50
25
0
B
Accuracy (%)
100
90
80
70
60
50
Hypertension
Hyperlipidemia
CAD
CHF
Diabetes
CKD
Smoking Habit
MA
LVH
OD
C
Sensitivity
1 - Specificity
Hypertension AUC 0.898
Hyperlipidemia AUC 0.818
CAD AUC 0.944
CHF AUC 0.945
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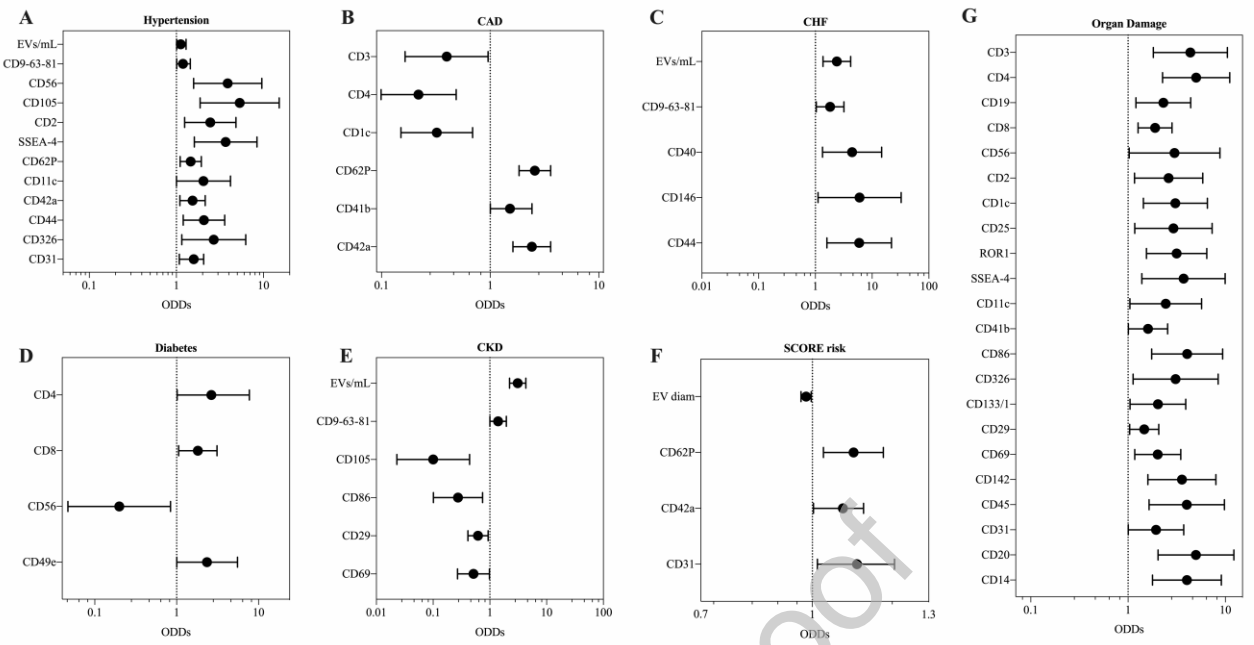
MA AUC 0.931
LVH AUC 0.959
OD AUC 0.973

Fig. 3.



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