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EXPERT REVIEWS

The diagnostic and prognostic potential of plasma extracellular vesicles for cardiovascular disease

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Ingrid EM Bank[1,2], Leo Timmers[2,3,4], Crystel M Gijsberts[1,2], Ya-Nan Zhang[5,6], Arend Mosterd[7], Jiong-Wei Wang[5,6], Mark Y Chan[4], Vince De Hoog[1,2], Sai Kiang Lim[8], Siu Kwan Sze[9], Carolyn SP Lam[10] and Dominique PV De Kleijn*[1,2,5,6]

[1]Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]The Netherlands Heart Institute (ICIN), Utrecht, the Netherlands
[3]Department of Cardiology, UMC Utrecht, Utrecht, the Netherlands
[4]Cardiac Department, National University Heart Centre, National University Hospital, Singapore, Singapore
[5]Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
[6]Cardiovascular Research Institute, National University Health System, Singapore, Singapore
[7]Department of Cardiology, Meander Medical Center, Amersfoort, The Netherlands
[8]Institute of Medical Biology, A-Star, Biopolis, Singapore, Singapore
[9]School of Biological Sciences, Nanyang Technological University, Singapore, Singapore
[10]National Heart Centre Singapore, Singapore General Hospital, Duke-National University, Singapore, Singapore
*Author for correspondence:Department of Surgery, NUS & Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands
surdvp@nus.edu.sg

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Cardiovascular disease (CVD) is the leading cause of death worldwide and its prevalence is expected to rise rapidly worldwide in the coming decades. Atherosclerosis, the syndrome underlying CVD, is a chronic progressive disease of the arteries already present at a young age. Strokes, heart attacks and heart failure are acute CVD events that occur after decades, however, and require timely diagnosis and treatment. Plasma extracellular vesicles (EVs) are microstructures with a lipid bilayer membrane involved in hemostasis, inflammation and injury. Both EV-counts and EV-content are associated with CVD and the identification of plasma EVs is a novel source of blood-based biomarkers with the potential to improve diagnosis and prognosis of CVD. Presented in this review is an overview of the current use of EVs in CVD and a discussion of the need for robust and easy isolation technologies for plasma EV subsets. This is needed to bring this promising field towards clinical application in the patient.

KEYWORDS: Plasma extracellular vesicles • vesicle counts • vesicle content • microparticles • biomarker • cardiovascular disease • flow cytometry

The burden of CVD

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide. In 2012, approximately 17.5 million people died from CVDs. An estimated 7.4 million of these deaths were caused by coronary heart disease, and 6.7 million were caused by stroke.[1] In 2008, 36 million deaths out of 57 million global deaths were due to non-communicable diseases (NCD), with 48% of the NCDs due to CVD.[2] Although cardiovascular mortality has gradually declined in the past decades in Western countries, the global death rates from CVD are rising. By 2030, more than 23 million people are projected to die from CVDs. This rise is mainly attributable to an increase of CVD deaths in low- and middle-income countries in Africa, South-East Asia and Eastern Mediterranean regions. Nowadays, over three quarters of CVD deaths occur in low- and middle-income countries, with a large proportion of deaths under the age of 70. (Figure 1) People in these lower-income regions do, for instance, often not have access to primary health care programs for early detection and treatment of people with risk factors for the development CVD.[1,3]

Atherosclerosis is the underlying cause in most CVDs. Inflammation of blood vessels and subsequent lipid deposition play a pivotal role in atherosclerosis. Activation of the endothelium by inflammatory mediators leads to the recruitment of circulating inflammatory cells, which drives atherosclerotic plaque formation and progression. Traditional risk factors such as hypertension, tobacco smoking, diabetes mellitus and hyperlipidemia accelerate this process. Atherosclerosis is a chronic progressive disease and is already present at a young age. The clinical manifestations of CVD (e.g. myocardial infarction (MI), heart failure and cerebrovascular accidents) are usually acute events that often occur decades later, but require timely diagnosis and intervention. Identification of the people that are at high risk for an adverse cardiovascular event is challenging in a background of

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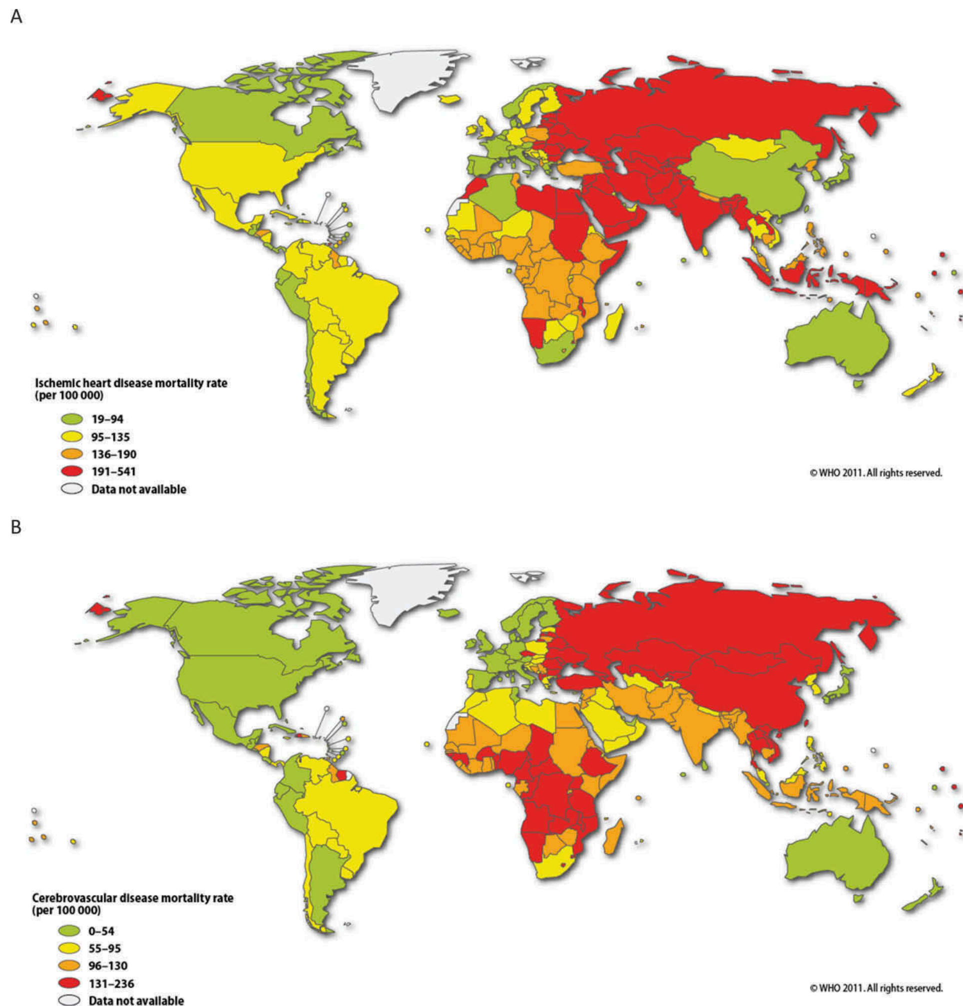

Figure 1. **World maps showing the global distribution of ischemic heart disease mortality rates (A) and cerebrovascular mortality rates (B) in males (age standardized, per 100,000). Especially when both ischemic heart and cerebrovascular disease are taken into account, mortality is high in low- and middle-income countries in Africa, South-East Asia and Eastern Mediterranean regions. Large differences between ischemic heart disease and cerebrovascular disease do exist when comparing regions (Middle East, Africa) or countries (China, India). Maps for females show similar results.**
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atherosclerosis already being present for decades. Therefore, in a world population consisting of a variety of ethnicities and differing risk profiles, novel biomarkers for diagnosis and prognosis of CVD are desperately needed.

Role of extracellular vesicles in CVD

Extracellular vesicles (EVs) are microstructures with a lipid bilayer membrane, which are abundantly present in human body fluids such as blood, urine, saliva and breast milk.[4] Most cell types, including circulating cells, vascular cells and cardiomyocytes, are capable of releasing EVs of different sizes, composition and subcellular origin. EVs can roughly be classified into three types: (1) microvesicles (or microparticles/ectosomes), which are mainly produced by outward budding of the plasma membrane and usually have a diameter of 50–1000 nm; (2) exosomes (diameter ≈40–100 nm), which are formed within the endosomal network and released upon exocytosis of multi-vesicular bodies; (3) apoptotic bodies, which are released during the late steps apoptosis (diameter up to 5 µm). Definitions of these subgroups, however, are not abided very strictly leading to inconsistency in the literature.

Since the discovery of this 'cell dust' in the 1960s, the scientific interest in EVs has increased substantially. Detection techniques and isolation protocols have improved, although standardization of methods remains limited. Several studies now show that EVs play a significant role in both physiological and pathophysiologic conditions; EVs contribute to coagulation and inflammation, as well as to intercellular (including cardiomyocyte) communication, endothelial function, angiogenesis, cellular survival and even to the progression of atherosclerosis.[5–9] EV release increases under inflammatory conditions, which is in accordance with higher numbers of circulating EVs (subsets) associated with the presence of CVD.[10]

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During their formation, EVs can maintain surface molecules from their parent cells, as well as selected cytosolic content (proteins, lipids, RNA, microRNA), often referred to as a 'liquid biopsy'. Therefore, both EV-counts and EV-content hold the potential to be of use as disease-specific biomarkers for CVDs. In addition, EVs have gained considerable interest as potential therapeutic tools in cardiovascular and regenerative medicine, due to their pro-angiogenic and cardio-protective properties and the opportunities to deliver specific agents to target cells of the cardiovascular system.[11] In this review, we firstly aimed to summarize the current knowledge of the potential of plasma EV-counts and EV-content as biomarkers in diagnosis and prognosis of CVDs. Secondly, we aimed to discuss emerging technologies and future directions in isolation, detection and application of EV biomarkers.

Current evidence on the diagnostic and prognostic potential of EVs in CVDs

Potential of plasma extracellular vesicle counts as a biomarker of CVDs

Flow cytometry is a standardized and well-accepted instrument for cell identification and detection, but the standard instruments need special attention when measuring EVs <200 nm.[12] Importantly, the EV concentration obtained is hugely affected by the calibration, scatter angle and minimum detectable EV size of the flow cytometer used. In polydisperse samples as blood plasma, EV enumeration depends both on counts of single EVs larger than the detection limit and on swarm detection of multiple smaller EVs.[13] Nevertheless, the flow cytometer provides the possibility to measure EVs directly in plasma samples and to analyze EV-subsets. Although the majority of EVs is smaller than 200 nm, it was recently shown [10] that EVs >200 nm also contain information on CVD, as endothelial cell EVs were associated with cardiometabolic risk factors. This additionally identifies the larger plasma EVs subsets as a potential biomarker source for CVD, without the need for high-resolution flow.

However, the group of Wauben showed that high-resolution flow cytometry and detection of EV subsets <200 nm is feasible, but requires special conditions of the flow cytometer, sample concentration and event rate.[12] Furthermore, Headland *et al.* recently reported that combining high-resolution flow cytometry with visual interrogation (proprietary charge coupled devices) can detect fluorescent signals even below the physical optical resolution cut-off of 200 nm.[14] This technology holds great potential for EV markers in CVDs, especially when EV subsets can be sorted and used for content analysis.

Diagnosis (vesicle counts)

Both the cellular origin and the nature of the trigger influence the number and phenotype of EVs released into the circulation. Accordingly, elevated levels of circulating EVs, expressing (parent-cell-)specific cell adhesion molecules (CAMs) or transmembrane receptors, have been shown to be associated with the presence of various CVDs. These CVDs include acute coronary syndrome (ACS) (concerning both MI and unstable angina),[15–22] subclinical atherosclerosis [23,24] and coronary artery disease (CAD),[25] acute stroke,[26–28] peripheral artery disease,[29] atrial fibrillation,[30] venous thromboembolism,[31] peripartum cardiomyopathy[32] and cardiovascular risk factors like smoking, diabetes and hypertension (Table 1).[5,10,33–35]

Although most of the studies observed elevated EV levels in diseased individuals, results are not entirely consistent across the different studies. Regarding the diagnosis of ACS, for example, most studies reported the plasma levels of platelet- and/or endothelial cell-derived EVs to be higher in diseased individuals as compared to healthy, or age- and gender-matched controls.[15–17,22] In contrast, Giannopoulos *et al.* and Huisse *et al.* observed no difference in platelet- or endothelial-derived plasma EV levels, but reported, respectively, levels of erythrocyte-derived and tissue factor exposing EVs, to be elevated in case of ST-segment elevation myocardial infarction, that is, acute myocardial infarction with electrocardiographic (ECG) changes highly suggestive of acute coronary occlusion.[36–38] In an observational cohort study of patients referred for dobutamine stress echocardiography, Augustine *et al.* observed an increase in procoagulant-, platelet- and endothelial-EVs immediately after cardiac stress initiation, but only in patients lacking signs of myocardial ischemia.[39] These data suggest a physiological, rather than a pathophysiological, cardiac stress-induced release of EVs.

Comparing EV-counts between studies is difficult, as clinical settings and study design vary. Also, differences in sample processing and centrifugation protocols cause variability in EV measurements.[40] Furthermore, the minimal detection limit of the technique used has a major effect on the observed number of EVs. But above all, various strategies, incorporating different surface markers and monoclonal antibodies, have been used in an attempt to quantify EVs with the same cellular origin. For example, numerous different combinations of Annexin V+, CD31+, CD105+, CD62E+ surface markers have been used to select the subfraction of endothelial cell-derived EVs. Furthermore, within the subfraction of endothelial EVs, less CD62E+ EVs have been observed in patients diagnosed with diabetes mellitus type II as compared to healthy controls and patients with metabolic syndrome.[41] With respect to this, we should be very cautious to use cellular markers to identify the origin of the EVs. For example, the monocyte marker CD14 is an excellent marker for monocytes as it is highly abundant on these cells, but it is also present in other myeloid and non-myeloid cells.[42] We simply do not know whether the EVs released from monocytes are more abundant in CD14 than EVs from other myeloid or non-myeloid cells.

The identification and enumeration of highly specific EVs holds great potential as biomarkers for CVDs, as long as they are investigated in the appropriate clinical setting (e.g. Walenta *et al.* [32]) and their diagnostic value is validated in external cohorts using standardized isolation and detection protocols.

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Table 1. Summary of publications on plasma extracellular vesicle (EV) counts in relation to the presence of (subclinical) cardiovascular disease, with emphasis on the different EV subsets, as well as the cell surface markers and flow cytometry settings used.

	Study characteristics			Extracellular vesicle counts (subsets)						Flow cytometry parameters	
Disease entity	Author, year	Study design	N	Total EV count	Platelet EVs	Red blood cell EVs	White blood cell EVs	Endothelial EVs	Size	Beads	Scatter
Acute coronary syndrome	Mallat, 2000	cc	39		= Annexin V +/GP Ib+		= Annexin V +/CD11a+	↑ Annexin V+/CD31+	NR	NR	NR
							= Annexin V +/CD3+	↑ Annexin V +/CD146+			
	Bernal-Mizrachi, 2003	cc	126		↑ CD31+/CD42 +			↑ CD31+/CD42-	<1.5 μm	NR	FSC
								= CD51+			
	Matsumoto, 2004	cc	83		↑ Annexin V +/GP IX+		↑ Annexin V +/CD14+		NR	NR	FSC
	Skeppholm, 2012	cc	112		↑ Phallodoin-/PS+/CD61+				<1.0 μm	Megamix	NR
					↑ Phallodoin-/PS-/CD61+						
					↑ Phallodoin-/PS+/CD62P+						
					↑ Phallodoin-/PS-/CD62P+						
					↑ Phallodoin-/PS+/CD142+						
					↑ Phallodoin-/PS-/CD142+						
	Montoro-Garcia, 2013	cc	127	↑ Ann exin V+ (NSTEMI)	= Annexin V +/CD4b-		↑ Annexin V +/CD14+	↓ Annexin V +/CD144+	<1.0 μm	Polystyrene	Small angle
				↓ Ann exin V+ (STEMI)						&silica beads	Light scatter
	Giannopoulos, 2014	cc	101		= Annexin V +/CD41+	↑ Ann exin V+/CD 235a+			0.5 μm	Megamix	FlogSC

(continued)

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Table 1. (continued).

Disease entity	Study characteristics			Extracellular vesicle counts (subsets)						Flow cytometry parameters	
	Author, year	Study design	N	Total EV count	Platelet EVs	Red blood cell EVs	White blood cell EVs	Endothelial EVs	Size	Beads	Scatter
	Van der Zee, 2006	cc	61		↑ Annexin V +/CD61+/P-selectin+	= Annexin V+/CD 235a+	= Annexin V +/CD4+	= Annexin V +/CD62e+	NR	NR	FSC/ SSC
					↑ Annexin V +/CD61 +/CD63+		= Annexin V +/CD8+				
							= Annexin V +/CD14+				
							= Annexin V +/CD20+				
							= Annexin V +/CD66e+				
Myocardial ischemia	Augustine, 2014	cohort	119	↓ Annexin V+	↓ CD31+/ CD41+	↓ CD235a+		↓ CD31+/CD41-	NR	1.0 µm Sigma L-2778	
Coronary artery disease	Bernal-Mizrachi, 2003	cc	126		↑ CD31+/ CD42+			↑ CD31+/CD42-	<1.5 µm	NR	FSC
	Koga, 2005	cc	232					↑ CD144+/CD42b-	<1.5 µm	4.2 µm standard beads	FSC/ SSC
	Jayachandran, 2008	cc									
	33	↑	Annexin V+	↑ Annexin V +/CD 61+		= Annexin V+/CD14+	↑ Annexin V +/CD62e+	NR 1.0 & 2.0 µm latex, TRUCOUNT 4.2 µm	NR		
	Hu, 2014	cc	33					↑ CD31+/CD62e+	<0.5 µm	0.46 µm Sigma	NR
								= CD31+/CD42b-			
Cerebrovascular accident	Simak, 2006	cc	64		= CD41a +/CD105-/ CD45-	= CD235a	= CD45+	= CD105+/CD41a-/ CD45-	NR	0.2-3.0 µm standard	SSC
								= CD105+/CD144+			

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Table 1. (continued).

Disease entity	Study characteristics: Author, year	Study design	N	Extracellular vesicle counts (subsets): Total EV count	Platelet EVs	Red blood cell EVs	White blood cell EVs	Endothelial EVs	Size	Flow cytometry parameters: Beads	Scatter
								↑ CD105+/PS +/CD41a-			
								= CD105+/CD54 +/CD45-			
	Williams, 2007	cc	20					= CD31+/CD42b-	<2.0 µm	Sigma beads	FSC
								= CD62e+/CD42b-			
	Lee, 1993	cc	122		↑ GP Ib+				<1.0 µm	0.15-5 µm Poly Science	NR
Carotid plaque instability	Sarlon, 2013	cohort	42				↑ CD11b +/CD66b+		NR	0.3-0.9 µm megamix	NR
							↑ CD15+				
Subclinical atherosclerosis	Chironi, 2006	cohort	216	= Annexin V+	= GP Ib+		↑ CD11a+	= CD105+	NR	NR	NR
Peripheral artery disease	Van de Zee, 2006	cc	61		↑ Annexin V +/CD61+/P-selectin+				NR	NR	FSC/ SSC
					↑ Annexin V +/CD61 +/CD63+						
Atrial fibrillation	Choudhury, 2007	cc	149		↑ CD42b +/CD61+						
(vs. healthy controls)					<1.01 µm	latex beads <1.01 µm NR					
					= CD42b +/CD61+						
(vs. diseased controls)											
Venous trombo-embolism	Chirinos, 2005	cc	50		= CD31 +/CD42+			↑ CD31+/CD42-	NR	NR	NR

(continued)

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Table 1. (continued).

Study characteristics				Extracellular vesicle counts (subsets)						Flow cytometry parameters	
Disease entity	Author, year	Study design	N	Total EV count	Platelet EVs	Red blood cell EVs	White blood cell EVs	Endothelial EVs	Size	Beads	Scatter
								↑ CD62e+/CD42-			
Peripartum	Walenta, 2012	cc	158		↑ Annexin V +/CD42 +/CD62P+		↑ Annexin V +/CD14+	↑ CD31+/CD144 +/Annexin V+	NR	LB-30 Sigma	FlogSC
cardiomyopathy							= CD45+	= CD31+/CD144 +/Annexin V-			
								↑ CD31+/Annexin V +			
CVD risk factors	Amabile, 2014	cohort	844					↑ CD144+	0.1-1.0 µm	0.5-3.0 µm megamix	FSC/SSC
								↑ CD31+/CD41-			

↑: EV levels higher in diseased versus non-diseased individuals, ↓: EV levels lower in diseased versus non-diseased individuals, = : no difference in EV levels in diseased versus non-diseased individuals. cc: case–control study, cohort: cohort study, NR: not reported.

Prognosis (vesicle counts)

In addition to the current cardiovascular risk stratification tools using established risk factors (i.e. Framingham risk score), several circulating biomarkers have been shown to be associated with adverse cardiovascular outcomes. These prognostic biomarkers include brain natriuretic peptide (BNP), high-sensitivity cardiac troponin (hs-cTn) and high-sensitivity C-reactive protein (hs-CRP). Furthermore, recent studies suggest endothelial dysfunction to be an independent predictor for future cardiovascular events including heart failure, beyond the classical risk factors. However, none of these biomarkers have been incorporated in the clinical guidelines yet.

Higher levels of EVs have been reported both in patients with subclinical atherosclerosis, cardiovascular risk factors and endothelial dysfunction, and in (the diagnosis of) patients with clinically manifest CVDs. Considering this, and the involvement of EVs to central pathophysiological processes in atherosclerosis, it was hypothesized that high EV-levels may be predictive of future adverse cardiovascular events. Thus far, studies evaluating the prognostic value of EV-counts have mainly focused on endothelial-cell-derived EVs.

In a heterogeneous population of n = 488 patients, with and without documented CAD, Nozaki *et al*. observed the level of CD144+-(endothelial) EVs to be an independent predictor for cardiovascular events. CD144+-EV levels also had an incremental value in a clinical prediction model containing established risk factors, hs-CRP and BNP.[43] Likewise, Sinning *et al*. reported the level of CD31+/Annexin V+ (endothelial) EVs to be an independent risk factor for major adverse cardiac events (MACE) and cardiovascular death in patients with angiographically proven stable CAD (6-year follow-up), and CD31+/Annexin V+ levels to add predictive value to a classical risk factor model (c-statistic from 0.637 to 0.702, p = 0.03).[44] Lee *et al*. observed the level of CD62E+-endothelial EVs to predict shorter event-free survival (MACE) in a cohort of n = 300 patients with prior stroke (>3 months), whereas CD31+/AnnexinV+ and CD31+/CD42− endothelial-derived EVs were not predictive for adverse cardiovascular events.[28] Thus far, endothelial-derived EVs have shown to provide information about prognosis and the risk of future adverse cardiovascular outcomes, beyond that obtained by classical cardiovascular risk factors and, therefore, have the potential to contribute to risk stratification in (suspected) stable CAD patients.[45] In addition, Berezin *et al*. have observed that in patients with chronic heart failure (NYHA class III/IV) that a higher ratio of plasma CD31+/Annexin V+ EVs to mononuclear progenitor cells is related to all-cause mortality.[46]

Potential of plasma extracellular vesicle content as a biomarker of CVDs

The type of trigger driving the release of EVs is reflected not only in the number and surface markers of the EVs released, but also in the composition of their cargo – proteins, lipids, DNA, mRNA and/or microRNA. EVs are able to transfer this specific molecular information to other cells, thereby affecting recipient

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cell function.[6] This role of EVs in cell–cell communication might reflect disease state. Plasma EV-content has therefore been described as a source for nucleotide markers [47] as well as protein markers [48,49] for CVD. Detecting EV-content has gained interest as a tool to unbiasedly discover new disease-specific biomarkers. Measuring EV nucleotide content has the advantage of using a robust and highly specific (single nucleotide mismatch detection) PCR or hybridization technology. Protein measurements are usually performed by means of ELISA and/or (Luminex) bead assays that are well accepted in clinical laboratories, but these assays demand antibodies that are not always available, or antibodies that are less specific. With respect to this, mass spectrometry-based multiple reaction monitoring may be a valid alternative for antibody-based assays.[50]

Proteins (diagnosis)

In the field of CVDs, only one study has performed a proteomics discovery approach on EVs to identify potential diagnostic biomarkers. In a chest pain cohort, De Hoog *et al.* identified EV pIgR, cystatin C and C5a as potential biomarkers for diagnosing ACS. Discovery was performed by comparing quantitative proteomics results of EVs from a pooled serum sample of 30 patients diagnosed with ACS with the results obtained in a pooled serum sample of 30 sex- and age-matched controls. In the total cohort of 471 patients with suspected ACS, mean EV concentrations in precipitated plasma EVs of all of these individual biomarkers were significantly associated with ACS. Interestingly, this association was markedly stronger in males as compared to females.[48] These results need to be validated in an external cohort, but suggest that EV biomarker research may benefit from discovery stratified by sex.

Proteins (prognosis)

Regarding prognostic CVD biomarkers, Kanhai *et al.*, based on a proteomics discovery approach, identified Cystatin C, Serpin F2, Serpin G1, and CD14 - EV levels, after precipitation of plasma EVs, to be related to an elevated risk for future vascular events and mortality in patients with clinically manifest vascular disease in a large cohort (n = 1060), with long-term follow-up (median 6.4 years). A one standard deviation increase in, respectively, the cystatin C or CD14 EV level was related to an increased risk for vascular events (hazard ratio (HR): 1.27; 95%CI: 1.07–1.52 and HR: 1.32; 95%CI: 1.12–1.55) and to an increased risk for all-cause mortality (HR: 1.41; 95%CI: 1.18–1.69 and HR: 1.36; 95%CI: 1.15–1.62) These HRs were corrected for age, gender and eGFR.[49] Two of these biomarkers, that is, the EV-Cystatin C and CD14 levels, were also related to progression of cerebral atrophy (as assessed using MRI) in patients with manifest vascular disease.[51] Moreover, EV-Cystatin C levels were positively related to the development of diabetes mellitus type II, while higher EV-CD14 levels were associated with a lower risk of developing type II diabetes.[52]

Similarly, Datta *et al.* performed a proteomics discovery approach on pooled samples of patients with lacunar cerebral infarction and different adverse outcome events (i.e., cognitive decline, recurrent vascular events) versus healthy controls, in order to identify candidate prognostic biomarkers and get insight into mechanisms of structural brain changes. In this small study of 45 patients versus 17 controls, several brain-specific proteins en proteins involved in the complement system, coagulation and integrin signaling were up-regulated.[53] Unfortunately, the levels of specific EV-proteins were only quantified in the pooled samples, not in the samples of individual patients.

MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNAs, capable of fine-tuning gene expression by serving as post-transcriptional modulators. Their role in both physiology and pathophysiology in the cardiovascular system has been recognized, and specific patterns of circulating miRNAs are observed to correlate with different CVDs.[54,55] Highly specific miRNA patterns with potential use as biomarkers have been identified in, for example, unstable angina,[56] ACS [57] and heart failure.[58] Recent evidence suggests that EVs serve as important transport carriers for miRNAs, protecting them from degradation from ribonucleases, and facilitating the uptake of miRNAs in target cells. Interestingly, Wang *et al.* demonstrated that expression patterns of circulating microRNAs are remarkably different as compared to miRNA profiles within EVs.[47] In contrast to the large number of publications concerning the association of plasma circulating miRNAs and CVDs, only a few studies have been performed on the topic of EV-miRNA profiles in relation to diagnosis or prognosis of CVDs. In a small case–control study, for example, Badrnya *et al.* observed a smoking-related decrease of EV-associated miR-223, and an increase in miR29b and RNU6-2.[59] In an elegantly performed study by Jansen *et al.*, 10 selected 'vascular' miRNAs were quantified both in plasma and in EVs of 181 patients with stable CAD. At a median follow-up duration of 6.1 years, increased miR-126 and miR-199a EV-levels were significantly associated with a lower major cardiovascular adverse event rate, while none of the plasma miRNA levels were predictive of CV events.[60]

Clinical applications

Plasma protein biomarkers are well established in the guidelines for the diagnosis of certain CVDs, like troponin for the diagnosis of ACS and B-type natriuretic peptide for the diagnosis of heart failure.[61,62] In other CVDs, however, plasma markers are not implemented in clinical practice. In the case of stroke diagnosis, for example, blood-based biomarkers currently have no place in clinical evaluation. Blood-derived bio markers that are either able to distinguish between ischemic and hemorrhagic strokes, or able to distinguish between strokes and stroke-mimicking neurological deficits, would be very helpful in stroke diagnosis, and could be complementary to neurovascular imaging.[63]

Within the scope of ACS, the treatment and risks of disease are similar for both non ST-segment elevation myocardial infarction (NSTEMI) and unstable angina (UA) patients. The

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diagnosis of NSTEMI is based on clinical symptoms together with a rise and/or fall in troponin levels, with at least one value above the assay's 99th percentile, and no ST-elevation on the ECG. In contrast, patients presenting with exactly the same chest pain complaints, but lacking elevated troponin levels, are diagnosed with UA, if there is evidence for myocardial ischemia on functional testing or angiography.

Diagnosing UA by means of using blood-based markers would be very helpful to guide immediate treatment in the emergency department, and to identify patients, without elevated troponins, who are actually at high risk for future cardiovascular events.

Another example for of application of novel EV markers is the identification of patients at risk for either a primary or a secondary cardiovascular event. Even after reducing LDL via statin treatment, patients have still a residual risk for future cardiovascular events.[64]

Expert commentary

Both plasma EV-counts and plasma EV-content already showed their potential as biomarkers in diagnosis and prognosis of CVD. While not yet for CVD, an EV-based test for prostate cancer shows clinical potential, and will be launched commercially in 2016.[65] With an increasing cardiovascular mortality [1,3] and worldwide demand for early and accurate diagnostic and prognostic CVD markers, together with the established potential for EVs in the field of CVD and the launch of the first clinically applicable EV-test, the stage is set for clinical evaluation and implementation of EV-based tests for CVD.

Analyzing content of extracellular vesicle sub fractions

High-resolution flow cytometry techniques hold great potential for EV markers in CVDs, especially when EV subsets can be sorted and used for content analysis. Isolation of EV subsets enables analyzing the content of EVs that originate from specific cells and tissues involved in disease pathophysiology, thereby increasing specificity of the information obtained. This might be key in identifying those patients at risk for a cardiovascular event among all patients sharing a common atherosclerotic background. Standardization and automation, however, are needed to quickly and reliably measure large numbers of samples in large CVD cohorts, which is well described in the ISEV position paper.[66] An EV isolation method that is not only reproducible, but also easy to use and feasible in small sample volumes is required. Precipitation of plasma EVs has been shown to be a reproducible technology for all types of plasma EVs.[48,49] Flow cytometry followed by sorting of the EVs can achieve interrogation of EV subset content.[14] Commercial kits that make use of EV surface proteins like CD9, CD63, CD81 and EpCAM to isolate subsets of EVs in a 96-well-based format recently became commercially available. Using the content of EV subsets as biomarker source in large CVD cohorts, however, has not been explored yet.

Biobanking of CVD patients

CVD is a compilation of disease entities that require specific diagnosis, treatment and risk profiling. With atherosclerosis as the common underlying syndrome, it is very difficult to find markers that can be applied to a specific CVD entity. To select the appropriate patients for biomarker discovery and subsequent validation, accurate and standardized diagnosis of the CVD entities (diagnosed by an adjudication panel) is needed. To accomplish this, biobanking of cardiovascular patients with accurate and diagnosis and lengthy follow-up is essential. Major efforts are made for this. We collect blood samples from, among others, atherectomy patients, acute and non-acute chest pain patients, and heart failure patients in both the Netherlands and Asia.[67–70] These cohorts are well defined, and can be used for identification and validation of novel EV markers in CVD.

Five-year view

The identified potential of EV markers in CVD, together with the first clinical EV-based test (for prostate cancer diagnosis) coming to the market in the near future, will move EV markers in the next 5 years from the academic discovery setting toward clinical application in CVD patients.

The critical hurdle is to develop a good and reproducible technology that can identify subsets of EVs, and is able to quantify biomarkers within these subsets. Flow cytometry has the potential, but will, due to costs and the required expertise, be linked to the clinical laboratories. Especially the identification of patients at risk for a primary cardiovascular event should be performed by a simple and cheap blood test that can be applied worldwide. Ideally, this test can be performed by the general practitioner, or even by the patients themselves. Other technologies like surface plasmon resonance and nuclear magnetic resonance techniques are emerging but are still in development before reaching clinical applications in large patient numbers. Plasma EV subset isolation can, especially when automated isolation is possible, for example, based on precipitation and measurement using emerging "Lab on Chip" technology, achieve this.[71] This opens new possibilities for easily accessible tests that can be developed toward point-of-care applications that can be performed outside the clinical laboratory. This is not only important for the field of CVD, but for every disease that needs EV-based liquid biopsy testing to be performed worldwide.

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Key issues

- Cardiovascular disease (CVD) is the leading cause of death worldwide. The burden of disease is rising globally, affecting many people under the age of seventy in low- and middle-income countries.
- Atherosclerosis, the underlying cause of most CVDs, is a chronic progressive disease of the arteries and is already present at a young age. The clinical manifestations of CVD (e.g. myocardial infarction, heart failure and cerebrovascular accidents) are acute events that often occur decades later, but require timely diagnosis and intervention
- Novel blood-based markers are desperately needed to diagnose people with CVD and identify people at high risk for an adverse cardiovascular event, against a background of atherosclerosis already being present for decades.
- Plasma extracellular vesicles (EVs) are microstructures with a lipid bilayer that have been identified as a source for nucleotide and protein markers for CVDs. Also, EV-counts have been identified as diagnostic and prognostic markers of CVD.
- Robust, easy and reproducible plasma EV isolation technologies are needed to isolate plasma EV (subsets), with flow cytometry and precipitation as the most promising technologies.

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