TITLE: Microvesicles and exosomes: new players in metabolic and cardiovascular disease

AUTHORS: Lawson, Charlotte (Royal Veterinary College, Comparative Biomedical Sciences); Vicencio, Jose (University College London, The Hatter Cardiovascular Institute); Yellon, Derek M (Hatter Institute of Cardiovascular Research, Medicine 67 Chenies Mews London, UK WC1E 6HX, Medicine); Davidson, Sean (Hatter Institute of Cardiovascular Research, Medicine)

JOURNAL TITLE: Journal of Endocrinology

PUBLISHER: BioScientifica

PUBLICATION DATE: 1 February 2016

DOI: 10.1530/JOE-15-0201

Microvesicles and exosomes - new players in metabolic and cardiovascular disease

Charlotte Lawson[1], Jose M. Vicencio[2], Derek M Yellon[2], Sean M. Davidson[2].

[1]Department of Comparative Biomedical Sciences, Royal Veterinary College, London NW1 0TU, UK; [2]The Hatter Cardiovascular Institute, University College London, London WC1E 6HX, UK.

Running Title: Extracellular vesicles in cardio-metabolic disease

Correspondence to: Dr Charlotte Lawson, Department of Comparative Biomedical Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK, Tel +44 (0)20 7468 1216, Fax +44 (0)20 7468 5204, chlawson@rvc.ac.uk

Keywords: diabetes, heart, endothelium, microvesicles, nanoparticles, exosomes, microRNA.

Disclosure statement: the authors declare no disclosures.

Abbreviations: CMD, Cardio-metabolic disease; CPC, Cardiac progenitor cells; CVD, Cardiovascular disease; EV, Extracellular vesicle; FCM, Flow cytometry; IR, Ischaemia-reperfusion; MV, microvesicles; MVB, Multi-vesicular body; NTA, Nanoparticle tracking analysis; PPP, Platelet-poor plasma; PS, Phosphatidyl serine; RIC, Remote ischaemic conditioning; Shh, Sonic hedgehog; T2DM, Type-2 diabetes mellitus; TF, Tissue factor.

Abstract

The past decade has witnessed an exponential increase in the number of publications referring to extracellular vesicles (EVs). For many years considered to be extracellular debris, EVs are now seen as novel mediators of endocrine signalling via cell-to-cell communication. With the capability of transferring proteins and nucleic acids from one cell to another, they have become an attractive focus of research for different pathological settings and are now regarded as both mediators and biomarkers of disease including cardio-metabolic disease. They also offer therapeutic potential as signalling agents capable of targeting tissues or cells with specific peptides or miRNAs. In this review, we focus on the role that microvesicles and exosomes, the two most studied classes of EV, have in diabetes, cardiovascular disease, endothelial dysfunction, coagulopathies and polycystic ovary syndrome. We also provide an overview of current developments in microvesicle/exosome isolation techniques from plasma and other fluids, comparing different available commercial and non-commercial methods. We describe different techniques for their optical/biochemical characterization and quantitation. We also review the signalling pathways that exosomes and microvesicles activate in target cells and provide some insight into their use as biomarkers or potential therapeutic agents. In summary, we give an updated focus on the role that these exciting novel nanoparticles offer for the endocrine community.

Introduction

It is well established that patients with metabolic diseases, in particular insulin resistance and type two diabetes mellitus (T2DM), are more than twice as likely to develop accelerated cardiovascular disease (CVD) including atherosclerosis, stroke and coronary artery disease (reviewed in (Rask-Madsen and King 2013)). Coronary artery disease is a major cause of morbidity and mortality worldwide, and is a leading cause of death in T2DM, with excess risk of fatality in women compared to men (Peters, et al. 2014). Extensive coronary artery disease can result in myocardial infarction, severe loss of cardiac function, and subsequently lead to the development of heart failure (Hausenloy and Yellon 2013). A cluster of risk factors have recently been defined by the American Diabetes Association and the American College of Cardiology Foundation as reliable indicators of a patient's risk for T2DM and CVD, and has been defined as cardiometabolic risk (CMR; (Brunzell, et al. 2008)). These risks include obesity, hyperglycemia, hypertension, insulin resistance and dyslipidemia. The presence of secondary cardiovascular disease in patients with IR or T2DM may be referred to as cardio-metabolic disease (CMD). Given its increasing prevalence and severe consequences, new approaches are needed to diagnose and treat CMD.

Extracellular vesicles (EVs) are small (50 nm to 2 μm) vesicles released from the surface of many different cell types into different bodily fluids, including plasma, milk, saliva, sweat, tears, semen and urine. There are several classes of EV, including exosomes, microvesicles (MV) and apoptotic bodies, which are produced by different mechanisms. Attracting perhaps the most attention recently have been exosomes (50-100 nm), a homogenous population of EV which are released from cells when multivesicular bodies (MVB; sometimes called multivesicular endosomes, MVE) fuse with the plasma membrane in a highly regulated process and release their contents. Cells can also produce a more heterogeneous population of EVs up to 2 μm in diameter called microvesicles (MVs), which are formed by budding and shedding of the cell membrane, a process that involves calcium dependent signalling and enzyme activity. Cells undergoing apoptosis also typically release EV of 1-5 μm in diameter which are referred to as apoptotic bodies (Colombo, et al. 2014; Dignat-George and Boulanger 2011; van der Pol, et al. 2012) (Figure 1).

In some literature, MVs isolated by centrifugation are referred to as "microparticles", particularly those isolated from platelets or endothelial cells. For clarity, this review will refer to EVs simply as exosomes or MV on the basis of the mechanism of their cellular production and their size range - an approach that has been taken by others (Thery, et al. 2009), with the caveat that most isolation methods do not provide a pure populations of vesicles. It is important to note that the size ranges of EVs may overlap and in particular, the size of

microvesicles could overlap with the exosomal size range. Where a mixture of exosomes and MV is likely, for example when plasma vesicles are isolated by high speed (~100,000 g) ultracentrifugation, we refer to them more broadly as EV. These EV are sometimes also referred to as “exosome-like vesicles”.

One of the characteristic markers of all EVs is the presence on the outer surface of phosphatidyl serine (PS), due to loss of membrane asymmetry during blebbing (apoptotic bodies) or budding (MV) and inward folding of the membrane during vesicle formation in MVBs (exosomes). This can be identified by binding of labelled annexin V, a reagent often used for flow cytometric analysis of apoptotic cells. However, more recently several groups have identified MVs lacking phosphatidyl serine (PS) on the outer membrane, suggesting that this is not essential for MV formation (Hou, et al. 2014; Larson, et al. 2012).

Both exosomes and microvesicles characteristically carry a cargo, which they are able to deliver to cells in remote locations. The cargo can include genetic material such as mRNA, microRNA (miRNA) or even small amounts of DNA (Moldovan, et al. 2013), and proteins including transcription factors, cytokines and growth factors, have also been described. Importantly, MVs also carry cellular receptors and transmembrane proteins on their surface characteristic of the cells from which they were released. This aids in their identification but also means that they can interact with specific target cells instigating signalling cascades via receptor interactions (rececrine signalling – akin to cell-cell interactions) and also increasing specificity of cargo delivery. On the other hand, exosomes are characteristically decorated with markers including Alix, HSP70, and the tetraspanins CD9 and CD63, which may be associated with beta-2 integrin binding and intercellular communication. Although these are commonly used as markers of exosomes, they are not exclusive to exosomes and may be found on other EVs. Furthermore, not all EVs express CD63 and different sub-populations of exosomes may express different markers (Thery et al. 2009). It is important to consider that exosomes do not necessarily express the same marker proteins as their parent cells. For example, we found that the common endothelial marker CD144 is absent on exosomes from human umbilical vein endothelial cells (HUVECs)(Figure 2). Recent work has further defined plasma EV and exosome surface marker expression by using extensive antibody profiling which showed that exosomes can express surface membrane markers such as CD146, CD4, CD3 and CD45 (Jorgensen, et al. 2015a). There is some evidence that the protein and RNA content of exosomes depends on the state of the source cell (de Jong, et al. 2012).

The mechanism behind the formation of exosomes and selective packaging of proteins, lipids and RNA is not completely understood but is gradually becoming revealed. The Endosomal Sorting Complex Responsible for Transport (ESCRT) pathway does not seem to

be required for exosome biogenesis, although some components are involved in their formation, particularly Alix (Baietti, et al. 2012; Raposo and Stoorvogel 2013; Trajkovic, et al. 2008). Other molecules that are enriched in exosomes such as tetraspanins and ceramide have also been implicated in exosome biogenesis. For example, inhibitors of neutral sphingomyelinase, an enzyme involved in ceramide production, inhibits exosome production (Trajkovic et al. 2008). Less well understood is the mechanism of exosome release. Certain members of the Rab GTPase family are required for efficient release of exosomes, although the exact members involved appears to depend on the cell type and experimental design, and may reflect different subtypes of exosomes relating to the stage (early or late) of endosome/MVB formation (Colombo et al. 2014).

Purification of EVs from different bodily fluids

Although MVs and exosomes are produced by distinct mechanisms, their sizes overlap, and most isolation protocols do not isolate a pure population. Therefore, in order to evaluate published experiments it is important to understand what type of EV is most likely to be isolated by different protocols.

A number of different protocols have been optimised for purification of different classes of EVs from different sources, with isolation from plasma being the best described (reviewed in(Lobb, et al. 2015; Witwer, et al. 2013)). The isolation of EVs from blood requires its rapid collection with an anti-coagulant – citrate is now generally advised (Lacroix, et al. 2012). The most straightforward technique for isolation of EVs involves sequential steps of centrifugation. After the collection of plasma by centrifugation at 1500 x g for 15 minutes, the supernatant contains platelet-rich plasma and EVs (MVs and exosomes). This is followed by a further centrifugation at 13,000 x g for 30 min to pellet the platelets, with the remaining EVs in the platelet poor plasma (PPP) supernatant. PPP may be snap frozen at -80 °C until analysis, or analysed immediately, using one of the methods outlined below. For further purification the PPP can be centrifuged at 17,000 x g to pellet the larger MVs, which can then be used for analysis. The supernatant can also be further ultracentrifuged at 100,000 x g to pellet the remaining EVs (Thery, et al. 2006). Although the resultant EVs are sometimes referred to as exosomes, this population is not completely pure and in addition to exosomes is likely to contain MVs and possibly lipoproteins. Density gradient centrifugation may be used to further purify the exosomal population (Thery et al. 2006), but recent evidence suggests that this still does not completely remove contamination by lipoproteins. Several newer methods have recently been described using commercially available columns and magnetic separation techniques, either directly from plasma or after initial ultracentrifugation

to pellet the EV fraction, typically based on CD9 or CD63, but a consensus has not yet developed on which technique is the most promising.

Several companies produce reagents designed to precipitate exosomes from plasma or tissue culture medium, though purity using these techniques is generally low, particularly from plasma. Affinity purification using antibodies bound to columns or beads results in much higher purity of EVs but by definition selectively purifies only EVs expressing the marker protein of interest. Size-exclusion chromatography is increasingly popular as a technique to purify exosomes, having been demonstrated to result in isolates relatively pure of contaminating lipoproteins and protein complexes (Boing, et al. 2014; Welton, et al. 2015). Alternatively, new approaches on the horizon include the use of antibody arrays to directly identify and quantify exosomes in body fluids bypassing the need for purification all together (Jorgensen, et al. 2015b).

Since the results of EV isolation procedures may vary, it is important to characterize the particular population being used as much as possible.

Methods for the identification and characterization of EVs

The small size of EVs makes their identification a challenge, indeed until relatively recently they were considered to be debris and not of any functional significance. Use of electron microscopy enables accurate sizing of all different classes of EVs, and is the gold standard to demonstrate presence of EVs, however this method is time consuming, not quantitative and not suitable for phenotyping (Figure 3; for review of methodology see (van der Pol, et al. 2010)). Other non-optical methods have been used, notably atomic force microscopy, which enables accurate size detection and can also be used in after antibody labeling of vesicles enabling phenotyping. Once again, however, the technique is time consuming and requires concentration of the sample meaning that it is not quantitative. A number of optical methods have been used for detection of EVs, the most widely reported of which is flow cytometry, however detection is limited to particle sizes above ~200 nm, so exosome analysis is not possible with standard configurations and techniques. However, recent exciting developments have enabled direct visualization and characterization of microvesicles in whole blood, platelet-rich and platelet-free plasma using Image stream technology (Headland, et al. 2014).

A number of sophisticated protocols have been described to differentiate MVs from background noise during detection using this method, and standardised guidelines have now been published for optimised collection of plasma for detection of MVs (Lacroix et al. 2012).

Techniques are being developed which may even allow the detection of individual exosomes using dedicated flow cytometers with special labelling methods (Pospichalova, et al. 2015). An alternative and more widespread approach is to bind exosomes to carrier latex beads, which are easily detectable by flow cytometry (Thery et al. 2006) (Figure 3).

Important considerations for detection of MVs by flow cytometry are that accurate sizing and enumeration of the MV population may be hampered by the light scattering of small particles compared to larger cells, for which flow cytometers are usually used. However, inclusion of commercially available pre-calibrated counting beads in all samples as internal controls and use of sizing beads can enable standardisation of measurements between samples in the same study (Figure 3) – although caution should be used when directly comparing data from flow cytometry with other methods of counting MV. The newer generations of flow cytometers have been optimised to enable detection of smaller particles. The use of surface markers for phenotyping MV has been reviewed elsewhere (Lacroix and Dignat-George 2012; Macey, et al. 2011).

Flow cytometry is very useful for detection of different phenotypic markers on the surface of MVs and enables accurate characterisation of the source of circulating EVs in bodily fluids, however this technique is not suitable for detection of smaller exosomes and several alternative methodologies have been developed, each with its own instrumentation. These include dynamic light scattering (DLS), nanoparticle tracking analysis (NTA, Figure 3) and tunable resistive pulse sensing (TRPS) (van der Pol et al. 2010). These methods have greater size discrimination compared to flow cytometry (down to below 50 nm diameter) and so enable quantitation of exosomes and smaller MV more efficiently (cost and time) than by EM or atomic force microscopy, however, they are limited by lack of multiple laser capabilities to enable accurate phenotyping, as well as sometimes requiring lengthy purification protocols to ensure that only exosomes are quantified. Importantly, they cannot distinguish EVs from other particulate matter such as protein aggregates, so confirmatory techniques are required to validate EV presence. Raman spectroscopy has also been used to define EV populations. This is a highly sensitive technique for analysis of the biochemical composition of EVs without labelling, and can provide quantitative data, however it is very time consuming. Direct detection of marker proteins on exosomes is challenging using these techniques.

Extracellular vesicles can transfer proteins and RNA

The field of EV research was greatly invigorated by the demonstration that they are able to deliver proteins and RNA to recipient cells. The first evidence for this was obtained in

platelets, which released tissue factor (TF), which was subsequently functionally transferred via microvesicles to monocytes and other cells where TF was able to exert its biological effects (Del Conde, et al. 2005; Scholz, et al. 2002). Microvesicles from tumour cells were shown to be capable of transferring a truncated, oncogenic form of the epidermal growth factor receptor between cells, activating signalling pathways (MAPK and Akt) and thereby transferring the associated transformed phenotype (Al-Nedawi, et al. 2008). Microvesicles can also deliver mRNA (Skog, et al. 2008).

Exosomes can also deliver molecules into the membrane of recipient cells. This appears to be part of their normal function in helping to establish morphogen gradients during development. For example, exosomes can transfer the Notch ligand Delta-like 4 (Dll4) between endothelial cells, where it is incorporated into the membrane of the target endothelial cells, and inhibits Notch signalling altering angiogenesis (Sheldon, et al. 2010). Interestingly, some cytoprotective proteins have been shown to be transferred between cells. αB crystallin is secreted from human retinal pigment epithelium in exosomes, and taken up by adjacent photoreceptors, protecting them from oxidative stress (Sreekumar, et al. 2010).

In a seminal paper, Valadi et al, were first to show that exosomes can also transfer mRNA and miRNA between cells (Valadi, et al. 2007). In this study, mast cells were demonstrated to transfer functional mRNAs between cells that were subsequently translated. Importantly when exosomes were pre-treated with RNAse and trypsin, the effect was no longer observed, demonstrating that the mRNA was protected within the vesicles and not simply associated or co-purified.

The profile of miRNAs contained within exosomes appears to depend on the cell type of origin. The miRNA profile is different in exosomes released from C2C12 myoblasts compared with those released by C2C12 cells once they have differentiated into myotubes (Forterre, et al. 2014). The miRNA profile within exosomes was also found to differ from the parent C2C12 cells, which indicates that there is selective sorting of miRNA into exosomes (Forterre et al. 2014). The mechanism for this is only beginning to be unravelled, but appears to involve recognition of particular sequence motifs by sumoylated heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) (Villarroya-Beltri, et al. 2013). When the exosomes secreted by C2C12 myotubes were taken up by myoblasts they suppressed expression of Sirt1, potentially modulating metabolic homeostasis and the commitment of myoblasts during differentiation (Forterre et al. 2014).

There is also evidence that exosomes are used by some cells in the heart to communicate to each other. Cardiac fibroblasts secrete exosomes that are enriched in specific miRNAs, including miR-21-3p. Intriguingly, this particular miRNA is a “passenger strand” miRNA which normally undergoes intracellular degradation and was therefore believed to be non-functional (Bang, et al. 2014). However, when neonatal cardiomyocytes took up these exosomes, they increased in size indicating a hypertrophic response (Bang et al. 2014). Endothelial cells have also been shown to transfer miRNA via EVs, in this case transferring EV to smooth muscle cells after stimulation by shear stress, which is known to be atheroprotective (Hergenreider, et al. 2012). The EVs delivered functional miR-143/145 into smooth muscle cells in co-culture, which controlled the expression of target genes (Hergenreider et al. 2012). Importantly, when administered *in vivo* to ApoE(-/-) mice, they reduced atherosclerotic lesion formation in the aorta (Hergenreider et al. 2012). The vesicles in this study were referred to conservatively as “extracellular vesicles”, because a maximum centrifugation speed of 20,500 g was used to pellet them, and the size range of most of the vesicles on electron micrographs ranged between 60 and 130 nm, therefore they likely contained a mix of exosomes and microvesicles.

In view of the RNA content of EVs which is related to the cell type of origin, and can alter in pathological settings, they have become an attractive source of biomarkers for profiling and identification of disease markers (Cheng, et al. 2014; Jansen, et al. 2013; Kruger, et al. 2014), as has been reviewed elsewhere (Gaceb, et al. 2014).

The role of EVs in diabetes and metabolic disease

T2DM is characterized by elevated fasting plasma glucose levels combined with insulin resistance. The metabolic syndrome additionally comprises abdominal (central) obesity, high blood pressure, insulin resistance, and lipid abnormalities (Perrone-Filardi, et al. 2015). It is present in 34% of the population, and greatly increases the risk of heart failure (Perrone-Filardi et al. 2015). There is accumulating evidence that EVs are elevated in these conditions and can contribute to some of the pathophysiology, including vascular complications, inflammation and alterations in blood coagulation (recent review Lakhter (Lakhter and Sims 2015)).

Exosomes and MVs from different cellular sources can be identified constitutively in plasma from normal individuals (Caby, et al. 2005; Raposo and Stoorvogel 2013), including MVs released from monocytes, lymphocytes, endothelial cells, erythrocytes and platelets. A number of studies have demonstrated that the numbers of circulating MVs is increased in insulin-resistant patients (Jayachandran, et al. 2011), and in patients with T2DM (Diamant,

et al. 2002; Omoto, et al. 1999). Levels are further increased in those with microvascular complications (Ogata, et al. 2006; Omoto et al. 1999), or secondary macrovascular CVD, including atherosclerosis (Diamant et al. 2002). Increased numbers of MV have also been linked to obesity (Stepanian, et al. 2013). Interestingly, a significant reduction in MV numbers has been described after calorific restriction or bariatric surgery in these patients (Cheng, et al. 2013). Increased EVs are also a hallmark of CVD including atherosclerosis (Feng, et al. 2010), hypertension (Chen, et al. 2012), and following stroke or myocardial infarction (D'Alessandra, et al. 2010; Kim, et al. 2012).

The role of chronic inflammation in progression of CVD and CMD has been highlighted in a number of studies (reviewed in (Hansson, et al. 2015);(Lindhardsen, et al. 2015)) and circulating EVs are increased in many inflammatory conditions (e.g. (Daniel, et al. 2006; Joop, et al. 2001; Suades, et al. 2015)). Their role in propagation of endothelial pro-inflammatory cascades is also increasingly recognized, and was first described by Mesri et al. They stimulated EVs *in vivo* in healthy volunteers by infusion of a chemotactic peptide and showed that these were able to induce cytokine and chemokine release from endothelial cells *in vitro* (Mesri and Altieri 1998). A number of other studies have reported similar findings using EVs from patients or animal models (Meziani, et al. 2010; Wang, et al. 2011). We have recently shown that EVs induced by long term feeding of a high fat diet in a rat model of insulin resistance and T2DM were able to induce VCAM-1 adhesion molecule expression and ROS production in rat cardiac endothelial cells *in vitro* (Heinrich, et al. 2015).

The same factors that increase the risk of cardiometabolic disease are also risk factors for polycystic ovary syndrome (PCOS)(Daskalopoulos, et al. 2015), the most common endocrine disorder in women aged 18-44, affecting up to 10% of the population, and which leads to reduced fertility (Teede, et al. 2010). Several studies have now shown that in accordance with these increased risk factors, PCOS patients have increased circulating levels of EVs, particularly pro-coagulant platelet MVs (Koiou, et al. 2011; Koiou, et al. 2013). Willis et al recently measured increased numbers of circulating EVs nearing the exosome size range (<150 nm), with a greater percentage of annexin V^{+ve} MV and 16 miRNA that were differentially expressed compared to matched controls (Willis, et al. 2014). However, a causal relationship has not yet been established between MVs and the other symptoms of PCOS which include excess androgen activity, oligo-ovulation or anovulation, and polycystic ovaries (Teede et al. 2010).

The role EVs in the function and dysfunction of healthy and diseased endothelium

A number of studies have demonstrated a correlation between the number of circulating endothelial ($CD31^+CD41^-$) MVs and endothelial dysfunction in patients with coronary artery disease (Chen et al. 2012; Wang, et al. 2014b; Werner, et al. 2006). Similarly, in T2DM patients higher numbers of endothelial MVs correlate with impaired endothelium function, as determined by the measurement of flow mediated dilatation in the brachial artery (Feng et al. 2010). In addition to their levels increasing with endothelial dysfunction, MVs may also have a direct effect on endothelial function. MVs isolated from T2DM patients by centrifugation have been shown to impair shear stress induced dilatation of mouse mesenteric arteries (Martin, et al. 2004) whilst aortic ring experiments have shown that endothelial derived EVs (obtained by ultracentrifugation at 100,000 x g) decrease nitric oxide (NO) and increase reactive oxygen species production, as well as impairing acetylcholine-mediated vasorelaxation (Brodsky, et al. 2004). Consequently, microvesicles have gained some notoriety as potentially detrimental factors contributing to cardiovascular disease.

On the other hand, EVs have also been observed to have some beneficial effects, particularly with regards to the stimulation of endothelial proliferation, migration and tube formation *in vitro* (Deregibus, et al. 2007; Jansen et al. 2013)(Vrijsen, et al. 2010). This effect has been observed with EVs isolated from apoptotic endothelial cells (Deregibus et al. 2007; Jansen et al. 2013) (and therefore presumably containing many apoptotic vesicles), as well as with more pure populations of MVs isolated from platelets (Brill, et al. 2005; Kim, et al. 2004), from endothelial progenitor cells (Deregibus et al. 2007; Vrijsen et al. 2010), or from ischemic muscle (Leroyer, et al. 2009). Exosomes isolated from cardiomyocyte progenitor cells (Vrijsen et al. 2010) or the conditioned medium of bone marrow $CD34^+$ stem cells (Sahoo, et al. 2011) have been shown to have a similar effect on endothelial cell proliferation and migration.

EVs can also stimulate endothelial repair. For example, endothelial EVs were isolated by centrifugation from human coronary artery endothelial cells undergoing apoptosis. When administered to mice in which a region of endothelium had been denuded, these EVs were found to be capable of repairing the endothelium via delivery of miR-126 (Jansen et al. 2013). It is significant, however, that this effect was abrogated in EVs isolated from cells which had been grown under hyperglycaemic conditions *in vitro* or isolated from patients with T2DM, since this suggests that this reparative property of EVs is altered by diabetes and may contribute to continued vascular damage and dysfunction (Jansen et al. 2013). Similarly, exosomes from the cardiomyocytes of non-diabetic rats were founds to be pro-angiogenic, stimulating endothelial proliferation, migration and tube formation *in vitro*, while those isolated from the cardiomyocytes of diabetic rats had the opposite effect (Wang, et al.

2014a).In this example, the detrimental effect was attributed to exosomal transfer of miR320 and the down-regulation of its target genes (IGF-1, Hsp20 and Ets2) (Wang et al. 2014a).

Various additional mechanisms have been implicated in the stimulatory effect of exosomes on endothelial cells. Platelet MVs appear to activate pro-angiogenic ERK and PI3K/Akt pathways (Brill et al. 2005; Kim et al. 2004) and may contain a contain a lipid growth factor (Kim et al. 2004), while EVs from endothelial progenitor cells appear to transfer mRNAs that activate PI3K/AKT and eNOS signaling in the recipient endothelial cells (Deregibus et al. 2007). The transfer of miR-214 has also been proposed to mediate induction of angiogenesis by endothelial exosomes by suppressing the expression of ATM in recipient cells (van Balkom, et al. 2013). Endothelial cells also communicate atheroprotective stimuli to smooth muscle cell via the transmission of miR-143/145 via EVs (Hergenreider et al. 2012). In this study, EV were purified by centrifugation at 20,500 g for 1 h, resulting in vesicles that were mostly between 60 and 130 nm.

In some cases, exosomes can also suppress hyperproliferative pathways such as those that contribute to hypoxia-induced pulmonary hypertension. Here, the beneficial effect of mesenchymal stromal cells was shown to be mediated by the release of exosomes which suppressed hyperproliferative pathways including those mediated by STAT3 and the miR-17 superfamily, in addition to increasing lung levels of miR-204 (Lee, et al. 2012).

Recently, pressure overload or stretch was shown to cause the release from cardiomyocytes of exosomes containing functional angiotensin II type 1 receptors, which are able to be transferred to skeletal muscle, mesenteric resistance vessels and cardiomyocytes, conferring responsiveness to angiotensin II (Pironti, et al. 2015). This exciting data suggests that exosomes may contribute to the *in vivo* tissue distribution of cell surface receptors such as angiotensin II, with functional consequences for the cardiovascular system.

The role of EVs in coagulopathies

When EVs were first described by Peter Wolf they were referred to as “platelet dust” (Wolf 1967) because they were thought not to be functionally significant. Despite there being some reports to the contrary (Tushuizen, et al. 2012), numerous studies have shown that platelet EVs are procoagulant due to the exposure of negatively charged PS which can enhance clot formation (for review see (Hargett and Bauer 2013)). Indeed, platelet EVs have more binding sites for the factors involved in the clotting cascade than do activated platelets themselves (Sinauridze, et al. 2007). More recent studies have revealed the presence of tissue factor (TF) on the surface of endothelial- and monocyte-derived EVs (Breitenstein, et al. 2010), as

well as P-selectin glycoprotein ligand-1 (PSGL-1) which can bind to P-selectin on the surface of activated platelets and become incorporated into the clot (Falati, et al. 2003). Other receptors including glycoprotein IIb/IIIa (Sommeijer, et al. 2005), factor VIII, factor Va (Nomura, et al. 1993) and protein disulphide isomerase (Raturi, et al. 2008) may also be present on the surface of EVs and participate in clot formation and thrombosis.

In addition to hyperglycemia, hyperinsulinemia can cause an increase in procoagulant TF-positive MVs (Boden and Rao 2007), and MVs are elevated in otherwise-healthy individuals with signs of metabolic syndrome (Agouni, et al. 2008; Ueba, et al. 2008). A correlation between circulating endothelial microparticles (MVs) and cardiometabolic risk factors (particularly dyslipidaemia), was also detected in the Framingham Heart Study cohort (Amabile, et al. 2014). The presence of hypertension, elevated triglycerides, and metabolic syndrome all increased circulating MVs, but dyslipidaemia had the most severe effect. Obesity has also been correlated with increased circulating endothelial MVs in children (Gunduz, et al. 2012). These increases may contribute to the disease, since MVs from individuals with metabolic syndrome have been shown to impair endothelium-dependent relaxation and decrease endothelial NO synthase expression when injected into mice (Agouni et al. 2008). Other cardiovascular risk factors such as uremia may also correlate with increased numbers of platelet MVs which may trigger thrombosis (Ando, et al. 2002). Elevated uric acid in chronic renal failure patients may also contribute to their increased risk of cardiovascular events (Faure, et al. 2006).

Tsimerman et al measured increased numbers of pro-coagulant TF-positive EVs in patients with T2DM, but MV coagulability was significantly increased only in those who also had macrovascular complications (foot ulcers and coronary artery disease) (Tsimerman, et al. 2011). EVs were isolated and evaluated for their ability to induce tube formation in endothelial cells *in vitro*. Endothelial tube formation was stimulated by MVs from healthy controls, but was defective when incubated with MVs from patients with macrovascular complications (Tsimerman et al. 2011).

Thus, hyperglycemia, dyslipidaemia and hyperinsulinemia as well as hyperuricemia and uremia appear to contribute to cardiometabolic disease via the procoagulant activity of MVs, but also due to their diminished ability to support endothelial function.

EVs as a potential therapy for cardiometabolic disease

The heart is essentially terminally differentiated, meaning that there is very little division of cardiomyocytes after injury (e.g. IR), and instead those that remain tend to undergo a

compensatory increase in size. The possibility of renewing the cardiomyocytes by stem cell therapy has been intensively investigated for a number of years, however, the results of this approach have been largely disappointing. Some improvements in cardiac function have been observed after stem cell therapy, although this is generally acknowledged to occur in the absence of new cardiomyocyte formation. Interestingly, similar levels of benefit could also be obtained experimentally after injecting medium that had been conditioned by stem cells. It was therefore proposed that stem cells release cytokines, growth factors and other proteins in a “paracrine” manner to improve survival and function of cardiomyocytes (Kim, et al. 2014; Menasche 2014; Yoon, et al. 2005).

In 2010, it was shown that exosomes purified from the conditioned medium of human ESC-derived mesenchymal stem cells (ESC-MSC) by HPLC size-exclusion fractionation, could protect the heart both *in vitro* and *in vivo* (Lai, et al. 2010). Cardiac function after 28 days was also improved (Arslan, et al. 2013). An increase in the activity of cardioprotective kinases Akt and GSK3α/β was observed 1 h after exosome administration until the following day (Arslan et al. 2013). These kinases are known to be highly cardioprotective (Hausenloy, et al. 2005). In another study, exosomes were isolated from MSC cells overexpressing GATA4, and these also restored cardiac contractile function and reduced infarct size when injected into rat hearts at the time of infarction (Yu, et al. 2014). Protection was attributed to an increase in the treated hearts of miR-19a, which targets PTEN, indirectly increasing Akt and ERK activation. However, with such experiments it is difficult to ascertain whether the miR-19a was transferred from the MSC exosomes or was a transcriptional response of the myocardium to the treatment (Yu et al. 2014). The ability to activate protective pathways does not appear to be restricted to exosomes, since microvesicles derived from human adult mesenchymal stem cells were also able to protect the kidney against ischaemia and reperfusion injury (Gatti, et al. 2011).

MSC are not the only type of stem cell that has been shown to release exosomes with beneficial cardiovascular effects. Intramyocardial injection of exosomes from murine cardiac progenitor cells (CPCs) reduced apoptosis after ischaemia and reperfusion (Chen, et al. 2013). In this study, however, exosomes were isolated by precipitation with polyethylene glycol (PEG) (Chen et al. 2013), which raises some uncertainty about the effects that the PEG might have itself. In another study EVs were isolated from CPCs derived from atrial appendage explants from patients undergoing heart valve surgery (Barile, et al. 2014). Injection of these CPCs-EVs into the hearts of rats subject to permanent coronary artery ligation reduced cardiomyocyte apoptosis and scar size, increased the amount of viable tissue in the infarct area, increased blood vessel density, and prevented the impairment of

ventricular function between day 2 and day 7 (Barile et al. 2014). In contrast, exosomes isolated from normal human dermal fibroblasts exhibited no benefit, suggesting that effects depend on cell type of origin (Barile et al. 2014). Intramyocardial injection of exosomes isolated from CPCs that had been exposed to hypoxia for 12 h improved cardiac function and also reduced fibrosis 21 days (Gray, et al. 2015). The exosomes released after hypoxia had an altered miRNA content, and co-regulated miRNA with a beneficial profile were identified (Gray et al. 2015). Although cardiac endothelial cells and fibroblasts took up fluorescently stained exosomes *in vitro,* uptake was minimal in primary rat cardiomyocytes (Gray et al. 2015), suggesting either that they deliver miRNA directly to the former cells types, or that they interact with surface receptors on cardiomyocytes without delivering miRNA intracellularly. Thus, the exact mechanism of functional benefit conferred by CPC-EVs remains unclear.

When a nonviral mini-circle plasmid carrying HIF1, a transcription factor that mediates adaptive responses to ischemia, was delivered into the endothelium of ischemic mouse myocardium, these cells were found to release exosomes with a higher content of miR-126 and miR-210. These exosomes could be taken up by CPCs administered to the heart, leading to the activation of pro-survival kinases and to a switch towards glycolysis. This resulted in them having an increased tolerance against hypoxic stress (Ong, et al. 2014) and suggests the interesting possibility that endothelial cells can support CPC survival by exosomal transfer of miRNA.

An attractive aspect of using EVs for therapy is the potential for altering their cargo to augment their protective capabilities. In a study by Mackie et al, $CD34^+$ cells or their exosomes showed no benefit after injection into ischaemic mouse hearts. However, $CD34^+$ cells were then genetically modified to to express the sonic hedgehog (Shh) protein, in order to enhance the angiogenic quality of $CD34^+$ cells. When $CD34^+$Shh cells were injected into the infarct border zone in mice, infarct size was reduced, border zone capillary density was increased, and ventricular dilation and cardiac function were improved 4 weeks later (Mackie, et al. 2012). *In vitro* studies in cells were performed to demonstrate that Shh was released from the $CD34^+$Shh cells in exosomes, and could be transferred to recipient cells and (modestly) activate transcription. Injection of the exosomes from $CD34^+$Shh cells had the same benefit, though exosomes from $CD34^+$ cells wihout Shh showed no benefit (Mackie et al. 2012).

Strikingly, it has been shown that there are on the order of 10^{10} EVs per ml present in the blood of all individuals, after isolation using the technique of differential ultracentrifugation, (Caby et al. 2005), and these could potentially be continually delivering different miRNA or

receptor-ligand mediated signals to the heart. This possibility was addressed by isolating plasma exosomes from rats or healthy individuals by differential ultracentrifugation and testing whether they were cardioprotective in *in vitro*, *ex vivo* and *in vivo* models of IR (Vicencio, et al. 2015). Indeed, exosomes from plasma were strongly cardioprotective, activating the cardioprotective ERK1/2 kinase and reducing infarct size (Vicencio et al. 2015). Plasma exosomes were similarly protective in an isolated perfused rat heart model and in primary cardiomyocytes, suggesting a direct effect of the exosomes at the plasma membrane level, although interestingly exosomes did not appear to be taken up by the cardiomyocytes but they were endocytosed by endothelial cells (Vicencio et al. 2015). This study also showed that the number of exosomes in the plasma was increased by short (5 min) cycles of limb IR. This manipulation is under investigation of a means of inducing protection of the heart and other organs via a phenomenon known as “remote ischaemic preconditioning (RIC)” (Hausenloy and Yellon 2008). As yet, the mechanism of RIC is unknown although evidence for several mediators has been presented, including SDF-1α and Il-10 (Cai, et al. 2012; Davidson, et al. 2013). As vehicles able to deliver multiple signals between cells, EVs had been proposed as possible candidates for carriers of the cardioprotective factor released by RIC (Yellon and Davidson 2014). A study by Giricz et al. suggested that this may be the case, since RIC was not effective when EVs were removed from medium containing the factor (Giricz, et al. 2014). However, in a dose-response experiment conducted using primary adult rat cardiomyocytes the EVs released after RIC were found not to be significantly more protective that exosomes from baseline (Vicencio et al. 2015).

On the other hand, the observation that plasma EVs themselves were cardioprotective is important and may suggest that they signal continuously to the heart, modulating the protective state. Protection was shown to involve HSP70 in the exosome membrane, which binds to TLR4 on cardiomyocytes, activating ERK1/2, p38MAPK and downstream phosphorylation of the small heat shock protein HSP27 (Vicencio et al. 2015). TLR4 is part of the innate immune system, and strong activation by its ligands from bacteria leads to a cell damage response and can cause cell death. However, mild activation is known to be protective (Mathur, et al. 2011; Zhang, et al. 2013). Other studies have suggested a link between body fluid exosomes and TLR-dependent signaling pathways, possibly mediating immunosuppressive and anti-inflammatory pathways (Bretz, et al. 2013; Zhang, et al. 2014).

Conclusion

With T2DM reaching epidemic proportions and cardiovascular disease being the major cause of death worldwide, novel therapeutic strategies are urgently needed to offer cell and tissue repair mechanisms to the myocardium and also diseases characterized by endothelial dysfunction. EVs including MVs and exosomes have emerged over the past decade to attract immense interest due to their potential either as biomarkers or mediators of disease. Increased MVs in plasma can be observed in patients with insulin resistance, T2DM, atherosclerosis and also after stroke or myocardial infarct. MVs have been also described as mediators of inflammation and to be involved in the pro-coagulant actions of platelets. The protein or RNA cargo of EVs offers additional potential not only for their use as biomarkers but also for their use as vehicles for delivering bioactives. As such, they offer the capability of delivering multiple signals to target tissues. Stem cells are the best-explored example of cells that deliver miRNA via exosomes with beneficial effects on the heart, kidneys and the endothelium. Exosomes and MVs have also been implicated in protecting the heart from infarction and have been proposed as potential mediators of ischaemic conditioning. EVs therefore represent one of the most exciting and promising research areas for the endocrine community. However, there is still much left to understand regarding the mechanisms of EV formation and their specific targeting to a selective tissue. Although current research has provided valuable insight to the mechanisms of EV release, we are only beginning to understand mechanisms of RNA/protein loading into exosomes for instance, and exploring these mechanisms is essential to design efficient therapeutical strategies involving EVs.

Acknowledgements

This work was funded by a grant from the Medical Research Council (MR/K002066/1) and the British Heart Foundation (RG/08/015/26411).

553 **References**

554 **References**

555

556 Agouni A, Lagrue-Lak-Hal AH, Ducluzeau PH, Mostefai HA, Draunet-Busson C, Leftheriotis
557 G, Heymes C, Martinez MC & Andriantsitohaina R 2008 Endothelial dysfunction caused by
558 circulating microparticles from patients with metabolic syndrome. *American Journal of*
559 *Pathology* **173** 1210-1219.
560 Al-Nedawi K, Meehan B, Micallef J, Lhotak V, May L, Guha A & Rak J 2008 Intercellular
561 transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells.
562 *Nature Cell Biology* **10** 619-624.
563 Amabile N, Cheng S, Renard JM, Larson MG, Ghorbani A, McCabe E, Griffin G, Guerin C,
564 Ho JE, Shaw SY, et al. 2014 Association of circulating endothelial microparticles with
565 cardiometabolic risk factors in the Framingham Heart Study. *European Heart Journal* **35**
566 2972-2979.
567 Ando M, Iwata A, Ozeki Y, Tsuchiya K, Akiba T & Nihei H 2002 Circulating platelet-derived
568 microparticles with procoagulant activity may be a potential cause of thrombosis in uremic
569 patients. *Kidney International* **62** 1757-1763.
570 Arslan F, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, Timmers L, van Rijen HV,
571 Doevendans PA, Pasterkamp G, et al. 2013 Mesenchymal stem cell-derived exosomes
572 increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance
573 myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion
574 injury. *Stem Cell Res* **10** 301-312.
575 Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, Ivarsson Y, Depoortere
576 F, Coomans C, Vermeiren E, et al. 2012 Syndecan-syntenin-ALIX regulates the biogenesis
577 of exosomes. *Nature Cell Biology* **14** 677-685.
578 Bang C, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, Just A, Remke J,
579 Zimmer K, Zeug A, et al. 2014 Cardiac fibroblast-derived microRNA passenger strand-
580 enriched exosomes mediate cardiomyocyte hypertrophy. *Journal of Clinical Investigation*
581 **124** 2136-2146.
582 Barile L, Lionetti V, Cervio E, Matteucci M, Gherghiceanu M, Popescu LM, Torre T, Siclari F,
583 Moccetti T & Vassalli G 2014 Extracellular vesicles from human cardiac progenitor cells
584 inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction.
585 *Cardiovascular Research* **103** 530-541.
586 Boden G & Rao AK 2007 Effects of hyperglycemia and hyperinsulinemia on the tissue factor
587 pathway of blood coagulation. *Current Diabetes Reports* **7** 223-227.
588 Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A & Nieuwland R 2014
589 Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell*
590 *Vesicles* **3**.
591 Breitenstein A, Tanner FC & Luscher TF 2010 Tissue factor and cardiovascular disease: quo
592 vadis? *Circulation Journal* **74** 3-12.
593 Bretz NP, Ridinger J, Rupp AK, Rimbach K, Keller S, Rupp C, Marme F, Umansky L,
594 Umansky V, Eigenbrod T, et al. 2013 Body fluid exosomes promote secretion of
595 inflammatory cytokines in monocytic cells via Toll-like receptor signaling. *Journal of*
596 *Biological Chemistry* **288** 36691-36702.
597 Brill A, Dashevsky O, Rivo J, Gozal Y & Varon D 2005 Platelet-derived microparticles induce
598 angiogenesis and stimulate post-ischemic revascularization. *Cardiovascular Research* **67**
599 30-38.
600 Brodsky SV, Zhang F, Nasjletti A & Goligorsky MS 2004 Endothelium-derived microparticles
601 impair endothelial function in vitro. *American Journal of Physiology: Heart and Circulatory*
602 *Physiology* **286** H1910-1915.
603 Brunzell JD, Davidson M, Furberg CD, Goldberg RB, Howard BV, Stein JH & Witztum JL
604 2008 Lipoprotein management in patients with cardiometabolic risk: consensus conference

report from the American Diabetes Association and the American College of Cardiology Foundation. *J Am Coll Cardiol* **51** 1512-1524.
Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G & Bonnerot C 2005 Exosomal-like vesicles are present in human blood plasma. *International Immunology* **17** 879-887.
Cai ZP, Parajuli N, Zheng X & Becker L 2012 Remote ischemic preconditioning confers late protection against myocardial ischemia-reperfusion injury in mice by upregulating interleukin-10. *Basic Research in Cardiology* **107** 277.
Chen L, Wang Y, Pan Y, Zhang L, Shen C, Qin G, Ashraf M, Weintraub N, Ma G & Tang Y 2013 Cardiac progenitor-derived Exosomes protect ischemic myocardium from acute ischemia/reperfusion injury. *Biochemical and Biophysical Research Communications*.
Chen Y, Feng B, Li X, Ni Y & Luo Y 2012 Plasma endothelial microparticles and their correlation with the presence of hypertension and arterial stiffness in patients with type 2 diabetes. *Journal of Clinical Hypertension (Greenwich, Conn.)* **14** 455-460.
Cheng L, Sharples RA, Scicluna BJ & Hill AF 2014 Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. *J Extracell Vesicles* **3**.
Cheng V, Kashyap SR, Schauer PR, Kirwan JP & McCrae KR 2013 Restoration of glycemic control in patients with type 2 diabetes mellitus after bariatric surgery is associated with reduction in microparticles. *Surgery for Obesity and Related Diseases* **9** 207-212.
Colombo M, Raposo G & Thery C 2014 Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annual Review of Cell and Developmental Biology* **30** 255-289.
D'Alessandra Y, Devanna P, Limana F, Straino S, Di Carlo A, Brambilla PG, Rubino M, Carena MC, Spazzafumo L, De Simone M, et al. 2010 Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *European Heart Journal* **31** 2765-2773.
Daniel L, Fakhouri F, Joly D, Mouthon L, Nusbaum P, Grunfeld JP, Schifferli J, Guillevin L, Lesavre P & Halbwachs-Mecarelli L 2006 Increase of circulating neutrophil and platelet microparticles during acute vasculitis and hemodialysis. *Kidney International* **69** 1416-1423.
Daskalopoulos G, Karkanaki A, Piouka A, Prapas N, Panidis D, Gkeleris P & Athyros VG 2015 Excess Metabolic and Cardiovascular Risk is not Manifested in all Phenotypes of Polycystic Ovary Syndrome: Implications for Diagnosis and Treatment. *Current Vascular Pharmacology*.
Davidson SM, Selvaraj P, He D, Boi-Doku C, Yellon RL, Vicencio JM & Yellon DM 2013 Remote ischaemic preconditioning involves signalling through the SDF-1alpha/CXCR4 signalling axis. *Basic Research in Cardiology* **108** 377.
de Jong OG, Verhaar MC, Chen Y, Vader P, Gremmels H, Posthuma G, Schiffelers RM, Gucek M & van Balkom BWM 2012 Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. *Journal of Extracellular Vesicles* **1**.
Del Conde I, Shrimpton CN, Thiagarajan P & Lopez JA 2005 Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood* **106** 1604-1611.
Deregibus MC, Cantaluppi V, Calogero R, Lo Iacono M, Tetta C, Biancone L, Bruno S, Bussolati B & Camussi G 2007 Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* **110** 2440-2448.
Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW & Radder JK 2002 Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. *Circulation* **106** 2442-2447.
Dignat-George F & Boulanger CM 2011 The many faces of endothelial microparticles. *Arteriosclerosis, Thrombosis, and Vascular Biology* **31** 27-33.
Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, Celi A, Croce K, Furie BC & Furie B 2003 Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *Journal of Experimental Medicine* **197** 1585-1598.

Faure V, Dou L, Sabatier F, Cerini C, Sampol J, Berland Y, Brunet P & Dignat-George F 2006 Elevation of circulating endothelial microparticles in patients with chronic renal failure. *Journal of Thrombosis and Haemostasis* **4** 566-573.
Feng B, Chen Y, Luo Y, Chen M, Li X & Ni Y 2010 Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus. *Atherosclerosis* **208** 264-269.
Forterre A, Jalabert A, Chikh K, Pesenti S, Euthine V, Granjon A, Errazuriz E, Lefai E, Vidal H & Rome S 2014 Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. *Cell Cycle* **13** 78-89.
Gaceb A, Martinez MC & Andriantsitohaina R 2014 Extracellular vesicles: new players in cardiovascular diseases. *International Journal of Biochemistry and Cell Biology* **50** 24-28.
Gatti S, Bruno S, Deregibus MC, Sordi A, Cantaluppi V, Tetta C & Camussi G 2011 Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. *Nephrology, Dialysis and Transplantation* **26** 1474-1483.
Giricz Z, Varga ZV, Baranyai T, Sipos P, Paloczi K, Kittel A, Buzas E & Ferdinandy P 2014 Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles. *Journal of Molecular and Cellular Cardiology* **68** 75-78.
Gray WD, French KM, Ghosh-Choudhary S, Maxwell JT, Brown ME, Platt MO, Searles CD & Davis ME 2015 Identification of therapeutic covariant microRNA clusters in hypoxia-treated cardiac progenitor cell exosomes using systems biology. *Circulation Research* **116** 255-263.
Gunduz Z, Dursun I, Tulpar S, Bastug F, Baykan A, Yikilmaz A, Patiroglu T, Poyrazoglu HM, Akin L, Yel S, et al. 2012 Increased endothelial microparticles in obese and overweight children. *Journal of Pediatric Endocrinology and Metabolism* **25** 1111-1117.
Hansson GK, Libby P & Tabas I 2015 Inflammation and plaque vulnerability. *Journal of Internal Medicine*.
Hargett LA & Bauer NN 2013 On the origin of microparticles: From "platelet dust" to mediators of intercellular communication. *Pulm Circ* **3** 329-340.
Hausenloy DJ, Tsang A & Yellon DM 2005 The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. *Trends Cardiovasc.Med.* **15** 69-75.
Hausenloy DJ & Yellon DM 2008 Remote ischaemic preconditioning: underlying mechanisms and clinical application. *Cardiovascular Research* **79** 377-386.
Hausenloy DJ & Yellon DM 2013 Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *Journal of Clinical Investigation* **123** 92-100.
Headland SE, Jones HR, D'Sa AS, Perretti M & Norling LV 2014 Cutting-edge analysis of extracellular microparticles using ImageStream(X) imaging flow cytometry. *Scientific Reports* **4** 5237.
Heinrich LF, Andersen DK, Cleasby ME & Lawson C 2015 Long-term high fat feeding of rats results in increased numbers of circulating microvesicles with pro-inflammatory effects on endothelial cells. *British Journal of Nutrition* 1-8.
Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, et al. 2012 Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nature Cell Biology* **14** 249-256.
Hou S, Grillo D, Williams CL, Wasserstrom JA, Szleifer I & Zhao M 2014 Membrane phospholipid redistribution in cancer micro-particles and implications in the recruitment of cationic protein factors. *J Extracell Vesicles* **3**.
Jansen F, Yang X, Hoelscher M, Cattelan A, Schmitz T, Proebsting S, Wenzel D, Vosen S, Franklin BS, Fleischmann BK, et al. 2013 Endothelial Microparticle-Mediated Transfer of MicroRNA-126 Promotes Vascular Endothelial Cell Repair via SPRED1 and Is Abrogated in Glucose-Damaged Endothelial Microparticles. *Circulation* **128** 2026-2038.
Jayachandran M, Litwiller RD, Lahr BD, Bailey KR, Owen WG, Mulvagh SL, Heit JA, Hodis HN, Harman SM & Miller VM 2011 Alterations in platelet function and cell-derived

microvesicles in recently menopausal women: relationship to metabolic syndrome and atherogenic risk. *Journal of Cardiovascular Translational Research* **4** 811-822.
Joop K, Berckmans RJ, Nieuwland R, Berkhout J, Romijn FP, Hack CE & Sturk A 2001 Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. *Thrombosis and Haemostasis* **85** 810-820.
Jorgensen MM, Baek R & Varming K 2015a Potentials and capabilities of the Extracellular Vesicle (EV) Array. *J Extracell Vesicles* **4** 26048.
Jorgensen MMl, Baek R & Varming K 2015b Potentials and capabilities of the Extracellular Vesicle (EV) Array. *2015*.
Kim HK, Song KS, Chung JH, Lee KR & Lee SN 2004 Platelet microparticles induce angiogenesis in vitro. *British Journal of Haematology* **124** 376-384.
Kim SJ, Moon GJ, Cho YH, Kang HY, Hyung NK, Kim D, Lee JH, Nam JY & Bang OY 2012 Circulating mesenchymal stem cells microparticles in patients with cerebrovascular disease. *PloS One* **7** e37036.
Kim SW, Houge M, Brown M, Davis ME & Yoon YS 2014 Cultured human bone marrow-derived CD31(+) cells are effective for cardiac and vascular repair through enhanced angiogenic, adhesion, and anti-inflammatory effects. *Journal of the American College of Cardiology* **64** 1681-1694.
Koiou E, Tziomalos K, Katsikis I, Kalaitzakis E, Kandaraki EA, Tsourdi EA, Delkos D, Papadakis E & Panidis D 2011 Circulating platelet-derived microparticles are elevated in women with polycystic ovary syndrome diagnosed with the 1990 criteria and correlate with serum testosterone levels. *European Journal of Endocrinology. Oslo* **165** 63-68.
Koiou E, Tziomalos K, Katsikis I, Papadakis E, Kandaraki EA & Panidis D 2013 Platelet-derived microparticles in overweight/obese women with the polycystic ovary syndrome. *Gynecological Endocrinology* **29** 250-253.
Kruger S, Abd Elmageed ZY, Hawke DH, Worner PM, Jansen DA, Abdel-Mageed AB, Alt EU & Izadpanah R 2014 Molecular characterization of exosome-like vesicles from breast cancer cells. *BMC Cancer* **14** 44.
Lacroix R & Dignat-George F 2012 Microparticles as a circulating source of procoagulant and fibrinolytic activities in the circulation. *Thrombosis Research* **129 Suppl 2** S27-29.
Lacroix R, Judicone C, Poncelet P, Robert S, Arnaud L, Sampol J & Dignat-George F 2012 Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. *Journal of Thrombosis and Haemostasis* **10** 437-446.
Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, et al. 2010 Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem cell research* **4** 214-222.
Lakhter AJ & Sims EK 2015 Emerging Roles for Extracellular Vesicles in Diabetes and Related Metabolic Disorders. *Molecular Endocrinology* me20151206.
Larson MC, Woodliff JE, Hillery CA, Kearl TJ & Zhao M 2012 Phosphatidylethanolamine is externalized at the surface of microparticles. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1821** 1501-1507.
Lee C, Mitsialis SA, Aslam M, Vitali SH, Vergadi E, Konstantinou G, Sdrimas K, Fernandez-Gonzalez A & Kourembanas S 2012 Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* **126** 2601-2611.
Leroyer AS, Ebrahimian TG, Cochain C, Recalde A, Blanc-Brude O, Mees B, Vilar J, Tedgui A, Levy BI, Chimini G, et al. 2009 Microparticles from ischemic muscle promotes postnatal vasculogenesis. *Circulation* **119** 2808-2817.
Lindhardsen J, Kristensen SL & Ahlehoff O 2015 Management of Cardiovascular Risk in Patients with Chronic Inflammatory Diseases: Current Evidence and Future Perspectives. *American Journal of Cardiovascular Drugs*.
Lobb RJ, Becker M, Wen SW, Wong CS, Wiegmans AP, Leimgruber A & Moller A 2015 Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J Extracell Vesicles* **4** 27031.

767 Macey MG, Enniks N & Bevan S 2011 Flow cytometric analysis of microparticle phenotype
768 and their role in thrombin generation. *Cytometry. Part B: Clinical Cytometry* **80** 57-63.
769 Mackie AR, Klyachko E, Thorne T, Schultz KM, Millay M, Ito A, Kamide CE, Liu T, Gupta R,
770 Sahoo S, et al. 2012 Sonic hedgehog-modified human CD34+ cells preserve cardiac
771 function after acute myocardial infarction. *Circulation Research* **111** 312-321.
772 Martin S, Tesse A, Hugel B, Martinez MC, Morel O, Freyssinet JM & Andriantsitohaina R
773 2004 Shed membrane particles from T lymphocytes impair endothelial function and regulate
774 endothelial protein expression. *Circulation* **109** 1653-1659.
775 Mathur S, Walley KR, Wang Y, Indrambarya T & Boyd JH 2011 Extracellular heat shock
776 protein 70 induces cardiomyocyte inflammation and contractile dysfunction via TLR2.
777 *Circulation Journal* **75** 2445-2452.
778 Menasche P 2014 Stem cells in the management of advanced heart failure. *Current Opinion*
779 *in Cardiology*.
780 Mesri M & Altieri DC 1998 Endothelial cell activation by leukocyte microparticles. *Journal of*
781 *Immunology* **161** 4382-4387.
782 Meziani F, Delabranche X, Asfar P & Toti F 2010 Bench-to-bedside review: circulating
783 microparticles--a new player in sepsis? *Critical Care (London, England)* **14** 236.
784 Moldovan L, Batte K, Wang Y, Wisler J & Piper M 2013 Analyzing the circulating microRNAs
785 in exosomes/extracellular vesicles from serum or plasma by qRT-PCR. *Methods in*
786 *Molecular Biology* **1024** 129-145.
787 Nomura S, Komiyama Y, Murakami T, Funatsu A, Kokawa T, Sugo T, Matsuda M &
788 Yasunaga K 1993 Flow cytometric analysis of surface membrane proteins on activated
789 platelets and platelet-derived microparticles from healthy and thrombasthenic individuals.
790 *International Journal of Hematology* **58** 203-212.
791 Ogata N, Nomura S, Shouzu A, Imaizumi M, Arichi M & Matsumura M 2006 Elevation of
792 monocyte-derived microparticles in patients with diabetic retinopathy. *Diabetes Research*
793 *and Clinical Practice* **73** 241-248.
794 Omoto S, Nomura S, Shouzu A, Hayakawa T, Shimizu H, Miyake Y, Yonemoto T, Nishikawa
795 M, Fukuhara S & Inada M 1999 Significance of platelet-derived microparticles and activated
796 platelets in diabetic nephropathy. *Nephron* **81** 271-277.
797 Ong SG, Lee WH, Huang M, Dey D, Kodo K, Sanchez-Freire V, Gold JD & Wu JC 2014
798 Cross Talk of Combined Gene and Cell Therapy in Ischemic Heart Disease: Role of
799 Exosomal MicroRNA Transfer. *Circulation* **130** S60-69.
800 Perrone-Filardi P, Paolillo S, Costanzo P, Savarese G, Trimarco B & Bonow RO 2015 The
801 role of metabolic syndrome in heart failure. *European Heart Journal*.
802 Peters SA, Huxley RR & Woodward M 2014 Diabetes as risk factor for incident coronary
803 heart disease in women compared with men: a systematic review and meta-analysis of 64
804 cohorts including 858,507 individuals and 28,203 coronary events. *Diabetologia* **57** 1542-
805 1551.
806 Pironti G, Strachan RT, Abraham D, Mon-Wei Yu S, Chen M, Chen W, Hanada K, Mao L,
807 Watson LJ & Rockman HA 2015 Circulating Exosomes Induced by Cardiac Pressure
808 Overload Contain Functional Angiotensin II Type 1 Receptors. *Circulation* **131** 2120-2130.
809 Pospichalova V, Svoboda J, Dave Z, Kotrbova A, Kaiser K, Klemova D, Ilkovics L, Hampl A,
810 Crha I, Jandakova E, et al. 2015 Simplified protocol for flow cytometry analysis of
811 fluorescently labeled exosomes and microvesicles using dedicated flow cytometer. *2015*.
812 Raposo G & Stoorvogel W 2013 Extracellular vesicles: exosomes, microvesicles, and
813 friends. *Journal of Cell Biology* **200** 373-383.
814 Rask-Madsen C & King GL 2013 Vascular complications of diabetes: mechanisms of injury
815 and protective factors. *Cell Metab* **17** 20-33.
816 Raturi A, Miersch S, Hudson JW & Mutus B 2008 Platelet microparticle-associated protein
817 disulfide isomerase promotes platelet aggregation and inactivates insulin. *Biochimica et*
818 *Biophysica Acta (BBA) - Bioenergetics* **1778** 2790-2796.
819 Sahoo S, Klychko E, Thorne T, Misener S, Schultz KM, Millay M, Ito A, Liu T, Kamide C,
820 Agrawal H, et al. 2011 Exosomes from human CD34(+) stem cells mediate their
821 proangiogenic paracrine activity. *Circulation Research* **109** 724-728.

Scholz T, Temmler U, Krause S, Heptinstall S & Losche W 2002 Transfer of tissue factor from platelets to monocytes: role of platelet-derived microvesicles and CD62P. *Thrombosis and Haemostasis* **88** 1033-1038.
Sheldon H, Heikamp E, Turley H, Dragovic R, Thomas P, Oon CE, Leek R, Edelmann M, Kessler B, Sainson RC, et al. 2010 New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood* **116** 2385-2394.
Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV & Ataullakhanov FI 2007 Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thrombosis and Haemostasis* **97** 425-434.
Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT, Jr., Carter BS, Krichevsky AM & Breakefield XO 2008 Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nature Cell Biology* **10** 1470-1476.
Sommeijer DW, Joop K, Leyte A, Reitsma PH & ten Cate H 2005 Pravastatin reduces fibrinogen receptor gpIIIa on platelet-derived microparticles in patients with type 2 diabetes. *Journal of Thrombosis and Haemostasis* **3** 1168-1171.
Sreekumar PG, Kannan R, Kitamura M, Spee C, Barron E, Ryan SJ & Hinton DR 2010 alphaB crystallin is apically secreted within exosomes by polarized human retinal pigment epithelium and provides neuroprotection to adjacent cells. *PloS One* **5** e12578.
Stepanian A, Bourguignat L, Hennou S, Coupaye M, Hajage D, Salomon L, Alessi MC, Msika S & de Prost D 2013 Microparticle increase in severe obesity: not related to metabolic syndrome and unchanged after massive weight loss. *Obesity (Silver Spring)* **21** 2236-2243.
Suades R, Padro T & Badimon L 2015 The Role of Blood-Borne Microparticles in Inflammation and Hemostasis. *Seminars in Thrombosis and Hemostasis* **41** 590-606.
Teede H, Deeks A & Moran L 2010 Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. *BMC Medicine* **8** 41.
Thery C, Amigorena S, Raposo G & Clayton A 2006 Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current Protocols in Cell Biology* **Chapter 3** Unit 3 22.
Thery C, Ostrowski M & Segura E 2009 Membrane vesicles as conveyors of immune responses. *Nature Reviews: Immunology* **9** 581-593.
Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brugger B & Simons M 2008 Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* **319** 1244-1247.
Tsimerman G, Roguin A, Bachar A, Melamed E, Brenner B & Aharon A 2011 Involvement of microparticles in diabetic vascular complications. *Thrombosis and Haemostasis* **106** 310-321.
Tushuizen ME, Diamant M, Peypers EG, Hoek FJ, Heine RJ, Sturk A & Nieuwland R 2012 Postprandial changes in the phospholipid composition of circulating microparticles are not associated with coagulation activation. *Thrombosis Research* **130** 115-121.
Ueba T, Haze T, Sugiyama M, Higuchi M, Asayama H, Karitani Y, Nishikawa T, Yamashita K, Nagami S, Nakayama T, et al. 2008 Level, distribution and correlates of platelet-derived microparticles in healthy individuals with special reference to the metabolic syndrome. *Thrombosis and Haemostasis* **100** 280-285.
Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ & Lotvall JO 2007 Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology* **9** 654-659.
van Balkom BW, de Jong OG, Smits M, Brummelman J, den Ouden K, de Bree PM, van Eijndhoven MA, Pegtel DM, Stoorvogel W, Wurdinger T, et al. 2013 Endothelial cells require miR-214 to secrete exosomes that suppress senescence and induce angiogenesis in human and mouse endothelial cells. *Blood* **121** 3997-4006, S3991-3915.
van der Pol E, Boing AN, Harrison P, Sturk A & Nieuwland R 2012 Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacological Reviews* **64** 676-705.

van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG & Nieuwland R 2010 Optical and non-optical methods for detection and characterization of microparticles and exosomes. *Journal of Thrombosis and Haemostasis* **8** 2596-2607.
Vicencio JM, Yellon DM, Sivaraman V, Das D, Boi-Doku C, Arjun S, Zheng Y, Riquelme JA, Kearney J, Sharma V, et al. 2015 Plasma exosomes protect the myocardium from ischemia-reperfusion injury. *J Am Coll Cardiol* **65** 1525-1536.
Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, Martinez-Herrera DJ, Pascual-Montano A, Mittelbrunn M & Sanchez-Madrid F 2013 Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat Commun* **4** 2980.
Vrijsen KR, Sluijter JP, Schuchardt MW, van Balkom BW, Noort WA, Chamuleau SA & Doevendans PA 2010 Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. *Journal of Cellular and Molecular Medicine* **14** 1064-1070.
Wang JG, Williams JC, Davis BK, Jacobson K, Doerschuk CM, Ting JP & Mackman N 2011 Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner. *Blood* **118** 2366-2374.
Wang X, Huang W, Liu G, Cai W, Millard RW, Wang Y, Chang J, Peng T & Fan GC 2014a Cardiomyocytes mediate anti-angiogenesis in type 2 diabetic rats through the exosomal transfer of miR-320 into endothelial cells. *Journal of Molecular and Cellular Cardiology* **74** 139-150.
Wang Y, Chen LM & Liu ML 2014b Microvesicles and diabetic complications--novel mediators, potential biomarkers and therapeutic targets. *Acta Pharmacologica Sinica* **35** 433-443.
Welton JL, Webber JP, Botos LA, Jones M & Clayton A 2015 Ready-made chromatography columns for extracellular vesicle isolation from plasma. *J Extracell Vesicles* **4** 27269.
Werner N, Wassmann S, Ahlers P, Kosiol S & Nickenig G 2006 Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26** 112-116.
Willis GR, Connolly K, Ladell K, Davies TS, Guschina IA, Ramji D, Miners K, Price DA, Clayton A, James PE, et al. 2014 Young women with polycystic ovary syndrome have raised levels of circulating annexin V-positive platelet microparticles. *Human Reproduction* **29** 2756-2763.
Witwer KW, Buzas EI, Bemis LT, Bora A, Lasser C, Lotvall J, Nolte-'t Hoen EN, Piper MG, Sivaraman S, Skog J, et al. 2013 Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* **2**.
Wolf P 1967 The nature and significance of platelet products in human plasma. *British Journal of Haematology* **13** 269-288.
Yellon DM & Davidson SM 2014 Exosomes: nanoparticles involved in cardioprotection? *Circulation Research* **114** 325-332.
Yoon YS, Wecker A, Heyd L, Park JS, Tkebuchava T, Kusano K, Hanley A, Scadova H, Qin G, Cha DH, et al. 2005 Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *Journal of Clinical Investigation* **115** 326-338.
Yu B, Kim HW, Gong M, Wang J, Millard RW, Wang Y, Ashraf M & Xu M 2014 Exosomes secreted from GATA-4 overexpressing mesenchymal stem cells serve as a reservoir of anti-apoptotic microRNAs for cardioprotection. *International Journal of Cardiology* **182C** 349-360.
Zhang B, Yin Y, Lai RC, Tan SS, Choo AB & Lim SK 2014 Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev* **23** 1233-1244.
Zhang Y, Zhang X, Shan P, Hunt CR, Pandita TK & Lee PJ 2013 A protective Hsp70-TLR4 pathway in lethal oxidant lung injury. *Journal of Immunology* **191** 1393-1403.

Figure legends

Figure 1

(A) Timeline (1956-2014) of the publications referring to extracellular vesicles (black line), microvesicles (blue line) and exosomes (red line). (B) Schematic representation of the mechanisms of formation of microvesicles, exosomes and apoptotic bodies. Microvesicles (0.2 – 2.0 μm) originate via budding and shedding from the plasma membrane of cells and therefore may contain specific surface markers from the cell of origin. Exosomes (50 - 100 nm) on the other hand originate intracellularly through a sorting pathway involving intermediate organelles such as the early endosome and a late multivesicular body, which fuses with the plasma membrane to release exosomes via exocytosis. Apoptotic bodies (1 - 2 μm) originate via blebbing of the plasma membrane.

Figure 2

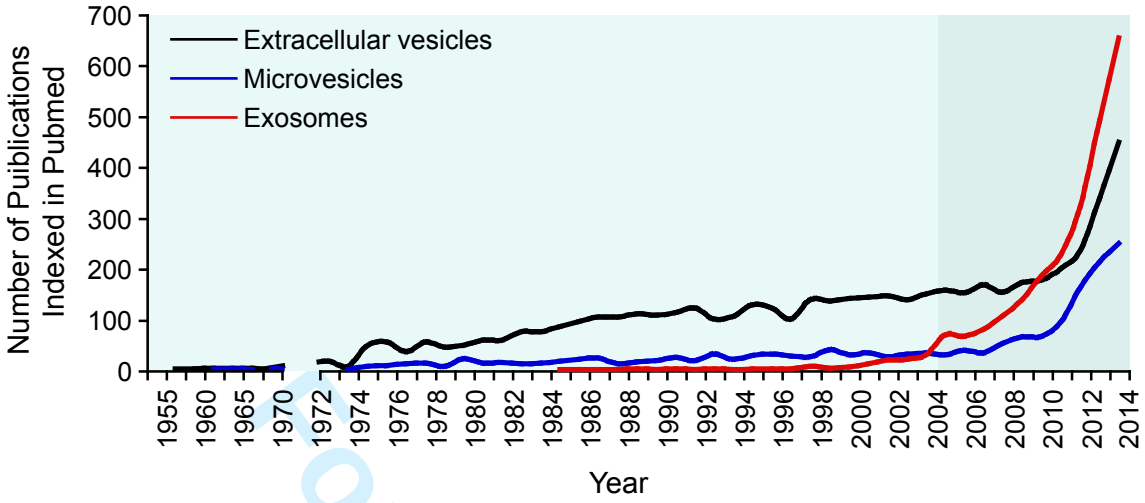
The endothelial cells marker CD144 is absent from exosomes isolated from HUVEC endothelial cells (A), despite being detectable on the parent cells (B). HUVEC cells or HUVEC exosomes bound to 4 μm beads were labelled with anti-CD144 and fluorescent secondary antibody, before fluorescent detection using a BD AccuriC6 flow cytometer.

Figure 3

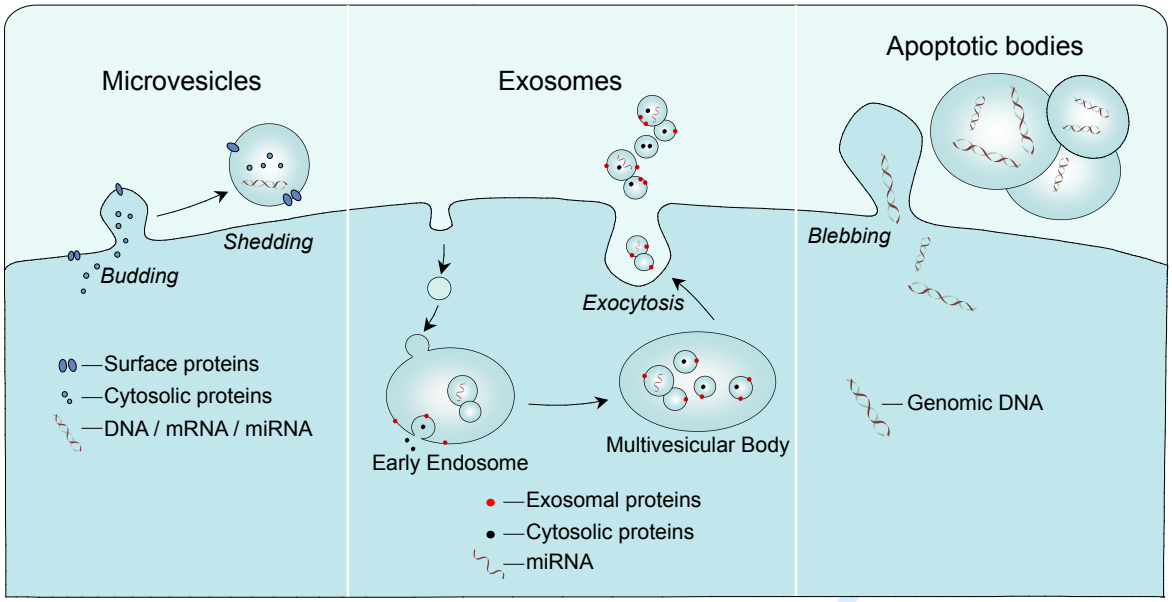
Flow cytometry (FCM) allows direct analysis of microvesicles (MVs) and indirect (conjugated) analysis of exosomes. Nanoparticle tracking analysis (NTA) is the preferred technique for EV quantitation. Electron microscopy (EM) is the golden standard for EV visualization. (A) Direct flow cytometric analysis of MVs in plasma of rats fed chow or high fat diets (HFD; Heinrich et al. 2015) after staining for phosphatidyl serine exposure (Annexin V PE-Cy7.7) and CD106 (PE) to determine MV release from activated endothelial cells. Enumeration beads (red) and 1,1 μm sizing beads (green) were added as internal controls. (B) NTA of MVs from rats fed chow or HFD. (C) Indirect flow cytometric analysis of exosomes bound to aldehyde sulphate beads (4 μm) after staining for the tetraspannin CD63 and surface HSP70 (Vicencio et al. 2015). (D) NTA of human plasma exosomes isolated via ultraceintrifugation (black line) or using the Exo-spin™ (Cell Guidance Systems) commercial kit (red line). (E) Electron micrograph of MVs and exosomes.

Figure 1

A


Number of Puiblications Indexed in Pubmed
700
600
500
400
300
200
100
0
Extracellular vesicles
Microvesicles
Exosomes
1955
1960
1965
1970
1972
1974
1976
1978
1980
1982
1984
1986
1988
1990
1992
1994
1996
1998
2000
2002
2004
2006
2008
2010
2012
2014
Year

B


Microvesicles
Exosomes
Apoptotic bodies
Shedding
Budding
Exocytosis
Blebbing
—Surface proteins
—Cytosolic proteins
—DNA / mRNA / miRNA
Early Endosome
Multivesicular Body
—Genomic DNA
—Exosomal proteins
—Cytosolic proteins
—miRNA

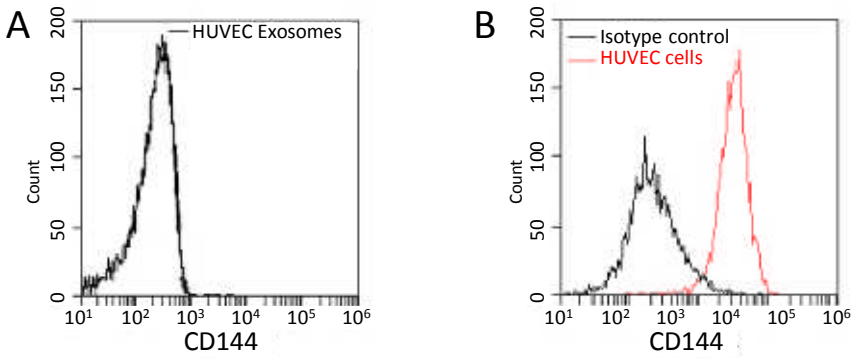
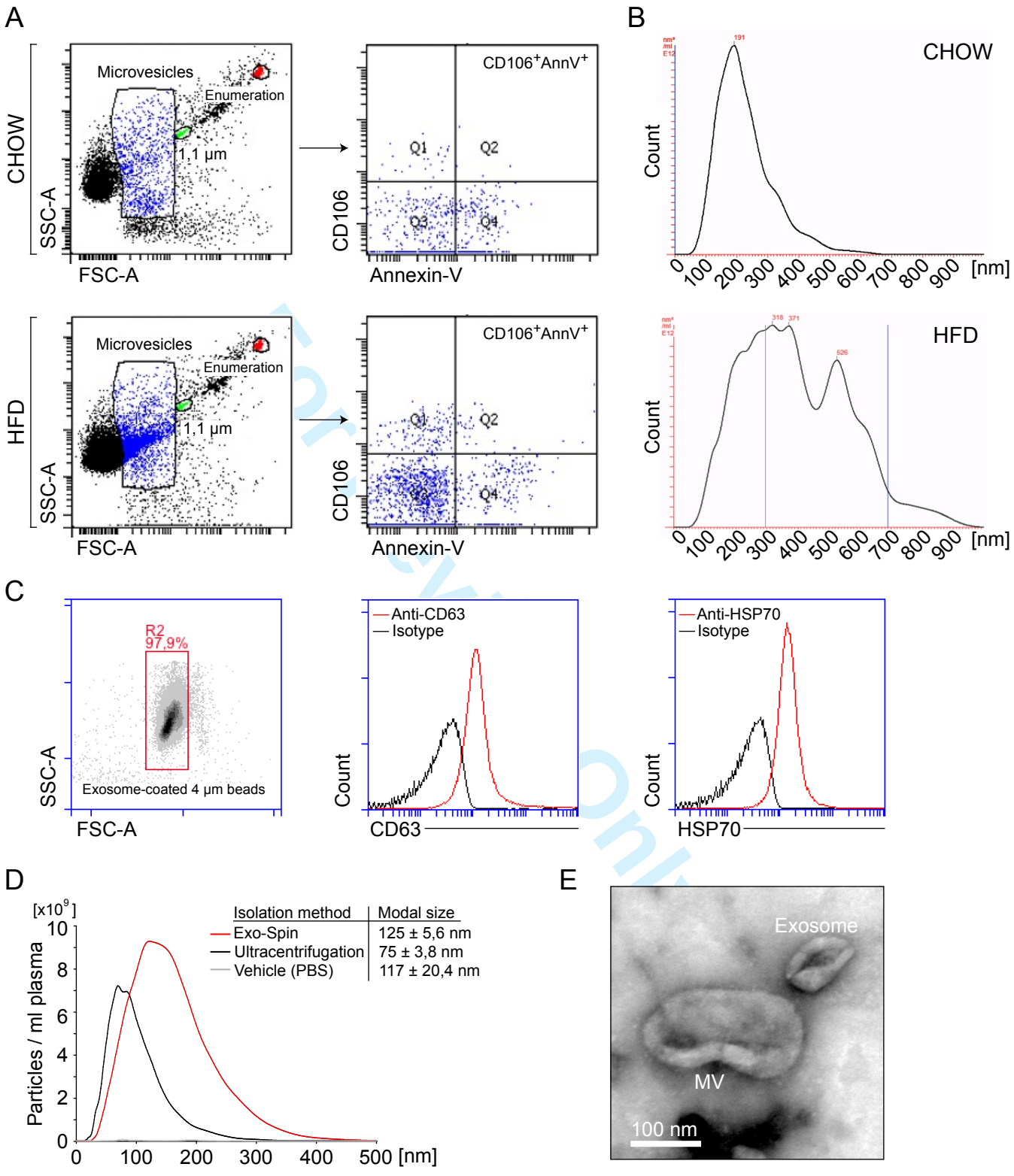


Figure 2.
The endothelial cells marker CD144 is absent from exosomes isolated from HUVEC endothelial cells (A), despite being detectable on the parent cells (B). HUVEC cells or HUVEC exosomes bound to 4 µm beads were labelled with anti-CD144 and fluorescent secondary antibody, and fluorescence detected using a BD Accuri C6 flow cytometer.

Figure 2


A
CHOW
Microvesicles
Enumeration
1,1 µm
SSC-A
FSC-A
CD106+AnnV+
Q1
Q2
Q3
Q4
CD106
Annexin-V
B
CHOW
Count
191
0 100 200 300 400 500 600 700 800 900 [nm]
HFD
Microvesicles
Enumeration
1,1 µm
SSC-A
FSC-A
CD106+AnnV+
CD106
Annexin-V
HFD
Count
318 371
526
0 100 200 300 400 500 600 700 800 900 [nm]
C
R2
97,9%
Exosome-coated 4 µm beads
SSC-A
FSC-A
Anti-CD63
Isotype
Count
CD63
Anti-HSP70
Isotype
Count
HSP70
D
[x10^9]
Particles / ml plasma
10
8
6
4
2
0
0 100 200 300 400 500 [nm]
Isolation method | Modal size
Exo-Spin | 125 ± 5,6 nm
Ultracentrifugation | 75 ± 3,8 nm
Vehicle (PBS) | 117 ± 20,4 nm
E
Exosome
MV
100 nm