Microvesicles and exosomes: new players in metabolic and cardiovascular disease

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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 (MVs) 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 MV/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 MVs 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.

Keywords
- diabetes
- heart
- endothelium
- microvesicles
- nanoparticles
- exosomes
- microRNA

Journal of Endocrinology (2016) **228**, R57–R71

Journal of Endocrinology

Introduction

It is well established that patients with metabolic diseases, in particular insulin resistance and type 2 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 & 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 with 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 & 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 CVD in patients

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DOI: 10.1530/JOE-15-0201

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with ischaemia-reperfusion (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 (MVs), 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 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 (Dignat-George & Boulanger 2011, van der Pol *et al*. 2012, Colombo *et al*. 2014) (Fig. 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 MVs 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 PS on the outer membrane, suggesting that this is not essential for MV formation (Larson *et al*. 2012, Hou *et al*. 2014).

Both exosomes and MVs 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) (Fig. 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*. 2015). 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 pathway does not seem to be required for exosome biogenesis, although some components are involved in their formation, particularly Alix (Trajkovic *et al*. 2008, Baietti *et al*. 2012, Raposo & Stoorvogel 2013). 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

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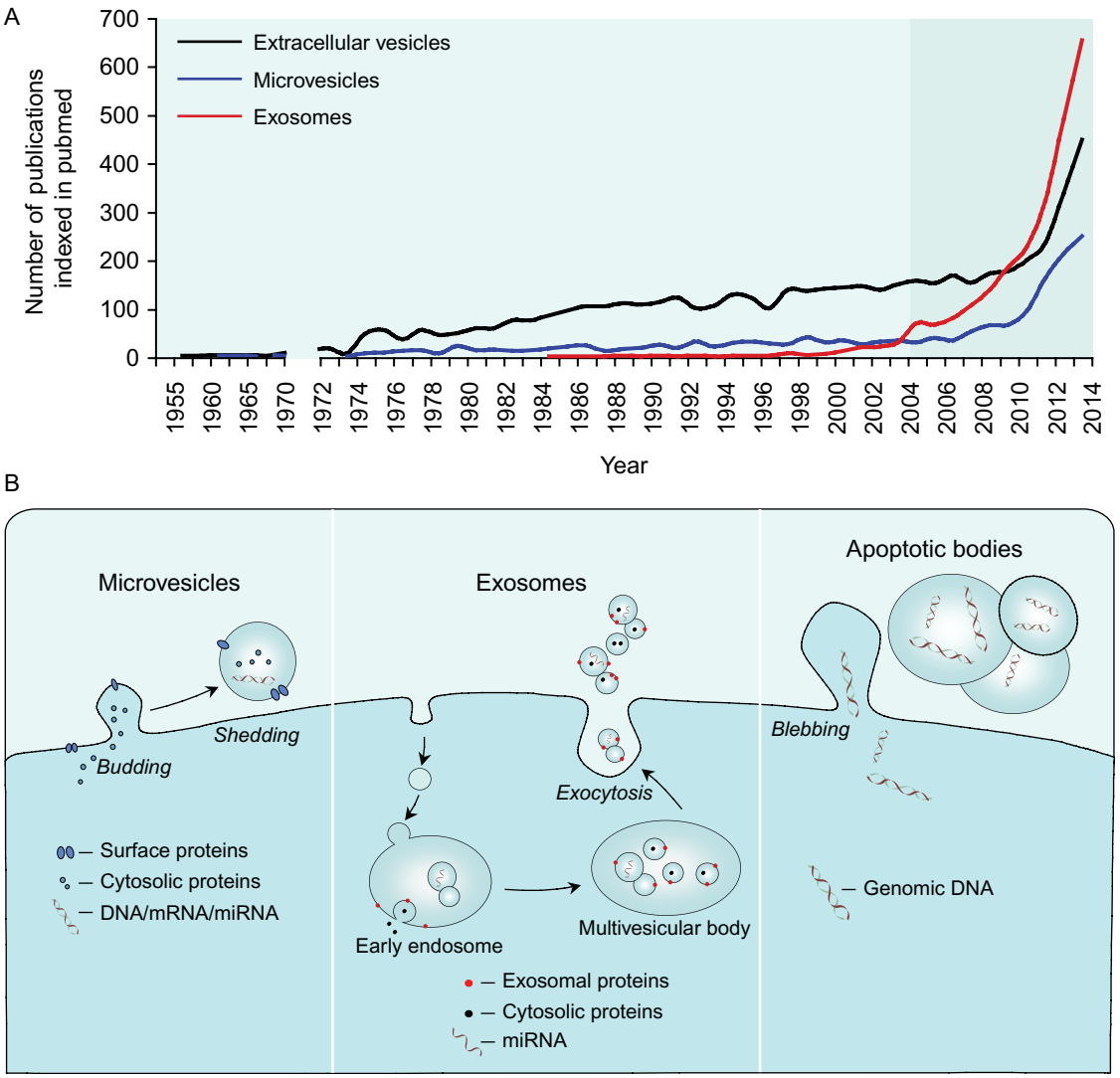

Figure 1
(A) Timeline (1956–2014) of the publications referring to extracellular vesicles (black line), microvesicles (blue line), and exosomes (red line). (B) Schematic representation of the mechanisms of formation of microvesicles, exosomes, and apoptotic bodies. Microvesicles (0.2–2.0 μm) originate 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.

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 optimized for purification of different classes of EVs from different sources, with isolation from plasma being the best described (reviewed in Witwer *et al*. (2013) and Lobb *et al*. (2015). 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

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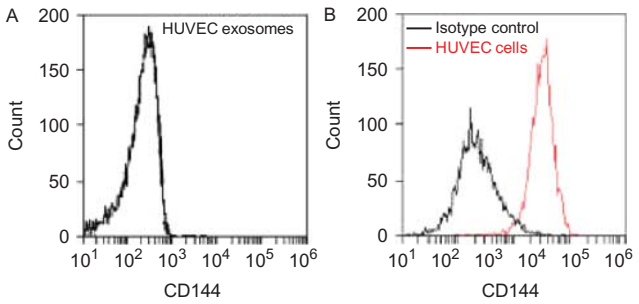

Figure 2
The endothelial cells marker CD144 is absent from exosomes isolated from HUVEC endothelial cells (A), despite being detectable on the parent cells (B). HUVEC cells or HUVEC exosomes bound to 4 μm beads were labelled with anti-CD144 and fluorescent secondary antibody, before fluorescent detection using a BD AccuriC6 flow cytometer.

centrifugation at 1500× ***g*** for 15 min, the supernatant contains platelet-rich plasma and EVs (MVs and exosomes). This is followed by a further centrifugation at 13 000× ***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× ***g*** to pellet the larger MVs, which can then be used for analysis. The supernatant can also be further ultracentrifuged at 100 000× ***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.* 2015).

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 (Fig. 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 MVs 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 optimized 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) (Fig. 3).

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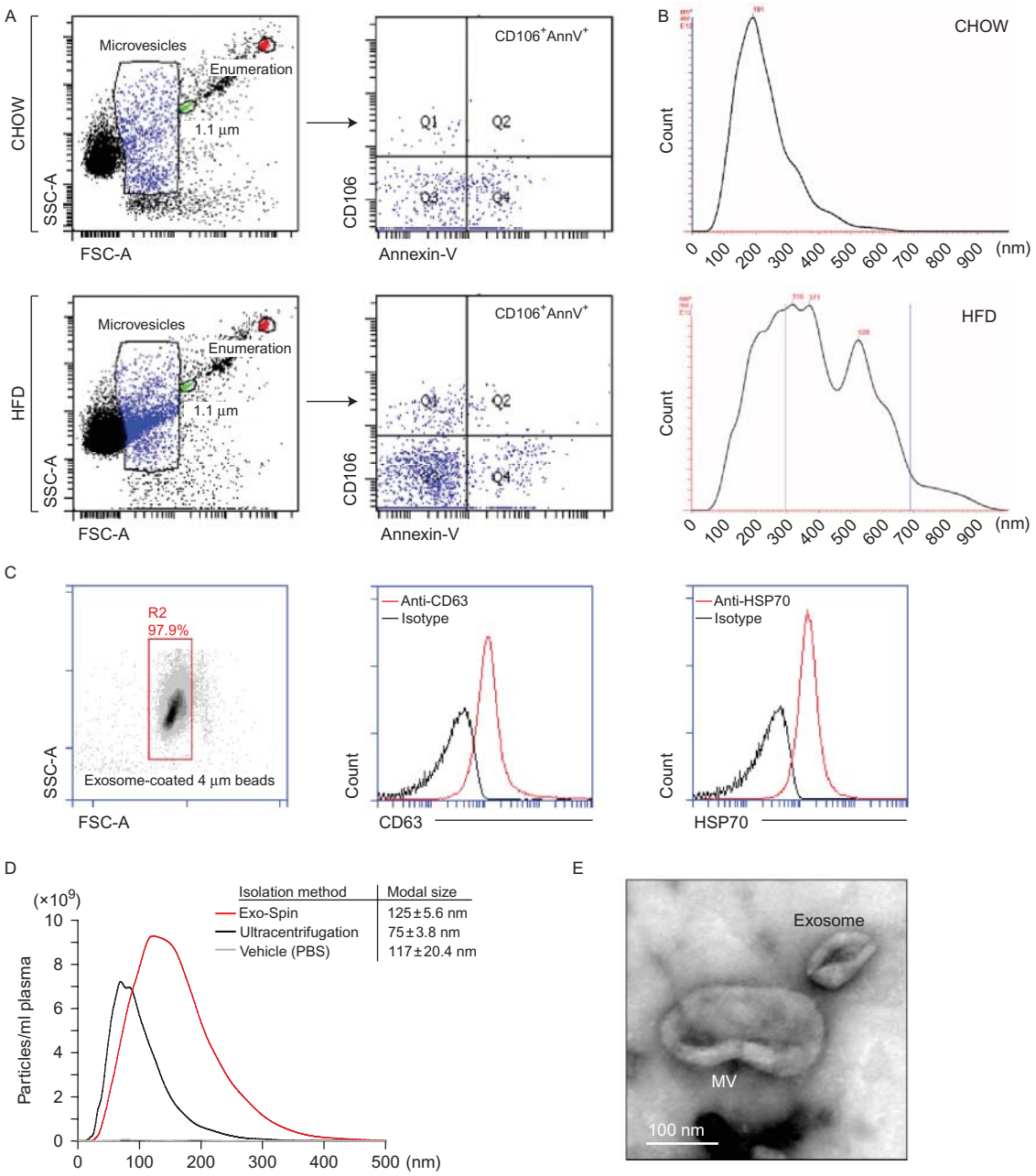

Figure 3

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 ultracentrifugation (black line) or using the Exo-spin (Cell Guidance Systems, Cambridge, United Kingdom) commercial kit (red line). (E) Electron micrograph of MVs and exosomes.

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 with 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

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in the same study (Fig. 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 optimized to enable detection of smaller particles. The use of surface markers for phenotyping MV has been reviewed elsewhere (Macey *et al*. 2011, Lacroix & Dignat-George 2012).

Flow cytometry (FCM) 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, nanoparticle tracking analysis (NTA, Fig. 3), and tunable resistive pulse sensing (van der Pol *et al*. 2010). These methods have greater size discrimination compared with 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.

EVs 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 MVs to monocytes and other cells where TF was able to exert its biological effects (Scholz *et al*. 2002, Del Conde *et al*. 2005). MVs 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). MVs 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 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*. (2007), were first to show that exosomes can also transfer mRNA and miRNA between cells. 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 (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

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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 'EVs,' 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 MVs.

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 (Jansen *et al.* 2013, Cheng *et al.* 2014, 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 & Sims (2015)).

Exosomes and MVs from different cellular sources can be identified constitutively in plasma from normal individuals (Caby *et al.* 2005, Raposo & 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 (Omoto *et al.* 1999, Diamant *et al.* 2002). Levels are further increased in those with microvascular complications (Omoto *et al.* 1999, Ogata *et al.* 2006), 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) and Lindhardsen *et al.* (2015)) and circulating EVs are increased in many inflammatory conditions (e.g., (Joop *et al.* 2001, Daniel *et al.* 2006, Suades *et al.* 2015)). Their role in propagation of endothelial pro-inflammatory cascades is also increasingly recognized and was first described by Mesri and Altieri. 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 & 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.* (2014) 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 with matched controls. 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

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patients with coronary artery disease (Chen *et al*. 2012, Wang *et al*. 2014*a*, 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) while aortic ring experiments have shown that endothelial derived EVs (obtained by ultracentrifugation at 100 000× ***g***) decrease nitric oxide (NO) and increase reactive oxygen species production, as well as impairing acetylcholine-mediated vasorelaxation (Brodsky *et al*. 2004). Consequently, MVs have gained some notoriety as potentially detrimental factors contributing to CVD.

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, Vrijsen *et al*. 2010, Jansen *et al*. 2013). 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 (Kim *et al*. 2004, Brill *et al*. 2005), 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*. 2014*b*).In this example, the detrimental effect was attributed to exosomal transfer of miR-320 and the down-regulation of its target genes (IGF-1, Hsp20, and Ets2) (Wang *et al*. 2014*b*).

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 (Kim *et al*. 2004, Brill *et al*. 2005) 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 suggest 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

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procoagulant due to the exposure of negatively charged PS which can enhance clot formation (for review see Hargett & 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 TF on the surface of endothelial- and monocyte-derived EVs (Breitenstein *et al*. 2010), as well as P-selectin glycoprotein ligand-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 & 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 microvesicles and CMR 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*. (2011) 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). 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 (Yoon *et al*. 2005, Menasche 2015, Yu *et al*. 2015).

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*. 2015). 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*. 2015). The ability to activate protective pathways does not appear to be restricted to exosomes, since MVs derived from human adult mesenchymal stem cells were also able to protect the kidney against ischaemia and reperfusion injury (Gatti *et al*. 2011).

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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.* (2012), $CD34^+$ 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. *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 & 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 & Davidson 2014). A study by Giricz *et al.* (2014) suggested that this may be the case, since RIC was not effective when EVs were removed from medium containing the factor. However, in a dose–response experiment conducted using primary adult rat cardiomyocytes the EVs released after RIC were

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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 CVD 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.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

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

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Received in final form 2 October 2015
Accepted 19 November 2015

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0201

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