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Review article

Extracellular vesicles in cardiomyopathies: A narrative review

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ABSTRACT

Extracellular vesicles (EVs) are membrane-bound particles released by all cells under physiological and pathological conditions. EVs constitute a potential tool to unravel cell-specific pathophysiological mechanisms at the root of disease states and retain the potential to act as biomarkers for cardiac diseases. By being able to carry bioactive cargo (such as proteins and miRNAs), EVs harness great potential as accessible "liquid biopsies", given their ability to reflect the state of their cell of origin. Cardiomyopathies encompass a variety of myocardial disorders associated with mechanical, functional and/or electric dysfunction. These diseases exhibit different phenotypes, including inappropriate ventricular hypertrophy, dilatation, scarring, fibro-fatty replacement, dysfunction, and may stem from multiple aetiologies, most often genetic. Thus, the aims of this narrative review are to summarize the current knowledge on EVs and cardiomyopathies (*e.g.*, hypertrophic, dilated and arrhythmogenic), to elucidate the potential role of EVs in the paracrine cell-to-cell communication among cardiac tissue compartments, in aiding the diagnosis of the diverse subtypes of cardiomyopathies in a minimally invasive manner, and finally to address whether certain molecular and phenotypical characteristics of EVs may correlate with cardiomyopathy disease phenotype and severity.

1. Introduction

Extracellular vesicles (EVs) are small lipid bilayer-delimited particles released from all cell types, known to mediate intercellular communications, and play a pivotal role in various physiological and pathological processes. During their formation, EVs are equipped with surface molecules deriving from their parent cells, as well as selected cytosolic content. Secreted by various tissues into most biological fluids (*i.e.*, blood, saliva, lymph, pericardial fluid, urine), EVs transport molecular cargo consisting mainly in proteins, microRNA, and lipids, whose abundance and composition vary according to the physiological state of the releasing cell [1] (Fig. 1). Owing to this feature, EVs possess significant potential as non-invasive biomarkers (referred to as a liquid biopsy), providing diagnostic and prognostic insight into pathophysiological processes in organs and tissues that are generally difficult to access [1]. While considerable challenges persist in terms of detection methodologies and clinical translation, there is a growing recognition of the

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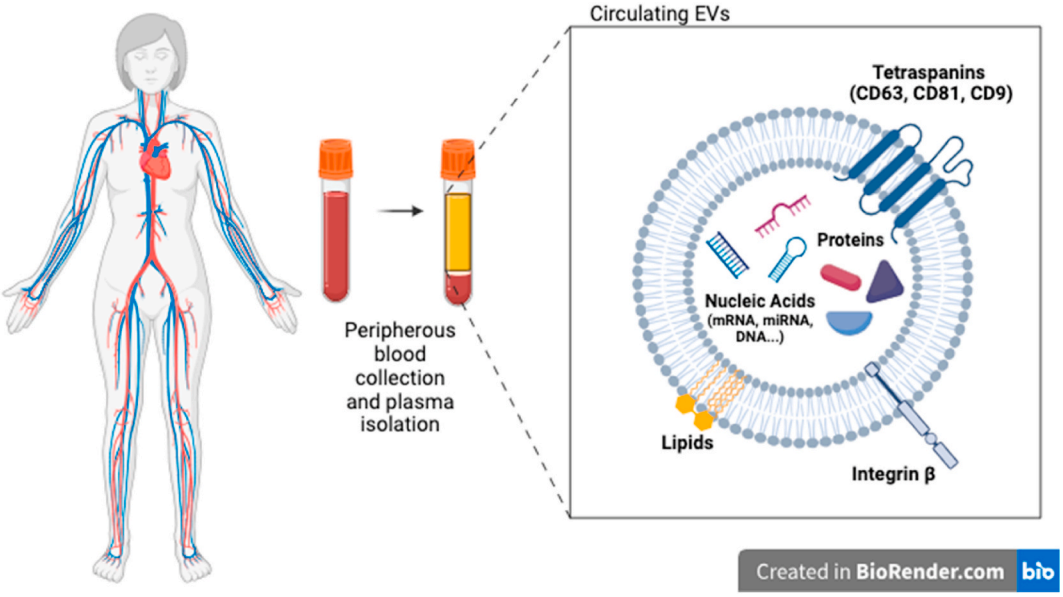

Fig. 1. *Circulating Extracellular vesicles.* Extracellular vesicles (EVs) are phospholipid-bilayer membrane-enclosed nanoparticles released by all cell types into most biological fluids. They constitute a readily accessible snapshot of the physiological state of their originating donor cell, owing to their ability to carry cell-specific molecular cargo (*e.g.,* nucleic acids and proteins). CD, cluster of differentiation.

relevance of EVs in cardiovascular research. Indeed, EVs have been related to atherosclerosis, coronary artery disease, ischemia, myocardial infarction, graft occlusion, and heart failure [2,3]. Conversely, evidence regarding the role of EVs in cardiomyopathies is lacking.

Cardiomyopathies refer to myocardial disorders affecting the heart muscle, in which structural and functional abnormalities are observed, in the absence of diseases such as coronary artery disease, hypertension, valvular disease, or congenital heart disease that could account for the observed abnormalities. According to the 2023 European Society of Cardiology (ESC) guidelines for the management of cardiomyopathies, morphological traits (ventricular hypertrophy: left and/or right; ventricular dilation: left and/or right; non-ischemic ventricular scar) and functional characteristics (global and/or regional ventricular systolic and/or diastolic dysfunction) are clinically employed to categorize five distinct cardiomyopathy phenotypes: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), non-dilated left ventricular cardiomyopathy (NDLVC), arrhythmogenic right ventricular cardiomyopathy (ARVC), and restrictive cardiomyopathy (RCM). Furthermore, the guidelines also specify the existence of syndromic and metabolic cardiomyopathies, including Anderson–Fabry disease, RASopathies, Friedreich ataxia, and Glycogen storage disorders [4].

Whilst this phenotypic description is essential in paving the diagnostic and therapeutic pathway, the exact evolving nature of cardiomyopathies, along with their underlying aetiological complexities, are yet to be fully elucidated [5]. Within this context, biomarkers represent putative tools for identifying high-risk patients in a prompt manner, unveiling potential risk associations with disease progression and outcomes, and may also provide insights on unexplored molecular mechanisms at the basis of the pathophysiology of these disorders.

Thus, the aim of the present narrative review is to summarize the current knowledge on EVs in the setting of cardiomyopathies and to elucidate whether certain molecular and phenotypical characteristics of EVs (*e.g.*, miRNA content) may correlate with cardiomyopathy phenotypes and severity.

2. Extracellular vesicles – an umbrella term

Within the cellular microenvironment, cell-to-cell communication plays a crucial role in the healthy state and during pathophysiological stress [6]. Inter- and extracellular communication is a phenomenon based on paracrine and endocrine signals, direct cell-to-cell contact, and also is recently known to depend on a heterogeneous family of small, membrane-limited vesicles, known as 'extracellular vesicles' (EVs). According to the 2018 position statement for the study of extracellular vesicles defined by the International Society for Extracellular Vesicles (ISEV) [7], EVs is a term used to refer to particles naturally released from almost all cell types.

EVs are delimited by a lipid bilayer, devoid of a functional nucleus, capable of transporting nucleic acids (*e.g.*, DNA, RNA and noncoding-RNA), proteins, lipids and whole and/or fragmented cellular organelles (*e.g.*, mitochondria) [8]. Initially described as a means of eliminating cellular waste, EVs are now gaining growing interest given their implications in both physiological and pathological processes [9,10]. The ability of delivering biologically active cellular cargo from a 'donor' cell to a 'recipient' cell supports the involvement of EVs in many intercellular communication networks, regulating specific cellular processes and inducing distinct target

cell responses [11].

Although copious amounts of literature mainly rely on size- and biogenesis-based definitions for EV description (*e.g.,* exosomes, microvesicles and apoptotic bodies), the overlapping range of dimensions, similar morphology and variable composition among EV subtypes renders this classification unfit for precise discrimination and has been found to be burdened by contradictory definitions and inaccurate experimental expectations. It is for these reasons that the ISEV recommend that EV description be performed according to the following operational terms: a) size, according to which EVs may be defined as small ('sEVs', <100 nm or <200 nm) or medium/large ('mEVs'/'lEVs', >200 nm); b) density range (low, middle, or high); biochemical composition, referring to the presence of specific transmembrane proteins known as tetraspanins which certain EVs are highly enriched in, such as Cluster of Differentiation 63 (CD63), CD81, CD9 or Annexin A5; c) state or conditions of the cell of origin (*e.g.,* 'hypoxic EVs', 'cardiomyocyte-derived EVs') [7]. EVs isolation is achieved according to one and/or more of these characteristics by a multitude of techniques.

3. Extracellular vesicles: isolation, quantification, and characterization

Despite the advances in knowledge from both theoretical and technical standpoints within the field of EVs, some drawbacks still stand. For instance, lack of standardization in the methods of isolation and in the identification of markers able to accurately distinguish vesicle subsets are present issues in this specific research field.

3.1. Blood collection for EVs

When it comes to exploring the potential EVs yield as biomarker elements, peripheral venous blood is generally considered the ideal source, given its straightforward collection and being a minimally invasive alternative to tissue biopsies. The method chosen for EV isolation determines the final sample's purity and quantity, and has important impact on downstream results [12].

For ideal plasma preparation, blood must undergo anticoagulation followed by centrifugation to remove cellular components such as erythrocytes, leukocytes, and platelets. This step is crucial to avoid excess platelet-derived EV production which occurs during platelet activation and clot formation, often an issue when performing serum preparations [13]. The quantity and characteristics of EVs separated from plasma may depend on the anticoagulant which is used at the time of blood collection (*i.e.*, EDTA or citrate), and great care is required to avoid haemolysis of the samples, which can influence EV measurements. Blood draw should be performed on fasting subjects, and once collected, the first 2 mL should be discarded [14]. Samples should be kept at room temperature and placed vertically until further processing. The time interval between blood collection and the first step of EV isolation should be kept to a minimum, or, to limit the effects on the concentration and functional activity of EVs, these time intervals should be kept constant [15].

3.2. EV isolation

EVs can be isolated according to different biophysical and biochemical properties, such as size, density, and antigen exposure. The isolation method chosen depends on the biological source and can affect the concentration of EVs in the final preparation.

Differential centrifugation is very commonly used to clear the starting sample from cells and debris [16]. Its principle is based on the difference in particle sedimentation coefficient (which depends on size and density), and so particles that have different sedimentation coefficients pellet at different centrifugation speeds. Differential centrifugation (1000, 2000 and 3000 g) followed by ultracentrifugation (between 100,000 and 200,000 g) is the most commonly employed approach for EV isolation, being easy to use and accessible, but is also quite prone to lipoprotein contamination [17].

In the context of employing EV as biomarker elements, isolation of EVs from blood plasma remains a complex task due to the presence of soluble proteins, protein aggregates and lipoproteins whose biophysical features, such as size and density, intersect greatly with those of EVs. Lipoproteins outnumber circulating EVs by several orders of magnitude, and therefore represent the most common co-isolate in blood derived-EV preparations [18]. Depending on the lipidic and protein content, size and density, circulating lipoproteins may be classified as high-density lipoproteins (HDLs), low density lipoproteins (LDLs), very low-density lipoproteins (VLDLs), lipoprotein a (Lp(a)), and chylomicrons. Lipoproteins further complicate the sole analysis of EV-associated RNAs, as they also are capable of transporting miRNAs [19]. While guidelines recommend to remove lipoprotein contamination to the greatest extent from EV preparations in order to avoid artefacts in downstream analyses, efficient separation of lipoproteins from EVs with the available techniques is still far from being a feasible procedure.

Density gradient centrifugation (DGC) is based on the difference in sedimentation and flotation coefficients of the particles. DGC applies a density gradient to isolate EVs, which distribute throughout a density gradient matrix according to their particular sedimentation and flotation index [20].

Size exclusion chromatography (SEC) is a size- and shape-based method for EV isolation. SEC is able to remove 99 % of soluble plasma proteins, along with >95 % of HDL, retains EV integrity and does not impact their biological activity. This method has the advantage of being fast and straight-forward and does not require large starting sample volumes. Whilst the frequency of its use is increasing, particles above the size cut-off, including chylomicrons, VLDLs and LDLs tend to co-isolate with the final EV isolate [21].

Ultrafiltration is an isolation method based on particle size. The use of membrane filters provides enrichment of EVs based on their size in relation to membrane pore size, enabling their separation from soluble components. This method is however incapable of removing lipoprotein contamination [22].

Immunoaffinity is based on the interaction between specific EV surface proteins and antibodies, which can be employed to select desired EV populations (immuno-enrichment) or to trap unwanted EV populations (negative selection or immuno-depletion). Through

this technique all particles, apart from those bound to the protein of interest, are removed efficiently. The main drawback of this methodological approach consists in its high costs, low scalability and also the poor biological activity of the isolated EVs [23].

3.3. Quantification and characterisation of EVs

According to the MISEV 2018 guidelines, it is recommended that EV preparations be quantified relative to their source (*e.g.*, number of secreting cells, biofluid volume, mass of tissue) and that their abundance is to be determined according to total particle number and/or protein or lipid content of the EV preparation [7].

Methods used for EV characterisation may include the following: Optical single particle tracking (Nanoparticle Tracking Analysis (NTA)), Electron Microscopy, Atomic force microscopy, and Flow cytometry [24].

Among methods for EV quantitation, *NTA analysis* is the most common. It allows an estimation of the size and concentration of EVs within a given sample by tracking their Brownian motion with a microscope. The main limitation of this method consists in the difficulty in distinguishing EVs from non-EV particles, along with the inability to highlight biochemical composition or cellular origin [25].

Flow Cytometry is a method of growing interest for both qualitative and quantitative analysis of EVs, however is seldom straightforward. The employment of this method for EV analysis is ridden with complex technicalities, owing mainly to the small size of EVs which renders their analysis challenging [26].

For the characterisation of the vesicular structure, *Transmission Electron Microscopy* and *Atomic Force Microscopy* allow visualization at the single EV level, permitting assessment of their size, the presence of contaminants, integrity and morphology [24].

3.4. EV cargo

The nature and abundance of EV cargoes are influenced by the type and state of the cell from which they derive, along also with the stimuli that modulate their release, whether they be of physiological or pathological nature [27].

EVs can carry a wide variety of bioactive molecules, including proteins, RNA, and lipids, that reflect the state of the originating cell and may be transferred to specific recipient cells, harnessing phenotypic and functional effects. In light of their functional relevance in intercellular signalling and cell-to-cell communication, along with the peculiarities of their intraluminal molecular content, EVs are spurring great interest as putative biomarkers for disease detection and monitoring [28].

Particularly, EVs are enriched in multiple subtypes of RNA molecules. These include messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA fragments (rRNA), circular RNA (circRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), small interference RNA (siRNA), long-non-coding RNA (lncRNA), microRNA (miRNA) and Retrotransposons (for a more detailed description refer to Toribio et al. [29]).

In 2007, Valadi et al. were the first to identify the presence of mRNAs and miRNAs in mast cell-derived EVs, along with their ability to target the transfer of RNA to recipient cells [30]. miRNAs are short non-coding RNA molecules made up of around 19–22 nucleotides which regulate post-transcriptional gene expression by binding to certain mRNAs and inducing their degradation and/or translational inhibition [31]. In this manner, miRNAs contribute to physiological and pathological processes, and therefore yield great biomedical interest as disease biomarkers. EVs are known to encompass miRNAs in the peripheral circulation to shield them from circulating ribonucleases, which would otherwise catalyse their degradation [32]. The implication of EV-miRNAs in modulating biological activities at the molecular level within recipient cells is a debated topic and remains unclear. Chevillet et al., reported a stoichiometric and quantitative analysis of miRNA content of EVs, in which EVs of multiple biological and cellular sources were quantified and compared to the number of miRNA molecules. Regardless of the source, less than one molecule of miRNA was identified per EV particle on average, suggesting the lack of biological and functional significance of EV-derived miRNA in intercellular communication [33]. This hypothesis was further supported by a different group, which demonstrated how the concentration of miRNAs contained within EVs was far below assumed functional levels, and so stating the implausibility that EV-derived miRNAs are capable of modulating target transcript levels in recipient cells [34]. Overall, the role of EV-miRNAs remains enticing when it comes to their potential in acting as predictors of certain disease states, given that also their expression patterns have been associated with cardiac diseases [35].

According as to whether they may be membrane-bound, present in the EV transmembrane or kept within the EV lumen, proteins also represent important elements which may be exploited for EV sub-classification and/or as indices of physiological and pathological states. Some of the common proteins shared by different EV subtypes consist in actin, ezrin, moesin, Heat Shock Cognate 71 kDa (HSC70), Heat shock protein 70 (HSP70), annexin II, CD63, CD9, integrins and more [36]. Even if multiple proteomic studies have indicated Alix (programmed cell death 6-interacting protein), TSG101, CD63 and CD9 as exosomal markers, the field of EV research has not matured to the point that a list of EV-specific ‘‘markers’’ that distinguish EVs from each other can be proposed, as different subsets of EVs contain many common markers. What is interesting is that the EV protein profiles vary even in early stages of disease, further underlying the potential of these membrane-bound vesicles of early biomarkers [37].

While lipids consist in invariable structural components of EVs and are fundamental for their formation and stability within the extracellular environment, bioactive lipid mediators shuttled by EVs also cover a significantly relevant role in EV biology. Indeed, EVs constitute of membrane lipids organised in a bilayer structure but can also carry various bioactive lipids. Among the entire pool of cellular lipids, cholesterol has been found to be the most enriched within EVs, displaying significant roles in guiding EV biogenesis, release, and uptake in recipient cells [38].

4. EVs and cardiomyopathies

In the setting of cardiomyopathies, the heart undergoes extensive cardiac remodelling that results in cardiac fibrosis deposition, contributing in turn to electrical and structural remodelling, ultimately leading to decreased cardiac function and, in some cases, heart

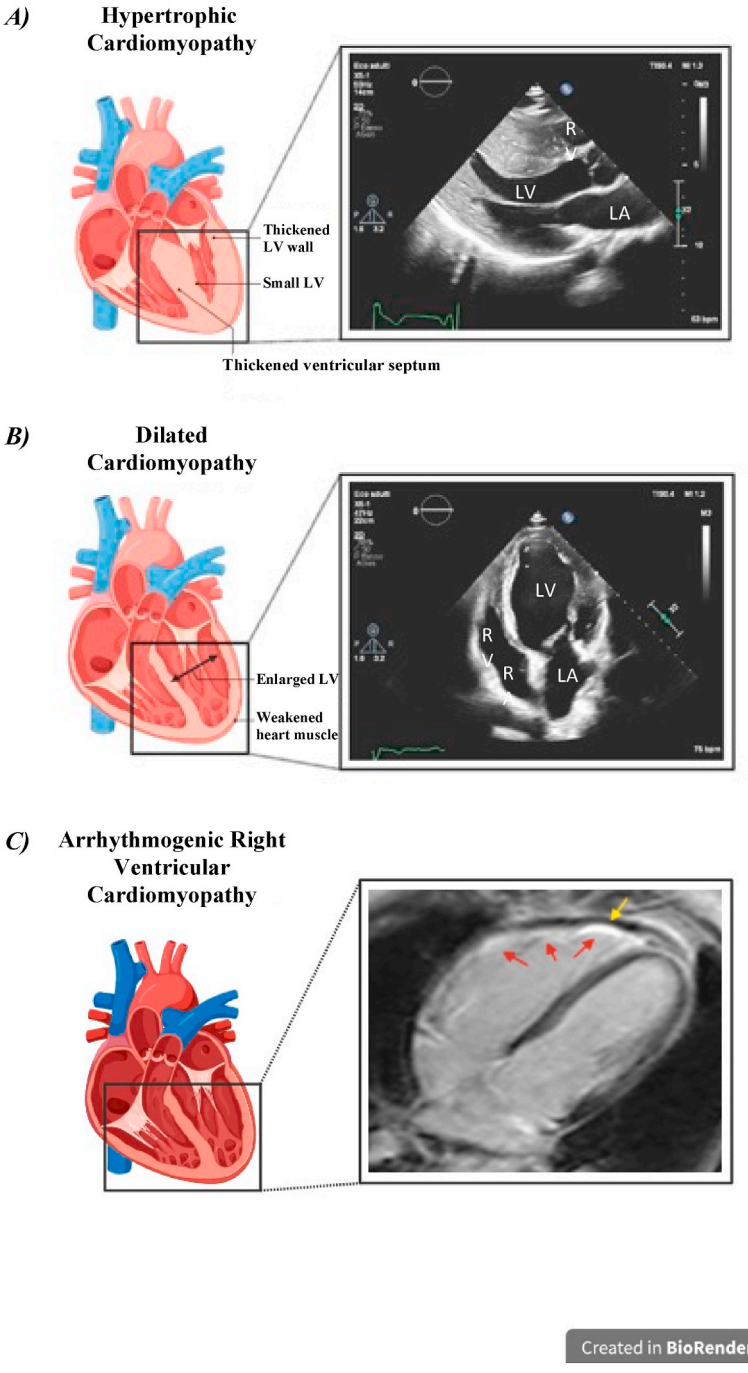

Fig. 2. *Echocardiographic representation of HCM, DCM and ARVC phenotypes*. Panel A displays a parasternal long-axis echocardiographic view showing a patient affected by severe HCM with typical asymmetric hypertrophy involving the interventricular septum. Panel B displays an apical four-chamber echocardiographic view showing the heart of a patient with DCM, with an extremely dilated left ventricle (volume was 142 mL/m^2). Panel C displays late gadolinium enhancement by CMR imaging of a patient affected by ARVC. A dilated right ventricle with extensive wall fibrosis (yellow arrow) and free-wall bulging (red arrows) can be appreciated. HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; ARVC, arrhythmogenic right ventricular cardiomyopathy; LV, left ventricle, LA, left atrium; RA, right atrium; RV, right ventricle.

failure [39]. It is well established that the majority of cases of cardiomyopathies are of genetic aetiology, specifically determined by the presence of mutations in genes encoding cardiac proteins (*e.g.*, sarcomeric, cytoskeletal and nuclear envelop proteins). This section particularly refers to HCM, DCM and ARVC phenotypes, of which the literature concerning the association with EVs is most abundant. For other phenotypes defined recently according to the 2023 ESC clinical classification (*e.g.*, NDLVC, RCM and syndromic and metabolic cardiomyopathies) [4], data on the role of EVs is currently lacking.

4.1. EVs in HCM

4.1.1. Clinical definition

With a prevalence of one in 200–500 adults in the general population worldwide, HCM is the most common monogenic cardiovascular disorder, often underrecognized in clinical practice [40]. HCM is characterized by hypertrophied, nondilated left ventricle, occurring independently of altered loading conditions (*e.g.*, hypertension or aortic valve stenosis) that would typically induce such a degree of hypertrophy (Fig. 2A). Cardiac hypertrophy is normally asymmetrical, mainly involving the basal interventricular septum, close to the aortic valve, sometimes leading to left ventricular (LV) outflow tract obstruction and/or systolic anterior motion of the mitral valve. Hypertrophy is also observed at the cellular level, as cardiac myocytes are visibly enlarged and disorganized. For suspected cases of HCM, the initial tests rely on an abnormal 12-lead ECG and echocardiography, with the latter serving as the primary imaging technique for confirming the diagnosis. Cardiac magnetic resonance or computed tomography are employed as supplementary diagnostic tools in cases where echocardiography produces inconclusive or suboptimal quality images, or when the extent of hypertrophy is borderline. A diagnosis is made when wall thickness is $\geq$ 15 mm in one or more LV myocardial segments, or $\geq$ 13 mm in the presence of a definite family history of HCM [4,41]. Furthermore, cardiac magnetic resonance is the gold standard in evaluating ventricular volumes, myocardial mass, wall thickness, biventricular function, and identifying specific features of HCM such as trabeculations, crypts, aneurysms, and non-ischemic fibrotic tissue. Indeed, the peculiar accumulation of late gadolinium enhancement within the myocardium (usually mid-wall and/or subepicardial) corresponds to the presence of focal fibrosis, while diffused fibrosis can be assessed using T1 mapping sequences and by determining the extracellular volume fractio

Up to 60 % of patients with HCM have an underlying genetic aetiology. In this case, HCM is a monogenic disorder with an autosomal dominant pattern of inheritance and incomplete penetrance, associated with 11 or more genes encoding for proteins of thick and thin myofilament contractile components of the cardiac sarcomere or Z disk. Beta-myosin heavy chain and myosin-binding protein C genes are the most involved [42]. The two most well-established causal genes for HCM which have been identified are *MYH7* (encoding for the beta-myosin heavy chain) and *MYBPC3* (myosin-binding protein C), identified in 70 % of variant-positive patients. Other known genes are *TNNI3*, *TNNT2* (cardiac troponin I), *TPM1* (α-tropomyosin), *MYL2* (myosin light chain 2), *MYL3* (myosin light chain 3), *ACTC1* (cardiac α-actin), each accounting for a small proportion of HCM cases (1 %–5 %) [43]. Thus, genetic screening plays a fundamental role in the diagnosis and management of HCM in both patients and their families. If a causative mutation is identified in a proband, clinically phenotypic screening and cascade genetic testing of the specific disease-causing variant should be offered to first-degree relatives to identify other relatives who could be at risk of disease.

4.1.2. EVs

Alterations in miRNA levels are associated with dysfunctional gene expression profiles observed in various cardiovascular disease conditions, including heart failure and cardiac hypertrophy. EV-derived miRNAs (specifically miRNA passenger strands which normally undergo degradation) were described as paracrine signalling mediators between cardiac fibroblasts and cardiomyocytes and were involved in the development of cardiomyocyte hypertrophy [44]. Cardiac fibroblasts represent one of the largest cell populations in the heart, contributing to structural, biochemical, mechanical and electrical properties of the myocardium. Fibroblast EV-derived miR-21_3p (miR-21*) induces cardiomyocyte hypertrophy, possibly by targeting sorbin and SH3 domain-containing protein 2 (SORBS2) and PDZ and LIM domain 5 (PDLIM5). To corroborate this hypothesis, when SORBS2 or PDLIM5 were silenced, the hypertrophy of cardiomyocytes was induced [44]. SORBS2 is a structural component of the sarcomere Z-line and is required for cardiomyocyte differentiation and the integrity of the sarcomere structure [45]. Cardiomyocyte-specific PDLIM5 knockout mice develop cardiomyopathy, suggesting PDLIM5 to play a major role in cardiac muscle structure and function [46].

In line with this finding, passenger strand miRNA-27a*-enriched EVs were hypothesized to act as drivers of cardiac hypertrophy. Indeed, following treatment with Ang II primary, rat cardiac fibroblasts release EVs enriched in miRNA-27a* that once taken up by cardiomyocytes lead to downregulation ofPDLIM5 protein [47]. Conversely, exogenous administration of miRNA27a* mimics, in cardiomyocytes, inhibits PDLIM5 translation whereas a miRNA27a* inhibitor enhanced the expression of PDLIM5.

In search of mechanistic pathways explaining cardiac hypertrophy, it has been demonstrated that Ang II induces an enrichment of myocyte-derived EVs with Hsp90 and IL-6 whose fate is to transmigrate to fibroblasts. This was seen to lead to the activation and maintenance of signal transducer and activator of transcription 3 signalling in cardiac fibroblasts, resulting in excessive collagen synthesis and deposition in the myocardium during cardiac hypertrophy [48]. When isolated from obese and hypertensive individuals (comorbidities for cardiomyopathy), endothelial derived-EVs caused an increase in the expression of key intracellular proteins involved in cardiomyocyte hypertrophy and fibrosis (*e.g.*, cTnT and α-actinin), determining a reduction in cardiomyocyte eNOS activation and NO production [49].

Among the broad spectrum of small noncoding RNAs transported by EVs, a differential cargo of small nucleolar RNAs have been identified in patients with a HCM-associated mutation ($c.ACTC1^{G301A}$) [50]. Specifically, the differential expression of 12 snoRNAs was observed in HCM human induced pluripotent stem cell (hiPSC)-cardiomyocyte-derived EVs compared to a control counterpart (WT hiPCS-cardiomyocyte-derived EVs). These included 10 SNORDs and 2 SNORAs. Furthermore, HCM hiPSC-CM EVs showed an increase

in transcripts associated with epidermal growth factor and fibroblast growth factor signalling, 5-HT receptor-mediated signalling, angiotensin receptor signalling, beta 1 and beta 2 adrenergic signalling, and G protein receptor signalling [50]. Table 1 summarizes the main data reported in this section.

4.2. EVs in DCM

4.2.1. Clinical definition

DCM is defined by the presence of LV dilatation and systolic dysfunction (LV ejection fraction <50 %) unexplained solely by abnormal loading conditions or coronary artery disease. LV dilatation is defined by LV end-diastolic dimensions or volumes >2 z-scores above population mean values corrected for body size, sex, and/or age. For adults this represents an LV end diastolic diameter >58 mm in males and >52 in females and an LV end diastolic volume index of ≥75 mL/m2 in males and ≥62 mL/m^2 in females measured by echocardiography [50] (Fig. 2B). Pathophysiological changes include a decrease in stroke volume and cardiac output, impaired ventricular filling, and an increase in end-diastolic pressure. While genetics play a predominant role in their aetiology (up to 35–50 %), the contribution of nongenetic factors to the development of cardiomyopathies must not be ignored. Namely, inflammation of the myocardium due to an infection (mostly viral), exposure to drugs, toxins or allergens, and systemic endocrine or autoimmune diseases are all well-established causes [51]. A precise diagnosis of the underlying aetiology requires a wide array of non-invasive and invasive examinations (*e.g.*, genetic testing and comprehensive cardiac imaging), whereas if no identifiable cause is found (except genetic), a clinical diagnosis of 'idiopathic DCM' is assigned. Cardiac magnetic resonance is necessary to ensure a thorough diagnosis and to stratify the prognosis through precise measurement of clinical features such as volumes, identification of late gadolinium enhancement, myocardial oedema, fibro-fatty degeneration, and accurate quantification of any associated functional valvulopathy (*e.g.*, mitral and/or tricuspid regurgitation) [52].

As DCM remains asymptomatic until severe disease development, an early identification of the condition in at-risk family members offers the chance to start any treatment before the disease progresses to its later phases. Thus, when a causative genetic mutation is found in a proband, a family based genetic cascade evaluation of first-degree relatives is recommended, along with cardiovascular imaging screening to assess LV volume and define the phenotype [53].

Regarding the genes associated with DCM, mutations in those encoding cytoskeletal, sarcomeric, mitochondrial, desmosomal, nuclear membrane, and RNA-binding proteins have been the most frequently identified [54]. Mutations of the genes encoding for sarcomere proteins, titin (*TTN*) truncating mutations and lamin A/C (*LMNA)*, which are inherited in an autosomal dominant manner, are considered the major genetic factors responsible for the development of DCM. They account, respectively, for 15–25 % and 5–10 % of DCM cases(66). Titin-truncating mutations lead to decreased length-dependent activation and increased elasticity of myofibrils whereas mutations in *MYH7* (β-myosin heavy chain), *TNNT2* (cardiac troponin T), and *TPM1* (α-Tropomyosin) are found in roughly 2 %–4 % of DCM cases. On the other hand, mutations in *MYBPC3* (Myosin-binding protein C) are rare. Regarding mutations in *LMNA*, these lead to disturbances in several structural and cytoskeletal components of the cell, including microtubules, cytoskeleton actin, and intermediate filaments. Concerning the genes encoding cardiac cytoskeletal proteins, *FLNC* (filamin C) mutations are the most frequently described in DCM. Compared to the genetic screening of patients with HCM, in the case of DCM, genetic screening serves not only as a diagnostic tool but also provides useful prognostic information for risk stratification, especially for arrhythmic risk. This can aid in clinical and therapeutic decision-making. Patients with *LMNA, DES, DSP, PLN, TMEM43, RMB20* and *FLNC* mutations, particularly in the case of nonsense mutations, generally have a worse prognosis, with a high risk of sudden cardiac death due to conduction defects and/or ventricular arrhythmias [54].

It is important to note that according to the recently published 2023 ESC guidelines for the management of cardiomyopathies,

Table 1
Extracellular vesicles in the context of hypertrophic cardiomyopathy.

Experimental model	Finding	Reference
Neonatal rat cardiac fibroblasts and cardiomyocytes were isolated from newborn rats by enzymatic digestion.	EV-derived miR-21* regulates the expression of SORBS2 and PDLIM5 in cardiomyocytes, a structural component of the sarcomere Z-line.	Bang [44]
Neonatal cardiomyocytes and fibroblasts were isolated from left ventricles of heart of postnatal day 3 rat pups (Sprague-Dawley rats).	miRNA-27a*-enriched EVs derived from fibroblasts were hypothesized to act as drivers of cardiac hypertrophy by regulating PDLIM5 in cardiomyocytes.	Tian C [47]
Neonatal myocytes were isolated from the hearts of 2- to 3-day-old rat pups; Cardiac fibroblasts were isolated from the hearts of 28-week-old rats.	Myocyte-derived exosomes are responsible for the excess collagen synthesis by cardiac fibroblast.	Datta [48]
Endothelial cell-derived EVs from obese/hypertensive adults.	Endothelial derived-EVs caused an increase in the expression of key intracellular proteins involved in cardiomyocyte hypertrophy and fibrosis (*e.g.*, cTnT and α-actinin).	Fandl [49]
hiPSC lines were generated from patients with a HCM-associated mutation (c.*ACTC1*^{G301A}) and isogenic controls created by correcting the mutation using CRISPR/Cas9 gene editing technology.	Omics analysis of HCM hiPSC-cardiomyocytes EVs, isolated from *c.ACTC1*^{G301A}, show a different pattern of differential snoRNA cargo and increased in transcripts associated with EGF and FGF signalling, 5-HT receptor-mediated signalling, angiotensin receptor signalling, beta 1 and beta 2 adrenergic signalling, and G protein receptor signalling.	James [50]

ACTC1, actin alpha cardiac muscle 1; cTNT, Cardiac Troponin T; EGF, epidermal growth factor; FGF, fibroblast growth factor; hiPSC, Human-induced pluripotent stem cell; HCM, hypertrophic cardiomyopathy; PDLIM5, PDZ and LIM domain 5; SORBS2, Sorbin and SH3 Domain Containing 2.

forms which have been variably classified as DCM to date may now fall under the newly defined phenotype known as NDLVC, defined as the presence of non-ischaemic LV scarring or fatty replacement regardless the presence of global or regional wall motion abnormalities, or isolated global LV hypokinesia without scarring [54]. Since this new phenotype has just been introduced into the classification, to date there are no data available regarding the involvement of EVs in this precise setting.

4.2.2. EVs

Considering that mechanisms underlying fibroblast activation in patients with familial DCM are still unclear, EVs from patients with familial DCM have been isolated and characterized to explore their putative involvement in promoting fibrogenesis. Compared to EVs isolated from healthy controls, those isolated from plasma of patients with DCM caused the upregulation of fibrotic gene expression in cardiac fibroblasts. DCM-derived EVs led to an increase in the expression of collagen I, collagen III, and to connective tissue growth in the heart, promoting cardiac fibrogenesis when injected in CD-1 mice. At the molecular level, miR-218-5p was identified as one of the key regulators of these processes through the activation of TGF-β cascade, responsible for suppressing the expression of the inflammatory inhibitor TNFAIP3. As a proof-of-concept, the silencing of miR-218-5p ameliorated the profibrotic effect mediated by DCM-derived EVs on cardiomyocytes [55]. In general, an inflammatory environment favours the infiltration of macrophages into the damaged heart, which further fuel inflammation, angiogenesis, and cardiac remodelling. In this context, the injection of mesenchymal stem cell-derived EVs can regulate the balance between M1 and M2 via the JAK2/STAT6 axis, a phenomenon which may favour an anti-inflammatory environment, and consequently cardiac repair. These were the results of a study in which mice with an established doxorubicin-mediated DCM received an intravenous injection of mesenchymal stem cell-derived EVs. They displayed improved cardiac function, attenuated cardiac dilatation, and reduced apoptosis in cardiomyocytes [56]. Also cardiovascular progenitor cell-derived EVs, when injected in a mouse model of chronic heart failure, were found to reduce the levels of pro-inflammatory cytokines and increased the levels of anti-inflammatory cytokine IL-10 [57]. Chronic activation of myocardial renin angiotensin system increases the local levels of angiotensin II (Ang II), inducing pathological cardiac hypertrophy. In rat neonatal cardiac myocytes and fibroblasts, Ang II stimulated cardiac fibroblasts to release EVs, which acted as paracrine mediators in the upregulation of renin, angiotensinogen, angiotensin receptor 1 and 2, and in the downregulation of Angiotensin Converting Enzyme-2 (ACE2), as well as causing an increased production of Ang II in cardiomyocytes. This could potentially explain the mechanism underlying the intercellular contact between cardiac fibroblasts and cardiomyocytes in the context of myocardial development and remodelling, This EV-mediated modulation intensified the autocrine activation of the Ang II–angiotensin receptor 1 axis, contributing to pathological cardiomyocyte hypertrophy [58]. At the molecular level, it seems that EVs released by cardiac fibroblasts activate mitogen-activated protein kinases (MAPK) and Akt pathways. To unravel the role of EVs in the paracrine communication between cardiac fibroblasts and cardiomyocytes, EV inhibitors (GW4869 and dimethyl amiloride) were administered in adult mice previously treated with Ang II. As a consequence, the Ang II-induced release of EVs was blocked and Ang II-induced myocardial hypertrophy and cardiac fibrosis were prevented [58].

The proteomic signature of plasma derived-EVs isolated from patients with DCM showed that 51 proteins were exclusively expressed in individuals with DCM compared to EVs isolated from 15 controls, matched for age and sex. Fibrinogen (α, β and γ chain), serotransferrin, α-1-antitrypsin, and a variety of apolipoprotein family members (C–I, C-III, D, H or β-2-glycoprotein, and J or clusterin) were clustered in EVs derived from DCM patients. A rise in stress response was also observed [59]. In paediatric patients with heart failure, DCM is the most common indication for heart transplantation in children over the first year of age. LV systolic pump function is impaired, leading to progressive cardiac enlargement and hypertrophy. Treating primary cardiomyocytes with EVs isolated from patients with DCM directs their gene expression towards a pattern similar to that found in diseased hearts (atrial natriuretic peptide and B-type natriuretic peptide were raised). This effect was reverted when the receiving primary cardiomyocytes (the receiving cells) were pre-treated with cytochalasin D, an inhibitor of the uptake of EVs [60]. The results of this study describe an independent contribution of EV cargo on pathological hypertrophy in cardiomyocytes, regardless the involvement renin-angiotensin-aldosterone system.

Additionally, a significant increase of serum exo-miR-92b-5p was found in patients affected by DCM, along with noticeable correlations to ultrasound indices often used in the clinical setting (*e.g.*, left atrial diameter, LV diastolic diameter and LV ejection fraction), indicating the potential use of this EV-miR as a biomarker for the diagnosis of DCM, given also its cost-effectiveness when compared to the usual indices that are employed [61].

Considering that endothelial cells are among the 5 major cell types of the heart, the role of endothelial cell-derived-EVs cannot be underestimated. Cardiomyocyte-derived EVs can be considered as key components of the cardio-endothelial communication system, enabling close-contact trafficking of glucose and metabolites from the blood stream between the two cellular compartments. Indeed, under conditions of energetic stress (such as glucose deprivation), cardiomyocytes release EVs loaded with glucose transporters that, after interaction with endothelial cells, induce glucose uptake and activate glycolytic pathways in recipient cells. These EVs had an increased lactate dehydrogenases activity, which lead to an increase in pyruvate production within the endothelium [62].

EVs isolated from endothelial cells overexpressing Kruppel-like family of transcription factor 2 (KLF2) improved cardiac function and inhibited ventricular remodelling when administered to a mouse model of doxorubicin-induced DCM [63]. KLF2 is enriched in the endothelium, is regulated by shear stress and cytokines, and directly mediates the expression of key endothelial genes (such as endothelial nitric oxide synthase). Furthermore, EV enriched in KLF2 determined an increase in the expression of anti-inflammatory factors and a decrease in proinflammatory factors in human umbilical vein endothelial cells [63]. In line with these findings, changes in miRNAs content of EVs isolated from patients receiving doxorubicin was a step preceding the development of the late onset cardiomyopathy phenotype. Gene ontology analyses show that the miRNAs mostly affected by doxorubicin were those involved in TGF-β signalling and in the processes linked to therapy-triggered fibrosis and senescence [64].

Table 2 summarizes the main data reported in this section.

4.3. EVs in ARVC

4.3.1. Clinical definition

According to the 2023 ESC guidelines, ARVC is a cardiomyopathy (population frequency estimated to be 1:1000 to 5000) characterized structurally by a progressive myocardial atrophy with fibro-fatty replacement of the RV myocardium. Lesions can also be present in the LV myocardium and predominant LV disease can coexist in the same family. For this reason, some authors still prefer to adopt the "umbrella term" arrhythmogenic cardiomyopathy to define the broader spectrum of the phenotypic expressions of this disease [64].

The main histological feature consists in myocyte depletion with fibrofatty ventricular myocardium replacement. Myocardial atrophy initiates within the epicardium and extends toward the endocardium, becoming transmural is a process. This process occurs over time and results in gradual thinning of the heart wall. The clinical picture is characterized by ventricular arrhythmias and impairment of ventricular systolic function. Genetic aetiology accounts for the majority of ARVC cases and, although most non-syndromic ARVC is inherited in an autosomal dominant pattern, recessive patterns also exist [65] (Fig. 2C). ARVC is typically genetically determined by abnormalities of cardiac desmosomes, which determine the detachment of myocytes and the alteration of intracellular signal transduction. Indeed, the most recognized genes that account for the majority of pathogenic or likely pathogenic variants in ARVC include those encoding cardiac desmosomal proteins (*e.g.*, plakophilin (PKP2), desmoplakin (DSP), desmoglein (DSG2) and desmocollin (DSC2)) or non-desmosomal proteins (*e.g.*, such as phospholamban (PLN), filamin C (FLNC), desmin (DES)) [66]. The clinical diagnosis workout includes electrophysiological parameters (ECG), cardiac imaging findings (cardiac magnetic resonance is the gold standard), genetic factors, and histopathologic features.

Despite the major achievements obtained regarding the understanding of the aetiology of ARVC, the molecular mechanisms underlying its pathogenesis remain unclear. This knowledge gap reflects the lack of efficient mechanism-driven therapies.

4.3.2. EVs

Despite major achievements in the understanding of the disease aetiology, the mechanism underlying ARVC pathogenesis remains unclear. Wnt/β-catenin, as well as Hippo and transforming growth factor-β pathways have been implicated in this disease. The impact of EVs in ARVS has been investigated in knock-in mutant desmoglein ($DSG2^{mt/mt}$) mice, a model which encompasses both human genetics and the clinical phenotype of arrhythmogenic cardiomyopathy, including myocardial inflammation [67]. Lin et al. isolated EVs from immortalised cardiosphere-derived cells that were previously engineered to express high levels of β-catenin, a protein which plays a crucial role in the pathogenesis of ARVC. EVs carrying high levels of β-catenin were intravenously injected for 1 month (when myocardial damage is undetectable) into $DSG2^{mt/mt}$ mice and displayed the potential to mitigate left and right ventricular dysfunction as well as fibrous tissue deposition and mitigated intercalated disc remodelling; the rate of cell death and immune response was also reduced. Looking at the electrocardiographic abnormalities, QRS duration and QTc interval were reduced in EV-treated $DSG2^{mt/mt}$ mice, there was a decreased burden and inducibility of ventricular arrhythmias with an accelerated repolarization and a faster conduction. Upon treatment with EVs, mice displayed a reduced expression of pro-inflammatory cytokines (*e.g.*, IL-1α, IL-1β, IL-2, IL-4, IL-13, TNF-α, chemokine ligand-5 and monocyte chemoattractant protein-1), as in the case of the nuclear translocation of nuclear factor-κB. Among the possible mechanisms driving the latter salutary effect, the transfer of EV-derived hsa-miR-4488 to

Table 2
Extracellular vesicles in the context of dilated cardiomyopathy.

Experimental model	Finding	
Cardiac fibroblasts; Healthy and DCM (*TNNT2*^{R173W}) iPSCs.	DCM-derived EVs, enriched in miR-218-5p, increase the expression of collagen I, collagen III, and of connective tissue growth, promoting cardiac fibrogenesis when injected in CD-1 mice.	Fu [55]
Rat neonatal cardiac myocytes and fibroblasts, and mouse adult cardiac fibroblasts.	Ang II stimulates cardiac fibroblasts to release exosomes, which in turn increase Ang II production and its receptor expression in cardiomyocytes, thereby intensifying Ang II-induced pathological cardiac hypertrophy.	Lyu [58]
Twenty DCM patients and 15 age- and sex-matched healthy controls with no cardiovascular disorders were recruited for peripheral venous blood extraction.	Proteomic analysis has shown that fibrinogen (α, β and γ chain), serotransferrin, α-1-antitrypsin, and a variety of apolipoprotein family members (C–I, C-III, D, H or β-2-glycoprotein, and J or clusterin) were clustered in EVs derived from DCM patients.	Roura [59]
iPSC-derived cardiomyocytes and neonatal rat ventricular myocytes; serum from pediatric patients with DCM.	DCM serum EVs mediate pathological responses in cardiomyocytes and may propagate the paediatric heart failure disease process.	Jiang [60]
43 patients with DCM and 34 healthy volunteers.	Serum EV-miR-92b-5p are increased in patients affected by DCM.	Wu [61]
Wistar rats were used as breeders, and pups were used for the isolation of neonatal rat cardiomyocytes and endothelial cells. Neonatal transgenic RFP mice were used also for the isolation of endothelial cells.	Under conditions of energetic stress (such as glucose deprivation), cardiomyocytes release EVs loaded with glucose transporters that, after interaction with endothelial cells, induce glucose uptake and activate glycolytic pathways in recipient cells.	Garcia [62]
C57BL/6 male mice; Human umbilical vein endothelial cells; recombinant lentivirus vector Lv-KLF2 (KLF2-transfected endothelial cells).	EVs isolated from KLF2-overexpressing endothelial cells inhibit ventricular remodelling when administered to a mouse model of doxorubicine-induced DCM. KLF2 regulates endothelial biology.	Zhang [63]

DCM, dilated cardiomyopathy; $DSG2^{mt/mt}$, homozygous knock-in mutant desmoglein-2; KLF2, Kruppel-like factor 2; TNNT2, troponin T.

cardiomyocytes constituted the most compelling evidence. The retro-orbital injection of antagomir-4488 in Dsg2$^{mt/mt}$ mice prior to each EV therapy blunted the beneficial effects of EVs, leading to deterioration of left and right ventricular function [67]. However, as highlighted in the accompanied editorial, while the role of miRNA as biomarkers has been demonstrated in several studies, none of them has yet provided compelling evidence of a pathogenetic significance [68].

Finally, since ARCV and Duchenne muscular dystrophy share many histological and molecular/cellular pathogenic mechanisms, it is worth mentioning that intramyocardial and intravenous injection of cardiosphere-derived EVs reverted many features of Duchenne muscular dystrophy by attenuating oxidative stress, mitochondrial dysfunction, inflammation, and fibrosis [69].

5. Future perspectives

Given their ability to carry specific molecular cargo reflecting the state of the releasing cell, EVs represent promising candidates for multiple clinical applications. While methodological and technical challenges have yet to be overcome (*e.g.*, the lack of biological reference material [70]), EVs yield potential as predictive markers in the diagnosis and prognosis of pathological conditions and are attracting much attention within the field of precision medicine in cardiology. The rarely obtainable insight on the human heart due to its inaccessibility for biopsy, coupled with the largely unexplored roles of sex, age, comorbidities and comedications and the lack of preclinical models encompassing the complexity of cardiomyopathies collectively pose significant challenges in both the clinical practice and scientific exploration of these diseases. Thus, the characterization of EVs, especially those derived from cardiomyocytes, constitutes a unique opportunity in the context of establishing clinically relevant predictive markers. Ideally, this approach would require that the information provided by circulating EVs be clustered in a multiple biomarker-based strategy, in order to perform patient risk stratification and reclassification (*e.g.*, as in the case of the HCM risk score for sudden cardiac death). However, future studies will be necessary, along with improvements in EV isolation techniques, to develop a highly multiplexed and targeted proteomic and lipidomic assessment of EV for biomarker discovery.

Another emerging application for EVs consists in their use as pharmacological vectors. EVs isolated from induced pluripotent stem cell-derived cardiomyocytes contained miRNA and proteins associate with angiogenesis, antifibrosis, promotion of M2 macrophage polarization, cell proliferation, and anti-apoptosis [71]. EVs are esteemed as promising delivery vehicles for a diverse range of genetic therapeutics, as they can be loaded with molecules either naturally, during their biogenesis, or following EV isolation using physical or chemical methods [72]. They are also relatively inert, non-immunogenic, biodegradable, and biocompatible. Recent advances have demonstrated the feasibility of loading molecular cargo into and/or onto EVs through endogenous engineering approaches. However, the employment of EVs in therapy still presents several challenges (*e.g.*, the relative short half-life indicates that multiple EV administrations would be required for therapy). These gaps hinder the definition of a regulatory framework governing EVs as an actual class of biological pharmaceutics. Additionally, the interactions which occur between EVs and the various *in* vivo biological matrices have yet to be fully comprehended, and mechanisms such as cell-specific targeting and uptake need to be further investigated. Surely, the incremental amount of knowledge gained from the study of basic EV biology will favour further understanding of future applications, hopefully also in the heterogenous and complex field of cardiomyopathies.

Additional information

No additional information is available for this paper.

Data availability statement

This review article was prepared using readily available data in the literature.

CRediT authorship contribution statement

A.S. Rizzuto: Writing – review & editing, Writing – original draft, Conceptualization. **A. Faggiano:** Writing – original draft, Conceptualization. **C. Macchi:** Writing – review & editing. **S. Carugo:** Writing – review & editing. **C. Perrino:** Writing – review & editing, Conceptualization. **M. Ruscica:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Massimiliano Ruscica reports financial support was provided by Banca d'Italia. Massimiliano Ruscica reports financial support was provided by Ministry of Education and Merit. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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