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Review article

Mitochondrial Extracellular Vesicles (mitoEVs): Emerging mediators of cell-to-cell communication in health, aging and age-related diseases.

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ABSTRACT

Mitochondria are metabolic and signalling hubs that integrate a plethora of interconnected processes to maintain cell homeostasis. They are also dormant mediators of inflammation and cell death, and with aging damages affecting mitochondria gradually accumulate, resulting in the manifestation of age-associated disorders. In addition to coordinate multiple intracellular functions, mitochondria mediate intercellular and inter-organ cross talk in different physiological and stress conditions. To fulfil this task, mitochondrial signalling has evolved distinct and complex conventional and unconventional routes of horizontal/vertical mitochondrial transfer. In this regard, great interest has been focused on the ability of extracellular vesicles (EVs), such as exosomes and microvesicles, to carry selected mitochondrial cargoes to target cells, in response to internal and external cues. Over the past years, the field of mitochondrial EVs (mitoEVs) has grown exponentially, revealing unexpected heterogeneity of these structures associated with an ever-expanding mitochondrial function, though the full extent of the underlying mechanisms is far from being elucidated. Therefore, emerging subsets of EVs encompass exophers, migrasomes, mitophers, mitovesicles, and mitolysosomes that can act locally or over long-distances to restore mitochondrial homeostasis and cell functionality, or to amplify disease. This review provides a comprehensive overview of our current understanding of the biology and trafficking of MitoEVs in different physiological and pathological conditions. Additionally, a specific focus on the role of mitoEVs in aging and the onset and progression of different age-related diseases is discussed.

1. Introduction

Mitochondria are highly dynamic and multifunctional organelles that regulate a wide array of different yet interconnected processes, including ATP synthesis and fatty acid ß-oxidation, lipid metabolism, iron homeostasis, calcium signalling, cell death and immune activity, metabolic reprogramming, cell cycle, proliferation and differentiation (Antico Arciuch et al., 2012; Monzel et al., 2023).

As metabolic and signalling hubs, mitochondria integrate these functions across multiple size scales. Therefore, proper spatiotemporal regulation of mitochondrial network architecture, tightly integrated with different subcellular compartments (e.g., endoplasmic reticulum, nucleus, and peroxisomes) and cell structures, contributes to transducing local information. Beyond cell boundaries, a tissue-specific repertoire of specialized mitochondrial phenotypes mediates intercellular and inter-organ communication in many physiological contexts and stress conditions, thus expanding mitochondrial activities systemically (Al Amir Dache and Thierry, 2023; Picard and Shirihai, 2022).

Consistent with their pleiotropic functions, mitochondria are key determinants for maintaining cell and tissues homeostasis. It is therefore expected that loss of mitochondrial function contributes to systemic organismal decline and to the onset of several non-communicable diseases that are closely related to oxidative stress and inflammation, including neurodegenerative and metabolic disorders, cardiovascular diseases, and cancer (Di Gregorio et al., 2022; Guo et al., 2023; Hernandez-Segura et al., 2018; Lima et al., 2022).

The ever-changing cellular milieu, especially during aging, constantly exposes mitochondria to multiple stressors causing impairment of oxidative phosphorylation and generation of oxidative stress, alongside with proteotoxicity (Iorio et al., 2021; Iorio et al., 2015; Petricca et al., 2019, 2022). When stimulated with cellular stress, mitochondria can release mitochondrial damage-associated molecular

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patterns (mtDAMPs) to elicit significant inflammatory reactions resulting in a negative impact on cellular homeostasis and stress resilience (Becker et al., 2022; Marchi et al., 2023).

Cells have evolved a set of mitochondrial quality control (MQC) mechanisms, including ROS scavenging, the ubiquitin–proteasome system, chaperones and proteolytic enzymes, along with the so-called mitochondrial unfolded protein response, to finely regulate mitochondrial homeostasis and turnover. Besides this nano-scale organization, other hierarchical levels of protection involve mitochondrial dynamics and biogenesis, and lysosome-related processes, including mitochondrial-derived vesicles (MDVs) and mitophagy pathways (Iorio et al., 2022; Ng et al., 2021).

In addition, the mitochondrion is involved in complex forms of mitochondrial transfer between different cell types, including tunnelling nanotubes (TNTs), gap junction channels, transfer of free mitochondria (via extrusions and internalization), and extracellular vesicles (EVs) (Borcherding and Brestoff, 2023; Iorio et al., 2024).

EVs are closed-shell, lipid bilayer structures that originate from endosomal system (exosomes) or bud directly from the plasma membrane, a process known as ectocytosis. The plasma-derived EVs are called with various names, such as microvesicles, microparticles, or ectosomes. In particular, exosomes (~30–150 nm diameter) are formed by endogenous budding where the cell membrane is endocytosed to form early endosomes, and then mature into late endosomes or multivesicular bodies (MVBs). MVBs further fuse with the plasma membrane and release EVs into the extracellular space. On average, exosomes are considered to be smaller than microvesicles, as their diameter can reach 1000 nm. All these EVs exhibit a high degree of structural and functional heterogeneity, and contribute to the cellular secretome by delivering many types of bioactive molecules, including nucleic acids, lipids, and proteins to target cells (Jeppesen et al., 2019, 2023; Santonocito et al., 2014).

In this context, much of the interest in EVs has recently linked to the evidence that EVs are sorting vehicles of mitochondria (Hayakawa et al., 2016; Hough et al., 2018; Ikeda et al., 2021; Thomas et al., 2022), mitochondria components (D'Acunzo et al., 2021; Peruzzotti-Jametti et al., 2021; Rabas et al., 2021) or mitochondrial microRNAs (MitomiRs) (Chen et al., 2024). Collectively, we termed these mitochondrial contents-containing EVs as mitoEVs. In addition to their multifaced biological roles, mitoEVs have been reported to be altered under various pathological conditions, arising the interest as potential therapeutic tools or possible biomarkers (Beatriz et al., 2022; Byappanahalli et al., 2023; D'Acunzo et al., 2021; Popov, 2022; Zecchini et al., 2023; H.-J. Zhang et al., 2023).

In this review, after summarising the current knowledge on mitoEVs biogenesis and trafficking, we highlight their role in intercellular communication, homeostatic maintenance, and cell development, with a specific focus on the mitochondrial quality control, immune modulation, and bioenergetic remodelling processes. Then, we provide an overview of the pathogenic and therapeutic potential of mitoEVs in aging and age-related diseases, including neurological disorders, cardiovascular disease, and cancer.

2. The expanding universe of mitoEVs: an overview

Recent discoveries have revealed the great complexity and diversity of mitoEVs in terms of origin, size (~30–4000 nm), markers and mitochondrial contents (Clancy et al., 2021; Q. Zhang et al., 2021).

Under physiological and stress conditions, distinct cell types can release many subsets of mitoEVs, including exosomes (Guescini et al., 2010; Konaka et al., 2023; Sansone et al., 2017), and microvesicles (D'Souza et al., 2021; Phinney et al., 2015). Mitochondrial cargo can be delivered also by exophers (3500–4000 nm), very large EVs sharing features with microvesicles (Melentijevic et al., 2017; Nicolás-Ávila et al., 2020), migrasomes (up to 3000 nm), newly discovered EVs whose production depends on cell migration (Jiao et al., 2021; X.-Y. Ma et al., 2023), mitophers (~ 720 nm), a peculiar type of microvesicle containing a single healthy mitochondria (Liu et al., 2023), and mitolysosomes, lysosomes that engulfe intact mitochondria, which are then released by exocytosis (Bao et al., 2022) (Table 1).

In the brain, a subpopulation of peculiar mitoEVs (termed mitovesicles, 100–300 nm) have been isolated with abundant mitochondrial components and ATP production ability in vitro, although lacking conventional EV markers (D'Acunzo et al., 2021).

In addition to cultured cells and tissues, mitoEVs have been isolated from several types of human body fluids (e.g., sweat, plasma, and endometrial fluid) (Bart et al., 2021; Bolumar et al., 2023; Garcia-Martinez et al., 2016; Mobarrez et al., 2019; Y. Wang et al., 2020; H.-J. Zhang et al., 2023).

Depending on the cellular context and on the triggering signalling pathways, mitoEVs play a divergent role by restoring homeostasis or amplifying disease conditions.

In the next chapters we will provide evidence that mitoEVs represent an alternative cargo disposal mechanism that finely regulates mitochondrial quality and homeostasis (Pan et al., 2023), operating as compensatory mechanism when internal degradation is compromised or overwhelmed (Liang et al., 2023). Besides alleviating the degradative pressure, mitoEVs contribute to the bioenergetic crosstalk between cells, maintenance of tissue homeostasis and immune regulation (Todkar et al., 2021; Pelletier et al., 2023; Zecchini et al., 2023).

3. Exploring the biology and trafficking of MitoEVs

3.1. MitoEVs biogenesis and sorting mechanisms: role in mitochondrial quality/quantity control

MitoEVs biogenesis is linked to distinct forms of mitochondria-specific export routes (Fig. 1), and to a specific MDVs trafficking pathway that contribute to mitoEVs generation via exosomes/EVs (Fig. 2) (Crewe et al., 2021; Liang et al., 2023; Peng et al., 2022; Rosina et al., 2022; Suh et al., 2023; Todkar et al., 2021; Vasam et al., 2021). A complete transfer of healthy/damaged mitochondria requires MDV-independent distinct cellular pathways and different timing of biogenesis (Liu et al., 2023). These include transmitophagy, the transfer of damaged mitochondria to neighboring cells for degradation; mitocytosis, the process by which migrating cells expel dysfunctional mitochondria packaged in migrasomes; mitolysosome exocytosis, and exophergenesis, which is the release of exophers. In contrast to these mechanisms of elimination of damaged/dysfunctional mitochondria, mitoEVs in the form of mitophers allow the transfer of healthy mitochondria (a process termed mitopherogenesis), firstly identified during sperm development which would represent a control mechanism of mitochondrial quantity (Liu et al., 2023) (Fig. 1).

Despite differing in terms of cargo, size, and mechanism of formation, mitoEV-mediated transfer of mitochondrial-derived cargos would represent the main mechanism that contributes to the MQC process, as well as to fine regulation of mitochondrial quantity. The contribution of different types of mitoEVs to MQC will be detailed and discussed specifically in the sections below.

3.1.1. Mitochondria- derived vesicles (MDVs)

Under baseline and mild stress conditions, the generation and release of single/double membrane MDVs budding from mitochondrial surface (Fig. 2) has emerged as an early and additional mechanism of autophagy-independent MQC (König et al., 2021; König and McBride, 2024; Sugiura et al., 2014).

On the one hand, selective targeting of damaged, oxidized mitochondrial components directly into peroxisomes or late endosomes/lysosomes, by distinct MDV subtypes (70–150 nm in diameter), alleviates mitochondrial damage, thereby preventing extensive mitophagy activation (McLelland et al., 2014; Neuspiel et al., 2008; Soubannier et al., 2012; Sugiura et al., 2014). On the other hand, MDVs can also

Table 1
Distinct subsets of mitoEVs.

mitoEVs	Formation/ biogenesis	Mitochondrial cargo and proteins	Role/biological function	Canonical markers	References
Exosomes (~ 10-150 nm)	exocytosis	mtDNA,	Intercellular communication	Tsg101, CD9, Alix,	Guescini et al., 2010
		mtDNA	Inflammatory response	CD63, CD9, Flotilin	Konaka et al., 2023
		mtDNA, ATP5A1, VDAC1, mtSOD	Oncogenic signal	CD63	Sansone et al., 2017
		VDAC, HSP60, COX4	Cardio-protection through hormesis	Alix, CD63, CD9	Crewe et al., 2021
		Fatty acid oxidation enzymes	Metabolic reprogramming and tumour progression	Alix, Tsg101, Flotilin-1	Lazar et al., 2016
		mtDNA, TOMM20, HSP70, NDUFA9, OPA1	Mitochondrial quality control	Alix, CD9	Todkar et al., 2021
		mtDNA, GLUD1, VDAC1, cyclophilin D	Tumour invasion	CD63	Rabas et al., 2021
		VDAC	Acquisition of chemoresistance	Tsg101	Abad & Lyakhovich, 2022
		mtDNA	Immunosuppression	Alix, Tsg101, CD9	Ko et al., 2023
		mtDNA, polarized mitochondria	Bioenergetic remodelling	CD9, CD63, CD81, Tsg101	Hough et al., 2018
Microvesicles (~150-1000 nm)	ectocytosis	mitochondria partially depolarized	disposal of damaged mitochondria and bioenergetic remodelling	Tsg101, ARRDC1	Phinney et al., 2015
		polarized mitochondria, ATP5A	Bioenergetic remodelling	–	Islam et al., 2012; D'Souza et al., 2021
		Mitochondria, COX4, TOMM22, Bcl-2	Inflammatory response	–	Puhm et al., 2019
Exophers (3500-4000 nm)	exo-pherogenesis	Damaged mitochondria	Disposal of damaged mitochondria (MQC mechanism)	–	Melentijevic et al., 2017; Nicolás-Ávila et al., 2020
Migrasomes (up to 3000 nm)	mitocytosis	Damaged mitochondria	Disposal of damaged mitochondria (MQC mechanism)		Jiao et al., 2021; Ma et al., 2023
Mitophers (~720 nm)	mito-pherogenesis	A single healthy mitochondrion	Control of mitochondrial quantity in sperm cells	–	Liu et al., 2023
Mitolysosome	exocytosis	Damaged mitochondria	Disposal of damaged mitochondria (?)	–	Bao et al., 2022
Mitovesicles (~100-300 nm)		VDAC, OPA1, MAO-A, MAO-B, PDH-E1α, HSP60, ETC complexes, PINK-1-Δ2	Disposal of damaged mitochondrial in the brain (?); impairment of synaptic plasticity; regulation of monoamine levels in the context of neurodegenerative disorders (?)	–	D'Acunzo et al., 2021, 2022, 2023, 2024

compensate for inactivation of conventional mitophagy to preserve mitochondrial health (Towers et al., 2021). Interestingly, when MDVs exceed lysosomal functional capacity or when lysosomal activity is compromised, they are incapsulated into MVBs (similarly to exosomal secretion) and released into extracellular space as mitoEVs, thereby limiting cardiotoxicity, and influencing osteogenesis, metabolic status, as well as hormetic and innate immune responses (Crewe et al., 2021; D'Souza et al., 2021; Rosina et al., 2022; Liang et al., 2023; Peng et al., 2022; Suh et al., 2023; Todkar et al., 2021; Vasam et al., 2021).

The specific detection of mitochondrial constituents, including inner mitochondrial membrane that characterizes double-membrane MDVs, outer mitochondrial membrane that characterizes single- and double-membrane MDVs, mitochondrial matrix proteins including proteins involved in metabolic and redox processes (Vasam et al., 2021) and mtDNA (Picca, Beli, et al., 2020) ensures to discriminate distinct subtypes of MDVs from microvesicles, whose secretion is MVB-independent, and exosomes. Furthermore, translocase of outer mitochondrial membrane 20 (TOMM20), mitochondria-anchored protein ligase (MAPL or Mul1), and β-barrel proteins are specific markers for single-membrane MDVs (Braschi et al., 2010; König et al., 2021; Ryan et al., 2020), whereas pyruvate dehydrogenase (PDH), small GTPase Rab7, and Rab9 are the conventional markers for double-membrane MDVs (Table 2). In this regard, MDVs incorporating mitochondrial matrix proteins are also involved in mitochondrial antigen presentation on the membrane surface (Matheoud et al., 2016).

Different molecular models of MDV biogenesis have been proposed, including PTEN-induced putative protein kinase 1 (PINK1)/Parkin-dependent signalling or dynamin-related protein 1 (DRP1)-dependent mechanism. Independently of DRP1 activity, the release of MDVs is initiated by the stabilization of PINK1 in the outer mitochondrial membrane and by recruitment of Parkin during slight stress conditions or mild mitochondrial dysfunction (Mondal and Towers, 2022; Peng et al., 2022; Popov, 2022; Sugiura et al., 2014). Consistently with these results, cannabidiol can activate the PINK1/Parkin-dependent MDV production (Ramirez et al., 2022). Other studies, however, do not support this hypothesis as the loss of Parkin does not lead to reduction in MDV formation (Choong et al., 2021) and neither PINK1 nor Parkin are required for MDV formation under steady-state conditions, suggesting that these regulators are essential only in stress-induced MDVs (König et al., 2021). Therefore, MDV biogenesis relies on microtubule-associated motor proteins, MIRO1/2, and is dependent on the catalytic activity of DRP1 at constriction sites. After MIROs-dependent mitochondrial-membrane tubulation along microtubule filaments, MDV scission depends on the DRP1 recruitment near the tip of a thin extending tubule by the mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 and 51 kDa (Mid49/Mid51) (König et al., 2021) (Fig. 2).

As described above, the different MDV subtypes are directed to distinct degradative pathways, including peroxisome, and the late endosomes/MVBs, where cargo mitochondrial proteins can be routed to lysosomes, or cell surface where they fuse with the plasma membrane, releasing mitoEVs in the form of exosomes into the extracellular space (Fig. 2). Microvesicles can bud directly from the plasma membrane and have larger dimensions (Fig. 1). Therefore, MDVs are shuttled to peroxisomes and endo-lysosomal system by specific sorting mechanisms (Braschi et al., 2010; Ryan et al., 2020) (Fig. 2). Different molecular

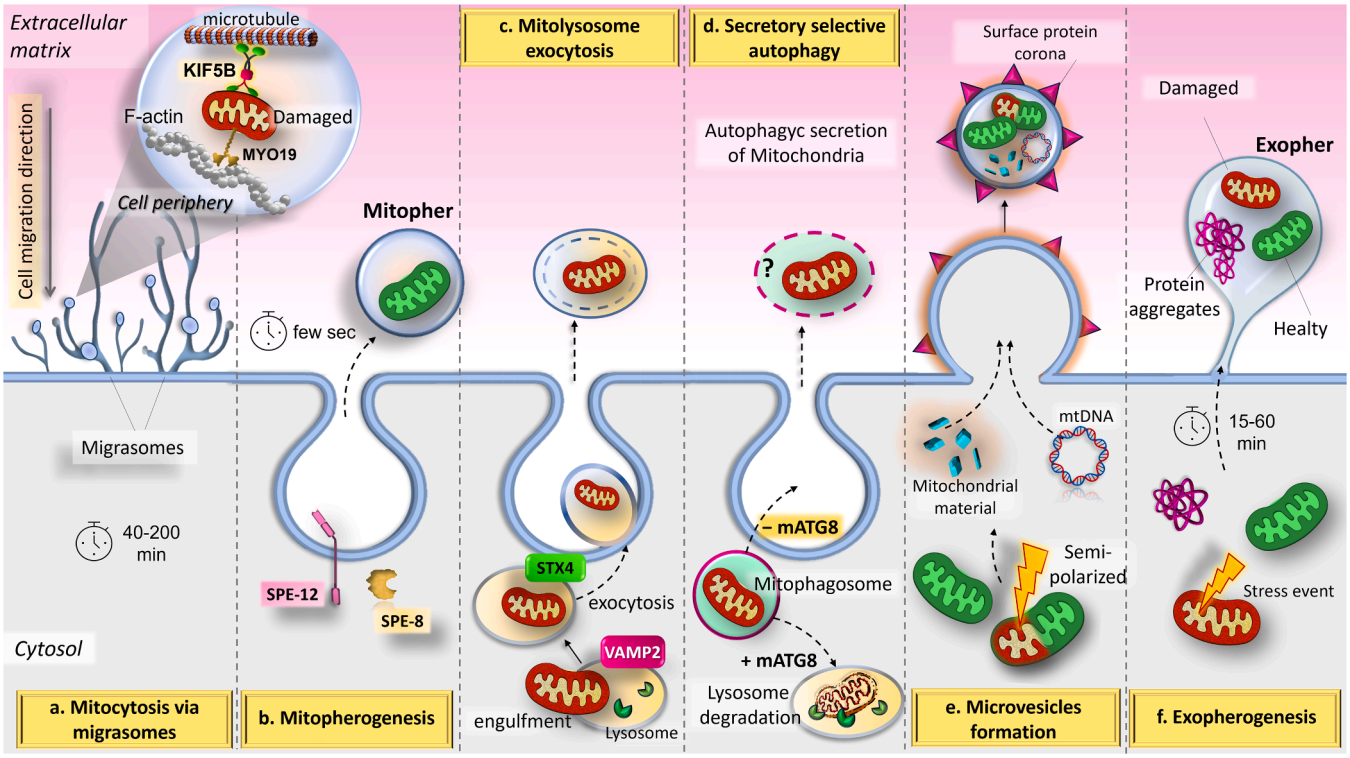

Fig. 1. Distinct forms of mitochondrial export, acting as spatio-temporal mechanisms of mitochondrial quality and quantity control. Mitochondrial export can occur through different routes, including (a) mitocytosis via migrasomes, (b) mitopherogenesis, (c) mitolysosome formation, (d) secretory autophagy of mitochondria, (e) microvesicles exocytosis, and (f) exophergenesis. (a) In migratory cells, mild mitochondrial stress promotes disposal of dysfunctional mitochondria by migrasomes to preserve cellular homeostasis. The outward motor protein kinesin family member 5B (KIF5B) is required for positioning of unhealthy mitochondria at cell edge. Here, damaged organelles are associated with cortical actin by actin-based motor myosin 19 (MYO19) before incorporating into migrasomes. (b) In male C. elegans, sperm mitochondrial quantity and fertility are finely regulated by a mechanism of mitopherogenesis, whereby spermatids release a single healthy mitochondrion into mitophers through the activity of SPE-8 and SPE-12 tyrosine kinases. (c) In neurons and astrocytes, disposal of damaged mitochondria can occur regardless of autophagosome formation. Therefore, damaged mitochondria are incorporated intact into lysosomes to form mitolysosomes, and subsequently released into extracellular milieu through a vesicle-associated membrane protein 2 (VAMP2)- and syntaxin-4 (STX4)-mediated exocytosis. (d) A putative MQC mechanism may include the autophagic secretion of mitochondria. During mitophagy, in the absence of mammalian ATG8-conjugation system, the mitochondrial clearance may occur independently of lysosomal degradation and through a mechanism involving autophagosome-plasma membrane fusion. (e) Microvesicles (MVs) bud directly from the plasma membrane. Therefore, healthy/damaged mitochondria and mitochondrial components can be routed to cell surface and release into extracellular milieu via MVs. (f) During physiological conditions and in response to different stressors, exhophers can mediate extracellular transfer of proteotoxic material and damaged mitochondria. Mammalian exophers appear to generate by outward extensions of the plasma membrane like TNTs. Following mitochondrial exocytosis, mitoEVs may have many putative fates, including the engulfment and degradation by surrounding cells via transmitophagy mechanism. (Some icons from BioRender.com).

mechanisms and players can mediate the fusion of MDVs to MVBs and operate their subsequent release as mitoEVs into the extracellular environment, such as cluster of differentiation 38 (CD38)/cyclic ADP ribose signalling (Suh et al., 2023), the optic atrophy 1, and sorting nexin 9 (Todkar et al., 2021). This route is also promoted by Parkin inhibition (Todkar et al., 2021).

Therefore, MDVs formation acts as a MQC mechanism in different cells. In cardioblasts, it operates under baseline and stress conditions (Cadete et al., 2016; B. Li et al., 2020). When lysosomal function is impaired, the release of mitoEVs originated from fusion of MDVs into MVBs increases through a mechanism involving the ablation of Rab7 and independently of autophagy (Liang et al., 2023). In this scenario, cardiac macrophages ensure phagocytosis of released cargo by mitoEVs reducing inflammation (Fig. 2).

MitoEVs biogenesis from MDVs is also involved in MQC of brown adipocytes (Rosina et al., 2022). Under resting conditions and thermogenesis-mediated metabolic stress, brown adipocyte-derived mitochondrial ROS stimulate PDH+ MDV production through a PINK1-mediated mechanism. The extracellular release of damaged parts of mitochondria, via mitoEVs, ensures the disposal of mitochondrial waste by resident macrophages, thus allowing for the right conditions that ensure brown adipose tissue thermogenesis. In particular, constitutive biogenesis of TOMM+/PDH− MDVs may contribute to MQC under baseline conditions, whereas stress conditions would stimulate the selective formation of TOMM−/PDH+ MDVs (König et al., 2021; McLelland et al., 2014).

Secreted mitoEVs can travel great distances and stimulate target tissues, such as the heart, to increase the level of protection against stressogenic stimuli. Therefore, in some conditions of cellular stress the transfer of damaged mitochondria (MDVs) into recipient cells (via mitoEVs) escapes the clearance by macrophages to contribute to an "inter-organ mitohormesis".

In particular, the release of circulating mitoEVs by palmitate-stressed adipocytes (mitoEVs^{PA}; with respiration-competent mitochondrial particles) stimulates a transient cardiomyocyte ROS generation followed by a compensatory antioxidant response, thereby highlighting a mitoEVs-mediated metabolic pre-conditioning of the heart (Crewe et al., 2021) (Fig. 2). Additionally, in mice pre-treated with mitoEVs^{PA} the cardiac ischemia/reperfusion injury is reduced (Crewe et al., 2021). Interestingly, mitoEVs derived from Parkin$^{-/-}$ -adipocytes exhibit a reduction of mitochondrial cargo and do not induce cardioprotective effects.

It has recently been suggested that MDVs may also be released into the extracellular milieu as mitovesicles, a formerly unknown population of mitoEVs isolated from murine and human Down syndrome and

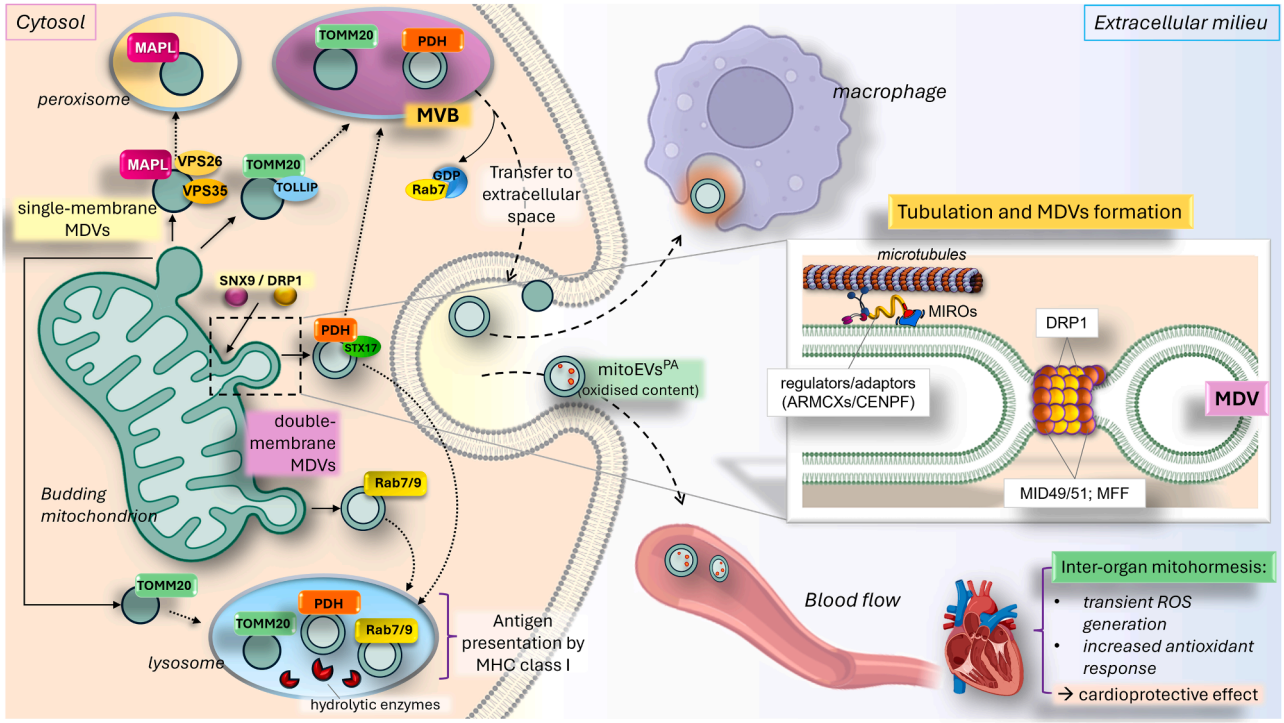

Fig. 2. A schematic model representing the biology and trafficking of distinct subsets of mitochondrial-derived vesicles (MDVs). Single- and double-membrane MDVs budding from the mitochondrial surface can follow distinct routes, such as peroxisomes or late endosomes/lysosomes. According to membrane-associated proteins and cargo, translocase of outer mitochondrial membrane 20 (TOMM20) and mitochondria-anchored protein ligase (MAPL) are the specific protein markers for single-membrane MDVs, whereas pyruvate dehydrogenase (PDH), small GTPase Rab7, and Rab9 are the common markers for double-membrane MDVs. The transport of MAPL+ MDVs to the peroxisome is assisted by the vacuolar protein sorting-associated proteins VPS35 and VPS26, whereas Tollip and syntaxin 17 (STX17) mediate the trafficking of TOMM+/PDH- and TOMM-/PDH+ MDVs to endo-lysosomal system. Different microtubule-related proteins, such as MIRO1/2 (mitochondrial Rho GTPases), centromere protein F (CENP-F), and armadillo repeat-containing proteins on the X chromosome (ARMCX) proteins catalyze MDVs tubulation, whereas MDV scission require the recruitment of DRP1 at constriction sites by adaptor proteins, including the mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 and 51 kDa (Mid49/Mid51). Alternatively, MDVs biogenesis can rely on SNX9-dependent mechanism. In response to energetic stress (e.g., palmitate; PA), circulating mitoEVsPA derived from adipocytes are taken up by cardiomyocytes and integrate with mitochondrial network. This condition results in transient oxidative stress, thereby leading to an antioxidant response and cardio-protective effects (inter-organ mitohormesis). When lysosomal function is compromised or Rab7 is ablated, secretion of mitoEVs increases. The secreted mitoEVs undergo phagocytosis by surrounding macrophages, thereby resulting in mitochondrial clearance and absence of inflammation. (Some icons from BioRender.com).

Table 2
Specific markers for MDVs.

Types of Mitochondrial Derived Vesicles (MDVs)	Specific Markers
Single-membrane MDVs	• **TOMM20** • **MAPL/Mul 1** • **β-barrel protein**
Double-membrane MDVs	• **Pyruvate dehydrogenase (PDH)** • Small GTPase: o **Rab7** o **Rab9**

diploid control brains (D'Acunzo et al., 2021). In this context, however, some specific MDV markers are not found, and the mechanism underlying mitovesicles formation and secretion still needs to be established.

Differently from exosomes and microvesicles, mitovesicles exhibit specific mitochondrial markers, including the monoamine oxidase (MAO; type A, commonly found in neurons, and B, primarily expressed in astrocytes and pyramidal neurons), distinct biophysical and biochemical properties, a double membrane, a specific lipidome, and capacity to generate ATP *in vitro* (D'Acunzo et al., 2021, 2022). Interestingly, mitochondrial dyshomeostasis in Down syndrome and during chronic cocaine exposure directly alters neuronal and astrocytic mitovesicles levels and cargo (D'Acunzo et al., 2021, 2023). Therefore, the massive release of enlarged mitovesicles may act as intracellular compensatory mechanism governing mitochondrial turnover to restore normal mitochondrial homeostasis.

However, very recently, in a murine model of Down syndrome, the brain mitovesicle MAO-B levels were found to be altered (D'Acunzo et al., 2024). Given the critical role of MAO-B in degrading different neuroactive monoamines by generating H_2O_2, an elevated MAO-B enzymatic activity can lead to alterations in long-term potentiation, thereby causing impairment of synaptic plasticity and depression. Therefore, mitovesicles may also have an extracellular active role in reverberating pathological conditions associated with neurodegenerative diseases (D'Acunzo et al., 2024).

3.1.2. Exophergenesis and transmitophagy, variants of a common "evulsion" process?

Neurons and cardiomyocytes are long-lived cells, expressing a low renewal ability. They have a particularly high metabolic activity, due to the presence of an efficient mitochondrial network that meets the high energy demands shown by these cell types. To ensure proper cell function, cardiomyocytes and neuronal cells employee many types of protein/damaged organelles degradation systems, including transmitophagy and exhopher-mediated processes that share common cellular mechanisms. In this context, the proper uptake of neuronal and cardiac mitoEVs, containing damaged mitochondrial constituents, by astrocytes, or cardiac-resident macrophages, represents an atypical mode of phagocytosis that supports mitochondrial homeostasis and

quality control, as well as tissue fitness, in response to toxic proteostress and mitochondrial dysfunction. This mitoEVs-mediated mechanism of transcellular degradation is also critical for brown adipocytes (see the section described above on MDVs) (Rosina et al., 2022) and mesenchymal stromal/stem cells (MSCs) (Phinney et al., 2015). In particular, to maintain mitochondrial and redox homeostasis human MSC-derived microvesicles transfer depolarized mitochondria to macrophages in response to oxidative stress.

The term transmitophagy was first coined by Davis and co-workers in 2014 (Davis et al., 2014). These authors described a process found in the optic nerve head (ONH) of mice, where a high percentage of axonal mitochondria originating from the retinal ganglion cells were normally engulfed and degraded by resident astrocytes. Although this phenomenon also occurs elsewhere in the central nervous system, the ONH represents an elective intersection point between biochemical stress and axon damage in glaucoma. More recently, the physio-pathological role of neuron-astrocyte transmitophagy has also been demonstrated in *in vivo* models of Alzheimer's (AD) (Lampinen et al., 2022) and Parkinson's diseases (PD) (Morales et al., 2020), and in cone photoreceptors (Hutto et al., 2023). After 6-hydroxydopamine treatment, the fragmentation of dopaminergic neurons of rats and mice is followed by the accumulation of dysfunctional mitochondria in specific saccular structures (spheroids, 1–9 μm), where mitophagy is initiated but not completed. Spheroids are subsequently transferred to surrounding astrocytes, where they are linked to autophagosomes for lysosomal-mediated degradation. Therefore, the spheroid-mediated transmitophagy would prevent the release of circulating inflammatory mediators to the extracellular space, thus reducing the incidence of inflammatory responses in PD pathophysiology (Morales et al., 2020). The ability of human and mouse AD-affected astrocytes to internalize and degrade neuronal mitochondria has also been reported, though the increased mitochondrial transfer may be mediated by TNT-like structures rather than mitoEVs (Lampinen et al., 2022). Also, constitutive and stress-induced transmitophagy via vesicular connections occurs in the retina, from the ellipsoid region of cone photoreceptors to Müller glia cells, suggesting new specialized supporting functions for the latter (Hutto et al., 2023).

Very recently, it has been suggested that axonal transmitophagy may be a variant of exophers formation (Jeong et al., 2023), a type of giant membrane-bound EVs (containing protein aggregates and dysfunctional organelles, including mitochondria; Fig. 1) that are released out of cells into the extracellular milieu by the outward budding of the plasma membrane through a "pinching-off" event. Current models indicate cardio- and neuroprotective roles for exophers. Therefore, exophers generation increases in response to different stressors, including ROS, protein aggregation and inhibition of autophagy (Melentijevic et al., 2017), heat, increased osmotic stress, nutrient deprivation (Cooper et al., 2021), and spaceflight (Laranjeiro et al., 2021), and may be under the control of distinct non-cell autonomous signalling pathways, including lipid biosynthesis, and FGF/EGF-RAS/MAPK pathways (Cooper et al., 2021).

In the nematode *Caenorhabditis elegans*, this process is relevant for supporting offspring development (Turek et al., 2021) and neuronal function (Melentijevic et al., 2017). In particular, exophers can mediate transfer of damaged mitochondria from proteotoxically stressed mechanosensory neurons to the neighbouring hypodermis and more distant coelomocytes for degradation. Exopher production is therefore a MQC mechanism used to preserve neuronal homeostasis, as demonstrated by the fact that cells generating exophers function better than those that do not activate it (Melentijevic et al., 2017). Consistently with this idea, hypodermal skin cells may phagocyte neuronal exophers through the Arp2/3-mediated F-actin formation (Yu Wang et al., 2023). The hypodermal GTPase ARF-6 and its effector SEC-10/exocyst, along with the phagocytic receptor CED-1/Draper, are required for the biogenesis of neuronal exophers. Engulfed exophers into hypodermis are then initially processed in a lysosome-independent manner to produce smaller "starry night" vesicular structures that subsequently fuse with endosomes prior to lysosomal degradation. In this regard, distinct molecular factors are required for fission and degradation of exopher-phagosomes (Yu Wang et al., 2023).

Neuronal exophers share common traits with mammalian cardiac exophers interacting with cardiac-resident macrophages (Nicolás-Ávila et al., 2020). Within the myocardium of mice, different subsets of such cells form a functional network surrounding cardiomyocytes (the average macrophage: cardiomyocytes ratio is ~ 5:1). This pervasive distribution ensures a physiological and stress-induced phagocytic activity that has evolved into the mechanism of exopher uptake to better support cardiomyocyte function and heart homeostasis. In particular, the efficient uptake of cardiomyocyte-derived exophers (containing TOMM20+ mitochondria) by cardiac-resident macrophages, through the phagocytic receptor MER-tyrosine kinase (MERTK), prevents the accumulation of dysfunctional mitochondria in cardiomyocytes, the intensification of inflammatory responses, and ventricular alterations (Nicolás-Ávila et al., 2020). Consistently with this, the depletion of cardiac-resident macrophages or the absence of the receptor MERTK have a dramatic effect in mitochondrial quality and cardiac function (Nicolás-Ávila et al., 2020). Innate exophergenesis mechanism is also conserved in wild-type mouse neurons and human neuroblastoma cells (Siddique et al., 2021). As reported previously in *C. elegans* and mammalian cardiomyocytes, exhophers containing mitochondria and lysosomes generate by outward extensions of the plasma membrane and remain connected to the parent cell by TNT-like structures. Interestingly, two different exophers originating from distinct cells would be connected to each other, suggesting that they may mediate neuronal communication. When neurons are exposed to proteostatic stress (wild-type or mutant human tau) the number of the exophers increased substantially, thus suggesting a possible adaptive response (Siddique et al., 2021).

In the optic nerve of *Xenopus laevis,* mutations in optineurin (a mitophagy receptor) related to glaucoma lead to increased levels of axonal mitophagy. This process is associated with the shedding and degradation of the extra-axonal mitochondria and optineurin by surrounding cells, including astrocytes, microglia, and myeloid cells. The authors also conclude that this mechanism may be an evolved variant of exopher formation as the axonal structures containing mitochondria appear similar to axonal protrusions and evulsions described by Davis and colleagues (Davis et al., 2014). In addition, axonal transmitophagy shares similarities with structures that mediate exhopher biogenesis and extrusion, and the axonal components within astrocytes may be analogous to "starry night" vesicular structures (Yu Wang et al., 2023).

3.1.3. Mytocitosis, mitopherogenesis, and mitolysosome formation: new mechanisms of spatiotemporal regulation of mitochondrial quality and quantity

In addition to MDVs and exophers, recent data reveal new forms of mitoEVs-mediated mitochondrial export that involve migrasome, mitopher, and mitolysosome formation (Fig. 1).

Migrasomes are membrane-bound structures that can contain different cytosolic components (e.g., RNAs and proteins) into smaller vesicles (with diameters of ~ 50–100 nm) resembling opened pomegranates (L. Ma et al., 2015). Migrasomes originate in migrating cells at the cross points and tips of retraction fibers (RFs) and have been found to be extensively distributed in different *in vitro* cellular system, including cancer cells, and normal human, rat or mouse cells, as well as in *in vivo* models, such as human, rat, mouse, zebrafish, and chicken (Y. Zhang et al., 2022). They play a crucial role in different physiopathological processes, such as cell-to-cell communication, discard of dysfunctional mitochondria, lateral transport of mRNA and proteins, organ morphogenesis during zebrafish gastrulation, as well as in the onset and development of neurological diseases and cancer (Xide Zhang et al., 2023).

Following RF break-up, migrasomes can be released into the extracellular space at the retracting region of moving cells in a process called

"migracytosis". Migrasomes can be taken up by surrounding cells and this process could mediate lateral and horizontal transfer of RNAs and proteins, also representing a source of signalling molecules to neighbouring cells (S. Yu and Yu, 2022).

Besides these biological functions, migrasomes-mediated mitocytosis safeguards cellular homeostasis by preventing the intracellular accumulation of defective mitochondria. Under basal conditions and mild mitochondrial stress, migratory cells promote a MQC process via the selective removal of dysfunctional mitochondria by migrasomes through a mytocitosis process (Jiao et al., 2021). In particular, mitochondria are sent to the cell periphery and subsequently loaded into migrasomes through key interactions with the cytoskeleton. The movement of damaged organelles on microtubules is mainly mediated by the selective intervention of the outward motor protein kinesin family member 5B (KIF5B), associated with a simultaneous reduction in mitochondrial binding with the inward motor protein dynein. At the peripheral edge of the cell, defective organelles are tethered to the plasma membrane-associated cortical actin by actin-based motor myosin 19 (MYO19) (Fig. 1). Here, subsequently to DRP1-mediated fission, fragmented mitochondria are sent to migrasomes for disposal into the surrounding environment. In this context, in *vitro* and *in vivo* evidence of the protective roles of mytocitosis have also been reported. Therefore, in macrophages and neutrophils, the knockout of the tetraspanin 9 gene, a key player in migrasome formation, decreases migrasome biogenesis, causes loss of mitochondrial membrane potential, and reduces cell migration and viability (Jiao et al., 2021).

Migrasome-mediated mytocitosis is also critical in mitigating the di-(2-ethylhexyl) phthalate -induced mitochondrial stress and damage in ovarian granulosa cells (GCs) in quails, suggesting a possible relationship between MQC in GCs and female reproductive performance (X.-Y. Ma et al., 2023).

In male *C. elegans*, during sperm development, it has recently described a mitochondria-specific export mechanism, that finely regulates the quantity of sperm mitochondria through the formation of the subsets of mitoEVs called "mitophers" (Liu et al., 2023). In this regard, spermatids can release a single membrane mitopher (average diameter: 720 nm) containing a single healthy mitochondrion (~ 600 nm) through the process of mitopherogenesis, a distinct and rapid plasma membrane outward-budding-off mechanism. This process is distinct from the previous mechanisms of mitoEV generation in terms of timing of biogenesis, vesicle size and cargo specificity (cytosolic components, protein aggregates and damaged mitochondria compared with healthy mitochondria). Indeed, whereas mitopherogenesis occurs within a few seconds, the formation of exophers (15–60 min) and migrasomes (40–200 min) takes longer time (Fig. 1). Molecular analyses show that mitopher formation requires normal actin-filament dynamics, and the tyrosine kinases SPE-8 and SPE-12 partially mediate the extracellular protease-triggered mitopherogenesis. By contrast, Myosin VI (SPE-15 in *C. elegans*) has a negative impact in mitopher production, probably by inhibiting the outward mitochondrial traslocation. In summary, the mitopherogenesis is pivotal in mitochondrial quantity modulation during development, and its impairment would lead to excessive accumulation of mitochondria. This condition may cause defects in sperm motility and function, thereby leading to male infertility (Liu et al., 2023).

Dysfunctional mitochondria can also be removed through other MQC pathways, including a novel form of lysosome-associated exocytosis that may be involved in the flunarizine (FNZ)-induced parkinsonism (Bao et al., 2022). FNZ is a piperazine derivative (known as a calcium channel blocker) widely used in medical practice for the prevention of migraine and treatment of vertigo, though it has been described as a potential inducer of parkinsonism-like symptoms (Lin et al., 2019).

In mice, FNZ treatment reduces striatal dopamine levels, and induces motor dysfunction and memory deficit along with depletion of mitochondria in brain (Bao et al., 2022). Therefore, FNZ can induce parkinsonism via mitochondrial elimination as significant loss of mitochondria has also been observed in FNZ-exposed nondopaminergic neurons and astrocytes. In this scenario, however, disposal of damaged mitochondria does not rely on ATG5- and RAB-9 mediated mitophagy. Indeed, damaged mitochondria are engulfed intact (independent of autophagosomes) into lysosomes to form mitolysosomes, and subsequently extruded through a vesicle-associated membrane protein 2 (VAMP2)- and syntaxin-4 (STX4)-mediated exocytosis. Therefore, the extracellular secretion of mitolysosome generates mitoEVs, that may be taken up by surrounding cells for disposal.

The critical importance of mitolysosome exocytosis as novel MQC mechanism has recently been highlighted in lung cancer cells, where nuclear factor erythroid 2–related factor 2 (Nrf2) depletion leads to the accumulation of dysfunctional mitochondria within mitolysosomes and the blockage of their processing (Dayalan Naidu et al., 2023). Therefore, Nfr2-mediated mitolysosomes processing is crucial for ensuring the proper mitochondrial clearance. Consistently with this, Nrf2 upregulation (by inactivation of Keap1) counteracts the oncogene-driven increase of ROS generation.

Finally, alternative MQC pathways for mitochondrial clearance may also include the autophagic secretion of mitochondria, an unconventional mechanism that occurs in the absence of mammalian ATG8-conjugation system (Tan et al., 2022). This process relies on autophagosome fusion with the plasma membrane and the extrusion of mitochondria from cells without the involvement of the lysosomal degradation. In recipient cells, however, extracellular mitochondria can trigger cGAS-STING signalling, thereby leading to the activation of pro-inflammatory responses. However, it must be said that mitochondria may not be secreted in EVs.

3.2. *Metabolic remodelling by mitoEVs*

Over the past years, an ever-growing number of evidence have indicated that mitochondrial constituents encapsulated into EVs can be incorporated into the recipient cell's mitochondrial network, thereby exerting powerful protective effects, including improvement of cellular metabolism, mitochondrial functions, and bioenergetics (Fig. 3).

In this regard, though much of interest has been focused on MSCs, mitochondrial transfer via EVs has been attributed to several other types of donor cells, such as platelets, endothelial progenitor cells, cancer cells, astrocytes, and cells of the endometrium.

In the lipopolysaccharide-induced acute lung injury mouse model, bone marrow-derived stromal cells can release mitochondria-containing microvesicles in a Ca^{2+} dependent fashion, thereby enhancing alveolar bioenergetics (Islam et al., 2012). Similarly, in response to oxidative stress, human MSC release depolarized mitochondria (via arrestin domain-containing protein 1-mediated microvesicles) to macrophages as pro-survival mechanism. The uptake of MSCs-derived mitoEVs improves macrophage homeostasis and functionality through the increased ATP production and a reduction in mitochondrial ROS (Phinney et al., 2015). In *in vitro* models of acute respiratory distress syndrome, MSCs polarize macrophages toward anti-inflammatory and phagocytic M2 phenotype, thereby alleviating lung injury. This effect can be attributed to MSCs-EVs-mediated mitochondrial transfer that leads to increased activity of macrophage oxidative phosphorylation (Morrison et al., 2017). Mitochondrial impairment has a critical role in airway smooth muscle dysfunction in chronic obstructive pulmonary disease (COPD). Therefore, mitochondrial transfer via EVs can take place between airway smooth muscle cells from healthy ex-smokers and those from COPD individuals, thus resulting in a positive impact on mitochondrial bioenergetics and mitochondrial biogenesis of recipient cells (Frankenberg Garcia et al., 2022). In line with these findings, MSCs-derived mitoEVs stimulate the repair of human distal lung epithelial cells and ameliorate the alveolar–capillary barrier properties via restoration of mitochondrial respiration and homeostasis (Dutra Silva et al., 2021; Fergie et al., 2019). Human MSCs can also transfer healthy mitochondria to chondrocytes, though the functional impact of

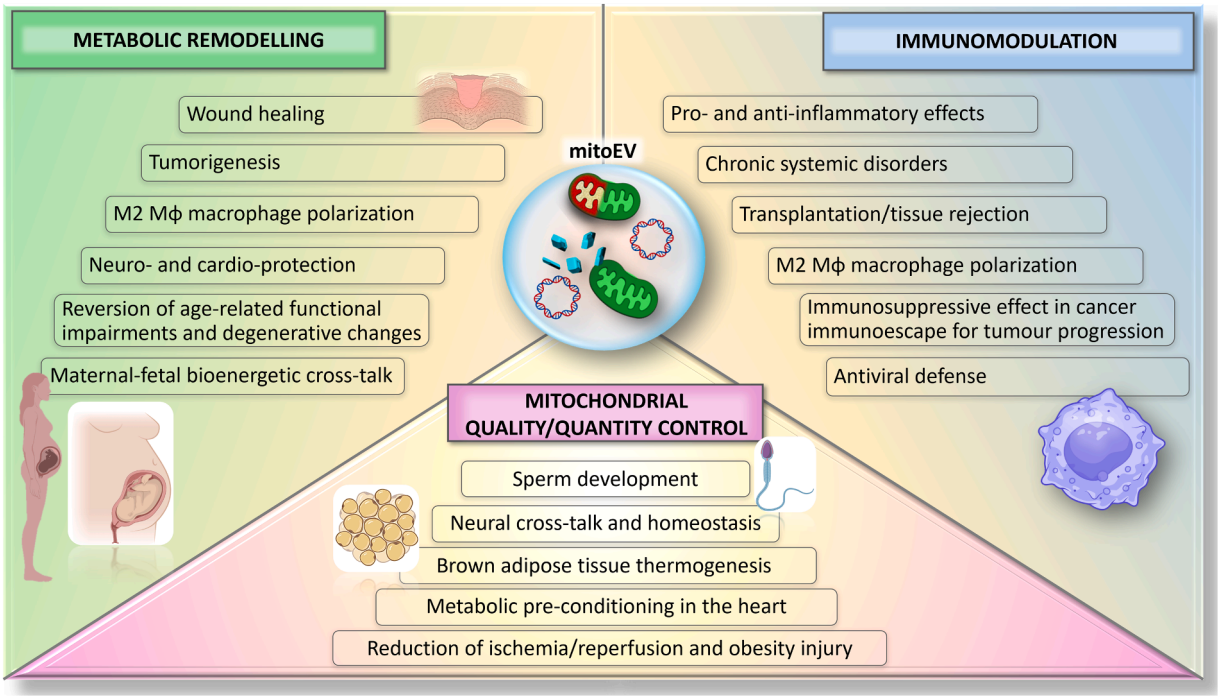

Fig. 3. Biological functions of mitoEVs. (Some icons from BioRender.com).

this process has not yet been investigated (Thomas et al., 2022). The release of mitoEVs from human induced pluripotent stem cell-derived cardiomyocytes improves cell survival, contractile profiles, mitochondrial functions, and intracellular bioenergetics upon hypoxia. Also, in *in vivo* murine myocardial infarction model, intramyocardial injection of induced cardiomyocytes-derived mitoEVs enhances cardiac functions (Ikeda et al., 2021). The hypoxic preconditioning enhances mitochondrial activity and reduces mitochondrial superoxide generation in MSCs. Therefore, the EVs-mediated transfer of hypoxia-treated healthy mitochondria to dysfunctional pancreatic acinar cells remodels their metabolic state, thereby leading to a more injury-resistant phenotype (Hu et al., 2023). MSCs-derived EVs restore Ca^{2+} homeostasis in dermal fibroblasts via transferring the transcription factor SP2. This mechanism, though it does not represent a specific example of mitoEVs-mediated mitochondrial transfer, also promotes Parkin-mediated mitophagy by delivering deubiquitinating enzyme USP9, thereby restoring mitochondrial function and promoting diabetic wound healing (M. Wang et al., 2024). Functional mitochondria from EVs can be released by neural stem cells and modulate the metabolism and physiology of target cells (Peruzzotti-Jametti et al., 2021). In particular, mitochondrial transfer restores the mitochondrial function and remodel metabolic profile of mtDNA-depleted L929 Rho^0 cells. Moreover, EVs-included mitochondria integrate into mitochondrial network of inflammatory mononuclear phagocytes Mφ, thereby restoring mitochondrial morphology and metabolism, and reducing the pro-inflammatory gene expression profile (Peruzzotti-Jametti et al., 2021). In *in vivo* model of multiple sclerosis, the functional transfer of mitochondria from neural stem cells to mononuclear phagocytes ameliorates clinical deficits (Peruzzotti-Jametti et al., 2021). The occurrence of bidirectional transfer of mitoEVs between MSCs and chondrocytes *in vitro* has also been described. Transferring MSCs-derived mitochondria leads to chondrogenic effects, including increase in DNA content and proteoglycan deposition (Korpershoek et al., 2022).

In a mouse model of focal cerebral ischemia, the astrocytic release of functional mitochondria via Ca^{2+}-dependent mechanisms, involving CD38–cyclic ADP ribose signalling, operates as neuroprotective mechanism, thereby supporting cell viability and bioenergetics of recipient neurons (Hayakawa et al., 2016).

Furthermore, in cell and animal models of renal injury, EVs from MSCs (containing transcription factor A mitochondria mRNA, mtDNA, and mitochondrial components) reduce renal mitochondrial damage and inflammation by improving mitochondrial transcription factor A (TFAM)-mtDNA complex stability in target cells (Zhao et al., 2021).

Activated platelets can release functional mitochondria (in EVs and isolated) that are taken up by MSCs via dynamin-dependent clathrin-mediated endocytosis. This transfer stimulates the *de novo* synthesis of fatty acids that in turn boosts the pro-angiogenic activity of MSCs (Levoux et al., 2021). In addition, monocytes and neutrophils incorporate functional mitochondria from platelet EVs, thus increasing their bioenergetics (Pelletier et al., 2023). Consistent with above results, platelet-derived mitochondria are taken up by vascular endothelial cells via dynamin dependent endocytosis. This transfer increases surviving expression, thereby reducing oxidative stress-induced apoptosis of endothelial cells and promoting vascularization and wound healing (Jin et al., 2023). Platelet-derived mitoEVs improves cell proliferation, ATP generation, and oxygen consumption rate of breast cancer cells (MDA-MB-231). Interestingly, mtDNA-deficient MDA-MB-231 cells recover cell proliferation and restore mitochondrial bioenergetics upon platelet-derived mitochondria internalization, suggesting a mtDNA replenishment by acquired exogenous mitochondria (Cereceda et al., 2024).

In *in vitro* conditions, renal scattered tubular cells (STC-like cells) can exert protective effects on damaged tubular epithelial cells via EVs-mediated mitochondrial transfer. Also, in ischemic kidney injury *in vivo*, STC-like cells-mitoEVs mitigate renal stenosis and modulate mitochondrial pathways (Zou et al., 2018). Recently, it has been shown that packaged mitochondria in microvesicles originating from a human brain microvascular endothelial cell line (hCMEC/D3) significantly enhance intracellular ATP levels in the recipient hCMEC/D3 cells (Dave et al., 2021). The authors further demonstrated that hCMEC/D3-derived mitochondria-containing medium-to-large EVs (m/lEVs) are taken up by recipient human brain microvascular endothelial cells (HBMECs) and merge with the host mitochondrial network. This results in enhanced bioenergetics, mitochondrial activity, and glycolytic capacities of target cells. In a mouse model of ischemic stroke, injected m/lEVs also show neuroprotective effects (Dave et al., 2023). In addition, human brain

endothelial cells can transfer functional mitochondria via mitoEVs to the ischemic cellular counterparts (increasing ATP levels, mitochondrial functions, and cell survival), and neurons in mice acute brain cortical and hippocampal slices (D'Souza et al., 2021).

As described above, mitoEVs can contribute to the inter-organ mitohormesis, an energetic stress-induced metabolic modulation that specifically involves the crosstalk between cardiomyocytes and adipocytes resulting in cardio-protection (Crewe et al., 2021).

Furthermore, in a model of allergic airway disease, mitoEVs derived from bronchoalveolar lavage fluid of asthmatics or originating from myeloid-derived regulatory cells are internalized by peripheral T cells. Subsequently, fusion of the transferred mitochondria with the recipient's mitochondrial network reshapes its bioenergetic and/or redox profile (K. P. Hough and Deshane, 2019; Kenneth P. Hough et al., 2018).

Interestingly, recent findings have suggested that mitoEVs may have key role in mediating the maternal-fetal bioenergetic crosstalk during peri-implantation development (Bolumar et al., 2023).

In particular, human endometrium can secrete different subsets of mitoEVs containing mtDNA (e.g., apoptotic bodies, microvesicles in particular, and exosomes) into uterine lumen fluid. Therefore, a maternal mtDNA vertical transfer process may modulate embryo metabolism and bioenergetics as endometrial-mitoEVs impact ATP turnover in the trophectoderm of recipient murine embryos *in vitro*.

Very recently, *in vitro* and *in vivo* evidence have demonstrated a crucial role of circulating young human and mouse mitoEVs in reversing age-related dysfunction through enhancing mitochondrial energy metabolism (Chen et al., 2024). Notably, the representative cargo of young mitoEVs are mitomiRs, including miR-144–3p, miR-149–5p, and miR-455–3p, factors that positively impact on metabolic remodelling through increased expression of PGC-1α, a critical master regulator of mitochondrial biogenesis (Chen et al., 2024).

MitoEVs may induce not only important protective effects but also exert remarkable influence on the pathogenesis of diseases. Therefore, MitoEVs can be mediators of oncogenic signals that are involved in the metabolic reprogramming of cancer cells, thus supporting tumor progression and resistance to therapy. Generally, mtDNA depletion/mtDNA mutations lead to oxidative phosphorylation reduced activity and low tumorigenic potential. Therefore, EVs from stromal cells can transfer mtDNA to breast cancer cells, thereby promoting the transition from therapy-induced metabolic quiescence state to hormonal therapy-resistant phenotype via modulating their metabolism/oxidative phosphorylation capacity (Sansone et al., 2017). Furthermore, the horizontal transfer of mtDNA via EVs from colon cancer patients promotes metabolic reprogramming in surrounding normal colonic epithelial cells (CECs). In this scenario, CECs exhibit increases in mitochondrial respiration and ROS generation that leads to NF-κB-mediated upregulation of the cancer-associated cytokine TGFβ1 that subsequently drives malignancy in colon cancer cells through the activation of the TGFβ/Smad signalling pathways (Guan et al., 2024).

After releasing EVs containing inflammatory mitochondria by M1 macrophages, recipient pancreatic beta cells undergo apoptosis via the ferroptosis pathway (Gao et al., 2024). The merge of inflammatory mitochondria with the mitochondrial network of beta cells induces a significant decrease of ATP production and mitochondrial respiration, along with the intracellular accumulation of Fe^{2+} and release of mtDNA that leads to cell death through the activation of cGAS-STING pathway.

3.3. Modulation of immune responses by mitoEVs: two side of the same coin

MitoEVs have been shown to play ambivalent roles in immunity. As a result of the presence of mtDAMPs, it has been well assumed that mitoEVs can intrinsically trigger or heighten inflammation, especially when are targeted to immune cells. On the other hand, EV-mediated mitochondrial delivery is also effective in driving and resolving inflammation, thus exerting therapeutic effects (Fig. 3).

Therefore, activated platelets can release EVs-embedded functional mitochondria that are hydrolysed by the bactericidal secreted phospholipase A2-IIA (generating bioactive mediators, including mtDNA, fatty acids, and lysophospholipids) to elicit neutrophil proinflammatory responses (Boudreau et al., 2014). As described above, polarized mitochondria incorporated in exosomes isolated from myeloid-derived regulatory cell integrate with mitochondrial network of T cells, thereby leading to bioenergetic remodelling of recipient cells. The resulting functional and differentiative reshape may be relevant under chronic inflammatory conditions, including asthma (Kenneth P. Hough et al., 2018). Furthermore, lipopolysaccharide-stimulated monocytes can release microvesicles containing damaged mitochondrial constituents that can elicit inflammatory reactions in endothelial cells via type-I interferon (IFN) and tumour necrosis factor signalling pathways (Puhm et al., 2019). In the setting of organ transplantation, increased levels of membrane-bound mitochondria in donor plasma directly correlate with neutrophil activation and generation of inflammation-associated cytokines. Therefore, circulating mitoEVs may trigger a pro-inflammatory condition in the liver post-transplant environment, contributing to tissue dysfunction and early rejection (Pollara et al., 2018).

Macrophages treated with different activators of NLRP3 inflammasome can release large EVs containing mitochondrially encoded mRNAs that may contribute to the pathogenesis of complex chronic inflammatory diseases, though this hypothesis remains to be determined (Budden et al., 2021). Activated platelets can release EVs carrying mtDAMPs that play a significant role in the pathogenesis of systemic lupus erythematosus (Linge et al., 2018; Mobarrez et al., 2019). EVs embedding mtDNA from antiphospholipid-exposed placental explants elicit endothelial proinflammatory responses that may lead to pre-eclampsia pathogenesis (Linge et al., 2018). High levels of circulating mitoEVs have also been observed in mouse models of hepatic inflammation (Garcia-Martinez et al., 2016), and patients with autism spectrum disorder where they promote the secretion of IL-1β by human microglia (Tsilioni and Theoharides, 2018). Similarly, individuals with sepsis exhibit increased number of circulating mitoEVs, probably contributing to endothelial activation via stimulation of type-I IFN signalling pathway (H.-J. Zhang et al., 2023). In addition, patients with Behçet's syndrome (a chronic systemic inflammatory disorder) show enhanced levels of circulating mtDNA encapsulated into EXOs. In this setting, mitoEVs can propagate and heighten inflammation by inducing leukocyte mobilization along with the production of cytokines (IL-1β and IL-23), and the IL-17-mediated neutrophil activation that causes the characteristic symptomatology of Behçet's syndrome (Konaka et al., 2023). In the same line, mtDNA and inflammatory proteins in EVs from frail individuals are altered, thus suggesting that mitoEVs may contribute to the chronic inflammatory state related to frailty (Byappanahalli et al., 2023). Circulating MitoEVs are also identified in individuals with PD exhibiting a specific inflammatory state (Picca, Guerra, et al., 2020).

In a model of alcoholic hepatotoxicity, hepatocytic mitochondrial double-stranded RNA and mtDNA are transferred to surrounding macrophages/Kupffer cells via exosomes and microvesicles. Consequently, the increased IL-1β expression by Kupffer cells promotes the production of IL-17 by γδ T cells at the early stage of alcoholic liver disease (ALD) (Lee et al., 2020). Interestingly, to prevent the mtDAMPs-induced inflammation cells can prevent the export of EVs containing oxidized mitochondrial proteins. Under basal conditions, MDVs transport functional mitochondrial components to EVs through optic atrophy 1 (OPA1) and sortin nexin 9 (SNX9) involvement. Following mitochondrial damage, however, this mechanism is blocked by PINK/Parkin pathway activation, and MDVs are transported to lysosomes to prevent the extracellular release of pro-inflammatory mtDAMPs (Todkar et al., 2021).

Although mitochondrial release can also contribute to the activation of host-protective immune responses (Mistry et al., 2019), there are many studies suggesting an anti-inflammatory role related to mitoEVs

(Dutra Silva et al., 2021; Morrison et al., 2017; Peruzzotti-Jametti et al., 2021; Zhao et al., 2021). Therefore, exosomes incorporating mitochondrial proteins derived from bone marrow-derived stromal cells suppress inflammatory markers generation and NLRP3 inflammasome activation in stressed nucleus pulposus cells (Xia et al., 2019). In this context, the refill of mitochondrial proteins also restores mitochondrial homeostasis and exerts therapeutic effects ameliorating the intervertebral disc degeneration *in vivo*.

Interestingly, mitoEVs-induced immunosuppressive effects are also involved in mediating cancer immunoescape and tumor progression *in vivo* (Cheng et al., 2020). In particular, Lon-overexpressing colon cancer cells can release EVs carrying mtDNA and immunosuppressive protein programmed death ligand 1 (PD-L1) that induce IL-6/13 and IFN-β secretion from M2 macrophages, thereby inhibiting T-cell function and promoting tumorigenesis. Furthermore, mitoEVs boost the ability of antigen-bearing dendritic cells (DCs) to orchestrate an immune response. After contacting DCs, activated T cells deliver mtDNA incorporated in EVs to DCs, thereby triggering the antiviral innate immune response in recipient cells via activation of cGAS/STING-IRF3 pathway (Torralba et al., 2018). Also, the uptake of MSC-derived mitochondria-containing EVs by macrophages leads to M2 phenotype, resulting in increased phagocytic activity and suppression of immune response (through T cells) (J. H. Ko et al., 2020).

Previous studies have indicated an increased production of EVs by virus-infected cells, suggesting that mitoEVs may be involved in triggering antiviral defence responses as a mechanism for cell homeostasis. Therefore, EVs containing mtDNA from Kaposi's Sarcoma-associated herpesvirus-infected endothelial cells induce the expression of interferon-stimulated genes through the cGAS-STING pathway in uninfected bystander cells, thus mediating antiviral response (Jeon et al., 2019).

4. MitoEVs in aging and aging-related diseases

Aging ensues from the gradual deterioration of biological functions and the organism's waning ability to adapt to metabolic stress over time, heightening the risk of developing various adverse health conditions and diseases (Cesari et al., 2017). Unveiling the intricacies of the aging process is pivotal for devising strategies to delay its onset and prevent age-associated diseases. Proposed drivers of aging encompass loss of proteostasis, genomic instability, telomere attrition, deregulated nutrient sensing, epigenetic alterations, altered intercellular communication, stem cell exhaustion, cellular senescence, and mitochondrial dysfunction (de Magalhães, 2024; López-Otín et al., 2023), thereby representing the so-called hallmarks of aging useful to develop age-delaying therapeutic approaches. Depending on their different causal roles and hierarchy temporal manifestation in aging, these signatures have been categorized in primary, antagonistic, and integrative. Further, hallmarks of aging are recognized to be interconnected, thereby leading to the more consistent concept of "aging network". As described above, aging is a driving factor of various age-related pathologies, including neurodegenerative and cardiovascular diseases, and cancer. In this regard, these pathologies are strongly associated with specific hallmarks of aging such as mitochondrial dysfunction, altered intercellular communication, and chronic inflammation (López-Otín et al., 2023).

The role of EVs in aging and aging-associated diseases, including neurodegeneration (AD and PD), cardiovascular diseases and reproductive diseases (ovarian aging and uterine aging) (Battaglia et al., 2020; Mas-Bargues and Alique, 2023; Shomali et al., 2020) has come forward leading to the proposal of alteration in EV secretion (levels, cargo, and effects on target cells) as a new hallmark of aging as reported by Manni et al. (Manni et al., 2023). Many evidence would support this concept. The release of EVs from senescent cells can have several roles. EVs and their different cargos can mediate cell-to-cell communication and alter the flogosis-, apoptosis-, proliferation-related status of surrounding cells (Al Suraih et al., 2020; Choi et al., 2020; Terlecki-Zaniewicz et al., 2018). On the other, some authors demonstrated that the inhibition of EV secretion caused the accumulation of nuclear DNA within the cytoplasm, thus activating ROS-dependent DNA damage response cell-cycle arrest (Takahashi et al., 2017). Senescent fibroblasts have been found to produce higher levels of EVs, as compared to the non-senescent counterpart, and this negatively impacted on the differentiation (Choi et al., 2020). Similar findings were reported by other researchers in endothelial cells (Mensà et al., 2020; Riquelme et al., 2020). Interestingly, some authors showed that EV release in senescent cells is dependent on p53, which responds to telomere erosion, a primary hallmark of aging (Manni et al., 2023; Roake and Artandi, 2017; Tesei et al., 2021; X. Yu et al., 2006). In aging mice, plasmatic circulating EVs exhibited augmented levels of specific miRNAs with effects on -immune function (Alibhai et al., 2020). Senescent cholangiocyte-derived EVs were shown to contain multiple growth factors, including EGF (Al Suraih et al., 2020). Interestingly, intravenous injection of young EVs into aged mice extends their lifespan, mitigates senescent phenotypes and ameliorates age-associated functional declines in multiple tissues (Chen et al., 2024).

In the context of EVs, some literature provide evidence that mitoEVs are emerging as a subset of EVs with a potential role in aging and aging-related processes.

4.1. Aging

During aging, diminished cellular degradation mechanisms, including mitophagy, impede the removal of damaged cytosolic materials, thereby leading to altered cellular homeostasis. In accordance with this concept, an increased release of mitoEVs has been detected in the aged heart (Liang et al., 2023) as well as in Huntington's disease (Beatriz et al., 2022) and it has been ascribed to impairment of the lysosome-endosome pathway. According to their biogenesis and markers, these mitoEVs are in the form of large EVs (~200 nm). Age-related declines of functional mitochondria in specific plasma EV subpopulation classified as exosomes have been reported and interpreted as evidence of age-associated defects in mitophagy of their parent cells during immunosenescence and/or inflammaging (Xin Zhang et al., 2020).

In the road towards the role of mitoEVs in the aging process, an important goal is the finding that, in contrast to peripheral blood mononuclear cells where a diminished mtDNA was found (Mengel-From et al., 2014), circulating cell-free mtDNA originating from cellular damage or stress progressively rises from childhood to middle age, followed by a gradual increase in the elderly post the age of 50 (Picca et al., 2017; Pinti et al., 2014). Afterwards, Lazo et al. reported the presence of mtDNA encapsulated in plasma EVs, whose level changed with age. This suggested that mitoEVs may signal and/or contribute to various physiological and pathological conditions associated with aging, with age-dependent packaging of EVs potentially playing a crucial role in these processes (Lazo et al., 2021). The increase in dysfunctional mitochondria in EVs produced by immune cells in plasma is also correlated with aging (Xin Zhang et al., 2020). In elderly individuals with frailty and sarcopenia, mitochondrial protein levels in serum EVs may serve as predictors to distinguish them from those without frailty and sarcopenia (Picca, Beli, et al., 2020).

Recently, in an effort to characterize the effects of aging on plasma EV cargo, some circulating miRNAs differentially expressed in young and aged mice and humans were found to be present in plasma EVs (Chen et al., 2024). In particular, miR-144–3p, miR-149–5p and miR-455–3p were considered as the representative cargoes of EVs at the young state, whereas miR-29a-3p, miR-29c-3p and miR-34a-5p were characteristic of EVs at the aged state. Based on different studies, these miRNAs are MitomiRs with specific roles in aging-related pathologies (Saikia et al., 2023) by opening up a new frontier in the characterization of mitoEVs as aging hallmarks.

4.2. Parkinson's disease

PD is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta and subsequent dopamine depletion in the striatum, which is a primary PD hallmark (Alexander, 2004). Similarly to other neurodegenerative disorders, PD is intricately linked with mitochondrial dysfunction (Picca, Calvani, et al., 2020), including severe disruption of mitophagy due to recessive mutations of PINK1 and Parkin, normally working together in the same pathway to govern mitochondrial quality control (Whitworth and Pallanck, 2017). In this context, a possible role of mitoEVs in PD onset and progression has emerged. Elevated levels of serum EVs attempting to expel damaged organelle components are described in PD patients (Picca et al., 2023). According to the EXosomes in PArkiNson Disease (EXPAND) study, EVs/exosome cargo from PD patient sera is characterized by mitochondrial proteins (ATP5A, NDUFS3, and NDUFB8) suggesting that decreased mitophagy has stimulated MDV generation as an alternative mechanism for MQC. In addition, PD exosomes contained a reduced content of mitochondrial proteins when compared to healthy controls. These alterations in mitoEV contents may mirror mitochondrial defects and may serve as prognostic indicators for disease progression (Picca et al., 2019; Picca, Guerra, et al., 2020). In in vivo models of PD it has been reported that dopaminergic neurons exhibit fragmented mitochondria that accumulate in spheroids, where mitophagy begins but is not completed. These spheroids are then transferred to surrounding astrocytes, where they are linked to autophagosomes for lysosomal degradation, preventing the release of inflammatory mediators and reducing inflammatory responses in PD (Morales et al., 2020). Finally, evidence for the role of mitoEVs in PD pathogenesis derives from the observation that, as mentioned above (Section 3.1), FNZ may induce parkinsonism through excessive mitochondrial elimination mediated by mitolysosome formation in the brain (Bao, Zhou, Xiao, et al., 2022). In particular, damaged mitochondria are engulfed intact into lysosomes to form mitolysosomes, which are subsequently extruded via vesicle-associated membrane protein 2 (VAMP2)- and syntaxin-4 (STX4)-mediated exocytosis. This extracellular secretion of mitolysosomes generates mitoEVs, which may be taken up by surrounding cells for disposal (Bao et al., 2022). Overall, the characterization of mitoEVs in PD may provide an important contribution to the pathophysiology of this neurodegenerative disease and the development of prognostic tools.

4.3. Alzheimer's Disease

AD is a neurodegenerative condition characterized by dementia-like symptoms. It manifests in two forms: a late-onset sporadic type (SAD) and an early-onset familial variant (FAD). Key pathological features include amyloid-β (Aβ) plaques, tau-containing neurofibrillary tangles, and neuronal inflammation/loss, leading to brain atrophy (DeTure and Dickson, 2019; Weller and Budson, 2018). The involvement of mitoEVs in AD arises from the finding that plasma EVs from AD patients exhibit reduced levels of mitochondrial electron transport chain complexes I/III/IV and ATP synthase compared to controls (Yao et al., 2021). Moreover, astrocytes, microglia, and neurons exposed to Aβ aggregates and oxidative stress release EVs containing mitochondrial structures as well as mtRNA and proteins, suggesting a role for MDVs in exporting harmful mitochondrial components from damaged mitochondria. Interestingly, Villar-Vesga *et al.* (Villar-Vesga et al., 2020) reported that systemic EVs from postmortem samples of SAD patients showed elevated endothelial- and leukocyte-derived EVs containing mitochondrial markers. Additionally, Kim et al. (M. Kim et al., 2020) reported increased mtRNAs in plasma EVs from individuals with mild cognitive impairment and AD compared to healthy controls. Finally, possible involvement of mitoEVs in neuronal-astrocytes transmitophagy observed in human and mouse cellular and animal AD models, remains to be demonstrated (Lampinen et al., 2022). Based on these consideration, further studies on mitoEV in AD may contribute to the ongoing characterization of EVs as AD biomarkers. (Kapogiannis et al., 2019; Kim et al., 2020; Pulliam et al., 2019).

4.4. Cardiovascular diseases

Cardiovascular diseases, exemplified by coronary artery disease (CAD), primarily stem from significant vascular injury and inadequate blood flow to the heart. Despite being a leading cause of global patient mortality, early detection of CAD remains challenging. While the precise determinants are yet to be fully understood, the potential utility of EVs as biomarkers for CAD has garnered attention, with a specific focus on mitoEVs. Notably, EVs generated by monocytes mostly as exosomes in the serum of CAD patients were found to contain mitochondrial RNA, with reduced levels observed in individuals experiencing new cardiovascular events when compared to those without such events (Holvoet et al., 2016). Kaplan-Meier survival analysis further revealed that CAD patients in the lowest tertile of mitochondrial RNA levels in plasma EVs exhibited earlier development of cardiovascular adverse events (Holvoet et al., 2016). These findings underscore the potential of circulating mitoEVs as predictors for cardiovascular disease outcomes. The importance of mitoEVs in cardiac pathophysiology has also been reported by Liang and colleagues (Liang et al., 2023) who increased levels of mitoEVs in the cardiac tissue of Danon disease patients in concomitance with defective autophagic-lysosomal-mediated degradation.

4.5. Cancer

Although the distinctive high glycolytic rate of tumor cells (Warburg, 1956) mitochondria are recognized as key mediators of cancer behavior in several steps of tumorigenesis (Danhier et al., 2017; Solaini et al., 2011; Vyas et al., 2016) as well as in acquired resistance to therapy (Falone et al., 2019; Herst et al., 2018; Ponzetti et al., 2022; Schulze and Harris, 2012; Song et al., 2013). The symbiotic relationship between neoplastic cells and the tumor microenvironment (TME) via transport of mitochondria is believed to be relevant to survival, progression and even development of resistance to therapy in tumor cells (Hekmatshoar et al., 2018; Sahinbegovic et al., 2020; Singh et al., 2017). In recent years, intriguing experiments provide evidence that cancer cell biology can also be modified by exchanging mitochondrial contents via EVs (Takenaga et al., 2021). As a matter of fact, EVs derived from tumor and stromal cells have been established to serve as powerful regulators of tumor progression and resistance to treatments by moving their cargo (proteins, lipids, and RNA) into target cells (Sohal and Kasinski, 2023). Accordingly, specific EVs types are currently used as valid biomarkers to detect some cancers at early stages (Allenson et al., 2017; Madhavan et al., 2015; Yang et al., 2017). Tumor-derived EVs harbor full mitochondrial genome (Guescini et al., 2010; Li et al., 2020) and are released to repress T-cell-mediated immune response (Hong et al., 2016), or to restore metabolism in impaired cancer cells (Sansone et al., 2017). Sansone et al. (2017) showed that cancer-associated fibroblast-originated EVs were able to transfer mtDNA to dormant breast cancer cells, promoting resistance to endocrine therapy, and favoring an efficient clonal expansion (Sansone et al., 2017). Such remarkable effects seemed strictly related to the recovery of oxidative phosphorylation in hormonal therapy-impaired cancer cells (Sansone et al., 2017). Another interesting study demonstrated that breast cancer cells can release mitoEVs enriched with mtDNA enhancing the invasive competence in recipient breast cancer cells (Rabas et al., 2021). MitoEV-mediated release of mtDNA and programmed cell death-ligand 1 (PD1) into the tumor microenvironment has cancer-promoting and immune-suppressive effects (H. Ko et al., 2023). In addition, Abad and Lyakhovich found that the conditioned medium enriched with mitochondria-containing exosomes from chemoresistant triple-negative breast cancer cells promoted the development of chemoresistance in sensitive cells by transferring mitochondria with a mutated form of the mtND4 gene (Abad and Lyakhovich, 2022). In 2022, Zeng and colleagues reported that endometrial

carcinoma cells released EV-encapsuled mtDNA and PD-L1 upon IL-6 stimulation, and that such event led to tumor immune escape via interaction with T cells (Zeng et al., 2022). As described above, mitolysosome exocytosis is a crucial MQC mechanism highlighted in lung cancer cells (Dayalan Naidu et al., 2023). In colon cancer, mtDNA transfer via mitoEVs promotes metabolic reprogramming in nearby normal colonic epithelial cells with increase of mitochondrial respiration and ROS generation which drive cancer progression (Guan et al., 2024). Additionally, mitoEVs releasing mtDNA and the immunosuppressive protein PD-L1 from Lon-overexpressing colon cancer cells. These EVs stimulate M2 macrophages to secrete IL-6/13 and IFN-β, inhibiting T-cell function and promoting tumor growth (Cheng et al., 2020). In addition to mitoEV production, cancer cells are recipients of extracellular mitochondria. Platelet-derived mitoEVs improves cell proliferation, ATP generation, and oxygen consumption rate of breast cancer cells (MDA-MB-231). Interestingly, mtDNA-deficient MDA-MB-231 cells recover cell proliferation and restore mitochondrial bioenergetics upon platelet-derived mitochondria internalization (Cereceda et al., 2024). Also, mitochondrial low-level transfer by secretion from astrocyte to glioblastoma was suggested to be involved in the increased mitochondrial respiration and tumorigenicity (Watson et al., 2023). Although it emerges the importance of mitoEVs in regulating cancer biology, mitochondrial intercellular trafficking takes place via TNTs as in the case of mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemia cells in response to superoxide (Marlein et al., 2017). Other mediators promoting mitochondrial transfer include metalloproteinase-1, nestin, and key proinflammatory cytokines (Berridge and Tan, 2010). There is much evidence that mitochondrial DNA acquisition by cancer cells increases their respiratory capacity and ATP production, which was linked to enhanced proliferation and invasion (Caicedo et al., 2015; Dong et al., 2017; Ippolito et al., 2019; Marlein et al., 2017; Tan et al., 2015). Blocking mitochondrial transfer in mice transplanted with myeloma cells resulted in increased survival, indicating the critical role of mitochondrial trafficking in tumor progression (Marlein et al., 2017).

5. Isolation and characterization of mitoEVs: a brief overview of current methods

As this topic has been extensively discussed elsewhere (Mas-Bargues and Alique, 2023) here we report a concise overview of the most common methods for mitoEV isolation. General characterization of EV size, morphology and markers is described by MISEV2018 and 2023 guidelines, the official repository of standardised isolation methods (Théry et al., 2018; Welsh et al., 2024). Nevertheless, a unique/exclusive method for the selective isolation of mitoEVs is not available. Rather, the techniques proposed by different authors seems to show advantages or disadvantages in relation to specific purposes (Mas-Bargues and Alique, 2023; Q. Zhang et al., 2023).

An important challenge in mitoEV isolation is to achieve a high level of purification of all the EVs in order to subsequently identify mitoEVs with specific techniques. In this regard, a reliable method using differential ultracentrifugation, filtration, concentration, and high-resolution density-gradient fractionation for sequential isolation of distinct populations of EVs and nonvesicular (NV) extracellular nanoparticles (NVEPs), including exomeres and supermeres, has recently been developed (Q. Zhang et al., 2023). Other methods routinely used include density-gradient centrifugation, size exclusion liquid chromatography, ultrafiltration, commercial kits based on polyethylene-glycol (e.g. ExoQuick or Total Exosome Isolation Kit) and immunoaffinity capture based methods, used alone or in association to combine the different separation capacity (Mas-Bargues and Alique, 2023; Sidhom et al., 2020). After isolation, multiple approaches are necessary for proper characterization and quantification of mitoEVs. The general techniques used are based on laser light microscopy and correlation of the light scattered with the EV dimensions, Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering (DLS) (Bağcı et al., 2022). The microstructure of EVs is obtained by transmission or scanning electron microscopy (Brennan et al., 2020; Konoshenko et al., 2018); in this regard, cryo-electron microscopy is considered more efficient in preserving morphological features and inner cargo when compared with chemical fixation. Large-scale quantification methods are based on the determination of total proteins amount with the classical Bradford or Bicinchoninic acid assays. Finally, flow-cytometric analyses with a high throughput capacity of discrimination efficiency have been developed. However, the achievement of good levels of resolution and sensitivity still remains challenging (Brennan, 2020). For a relatively cheap and easy mitoEV characterization, selective markers can be detected by western blot analyses. A further challenge in mitoEV isolation is the accumulation of impurities and contaminants during the process (Phinney et al., 2015; Todkar et al., 2021), To avoid this, some authors have proposed to use sucrose-based methods which at the one hand show higher resolution power of the fractions, but on the other have some limitations referred to osmolarity changes and loss of the physiological features (Soubannier et al., 2012). Regarding MDVs, it has been reported their *in vitro* isolation from purified budding-mitochondria (Heyn et al., 2023) or by immunoaffinity-based techniques using specific markers of MDVs, such as TOMM20 and MAPL (König et al., 2021). Interestingly, D'Acunzo's research group proposed a modified version of this ultracentrifugation method, based on the innovative use of iodixanol-high-density gradient, providing the isolation of mitovescicles from solid tissues; in particular, after the enzymatic digestion of mouse and human brains tissues, a whole EVs pellet was obtained and subsequently submitted to an iodixanol-gradient density column step. This approach has improved the maintenance of physiological features and avoided vesicle shrinkage related to sucrose-based methods (D'Acunzo et al., 2021, 2022, 2024). Very interestingly, the extension of this pioneer method to a variety of other biological fluids may pave the way to further studies on the relationship existing between mitoEVs and different physio-pathological conditions.

6. Conclusions and future directions

The field of EVs-mediated mitochondrial delivery is an emerging area of research that sheds new light in the ability of mitochondria to participate in an intercellular connectivity in various physiological and pathological states. In this regard, mitoEVs play a key role in multiple biological functions (Fig. 3). The release of mitoEVs represents an alternative MQC mechanism contributing to diversify the cellular responses to distinct stressing conditions. A small part of MDVs can be routed to cell surface, generating mitoEVs (Todkar et al., 2021) within a few minutes upon stress (Rosina et al., 2022). As described above, mitoEVs are critical for cardiac homeostasis (Liang et al., 2023) and metabolic regulation induced by energetic stress in adipocytes (Crewe et al., 2021; Rosina et al., 2022). In these scenarios, mitoEVs are destined for engulfment and digestion by tissue resident macrophages or act as warning signal for the heart. Therefore, this type of mitoEVs stimulate cell survival protecting recipient cells through a hormetic mechanism (Crewe, 2023). MitoEVs derived from MDVs also provide a form of quality control when lysosomal function is impaired and mitophagy is inactive in cancer cells (Towers et al., 2021). In this condition, mitoEVs production increases to maintain efficient cancer cell growth. Therefore, from a health perspective, targeting MDV pathways may make cancer therapy more effective. Similar but distinct mechanisms for mitochondrial extrusion contributing to MQC have also been reported. Stressed neurons and cardiomyocytes release damaged mitochondria through LC3-positive membrane vesicles called exophers, suggesting protective roles. Innate exophergenesis mechanism is conserved in the nematode *Caenorhabditis elegans*, being crucial for supporting offspring development (Turek et al., 2021) and neuronal function (Melentijevic et al., 2017). Alternative MQC pathways for mitochondrial clearance can also include the transmitophagy. As

described above, this mechanism may be an evolved variant of exophers formation as it shares similar axonal protrusions and evulsions containing mitochondria. Indeed, in both contexts, mitoEVs are taken up and degraded by tissue resident cells, including macrophages and astrocytes. In migrating cells, under basal conditions and in response to mild stress, dysfunctional mitochondria are selective transferred to migrasomes and transported outside cell through mytocitosis, a process that required 40–200 minutes. In terms of timing, mytocitosis along with MDVs production may represent early MQC mechanisms under mild stress, thereby limiting damage propagation to the entire mitochondria. By contrast, mitophagy would operate under more severe stress conditions to remove a large quantity of dysfunctional mitochondria. Differently from these selective processes, the exocytosis of mitolysosome is a non-selective removal of damaged mitochondria generating mitoEVs containing even the whole mitochondrial content of the cell (Bao, Zhou, Xiao, et al., 2022). MitoEV processes included in MQC may involve mitopherogenesis. However, mitophers contain healthy mitochondria and operate for eliminating the excessive mitochondria. This finding could expand the concept of MQC introducing the need to control the homeostasis of the cell mitochondrial during development.

Depending on the specific conditions of the donor cells and mitochondrial biology, the structural and functional nature of mitoEVs varies. This condition can result in reverberant specific signals within cells and across the organism that leads to either survival or death of the recipient cells. This complex metabolic interplay highlights the essential properties of EVs-mediated mitochondrial transfer and may pave the way for the use of mitoEVs as potential biomarkers or therapeutic tools. Indeed, the existing literature shows an emerging role of mitoEVs in aging, neurodegenerative disease and cancer biology. Thus, mitoEVs may represent an intriguing aspect of the concept of EVs as hallmark of aging (Manni et al., 2023) and offer a relevant contribution to the search for “geroprotectors” against mitochondrial dysfunction. Current challenges in this direction include a better resolution of mitoEV heterogeneity by improving methodology for vesicles isolation and the dynamic mapping of stress-triggered mitoEV systemic signatures at different time scales. This would allow to conceive and validate robust and reliable prognostic tools. Furthermore, mitoEVs-mediated inter-cellular communication require elucidation of the mechanisms involved in the selective packaging of mitochondrial constituents and sorting processes. In this regard, CD38/cADPR signalling (Suh et al., 2023), OPA1, and SNX9 have been demonstrated to mediate the extracellular release of mitochondria (Todkar et al., 2021) in astrocytes, osteoblasts, fibroblasts, and epithelial cells. Recently, it has been shown that mitochondrial fragmentation stimulates MDVs generation and extrusion in osteoblasts (Suh et al., 2023). However, whether alterations in mitochondrial dynamics are a universal prerequisite for biogenesis of mitoEVs remain to be determined. As described above, mitoEVs can be taken up by recipient cells and merge with the host mitochondrial network. In this regard, the mechanisms of recipient cell contact and uptake of extracellular mitochondria, as well as the mitochondrial fusion process are beginning to be understood. Finally, although mitoEVs from functional cells (especially MSCs) have shown to mitigate mitochondrial dysfunction and inflammation in different cell types and animal models of organ injury, it will be important to discriminate the specific role of mitochondrial components compared to other active compounds (such as miRNA, lipids, proteins, and small biomolecules) carried by mitoEVs in the same way. Future understanding of these concepts may allow to further resolve the relationship between systemic mitochondrial communication and the conditions of health, aging and disease.

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Declaration of Competing Interest

The authors 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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Abbreviations

extracellular vesicles (EVs), mitochondrial extracellular vesicles (mitoEVs); mitochondrial derived vesicles (MDVs); mitochondrial quality control (MQC); non-communicable diseases (NCDs); mitochondrial damage-associated molecular patterns (mtDAMPs); reactive oxygen species (ROS); tunnelling nanotubes (TNTs); arrestin domain-containing protein 1-mediated microvesicles (ARMMs); mitochondrial microRNAs (MitomiRs); protein kinesin family member 5B (KIF5B); vesicle-associated membrane protein 2 (VAMP2); syntaxin-4 (STX4); translocase of outer mitochondrial membrane 20 (TOMM20); mitochondria-anchored protein ligase (MAPL); pyruvate dehydrogenase (PDH); centromere protein F (CENP-F); mitochondrial dynamics proteins of 49 and 51 kDa (Mid49/Mid51); cyclic ADP ribose (cADPR); optic atrophy 1 (OPA1); sorting nexin 9 (SNX9); monoamine oxidase (MAO); optic nerve head (ONH); Alzheimer disease (AD); Parkinson’s disease (PD); receptor MER-tyrosine kinase (MERTK); retraction fibers (RFs); myosin 19 (MYO19); granulosa cells (GCs); flunarizine (FNZ); bone marrow-derived stromal cells (BMSCs); acute respiratory distress syndrome (ARDS); induced pluripotent stem cells (iPSCs); pancreatic acinar cells (PACs); neural stem cells (NSCs); oxygen consumption rate (OCR); medium-to-large EVs (m/lEVs); oxidative phosphorylation (OXPHOS); colonic epithelial cells (CECs); circulating cell-free mtDNA (ccf-mtDNA); electron transport chain (ETC); EXosomes in PArkiNson Disease (EXPAND); sporadic type of AD (SAD); familial variant of AD (FAD); dihydroorotate dehydrogenase (DHODH); respiratory complex I (CI); Programmed death 1 ligand (PD-L1); colonic epithelial cells (CECs).

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