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Large extracellular vesicles: Size matters in tumor progression

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ABSTRACT

Extracellular Vesicles (EVs) represent a heterogeneous population of particles naturally released from all cells, delimited by a lipid bilayer and able to horizontally transfer their cargos to recipient cells. These features imply the growing interest on EVs in cancer biology as biomarkers and therapeutic targets. In this review, we will highlight the specific process related to biogenesis and release of large EVs (L-EVs) derived from the plasma membrane (PM) compared to the small and well described exosomes, generated through the classical endosome-multivesicular body (MVB) pathway. The control of PM rigidity by cells depends on lipid/protein composition, cytoskeleton dynamics, cytoplasmic viscosity, ions balance, metabolic reprogramming and specific intracellular signaling pathways, all critical determinants of L-EVs biogenesis. We will focus in details on a specific class of L-EVs, named Large Oncosomes (LO), exclusively shed by cancer cells and with a size ranging from 1 μm up to 10 μm. We will examine LO specific cargos, either proteins or nucleic acids (i.e. mRNA, microRNAs, single/double-stranded DNA), as well as their functional role in cancer development and progression, also discussing the mechanisms of L-EVs internalization by recipient cells. Overall we will highlight the potential of LO as specific diagnostic/prognostic cancer biomarkers discussing the associated challenges.

1. Introduction

"Extracellular Vesicles" (EVs) is the generic term indicating "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate" [1], basically shed from all cell types.

When we look at EVs and especially in cancer, a plethora of studies described the relevance of highly heterogeneous EVs population, based on their origin, size and peculiar markers, in the horizontal transfer of aggressive properties to cancer/non cancer recipient cells. However, consulting the literature on EVs may generate confusion, because the EVs-specific population nomenclature results not always informative and effective [2]. To avoid this confusion that can also generate non-reproducible data, it is critical to refer to the International Society for Extracellular Vesicles (ISEV) guidelines on minimal information for studies of extracellular vesicles (MISEV) [1,2].

The exosomes are the smallest among EVs categories (30–100 nm), generated through the classical endosome-multivesicular body (MVB) pathway followed by fusion to the plasma membrane (PM). The process has been discovered around 60 years ago and previously reviewed in details [3]. However, also exosomes themselves are emerging as a quite heterogeneous population of EVs. Zhang H et al. described a distinct non-membranous exosomes population named exomeres (~35 nm) [4]. Similarly, a recent report suggests the occurrence of a small EVs population distinguished from "exosomes" in protein contents and biogenesis pathway [5].

EVs larger than exosomes and mostly derived from the PM range from hundred nanometers to few microns. Among these, apoptotic bodies [6] and ectosome/microvesicles (MV) [7] are the most studied.

Abbreviations: EVs, extracellular vesicles; L-EVs, large extracellular vesicles; PM, plasma membrane; MVB, multivesicular body; MV, microvesicles; LO, large oncosomes; ISEV, International Society for Extracellular Vesicles; MISEV, minimal information for studies of extracellular vesicles; DIAPH3, diaphanous-related formin 3; MAT, mesenchymal to amoeboid transition; Ca^{2+}, calcium ions; PS, phosphatidylserine; VAMP3, vesicle-associated membrane protein 3; ROCK, Rho-associated coiled-coil containing kinases; ESCRT, endosomal sorting complex required for transport; EGFR, epidermal growth factor receptor; ARF6, GTPase ADP-ribosylation factor 6; CK18, keratin 18; MMPs, matrix metalloproteinases; PCa, prostate cancer; αV-integrin, integrin alpha V; uPAR, urokinase-type plasminogen activator receptor; eEF1γ, eukaryotic elongation factor 1 gamma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPI, glucose phosphate isomerase; LDHB, lactate dehydrogenase B; HSPA5, heat shock 70 kDa protein 5; MDH, malate dehydrogenase; GOT, aspartate transaminase; GLS, glutaminase; mRNA, messenger RNA; ncRNA, non-coding RNA; CAV2, caveolin-2; GSTP1, gene encoding Glutathione S-transferases protein; CAFs, cancer-associated fibroblast; MyrAkt1, myristoylated Akt1; BDNF, brain-derived neurotrophic factor; CXCL12, C-X-C motif chemochine 12; NAF, normal fibroblast; IL6, interleukin 6 EIPA, 5-(N-Ethyl-N-isopropyl)amiloride; HUVEC, human endothelial cells

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Large Oncosomes (LO) have been described 10 years ago for the first time as exclusively shed from cancer cells and extremely variegated at least in the size range, starting from 1 μm up to 10 μm [8]. In oncology, the number of publications on EVs other than exosomes is negligible compared to those on exosomes. One reason might be that large EVs (L-EVs), referring to MV that are basically larger than exosomes and LO, collected from cancer cells, were described as less abundant than exosomes ([9,10] and *unpublished observations*), thus resulting in less material to work with. However, since purification strategies have not been always accurate, those vesicles defined as exosomes could represent mixed populations, including MVs. In addition, LO have been identified only recently compared to exosomes, thus have not been investigaterd in all tumor models yet.

Indeed, most of the extraction kits available are thought to isolate exosomes, making the focus on smallest population of EVs faster compared to other populations. Moreover, even the nomenclature issues mentioned above (and highlighted by C.Thery and K.W. Witwer in [2]) contribute to the “exosomes supremacy”. The process of biogenesis and release of PM derived Large EVs (L-EVs), referring again to MV and LO, has been correlated to several signaling involving either biochemical or mechanical processes. Taken together, these two perspectives resulted in a complex series of events that are very sensitive to microenvironment conditions; thus explaining, at least in part, the huge variability in data generated on both biogenesis and function of L-EVs. The only exception is apoptotic bodies whose genesis is exclusively linked to the condition of "programmed cell death".

In this review we will discuss the process related to biogenesis and release of EVs larger than exosomes and particularly focusing on LO distinct cargos and functions highlighting their potential to be used as specific cancer biomarkers.

2. L-EVs biogenesis: fusing the biochemical and mechanical knowledge

L-EVs have been described in several tumors: prostate cancer [11], breast cancer [12], glioblastoma [13], glioma [14], pancreatic cancer [15], colon cancer [16], melanoma [17], leukemia.[1] Overall, all cancer cells shed L-EVs, whose composition is peculiar compared to those shed from the normal counterparts and/or tumor microenvironment [18]. However only few populations of L-EVs, such as LO, have been described as released exclusively by cancer cells [8].

Both biochemical composition and biophysical mechanisms contribute to signaling processes, triggering L-EVs biogenesis and shedding. One of the main aspect to take in account in the studies on L-EVs PM-derived biogenesis, is the control of PM rigidity by cells, which depends on different coexisting lipid/protein combinations., The result is a dynamic signaling platform, differently composed in healthy compared to cancer cells and highly sensitive to stimuli from either inside (i.e. gene mutations) and outside (i.e. oxygen or metabolites deprivation, inflammation) [11,19,20]. The first evidences of EVs shedding from cancer cells [21] as well as of PM organization in micro domains [22] were not investigated as related events. Indeed, the hypothesis that PM lipid domains may work as platforms for EVs biogenesis and shedding is very recent [23].

H. Pollet and colleagues reviewed mechanisms involved in L-EVs biogenesis from Red Blood Cells, individuating 4 main determinants: 1) cytoskeleton dynamics 2) cytoplasmic viscosity 3) ions balance 4) metabolic processes. All these determinants belong also to nucleated-cancer cells lifespan and progression, and some of them have been associated to L-EVs shedding in recent publications. However, not all studies detailed the molecular mechanisms determining the enhanced L-EVs shedding by cancer cells.

[1] Minciacchi V.R. et al. abstract #OT05.02 (https://doi.org/10.1080/20013078.2018.1461450).

It was reported that changes in cytoskeleton dynamics related to the acquisition of aggressive features of PCa cells resistant to mevalonate pathway inhibitors [20] and increased cholesterol in the PM (unpublished observations) afterwards resulted concomitant to the increased shedding of Large Oncosomes (LO) [11]. Also the amoeboid phenotype has been demonstrated at the bases of non-apoptotic blebbing and LO release, through the activation of RhoA/ROCK [24]. Notably, LO shedding has been shown as promoted by silencing of the gene encoding the cytoskeletal regulator Diaphanous related formin-3 (DIAPH3), which in turn promotes the amoeboid phenotype [25]. Indeed, DIAPH3 loss induces a transition from a mesenchymal phenotype, characterized by pericellular proteolysis, to a rapid amoeboid migratory phenotype, a transition named mesenchymal to amoeboid transition (MAT). However, transitions between these phenotypes are mediated by the Rac1/RhoA circuit that responds to external signals such as HGF/SF via c-MET pathway [26]; both phenotypes have been associated to stem features [20,27,28] thus contributing to the plasticity of cancer cells [28]. Recently, a report from Brassart B. and colleagues demonstrated that extracellular matrix degradation products are able to influence intracellular calcium influx and cytoskeleton reorganization, favoring a tumor amoeboid phenotype [29]. Initially cell viscosity has been investigated in tumor cells compared to normal cells [30,31]; and only recently it was associated to chemo-resistance development coupled to augmented EVs shedding [32]. About ions balance, it is very well established that intracellular Ca^{2+} levels determined L-EVs shedding upon the activation of flippases and other proteins involved in the lipid bilayer asymmetry [17,33]. The Ca^{2+} dependent activation of calpains determines the formation of membrane protrusions and the PS exposition to the external leaflet [23]. To date, even if tetraspanins are often proposed as exosome markers, specialized tetraspanins can also induce PM curvature [34], and their presence in shedding vesicles has been reported [35]. Additional proteins could also actively help sorting other proteins into L-EVs such as matrix metalloproteases delivered to nascent L-EVs through the association of vesicle-associated membrane protein 3 (VAMP3) with tetraspanin CD9 [36].

Although the mechanisms underlying L-EVs biogenesis involve multiple partners, depending on cell type and stimulation as mentioned above, it is essential to mention that Ras superfamily GTPases are postulated to be major mediators of L-EVs formation. Indeed, activated RhoA promotes actin-myosin contraction that is required for L-EVs formation through the downstream signaling of ROCK (Rho-associated coiled-coil containing kinases) and ERK (extracellular signal-regulated kinases) [37] and similar mechanisms have been shown specifically for LO biogenesis [24].

It is also worth to mention that the endosomal sorting complexes required for transport (ESCRT) may be involved in L-EVs biogenesis. This pathway was initially thought to play critical role only into exosome biogenesis from the endosomal membrane. However, it was described that some proteins from the ESCRT (named TSG101 and VPS4 ATPase) can be relocated from the endosomal membrane to the PM where they mediate the release of L-EVs [38].

Finally, LO shedding has also been associated to activation of the epidermal growth factor receptor (EGFR) and overexpression of a membrane-targeted, constitutively active form of Akt1 [8,39].

Overall, due to the PM origin related to both LO and MVs, some pathways might also contribute to the biogenesis of both populations [40] and have been summarized in Fig. 1. However, some of them has been reported as peculiar for LO release (*see above*).

3. L-EVs content may be useful for cancer biomarkers discovery

Due to the potential use of EVs as source of both diagnostic and prognostic biomarkers in cancer, many efforts are nowadays focusing on the characterization of EVs specific cargos, in order to select new molecular markers. Indeed, proteins, lipids and nucleic acids vehicled

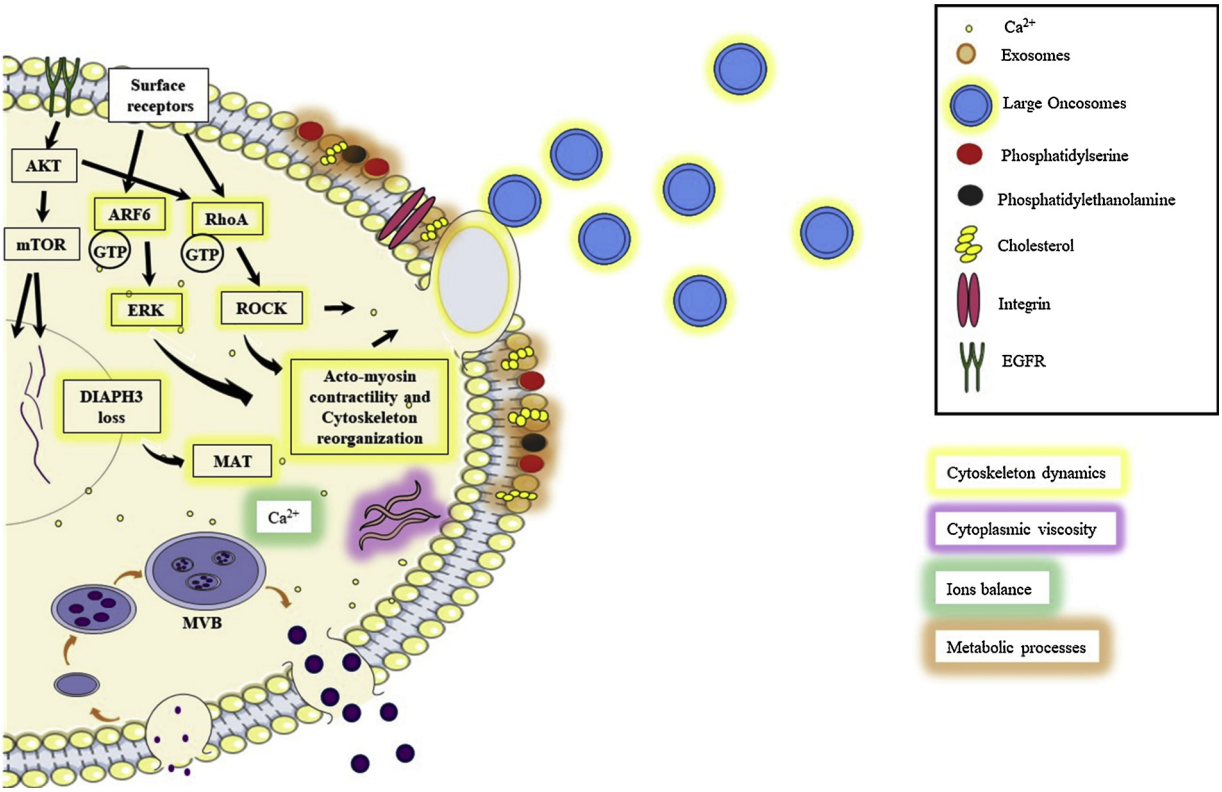

Fig. 1. Biogenesis and shedding of PM-derived and LO: molecular determinants involved in MV/LO biogenesis are summarized.

by EVs are all of growing interest in biomarker discovery. However, several remarks need to be taken in account on the definition of EVs-related biomarkers: a) most of EVs populations are shed from all cell types in the organism; b) molecular determinants contained in EVs are dependent on cells/tissues of origin; c) however the specific EVs cargo (i.e. proteins, miRNAs) is not always coupled to the overexpression in the cells of origin ; d) molecular cargos in EVs can be affected by microenvironment conditions (inflammation, oxygen deprivation, metabolic balance); e) EVs size may affect their content.

Among L-EVs, LO have been well characterized in the last decade because their size and their origin (exclusively by PM of cancer cells), make them an ideal source of information applicable to liquid biopsy. Notably, LO are ~1000 folds the size of exosomes, therefore they can virtually include a larger number of tumor-derived molecules, with a distinct impact on the tumor microenvironment [41]. Below, we will review the literature on LO content (Table 1), suggesting that they could represent specific cancer biomarkers.

3.1. Proteins

Studies focusing on proteins EVs-associated are both investigating their differential expression among EVs population and exploring the possibility to detect their amount in body fluids with the aim to define novel cancer biomarkers as well as therapeutic targets.

A significant overlap of proteins expressed by different population of EVs does exist, and a unique signature for L-EVs is hard to define, due to the huge amount of proteins identified and their PM-origin.

LO have been reported to contain the GTPase ADP-ribosylation factor 6 (ARF6) [8] that, once activated, promotes the recruitment of ERK to the PM, allowing the contraction of actomyosin at the necks of L-EVs and thus their release [42].

LO also carry caveolin-1 [39] and metalloproteinases 2–9 (MMP2, 9) [11,39], accordingly to older reports on PM-derived L-EVs [43,44]. In addition, keratin 18 (CK18, a cytokeratin type I) appears to be highly abundant in L-EVs, and has been used to show LO-like structure *in situ* in human PCa tissues and body fluids [11,25,39,45]. Intriguingly, LO from PCa cell lines have been recently shown as enriched in a set of peculiar enzymes involved in cancer cell metabolism, compared to the exosomes counterpart from the same source, such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), glucose phosphate isomerase (GPI), lactate dehydrogenase B (LDHB), heat shock 70 kDa protein 5 (HSPA5), malate dehydrogenase (MDH) and aspartate transaminase

Table 1
Main molecules vehicled by LO.

Enriched in LO	Molecules and functions	References
Proteins	ARF6 (recruitment of ERK to the PM allowing the contraction of actomyosin at the necks of L-EVs and their release).	[8,42]
	Caveolin-1, CK18. MMP 2-9 scaffolding protein/ cytoskeleton components and gelatinase activity	[11,39,45];
	GAPDH, GPI, LDHB, HSPA5, MDH, GOT, GLS (metabolic enzymes); V-ATPase subunit V1G1 (pump for delivering of oncogenic signals)	[57]
	αV-integrin (transfer of both adhesion and invasion properties from the PCa aggressive cell line to the less aggressive counterpart).	[11]
	Urokinase-type plasminogen activator receptor, eukaryotic elongation factor 1 gamma (eEF1γ) and AKT1: promotion of cancer progression when released by the aggressive counterpart.	[9,11]
Nucleic Acids	Circulating DNA and copy number variations of genes frequently altered in metastatic PCa (i.e. MYC, AKT1, PTK2, KLF10 and PTEN): favor cancer cell progression; miR-1227 overexpressed, by RWPE-2 PCa cells, modulator of CAFs migration	[10,56]

(GOT) [25]. Interestingly glutaminase (GLS), a cytoplasmic enzyme that converts glutamine to glutamate, was identified among the protein uniquely expressed in L-EVs [25].

The metabolic enrichment described above may be due to the reprogramming of glucose, glutamine and amino acid metabolism, all metabolic processes altered in the dynamics of cancer progression [46] and should be further explored because could be associated to both the biogenesis and function of L-EVs.

To date, the amount of membrane proteins was found to represent 30% of the proteins identified in both large and small populations of EVs [25,47,48]. Among them, although tetraspanins and cell adhesion proteins resulted enriched in nano-sized EVs, we have recently reported that LO from an aggressive model of PCa, resistant to mevalonate pathway inhibitors, carried a functionally bioactive alpha V (αV)-integrin on their surface [11]. This LO-associated αV integrin is involved in the transfer of both adhesion and invasion properties from the PCa aggressive cell line to the less aggressive counterpart [11]. Moreover we also showed that LO are enriched in urokinase-type plasminogen activator receptor (uPAR) and eukaryotic elongation factor 1 gamma (eEF1γ), two proteins related to cancer progression when released by the aggressive counterpart [11].

Although the dynamics of protein cargo in different population of EVs is far to be elucidated, we can speculate that aggressive PCa cells may preferentially load some proteins in LO more than in small EVs, in order to improve cell-to-cell messages aimed to cancer progression. However, further investigations are necessary to explore this suggestion. Moreover, in the context of PCa, LO have been also shown to harbor sustained AKT1 kinase activity, corroborating this population as an active signaling platform [9]. Notably, active AKT1 was detected in circulating EV from the plasma of metastatic prostate cancer patients and was LO specific [9].

3.2. Nucleic acids

Numerous studies focusing on EVs content in terms of nucleic acids demonstrated a variegated RNAs cargo such as mRNAs, microRNAs, and ncRNAs. In particular, a recent report comparing the mRNA content between L-EVs (MV) and exosomes from different PCa cell lines, demonstrated that L-EVs were enriched in caveolin-2 (CAV2), glutathione S-transferase pi 1 gene (GSTP1) transcripts compared to the exosomes counterpart, corroborating the idea that mRNA cargo could differ not only based on the EV cellular origin but also on the subpopulation analyzed, each of them conveying a different proportion of the cell transcriptome [49]. Indeed, distinct microRNAs-enrichment signatures have been identified analyzing different EVs subtypes released from a human colon cancer cell line [50,51]. However, the study suggest that microRNA biogenesis may be interlinked specifically with endosomal/exosome processing [51].

The detection of retrotransposon [52,53] and amplified oncogene sequences, have been also reported in L-EVs beyond exosomes [52], while single-stranded DNA (ssDNA) and mitochondrial DNA have been investigated mostly in exosomes [54]. Among the studies reporting the presence of double stranded genomic DNA (gDNA) in diverse types of EVs, the suggestion that specific EV types might package different parts of the genome was already formulated by one of the first report on DNA in EVs, showing that different EV populations harbor either *TP53* or *PTEN* mutations [55].

LO in particular, have been shown to contain miRNA, mRNA and DNA [39] suggesting that, similarly to other EVs, they may mediate horizontal transfer of diverse nucleic acid species within and across tissue compartments and to distant sites through the circulation [56]. In 2013, a report showed that the overexpression of miR-1227 in RWPE-2 PCa cells, determined its loading preferentially into LO vs. smaller EV, and that its overexpression in LO was a novel modulator of the migration of cancer-associated fibroblast –CAFs- [56]. More recently, it has been shown that circulating DNA is almost exclusively enclosed in L-EVs derived from PCa patient plasma [10]. In addition the same group showed that genetic aberrations belonging to the cell of origin, including copy number variations of genes frequently altered in metastatic PCa (i.e. MYC, AKT1, PTK2, KLF10 and PTEN), were reflected in L-EVs, suggesting the use of LO as biomarkers within a liquid biopsy approach.

4. LO functions and internalization mechanisms serving to L-EVs fusion into recipient cells

The functional effects exerted by LO varies from a direct proteolytic activity [11,39], to the activation of pro-tumorigenic programs into different types of target cells including other tumor cells or cells of tumor microenvironment [9,11,39,56–58]. In details, LO have been shown to influence glutamine metabolism of PCa cells through the stimulation of the enzyme GOT1 that catalyzes the formation of glutamate from aspartate and α-ketoglutarate [25]. In addition, it has been recently shown that LO shed from an aggressive PCa cell line are able to induce both adhesion and invasion of PCa recipient cells by activating AKT through the αV-integrin expressed on LO surface [11]. The induction of cell migration has been also reported upon treatment with LO from LNCaP/MyrAkt1 cells, carrying an over-activated form of AKT [39]. Interestingly, in line with the above mentioned observations, tumor cell migration was also enhanced by fibroblasts previously treated with LO. The impact of LO on tumor microenvironment has been described for: a) stromal cells, in which LO treatment can induce the expression of metastasis-associated factors, such as brain-derived neurotrophic factor (BDNF), C-X-C motif chemokine 12 (CXCL12) and osteopontin [39]; b) endothelial cells, in which tube branching was induced by LO and whose migration resulted enhanced by treatment with LO purified from the circulation of mice with metastatic disease [39]; c) normal fibroblast (NAF) where LO treatment caused enhanced expression of interleukin 6 (IL6), MMP 9 and α-smooth muscle actin (α-SMA), thus favoring a provascularization phenotype through Myc activation [9]. Finally, it has been recently demonstrated that Glioblastoma neurospheres influence their non-neoplastic microenvironment by delivering the V-ATPase subunit V1G1 and the homeobox genes HOXA7, HOXA10, and POU3F2 to recipient cells via LO [57].

LO can exert all the functions described above by an autocrine mechanism (intuitively confined to tumor cells) and/or a paracrine effect (affecting both tumor and tumor microenvironment cells). Although not directly proved yet, we can hypothesize that LO can also exert a systemic/endocrine effect, as shown for other EVs such as exosomes [59,60]. Moreover, it is important to define whether the EVs docking event on the recipient cell is sufficient to initiate a signaling pathway that causes functional changes and/or EVs docking is always followed by the transferring of their cargo inside the recipient cells. In this regard, it has been recently shown that AKT activity is critical for MYC-dependent reprogramming of stroma recipient cells upon the uptake of PCa-derived LO [9]. Interestingly, the authors have also found an activated AKT in LO isolated from PCa cells and from PCa patients' plasma. Similarly, in a recent report we demonstrated a functional role of αV-integrin on LO surface to affect tumor recipient cells via AKT activation, and we cannotexclude selective LO internalization [11]. Anyhow whether AKT activation in recipient cells was the consequence of LO cargo release or associated with the endogenous activation of AKT upon LO docking/uptake, remains an open question [9,11].

Mechanisms regulating L-EVs internalization have received less attention compared to their biogenesis or content. In addition, the functional assays mentioned above, exploring L-EVs effects on recipient cells, often do not explore the type interaction mediating the L-EVs biological activity. This is not a trivial topic, if we consider that L-EVs time/mechanisms of uptake may also influence their availability in the circulation for liquid biopsy.

In our opinion, all the effects exerted by LO are probably due to both EVs docking and membrane-membrane contact, followed by L-EVs

uptake.

Generally, internalization of large (> 1 μm) particles occurs through a mechanism known as phagocytosis, although this is a type of endocytosis typically restricted to specialized professional phagocytes. In contrast, pinocytosis, is exhibited by all cells, and can involve different molecules (i.e. Arf-6, flotillin-1-, CDC42- and RhoA) [61]. Minciacchi et al. suggested that LO internalization is similar to a phagocytosis-like mechanism, demonstrating the efficient block of the process by Dynasore, non-competitive inhibitor of the GTPase activity of dynamin, which is a protein essential for endocytosis and phagocytosis, but not by EIPA (5-(N-Ethyl-*N*-isopropyl) amiloride) a macropinocytosis blocker [9].

However, if the mechanisms of L-EVs uptake are similar to the classic endocytosis processes, either receptor-dependent or the fluid-phase endocytosis (low efficient, non-specific process), is unclear. Notably, changes in the cellular microenvironment (i.e. pH.) can influence EV uptake, as shown by studies on exosomes [62,63].

LO internalization by heterologous cells has been shown in normal fibroblast primary cells generated from prostatectomy tissues not associated with prostate cancer (NAF), human endothelial cells (HUVEC), $CD8^+$ lymphocytes, and DU145 and LNCaP PCa cell lines. Each type of cell line internalized with different efficiency LO, implying a selective mechanism of uptake. In particular, the observation that LO uptake was almost completely impaired in $CD8^+$ lymphocytes [9] might suggest a specific tumor- or tumor-microenvironment-associated effects. Interestingly, a seminal paper by Chen G et al. showing elevated levels of PD-L1 on both exosomes and larger EVs from metastatic melanoma patients, functionally demonstrated a specific immunosuppressive effect mostly confined to exosomes through the direct interaction with effector CD8 T cells [64]. The authors also suggested that exosome expressing PD-L1 are better predictors of anti-PD-1 therapy efficacy compared to PDL1-positive L-EVs.

5. Conclusions

In conclusion, due to their atypical size and their specific release from cancer cells LO are promising source of both diagnostic and prognostic markers. Although some pathways involved in their biogenesis may be shared with MV, others resulted peculiar for this class of EVs. LO enrichment in specific proteins and nucleic acids could be exploited to define strategies for their detection in the circulation. Notably, although has been already demonstrated that LO detection can discriminate between healthy and cancer cells/tissues, particularly in PCa models [8,9,11,39], and that LO shedding is associated to aggressive features in PCa and glioblastoma models [57], further investigations are needed to define their role in other tumor models. Overall, all the approaches aimed to detect LO in both tumor tissues and patients body fluids need to be standardized in order to be implemented in clinical practice.

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CRediT authorship contribution statement

Chiara Ciardiello: Conceptualization, Writing - original draft. **Rossella Migliorino:** Conceptualization, Data curation. **Alessandra Leone:** Conceptualization, Writing - original draft. **Alfredo Budillon:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

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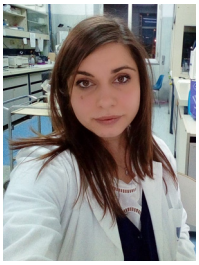

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